EMERGING ZOONOSES: ECO-EPIDEMIOLOGY, INVOLVED MECHANISMS AND PUBLIC HEALTH IMPLICATIONS

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EMERGING ZOONOSES: ECO-EPIDEMIOLOGY, INVOLVED MECHANISMS AND PUBLIC HEALTH IMPLICATIONS

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Male of Aedes albopictus, the Asian Tiger Mosquito (Author: Dr. Rubén Bueno-Marí).

Zoonoses are currently considered as one of the most important threats for public health worldwide. Zoonoses can be defined as any disease or infection that is naturally transmissible from vertebrate or invertebrate animals to humans and vice-versa. Approximately 75% of recently emerging infectious diseases affecting humans are diseases of animal origin; approximately 60% of all human pathogens are zoonotic. All types of potential pathogenic agents, including viruses, parasites, bacteria and fungi, can cause these zoonotic infections. From the wide range of potential vectors of zoonoses, insects are probably those of major significance due to their abundance, high plasticity and adaptability to different kinds of pathogens, high degrees of synanthropism in several groups and difficulties to apply effective programs of population control. Although ticks, flies, cockroaches, bugs and fleas are excellent insects capable to transmit viruses, parasites and bacteria, undoubtedly mosquitoes are the most important disease vectors. Mosquito borne diseases like malaria, dengue, equine encephalitis, West Nile, Mayaro or Chikungunya are zoonoses with increasing incidence in last years in tropical and temperate countries. Vertebrates can also transmit serious zoonoses, highlighting the role of

some carnivorous animals in rabies dissemination or the spread of rodent borne diseases in several rural and urban areas. Moreover, the significance of other food borne zoonoses such as taeniasis, trichinellosis or toxoplasmosis may not been underestimated.

According to WHO, FAO and OIE guidelines an emerging zoonotic disease can be defined as a zoonosis that is newly recognized or newly evolved, or that has occurred previously but shows an increase of incidence or expansion in geographical, host or vector range. There are many factors that can provoke or accelerate the emergence of zoonoses, such as environmental changes, habitat modifications, variations of human and animal demography, pathogens and vectors anomalous mobilization related with human practices and globalization, deterioration of the strategies of vector control or changes in pathogen genetics. To reduce public health risks from zoonoses is absolutely necessary to acquire an integrative perspective that includes the study of the complexity of interactions among humans, animals and environment in order to be able to fight against these issues of primary interest for human health. In any case, although zoonoses represent significant public health threats, many of them still remain as neglected diseases and consequently are not prioritized by some health international organisms.

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Table of Contents

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EDITORIAL
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Editorial: Emerging zoonoses: eco-epidemiology, involved mechanisms, and public health implications

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Keywords: zoonoses, infectious diseases, infectious diseases epidemiology, editorial, one health

Zoonoses are currently considered as one of the most important threats for Public Health worldwide. Zoonoses can be defined as any disease or infection that is naturally transmissible from vertebrate or invertebrate animals to humans and vice-versa. Approximately, 75% of recently emerging infectious diseases affecting humans are diseases of animal origin; approximately, 60% of all human pathogens are zoonotic. All types of potential pathogenic agents, including viruses, parasites, bacteria, and fungi, can cause these zoonotic infections. From the wide range of potential vectors of zoonoses, arthropods are probably those of major significance due to their abundance, high plasticity, adaptability, and coevolution to different kinds of pathogens, high degrees of synanthropism in several groups, and difficulties to apply effective programs of population control. Although ticks, flies, sandflies, cockroaches, bugs, and fleas are excellent vectors capable of transmitting viruses, parasites, and bacteria, undoubtedly mosquitoes are the most important human disease vectors, while ticks are the most important vectors of pathogens in domestic production animals. Mosquito borne diseases like malaria, equine encephalitis, or West Nile are zoonoses with increasing incidence in the last years in tropical and temperate countries. All these zoonoses are thoroughly discussed in the Research Topic (1–5). Moreover, several researches focused on new tools to fight against Dengue vectors (6), studies about mosquito biodiversity (7), or novel modeling techniques based on climatic factors to predict vector's incidence (8) can also be found in our compilation of research works related with zoonoses. Although it is well known that mosquitoes are the major vectors worldwide, probably ticks and tick-borne diseases are those that have aroused higher interest in epidemiologists and medical entomologists in recent years (9–12).

The problems related with zoonoses have different significance in developed and undeveloped countries. One example of a vector-borne disease relatively easy to combat with current pharmacological, preventive, and vector control tools but with a dramatic incidence in Central and South America is Chagas disease or American trypanosomiasis (13–19). In Africa and Asia, other neglected diseases like leishmaniasis or African trypanosomiasis have serious impact on human populations locally (20–22).

Not all zoonoses are vector borne, vertebrates can also transmit serious zoonoses, highlighting the role of some carnivorous animals in rabies dissemination, the spread of rodent borne diseases in several rural and urban areas, or some transmissible bacteria in cattle and other livestock (23).

According to WHO, FAO, and OIE guidelines, an emerging zoonotic disease can be defined as a zoonosis that is newly recognized or newly evolved, or that has occurred previously but shows an increase of incidence or expansion in geographical, host, or vector range. There are many factors that can provoke or accelerate the emergence of zoonoses, such as environmental changes, habitat modifications, variations of human and animal demography, pathogens and vectors anomalous mobilization related with human practices and globalization, such as the introduction of exotic mosquito

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species of which *Aedes albopictus* is the paradigm, deterioration of the strategies of vector control, or changes in pathogen genetics (24–26). To reduce Public Health risks from zoonoses, it is absolutely necessary to acquire an integrative perspective that includes the study of the complexity of interactions among humans, animals, and environment in order to be able to fight against these issues of primary interest for human health, hence the new "One Health" approach. In any case, although zoonoses represent significant public health threats, many of them still remain as neglected diseases and consequently are not prioritized by some national or international health organisms.

The aim of this Research Topic is to cover all related fields with zoonoses, including basic and applied researches, approaches to control measures, explanations of new theories or observations, opinion articles, reviews, etc. To deeply discuss these issues, a holistic and integrative point of view is obviously needed and guided by the "One Health" strategy. Editors are very proud to say that this ambitious goal for the Research Topic has been accomplished, thanks to the collaboration of researchers specialized in different fields as medical and veterinary entomologists, parasitologists, veterinarians, virologists, zoologists, microbiologists, ecologists, evolutionary biologists, and medicals specialized in epidemiology, public health, and animal health. The participation of multiple contributors and a multidisciplinary approach have been most important to comply with a knowledge demand of this issue of first-rate of scientific and medical interest.

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Emerging vector-borne diseases – incidence through vectors

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Vector-borne diseases use to be a major public health concern only in tropical and subtropical areas, but today they are an emerging threat for the continental and developed countries also. Nowadays, in intercontinental countries, there is a struggle with emerging diseases, which have found their way to appear through vectors. Vector-borne zoonotic diseases occur when vectors, animal hosts, climate conditions, pathogens, and susceptible human population exist at the same time, at the same place. Global climate change is predicted to lead to an increase in vector-borne infectious diseases and disease outbreaks. It could affect the range and population of pathogens, host and vectors, transmission season, etc. Reliable surveillance for diseases that are most likely to emerge is required. Canine vector-borne diseases represent a complex group of diseases including anaplasmosis, babesiosis, bartonellosis, borreliosis, dirofilariosis, ehrlichiosis, and leishmaniosis. Some of these diseases cause serious clinical symptoms in dogs and some of them have a zoonotic potential with an effect to public health. It is expected from veterinarians in coordination with medical doctors to play a fundamental role at primarily prevention and then treatment of vector-borne diseases in dogs. The One Health concept has to be integrated into the struggle against emerging diseases. During a 4-year period, from 2009 to 2013, a total number of 551 dog samples were analyzed for vector-borne diseases (borreliosis, babesiosis, ehrlichiosis, anaplasmosis, dirofilariosis, and leishmaniasis) in routine laboratory work. The analysis was done by serological tests – ELISA for borreliosis, dirofilariosis, and leishmaniasis, modified Knott test for dirofilariosis, and blood smear for babesiosis, ehrlichiosis, and anaplasmosis. This number of samples represented 75% of total number of samples that were sent for analysis for different diseases in dogs. Annually, on average more then half of the samples brought to the laboratory to analysis for different infectious diseases are analyzed for vector-borne diseases. In the region of Vojvodina (northern part of Serbia), the following vector-borne infectious diseases have been found in dogs so far borreliosis, babesiosis, dirofilariosis, leishmaniasis, and anaplasmosis.

Keywords: emerging diseases, zoonoses, vector-borne diseases, One Health, ELISA

INTRODUCTION

Vector-borne diseases use to be a major public health concern only in tropical and subtropical areas, but today they are an emerging threat in continental countries also. Nowadays, in intercontinental countries, there is a struggle with some emerging diseases, which have found their way to appear through vectors. Vector-borne zoonotic diseases occur when vectors, animal hosts, climate conditions, pathogens, and susceptible human population exist at the same time, at the same place. Global climate change is predicted to lead to an increase of vector-borne infectious diseases and disease outbreaks. It could affect the range and population of pathogens, host and vectors, transmission season, etc. Reliable surveillance for diseases that are most likely to emerge is required. There are countries where environmental conditions are not so favorable for certain vector populations, but immigration allows them to persist (1). Vector-borne pathogens have a considerable impact on human and animal health. Newly emerging pathogens and increasing case numbers of endemic diseases have received considerable public attention in Europe recently. Vector-borne diseases are a matter of Public Health, and therefore, it is important to establish a systematic approach for the understanding, analysis, assessment, communication, and the management risk associated with vector-borne diseases. Risk assessment of vector-borne diseases, however, is influenced by the fact that the infection depends on many factors, the probability of infection in human beings and animals, and the spread of pathogen, or vectors. These factors are climate change, change in human beings living habits, agricultural land usage, individual human behavior, travel, and global trade (2).

The complex epidemiology of vector-borne diseases creates significant challenges in the design and delivery of prevention and control strategies, especially in sight of rapid social and environmental changes. Many diseases are specially constrained, for example, vector-borne and zoonotic diseases occur where and when vectors, animal hosts, pathogens, and susceptible human

populations overlap (3). Global climate change is predicted to lead to an increase of infectious disease outbreaks. Reliable surveillance for diseases that are most likely to emerge is required. Climate changes could affect the range and population size of pathogens, hosts and vectors, the length of the transmission season, and the timing and persistence of out breaks (4). If there are outbreaks of infectious diseases that are considered to be eradicated from before or that are totally under control, they are called emerging infectious diseases (5). Emerging infectious diseases can be defined as infections that have newly appeared in a population, or are rapidly increasing in incidence or geographic range. Many of these diseases are zoonoses (6). From all the causative agents of emergent infectious diseases, 60–70% of them have a zoonotic potential (7). Zoonoses are infectious diseases, which can be transferred from animals (mammals) to human beings. Maps of expected distributions of vector existence are often presented as a risk of exposure to a pathogen. Global climate change is predicted to lead to an increase of vector-borne infectious diseases and disease outbreaks. It could affect the range and population of pathogens, hosts and vectors, transmission season, etc. Reliable surveillance for diseases that are most likely to emerge is required.

The factors of emergence are the following: changes in ecology, changes in demography and human behavior, changes and adaptations of microorganisms, improvement in technology, and changes in industry, international transport and trade, and incompliance of public health measures. Changes in ecosystem may lead to the increase of population in natural hosts, or vectors for certain emerging infectious disease. These factors are becoming increasingly prevalent, suggesting that infections will continue to emerge and probably increase. Strategy for dealing with this problem includes focusing of attention on promoting the emergence of diseases, especially in situations when animals and human beings are in contact and implementation of effective disease surveillance and control (6).

For practicing veterinarians, vector-borne diseases represent a constant challenge. The health of companion animals never played a more important role in a family life. It is expected from veterinarians to play a fundamental role in first of all prevention and then treatment of vector-borne diseases in dogs. Canine vector-borne diseases represent a complex group of diseases including anaplasmosis, babesiosis, bartonellosis, borreliosis, dirofilariosis, ehrlichiosis, leishmaniosis, and rickettsiosis. Some of these diseases cause serious clinical symptoms in dogs and some of them have a zoonotic potential with an effect to public health. Veterinarians are comfortable accepting that a multi-modal approach in disease diagnostics, including antigen and antibody testing combined with other diagnostic methods is ideal to diagnose some diseases. But, it is very important to highlight that increased detection of infection with, or exposure to, canine vector-borne diseases does not always confirm the disease cause. It is still the veterinarian who is responsible for the interpretation of the laboratory findings, having in mind clinical status, and response to the treatment. To optimize the clinical division, veterinarians in practice should always consider using panels that include serological and PCR assays in parallel to maximize their chances of detecting infection or exposure to canine vector-borne pathogens (8). Also, annual control of artropodes using ectoparasiticides with repellents, to block the interaction between vector and host is upon veterinarians to be recommended to the owners (9).

Vector-borne diseases such as tick-borne *Borrelia burgdorferi s.l*, tick- and flea-borne emerging and re-emerging *Rickettsia* species, and sandfly borne *L. infantum* are important infectious diseases of human and veterinary medicine across Europe (9). A tick species *Ixodes ricinus* is usually predominant among ticks originating form Serbia and is one of the most widely distributed. A significant presence of *B. burgdorferi sensu lato* was detected in *I. ricinus* ticks from Serbia, using dark field microscopy (10, 11). Also, multiple and mixed infections with different pathogens were found in different species of ticks in Serbia (12) with a highlight on *I. ricinus*, in which also mixed infections were found (13).

MATERIALS AND METHODS

During a 4-year period, from 2009 to 2013, a total number of 734 dog samples were analyzed for different diseases. For vector-borne diseases (borreliosis, babesiosis, ehrlichiosis, anaplasmosis, dirofilariosis, and leishmaniasis), 551 dog blood samples were analyzed in routine laboratory work, which makes 75% of total samples analyzed for different diseases. The analysis was done by serological tests – ELISA for borreliosis (Microgen IgG and IgM and Euroclone commercial ELISA set kits for detection of specific antibodies against *Borrelia*), leishmaniosis (Ingenaza commercial ELISA set kit for detection of specific antibodies against *Leishmania*), and dirofilariosis (IDEXX commercial ELISA set kit for detection of specific antibodies against *Dirofilria*). The procedure of analysis was done by the original manufacturer's instructions. Diagnostic of dirofilariosis was also done with modified Knott test for dirofilariosis. Diagnosis of babesiosis, ehrlichiosis, and anaplasmosis was done in a stained blood smear. The samples were collected when dogs came for a routine check-up, or when they were sent for a certain disease detection because of the clinical symptoms that could be found in the animal. Some of the dogs had clinical symptoms, which could be identified as clinical symptoms for borreliosis, leishmaniosis, or dirofilariosis. Some of the samples were sent for a complete check-up, with a panel of all six diseases, or different combination of more then one, depending on the needs of the patient. Fast and snap tests for diagnostics of one or combination of pathogens were not used at any time.

RESULTS AND DISCUSSION

The number of samples analyzed for vector-borne zoonoses: borreliosis, babesiosis, ehrlichiosis, anaplasmosis, leishmaniosis, and dirofilariosis represented 75% of total number of samples that were sent for analysis for different diseases. Annually, on average, more then half of the samples brought to the laboratory for analysis on different diseases are analyzed for vector-borne diseases. In the region of Vojvodina (northern part of Serbia), the following vector-borne infectious diseases have been found so far in dogs: borreliosis, babesiosis, dirofilariosis, leishmaniasis, and anaplasmosis. For babesiosis, ehrlichiosis, and anaplasmosis a routine diagnostic procedure started only in 2012 in our laboratory, so the number of these samples was taken into consideration since that period.

Table 1 | Total number and positive findings in dog samples for vector-borne diseases during the period 2009–2013.

	2009 (Positive %)	2010 (Positive %)	2011 (Positive %)	2012 (Positive %)	2013 (Positive %)
Borreliosis	13 (30.7%)	45 (24.4%)	30 (23.3%)	25 (16%)	77 (29.8%)
Babesiosis	–	–	–	17 (11.7%)	24 (12.5%)
Ehrlichiosis	–	–	–	30 (0%)	59 (0%)
Anaplasmosis	–	–	–	30 (0%)	59 (10.1%)
Leishmaniasis	5 (20%)	14 (14.28%)	53 (15.1%)	15 (13.3%)	133 (15%)
Dirofilariosis	7 (28.5%)	11 (27.7%)	15 (33.3%)	18 (38.8%)	101 (24.7%)

Table 2 | Total number of analyses and positive findings for the study period 2009–2013.

Total no. of analyses for the study period 2009–2013	
Borreliosis	190 (25.8%)
Babesiosis	41 (12.2%)
Ehrlichiosis	89 (0%)
Anaplasmosis	89 (6.74%)
Leishmaniasis	220 (15%)
Dirofilariosis	152 (27.6%)

In the same region, the following diseases have been diagnosed in human beings, so far borreliosis, dirofilariosis, and leishmaniosis (imported cases). Borreliosis exists as a common disease in human beings in Serbia. Several cases of dirofilariosis in human beings have been found during the last 4 years in Vojvodina and also cases of leishmaniasis have been found during the same period, but only as imported cases (unpublished data). Dirofilariosis has also been found in human beings Serbia during the last few years (14).

A total number of 551 dog samples were analyzed for different vector-borne diseases (borreliosis, babesiosis, ehrlichiosis, anaplasmosis, dirofilariosis, and leishmaniasis) in routine laboratory work. Total number of analyses done in these samples was 692. The results are presented in **Tables 1** and **2**.

During the observed period (2009–2013), 190 dogs were examined for Lyme borreliosis in routine work and 49 of them were found positive (25.8%). Seroprevalence for Lyme borreliosis in dogs was studied previously, for the earlier 3 year period (2006–2008) in a larger number of samples, with a randomly chosen group of samples and in the same region. Seroprevalence was found to be 25.81%. Also, the prevalence of Lyme borreliosis in ticks in northern part of Serbia was found to be 22.12% (15). In a study from Milutinovic et al., the highest prevalence rate found in different part of Serbia among ticks for *B. burgdorferi sensu lato* was 42.5%. So far, in Serbia presence of five *B. burgdorferi sensu lato* genospecies was found: *B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, *B. usitaniae*, and *B. valaisiana*. Also, coinfections were found in ticks with *B. burgdorferi sensu lato* and *A. phagocitophilum* (10).

Only 41 samples of dogs were examined for babesiosis. The reason for this small number of samples is that veterinarians perform the analysis for babesiosis themselves in their practices. Only complicated cases where they could not find positive result were sent for a conformation to our laboratory. Therefore, only five of those suspicious dogs with anemia were really positive for babesiosis.

Ehrlichiosis is an emerging disease coming to Serbia, with just a few positive findings within the country (16). In our study with the method of stained blood smear no positive samples were found.

Anaplasmosis is also a vector-borne disease new to veterinarians and owners in the region. Some fast tests are done in several practices, with very low incidence of the disease itself. In a study from different region of Serbia showed a prevalence of 5.40–6.60% (16). In our study, a similar prevalence was found and it was 6.74%.

The number of examined dog blood samples for leishmaniosis, during the period of study, was 220. The number of positive samples detected for leishmaniosis was 33, which makes 15%. A certain number of dogs have traveled abroad (Italy, Greece, or Montenegro) during a previous study period. Those dogs were examined after coming back to Serbia (21 dogs) for the presence of specific antibodies against *Leishmania*. From the total of 21 samples in 28.57%, a positive finding for leishmaniosis was detected and the dogs had clinical symptoms of leishmaniosis (17).

During the same period from 2009 to 2013, the number of 152 dogs was examined for dirofilariosis – with or without clinical symptoms, and in 69 samples (27.6%) the presence of microfilaria was detected. In a previous study period, the analysis was done on working and military dogs from a selected group, in the same region, where as a result a seroprevalence for dirofilariosis was found to be 18% (18).

Recently published data from Europe indicate that there is an urgent need for further research and more field studies about *Ixodes* ticks and tick-borne diseases. The epidemiology of *Ixodes* ticks, the dynamics of tick spreading into new habitats due to the change in climate and land use is not fully understood. Interaction in various habitats in Europe, between the vector, pathogen, and mammalian host (human beings and dogs) needs further research (9).

CONCLUSION

Human and animal health is connected today into a One Health concept, which focuses on zoonotic pathogens emerging from wild life, domestic animals, and companion animals. A role of companion animals influence to public health is more important over the years, because the interaction and connection between human beings and pets are growing and getting stronger over time. There should be an interaction between veterinary and human medicine for the benefit of domestic, wild animal, and human health. It should always be in our minds that there is an interaction between human health and domestic animal and wild life

health with global zoonotic disease pandemics and emerging infectious diseases, which came from these animal species. From the total of 734 dogs examined for different infectious diseases, 551 of them were tested for canine vector-borne diseases that exist in the region, in vectors and in human beings, and 692 analyses were done. From the total number of analysis, 23.4% of examined dogs blood samples were positive to one of the canine vector-borne diseases.

Borreliosis, babesiosis, leishmaniasis, dirofilariosis, and anaplasmosis are considered as major vector-borne infectious diseases that are shared by man and dogs in the region of the study, from the One Health concept point of view. There should be an interaction between veterinary and human medicine, with clinicians, researchers, and government working together for the benefit of domestic, wild animal, and human health and the global environment (19). Managing of canine vector-borne diseases requires a One Health approach and proper control of arthropod transmitted pathogens to both human beings and animals. This approach is only achievable when clinicians and researchers from different disciplines work joined. Legislative bodies nationally and on the European level should support this initiative (9).

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Vector-borne disease intelligence: strategies to deal with disease burden and threats

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Owing to the complex nature of vector-borne diseases (VBDs), whereby monitoring of human case patients does not suffice, public health authorities experience challenges in surveillance and control of VBDs. Knowledge on the presence and distribution of vectors and the pathogens that they transmit is vital to the risk assessment process to permit effective early warning, surveillance, and control of VBDs. Upon accepting this reality, public health authorities face an ever-increasing range of possible surveillance targets and an associated prioritization process. Here, we propose a comprehensive approach that integrates three surveillance strategies: population-based surveillance, disease-based surveillance, and context-based surveillance for EU member states to tailor the best surveillance strategy for control of VBDs in their geographic region. By classifying the surveillance structure into five different contexts, we hope to provide guidance in optimizing surveillance efforts. Contextual surveillance strategies for VBDs entail combining organization and data collection approaches that result in disease intelligence rather than a preset static structure.

Keywords: vector-borne diseases, surveillance, one health, disease burden, threat, emerging diseases

INTRODUCTION

Globalization and human population growth continue to put increasing pressures on human health and well-being. Emerging diseases have become a growing threat leading to the development of epidemic intelligence systems to aid public health authorities. The main aim of this epidemic intelligence is to encompass all activities that permit the early identification of potential health hazards, their verification, risk assessment, and investigation in order to inform and improve public health control measures in a timely manner (1). Epidemic intelligence integrates both an indicator-based and an event-based component; the former referring to structured collection of data through routine surveillance systems and the latter referring to data gathered from sources of intelligence of any nature (2). Finally, good epidemiologic judgment, the reasoning process that indicates when there is sufficient data on which to make public health decisions (1), is essential. This has been a challenging component in the past but even more so today with mandated transparency of public decisions, and a continuously changing interconnected world. Further, it has become apparent that an interdisciplinary approach to the prevention and control of zoonoses is invaluable. Cross-sector working ensures better preparedness and contingency planning, more efficient and effective surveillance systems, cost-sharing between sectors according to their benefits of control, increased health equity, and improved sharing of logistics and costs for service provision (3). In recent years, efforts to improve the collaboration between the public and veterinary health, has paid off (4). Since the transmission cycles of several zoonotic pathogens occur in nature, involvement of stakeholders of forest and nature management is a logical next step. This means combining organization and data collection approaches that result in disease intelligence (2) rather than a preset static structure.

In recent decades, vector-borne diseases (VBDs) have emerged as a significant threat to human health in temperate areas (5). In Europe, the incidence and geographical distribution of endemic VBDs, specifically Lyme borreliosis, are increasing in several areas (6). Also, West Nile fever is emerging in Europe (7). Other VBDs have appeared outside the regions where they originally circulated such as dengue in Madeira (8). And last but not least, novel VBDs have emerged or have recently been recognized, such as infections with the tick-borne *Borrelia miyamotoi* (9). Considering the already occurring disease burden and

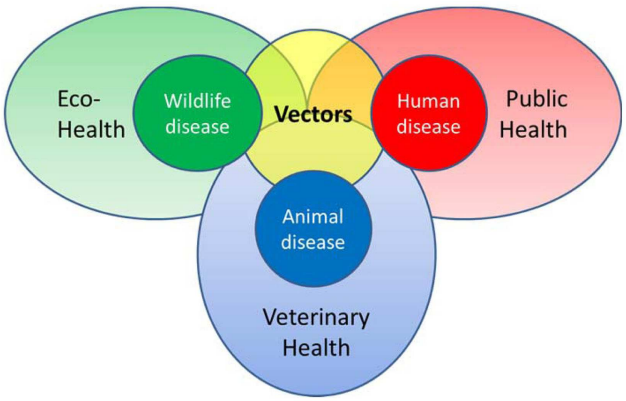


FIGURE 1 | Schematic representation of the various health disciplines involved in vector-borne diseases.

various emerging threats for the future, cost-effective VBD surveillance is challenging. Investment in networks keeping a close watch on the matters within their expertise, public health, veterinary, and ecohealth (**Figure 1**) is needed. Here, ecohealth is defined as the combined health effects of (changes to) the ecologic network in which we function. This also includes the implications of nature management decisions and land use on factors influencing VBD epidemiology. Each and every pathogen has its own disease ecology; therefore, the combined effect of changes affecting factors like, for instance, reservoir species distribution, vector densities, and human exposure, is inheritably complex.

We aim to develop a strategy for the surveillance of vector-borne pathogens, on a national as well as a Pan-European context. When developing and structuring VBD surveillance strategies, consideration of potential prospectives for action is important. Here, we present the building blocks of such contextual surveillance strategy. We expand the surveillance pyramids conventionally used as the basis of public health surveillance to pyramid assemblies, encompassing data from livestock (and pets), wildlife, and vector population. Next, we customize the pyramid assemblies to specific diseases, here focusing on those transmitted by mosquitoes and ticks. The contextual surveillance strategy of VBDs is further illustrated using examples from the Netherlands. Finally, we link the national with Pan-European VBD surveillance strategies.

BUILDING BLOCKS

POPULATION-BASED SURVEILLANCE

To adequately monitor the disease burden of VBDs related to their occurrence, information is needed from different subgroups of the human population, represented by a disease burden pyramid. However, the forecasting, early detection, prevention and control of VBDs requires knowledge of other parameters. Acquiring data on the presence and density of (infected) vectors complements the disease burden pyramid for VBDs. In the case of a zoonotic VBD, an additional level is required to encompass the density of (infected) reservoir hosts (10). Actually, for each of these layers (10), a surveillance pyramid of their own can be drawn, which can be used when developing a contextual surveillance strategy. Separate surveillance pyramids can also be drawn for each of the additional population groups (e.g., sentinels or affected animal populations), highlighting all possible targets and levels of surveillance, forming a surveillance pyramid assembly (**Figure 2**). The distinction between wild and domestic animals is necessary to match the different health disciplines involved in the data collection (see also **Figure 1**). For all pyramids, the level of "infectious population" is added to better assess transmission risks. While all surveillance pyramids have the same organization, not all levels occur in every pyramid. For example, the top four surveillance levels of the pyramid do not exist for vectors. An additional point is that some pyramids might be absent in the surveillance pyramid assembly (see below).

DISEASE-BASED SURVEILLANCE

To accurately describe all possible targets of surveillance for a specific VBD, tailoring the surveillance pyramid assembly is important. Some vector-borne pathogens, such as malaria, dengue, and chikungunya, can be maintained in a human-vector-human cycle, with no involvement of animals in the transmission cycle. Most vector-borne pathogens, however, circulate among vectors and (wild) reservoir animals. Here, humans frequently act as dead-end hosts, from which pathogens are not transmitted to other susceptible hosts, in nature; non-natural routes of transmission (e.g., through blood transfusion) or "rare" routes (e.g., mother-to-child transmission) are not considered here. In these cases, the infectious level is omitted from the human surveillance pyramid (**Figures 3A–C** and **4B**). The same holds for animal species that are dead-end hosts (**Figures 3A** and **4B**). In some cases, the addition of a pyramid for animals, that are neither a reservoir nor a host, but may serve as a sentinel for exposure, can be useful. Spill-over of tick-borne encephalitis virus, for example, has been revealed in dogs and deer in Belgium (11, 12). Similarly, chickens may serve as sensitive sentinels for West Nile virus (WNV) without contributing to the virus transmission cycle. Vector surveillance usually entails data on the population sizes and the infection rates.

CONTEXT-BASED SURVEILLANCE

The concept of surveillance pyramid assemblies offers a systematic approach for identifying all possible targets and levels of surveillance of any VBD. The next step is to develop a cost-effective strategy for surveillance for VBD, which largely depends on the purpose of the surveillance. Selecting the particular layers of each population to include as sensible targets for surveillance depend on the contexts of VBDs. A surveillance strategy should be based on the identification of the involved levels for a certain disease and the technical/laboratory methods (defining the feasibility) that can support the surveillance of that level (10).

The presence of a vector is a prerequisite for the transmission of a VBD. While the absence of a vector prevents transmission, the presence of a vector does not imply that actual transmission of a pathogen is occurring or will occur in the future. Based on this premise, the status of a VBD can be described in five different contexts based on the presence or absence of the three facets of VBDs important for public health: human cases, pathogens, and vectors (**Table 1**) (10). In short, VBDs with disease burden in a

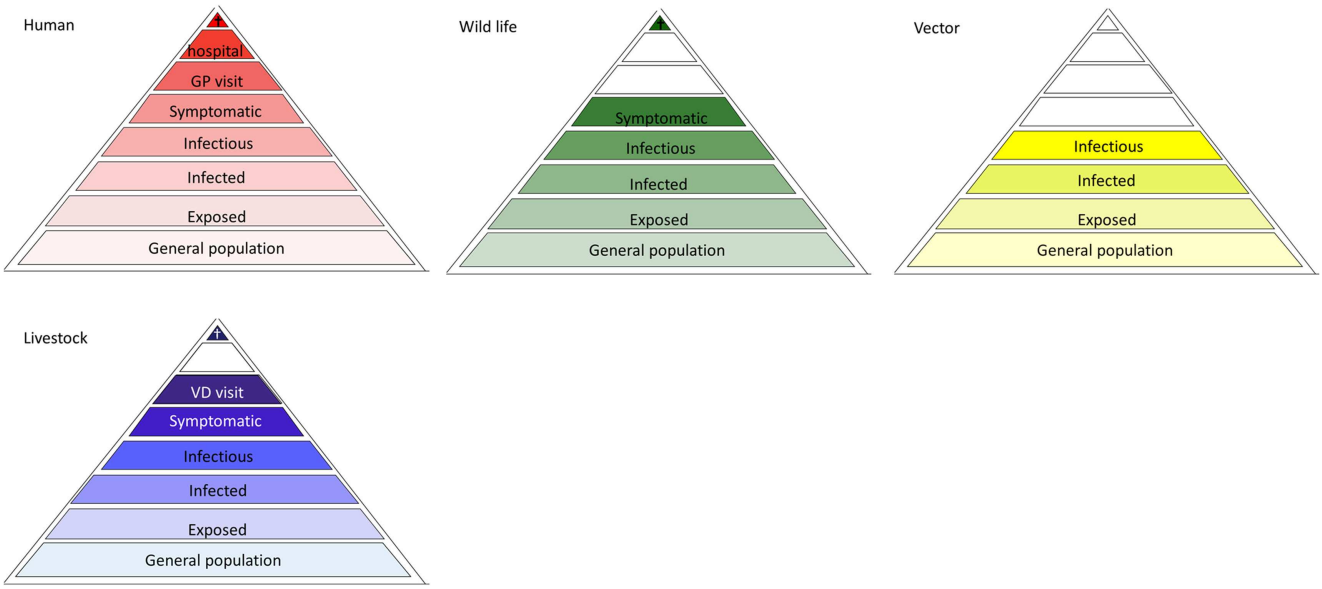


FIGURE 2 | Surveillance pyramid assembly of a hypothetical vector-borne zoonosis. Not all levels exist in surveillance pyramid of all populations. The color scheme used refers to **Figure 1**.

country fall under context 1 and the remainder fall under one of the other four contexts (Contexts 2–5).

To identify, assess the risk, communicate, and ultimately control VBDs, monitoring and surveillance tools, appropriate to the context, are needed (10). Generally, the current impact of a health problem is assessed through burden of disease calculations, while the impact of future outbreaks in an area is determined through quantitative risk assessments. Although applicable for endemic VBDs (context 1, **Table 1**), such quantitative assessments would not be sensible for the remaining VBD contexts without actual disease burden. Nevertheless, information on these so-called threats to human health is desirable (10). Consequently, surveillance efforts for VBDs, that are endemic and cause disease burden, need to have a different focus and structure than those for VBDs that are a threat. As a consequence, any surveillance strategy for a VBD needs to be developed considering its context.

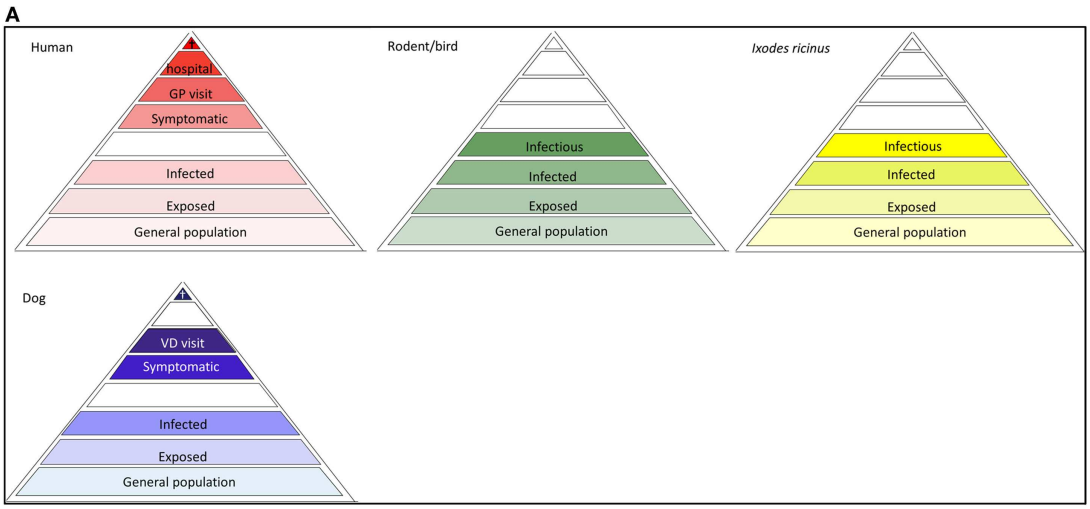
NATIONAL CONTEXT

When comparing tick-borne with mosquito-borne diseases in Netherlands (**Table 1**), for example, the most striking difference arises in context 1, i.e., endemic diseases. While the disease burden of mosquito-borne diseases is currently zero, the disease burden of tick-borne diseases, such as Lyme disease, is significant and growing (14). In the Netherlands, an increase in reported cases of tick bites and erythema migrans, the first clinical symptom of Lyme disease, has been reported over the last 15 years (14, 15).

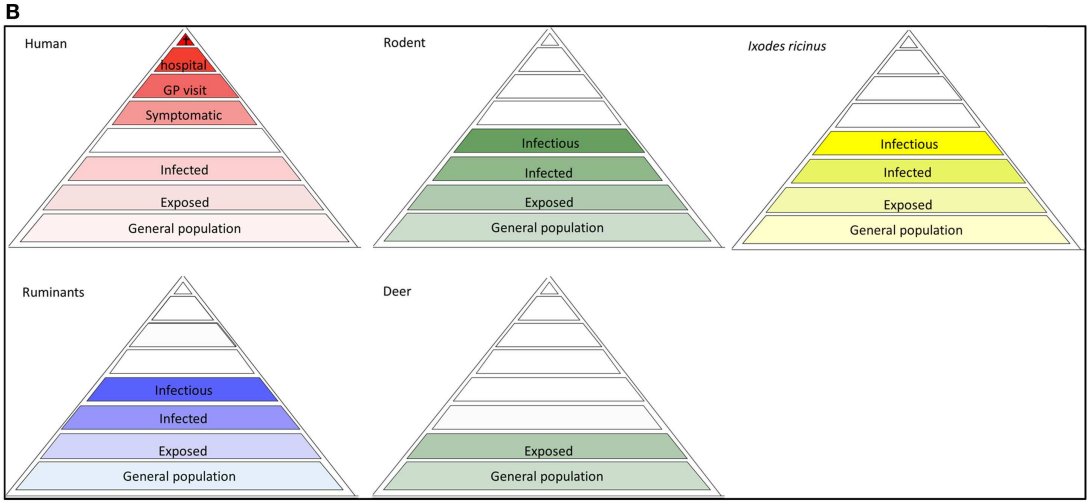
For mosquito-borne diseases in the Netherlands, risk estimates, preparedness, and early warning are the more important components of risk analyses (10). Risk estimates are very useful in providing insight into the complexity of VBD emergence, provided that their assumptions, uncertainties, and ambiguities are taken into account (16, 17). Between 30 and 40 species of mosquito are indigenous to north-west Europe, many of which act as potential vectors of pathogens. However, since the eradication of malaria in the 1960s, no mosquito-borne diseases have been reported. Still, several endemic mosquito species are proven vectors of zoonoses or malaria in the laboratory or in the field elsewhere in Europe. With this in mind, various mosquito-borne diseases are categorized as context 3. Whether actual transmission of mosquito-borne pathogens occurs after introduction depends upon the vector capacity. The consequences of pathogen introduction in a region with a competent mosquito vector population can be very quick and significant, as illustrated with WNV introduction in the United States in 1999 (18) or chikungunya virus introduction in the Caribbean in 2013 (19). Development of contingency plans (including outbreak exercises) for potential emergence and databases with background information are tools to improve preparedness. For early warning purposes, focus is placed on improving and updating detection tools for the laboratory (20) and field (21) to enable rapid pathogen detection in a vector, reservoir, or host, when introduced. To this end, knowledge of the contexts of VBDs in the rest of Europe and more globally through international collaboration and networks is essential to enhance preparedness. This also applies to non-endemic tick-borne pathogens (22).

Here, we further describe surveillance strategies when placed in context, in reverse order (Contexts 5–1). Overviews of surveillance strategies for a mosquito- and tick-borne example (when available) in the Netherlands are shown in a box.

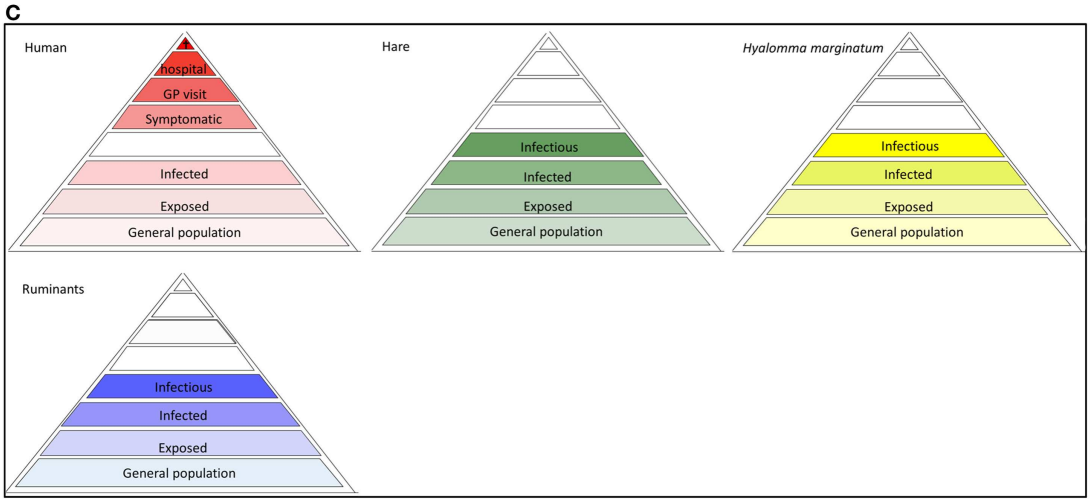
Context 5 deals with VBDs that currently do not pose any risk to the country owing to the current absence of both the pathogen and vector. The main concern centers therefore on the future establishment of the vector upon its introduction. Surveillance of pathogens and/or human cases is not a priority at this stage.



Lyme borreliosis



Tick-borne encephalitis *(NB: milk(-products) of ruminants can become infectious to humans through consumption)*



Crimean Congo hemorrhagic fever. (NB: Blood of infected ruminants can be infectious to humans)

FIGURE 3 | Disease depended surveillance pyramids of tick-borne diseases, (A) Lyme borreliosis (B) tick-borne encephalitis, (C) Crimean Congo hemorrhagic fever.

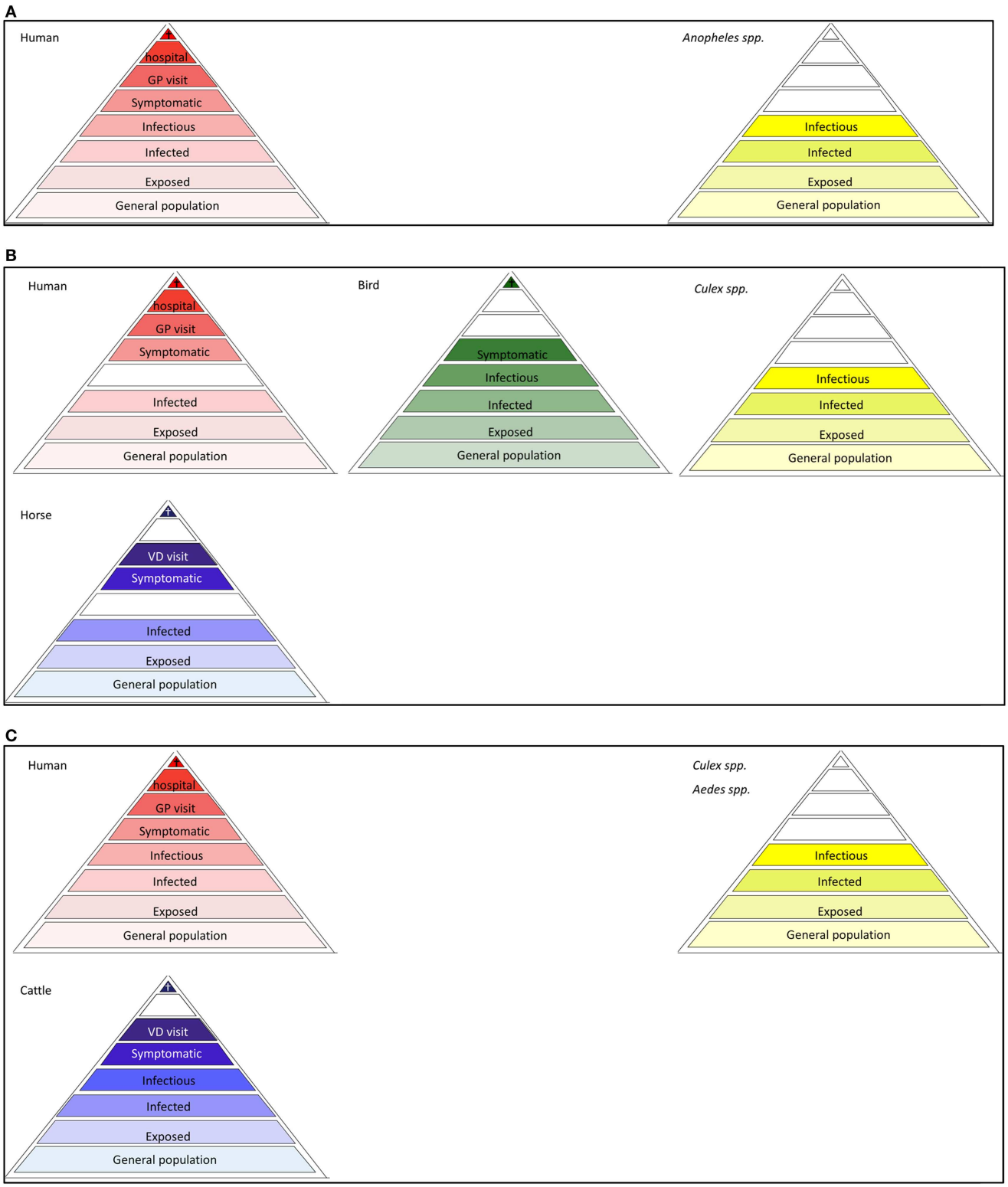


FIGURE 4 | Disease dependent surveillance pyramids of mosquito-borne diseases (A) Malaria, (B) West Nile fever, (C) Rift Valley fever. The pyramid assemblies for dengue and chikungunya resemble that of malaria, provided that *Anopheles* spp is replaced by *Ae. albopictus* and *Ae. aegypti* as vector.

Table 1 | Current status of vector-borne diseases in the Netherlands.

Context	Disease burden[1]	Pathogen[2]	Vector	Tick-borne pathogens	Mosquito-borne pathogen
1a	√ (every year)	√	√	*Borrelia burgdorferi spp.*	No examples
1b	√ (not every year)	√	√	*Borrelia miyamotoi*	No examples
2	–	√	√	*Rickettsia helvetica, Neoehrlichia mikurensis, Anaplasma phagocytophilum, Ehrlichia & Babesia, Bartonella*	*Plasmodium* spp
3	–	–	√	Tick-borne encephalitis virus	*Dirofilaria* spp., Mosquito-borne viruses (Batai, Inkoo, Rift Valley, Sindbis Snowshoe hare, Tahyna, Usutu, West Nile)
4	–	√	–	*Coxiella burnetti, Francisella tularensis*	Chikungunya virus; Dengue virus
5	–	–	–	Crimean Congo Hemorrhagic Fever virus	Japanese encephalitis virus[3]

[1] *Locally acquired human case.*

[2] *Imported human case, infected animal reservoir, or vector.*

[3] *Potentially European mosquitoes are competent to transmit JEV (13), but this has not been validated.*

If vector establishment on the basis of climatic or environmental constraints is impossible or highly improbable, no surveillance activities are recommended. However, this assessment needs to be iterative accounting for changes in the geographic distribution of the vectors in Europe.

Japanese encephalitis risk to the Netherlands (Context 5)

Japanese encephalitis is endemic to South East Asia. The principal vectors are *Culex* mosquitoes, and to a lesser degree, *Aedes*. The main vector in Asia, *Cx. tritaeniorhynchus,* is not present in Europe. Recently, an ambiguous report on the detection of fractions of this virus in *Cx. pipiens* in Italy appeared (13). Until there is conclusive evidence of Japanese encephalitis virus in Europe, this virus and its main vector are considered absent in Europe (23). Therefore, no surveillance activities are recommended.

Crimean Congo Hemorrhagic fever risk to the Netherlands (Context 5)

The main vector of Crimean Congo hemorrhagic fever virus is the hard tick *Hyalomma marginatum* and its current known European distribution is shown in **Figure 5**. Transmission only occurs in regions within the vector's geographic distribution. These ticks have been occasionally imported to Northwestern Europe on migratory birds (24, 25). Modeling suggests that the current climate of the Netherlands is unsuitable to sustain a population of these ticks, with little prospect of their establishment in the foreseeable future (26, 27), making any surveillance activity in the surveillance pyramid assembly obsolete. Nevertheless, source-finding activities in response to incidental findings of specimen to confirm absence of a local population should be considered.

Context 4 deals with VBDs where although the vector is absent, the pathogen is regularly introduced. In comparison to context 5, owing to the introduction of the pathogen, assessments whether or not the vector can establish, upon introduction, has priority. If local climatic and environmental conditions permit establishment, surveillance focusing on detecting the introduction of the vector at potential ports of entry/risk locations is required. Only the vector pyramid within the surveillance pyramid assembly needs to be considered, preferentially also in an international context.

Dengue virus risk in the Netherlands (Context 4) – excluding current risks in the Dutch Overseas Territories

Dengue virus is regularly introduced with infected humans returning from their travel to dengue endemic countries. However, there are no established populations of the principal vector, *Ae. aegypti* nor of *Aedes albopictus*, in the Netherlands, although *Ae. albopictus* has been regularly introduced, but intercepted (28). The current European geographic distributions of these mosquito species are shown in **Figure 5**. The climate is suitable for establishment of *Ae. albopictus* in Northwestern Europe, but is unsuitable for *Ae. aegypti*. Expansions of the latter in Europe is expected, but even with climate change it is not anticipated to establish in Northwestern Europe, where the temperature will remain unsuitable (29). Upon establishment of *Ae. albopictus*, the risk status will change from context 4 to 2. The current Dutch policy is therefore to prevent establishment of vectors by active surveillance and control using biocides at high-risk areas, since exotic mosquitoes are frequently imported.

Context 3 deals with VBDs that already have established putative vector populations, but so far there is no evidence of either pathogen circulation nor pathogen introductions. Surveillance is therefore focused on detecting the introduction or circulation of the disease causing pathogen as early as possible to enable adequate preventive and control measures. This may necessitate surveillance of the reservoir or sentinels (which may be humans or animals depending on the disease) and vector pyramids. In instances, where human cases of vector-borne zoonoses have been detected earlier than pathogen circulation in an enzootic cycle, human surveillance needs to be added to the surveillance strategy. In other cases, the availability or costs of samples determines the focus of surveillance. For all diseases belonging to this context,

monitoring on the geographic distribution of the diseases in Europe is advisable.

West Nile fever risk in the Netherlands (Context 3)

Since human cases (humans as well as horses are dead-end hosts **Figure 4B**) can only occur following virus amplification cycles between bird-biting mosquitoes and birds. Given that endemic mosquitoes are putative WNV vectors, potentially infected populations of both birds (the host) and mosquitoes (the vector) are targeted for surveillance in high-risk areas suitable for transmission from an environmental/climatic point of view. Upon detection of an enzootic cycle of WNV, adequate preventive measures including public awareness campaigns, veterinary horse vaccination campaigns, and mosquito control activities may be implemented. In the Netherlands, a combined mosquito and WNV surveillance in the Oostvaardersplassen was started in 2009 and continued in 2010 with a wider screening for arboviruses, namely WNV, Usutu virus, Sindbis virus, Tahyna virus, and Batai virus (20, 30) and potential vector composition (30). In 2012, dozens of wild songbirds that were found dead in the Netherlands were tested for Usutu virus, but found negative (31). Up to now, no evidence of mosquito-borne virus circulation has been found in the Netherlands. Cerebrospinal liquors of humans with encephalitis of unknown cause were tested for the presence of flavivirus (32, 33). In collaboration with the Dutch Animal Health Service, a similar survey was performed on horses. In the UK, routine mosquito surveillance detected the presence of an additional WNV mosquito vector, *Culex modestus* in north Kent. To ensure an updated risk assessment on WNV for the UK, enhanced surveillance for the mosquito, and virus testing in mosquitoes and birds was implemented. The mosquito has shown evidence of expansion, but there is so far no evidence of the virus (34).

Tick-borne encephalitis risk in the Netherlands (Context 3)

While its main vector, the sheep tick *I. ricinus,* occurs commonly, no autochthonous cases of tick-borne encephalitis (TBE) has been reported, nor any evidence of this virus circulating in an enzootic cycle in Netherlands. A closely related tick-borne flavivirus, named louping ill virus (LIV), however, is causing disease, predominantly in animals in upland areas of the UK and Ireland (35). In comparison with mosquito-borne disease outbreaks, the consequences of pathogen introduction in a region with a competent tick vector population are generally much more transient. In endemic areas, the permissive locations for transmission of tick-borne encephalitis virus are very local and patchy. In addition, the transmission of the virus between vectors occasionally occurs through co-feeding of immature stages of the tick on a non-viremic rodent host. Consequently, the detection of the pathogen soon after introduction is difficult and requires a very costly and intensive surveillance system. Recent publications suggest that animals that are neither a reservoir nor a sensitive host can act as sentinels for the circulation of tick-borne encephalitis virus, as shown with dogs and deer in Belgium (11, 12). However, the fact that a safe human vaccine against tick-borne encephalitis exists enables public health authorities to take effective preventive measures upon detection the first human case to protect the population for additional human cases, but not to prevent circulation. Such surveillance strategy aiming at an early detection of the first human case may be the most cost-effective one.

Context 2 includes VBDs with enzootic circulation of the pathogen, which so far has not resulted in reported human cases in the country and VBDs with frequent introduction of infectious reservoirs into the country, which so far has not resulted in autochthonous human cases. The presence of an established vector population for both categories is a requirement within this context.

Malaria risk in the Netherlands (Context 2)

The indigenous mosquito species, *Anopheles atroparvus*, is capable of transmitting malaria parasites *Plasmodium vivax,* but transmission has ceased in the second half of the last century through reduction of the vector population, changes in farm management, improved health, and eradicating the pathogen (36). In recent years, intense mosquito nuisance caused by *An. plumbeus*, a putative vector for *Plasmodium falciparum,* has been reported regularly in local agricultural area in the Netherlands and Belgium. Until recently, this species had been breeding only in tree holes, but has adapted to new breeding ground, namely manure gutters under abandoned pig stables in Netherlands and Belgium (37). Although human cases are imported, there is no autochthonous transmission to humans. The chance of malaria transmission is much more unlikely than arboviral transmission given the non-zoonotic nature of malaria. While dengue cases are infectious before or even without developing symptoms, patients infected with *falciparum*-malaria become infectious only after developing symptoms, which permits an opportunity to prevent subsequent transmission.

Non-*Borrelia* tick-borne disease risk in the Netherlands (Context 2)

The sheep tick *I. ricinus* is responsible for the vast majority of human tick bites. In ticks in the Netherlands, a total of six (groups of) pathogens can be distinguished. For four groups, no association with human cases has been found (yet) (**Table 2**) (38). The latter may be due to one of the following causes. First, humans do not develop symptoms when infected with these pathogens (in this case, microorganisms would be the correct term). Second, humans do develop symptoms when infected with these pathogens, but these symptoms are mild and go unnoticed. Third, humans do develop symptoms when infected with these pathogens, but these symptoms are atypical and ambiguous and diagnosed under another cause. Fourth, humans do develop symptoms when infected with these pathogens, but these infections are travel-related. *Rickettsia helvetica*, can infect humans, but whether it causes symptoms and therefore disease burden is unclear.

Context 1 deals with VBDs that result in human cases or disease burden every year (Context 1a) or infrequently (Context 1b) (**Table 1**). In the Netherlands, as mentioned earlier, there are two tick- and no mosquito-borne diseases belonging to these contexts. To halt the increase in disease incidences, management (control and intervention) strategies for one or more of the three components (disease burden, pathogen, and vector) and the environment are important.

***Borrelia miyamotoi* risk in the Netherlands (Context 1b)**

In 2012 in the Netherlands, a tick-borne disease switched from context 2 to context 1b, as a result of a case of meningoencephalitis caused by relapsing fever spirochete *Borrelia miyamotoi* (9). Since there are no diagnostics available yet, the possibilities for surveillance are limited. Development of diagnostics has been prioritized to help to investigate the form of the human disease surveillance pyramid. Possibly the disease belongs to context 1a, but until evidence is provided it will be assigned to context 1b.

Lyme disease risk in the Netherlands (Context 1a)

For the majority of Europe, including the Netherlands, Lyme disease belongs to context 1a. Because of the large and growing number of human infections, the epidemiology, ecology, and prevention of Lyme borreliosis is receiving vast public, political, and scientific interest in the Netherlands (14, 39–43). The strong increase in incidence has several biological, environmental, and societal reasons. The two main reasons for the increase are increases in the level of exposure of humans to ticks and increases in the abundance of infected ticks (44). Environmental change factors are also exacerbating the risk (45).

PAN-EUROPEAN CONTEXT

Public health authorities are required to prepare for future threats and call for predictions of the likely changes in public health risks. Usually, they focus their preparedness on their own geographical region. Whereas, the context of a disease is essential to design a surveillance strategy, assessing the context of VBDs is actually an issue for many public health authorities as the necessary information is not always readily available. In other words, in some cases surveillance data are required to assess the context, in which the subsequent surveillance strategy is prioritized. In addition, for all diseases belonging to contexts 3–5, national health experts are required to monitor the geographic distribution of the diseases, pathogens, and vectors in Europe.

To assist EU member states in this task, the European Centre for Disease Prevention and Control (ECDC) runs various programs to aggregate data to develop Pan-European maps on vector distribution and VBD incidence, to identify drivers of change and to provide guidance (10, 45–48) (**Figures 5** and **6**). Based on such data, assessing the context of a particular VBD in each European country seems to be a rather straightforward task. Central databases, however, on infections of wildlife with vector-borne pathogens are lacking or incomplete, making the assessment of the pathogens category difficult. When ignoring this category, context 2 and 4 are effectively omitted from the Pan-European maps on VBD contexts, hindering appropriate risk assessments on national and international level. Through the Eden FP6 and Edenext FP7

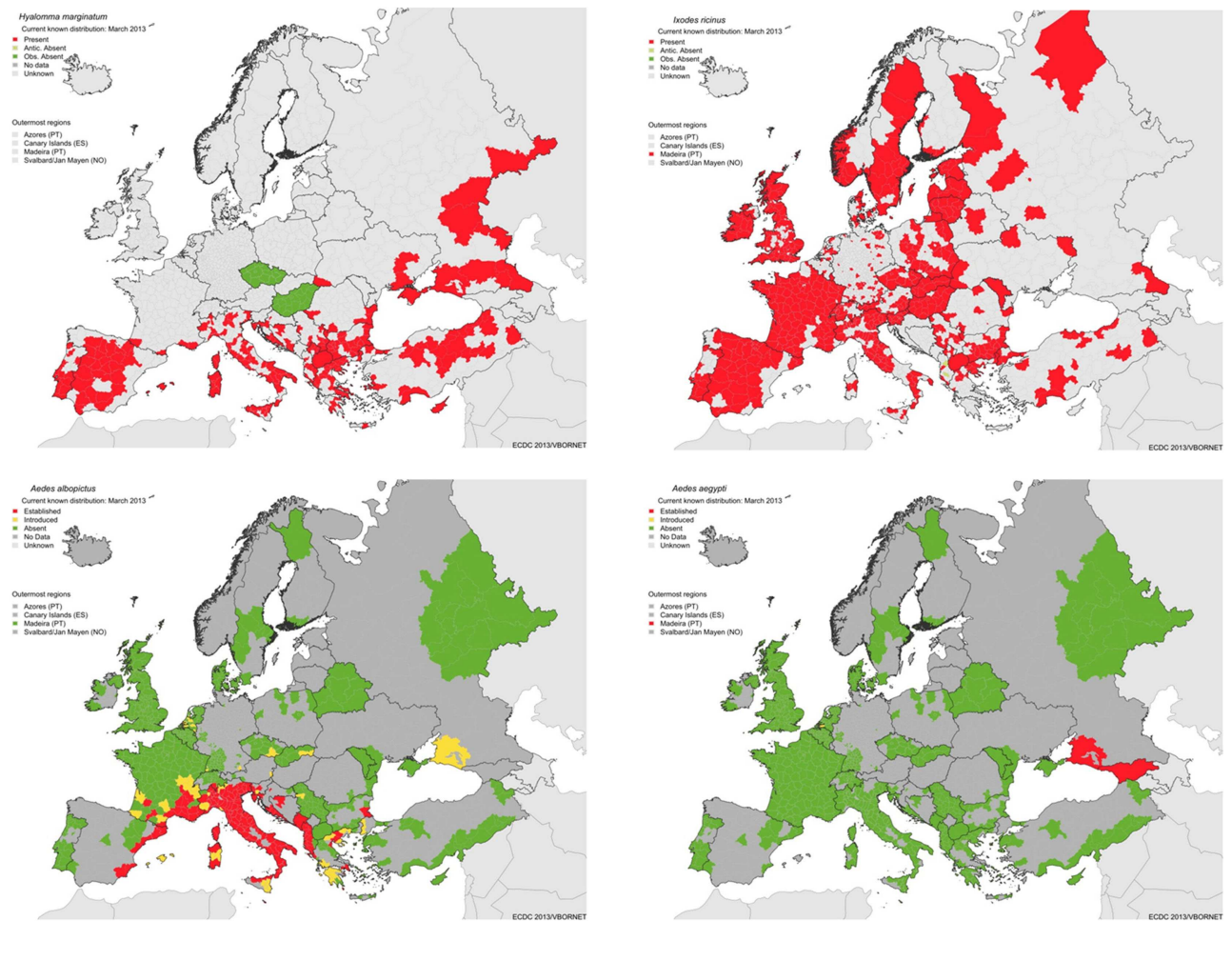


FIGURE 5 | Geographic distribution of major vectors in Europe: ticks: *Hyalomma marginatum*, vector of Crimean Congo hemorrhagic fever (top left), *Ixodes ricinus*, vector of Lyme borreliosis and tick-borne encephalitis (top right), and *Ae. albopictus* (bottom left) and *Ae. aegypti* (bottom right), vectors of dengue (source: ECDC).

programs, huge steps have been made with collecting data and identifying drivers for pathogen circulation (49). Following on from data collected on human cases and vectors, the aggregation of data on pathogen circulation in reservoir species is the final step in developing Pan-European maps of VBDs, based on their national context.

CONCLUSION

Cataloging all possible surveillance activities on VBDs without putting these into context does not assist public health authorities with assessing comparative risk. Here, we propose a way forward for public health authorities to assess potential surveillance approaches for VBD based on its context and on a country-by-country basis. Surveillance efforts for VBDs that are endemic have a different focus and structure than those for VBDs that do not pose any immediate risk as neither the pathogen nor vector is present. Within the context of a VBD, the best surveillance strategy depends on the potential prospects for action and the costs/benefit analysis. This is particularly important given economic constraints, and therefore, a focus on interventions that achieve the largest health gain per euro spent seems eminently appropriate. For some VBDs, taking action prior to a VBD becoming an issue is preferable. Once a decision for intervention to decrease the disease burden (or group/category of diseases) or to mitigate a threat has been made, surveillance should be implemented in order to measure the effectiveness of this intervention (10).

It has become apparent that an interdisciplinary approach to the prevention and control of zoonoses is invaluable. Cross-sector working also ensures better preparedness and contingency planning, more efficient and effective surveillance systems, cost-sharing between sectors according to the benefits of control, increased health equity and improved sharing of logistics and costs for service provision (3). The field of integration of animal and human disease surveillance is new, but growing (4). Since the

Table 2 | Pathogens found in Dutch *I. ricinus* ticks.

Pathogen	*I. ricinus* (n)	Tick infection rate (%)	Dutch human cases reported
Borrelia burgdorferi s.l.	628 (5308)	11.8	Yes
Rickettsia helvetica	1265 (4061)	31.1	No
Anaplasma phagocytophilum	44 (5343)	0.8	No
Babesia spp.	112 (4238)	2.0[b]	No
Neoehrlichia mikurensis[a]	300 (5343)	5.6	No
Borrelia miyamotoi[a]	6 (300)[b]	2.7	Yes

[a] *New to the Netherlands.*

[b] *tested in pools.*

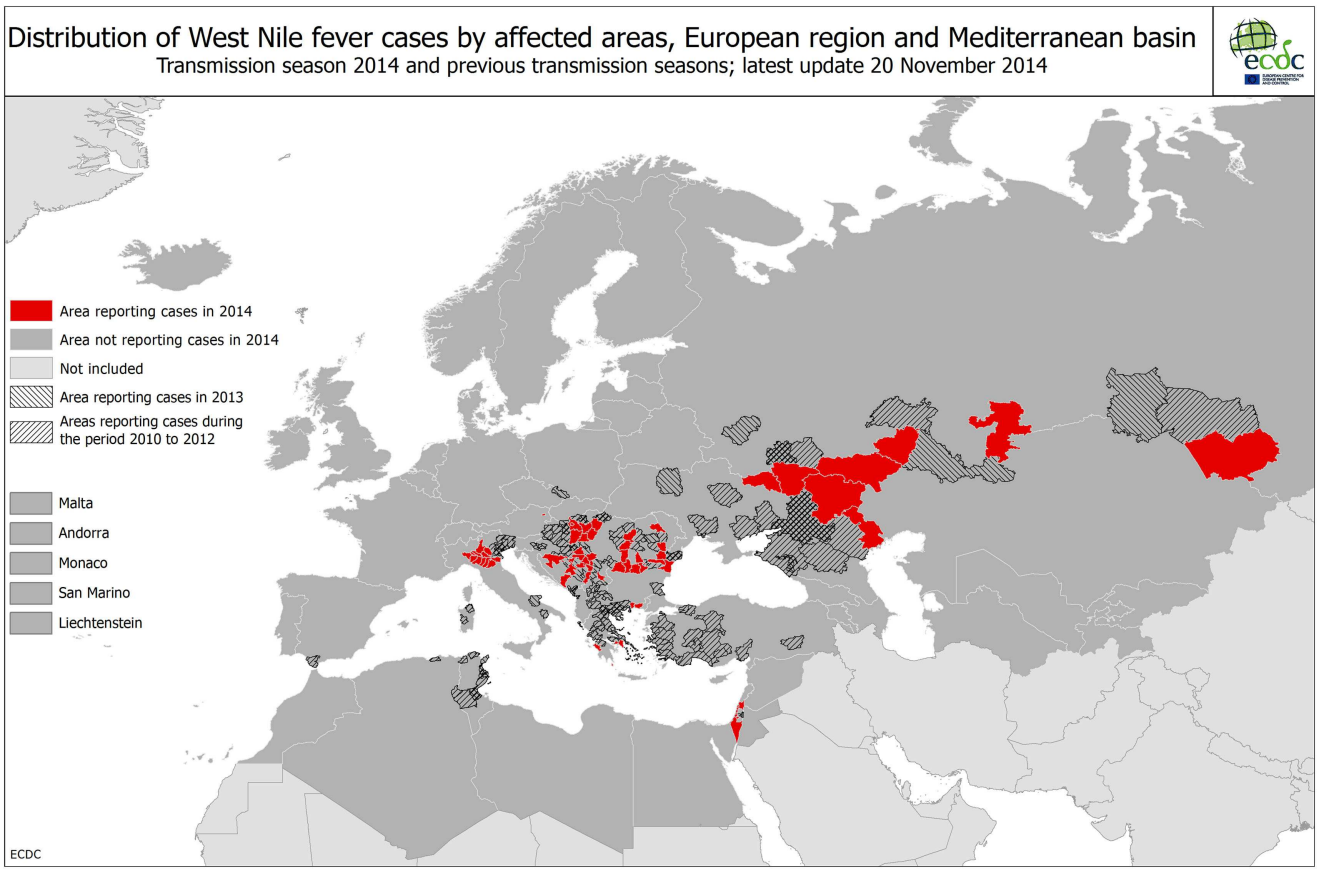


FIGURE 6 | West Nile fever map of Europe (Source: ECDC).

transmission cycles of several zoonotic pathogens occur in nature, involvement of stakeholders involved in environmental management and biodiversity-enhancing strategies is the next logical step. This requires an extension of the data collection approaches to further enhance disease intelligence (2) rather than a preset static structure. The described contextual surveillance for VBD extended with veterinary and wildlife health along with public health is highly applicable in a One Health approach.

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Emerging vector-borne zoonoses: eco-epidemiology and public health implications in India

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The diseases originating from animals or associated with man and animals are remerging and have resulted in considerable morbidity and mortality. The present review highlights the re-emergence of emerging mainly zoonotic diseases like chikungunya, scrub typhus, and extension of spatial distribution of cutaneous leishmaniasis from western Rajasthan to Himachal Pradesh, Kerala, and Haryana states; West Nile virus to Assam, and non-endemic areas of Japanese encephalitis (JE) like Maharashtra and JE to Delhi; Crimean–Congo hemorrhagic fever making inroads in Ahmedabad; and reporting fifth parasite of human malaria with possibility of zoonosis have been highlighted, which necessitates further studies for prevention and control. Emphasis has been given on understanding the ecology of reservoir hosts of pathogen, micro niche of vector species, climatic, socioeconomic risk factors, etc. Development of facilities for diagnosis of virus from insects, reservoirs, and human beings (like BSL4, which has been established in NIV, Pune), awareness about symptoms of new emerging viral and other zoonotic diseases, differential diagnosis, risk factors (climatic, ecological, and socioeconomic) and mapping of disease-specific vulnerable areas, and mathematical modeling for projecting epidemiological scenario is needed for preparedness of public health institutes. It is high time to understand the ecological link of zoonotic or anthroponotic diseases for updated risk maps and epidemiological knowledge for effective preventive and control measures. The public health stakeholders in India as well as in Southeast Asia should emphasize on understanding the eco-epidemiology of the discussed zoonotic diseases for taking preventive actions.

Keywords: zoonotic diseases, scrub typhus, cutaneous leishmaniasis, Public Health, India, chikungunya virus, *P. knowlesi*, vector-borne disease

INTRODUCTION

Most of the diseases of human beings, which are transmitted from man to man through an arthropod/insect or other invertebrate or vertebrates, are the diseases originated from animals. The socioeconomic development leading to clearing of forests, reclaiming wasteland for urbanization, etc. are resulting in ecological change leading to altered epidemiology of diseases. The changes in habitats of wild animals lead to man–vector contact, thus posing a risk to public health. In last two to three decades, many diseases, which were either forgotten or were restricted to a few foci, have re-emerged with vast spatial distribution. In recent years, there is increased awareness about resurgence of zoonotic or other re-emerging diseases in India (1). In the present review, the major vector-borne, zoonotic diseases of public health importance, which have re-emerged in India in last two to three decades, are being dealt with from the viewpoint of eco-epidemiology and public health implications. Of six major vector-borne diseases in India, i.e., malaria, dengue, chikungunya, filariasis, Japanese encephalitis (JE), and leishmaniasis, JE and cutaneous leishmaniasis (CL) are zoonotic. Malaria that was considered basically an anthroponotic disease has recently been investigated as zoonotic disease with evidence of *Plasmodium knowlesi* parasite from human cases from Southeast Asia. Scrub typhus and leptospirosis are spreading to new areas. Crimean–Congo hemorrhagic fever (CCHF) has been reported from India for the first time in 2010 while West Nile virus (WNV) is making inroads into new areas. Swine flu, which was not known till 2009, emerged in pandemic form throughout the world. The details of epidemiological aspects of malaria, CL, and scrub typhus as parasitic diseases; and JE, WNV, chikungunya, and CCHF as arboviral diseases in the context of changing ecology and transmission pattern are discussed in the present review so as to highlight the importance of eco-epidemiology, public health implications, and way forward for preparedness to combat their further spread. The general information in respect of seven vector-borne zoonotic diseases, which have re-emerged in last two to three decades are summarized in **Table 1**.

PARASITIC ZOONOTIC DISEASES

MALARIA

Malaria is a parasitic disease caused by four species of Plasmodium parasite, i.e., *P. vivax*, *P. falciparum*, *P. malariae*, and *P. ovale* and transmitted by anopheline mosquitoes. In addition, rodent malaria parasites (*P. berghei*, and *P. chabaudi*) and avian malaria parasite (*P. gallinaecium*) are also circulating in nature.

Singh et al. (8) in 2004 for the first time reported a naturally acquired focus of *P. knowlesi* in human beings in Malaysia. Further, the group (9) described the first focus of human malaria

Table 1 | Major emerging/re-emerging zoonotic diseases in India.

	Disease	First reporting in India	Geographic distribution	Pathogen	Vector species	Animals involved	Preferred ecological conditions
1	Malaria (zoonotic)	2013 [Tyagi et al. (2)]	Andaman and Nicobar Islands	*Plasmodium knowlesi*	Not known	Monkeys in Malaysia	Forest ecosystem
2	Cutaneous Leishmaniasis	1971 [WHO (3)]	Rajasthan, Himachal Pradesh, Kerala, Haryana	*Leishmania tropica*, *L. donovani* (in Himachal Pradesh)	*Phlebotomus salehi* and *P. papatasi/P. sergenti*	Rodent, dog	Zoonotic cycle in agricultural field with rodent burrows
3	Scrub typhus	1934 [From Vivekanandan et al. (4)]	Whole country is reportedly endemic	*Orientia (Rickettsia) tsutsugamushi*	*Leptotormbidium deliense*	*Rattus rattus*, mice	Moist, scrubby vegetation, coinciding with distribution of *Rattus rattus*
4	Japanese Encephalitis	1955 (www.icmr.nic.in/pinstitute/niv/JAPANESE%20ENCEPHALITIS.pdf)	Rice growing areas, Uttar Pradesh, Karnataka, West Bengal, Assam, Bihar etc. Report from Delhi in 2011.	JE virus	Culex spp. of mosquitoes, Anopheles	Pigs, egret birds	Rice fields
5	West Nile	1952 [Paramasivan et al. (5)]	Mainly southern India, recent reports from Assam, Maharashtra	WN virus	*Culex vishnui* group of mosquitoes	Birds	River fields
6	Chikungunya	1824 (http//www.searo.who.int/entity/emerging_diseases/topics/Chikungunya/en)	Mainly southern India	CHIK virus	*Aedes aegypti, A. albopictus*	Not known in India (monkeys in Africa, Malaysia)	Not fully understood
7	Crimean–Congo hemorrhagic fever	2010 [Patel et al. (6), Yadav et al. (7)]	Gujarat	CCHF virus	*Hyalomma anatolicum anatolicum*	Livestock	Rural environment in vicinity of livestock

infections with *P. knowlesi* and demonstrated from Kapit Division of Sarawak, Malaysian Borneo, that wild monkeys in the forest were infected with malaria parasite including *P. knowlesi*. The number of *P. knowlesi* genotypes per infection was much higher in monkeys than human beings, providing circumstantial evidence that *P. knowlesi* transmitted malaria is essentially a zoonotic disease. In such a scenario, deforestation and intrusion of man into forests may result into devastating outbreaks of malaria in Malaysia. Recently Tyagi et al. (2) while studying drug resistant-associated marker genes in *P. falciparum* in Andaman and Nicobar Islands (India), found co-infections with *P. knowlesi* up to the tune of 11.9% of which 5.3% were mono-infections with *P. knowlesi* only. The study emphasized that in South Asia, larger population might be at risk of *P. knowlesi* infection and such co-infections with *P. falciparum* would warrant reconsideration of malaria drug policy. However, it is assumed that in India also *P. knowlesi* infection is a zoonotic one. In Andaman and Nicobar Islands, the human dwellings are close to forest area, thus, necessitating the need for understanding role of monkeys in the epidemiology of *P. knowlesi* transmitted malaria in India for advocating preventive and control measures.

Spence et al. (10) demonstrated efficient and reproducible vector transmission of *P. c. chabaudi*, a rodent malaria parasite, through *Anopheles stephensi*. In view of changing ecological scenario, the malaria parasites of animals might be adapted to human akin to *P. knowlesi*.

LEISHMANIASIS

In India, there are two forms of leishmaniasis, visceral and cutaneous. Visceral leishmaniasis (VL), commonly known as Kala-azar, is basically an anthroponotic disease and is confined mainly to Bihar, Jharkhand, West Bengal, and Uttar Pradesh. The vector species is *Phlebotomus argentipes*. However, the occurrence of cases of VL from Himachal Pradesh (11), Uttarakhand (12), resurgence from Assam since 2004[1], and detection of *L donovani* from cases of CL from Himachal Pradesh (13) highlights the need for understanding the epidemiology of VL, which could be due to zoonotic reservoir in such erstwhile non-endemic areas of leishmaniasis.

[1] http://www.nvbdcp.gov.in

In India, the known focus of CL is western Rajasthan (3), wherein the reservoir of the infection is *Meriones hurrianae*, a desert rodent in rural areas, and the vector is *P. salehi*. On the other hand, in urban area, dogs are reservoir and *P. papatasi/P. sergenti* are the vectors. Man contracts CL accidentally while working/staying in agriculture fields. Owing to lack of knowledge about the signs and symptoms of CL, the incidence is not known resulting into neglect of the problem. The detection of a new focus of CL from Himachal Pradesh postulated the role of zoonotic reservoir, increased man to vector contact due to deforestation and construction activities in the area (14). Recording of stray cases of CL from Kerala, Assam, and the state of Haryana in India highlights the need to study the role of zoonotic reservoir in VL and CL transmission in Himachal Pradesh and epidemiological investigation of other foci from the viewpoint of ecological conditions for possible reservoir and sand fly vectors.

SCRUB TYPHUS

Scrub typhus is caused by a rickketsia, *Orientia tsutsugamushi* and transmitted by a trombiculid mite, *Leptotrombidium deliense*. As the name suggests, the disease is confined to scrubby/jungle areas and typhus word means typos (greek word), i.e., fever. The word tsutsugamushi is a Japanese word; tsutsuga means dangerous and mushi means insect/mite. The geographic distribution is confined to Southeast Asia including Japan and Korea. In India, the disease was considered to be prevalent among army troops and was reported long back in 1934 (4). The disease remains mostly undiagnosed due to lack of awareness among affected persons and non-availability of diagnostic facilities. The major symptoms of scrub typhus are non-specific ranging from high-grade fever of 1–2 weeks, a typical eschar/papule at the site of bite by mite, headache, myalgia, dry cough, lymphadenopathy, hepatosplenomegaly, apathy, breathlessness, and myocarditis. Mortality may reach up to 30% if not treated. The diagnosis is made by Weil–Felix test, Indirect Immunofluorescence Test, Quantitative ELISA, and polymerase chain reaction (15). Scrub typhus is a zoonotic disease and infection of rickettsia is maintained in small rodents in recently cleared forest/scrub, grassy areas. The nymph and adult mite do not feed on man and the transmission to man occurs through larval stage of mite accidentally. Transovarian transmission through larvae has been reported. When the ecological conditions in forest are disturbed due to deforestation, and human beings intrude into cleared forest, the larval mite attack man.

Tsai and Yeh (16) studied the association of climatic/environmental factors with distribution of scrub typhus in Taiwan. They found that some areas exhibit no climatic effect, whereas in another area, the incidence correlates positively with higher temperatures during the warm season, and the third area correlates positively with higher surface temperatures and longer hours of sunshine. The results also showed that scrub typhus is associated with farm worker population density, timber management, recreational forest, natural reserve, or other purpose. Though whole India has been reported to be endemic for scrub typhus (17), however, the reporting of scrub typhus has seen resurgence since 2004 from different parts of India, e.g., Himachal Pradesh (18–22), Darjeeling (23), Jammu and Kashmir (24, 25), Rajasthan (26), Andhra Pradesh (27, 28), Uttarakhand (29), Pondicherry (30), Goa (31), Delhi (32), Kerala (33, 34), and West Bengal (35) (**Figure 1**).

Figure 1 reveals that the areas reporting scrub typhus are forested or scrubby with moderate to high humidity. Based on the retrospective data, the preferred habitats of rodent reservoirs and mites may be mapped for effective preparedness to prevent the disease.

ARBOVIRAL DISEASES

JAPANESE ENCEPHALITIS

Japanese encephalitis is a viral disease transmitted mainly by *Culex triteaniorhynchus*, the *Culex vishnui* group of mosquitoes. The epidemiology involves pig as amplifier host while man is a dead end host[2]. Cattle egrets are also known to act as reservoir of infection. As the vector species prefers to breed in rice fields, the distribution of JE coincides with rice growing areas of eastern Uttar Pradesh, Andhra Pradesh, Tamil Nadu, Brahmaputra Valley in Assam, Karnataka, and West Bengal, etc. In India, the endemicity of JE was confined to seven states in 2008, while in 2011, it spread to 15 states and in 2013, 13 states reported JE cases with 1086 cases[1] indicating geographical spread. Though the typical biotope of JE endemic area is well defined, the occurrence of acute encephalitis syndrome is complicating the problem owing to ill-defined etiology and risk factors. In 2011, recording of nine cases of JE from Delhi[1] warrants detailed investigation of ecological features supporting biotope for JE transmission.

WEST NILE VIRUS

India is endemic for WNV with reported infection in human beings mainly from southern India. In nature, the natural cycle is maintained in bird and mosquito. Antibodies against WNV were first detected from Bombay in 1952 (5). Pigs and horse have also been found with antibodies against WNV. The symptoms are mild influenza, fever, general body ache, headache, nausea, and vomiting including encephalitis syndrome. The disease is transmitted mainly by *Culex vishnui* group of mosquitoes, and transovarial transmission has been reported. Till 2002, WNV was reported mainly from southern India, but in 2002, Thakare et al. (36) reported prevalence of WNV from non-endemic (Maharashtra and Rajasthan) and endemic area of JE (Goa and Orissa). Khan et al. (37) reported the WNV from Dibrugarh, Golaghat, Sivasagar, and Tinsukia districts of Assam. WNV may be a cause of acute encephalitis syndrome in JE endemic areas. Further studies are needed to delineate the WNV endemic and potential areas for better prevention, particularly in JE non-endemic areas.

CHIKUNGUNYA

Chikungunya fever is caused by CHIK virus and transmitted by *Aedes aegypti* mosquito. The disease resembling the symptoms of chikungunya was reported from India in 1824[3] but the outbreaks due to chikungunya were witnessed in 1963. Transmission cycle of CHIKV can be man–mosquito–man (urban cycle) or animal–mosquito–man (sylvatic cycle). In Africa, the transmission of chikungunya is maintained in a sylvatic cycle, i.e., monkeys,

[2]http://www.icmr.nic.in/pinstitute/niv/JAPANESE%20ENCEPHALITIS.pdf
[3]http://www.searo.who.int/entity/emerging_diseases/topics/Chikungunya/en

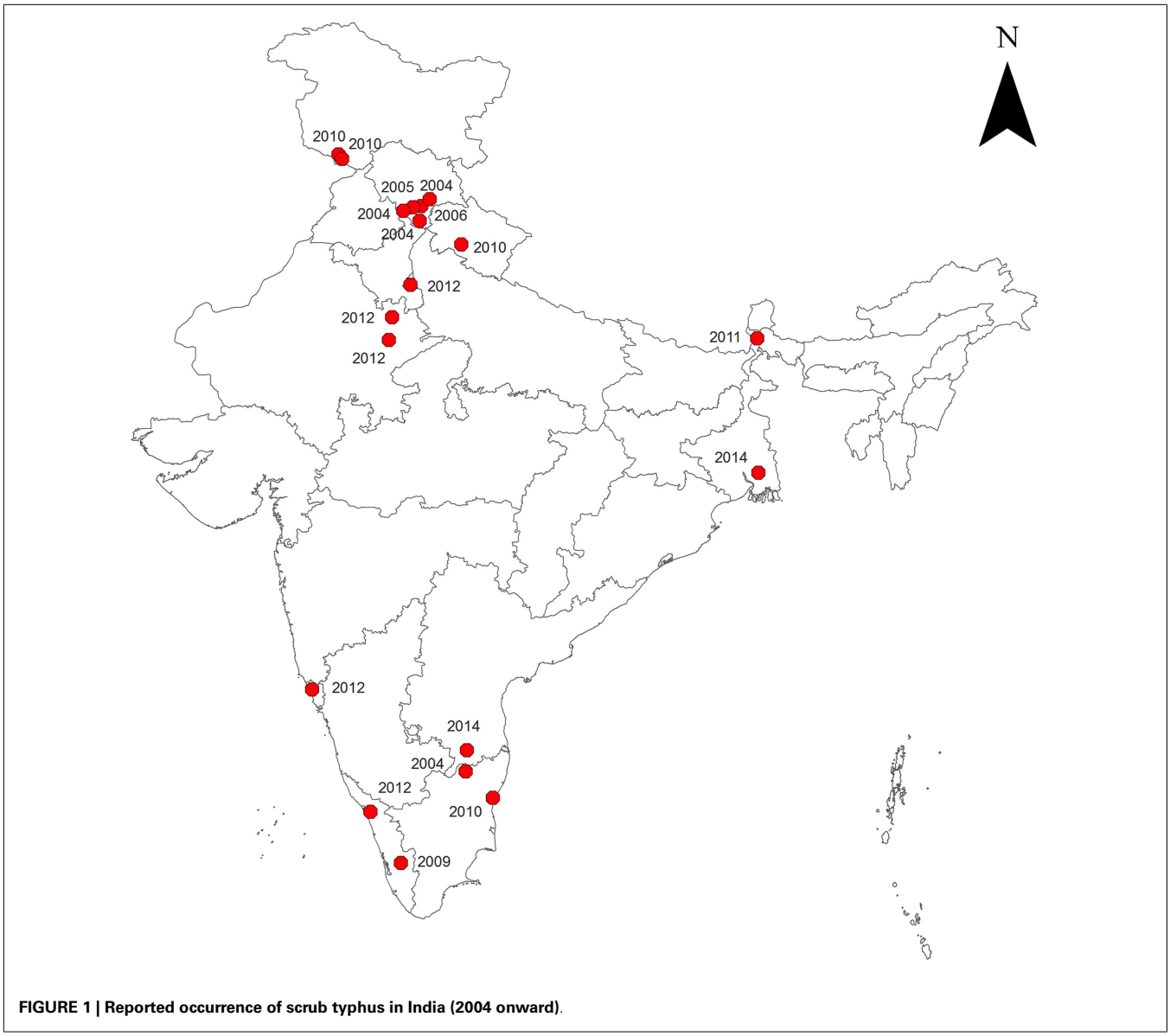

FIGURE 1 | Reported occurrence of scrub typhus in India (2004 onward).

mosquito, and man, while in India man-to-man transmission through *A. aegypti* mosquito is known. *A. albopictus* has also been reported as vector of chikungunya in Africa (38). In Malaysia (39), neutralizing antibodies were reported in wild monkeys in 1960s, providing a clue that CHIK virus may be maintained in nature through animals.

The outbreaks of chikungunya in India were recorded in 1960s and 1970s (40). Banerjee (41) in 1965 revealed that antibodies to CHIK virus were detected in the sera collected in 1956 from Madras state. Resurgence of chikungunya in India was witnessed in 2005 when severe outbreaks were reported from whole southern India and the spatial distribution gradually extended to northern India. Before the outbreaks of 2005, virus activity was detected in 2001 (42) by isolating CHIK virus from *A. aegypti* from Yawat town of Pune district. Since the outbreak in 2005, chikungunya cases have been reported from 13 to 18 states of India with 18,639 clinically suspected cases in 2013[1]. Most of the publications have reported the epidemiological profile of cases, but did not elucidate the reasons of outbreaks from the ecological, climatic, and socioeconomic development points of view. In view of reported role of *A. albopictus* mosquito in transmission, which prefers peri urban areas, there are possibilities of sylvatic cycle of chikungunya in India warranting in depth investigations.

CRIMEAN–CONGO HEMORRHAGIC FEVER

Crimean–Congo hemorrhagic fever is a viral disease transmitted by ticks of *Hyalomma anatolicum anatolicum* species or through direct contact with reservoir animals or human beings. Persons residing in rural area are at the risk of contracting CCHF as they live in vicinity of livestock and other wild animals like hare and hedgehog, which are reservoirs of the infection. Nosocomial outbreaks among healthcare staff have also been reported. The disease was first of all reported from Crimea, Russia in 1944 and is distributed in Africa, Asia, Europe, and Middle East, and the

symptoms are similar to dengue hemorrhagic fever with mortality rate up to 80% during outbreaks (43). The first case of CCHF was reported from Ahmedabad, India in 2010 (6), and in 2014, an outbreak was reported in a cluster in a village in Amreli and Patan district of Gujarat (7). IgG antibody positivity in the animals was up to 43.9% and Hyalomma ticks were also found positive by RT-PCR. There is need to create awareness among public health stakeholders for symptoms, diagnosis, and preventive measures for timely diagnosis and management of CCHF cases and for containing spread.

CONCLUSION

Zoonoses are a major challenge to public health in view of rapid deforestation, urbanization, population movement, changing climatic scenarios, etc. Various developed and developing countries have faced unknown as well as re-emerged diseases like chikungunya, scrub typhus, Swine flu, dengue, CCHF and CL. Studies undertaken so far have been of reactive type, offering little solution for preventive aspects in a long-term perspective. In view of life threatening nature of most of the diseases, there is need for understanding having eco-epidemiological approach in respect of each disease with emphasis on habitats of reservoirs of infection, micro niche of arthropod/insect vectors, and vulnerable areas from the climatic determinants point of view for development of pathogen and vectors. As there is no routine surveillance for most of the zoonotic diseases discussed here, emphasis should also be given to periodic serological surveys for detection of evidence of arbovirus infections in vulnerable areas in a systematic way. Reporting of epidemiological data in public domain should also be augmented for neglected diseases like scrub typhus, WNV, CCHF, and CL. With the advent of tools like satellite remote sensing, geographic information system, and mathematical modeling in better understanding of diseases epidemiology, it is possible to detect and identify ecological niche at finer resolutions and map and project the current as well as potential risk of the diseases, which have ecology-driven epidemiology. The risk factors also need to be ascertained for prevention from contracting the diseases. Risk maps of zoonotic diseases discussed here, creation of facilities for laboratory diagnosis of pathogens, awareness in the communities about symptoms of diseases, and health education for source reduction of vectors' breeding wherever possible, should be thrust areas for preventing vector-borne zoonotic diseases. This review should sensitize public health stakeholders to emphasize on understanding the eco-epidemiology of the discussed zoonotic diseases for taking preventive actions in India, as well as in Southeast Asia.

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Pathogenic landscape of transboundary zoonotic diseases in the Mexico–US border along the Rio Grande

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Transboundary zoonotic diseases, several of which are vector borne, can maintain a dynamic focus and have pathogens circulating in geographic regions encircling multiple geopolitical boundaries. Global change is intensifying transboundary problems, including the spatial variation of the risk and incidence of zoonotic diseases. The complexity of these challenges can be greater in areas where rivers delineate international boundaries and encompass transitions between ecozones. The Rio Grande serves as a natural border between the US State of Texas and the Mexican States of Chihuahua, Coahuila, Nuevo León, and Tamaulipas. Not only do millions of people live in this transboundary region, but also a substantial amount of goods and people pass through it everyday. Moreover, it occurs over a region that functions as a corridor for animal migrations, and thus links the Neotropic and Nearctic biogeographic zones, with the latter being a known foci of zoonotic diseases. However, the pathogenic landscape of important zoonotic diseases in the south Texas–Mexico transboundary region remains to be fully understood. An international perspective on the interplay between disease systems, ecosystem processes, land use, and human behaviors is applied here to analyze landscape and spatial features of Venezuelan equine encephalitis, Hantavirus disease, Lyme Borreliosis, Leptospirosis, Bartonellosis, Chagas disease, human Babesiosis, and Leishmaniasis. Surveillance systems following the One Health approach with a regional perspective will help identifying opportunities to mitigate the health burden of those diseases on human and animal populations. It is proposed that the Mexico–US border along the Rio Grande region be viewed as a continuum landscape where zoonotic pathogens circulate regardless of national borders.

Keywords: Lyme borreliosis, VEE, Hantavirus, *Babesia*, Chagas, *Leishmania*, pathogenic landscapes, global change

INTRODUCTION

The United States (US) and Mexico share a border spanning 3,100 km from the Gulf of Mexico to the Pacific Ocean. Approximately 14 million people reside within the area found roughly 100 km on either side of the international line between the two countries, with 7.3 million residing in the US and 6.8 million in Mexico (1). A bi-national effort is in place to protect the environment and public health in the US–Mexico border region that is consistent with sustainable development. The sector encompassing the border States of Texas, Tamaulipas, Nuevo León, and Coahuila includes at least 29 municipalities on the Mexican side and 168 cities and towns on the US side, covers portions of the Southern Texas Plains and Western Gulf Coastal Plain ecoregions of Texas, and lies within a zoonotic disease hotspot (1–3). It appears that conditions leading to the emergence of zoonotic diseases as a public health threat in the US and other parts of the world may be at play in the transboundary region covering south Texas and Northeast Mexico (4–7). Among these factors, we have poverty. For instance, in southern US, 16.5% of the population is in poverty, and 22% of children under 18 years old live in such conditions in the same region [National Center for children in poverty[1] (8)]. Texas has a poverty level (17.6%) higher than the national average (15%) calculated as a 3-year average. In addition, recent

[1] http://www.nccp.org

studies showed that migrants displaced due to adverse weather conditions related to climate change, are predicted to increase during the twenty-first century (9, 10). Economically, deficient areas will be highly impacted with these types of extreme weather events, making this population more vulnerable to emerging infectious diseases due to an increase in out-migration flow (9).

These scenarios acquire special relevance in the US–Mexico border, one the largest and longest-sustained routes of human migration. From the human migratory standpoint, the States of Chiapas and Tabasco in Mexico play a vital role as transit points for large numbers of people. In this regard, the ports of entry are the Ocosingo and Tapachula surroundings in Chiapas, and Tenosique in Tabasco. Veracruz is therefore the transition point for the nearly 400,000 individuals, representing approximately 50 nationalities, who traverse Mexico each year with the ultimate goal of reaching the US (Migration Department, Mexican Interior Ministry 2006). All these individuals are forced to cross areas that might be "hot spots" of Babesiosis, Venezuelan equine encephalitis virus (VEEV), and other pathogens that have incubation periods fluctuating between 3 and 10 days (3). Consequently, the "One Health" approach is required to enhance the ability to recognize zoonotic pathogens in humans, domestic, and wildlife reservoirs and the associated vectors in the US and Mexico transboundary region. This concept states simply that clinicians, researchers, agencies, and governments must work together seamlessly for the benefit of animal and human health as well as for the welfare of the global environment.

An international perspective on the interplay between disease systems, ecosystem processes, land use, and human behaviors is applied in this review paper to analyze landscape and spatial features of Venezuelan equine encephalitis (VEE), Hantavirus disease, Lyme disease (LD), Leptospirosis, Bartonellosis, Chagas disease, Babesiosis, and Leishmaniasis all of which can be considered, or have the potential to be emerging zoonotic infectious diseases of relevance in this transboundary region (**Table 1**) (11–15).

PATHOGENIC LANDSCAPE

GLOBAL CHANGE AND EMERGENCE OF VECTOR-BORNE ZOONOTIC DISEASES

As the world globalizes in terms of Nation's economies and increased travel, borders are opened for a constant flow of goods, products, and pathogen dissemination. Likewise, as human populations expand into new geographical regions, the possibility that humans will come into close contact with infectious agents' potential hosts, which can transmit pathogens to human beings, increases. Such factors, combined with increased human density and mobility, stand as a serious human health threat. Additionally, climate change is increasingly becoming a concern in the emergence of zoonotic infectious diseases (16). For the past 70 years, most of the newly emergent diseases have been identified as zoonoses (60.3% of EIDs), and the majority (71.8%) has originated in wildlife (17). Thus, according to the transboundary zoonotic disease concept, understanding how these pathogens emerge in different geographical regions will directly benefit global trade and public health. Here, we review several diseases that could impact a geographically strategic region in the US and Mexico border area. Insights gained understanding the pathogenic landscape of these zoonotic diseases could help enhance predictive tools, which might be applied to study the epidemiology of other transboundary pathogens. Pathogenic landscape is a term used to describe attributes of an ecosystem that influence spatial variations in disease risk or incidence (18).

Emission of greenhouse effect gasses has impacted global climate (19), increasing Earth's surface temperature 0.74°C on average (20). According to the 2002 World Health Organization (WHO) report, climate change has caused approximately five million disability-adjusted life years (DALYs) in the world (21). Moreover, the 2007 Intergovernmental Panel on Climate Change report (22) and the 2014 National Climate Assessment by the US Global Research Program (16), suggest that climate change will affect North America at multiple levels, such as public health, agriculture, water supply, and frequency of extreme weather events, among others (15). Increased temperatures, sea levels, precipitations, and droughts due to climate change can drastically change the epidemiology of vector-borne diseases (15, 21, 23, 24), as both vectors and pathogens are very sensitive to these climatic variables.

Climate change can potentially alter the spatial range of vector-borne diseases through shifts in geographical distributions of their vectors (14, 25, 26). Despite some positive developments, such as better access to clean drinking water, lower exposure to insect vectors, and higher-quality housing, projected changes in climate over the next decades may exacerbate infectious disease incidence even in developed regions such as North America (15). Habitat changes, alterations in water storage and irrigation habits, pollution, development of insecticide and drug resistance, globalization, tourism, and travel are additional factors that may aggravate this threat (26). For instance, in Europe, short winters appear to have influenced populations of *Dermacentor reticulatus*, the tick vector responsible for transmission of *Babesia canis*, to expand to the East (20). In Veracruz, Mexico, a study found an association between an increase in dengue cases and increased temperature and rainfall that followed El Niño Southern Oscillation (ENSO) events (27). Increased rainfall could create both microclimates, in which vectors can thrive, as well as cause high temperatures, which could allow for a rapid increase in vector densities and ultimately put humans at risk for vector-borne disease. Extreme-flooding events can cause outbursts of zoonotic diseases caused by infectious agents transmitted by rodents, as their pathogen-containing urine contaminates the water. This was the case in Nicaragua, in which a Leptospirosis epidemic followed a flooding event (28). Thus, humans might face increasing exposure to zoonotic diseases as naturally occurring phenomena like ENSO and flooding events are expected to become frequent due to climate change (23). In addition, climate change can also affect the epidemiology of zoonoses indirectly. For instance, the density of vegetation in a particular area increases during heavy rainfall seasons. This vegetation indirectly supports the reproduction of rodents, which can be infected with pathogens transmissible to humans (29).

Human migration and economic trade can exacerbate climate change influences on vector-borne diseases along the Texas–Mexico border. Ecological niche models, under climate change scenarios, showed an increased distribution of Leishmaniasis vectors and reservoirs in Texas and North Mexico (14). A recent study in the Texas–Mexico border identified the present and future

Table 1 | Transboundary zoonotic diseases, distribution, agents, vectors, and transboundary relevance in the US–México border region.

Disease	Distribution[a]	Etiologic agent	Vector	Transboundary relevance
VIRUS				
VEE	Meso-America, Southern Texas, and Northern Mexico	Venezuelan equine encephalitis virus	*Culex (Melanoconion) taeniopus* *Deinocerites pseudes* *Aedes (Ochlerotatus) taeniorhynchus* Mammalophilic mosquitoes	Shared vectors and reservoirs Human migration Livestock movement
HPS and HFRS	American continent, Europe, Asia, Africa likely worldwide	Hantavirus	Wild rodents of the Cricetidae and Soricidae families serve as reservoirs	Shared reservoir species across border Human migration Different public health preparedness Poverty (suboptimal housing)
BACTERIA				
Lyme disease	US, Mexico, Canada	*Borrelia burgdorferi*	Ticks *Ixodes scapularis* *I. pacificus*	Shared vectors and reservoirs Different public health policies
Leptospirosis	Worldwide	*Leptospira interrogans*	Wild rodents serve as reservoirs	Shared reservoir species across borders Human migration Livestock movement Different public health policies Poverty (suboptimal housing, sanitation, and hygiene)
Rocky mountain spotted fever/Brazil spotted fever	US, Mexico, Canada, Costa Rica, Panama, Colombia, Uruguay, Argentina, Brasil	*Rickettsia rickettssi*	Ticks *Dermacentor variabilis* *D. andersoni* *Rhipicephalus sanguineus* *Amblyomma cajennense* *Haemaphysalis leporispalustris*	Shared vectors and reservoirs
Human monocytic ehrlichiosis	US, Mexico	*Ehrlichia chaffensis* *E. ewingii*[e]	Ticks *A. americanum* *D. variabilis* *I. pacificus*	Shared vectors and reservoirs
Human granulocytic anaplasmosis	US	*Anaplasma phagocytophilum*	Ticks *I. scapularis* *I. pacificus*	Shared vectors and reservoirs
Bartonellosis[b]	Americas, Europe, Asia	*Bartonella henselae* *Ba. quintana* *Ba. bacilliformi* *Ba. vinsonii* subsp. *berkhoffii*	Ticks *I. pacificus* *I. scapularis* *I. ricinus* *I. persulcatus* *D. reticulatus* *D. marginatus* *R. sanguineus* *R. microplus* Sand flies *Lutzomyia verrucarum* *L. columbiana* *L. peruensis* Lice[c] *Pediculus humanus humanus* *P. capitis* Fleas[c] *Pulex irritans*	Shared vectors and reservoirs Human migration Livestock movement Poverty (poor sanitation, hygiene and crowded housing environments)

(Continued)

Table 1 | Continued

Disease	Distribution[a]	Etiologic agent	Vector	Transboundary relevance
PROTOZOA				
Human Babesiosis	US	*Babesia microti*	Ticks *I. scapularis* *I. pacificus* *I. texanus* *D. variabilis*	Shared vectors and reservoirs
Chagas[d]	American Continent	*Trypanosoma cruzi*	*Triatoma sanguisuga* *T. gerstaeckeri* *T. lenticularia*	Shared vectors and reservoirs Human migration Different public health policies Poverty (suboptimal housing)
Leishmaniasis	Americas	*Leishmania (Leishmania)* *Leishmania (Viannia)*	*Lutzomyia* sand flies	Shared vectors and reservoirs Human migration Different public health policies

[a] *Adapted from Ref. (173).*

[b] *With relevance to this review paper.*

[c] *Human body and head lice, and human fleas. Other species have been associated with wildlife and domestic animals (173).*

[d] *As per Ref. (298, 304).*

[e] *In the US only.*

potential distribution, under climate change scenarios, of the LD vector, *Ixodes scapularis* (13). The results of this study indicated that South Texas includes suitable habitat for *I. scapularis*. In a similar study, the potential future distribution of main Chagas disease vectors, *Triatoma gerstaekeri* and *T. sanguisuga*, is expected to increase in the Texas–Mexico border due to climate change (12). Temperature and precipitation played a major role in the models presented in these three studies.

The transboundary region between Mexico and the US is vulnerable to outbreaks of vector-borne diseases because some Southern States, such as Texas, share a legacy of neglected tropical diseases (NTD) (30) with Mexico. This situation highlights the urgency to develop and deploy active surveillance programs, which are necessary for optimal management and control of vector-borne diseases.

EFFECT OF EXOTIC WEEDS ON VECTOR POPULATIONS

Invasive weeds can change the ecology of, and induce a pathogenic landscape in which arthropod-borne disease transmission can increase. Mack and Smith (31) link invasive plants as catalysts for the spread of human parasites by documenting the escape and mast seed production of Asian frost-tolerant bamboos from cultivation in the Pacific Northwest to potential outbreaks of the omnivorous deer mouse *Peromyscus maniculatus* that carries Hantavirus. Invasive weeds also interact with ticks. Japanese barberry has been shown to increase the abundance of the black-legged tick, *I. scapularis* and the infection prevalence of *Borrelia burgdorferi* (LD) (32, 33). In India, Kyasanur forest disease of cattle and monkeys is attributed to disturbance of the native forest for tea plantations, which resulted in invasions of the invasive weed *Lantana camara* and outbreaks of *Haemaphysalis spiniger*, the vector for Kyasanur encephalitis-inducing virus within the *flavivirus* group (34). The clearing of the native forest in Argentina and the transition to exotic African grasses increased the impact of cattle fever ticks (CFT) by increasing the encounter rate with cattle (35). This same pathogenic landscape phenomenon appears to be happening in the permanent quarantine zone (PQZ) in the Texas–Mexico border region, with the invasion of the exotic and invasive weed species, *Arundo donax* known as the giant reed (**Figure 1**). Aerial remote sensing pictures of the Rio Grande taken in 2002 indicate that 62% or 5981 ha of riparian habitat on Rio Grande from Big Bend to Falcon Dam was infested with giant reed, which includes most of the PQZ (36). Zoonotic agents are not transmitted by CFT, but knowledge from the study of CFT ecology can be applied to understand how ecosystem shifts can influence spatial variation in disease risk or incidence for tick-borne disease systems of public health importance.

Giant reed impacts the USDA-APHIS Cattle Fever Tick Eradication Program (CFTEP) along the transboundary region of Texas–Mexico border. Giant reed indirectly affects CFT because survival is highest in giant reed as compared to native riparian vegetation or buffel grass pastures (37). Abiotic conditions within giant reed stands are cooler due to the heavy shade and high rates of evapotranspiration (ET), which appears to be cause of lower levels of CFT mortality. Biotic conditions in giant reed stands are also altered because the abundance of CFT arthropod predator species is reduced (37). In a review of the literature records of predation on ticks, Samish & Alekseev (38) documented that ground dwelling predators (e.g., ants, beetles, and spiders) are the major natural enemies of ticks. Preliminary pitfall trap surveys in the PQZ indicate that ground dwelling beetle populations, specifically the predaceous Carabidae and omnivorous Tenebrionidae species, are strongly reduced in giant reed compared to adjacent native plant communities (Goolsby, personal communication). Ants are also known to be important predators of ticks in Texas. Fleetwood et al. (39) documented reduced populations of Lone Star ticks,

FIGURE 1 | Invasion of exotic *Arundo donax*, giant reed, is facilitating the invasion of Cattle Fever Ticks in the transboundary region by creating a microclimate that is favorable for its survival. **(A)** *Arundo donax* on Rio Grande near Eagle Pass, TX, USA. **(B)** *Arundo donax* on Rio Grande near Del Rio, TX, USA.

Amblyomma americanum, in pastures with abundant red imported fire ants, *Solenopsis invicta*. Fire ant predation is generally believed to reduce the incidence of tick-vectored pathogens of livestock. In Louisiana, fire ant predation of *Ixodes* ticks was associated with a reduced incidence of Anaplasmosis in cattle (40). Preliminary studies in the PQZ found that ant diversity and abundance is low in giant reed stands, with the red imported fire ant, *Solenopsis invicta*, being the most common species. Comparative studies are needed to survey ant diversity throughout the PQZ to investigate their potential impact on CFT. Control of exotic giant reed and restoration of the native riparian vegetation could reduce the favorability of this habitat for CFT and lead to restoration of a more intact leaf litter insect predator community and in total a more robust biological barrier to invading CFT and other tick-borne zoonoses. Giant reed may also be creating a localized climatic refuge for CFT when conditions in the upland habitat are not favorable for survival. As giant reed declines, lower ET rates, increased ground temperatures, and lower humidity levels are expected in these riparian habitats and these conditions are known to reduce the survival of larval and adult CFT (41–45). Giant reed also indirectly impacts the CFTEP by reducing visibility in the PQZ along the Rio Grande. Heavy infestations of giant reed make it extremely difficult for mounted inspectors to detect and capture stray livestock.

Exotic weeds interact with disease vectors in the transboundary region between Texas and Mexico. These weeds create a landscape that is depauperate of beneficial predators of disease vectors and alter the microclimate, and as such, they can facilitate the invasion of these vectors and must be considered in their full ecological context.

ZOONOTIC INFECTIOUS DISEASES

VIRAL INFECTIOUS DISEASES

Venezuelan equine encephalitis

Venezuelan equine encephalitis viruses are members of the VEE complex and comprise the three major serogroups of New World alphaviruses (46, 47). Fourteen subtypes and varieties have been described within the VEEV complex (48). The IAB and IC viruses are designated "epidemic" or "epizootic" because they have been isolated only during equine and human outbreaks. They are distinct from enzootic strains (subtypes/varieties ID-F, II-VI) that circulate in sylvatic or swamp habitats, and occasionally cause disease in humans or domestic animals (48–50). Importantly, VEEV isolates identified to be of the IE subtype identified during epizootics in Mexico appear to be equine neurovirulent, but are unknown to produce high titer viremia (50). Transmission cycles have been described for most of the enzootic VEE subtypes/varieties [ID, IE, II, IIIA, and IIIB (Tonate)], except III C and V (48). Most of them are transmitted among rodents by mosquitoes in the subgenus *Culex* (*Melanoconion*) and few mammalophilic mosquitoes (51).

Venezuelan equine encephalitis virus was known to be circulating and producing illness in horses since the 1920s (49, 51), and in 1938 the virus was isolated from the brain of a horse that died of encephalitis in South America (52–54). Human cases with neurological complications were recorded in 1950 during an outbreak of febrile illness in Espinal, Colombia (55). VEEV outbreaks continued at regular intervals through the 1960s in South America affecting tens-to hundreds-of-thousands of people (50, 56). Between 1973 and 1992 no VEEV was documented, which led to the assumption that the epidemic-epizootic subtypes IAB and IC VEEV had disappeared (50). However, phylogenetic studies and renewed epidemic/epizootic VEE activity in Northwestern Venezuela during 1992–1993 revealed that these viruses remain a threat (57, 58). Two equine epizootics in the States of Chiapas and Oaxaca, Mexico caused by a subtype IE virus (59), and a major outbreak in Venezuela and Colombia during 1995 affecting about 100,000 people (60, 61) draw attention to the continued threat of VEEV. To date, VEEV viruses affecting humans and equids have been found in at least 12 countries of the Americas causing important social and economic damage mainly in Latin America (48).

Most VEEV epizootics and epidemics have taken place in Northern South America and in regions of Venezuela and Colombia. However, VEE has also affected North America on several occasions. In 1966, an equine epizootic in Northeastern Mexico

was reported in southern Tamaulipas and Northern Veracruz within the Panuco river basin (62). Although no viruses were isolated, VEE etiology was determined serologically. The lack of VEE vaccination in Mexico at this time suggests that this outbreak was caused by a local enzootic subtype IE strain. Another major Middle American outbreak and one of the most devastating VEEV pandemics of the Continent, began near the Guatemala-El Salvador border on the Pacific coast and spread through much of Central America, Mexico, and Texas during 1969–1972, and involved tens-of-thousands of equines and people. In Mexico, equine deaths from VEE were first reported in 1969 in mountainous regions of the State of Chiapas close to the Guatemalan border (63). By 1970, the epizootic had produced about 10,000 equine deaths in the States of Chiapas and Oaxaca (64). The outbreak then spread northward to the Gulf Coast and eventually reached Southern Texas. In Texas, approximately 1500 horses died of VEE, and several hundred human infections were documented. The Texas outbreak was halted by a massive equine vaccination program and aerial insecticide spraying costing about 15 million dollars (50, 65).

In the 1990s, two outbreaks of equine encephalitis occurred on the Pacific Coast of Southern Mexico. From June to July in 1993, 125 equine cases including 63 deaths were reported in Chiapas State. Three years later, from June to July in 1996, another equine epizootic occurred nearby in Oaxaca State, involving 32 horses with 12 deaths (59, 66). Epidemiological and serological data were consistent with VEE, and VEEV was isolated from encephalitic horses involved in each outbreak. No human cases were documented during these outbreaks. However, further serosurveys and VEEV isolations obtained in the same area of the 1990s outbreaks demonstrated that VEE has been endemic in this Southern region of Mexico for decades (67).

Two virus isolates from the Mexican outbreaks of the 90s were examined antigenically and genetically. All were VEE subtype IE by IFA and ELISA using monoclonal antibodies (68). Sequencing and phylogenetic studies indicated that the outbreak strains belong to one of three major subtype IE VEEV lineages. This lineage circulates on the Pacific Coast of Guatemala, and was sampled there from 1968 to 1980 (59, 66). The absence of previous Mexican isolates from this lineage suggests that the currently circulating Mexican strains originated from enzootic transmission foci on the Pacific Mexican Coast. The remaining IE lineages circulate on the Gulf and Caribbean Coasts of Central America (Nicaragua northward to the Gulf Coast of Mexico and close to the US border) and in Western Panama, and differ by up to 7% at the nucleotide sequence level.

Before the Chiapas–Oaxaca outbreaks, isolates of enzootic VEEV, including subtype IE, were traditionally believed to be avirulent for equines, and were not previously known to have epizootic potential (69–71). Experimental infection of horses with several IE strains from Mexico and Nicaragua showed that these viruses generally produced little viremia and disease (70, 71). Further studies using reverse genetics approaches demonstrated that *Aedes* (*Ochlerotatus*) *taeniorhynchus*, an abundant epizootic vector in coastal areas of Chiapas and Oaxaca, was more susceptible to isolates obtained during the 1993 and 1996 epizootics compared with closely related enzootic IE strains isolated previously in Guatemala (72). A mechanism of VEEV emergence was suggested showing that a single Ser → Asn amino acid substitution at position 218 of the E2 envelope glycoprotein was the major determinant of the increased *Ae. taeniorhynchus* infectivity. Viral adaptation to a vector that prefers to bite large mammals was suggested as the emergence mechanism in the 1990s outbreaks of Southern Mexico (72).

Subtype IE enzootic viruses are the only VEEV known to continuously circulate in Mexico both currently as well as prior to 1993 outbreak. They occur from Western Panama through Tamaulipas State in Mexico (48, 65, 73–75). The ecology of the IE viruses has been studied in detail and *Culex* (*Melanoconion*) *taeniopus* is the known primary enzootic vector of subtype IE viruses in Guatemala (76), and one of the principal vectors maintaining endemic VEEV cycles in southern Mexico, the Gulf Coast of Mexico (48, 73, 75), and Panama (65). It has been demonstrated that *Cx. taeniopus* found in estuarine areas of Chiapas is susceptible to both subtype IE VEEV isolates from the Pacific Mexican outbreaks of the 90s and to strains isolated from hamster sentinels during the 2000s in the same region (77). *Cx. taeniopus* feeds on a wide variety of hosts, mainly small rodents from the Cricetidae family such as cotton rats and rice rats, and seems to circulate subtype IE VEEV not only in the Mexican Pacific Coast but also in the Gulf Coast of Mexico (73, 77). The scenario in the Pacific Coast of Southern Mexico appears to involve VEEV transmission by *Ae. taeniorhynchus* to equids and possibly humans at inland locations (48). The threat of VEEV outbreaks in the Mexican Pacific region involving enzootic and epizootic vectors exists (48). Such region is linked to the East through Gulf river basins where several endemic VEEV foci are found, along the States of Tabasco, Veracruz, and Tamaulipas. The later of these States is adjacent to the US Texas border, and is a spot of subtype IE VEEV activity (73). Hotspot activity was characterized through serosurveys suggesting VEEV infections in cattle, equines, rodents, and humans, which was complemented with the isolation of IE VEEV (73). Findings of the study showed that at least one major urban region (Minatitlan in the State of Veracruz) has active enzootic VEEV transmission with *Cx. taeniopus* identified as the main VEEV vector (74; Estrada-Franco and Weaver, unpublished information). Several mosquito epizootic vectors of VEEV that were found infected and active during the VEEV pandemic of the 1970s are present on both sides of the border (Tamaulipas and Texas), such as *Ae. sollicitans*, *Psorophora confinnis*, and *Ae. taeniorhynchus* (63, 78). The risk of *Ae. taeniorhynchus* or other epizootic vectors adapting to endemic VEEV cycles elsewhere outside the Chiapas area, as was demonstrated with the IE VEEV strains isolated in the Pacific coast, could be of veterinary and public health significance. An epidemic strain of VEEV has the potential to arise from circulating endemic strains, which may be easily misdiagnosed for another febrile-causing disease if appropriate diagnostic assays are not routinely performed. The movement of epidemics by viremic individuals is a major concern, particularly in the Gulf Coast region of Mexico, which could threaten previously unaffected areas of Mexico and even the US. Moreover, direct human-to-human transmission of VEEV has also been suggested by the sudden appearance, rapid increase, and brief occurrence of human disease within affected communities (61, 79).

Reconstructing the historical incidence of VEE could facilitate the forecast of recurring patterns and help improve strategies for disease prevention, e.g., vaccine distribution logistics. Our hypothesis is that drivers of VEE outbreaks are responsive to heavy rainfall events but activity subsides as drier conditions return.

The historical time-series shows three repeating VEE outbreaks over the past century, recurring at approximately 30-year intervals, and spanning up to one decade. The gap of two decades following each outbreak period is conspicuous (**Figure 2**). Attention to details of each major outbreak show striking differences between epidemics. The first event (Outbreak I, 1935–1946) is exclusively an equine outbreak, with a locus in Columbia and Venezuela. By 1942, it had spread to Peru in the South and by 1943 to Trinidad in the East. The actual number of equines affected in this outbreak was not documented, and there are no records of human VEE cases; the latter do not appear as recorded observations until the 1960-decade. The gaps over the 1970–1980 decades (Gap b) and over the 1996–2013 interval (Gap c) contrast, at least in terms of recent serological evidence, and in terms of important climatic conditions.

The ocean indicators both show low levels of storm impact over the 1972–1992 (Gap b), and likely reflect the paucity of extreme rainfall events, which is in contrast with the high storm levels over the 1996–2013 interval (Gap c) (**Figure 2**). The latter suggests hurricane and ENSO events are now opportune to bring heavy rainfall into the VEE-affected region. This begs the question of why recent VEE levels remain relatively low and points to the need for further analysis of local rainfall and other weather events across the region.

There is uncertainty and considerable concern over the possible re-emergence of an outbreak of VEE. Our time-series model and its correlation with broad climate signals (**Figure 2**) offers a new and evocative look at VEE at the century and cross-regional scales. It opens a window on a conceptual framework for better understanding and managing outbreaks of the virus through both the tracking of accelerated climate change (e.g., satellite imagery), and use of long-term forecasting (**Figure 3**). Work is in progress using power spectrum analysis to quantify likely timing and magnitude of future outbreaks. This is important now that serological analyses show the widespread presence of the VEE virus in humans, equines, and populations of virus reservoirs like the rodent species mentioned above, which mosquito vectors feed on, that respond to unusually strong rainfall events.

The development and persistence of high Accumulated Cyclonic Energy Index (ACE) and equatorial Pacific Ocean Southern Oscillation Index (SOI) levels imply VEE outbreak activity currently and in the near future (**Figure 2**). Why VEE outbreak activity has remained low since 1966, when broad oceanic indicators suggest a high potential for outbreak, remains unknown, which indicates the need for further research. For example, additional research into local rainfall conditions could help understand how mosquito populations expand, which may drive VEEV activity as it has been shown in other mosquito-borne arboviral disease systems (80). Conditions since the 1972–1992 gap have changed, moving toward better virus monitoring and disease management approaches. These changes, taken with the movement of human populations into cities and a relative decline in equine populations, will also play a role in decreasing the likelihood or severity of a VEE outbreak. There is currently widespread mosquito control in response to dengue epidemics. Any of these developments may be limiting the magnitude of current and near-term VEE epidemics (**Figure 3**). Outbreaks in the 1930s and 1960s were weakly constrained, at best, and may offer a study in contrast to modern conditions with better knowledge and disease management infrastructure.

Hantavirus

The genus *Hantavirus*, from the family Bunyaviridae, is composed of viruses with a three-segment negative sense RNA genome (81,

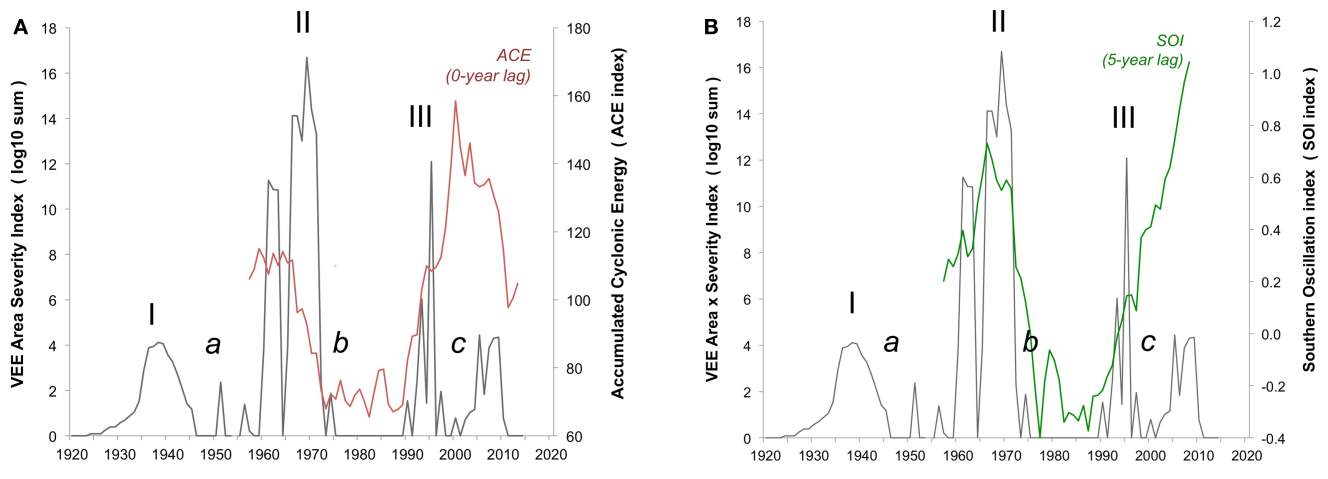

FIGURE 2 | Integrated VEE area-severity index. Integrated VEE area-severity index (*dark solid line*, left axis) based on historical reconstruction of areal extent and severity in equine and human populations over the 1920 through 2013 period. Three major VEE outbreaks (I, II, III) and three gap-intervals (*a*, *b*, *c*) of low VEE are shown. **(A)** Eleven-year running means of accumulated cyclonic energy index (ACE, *red font*) and **(B)** the Southern Oscillation Index (SOI, *green font*) given on right axis.

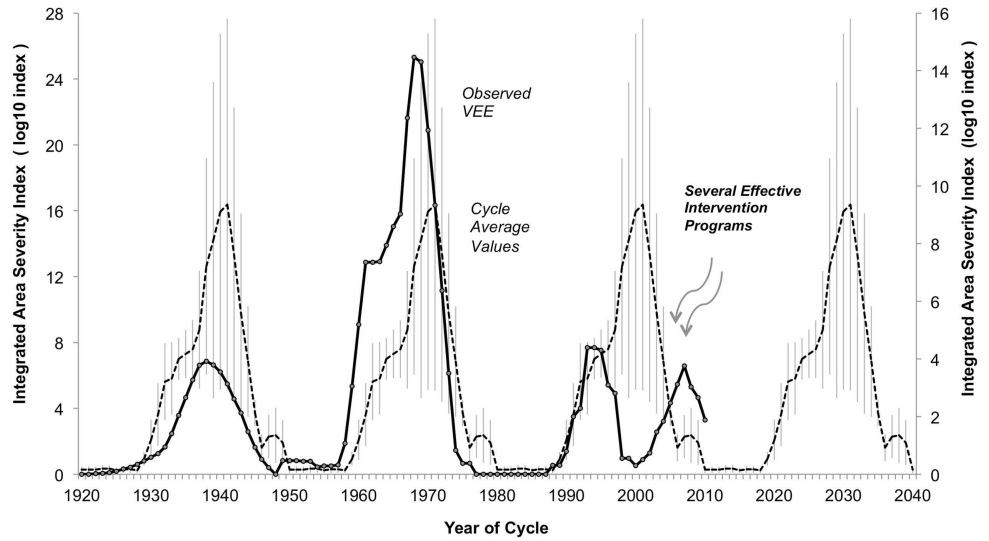

FIGURE 3 | Observed integrated area-severity index for Venezuelan equine encephalitis (VEE) in the Americas, 1920–2010. Integrated Area-Severity (IAS) index (*dark solid line*, left axis, log10 scale, 5-year point-centered moving average) for Venezuelan Equine Encephalitis (VEE) in the Americas, based on reconstruction of actual disease incidence reported in literature, 1920–2010. Hypothetical reconstruction of expected levels of VEE, 1920–2010, achieved by sequential repeat of the average IAS index levels over Outbreak Two (1950–1979) and Outbreak Three (1980–2009) (*dashed line*, right axis, log10 scale), with standard error of mean added (*light vertical lines*). Outbreak One (1920–1949) values excluded from average due to lack of solid data. Subdued levels of VEE over Outbreak Three could be the result of assertive mosquito control programs related to dengue fever epidemics, and/or improved VEE management approaches and infrastructure (see text for details).

82). In nature, these viruses are hosted by a variety of rodent and soricomorph species as persistent infections (81). Humans acquire *Hantavirus* infection by inhaling aerosolized particles from rodent excreta and urine, or via the bite of an infected animal (81). Some rodent-borne Hantaviruses are associated with two types of human disease, differing between the New and Old Worlds. In the Americas, *Hantavirus* pulmonary syndrome (HPS) has reached fatality rates of 60% and is characterized by elevated pyrexia, pulmonary dysfunction, and cardiac shock (83). In several regions of the Old World (Europe, Russia, China and Korea), hantaviral infections cause a hemorrhagic fever with renal syndrome (HFRS) characterized by high fever, renal dysfunction, and hemorrhage but with mortality rates usually lower than 12% (81, 83).

A relevant feature of Hantaviruses is their close association between a specific *Hantavirus* and its rodent host species, suggesting a strong relationship between Hantaviruses and their reservoirs (84, 85). Globally, these viruses occur in close association with rodent and shrews of the families Cricetidae and Soricidae, with a majority of New World Hantaviruses detected in reservoir rodent species of the sub-family Neotominae (82, 86). Throughout America, more than 30 Hantaviruses have been identified, but only a few have been associated to HPS (81). The New World geographic distribution of known hantaviral strains includes Canada, US, Mexico, Honduras, Costa Rica, Panama, Venezuela, Peru, Bolivia, Brazil, Argentina, Chile, and Paraguay (81, 83). Rodents with *Hantavirus* antibodies have been detected in Peru, Venezuela, Costa Rica, Honduras, and Mexico, although HPS in humans has not been documented in these countries (81, 87). The distribution of recorded cases of HPS and the distribution of *Hantavirus* seroprevalent rodents in the Americas is not coincidental for several reasons: (1) not all Hantaviruses have been associated with HPS; (2) our knowledge of hantaviral diversity present in the continent is likely incomplete; and (3) HPS may be confused with clinically similar diseases. In North America, five Hantaviruses known to cause HPS are the Sin Nombre virus (SNV), New York virus (NYV), Choclo virus (CHOV), Black Creek Canal virus (BCCV), and Bayou virus (BAYV) (83, 88). SNV is the major cause of HPS in the US and Canada, where *Peromyscus maniculatus* (deer mouse) is the primary rodent reservoir (81, 89).

Northeastern Mexico (Chihuahua, Coahuila, Nuevo León, and Tamaulipas) and Texas share a common biogeographic history, and thus a large number (23) of Cricetinae rodent species are shared among these States (90, 91). Because the abiotic environment and rodent assemblages of Northeastern Mexico are similar to those of areas in the adjacent US, it is likely that many viruses circulating in this region occur on both sides of the international border. However, specific information regarding the prevalence and spatial distribution of Hantaviruses within this region is scarce and incomplete. *Hantavirus* antibody-positive rodents from seven species have been found in Chihuahua, Nuevo León, and Tamaulipas. The only *Hantavirus* identified to date within these species is the SNV. However, because most of the rodent individuals tested were only analyzed by serology tests, it is possible that other Hantaviruses occur in Northeastern Mexico (88, 92, 93). In Texas, antibodies for Hantaviruses have been detected in 11 rodent species and 4 Hantaviruses (SNV; El Moro Canyon virus, ELMCV; Muleshoe virus, MULV; and BAYV) are known to occur in this state (94). No cases of HPS are known from Mexico, but up to 2006 a total of 28 confirmed cases were recorded in

Texas. Of these, 24 cases were associated to SNV and 3 to BAYV. Infection with SNV had a high mortality rate of 50% (12/24), but all of the three patients infected with BAYV survived (95). The geographic distribution of HPS cases in Texas shows the two groups with most cases (64%) are in the West and Panhandle areas, with the rest of the cases found along the Gulf of Mexico Coast area. This disjoint case distribution is due to rodent reservoir distributions. Cases along the Gulf Coast are associated to the BAYV, which is carried by the rice rat (*Oryzomys palustris*), whereas the cases in Western Texas have been related to SNV presence in the deer mouse (*P. maniculatus*) (95). *O. palustris* is present in Northeastern Mexico, but no seropositive individuals have been reported. Its role as a BAYV reservoir in Mexico is likely minor as the range of this rodent is limited to the extreme Northern corner of Tamaulipas (90). However, two rodent species (*P. maniculatus*, and the white-footed mouse, *Peromyscus leucopus*) pose a more serious risk for HPS along the transboundary States. Both species have wide ranges across Texas and Northeastern Mexico; within this region, SNV seroprevalent individuals have been recorded for both species (88, 94, 95). Moreover, based on the list of Hantaviruses identified in the Southwestern US, it is suggested that four Hantaviruses likely circulate in Northern Mexico: SNV, ELMCV, MULV, and Limestone Canyon virus (LSCV). These viral strains are hosted by rodent species of the *Peromyscus*, *Reithrodontomys*, and *Sigmodon* genera (88), which are ubiquitous throughout the transboundary region. This hypothesis needs to be confirmed with further work to ascertain the public health risk for human populations on both sides of the border. To achieve this goal, it is necessary to determine the geographic distribution patterns of *Hantavirus* sero-prevalence in rodent reservoir species and understand the mechanistic processes that determine these patterns.

Beyond knowing the specific situation in the Eastern transboundary region between Mexico and the US, further work needs to extend outside of this zone, as it might influence this region. Mexico has a very diverse mammalian fauna (~525 species) with rodents comprising almost half (235 spp., 44.8%) of the species (90). It is possible that many more species harbor Hantaviruses than those currently recognized (88). Specifically, the transboundary region could serve as a connection between Hantaviruses of Neartic origin with others from tropical regions. The State of Tamaulipas has a mammalian fauna that represents a mix of Neartic and Neotropical taxa (90) and the possibility exists that these rodent species of Neotropical affinities could harbor Hantaviruses found in tropical areas such as the Catacama virus (CATV) present in *O. couesi* from Honduras (83, 87). Moreover, evidence of the presence of *Hantavirus* seropositive rodents in regions of Southern Mexico and crucial human migration crossing pathways are elements to be considered in this complex equation (88). For instance, in the State of Chiapas' coastal and central valleys, there are clear indications for the presence of *Hantavirus* in wild life. After infection, the resultant disease can take up to 2 weeks to develop in the human host, allowing the disease to move with relatively low detection. Thus, the potential risk of these Neotropical Hantaviruses existing in the transboundary region needs to be evaluated.

BACTERIAL INFECTIOUS DISEASES

Tick-borne bacterial infections

Globally, ticks serve as vectors for a number of zoonotic bacterial pathogens, such as the spirochete *Borrelia burgdorferi*, the causative agent of LD, as well as the intracellular pathogens *Rickettsia rickettsii*, *Ehrlichia chaffeensis*, *E. ewiingii,* and *Anaplasma phagocytophilum* (**Table 1**), also known as tick-borne rickettsial diseases (TBRD) (96). These pathogens are maintained in natural cycles involving wild mammals and several species of hard ticks in the family Ixodidae. Foci of LD exist in the US, Europe, and Asia, and it is considered an emerging infection in those parts of the world (97–101). In the US LD is the most prevalent arthropod-borne infection with over 30,000 cases reported to the Centers for Disease Control and Prevention (CDC) in recent years. The increase in LD cases during the last few years has prompted its classification as an emerging infectious disease. Similar to other arthropod-borne diseases, LD is a complex system subject to shifts in ecological processes that influence vector biology and the epidemiology of *B. burgdorferi* infection in reservoir hosts and humans.

Hard tick species in the genus *Ixodes* are recognized generally as common vectors of *B. burgdorferi*. *I. scapularis* and *I. pacificus* are known competent vectors in the US, while *I. persulcatus* and *I. ricinus* are the documented vectors in Eurasia (99, 102–105). The pathogen is maintained in the environment by different vertebrate hosts with varying degrees of competence. The main reservoir, the white-footed mouse, *Peromyscus leucopus*, is found in the forests of Eastern North America (106, 107). There is an extensive bibliography on the molecular diversity and adaptation of *B. burgdorferi* to its natural environment, as well as on the impact of species diversity in a particular area on reducing LD risk (108–118). In addition, some of these studies have also considered the effect of climate change on the geographic distribution of *I. scapularis* in addition to its phenology in the US and Canada (119–121). Although LD in humans is more prevalent in Northeastern US, the lack of detailed studies in Southern US has prevented comparisons, and evaluations of ecological factors responsible for promoting the differential incidence of LD between these regions. Moreover, in some parts of the world the ecology and epidemiology of LD remain to be fully understood. Thus, LD is considered a transboundary zoonotic disease in that it can reach epidemic proportions in regions of the globe regardless of country borders (122). This is coupled with the fact that there is unequally distributed knowledge about the ecology of this disease among the regions in which it occurs.

Human risk of infection with *B. burgdorferi* across the continental US has been predicted using the density of *I. scapularis* infected nymphs (DIN) (123, 124). Under this scenario, Southern US States were considered as a low risk region given the non-appearance of host-seeking *I. scapularis* nymphs at sampled sites (123, 124). In striking contrast to the conclusion of this suggested null risk of acquiring LD in Southern States, a steady number of LD cases have been reported in these low risk areas every year (125, 126). Some of the caveats of these most recent studies include the lack of accounting for both human movement (some cases can be acquired in a region different from the one where they are

reported) and differences in tick phenology between geographic areas (124). These limitations might explain why the models utilized cannot explain the variation in distribution of the disease observed in low incidence areas. Drivers for the variation in distribution of disease cases observed in low incidence areas remain to be identified.

In Mexico, a national serosurvey of human serum samples reported a *B. burgdorferi* sero-prevalence of 1.1% (127). The Mexican States of Tamaulipas, Nuevo León and Coahuila in the Texas–Mexico border region presented the highest sero-prevalence (6.4%) for the country (128). Also, *Ixodes* ticks infected with *B. burgdorferi* sensu stricto occur in the same States (129), and recently the infection has been documented in white-tailed deer (130). Distribution models of potential tick vectors in Mexico point to a wide distribution range that overlaps not only Northeastern Mexican States along the border with the US, but also extend to central Mexico (131, 132). These studies, together with confirmed clinical cases of LD acquired in parks near Mexico City (133, 134), demonstrate the existence of a zoonotic cycle responsible for LD in Mexico.

TBRD are a group of zoonoses clinically similar, yet epidemiologically and etiologically distinct. In the US, these diseases include: (1) Rocky Mountain spotted fever (RMSF), (2) human monocytic ehrlichiosis (HME), (3) human granulocytic anaplasmosis (HGA) (135), (4) *Ehrlichia ewingii* infection, and (5) other emerging TBRD (**Table 1**). TBRD are common occurrences in the medical and veterinary clinical setting, and are gaining more attention from physicians and veterinarians since TBRD continue to cause severe illness and death in otherwise healthy individuals (136, 137). The epidemiology of these diseases reflects the geographic distribution and seasonal activities of vectors and reservoirs and human behavior that places persons at risk for infection through tick bite (13, 129, 137, 138). Environmental changes may alter the distribution of wild animals and arthropod vectors, which could extend their range to areas close to human populations where these pathogens could be transmitted (13). But demographic and sociologic factors also play a critical role in determining disease incidence.

Several ticks species are vectors of different rickettsiae causing TBRD. *R. rickettsii*, the causative agent of RMSF, is transmitted most frequently by the American dog tick (*Dermacentor variabilis*) in the Eastern, Central, and Pacific coastal US and the Rocky Mountains, while the wood tick (*D. andersoni*) transmits this pathogen in the Western US. The brown dog tick (*Rhipicephalus sanguineus*), a vector of RMSF in Mexico (129, 137, 139), was implicated in 2005 as vector of this disease in a confined geographic area in Arizona (140). *Rhipicephalus* ticks from Mexicali, Mexico have been recently genetically characterized, and found to be different from those isolated in the US (141). The cayenne tick (*Amblyomma cajennense)* is a common vector for RMSF in Central and South America (129, 132, 142), and its range extends into the US through Texas. *Ehrlichia chaffeensis* and *Ehrlichia ewingii* are transmitted to humans by the lone star tick (*Amblyomma americanum*). *E. ewingii* infections in dogs or ticks have been described in Missouri, Oklahoma, Tennessee, Arkansas, Texas, Florida, Georgia, Mississippi, North Carolina, and Virginia (143, 144). *A. phagocytophilum* is transmitted by the blacklegged tick (*I. scapularis*) and is distributed in New England, North Central, and recently, Southeast United States, in addition to the Northeast of Mexico (13, 129). The western blacklegged tick (*I. pacificus*) is the principal vector in Northern California. In the US, the estimated average annual incidence of RMSF was 2.2 cases per million people. In Mexico, the incidence from 1975 to 1987 was 12.59 cases per 100,000 people in North and Northwest States. From 2009 to 2011, there were 2616 reported cases with an incidence of 0.8 cases per 100,000 people. In 2012, there was an increase to 2875 cases in the States of Baja California and Coahuila in Northern Mexico. Due to the consistent increase and presence of this disease, Mexico started to officially report RMSF and other Rickettsial human cases in 2014 (145).

Ehrlichiosis was first recognized as a disease in the late 1980s, but did not become a reportable disease until 1999 in the US. The number of ehrlichiosis cases due to *E. chaffeensis* that have been reported to CDC has increased steadily since the disease became reportable, from 200 cases in 2000 to 961 cases in 2008 (138). The incidence increased from less than 1 to 2.5 cases per million people in 2000–2010. Both *E. chaffeensis* and *E. ewingii* are causes of human illness in the US, although the majority of reported cases identify *E. chaffeensis* as the causative agent of HME. HGA is more frequently reported than HME with an annual incidence of 1.6 cases per million during 2001–2002. In Mexico, the first Ehrlichiosis case was reported in 1999 (146). It is important to understand the involvement of dogs in the potential enzootic cycle for *Ehrlichia* infection acquired by humans in close contact with domestic dogs. In this sense, our research team found that human contact with *Ehrlichia* infected dogs have 14.9 times higher risk to become infected, and dogs infested with *Ehrlichia* infected ticks have 8.2 higher risk of being infected (128).

The understanding of vector-borne disease ecology has improved in recent years due to advancements in molecular biology, geographic information systems (GIS), and species distribution models (SDM) (147, 148). A recent study evaluated the presence of *I. scapularis* ticks in Texas and Northern Mexico, and forecasted the distribution of this tick species considering different climate change scenarios (13). It was observed that a geographic region could provide suitable environment where the competent vector for transmission of LD and other zoonotic pathogens would survive. The model presented in this study showed East Texas to include suitable habitat where established populations can exist (13), which agrees with findings from other studies (149). This model also showed expansion towards Central and to South Texas through a corridor along the Gulf Coast and Northern Mexico, forming a geographic continuum of habitat suitable for *I. scapularis* populations in the border region. Although no specific distribution model exists for *I. scapularis* in Mexico, a distribution model for the genus *Ixodes* generated with similar methodologies predicts a wide distribution covering Northeastern Mexico (132).

Variation in questing behaviors may significantly impact the type of hosts ticks encounter and may lead to differential host use within a particular study area (150). Therefore, further studies testing different sampling procedures, including different time of the day and season, would be needed to determine the phenology of *I. scapularis* in Southern US and Mexico and the questing behavior of the different developmental stages. These studies will

be critical to determine how questing behavior will affect the risk for LD in humans and companion animals in the transboundary region.

Leptospirosis

Leptospirosis is a zoonotic infectious disease of worldwide distribution that is endemic in tropical and temperate climates, with higher prevalence in tropical countries (151–153). Leptospirosis can be caused by *Leptospira interrogans*, which in cludes 200 serovars affecting both domestic and wild mammals, and humans (153, 154). The reservoirs for these pathogens are wild or domestic animals such as rodents, cattle, or dogs (155). In urban areas, rodents (mostly rats) are the main carriers of the disease (156), whereas the dog is considered a dead-end host (157, 158). However, due to their close contact, dogs pose a risk of infection for human beings (151, 159). It has been suggested that ticks are potential vectors of *Leptospira* spp. (160).

In the forest region of Indiana, a study was conducted with 34 raccoons (*Procyon lotor*) to determine the presence of *Leptospira*. In this study, cell culture techniques, microscopic agglutination test (MAT), and PCR were used. The results indicated the presence of *L. interrogans*, *L. kirschneri*, and *L. borgpetersen* in raccoons from this region. For *L. interrogans*, the serovars most frequently detected were Bratislava (38.2%) and Grippotyphosa (32.4%). This finding indicated that *L. interrogans* is circulating in the raccoon population, which is acting as a reservoir for the pathogen. The racoon is an abundant species in the south Texas – northeast Mexico region.

Cervids may be involved in the epidemiology of leptospirosis. Diversified livestock production comprises activities aimed to breed in sustainable manner wild animals, including native and exotic species of deer. Ranches in northeast Mexico have been managed to be units for the conservation, management, and sustainable use of wildlife (UMA), which are dedicated to the diversification of livestock. A cross-sectional epidemiological study on leptospirosis was conducted with cervids at an UMA in Tamaulipas, Mexico (161). Of the 37 animals sampled, eighteen individuals were Axis deer (*Cervus axis*) and 19 Fallow deer (*Cervus dama*). Seropositivity for *Leptospira* spp. in all the cervids sampled was 13.5%. Twenty-one percent of the Axis, and 5.5% of the Fallow deer were seropositive. Positive deer were reactive to serovars Bratislava and Muenchen, which confirms the presence of this pathogen in deer in Northern Mexico (161). Similar seroepidemiological findings for leptospirosis in cervids have been reported in the US and Spain (162–165).

It is important to understand how global change may alter the pathogenic landscape of leptospirosis because this zoonosis is considered a major bacterial NTD in Texas and Mexico (11). For example, the water buffalo (*Bubalus bubalis*) is originally from Asia and was introduced in Mexico as an alternative livestock species during the 1990s (166). Water buffaloes produce milk, meat, and are used as working animals. Although subclinical *Leptospira* infection has been documented in other parts of the world and is considered a health risk to humans, information is lacking on the prevalence of leptospirosis in water buffalo herd in Mexico. An epidemiological cross-sectional study was conducted with a sample size of 368 blood specimens in the Mexican state of Veracruz to fill this knowledge gap. The overall sero-prevalence for Leptospirosis was 53.5% (167). The most common serovars detected were Muenchen (44.3%), Pyrogenes (11.4%), Icterohaemorragiae (11.1%), and Hardjo (8.1%). In this study, the interaction between water buffaloes and dogs was identified to be a risk factor (167). This was the first study identifying seropositive buffaloes to *L. interrogans* and risk factors associated with Leptospirosis in Mexico (167).

Zoonoses common to pets threaten the health of humans, particularly children. A study conducted to determine the frequency of canine Leptospirosis in dogs from two shelters in the city of Veracruz, Mexico showed that 8.6% (8/92) were seropositive. The most frequent serovar was Canicola (168). Similar results were observed in Yucatan, Mexico where the serovars present were Canicola and Icterohaemorrhagiae (169). Even though leptospirosis occurs in the transboundary region, more research is required to understand its epidemiology and mitigate the burden of this neglected zoonosis on human and domestic animal populations (11).

Bartonellosis

Bartonella species are fastidious gram-negative, facultative intracellular bacteria that cause host restricted hemotropic infections in mammals. Normally, they infect erythrocytes, macrophages, and endothelial cells. In addition, a number of *Bartonella* spp. are transmitted by blood sucking arthropods such as sand flies, biting flies, lice, and fleas. More recently, ticks and mites have been suggested as potential vectors for zoonotic *Bartonella* spp. (170–175). In addition, *Bartonella* spp. affect a number of mammals, including humans, dogs, horses, cattle, cats, and even marine animals (176, 177).

The number of *Bartonella* spp. identified infecting a wide range of mammalian species has significantly increased. Currently, a total of 13 species or subspecies can cause disease in humans, and most of them are zoonotic (173, 176, 178). *Bartonella henselae*, the causative agent of cat-scratch disease (CSD) is the most recognized in the medical community together with *B. quintana*, the causative agent of Trench Fever, and *B. bacilliformes*, the causative agent of bacillary angiomatosis, Oroya fever and veruga peruana (176, 177). Moreover, most of the *Bartonella* species causing disease in humans and companion animals have a worldwide distribution, and are associated with poverty and overpopulation, as these bacteria thrive in crowded and unhygienic environments (172, 179–181). This aspect of *B. quintana* and *B. elizabethae* infections has been extensively reviewed elsewhere (180–184).

Cats have been classified as reservoirs for *B. henselae*, the causative agent of CSD, which is distributed worldwide. The prevalence of infection in felines is higher in warmer countries that it is in cold countries (178). In addition, cats tend to be bacteremic for months and in some instances for over a year (178). The cat flea (185), *Ctenocephalides felis*, is responsible for the transmission of the bacterial pathogen between cats. Studies by Finkelstein and collaborators (180) showed that *B. henselae* can remain viable in flea feces for over 72 h. Therefore, transmission to humans can occur via inoculation of *B. henselae* from infected flea feces into the skin via open wounds, such a scratch lesion. The CDC documents over 20,000 CSD cases annually. Human cases have been reported in Texas (186–189), Mexico, and other Latin American countries (190, 191). Domestic dogs, together with wild canids, have been

suggested as potential reservoirs for zoonotic *Bartonella* species, such as *B. vinsonii* subsp. *berkhoffii*, *B. henselae*, *B. clarridgeiae*, *B. wahoensis*, *B. rochalimae*, *B. quintana*, and *B. elizabethae*. In some cases, domestic dogs display a broad range of clinical signs (174, 176, 178, 192, 193) similar to those observed in humans. Consequently, domestic dogs might be considered sentinels for *Bartonella* infections (178) as they are for other vector-borne infectious diseases (194–196).

The number of annual cases due to Bartonella infection in the US–Mexico transboundary region remains unknown even though more epidemiological information is available relative to bacterial NTD like leptospirosis (197). *Bartonella* infections are not reportable in humans or animals in the US and Mexico. This situation makes it difficult to understand the epidemiology and pathogenic landscape for Bartonellosis in a geographic region where about 17.6% of the population lives in poverty[2].

PROTOZOAN INFECTIOUS DISEASES

Human babesiosis

Human Babesiosis (HB) is caused by several species of apicomplexan tick-borne protozoa of the genus *Babesia* and is a zoonotic emerging disease globally although it is endemic in the US (198–200). Surveillance for Babesiosis started in 2011 in 18 States. That year, there were 1,124 confirmed and probable human cases across the US (126). Most cases of naturally transmitted HB in the Northeastern and Midwestern US are caused by *B. microti* (201). Infection in other regions of the US and the world has been documented with *Babesia* species that remain to be fully characterized (202–204). In rural Mexico, human infection with *Babesia* spp. was documented during the 70s (205), where 37% of the tested individuals were seropositive but asymptomatic, and all volunteers recalled having tick bites.

HB is a complex zoonotic disease system whose relevance as an emerging public health concern is considered to be the result of aspects related to global change (206, 207). Most zoonotic *Babesia* species are maintained in wildlife reservoirs, but gaps exist in our knowledge of several epidemiological aspects of HB in various parts of the world (208–210). The vector(s) and reservoir hosts of *Babesia* species affecting humans in Mexico and other Latin American countries remain unknown (211). Accurate diagnosis of HB in sub-tropical and tropical parts of Latin America can be complicated because of malaria-like symptoms in the case of affected patients (212), asymptomatic infection in others, and the possibility that tick bite may have resulted in co-infection with other tick-borne pathogens (213, 214) coupled with the use of serodiagnostic tools that cross-react with other *Babesia* and *Plasmodium* hemoparasites (215, 216). Thus, it is important to understand how environmental change (217), including the knowledge of vectors and reservoir hosts, could influence the patterns of zoonotic *Babesia* transmission at the regional level to evaluate the risk for the emergence of Babesiosis among humans in the South Texas–Northeast Mexico transboundary region.

Although its known geographic range appears to be expanding, it is possible that HB remains under-reported in the South Texas – Northeast Mexico transboundary region (218–220). Increasing evidence suggests that reservoir species and vectors are present in the Eastern sector of the border between the US and Mexico, and our knowledge of wildlife and domestic animals harboring *Babesia* species with the potential to be pathogenic to humans is expanding (204, 221, 222). The infection of cotton rats (*Sigmodon hispidus*) and raccoons (*Procyon lotor*) with *B. microti* presents a risk for HB in Northeast Florida (223). Both mammals are abundant in Texas and Northeast Mexico (129, 142, 224, 225). *I. scapularis* is the known vector in the Northeastern US and its presence has been documented in the US–Mexico transboundary region (13), but Clark et al. (223) suggested that additional *Ixodes* species might be transmitting *B. microti* in the Southeastern US, like the raccoon tick (*I. texanus*) and other *Ixodes* ticks known to feed on the cotton rat. *I. texanus* is distributed in Texas and Nuevo León (142, 226, 227). Of the ticks species present in the US and Mexico, which are known to infest the cotton rat (13, 228–230), *D. variabilis* and *I. scapularis* have been shown to harbor *Babesia* spp. and bite humans in Southern latitudes (231, 232). Suspected vectors of a *Babesia* detected in the woodrat (*Neotoma micropus*) with a partial 18S rRNA sequence related to *B. conradae* included *A. inornatum* and *D. variabilis*, which are ticks reported to parasitize humans in Texas (233, 234). *A. inornatum* is found in the Mexican border States of Coahuila, Nuevo León, and Tamaulipas (142, 235). Blood donors from Texas were seropositive for *B. duncani*, but the vector and reservoir host species remain to be determined (236). Notably, four adult *A. americanum* and one adult *D. variabilis* PCR positive for *Babesia* spp. were removed from humans in Texas (231). One of the *A. americanum* was infected with a large *Babesia* molecularly resembling a large *Babesia* detected in an immunocompromised dog residing in Texas (237). Nevertheless, the zoonotic potential of the other *Babesia* infecting *A. americanum* remains to be determined.

Global change is altering the distribution of ticks and tick-borne diseases globally, and the South Texas–Northeast Mexico transboundary region is not immune to this process (13, 198, 238). Environmental drivers for the emergence of HB as a public health concern in other parts of the world seem to be at play in the Texas–Mexico border (219, 231, 232). Assessing the incidence of HB accurately requires knowledge of the rates for human-tick contact and infection in the vector population. However, HB is considered to be under-reported, even in states with a surveillance program and areas where the disease is known to be endemic (201). Diagnosis can be complicated because of co-infection with *B. burgdorferi* and *Plasmodium* spp. (239, 240). It remains to be determined if co-infection with *B. burgdorferi* and *Babesia* spp. affecting humans occurs in populations of *I. scapularis* inhabiting ecosystems spanning the US–Mexico border. However, the presence of potential vectors and reservoirs indicates that studies are required to determine if zoonotic Babesiosis is an unrecognized cause of illness among humans in that transboundary region. Tick-based surveillance has been proposed as an alternative approach to assess infection risk because it provides a more sensitive method for identifying areas where Babesiosis could be emerging, and could be used to estimate zoonotic prevalence in established areas (218). The adaptation of this strategy, with an international perspective and in the context of the One Health concept is suggested, as it was done recently for LD, to establish an

[2]http://www.census.gov/hhes/www/poverty/data/incpovhlth/2012/tables.html

early warning system for the emergence of HB in the Texas–Mexico border region (13, 241).

Leishmaniasis

Leishmaniasis is a vector-borne disease caused by *Leishmania* species of the family Kinetoplastidae, which is transmitted by sand flies of the genus *Lutzomyia* in the Americas and *Phlebotomus* in other regions of endemicity (242, 243). There are 98 countries where *Leishmania* is endemic, with the majority of cases occurring in developing nations (244). The distribution of competent vector species and leishmaniasis has expanded over the last decade as areas with suitable habitat for sand flies continue to increase due in part to shifts in climate (14). Of the 1.6 million new cases per year estimated to occur worldwide, approximately 600,000 are recorded (245). Moreover, leishmaniasis is estimated to affect about 12 million people in four continents (Africa, Americas, Asia, and Europe) (245, 246).

The leishmaniases have been divided in two main syndromes: Old World, and New World leishmaniasis (247). Old World leishmaniasis includes two clinical presentations: cutaneous leishmaniasis, which is confined to skin, and visceral leishmaniasis, which involves the bloodstream and inner organs. New World leishmaniasis' clinical presentation can manifest in a cutaneous form, or as a mucocutaneous syndrome, which involves mucous membranes in addition to the skin (245, 246). Presently, new terms are used to describe the clinical forms of leishmaniasis. The term mucosal leishmaniasis indicates the involvement of mucosal tissues such as mucous membranes of the upper respiratory tract and oral cavity, i.e. mucocutaneous leishmaniasis (246). Together with the cutaneous and diffuse cutaneous forms the mucocutaneous syndrome is one of the typical presentations of leishmaniasis in South America (248).

Twenty-one *Leishmania* species have been identified as human pathogens. They are systematically classified in four complexes. In the New World leishmaniasis is caused by species belonging to the subgenus *Leishmania* [such as *Leishmania (Leishmania) mexicana*, *L. (Leishmania) amazonensis*] and the subgenus *Viannia* [*L. (Viannia) braziliensis*, *L. (Viannia) panamensis*, and *L. (Viannia) guyanensis*] (246, 249). In Mexico and US, cutaneous leishmaniasis is caused by a number of *Leishmania* spp. with widespread distributions and a variety of location-specific reservoir species (242). Numerous species causing cutaneous leishmaniasis have been identified in multiple mammalian species. *L. mexicana* is found from Central America to the Yucatan peninsula in Mexico, and cases have been reported in Texas (250). In the Old World, *Leishmania major* is a predominant cause of cutaneous Leishmaniasis.

In Mexico, the first clinically documented records of cutaneous leishmaniasis were from forested parts of the Yucatan Peninsula (251, 252). Until 1989, only eight cases of visceral leishmaniasis were reported; all of them were in the Balsas River basin, which includes the States of Guerrero, Puebla, Morelos, and Oaxaca (253). In Chiapas State, the first case was documented in Tuxtla Gutiérrez in 1990. An increase in cases in several municipalities was observed in subsequent years. From 1990 to 2006, 89 cases of American visceral leishmaniasis were reported in Chiapas State (254). In the US, human cases ($n = 30$) of non-travel-related (or autochthonous) disease have been reported since 1903 in the epidemic focus located in South-Central Texas. In 2008, nine cases of non-travel-related cutaneous leishmaniasis in Northern Texas, specifically in suburbs and smaller towns near the Dallas-Ft. Worth metro area, were reported (255). Subsequently, four cases of autochthonous cutaneous Leishmaniasis were described in Northeastern Texas and Southeastern Oklahoma (256).

Several *Leishmania* species are transmitted zoonotically, and in the case of *L. infantum*, dogs are the main reservoir. In many settings, dogs may serve as a link between sylvatic and domestic cycles of visceral leishmaniasis. Dogs can cross forest-edge boundaries, thereby potentially bringing parasites to or from sylvatic systems and to and from other potential mammalian hosts (such as foxes, rodents, and opossums) (242). In Yucatan, Mexico, the prevalence of *L. mexicana*, *L. infantum*, and *L. braziliensis* in dog sera ($n = 218$) was 30.2, 11.9, and 8.2%, respectively (257). Antibody based prevalence of 10.5% for *L. mexicana* and 11.57% for *L. braziliensis* has also been reported in cats (258). Vertical transmission of leshmaniasis has been characterized for dogs and people, causing an increased risk for infants born to parasitemic mothers (259). There have also been a number of non-travel-associated reports of cutaneous leishmaniasis in companion animals in Texas (250). Many of these cases of zoonotic cutaneous leishmaniasis were in cats, which may be associated with an outdoor life-style (250). Until recently, visceral leishmaniasis was thought to be primarily an imported disease in North America. Infected dogs had usually been imported from regions in Southern Europe or South America where *L. infantum* and *L. chagasi* were enzootic (260, 261). Additional risk factors for humans are related to their immunologic status and their ability to clear infection or maintain an asymptomatic state. These factors include concurrent infection with HIV, co-infections with helminthic parasites, drug abuse, and other immunosuppressive conditions (262).

A serosurvey conducted in the US looked at over 12,000 foxhounds and other canids, as well as 185 people in 35 States, to determine geographic extent, prevalence, host range, and modes of transmission. This study showed that foxhounds infected with *Leishmania* spp. were present in 18 States. However, no evidence of infection was found in humans (263). While companion animal infection and transmission occur, the predominant sylvatic reservoir in Texas is the Southern Plains woodrat, *Neotoma micropus* (264). Given the presence of sand fly vectors throughout the Southern US, it is possible that disease rates associated with *L. mexicana* infection will increase in the US due to climate change (14). South American species causing cutaneous leishmaniasis, including *L. amazonensis*, *L. braziliensis*, *L. guyanensis*, and *L. panamensis*, have sylvatic reservoirs (242). Thus, human risk factors for zoonotic cutaneous leishmaniasis are dependent upon exposure to vector species and the presence of reservoir species. Urbanization and wilderness encroachment have resulted in increased interactions between humans, reservoir, and vector species and the establishment of peri-urban domestic life cycles rather than sylvatic ones (242).

In Mexico, *Lutzomyia olmeca olmeca*, *Lu. cruciata, Lu. shannoni, Lu. panamensis*, and *Lu. ylephiletor* have been incriminated as vectors of *Leishmania* spp. (14, 265). In Northern Mexico and US, sand fly species suspected of being involved in *Leishmania*

transmission to humans are *Lu. diabolica* and *Lu. anthophora* (266, 267). *Lu. diabiolica* is suspected of being a vector of *L. mexicana* and has been infected experimentally with *L. infantum*. In addition, *Lu. anthophora* was able to transmit *L. mexicana* experimentally to mice (268, 269). *Lu. shannoni* is a possible vector of *L. infantum*, and is present in the Midwestern, Southern, and Southeastern US (263). The sand fly vectors of *L. infantum* causing visceral leishmaniasis in Mexico include *Lu. longipalpis* and *Lu. evansi* (270). Visceral leishmaniasis cases have been reported in the States of Chiapas, Guerrero, Puebal, Oaxaca, morelos, and Veracruz where dogs are considered to be disease reservoirs (270).

The pathogenic landscape for leishmaniasis in the transboundary region remains to be fully understood (271). In Mexico and the US, the risk for leishmaniasis has been associated with forest habitat like pluvial rainforest and agricultural fields close to the forest where reservoir mammals share habitat with humans. However, the incidence of leishmaniasis is increasing in domestic habitats as a direct consequence of the spreading of sand fly vectors to urbanized areas, especially the outskirts of cities (272). Moo-Llanes and collaborators (271) studied the current and future niche of North and Central American sand flies and concluded that continued landscape modification and future climate change will provide an increased opportunity for the geographic expansion of sand flies and increased risk for human exposure to *Leishmania* infection. The One Health paradigm could also be applied to enhance our ability to recognize *Leishmania* spp. in humans, domestic and wildlife reservoirs, and sand fly vectors in the US-Mexico transboundary region (273).

Chagas

Chagas is a zoonosis caused by *Trypanosoma cruzi*, a protozoan parasite that is present in a variety of mammalian reservoirs. This disease is one of the most prevalent parasitic diseases in the world (274) and kills around 45,000 people annually (275). The parasite is transmitted by species of insect vectors, commonly known as kissing bugs, belonging to the sub-family Triatominae in the family Reduviidae (276, 277). During the blood meal, the Triatomine kissing bug defecates and sheds the parasite in the feces. The parasite then enters its host through the bite wound or through contact with mucous membranes. The parasite can also be transmitted through blood transfusion, organ transplants, ingestion of infected food, or congenital transfusion (277). However, 85–96% of *T. cruzi* transmission to humans occurs via contact with infected feces from Triatomine insects (278). The acute phase of the disease is rarely recognized since cases are typically subclinical and asymptomatic. Chagas disease can then enter the chronic phase, in which 30% of cases will develop a fatal cardiomyopathy around 10–30 years post-infection (274, 279). Anti-parasitic treatment is mostly effective during the acute phase, and in infants and individuals up to 15 years old, although the currently accepted therapeutic options have limited efficacy and can have disabling side effects. Current research includes efforts to develop a vaccine for Chagas (11).

Chagas is endemic in the Southern US and Latin America, where it affects more than 10 million people (280) and it is spreading rapidly to non-endemic areas (276). It is considered a NTD in Texas (11), and Chagas could have been present in hunting-gatherer native Americans as far back as 1200 years ago, as described in a case of megacolon found in a mummified ancient resident of what is now known as Texas (281, 282). Studies conducted in US blood donors have demonstrated that *T. cruzi* seropositive donors have persistent infection with demonstrable parasitemia long after acquisition of infection (283). In Texas, for example, there are an estimated 267,000 people infected (284, 285). This is only an estimate based on small sero-prevalence studies and risk modeling. The exact risk for infection and the number of Chagas cases in Texas is unknown (277). In January 2013, Chagas became a reportable condition in Texas, which is a critical step toward documenting cases and understanding the epidemiology of this critical NTD.

There are several Chagas endemic areas in Mexico, including the States of Yucatán, Chiapas, Guerrero, Oaxaca, Jalisco, Veracruz, Puebla, Hidalgo, and Morelos where the disease occurs mainly in rural areas (286). The highest prevalence was observed in the Northeastern region of the country, which corresponds to the central area of a tropical region comprising the States of Hidalgo, San Luis Potosí, Veracruz, and the US neighbor State of Tamaulipas (287, 288). Recent cases of Chagas have been reported in Coahuila (289, 290) where *T. cruzi* infection has been found also in blood donors (290). There have been new records of *T. gerstaeckeri* and *T. rubida* in Nuevo Leon and Coahuila (291). As in the US, *T. cruzi* is increasingly transmitted through blood transfusions partly due to migration from rural areas toward Mexico City (292). More than 180 domestic, synanthropic, and wild species of mammals, especially rodents and marsupials, are likely to be infected with *T. cruzi* and to be involved in the disease transmission cycle (293). In the Yucatan peninsula, the anti-*T. cruzi* antibody prevalence in dogs and cats were determined to be 14.76 and 4.21%, respectively (258, 294). In addition, an active canine *T. cruzi* transmission cycle with severe symptoms affecting a broad range of dog breeds and age groups was observed in several counties in Texas (295). Further, in regions of Central Mexico, studies have demonstrated that canine sero-prevalence is directly correlated to human sero-prevalence, demonstrating the importance of this host as a sentinel species (296). Serosurveillance in shelter dogs was found useful as a public health tool to assess the risk for *T. cruzi* infection (297).

Currently, 40 species of triatomine insects are known to be naturally infected with *T. cruzi* in North America. Twenty-eight species are found exclusively in Mexico, and eight are shared with the US (298). Considering the vectorial transmission capacity and widespread distribution in Mexico, important species include *Triatoma barberi*, *T. dimidiata*, *T. phyllosoma*, *T. longipennis*, *T. mazzottii*, *T. pallidipennis*, *T. picturata*, *T. mexicana*, and *T. gerstaeckeri* (299, 300). *T. gerstaeckeri* and *T. sanguisuga* are the most common triatomine species in the Southern US and might be involved in *T. cruzi* transmission. Different reports have revealed information about the major vectors in endemic areas of Mexico (287). In the State of Veracruz, the main recognized vectors are *T. dimidiata* and *R. prolixus*, and three vector species, *P. rufotuberculatus*, *R prolixus*, and *T. dimidiata*, have been identified in the State of Chiapas. Two important vectors in the Southern region of the State of Mexico are *T. pallidipennis* (97.4%) and *T. dimidiata* (2.6%), and 28.0% of the triatomines in that region were infected

with *T. cruzi* (299). Studies conducted in rural communities of Yucatan, Mexico found that 21.9% of *T. dimidiata* (23.9% of adults and 13% of nymphs) were infected with *T. cruzi* (301).

An accepted strategy to control and prevent Chagas disease is through the management of triatomine insect infestations in domestic areas based on government and population surveillance programs (302). These programs have been successful in South America, including Mexico. However, a surveillance program aimed at controlling triatomine vectors and preventing Chagas disease has not been attempted in the US, perhaps due to the lack of data on human cases and vector-parasite distribution. Triatomine insects can be found in domestic and sylvatic life cycles, which could make efforts to control insect infestations difficult in domestic habitats due to the continuity of insect populations (303).

Recent models of risk assessment have identified Hidalgo and Cameron counties as the areas of highest risk for human infection of Chagas disease in Texas (277). Hidalgo County has suitable climatic conditions for the vector (304). This risk factor could be compounded by a high poverty rate among the border population and substandard housing. In Hidalgo County alone, almost 130,000 people live in unincorporated residential areas known as *colonias*. Much of this population is characterized by low incomes (<$10,000 per year) and poorly constructed residences with substandard sanitation and drainage systems, which are landscape characteristics that provide suitable habitat for Triatomine insects (11, 277). Hidalgo and Cameron County form part of the Lower Rio Grande Valley (LRGV), one of the main points of entry for immigrants from Latin America, mainly Mexico. Since Chagas disease is endemic to tropical areas, it is possible that migrants can carry the disease into the US via the LRGV (11). Because Chagas became a reportable disease in Texas in 2013, it can be argued that sufficient epidemiologic data are lacking to implement a scientifically sound disease control and prevention program yet. To be effective, a management program to control and prevent Chagas disease in south Texas will probably need to be combined with government and population-based surveillance of insect infestation. Important challenges to overcome include a lack of knowledge among the local population about Chagas disease and how to identify insect vectors.

Climate change could drive enhanced transmission of *T. cruzi* (12). A potential Northern shift from current range due to climate change could occur with two of the most common triatomine *T. cruzi* vectors in the Southern US (*T. gerstaeckeri* and *T. sanguisuga*). Furthermore, an increase in temperature may have influenced the behavior of triatomine species (305, 306). When temperature exceeds 30°C and humidity is low, the insects increase their feeding rate to avoid dehydration. In addition, in domestic life cycles, when indoor temperatures increase, the insects may develop shorter life cycles and reach higher population densities (305). High temperatures could also speed up the development of *T. cruzi* in vectors (307).

Trypanosoma cruzi has three infective forms capable of infecting its host and currently six DTUs (discrete typing units) are recognized in the taxon. These DTUs correlate with mammalian hosts specific interactions in distinct time-space scales. We know relatively little about confirmed mammalian *T. cruzi* hosts in the US. More studies are needed to produce a comprehensive list of confirmed *T. cruzi* hosts as well as time-space scales for the operative interactions of hosts, vectors, and parasites. To better understand the epidemiology of Chagas disease in the Texas–Mexico transboundary region, ongoing research could focus on detecting *T. cruzi* infection status of vectors, potential role of the different reservoirs and hosts in the parasite cycles, and DTUs identification (12).

CONCLUDING REMARKS

The threat of zoonotic diseases to human and animal populations in the Mexico–US border along the Rio Grande is documented here. Attention is called to a gap in understanding of the pathogenic landscape for zoonotic vector-borne diseases in this transboundary region. Among other things, research on ecosystem processes, land use, and human behaviors is required because the region analyzed functions as a pathway for the movement of humans and animal migrations, and thus links Central America/Mexico with the US and Canada. The One Health approach for international collaboration on veterinary and public health research is proposed to generate the knowledge base that can translate into strategies to mitigate the risk of zoonotic diseases in the US–Mexico border.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fpubh.2014.00177/abstract

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Influenza: environmental remodeling, population dynamics, and the need to understand networks

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NEW CHALLENGES FOR PUBLIC HEALTH

Emergence of new pathogens has been the reality of the 21st century; the role of animal reservoirs and changes in human behaviors may be the main key factors for disease dynamics. Human population growth and territory expansion have lead to habitat sharing between human beings, domestic animals, wild animals, and their pathogens; bringing new opportunities for spill over. Risk assessment regarding the main factors associated with potential reassortment and transmission between species should get to a stage where the analysis of wildlife networks and interactions with domestic animals and human beings is well mapped. This last will allow an accurate prevention and control of hot spots of influenza transmission (1, 2).

The challenge for all public health professionals lies upon the integration of the analysis of environment, animal reservoirs, and human population as a whole and develop action plans accordingly to their interactions and not each of them separately (3–5).

New animal production systems and human population dynamics have lead to different sources of infection that are not well understood. Although surveillance systems have improved considerably, the need for better communications and relations between environmental, health, and agricultural sectors is essential for a precise prevention of disease appearance and dispersal. Interdisciplinary and Intersectorial help becomes imperative when assessing the risk factors; such as human interactions with animals, wildlife contact with animal production systems, and environmental remodeling that contribute to disease emergence (6). In addition, the assessment of hot spots of influenza transmission should be the tool to map animal habitats that are at most risk of encounters with domestic animals that might serve as a mixing vessel and as the source of infection for humans (7, 8).

The emergence of influenza viruses is just one example of many diseases that have social and environmental factors that enhance their appearance and dispersal. The new strains that have emerged have social and environmental issues in common, which contribute to the appearance of new viruses, or at least, to the spillover between species; and it is here where the efforts should focus (9, 10).

MULTICASUALITY, NETWORKS, AND DISEASE EMERGENCE – INTERDICIPLINARY CALL

The close interaction between human beings and animals has determined many social behaviors, food availability, and diseases present nowadays (11, 12). When humans domesticated animals they started to be in close contact not only with the animals but also with the pathogens that they hosted. Some of these pathogens may have been of low pathogenicity in animals but after they acquired the ability to infect humans, they became pathogenic and even fatal for some hosts. This last is true only for some viruses that have the molecular characteristics that allow them to jump from one species to another, where genetic rearrangements and mutations result in new strains that infect more than one host from different species (13–15).

Spill over is the term used when a pathogen acquires the ability to jump from one species to another, allowing it to move to other habitats, and finally establish within a new niche (16). Is in these new niches where animal networks should be well assessed in order to prioritize the risks areas and animals involved in the dispersal and transmission of influenza virus for instance. As a consequence, when these interactions are understood the whole marketing systems and live bird markets' (LBMs) chains can be mapped and controlled (17, 18).

Understanding of the complete commerce chain; starting with the poultry farm and ending in the LBMs have been addressed by Martin and his colleagues. This sort of studies will be enriched if the contacts between wild and domestic animals are mapped to pinpoint the hot spots of possible niches where reassortment of the virus or an outbreak might take place (17, 19).

As it has been mentioned before, environmental interactions between animals, pathogens, and human beings play a crucial role in disease dynamics and its emergence or re-emergence. However, it is not only the environmental surroundings that determine the contact of this last three: social interactions, economic activities, and food related preferences and trade, but also have an impact and should be well assessed when conducting control and surveillance actions (16, 20). For influenza viruses, there are two main facts that should be well addressed. In the first place, the role of

animal reservoirs such as migratory birds and bats and, in the second, the role of poultry farms, live animal markets, and how the animals are sold, transported, and maintained in these (16, 21, 22).

Yet the above reasons give rise to new research and partnership opportunities that will need the participation of many disciplines. For instance, these new challenges will allow the accurate integration of the one health concept in the new approach to disease prevention.

HOT SPOTS AND INFLUENZA TRANSMISSION – THE KEY FOR PREVENTION

Network dynamics both in human beings and animals will determine the new pathogens for human populations (23, 24). Since climate change, population growth, and expansion are phenomena that are the reality for this century, human and animal health professionals will need to work from a population based perspective, but this time assessing the environment in which the interactions take place. Tracking the possible strategic spots in which intervention measures can be conducted. Diseases have unique characteristics, and although they may be well understood nowadays the lesson arises when even in the 21st century, we encounter disease threats that have complex behaviors and that are caused by multicasuality (3, 25).

For instance, it will be of much use to have complete knowledge of the health status of wild animals that live close to animal production systems and/or human living areas (9, 26). Consequently active surveillance should be coupled with a better understating about animal behavior, distance traveled by the birds and/or bats, nesting, and resting sites. It will be of much use to establish if the birds are migratory or resident, and if migratory map their networks in both living sites. Lastly, not only endangered species should be treated carefully but also included in this type of studies; furthermore, sampling should be accompanied by mapping and census (27, 28).

We will be achieving the correct introduction of the one health concept into the production systems, economic chains, and disease dynamics if we sum up all the relations that intervene in the dynamic of the disease. Finally interdisciplinary work will allow for a better and broader analysis of all the risk factors that put the health status of a country at risk. Finally we will not be prepared to respond to a pandemic event until the understanding of the interactions and influence of these in disease dynamics are incorporated into the prevention measures.

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Tick-borne pathogen – reversed and conventional discovery of disease

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Molecular methods have increased the number of known microorganisms associated with ticks significantly. Some of these newly identified microorganisms are readily linked to human disease while others are yet unknown to cause human disease. The face of tick-borne disease discovery has changed with more diseases now being discovered in a "reversed way," detecting disease cases only years after the tick-borne microorganism was first discovered. Compared to the conventional discovery of infectious diseases, reverse order discovery presents researchers with new challenges. Estimating public health risks of such agents is especially challenging, as case definitions and diagnostic procedures may initially be missing. We discuss the advantages and shortcomings of molecular methods, serology, and epidemiological studies that might be used to study some fundamental questions regarding newly identified tick-borne diseases. With increased tick-exposure and improved detection methods, more tick-borne microorganisms will be added to the list of pathogens causing disease in humans in the future.

Keywords: tick-borne pathogens, public health, *Rickettsia*, *Neoehrlichia mikurensis*, *Ixodes ricinus*, *Borrelia miyamotoi*, emerging diseases

INTRODUCTION

Molecular methods, especially polymerase chain reaction (PCR), have brought huge changes to tick-borne disease research in the last two decades. A vast number of new microorganisms have been detected in ticks (1, 2), leading to an increase in reversed-disease discovery, where the microorganism is identified before its causal relationship with a disease is established (3–5). Several years can pass between the first detection of a microorganism in ticks and the first identification of a human case (4, 6). It is often unclear whether these novel tick-borne diseases were previously overlooked or if they were absent. Though molecular methods are not yet broadly used diagnostically, their increasing use in outpatient settings, as well as hospital settings, will improve the chance to identify novel tick-related microorganisms as causative agents of disease in future. There is a call for research on the growing lists of both new tick-related microorganisms with unknown pathogenicity and novel tick-borne pathogens for which the ecology, epidemiology, and full clinical picture are unknown, to elucidate their impact on public health.

DISCOVERY OF TICK-BORNE DISEASES

REVERSED DISCOVERY OF TICK-BORNE DISEASES

Modern molecular methods enable the exploration of bacterial and viral communities in ticks without needing culturing. Since the 1990s, many studies have identified microorganisms in ticks that are distinct from known pathogens but cluster genetically with them. Frequently encountered bacteria in ticks with (initially) unknown pathogenicity are relatives of *Anaplasma*, *Bartonella*, *Ehrlichia*, and *Wolbachia*, and an increasing number of *Rickettsia* species (1, 2, 7). Now, we try to identify diseases caused by known microorganisms instead of looking for microorganisms causing known diseases (8, 9). This has led to an increase in pathogens about which only a few case reports exist and the disease burden – including clinical picture, severity, and incidence – is unclear.

Examples of reversed-disease discovery include *Neoehrlichia mikurensis*, *Borrelia miyamotoi*, and some *Rickettsia* species. *N. mikurensis* was discovered in 1999 in ticks by PCR and has since been reported in several countries (7, 10–12). Since 2010, serious diseases in immuno-compromised patients and mild disease in otherwise healthy individuals were associated with this bacterium (4, 13–16). Similarly, *B. miyamotoi* was found to cause disease in patients with febrile illness, Lyme, or anaplasmosis-like diseases years after it had been detected in ticks (5, 17–22). *Rickettsia helvetica* and *Rickettsia monacensis* had also first been identified in ticks before disease cases were linked to them (3, 23). These examples show that tick-borne infections can go unnoticed for various reasons. They might resemble known diseases or be overlooked due to non-specific symptoms. Furthermore, a lower disease incidence, due to a low exposure rate or due to a small susceptible population, can contribute to delayed discovery.

Ongoing developments in the field of next generation sequencing will deliver more sequence data of microorganisms in ticks (24). From this pool of microorganisms with unknown pathogenicity, more tick-borne pathogens could arise.

CONVENTIONAL DISCOVERY OF TICK-BORNE DISEASES

In contrast to reversed-disease discovery, conventional disease discovery starts with the identification of cases and the correlation with tick bites is recognized afterwards. This is facilitated if one or several of the following properties characterize the illness:

serious disease course, temporal, or geographic clustering of cases or illness with characteristic symptoms (often a rash). These properties facilitate case definitions and epidemiological source tracing, thereby linking disease, tick-bite, and pathogen to a full etiological picture. Subsequently, the list of symptoms linked to the specific syndromes might be expanded, as demonstrated by the example of Lyme borreliosis.

In modern history, first correlations between tick bites and disease were observed around the turn of the last century (25). The first recognized tick-borne disease in humans was Rocky Mountain spotted fever (RMSF) (25), which drew attention since 1870s due to its high fatality rate, geographic and temporal clustering, and economic impact (26). Howard T. Ricketts identified the tick vector and the pathogen responsible for the disease (25, 26). Similarly to RMSF, tick-borne encephalitis (TBE) was identified in Russia due to the temporal clustering of cases that initiated an intensive search for the pathogen. The virus was isolated in 1937 (27). There have been severe cases with fatality rates between 1 and 40% depending on the subtype (28). Currently, the most commonly recognized tick-borne disease in humans is Lyme borreliosis, caused by members of the *B. burgdorferi* s.l. complex. Lyme borreliosis lacks the high mortality of RMSF and TBE but its typical rash, erythema migrans (EM) was recognized by Arvid Afzelius and other dermatologists in Europe in the early twentieth century (29, 30). There is a long list of differential diagnostics for other symptoms associated with Lyme borreliosis, including neurological, skeletomuscular, cardiac, and skin conditions (31). Therefore, the complete clinical spectrum of Lyme borreliosis was not recognized until 1970s, when an unusually high incidence of arthritis was observed in a small geographic area of the US (32). Tick-borne phleboviruses are the most recent pathogens identified following the conventional discovery route (33, 34). The first tick-borne phlebovirus was discovered in China after a small cluster of cases with thrombocytopenia and leukocytopenia provoked active surveillance for additional cases, identifying 285 patients. Cases were clustered in rural areas and a tick-borne etiology was soon suspected. The agent was then identified through metagenomic analysis of patient samples and later also detected in ticks (33). More examples for conventional discovery of tick-borne diseases are given in **Table 1**.

FROM NON-PATHOGENIC TO ESTABLISHED PATHOGEN

Microorganisms detected in ticks can have different implications for human health. Some have not been shown to cause disease in humans while others are established human pathogens. Non-pathogenic microorganisms detected by molecular methods in ticks include tick endosymbionts, commensal bacteria, and residual DNA from earlier blood meals (24, 41, 42). Established pathogens include agents such as *R. rickettsii*, TBE, and *B. burgdorferi* s.l., which are well described and known to cause disease.

When tick-borne diseases are identified following the reversed course of disease discovery, they progress from the category of non-pathogens to pathogenic microorganisms. However, as information about ecology, epidemiology, and clinical picture are initially lacking, further research is necessary to confirm pathogenicity, incidence, and geographic distribution. Currently, a number of novel tick-borne microorganisms fall in this category, including *R. helvetica*, *N. mikurensis*, and *B. miyamotoi* (34). The public health relevance of such suspected tick-borne pathogens is unknown and should be one of the key objectives of further studies. Some of these novel tick-borne pathogens might be involved in yet unexplained disease following tick bites or acute or chronic inflammation without known cause. To assess actual health impact and relevance, a causal relationship needs to be confirmed by a strong line of evidence, for example, following Koch's postulates (**Box 1**). Case definitions have to be established and prevalence of disease needs to be estimated.

METHODS FOR THE DISCOVERY OF NOVEL TICK-BORNE PATHOGENS AND THE ESTIMATION OF THEIR PUBLIC HEALTH IMPACT

Estimating the disease burden of novel tick-borne diseases and microorganisms with unknown pathogenicity should be the focus of research in this area. This requires studies on many levels.

Table 1 | Selection of tick-borne diseases in humans and characteristics associated with their discovery.

Disease	(Suspected) Pathogen	Disease first reported	Characteristic symptoms[a]	Temporal/ geographic clusters	First isolated from	Diagnostic tests[b]	
Rocky Mountain spotted fever	*Rickettsia rickettsii*	1896	Yes	Yes	Humans	Yes	(25)
Relapsing fever	*Borrelia hermsii, B. duttonii*	1904	Yes	No	Humans	Yes	(35)
Mediterranean spotted fever	*R. conorii*	1910	Yes	No	Humans	Yes	(36)
Lyme (erythema migrans)	*B. burgdorferi* sensu lato	1912	Yes	No	Humans	Yes	(29)
Tick-borne encephalitis	TBE virus	1937	Yes	Yes	Humans	Yes	(37)
Human babesiosis	*Babesia microti, B. divergens*	1969	No	No	Livestock	Yes	(38, 39)
Lyme (whole syndrome)	*B. burgdorferi* sensu lato	1977	No	(Yes)	Humans	Yes	(32)
Anaplasmosis	*Anaplasma phagocytophilum*	1994	No	No	Livestock	Yes	(40)
Rickettsiosis	*R. helvetica*	1999	No	No	Ticks	No	(3)
Neoehrlichiosis	*Neoehrlichia mikurensis*	2010	No	No	Ticks	No	(4)
Lyme-like illness	*B. miyamotoi*	2011	No	No	Ticks	No	(5)

[a] *Characteristic symptoms do not need to occur in all patients with the infection.*

[b] *Commercially available diagnostic tests for the specific age.*

Box 1 | Tick-borne diseases and Koch's postulates.

Providing evidence for a causal relationship between a tick-borne microorganism and a certain disease can be challenging. About 120 years ago, Jakob Henle and his student Robert Koch formulated three postulates to help prove a causal relationship between an infectious agent and a disease. If the following points are met, it can be concluded that the parasite has a causal relationship with the disease in question [freely translated from Ref. (43)]:

1. The parasite is found in every case of the disease in question, in circumstances under which it can account for pathological changes and the clinical course of the disease.
2. The parasite is not found in any other disease as a fortuitous and non-pathogenic parasite.
3. After complete isolation from the body and grown repeatedly in pure cultures, the parasite is again able to produce the disease.

To meet the postulates, the agent must be culturable and cause the same disease invariably in a new host (human or experimental animal). However, many pathogens, including some novel tick-borne microorganisms, cannot fulfill these premises or lack suitable animal models (44). Koch was the first to identify asymptomatic carriers of a pathogen and was thus aware of these limitations (43, 45) that also restrict the applicability of his original postulates for tick-borne pathogens. However, several alternatives have been formulated (46–49). The postulates of Fredericks and Relman (48) rely on sequence-based detection of pathogen DNA in tissue samples. Making no absolute statements, they emphasize the importance of higher amounts of DNA and higher incidence of DNA detection in cases compared to controls, while the DNA load should fall or rise with disease resolution or recurrence. Evans formulated several premises that should be met, including epidemiological measures (e.g., higher disease incidence in those carrying an organism), host response (e.g., serology), and effectiveness of preventative measures (46). Other authors acknowledge the value of direct visualization of infectious agent, strain differences, serology, epidemiology and, especially, combinations of these (46, 47). A conclusive line of evidence for the causal role of an infectious agent in a specific disease supported by classical or alternative postulates would be ideal, but might not be realistic for some of the novel tick-borne pathogens in the near future.

Information about the ecology of novel tick-borne diseases, including the vector, natural cycle, and reservoirs of the microorganism can help to identify high-risk regions and populations. In the long run, this information could also be helpful in identifying counteractions such as culling reservoir animals (if compatible with nature conservation efforts) or other ways to reduce tick density. Data about the epidemiology will help to identify peak periods, estimate disease incidence, and the overall public health impact of novel tick-borne pathogens (50). Finally yet importantly, knowledge about symptoms associated with novel tick-borne pathogens and knowledge about risk factors provide health practitioners with tools to identify potential cases. Identifying cases is important to request appropriate diagnostic tests and initiate appropriate treatment and request appropriate diagnostic tests. This in turn might help epidemiological data collection.

IDENTIFICATION OF POTENTIAL PATHOGENS IN TICKS AND POSSIBLE PITFALLS

Microorganisms in ticks are most commonly detected and identified by PCR and direct sequencing. 16S rDNA library and next generation sequencing methods have also been used (24, 51–53). With decreasing costs in the future, the latter will probably gain importance and open new doors to microbial discovery. Sequencing several genes of a novel tick-related microorganism can also give a preliminary estimation of the microorganism's pathogenic potential. Some genes, such as the surface protein OspC of *B. burgdorferi* sl., might be directly linked to pathogenicity (54). However, with novel microorganisms, such associations are usually unknown. A comparison of the microorganisms' overall genetic background with that of known pathogens might help. *Rickettsia* species, for example, are plentiful in invertebrates of which only a fraction is found in vector species (55). A first evaluation based on several gene sequences can help to determine whether a novel *Rickettsial* species clusters in one of two known groups that contain human pathogens: the typhus and the spotted fever group. A *Rickettsia* species not belonging to one of these pathogen-containing groups has therefore a lower chance to be pathogenic. More advanced predictions based on whole genome sequencing are also underway and might assist in the identification of tick-borne pathogens in the future (56). However, it should be noted that molecular techniques have weaknesses, including the inability to distinguish living and dead cells and the risk of contamination or PCR artifacts from various sources. Although not yet shown for ticks, in some cases the detection of a single gene might also be due to horizontal gene transfer (57). One source of misleading PCR results was recently discovered. Eggs of a parasitic wasp, *Ixodiphagus hookeri*, can be embedded in ticks collected in the field. The eggs contain *Wolbachia* but more bacteria or viruses might be present in them and lead to misleading PCR results (58, 59).

KNOWLEDGE ABOUT ECOLOGICAL FACTORS CAN GUIDE SEARCH FOR DISEASE

Studying novel tick-borne pathogens and the diseases they cause can be facilitated by knowledge of the microorganism's ecology. Tick species that can act as vectors include generalist species that readily bite humans (e.g., *I. ricinus* and *Amblyomma americanum*) but also opportunistic species that prefer other vertebrate hosts (e.g., *Rhipicephalus sanguineus*) (60). Environmental factors can influence tick densities and the prevalence of tick-borne microorganisms and can thus influence exposure risks for humans (61–67) (**Figure 1**). Understanding the relationships between ecological factors and the prevalence of tick-borne pathogens as well as mapping densities of infected ticks can help to identify high-risk areas for human exposure. Furthermore, knowledge about natural

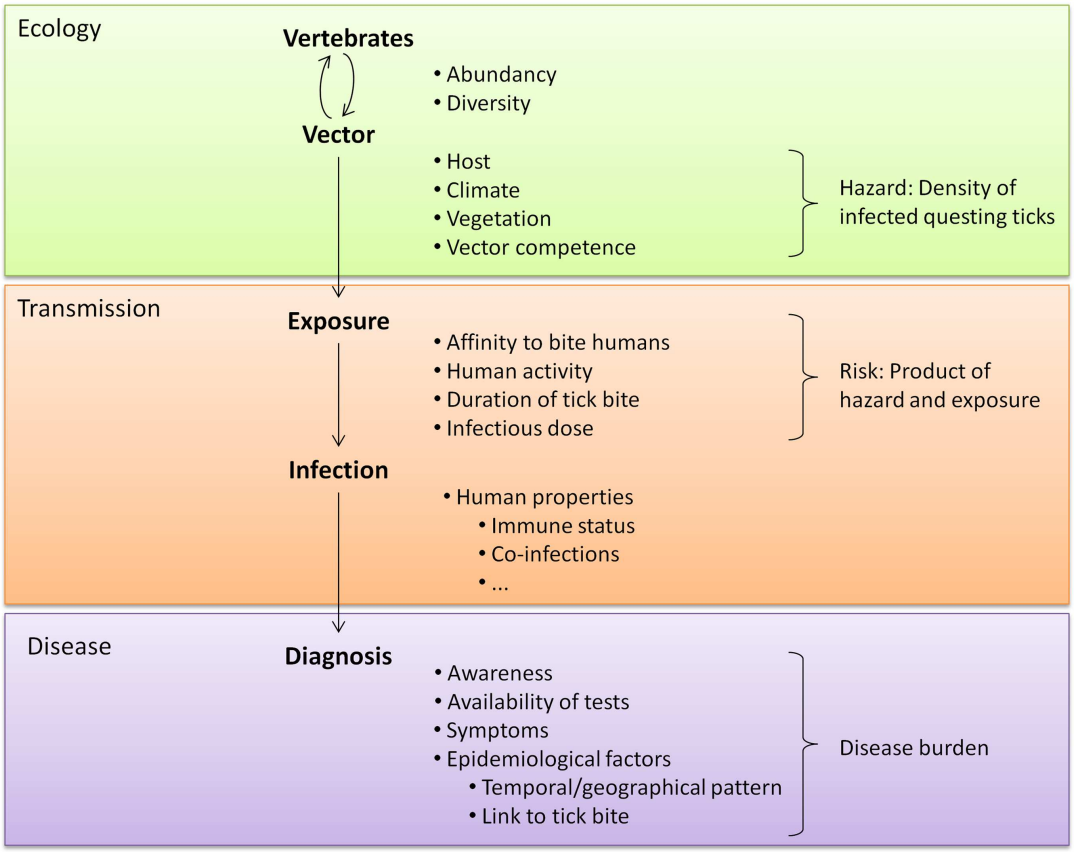

FIGURE 1 | A tick-related microorganism must take various steps to cause disease. Each step is influenced by many factors, including the characteristics of the microorganism. These characteristics affect every step of transmission and are therefore not listed separately.

cycles of vectors, hosts and pathogens might help to predict seasonal variations in pathogen prevalence (50). Even for pathogens transmitted by the same tick species, peak periods of disease cases can vary because disease incidence does not only depend on the questing activity of ticks. First of all, different tick-borne diseases can be transmitted by different tick stages (e.g., *Rickettsia* vs. *B. burgdorferi*) and these differ in their main questing period. Secondly, the infection rate of ticks with various pathogens can underlay different seasonal variations as was shown by Coipan et al. (50). However, if no data are available about seasonality of novel tick-borne pathogens, peaks in established tick-borne diseases vectored by the same tick species could indicate similar seasonal patterns of novel pathogens (50). Aligning the start and location of epidemiological studies and sampling periods of serological studies with such high-risk areas and high-risk periods could improve the chance to identify cases of a novel tick-borne disease.

SEROLOGY FOR IDENTIFICATION OF EXPOSURE AND CASES

Serological methods have a wide diagnostic window, as antibodies to a pathogen may persist for months or even years. This makes these assays valuable to investigate past exposure to tick-borne pathogens. Advantages of serology include the ease of obtaining samples and ability to detect current infections by seroconversion. Serology can be used to investigate infection with tick-borne pathogens in high-risk populations or those showing signs of disease with unknown cause. Examples of the latter are the investigation into possible rickettsial origins of chronic illness in Australian patients and of liver dysfunctions in Spanish patients (68, 69). In these studies, patient groups had a higher seroprevalence for rickettsial antigens than controls. Cochez and coworkers screened paired sera of 322 patients with suspicion of tick-borne infections for the presence of *Anaplasma phagocytophilum* antigen and found evidence of infection in about a third of the patients (70). Such investigations do not prove causal relationships of disease with a specific tick-borne pathogen but could be the key to identifying certain clinical entities.

Highly specific serology would facilitate this kind of study. However, for many tick-borne diseases, serological assays need improvement as they lack sensitivity or the specificity to differentiate between species or genotypes. For some of the newest tick-borne diseases, no serological assays are available at all. Fast development of serological assays often relies on culture for antigen production. In cases where cultures are not available, production of recombinant proteins or synthetic peptides offer an alternative (22, 71). Both require genetic information on potentially antigenic

proteins. A further bottleneck in the development of serological assays is the availability of suitable samples for validation. Especially if a microorganism is suspected to be involved in disease but positively identified cases are scarce or lacking, the availability of well-defined sera for validation purposes is limited. Disregarding the type of antigen used, the inability to distinguish asymptomatic infections from disease and lack of immune response under some circumstances limit the use of serology, as it can only be a measure for infection risk rather than disease risk (72, 73). For these reasons, the use of serology in novel tick-borne disease research is limited but can have increased value when combined with other methodologies such as molecular detection of pathogens or large epidemiological studies linking sero-conversion with clinical manifestations of disease.

MOLECULAR METHODS FOR IDENTIFICATION OF INFECTIONS

The development of serology for novel pathogens generally takes time, while PCR is often already available or can be set up quickly. Molecular methods enable testing for tick-borne pathogens for which no serological assays exist. The limitation of PCR on patient material, besides contamination risks, lies in the availability of suitable material. Tissue tropisms differ for different pathogens or clinical presentations (74). Therefore, the choice of the tissue to be tested is crucial for success, requiring a certain degree of knowledge about the pathogenesis of a microorganism. This can be derived from previous case reports, wildlife and animal studies, or tissue tropisms of related pathogens. Samples that might be available for testing include skin biopsies, tissues removed during medically required surgery, cerebrospinal, synovial fluids, and blood samples. Blood samples, being so readily available, will often be the first though potentially not always the best choice to test for tick-borne pathogens and have been successfully used (5, 75). Skin biopsies have been useful in the diagnosis of rickettsiosis, or for research purposes on various rashes, including EM (76, 77). Novel tick-borne diseases have not yet been identified by PCR on skin samples but this may change in future.

Detection of a microorganisms' DNA in a single patient does not prove a causal relationship. The microorganism might not be the causative agent of the observed disease but a mere asymptomatic co-infection. However, using molecular techniques on larger case numbers and analyzing the data according to specified parameters [e.g., with adapted Koch's postulates (48)] could help support causal relationships and formulate case definitions.

EPIDEMIOLOGICAL STUDIES TO DEFINE DISEASE INCIDENCE AND IDENTIFY CASES

Epidemiological studies to link tick bites with health outcome vary in their design from retrospective to prospective and from case–control to cohort studies. Prospective cohort studies have been performed to find associations between tick-borne pathogens and adverse health effects or serological response (78–82). Prospective cohort studies combine high precision-of-risk estimations, the ability to study several outcomes at once, and opportunity to include (molecular) data collected from ticks, if available. While past studies with 250–400 tick-bitten participants detected some Lyme borreliosis cases, they failed to identify cases caused by other pathogens that might have a lower incidence (either in due to lower prevalence in ticks or lower infectivity) (81, 82). This highlights the drawbacks: success of cohort studies depends on the size of the study population, the fraction of exposed individuals, and the frequency of the expected outcome in exposed individuals. Consequently, cohort studies are less suitable for uncommon pathogens or those with a low pathogenicity, unless the cohort is very large. Studies focusing on syndromic surveillance and diagnosis of high-risk patients, ideally coupled with case–control studies to identify causal factors, would be more suitable to detect rare tick-borne diseases. Identifying a patient group with symptoms or laboratory findings matching earlier case descriptions increases the chance of detecting novel tick-borne pathogens in patients (21). Focusing on patients with tick-exposure history or on areas with a high prevalence of the pathogen will further increase the chance of identifying cases. Identifying individual cases is crucial to answering some questions concerning novel tick-borne diseases, such as the full clinical picture and risk factors. In contrast to individual case studies, epidemiological studies can supply valuable data to help risk estimation and disease burden of newly identified tick-borne diseases.

SUMMARY

Tick-borne disease research has changed greatly since the age of molecular detection methods. An increasing number of novel tick-related microorganisms are being identified and this evolution will continue in future due to the increasing availability of new sequencing methods. Isolated cases of human diseases caused by novel tick-borne microorganisms can suggest that a microorganism is pathogenic but they do not provide sufficient proof of a causal relationship. A causal relationship of a novel pathogen with a disease would be supported by the use of Koch's postulates (**Box 1**).

The rigid criteria used in the original postulates might not be suitable, though, in which case modern adaptations of the postulates can demonstrate a causal role (48, 49). Such alternative postulates can rely on serology, molecular diagnostics, or epidemiology. Novel tick-borne pathogens could play a role in diseases with currently unknown etiology, such as chronic fatigue, skeletomuscular, and neurological symptoms (5, 20). They might also explain treatment-resistant symptoms in patients diagnosed with other tick-borne diseases (20). Knowledge about a pathogen's ecology could be used to guide such studies by identifying high-risk areas and populations. Ecological knowledge might also be useful to educate the public and take measures to reduce the density of infected ticks. Efforts to increase awareness among medical health professionals, providing diagnostic tools (case definition, serology, PCR, etc.) and recommending effective treatment options will further help to diagnose and treat cases.

The incidence of some tick-borne diseases showed an increase or fluctuations throughout recent decades due to various factors, mainly associated with increased tick-exposure (82–84). This upward trend might extend to newly identified tick-borne diseases as well, as these also depend on tick-exposure. People with co-morbidities are more likely to develop (severe) disease following infection with tick-borne pathogens and this sensitive group is

growing due to current medical and sociological developments (4, 21). It is likely that even in the healthy population many cases caused by novel tick-borne pathogens go unnoticed. The real number of cases could therefore be significantly higher than currently apparent. The actual incidence needs to be determined to help estimate public health impact.

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Predicting tick presence by environmental risk mapping

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Public health statistics recorded an increasing trend in the incidence of tick bites and erythema migrans (EM) in the Netherlands. We investigated whether the disease incidence could be predicted by a spatially explicit categorization model, based on environmental factors and a training set of tick absence–presence data. Presence and absence of *Ixodes ricinus* were determined by the blanket-dragging method at numerous sites spread over the Netherlands. The probability of tick presence on a 1 km by 1 km square grid was estimated from the field data using a satellite-based methodology. Expert elicitation was conducted to provide a Bayesian prior per landscape type. We applied a linear model to test for a linear relationship between incidence of EM consultations by general practitioners in the Netherlands and the estimated probability of tick presence. Ticks were present at 252 distinct sampling coordinates and absent at 425. Tick presence was estimated for 54% of the total land cover. Our model has predictive power for tick presence in the Netherlands, tick-bite incidence per municipality correlated significantly with the average probability of tick presence per grid. The estimated intercept of the linear model was positive and significant. This indicates that a significant fraction of the tick-bite consultations could be attributed to the *I. ricinus* population outside the resident municipality.

Keywords: lyme, risk mapping, ticks, Borrelia

INTRODUCTION

Borrelia burgdorferi s.l. is the bacteria that causes Lyme disease in humans. In Europe, the main vector is the tick *Ixodes ricinus*. In the Netherlands, Lyme disease is on the rise; there has been a threefold increase in consultations of general practitioners (GP) for tick bites and Lyme disease since 1994 (1). This rise can be partially explained by spatiotemporal increases in the abundance and activity of questing ticks, as the total area suitable for tick persistence including forest areas expanded in the Netherlands (2). The concomitant increase in these time series data sets indicates that tick activity might be explained based on environmental factors.

Risk mapping was used to predict the spatial distribution of tsetse flies in Africa based on environmental factors (3). The methodology also accurately delineated the areas of mosquito presence, both regionally and globally (4). It was the modeling tool of choice for identifying the distribution of malaria (5), tick-borne encephalitis (6), blue tongue epidemic in Europe (7), and Lyme disease in Belgium (8). While all surveillance targets a specific microorganism involved in a specific infectious disease, the algorithms, and the satellite images in the risk mapping methodology are generally applicable in a broad range of infectious disease contexts, including Lyme disease in the Netherlands. A complex interplay of vegetation, climatic conditions, and vertebrate hosts determine where the disease-transmitting vector, *I. ricinus*, can maintain its lifecycle. Nymphal and adult ticks for example, start questing after the winter season once the daily maximum temperatures during a week exceeds 7°C on average (9). Vegetation provides different degrees of shelter for ticks. Satellite images of vegetation and climatic variables are expected to provide necessary information to identify tick suitable areas.

I. ricinus requires three blood meals (choosing from a plethora of warm and cold blooded vertebrates) to complete their life cycle. Larvae feed primarily on small animals while nymphs and adults preferably feed on larger vertebrates such as hare and deer (10). The abundance of ticks greatly depends on the abundance of feeding and propagation hosts (11, 12). Here, regional roe deer population densities are utilized to identify the presence of *I. ricinus*, in addition to the satellite images.

Experts on ticks are able to estimate the tick density and activity for a particular land type. It is possible to quantify this prior knowledge. The methodology, expert elicitation, has been applied to a food-risk-assessment study (13). We applied the method to estimate the tick densities and activities for heterogeneous Dutch land surfaces, and used the estimates as Bayesian priors in our analyses of the field data.

Standard blanket dragging (14, 15) is the method to collect ticks searching for a blood meal. Although the blanket fails to catch molting, resting, and feeding ticks, it is currently the best method for measuring public health risk. Here, we predicted the presence of *I. ricinus* based on the field surveillance, satellite images, and host population densities. The number of tick-bite consultations by GP in the Netherlands (16) is a measure of tick presence, independent of field surveillance. The tick-bite consultation statistics

are an empirical input into the analysis to validate the predicted tick presence.

MATERIALS AND METHODS

DATA COLLECTION

Sampling of I. ricinus

Ticks were collected by blanket dragging (blanket 1 m × 1 m) at 677 distinct coordinates in the Netherlands between April 2000 and September 2013 (the full list of coordinates is available on request). The dataset in this analysis includes data described in three publications. First, the study conducted each month from April to October in the period 2000–2004 at forest, dune, heather, and City Park (17). Second, the study conducted each month from April to September in the period 2000–2008 at vegetation-rich dune, City Park, heather, and forest areas (18). Third, the study conducted each month from July 2006 to December 2007 at forest areas (19). The rest of the dataset consists of three additional tick collections. Firstly, a study conducted each month from April to July in the 3-year period 2011–2013 at randomly generated sampling coordinates over the whole nation. Only incomplete and scarce information is available about the flora and fauna present at these areas. Secondly, the studies conducted in June, July, and August in the 2-year period 2012–2013 at City Parks, forest, and the ground adjacent to a walking path. Thirdly, the studies conducted in June 2012 and in May, June, July, and September 2013 at dune, heath, and forest areas. Ticks were dragged over 200 m^2 after which an average per 100 m^2 was calculated. The sum of nymphal and adult ticks, the two active life stages of *I. ricinus*, was recorded into the database. A sampling coordinate is assigned a state "tick is absent" if the sum is below or equal to a set threshold (default zero), and "present" otherwise.

Satellite images

We downloaded the satellite imageries from the MODIS ftp site (20). Tile h18v3 covers the Netherlands. Satellite images from the period January 2005 to June 2012 were downloaded and used for subsequent analysis in this study. **Table 1** summarizes the images used.

To all satellite images and all following spatial maps, a water mask and a mask removing neighboring countries has been applied.

Roe deer population densities

Estimates on the regional roe deer population density per square kilometer (2008) was extracted from the roe deer database (Royal Dutch Hunting Association).

Soil moisture

Soil moisture maps were calculated by the hydrological bureau *FutureWater* by means of the hydrological SPHY-model on a spatial resolution of 250 m × 250 m (21). The soil moisture fraction is the result of the hydrological budget equation, with precipitation and upward seepage as incoming fluxes. Output fluxes are evaporation, run-off, drainage from root zone and sub-zone, and downward seepage. Furthermore, percolation and capillary rise are taken into account.

MATHEMATICAL ANALYSIS

Our aim is to classify pixels as either suitable for ticks (an event denoted C^+) or not suitable for ticks (C^-). This classification is made using the data collected in the form of maps. The absence–presence data of the ticks, with the values of the maps at their locations, constitute the training set. For the satellite images, we have many images per year. In order to obtain a manageable data set that retains some of the seasonality, we employ a Fourier analysis. The classification is based on a quadratic discriminant analysis (QDA), aided by a Bayesian inclusion of expert opinion data.

Fourier analysis

A Fourier analysis is a technique for decomposing a signal into oscillating components; we follow the exposition as detailed in Ref. (22), chapter 7.7, and also Ref. (4). In the current context, the signal is the time series of the satellite image at a pixel. Each of these components represents a cosine with a certain frequency, and has phase and amplitude coefficients. An efficient method for performing a Fourier analysis is the "fast Fourier transform" (FFT). It applies only to equidistant data points, and thus we linearly interpolate the signal to daily values. We excluded time series with more than 20% missing values (e.g., due to cloud cover), and set the pixel to the symbolic value "NA," this pixel does not contribute to the model any more.

For all other pixels, we extract the phase and amplitude of the yearly oscillation (emulating seasonal effects), the half-yearly oscillation, and the bi-yearly oscillation. Finally, we include the average, which has no phase, only amplitude. In total, the 8-year signal is now represented by seven components.

Quadratic discriminant analysis

Since the method of QDA is not widely used, we will outline the method briefly. The derivation is based on Ref. (23).

After the Fourier transform procedure, we evaluate the k Fourier components at each pixel of each satellite image, at the

Table 1 | Summary of the MODIS products used.

Name	Data	Short name	HDF layer	Resolution	Time granularity
EVI	Enhanced vegetation index	MOD13Q1	2	250 m^2	16 days
DLST	Daytime land surface temperature	MOD11A1	1	1 km^2	1 day
NLST	Nighttime land surface temperature	MOD11A1	5	1 km^2	1 day
MIR	Middle infra red	MCD43A4	7	250 m^2	16 days

See also the online resource https://lpdaac.usgs.gov/products/modis_products_table. Briefly, EVI is a measure of vegetation density, DLST and NLST measure temperature, and MIR is a measure of vegetation regrowth rate.

n presence points and at the m absence points. This yields vectors $x_1^+, \ldots, x_n^+$, each $x_j^+ \in R^k$ containing all Fourier coefficients taken at presence location j. Analogously for the absence points, we have vectors $x_1^-, \ldots, x_m^-$. We assume that each vector is a realization of a multivariate Gaussian distribution, one distribution for the presence points, and one for the absence points. Let X denote a vector of Fourier coefficients, then,

$$X|C^+ \sim N\left(\mu^+, \sum^+\right)$$
$$X|C^- \sim N\left(\mu^-, \sum^-\right)$$

We construct the matrix $X^+ \in R^{k\times n}$ by column wise concatenation of the vectors $x_1^+, \ldots, x_n^+$. Similarly, we define for the absence points a matrix $X^- \in R^{k\times m}$. We use these matrices for calculation of the estimators $\hat{\mu}^+$, $\widehat{\sum}^+$, and $\hat{\mu}^-$, $\widehat{\sum}^-$ of the means and covariance matrices.

Let f^+ and f^- denote the corresponding probability density functions. Now at a new point $x \in R^k$, corresponding to a location where no tick presence or absence observation is available, we wish to determine the probability of tick presence. Using Bayes' theorem, and the symbolic notation $P(C^+)$ for the probability of belonging to the positive group, we may write

$$P\left(C^+|\, X = x\right) = \frac{f^+(x)\, P\left(C^+\right)}{f^+(x)\, P\left(C^+\right) + f^-(x)\, P\left(C^-\right)} \qquad (1)$$

Under the assumption of common covariance matrices between groups, $\Sigma^+ = \Sigma^- \equiv \Sigma$, and uninformed priors $P(C^+) = P(C^-) = 1/2$, we arrive at linear discriminant analysis by checking if

$$\log\left(\frac{P\left(C^+|X=x\right)}{P\left(C^-|X=x\right)}\right) = \left(\mu^+ - \mu^-\right)^T \sum^{-1} x + \frac{1}{2}\left(\mu^+ + \mu^-\right)^T \sum^{-1} \left(\mu^+ - \mu^-\right)$$

is below or above zero. Thus, the decision boundary is a hyperplane, and the classifier is linear in x. We do not make these assumptions, and work with separate covariance matrices for absence and presence. Furthermore, we use expert opinions for the prior probabilities. Instead of a classifier based on probability ratios, we work with the QDA probability of presence, given by

$$P\left(C^+|x\right) = \frac{P\left(C^+\right)|\sum^+|^{-\frac{1}{2}} e^{-\frac{1}{2}D^2(x,C^+)}}{P\left(C^+\right)|\sum^+|^{-\frac{1}{2}} e^{-\frac{1}{2}D^2(x,C^+)} + P\left(C^-\right)|\sum^-|^{-\frac{1}{2}} e^{-\frac{1}{2}D^2(x,C^-)}}$$

with the Mahalanobis distance defined by

$$D^2\left(x, C^+\right) = \left(x - \mu^+\right)^T \left(\sum^+\right)^{-1} \left(x - \mu^+\right)$$

We estimate the mean and covariance matrices by the sample mean and covariance as stated above. A straightforward implementation of the algorithm would be computationally expensive, as the number of covariates and the number of pixels are both large. However, by diagonalizing the covariance matrices, and some further time saving application of linear algebra [detailed in Ref. (23)], the calculations simplify tremendously. Next, observe that

$$D^2\left(X, C^+\right) = \left(X - \mu^+\right)^T \left(\sum^+\right)^{-1/2} \left(\sum^+\right)^{-1/2} \left(X - \mu^+\right)$$

Thus, since conditional on C^+, X is normally distributed, observe that $Z = (X - \mu^+)^T(\Sigma^+)^{-1/2} \sim N(0,\ 1)$ and $D^2\left(Z, C^+\right) = Z^T Z = \sum_{i=1}^n Z_i^2$. Since the sum of n standard normal random variables is distributed as χ_n^2, we have a criterium for prediction uncertainty by comparing D^2 to a set percentile of the chi-squared distribution with n degrees of freedom. We exclude pixels below this percentile, and set them to a symbolic "no-prediction" value. Those pixels will be colored as white in the figures. We use a default of 90%, but evaluate other settings in the supplementary material.

Expert elicitation

Thirteen individuals were selected based either on their affinity to tick research or on their affiliation to landscapes where ticks are expected to be found (e.g., experts from forest services). We asked the experts a probable range of questing nymphal plus adult tick densities per 100 m^2 for a number of specific landscape types. The Netherlands is partitioned into 39 land types (24). The experts were asked to provide a range between 0 and 200 ticks per 100 m^2. At the start of the elicitation, we instructed an expert that the questionnaires contain one or more questions in order to calibrate the participant's expertise.

We represent the answer of expert i to question q by the vector, $v_{i,q} = (p_0, \ldots, p_{200})$ where p_j is the probability that the questing nymphal and adult tick density equals j. We set

$$P_{i,q,j} = \begin{cases} (b_{i,q} - a_{i,q} + 1)^{-1} & j \in [a_{i,q}, b_{i,q}] \\ 0 & \text{otherwise} \end{cases}$$

where $[a_{i,q}, b_{i,q}]$ is the response of expert i to question q.

First, we determine a weight for each expert by calibration using their answers $p'_{i,q}$ to control questions. Control questions are the measurements on the densities of nymphal plus adult ticks (H. Sprong, personal communication) in the following land types: salt water (0 ticks per 100 m^2), corn (<2 per 100 m^2), heather (2–20 per 100 m^2), and deciduous forest (20–200 per m^2). We represented a control question by the vectors c_1 to c_4. We determine a weight for each expert by,

$$W_i = \prod_{q=1}^{4} p'_{i,q} \cdot c_q$$

then normalizing the weight over all experts $w_i = \frac{W_i}{\sum_{j=1}^n W_j}$ (we use the lower case letter for the normalized weights). This is a strict weighting, which invalidates an expert who gave an answer disjoint to one of the control questions. Responses from the experts

to the actual questions were weighted by the w_i and combined to obtain an empirical probability density function for the number of ticks,

$$P\left(\text{tick density } = j \,|q\right) = \sum_{i=1}^{n} w_i p_{i,q,j}$$

Finally, from this probability we construct the Bayesian prior, by setting a threshold t to distinguish absence and presence (very low numbers may be accidental, non-endemic, presence),

$$P\left(C^+|q\right) = 1 - \sum_{j=0}^{t-1} P(\text{tick density } = j|q)$$

Our default threshold is $t = 1$ but we evaluate other scenarios in the supplementary material. For each pixel, we determine the land-use type, and use the corresponding $P(C^+ \mid q)$ as a prior.

Independent support using EM consultation statistics

Estimation of the number of tick-bite consultations in the Netherlands was as described elsewhere (1). In short, we use Dutch GP consultation data over 2009, regarding erythema migrans (EM), an expanding skin lesion occurring after several days or weeks at the site of the tick bite. This dataset was aggregated on a municipality level, and we used it to validate our predicted presence of ticks. For this purpose, we changed the spatial unit in our prediction to a municipality by averaging the probability of tick presence over all 1 km by 1 km grids enclosed by the municipality boundary. We only include pixels that were significant at the 90% level. We applied a linear model and calculated the P-value for the slope being significantly different from zero.

RESULTS

OBSERVED ABSENCE AND PRESENCE OF *I. RICINUS*

Ticks were sampled at 677 distinct geographic coordinates (**Figure 1**). Sampling was conducted only once at the majority of sampling coordinates and up to three repeated samplings at few sampling coordinates. Ticks were present at 252 distinct sampling coordinates: one tick (either nymphal or adult stage) or more were found on the blanket at these sampling coordinates. Ticks were absent at 425 distinct sampling coordinates. Some distinct sampling coordinates fell into the same pixel, and aggregating the sampling coordinates by pixel resulted in 177 presence pixels and 163 absence pixels. In one absence pixel, *I. ricinus* was absent at all sampling coordinates within the pixel.

PRIOR TICK PRESENCE PROBABILITIES PER LAND-USE TYPE

We applied expert elicitation to estimate a Bayesian prior for the probability of presence of nymphal plus adult ticks per unit area of a specific land type. The prior probability of tick presence was less than 0.5 at 12 land types, mainly cultivated areas and vegetation-poor grounds. Prior probability of tick presence was greater than 0.5 at 25 land types of a wider variety (**Figure 2**).

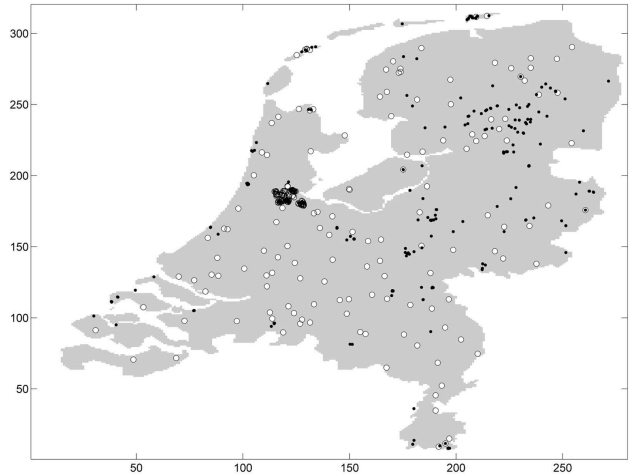

FIGURE 1 | Absence (white circle) and presence (black dot) of ticks in the Netherlands.

ESTIMATES ON TICK SUITABLE GRIDS

The blanket dragging, covering 100 m^2 each, at 677 distinct coordinates during our multi-year surveillance for *I. ricinus* is approximately equal to 7 ha of the investigated area in total. For the remaining land surface (99.98% of the total land surface), the presence of *I. ricinus* needs to be extrapolated from the outcomes of the sampling coordinates. Classifying all the sampling coordinates into either presence or absence, we estimated the probability that *I. ricinus* is present, for each 1 km by 1 km square grids enclosed by the nation border (**Figure 3**). Summing all the 1 km by 1 km grids by the weighs of the presence probabilities, we estimated that total tick suitable area is 20,698 km^2. By counting pixels, we estimate the total land surface of the Netherlands as 35,001 km^2. Hence, an estimated 54% of the land surface meets the conditions for maintaining the tick life cycle.

INDEPENDENT SUPPORT USING EM CONSULTATION STATISTICS

The number of tick-bite consultations by GPs in the Netherlands (16) is an alternative measure of tick presence, independent of field surveillance by the blanket-dragging method. Hence, the estimated probability of tick presence is expected to correlate positively with the consultation statistics. **Figure 4** shows the aggregation of EM incidence and predicted risk to the municipality level. To assess linear correlation we also performed a linear regression at this municipality level (**Figure 5**). The linear relationship between our prediction and the consultation statistics was positive (slope 137) and significant (P-value <0.001). The estimated intercept of the linear model was also positive (95) and significant (P-value <0.001).

ALTERNATIVE SCENARIOS

In order to assess the impact of modeling choices, we ran the model with other parameter settings, and an alternative corroborating scenario. Firstly, we varied the number of ticks below which a sampling occasion is marked as negative. The rationale for this is that a very low number of ticks may indicate ticks that are

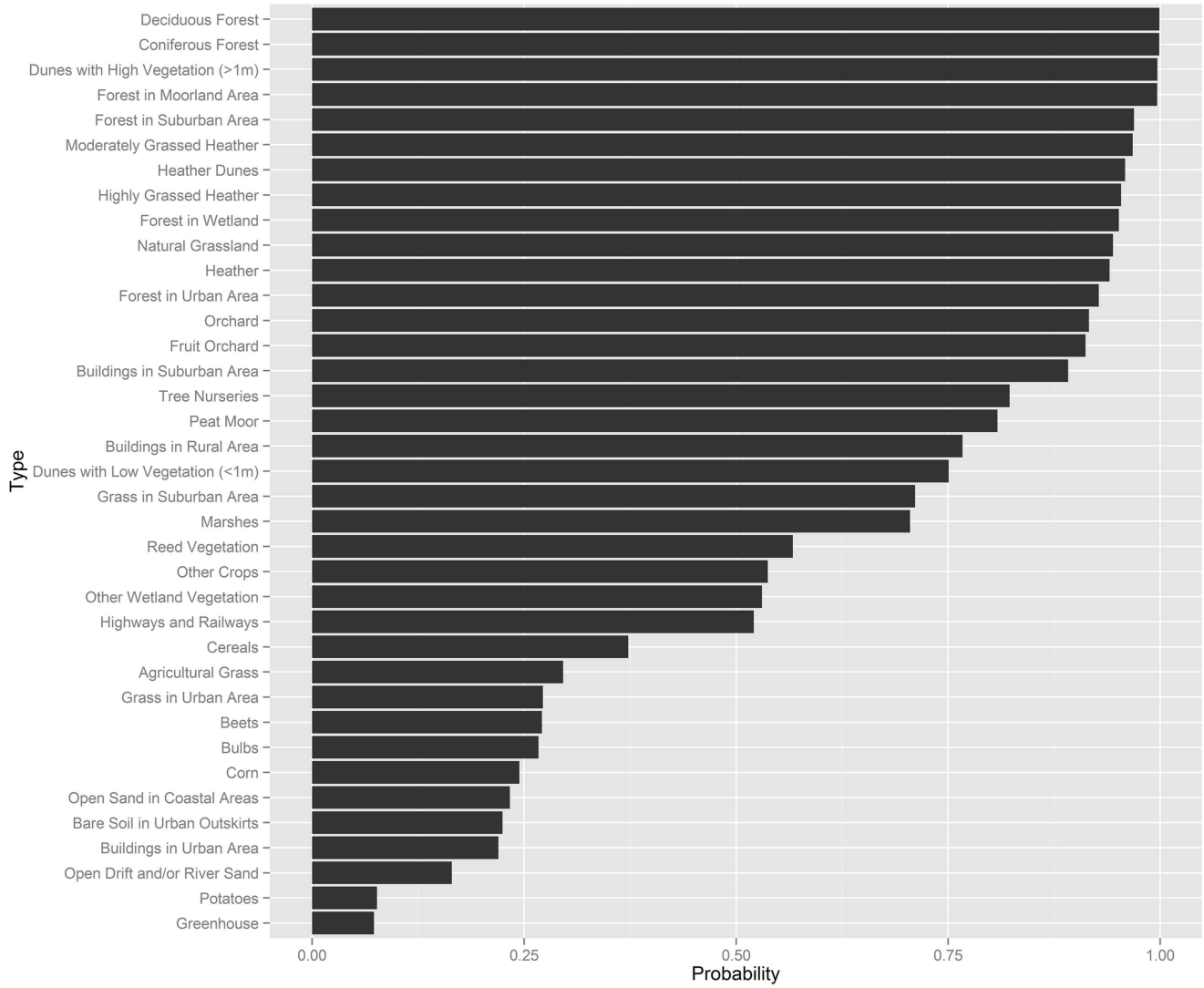

FIGURE 2 | Summary of the expert elicitation, showing the probability of tick presence for several land-use types.

not from an established population, but rather ticks that accidentally ended up in a tick-free area. Secondly, we varied the cutoff for the value for "no prediction." Table S1 of the supplementary material gives the results in terms of the *P*-value of the comparison with EM-cases. The tick presence threshold of zero is clearly superior. For the no-prediction cutoff, it seems that even very low cutoff values still yield good results. This is probably since the validation is at the municipality level, while inclusion or exclusion due to "no-prediction" is at the pixel level. Misclassifications may cancel out by this procedure. A second alternative scenario set consisted of replacement of the EM notifications by tick-bite notifications (with, or without EM). This yields the results as given in Table S1 of the supplementary material. We observe *P*-values much lower than for tick-bite consultation, indicating that tick presence correlates better with EM consultations than with tick bites in general. Figures associated to these tables may also be found in the supplementary material.

DISCUSSION

We applied a quadratic discriminant model (QDA) for predicting suitability for ticks, based on environmental covariates. The model was trained using a set a tick absence/presence points. Probabilities of tick presence were averaged over each municipality of the Netherlands, with the aim to validate the prediction with an independent measure of tick presence: estimated numbers of consultations of EM per municipality. The estimate for the intercept of the linear model was positive and significant. We observe that even in the municipalities where the mean probability of tick presence is near zero, tick bites were recorded, with an average of 95 consultations per 100,000 residents (the intercept of the linear model, **Figure 5**). In municipalities where the mean probability of tick presence is close to one, tick-bite consultations reached 232 consultations per 100,000 residents, almost a tripling compared to the municipalities where the mean probability of tick presence is predicted to be almost zero. We interpret the intercept as cases that

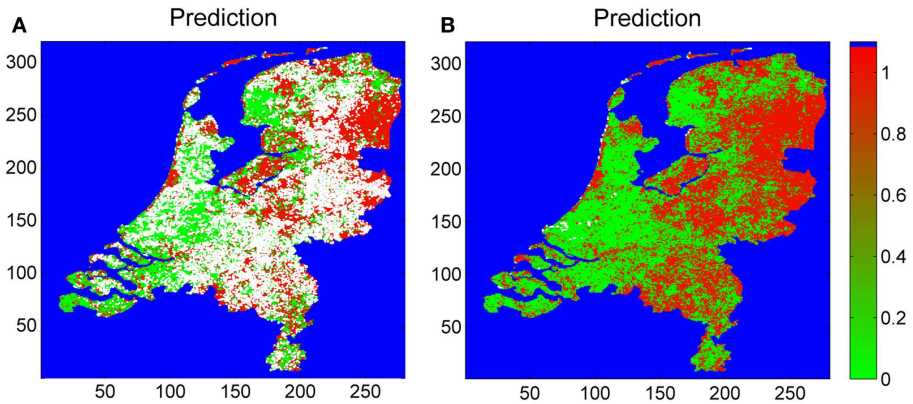

FIGURE 3 | Estimated map of tick presence based on the sampling coordinates (Figure 1), roe deer densities, soil moisture, and the satellite images: EVI, NLST, DLST, and MIR. The risk is indicated by colors ranging from 0 (green, no risk) to 1 (red, maximum risk). White pixels indicate "no prediction." Pixels not within Dutch land surface (e.g., water bodies) are indicated in blue. The leftmost **(A)** has the "no-prediction" pixels censored; the rightmost **(B)** shows all predictions.

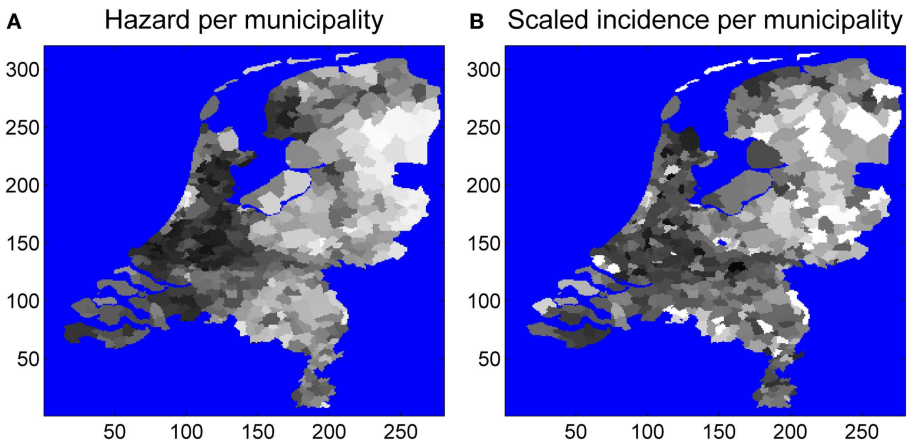

FIGURE 4 | EM consultations (A) and tick presence per municipality (B). Averages were taken over all pixels in a municipality, and numbers were scaled between zero and one.

obtained their tick-bite outside of the municipality of residence; hence this part of the risk of a tick-bite might not be explained by local risk factors. The increase over the entire range of the risk of a tick bite, 137 per 100,000 residents can be explained by the local risk factors. Roughly speaking, our model explains 2/3rd of the risk of a tick bite.

This study is a first attempt to map tick presence in the Netherlands using environmental and biological factors. The tick-bite incidence independently supports our predictions. Nonetheless, a high variability in incidence of tick bites per municipality remains un-accounted for by the presence of ticks only. To reduce the high variability, the methodology implemented in this study could be extended by considering additional biological and social factors that are missing in our current approach.

First, a potentially better proxy for the Lyme-disease incidence than the tick-presence would be the density of infected ticks. The density of infected ticks is equal to the sum of densities of larval, nymphal, and adult ticks weighted by instar-specific prevalence estimates of *Borrelia burgdorferi*. We leave tick abundance as an option to investigate at a later stage. The analysis will be more involved, since tick abundance will vary greatly over the year.

Next, as small mammals are important for the *Borrelia* life cycle their population density is a potential biological factor that might reduce the variability. However, nationwide estimates on local population densities for any specific rodent species in the Netherlands are currently unavailable. Lastly, an inclusion of social factors in our methodology (e.g., human activities) might help to reduce the high variability in predicted disease incidence. An example is an exposure map indicating where people are likely to receive a tick bite. A source of information regarding human activities is an on-going study in the Netherlands in which any person can report the location where they received a tick bite in a past day by visiting a website (www.tekenradar.nl).

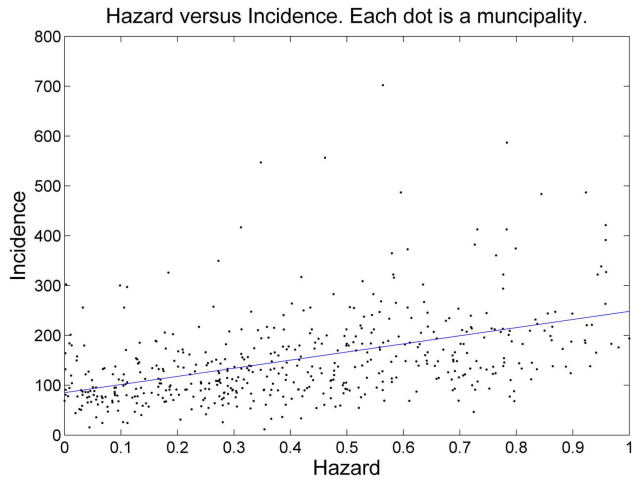

FIGURE 5 | Scatterplot of risk (*x*-axis) versus incidence (*y*-axis). The blue line is the linear regression line.

Note that in the current study, we do not attempt any model selection. In principle, using for example cross-validation, we could compare the explanatory power of models with different sets of satellite data. Also, techniques exist that assess the importance of individual variables within fixed models. However, this falls out of the scope of our current paper, which was simply to demonstrate that a riskmap may be constructed, which has good correspondence to independent incidence data. In the future, we hope to further pursue model selection methods.

It is common to set up surveillance solely for catching and identifying the disease-transmitting vector species. Due to this common practice, a statistical methodology to estimate the vector distribution necessarily assumes pseudo-absence, a set of geographic coordinates at which the vector is assumed absent. For us, it was straightforward to eliminate the pseudo-absence; we requested our trained volunteers to report absence when no *I. ricinus* tick attached to the blanket. Absence of *I. ricinus* on a blanket was recorded more than 400 times in our field surveillance database.

Empirical observations of absence have limitations. An absence record indicates that either: (1) the tick *I. ricinus* was absent at the sampling coordinates, or (2) by chance the blanket-dragging failed to catch any *I. ricinus*. An estimate on the fraction of false absence records in our database is lacking, but the most sensible interpretation of the hundreds of absence records in our database is the former explanation. We expect furthermore that the statistical algorithm is robust to a small fraction of false absent signals in our surveillance database. A positive and significant correlation with the independent indicator of tick presence, i.e., tick-bite consultations, further corroborates that this potential artifact in the data collection procedure is a marginal limitation in this study.

Bayesian prior probabilities of the tick presence were estimated from expert knowledge on all major land types in the Netherlands. Effects on the predicted *I. ricinus* distribution, however, were not visibly present. We might infer from this observation that our model contains at least as much information as the prior distribution. To test this hypothesis, we ran the riskmapping model with only the expert elicitation data, and used this as the only input. We find that the significance of the correlation with human cases is strongly reduced, but still highly significant (P-value <0.0001). Also, the risk is highly clustered around one, and the number of no-prediction points has grown to high numbers.

Concerning the modeling approach, we opted for QDA, a robust and proven algorithm for unsupervised classification. Alternatives are certainly possible. For example, logistic regression may be used to predict binary outcomes. However, since in logistic regression the logit of the probability of presence is modeled by a linear function, we expect the QDA algorithm to outperform logistic regression. Also, for future work, state-of-the art techniques like boosted regression trees, or random forests are promising candidates for classification.

In summary, we identified large-scale areas in the Netherlands where environmental conditions are likely to be suitable for maintaining the *I. ricinus* life cycle. An independent proxy for the tick presence, estimates on the number of tick bites on humans (1), is consistent with our identification based on satellite images and the host population densities. In conclusion, we presented a validated statistical approach to identifying areas where the human's exposure to the Lyme-disease transmitting vector *I. ricinus* is expected to be high.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fpubh.2014.00238/abstract

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Ixodes ricinus and its transmitted pathogens in urban and peri-urban areas in Europe: new hazards and relevance for public health

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Tick-borne diseases represent major public and animal health issues worldwide. *Ixodes ricinus*, primarily associated with deciduous and mixed forests, is the principal vector of causative agents of viral, bacterial, and protozoan zoonotic diseases in Europe. Recently, abundant tick populations have been observed in European urban green areas, which are of public health relevance due to the exposure of humans and domesticated animals to potentially infected ticks. In urban habitats, small and medium-sized mammals, birds, companion animals (dogs and cats), and larger mammals (roe deer and wild boar) play a role in maintenance of tick populations and as reservoirs of tick-borne pathogens. Presence of ticks infected with tick-borne encephalitis virus and high prevalence of ticks infected with *Borrelia burgdorferi* s.l., causing Lyme borreliosis, have been reported from urbanized areas in Europe. Emerging pathogens, including bacteria of the order Rickettsiales (*Anaplasma phagocytophilum*, "*Candidatus* Neoehrlichia mikurensis," *Rickettsia helvetica*, and *R. monacensis*), *Borrelia miyamotoi*, and protozoans (*Babesia divergens*, *B. venatorum*, and *B. microti*) have also been detected in urban tick populations. Understanding the ecology of ticks and their associations with hosts in a European urbanized environment is crucial to quantify parameters necessary for risk pre-assessment and identification of public health strategies for control and prevention of tick-borne diseases.

Keywords: ticks, *Ixodes ricinus*, tick-borne pathogens, urban habitats, Europe

INTRODUCTION

Tick-borne infections are arthropod-borne diseases frequently reported worldwide. Ticks are known to transmit a great variety of pathogenic agents producing the highest number of human disease cases compared to other vector-borne diseases in Europe (1, 2). In general, the eco-epidemiology of zoonotic vector-borne diseases is very complex. It depends on the interactions of the vectors with the reservoir hosts and the pathogenic agents, which are modulated by several abiotic and biotic factors that vary in space and time. Certain tick-borne infections have recently been emerging in new regions or re-emerging within endemic sites and create an increasing concern for public health, food security, and biodiversity conservation (3–5). Global warming obviously affects the spread of tick-borne diseases, but climate alone does not determine the geographical distribution of tick species, their population densities and dynamics, the likelihood of their infection with microorganisms pathogenic for humans and animals, nor the frequency of contacts of humans and domestic animals with infected ticks (4, 6, 7). Socio-demographic factors, agricultural and wildlife management, deforestation and reforestation, are known to exert a big impact on the transformation of biotopes, thus affecting tick host assemblages as well as tick infection rates (8–10).

Urbanization as one of the socio-demographic factors has increased worldwide in recent decades (11, 12). Currently, more than half of the world's population lives in urban areas, and it is expected that 70% will live in urban areas by 2050 (13). Nowadays, more than 75% of Earth's ice-free lands show evidence of alteration as a result of human residence and land use, with less than a quarter remaining as wildlands. Europe shows the highest level of urbanization worldwide (14). Urbanization, due to restriction of natural areas, is known to dramatically change the composition of wildlife communities and affect the associated tick populations. In European cities, public parks, gardens, peri-urban

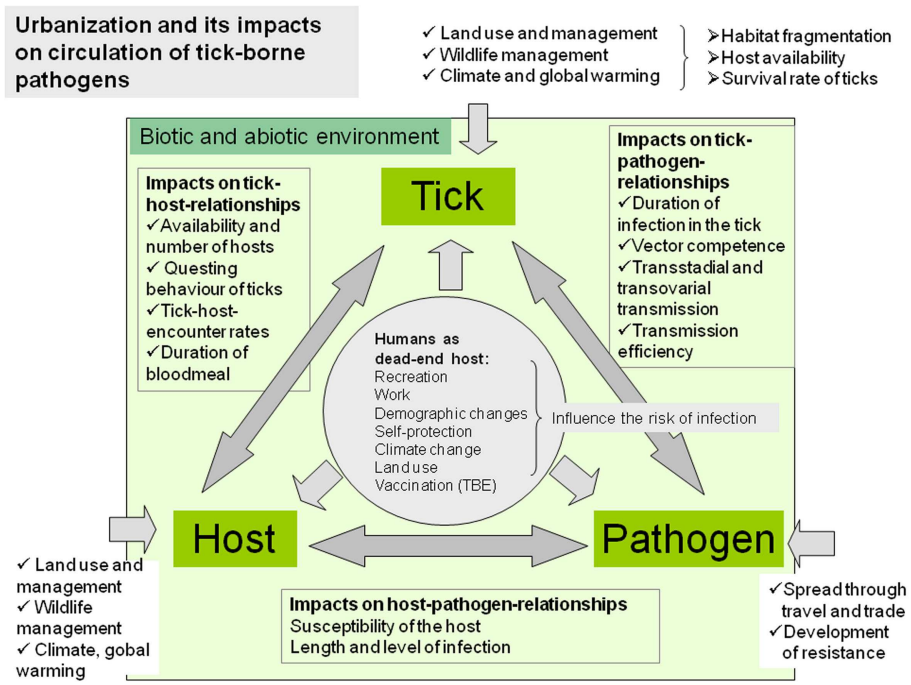

FIGURE 1 | Complex factors of the biotic and abiotic environment influence the tick–host–pathogen interaction and consequently the occurrence of tick-borne diseases in urban and peri-urban environments.

leisure-time areas, and cemeteries became particularly important places where humans and domesticated animals can encounter potentially infected questing ticks (2).

Urban areas are highly fragmented environments composed of a mosaic of patches of various sizes, vegetation, and land-use types. Urban and peri-urban habitats are generally characterized by lower biodiversity of wildlife species compared to natural ecosystems. Urbanization often produces a certain gradient of homogenization in densely built-up areas, where synanthropic species adapted to urban habitats can be found and where species richness is reduced (15). On the other hand, suburban habitats are also occupied by native species comprising medium-sized mammalian predators and ground-foraging, omnivorous, and frugivorous birds that produce abundant populations there. But urbanization can also result in variation of animal species composition, e.g., by introduction of non-native species that replace native ones (16, 17).

Majority of the wildlife species commonly found in urban and peri-urban sites can serve as tick-maintenance hosts and also as reservoirs of tick-borne pathogens (18, 19). Furthermore, the majority of these species are generalists and are able to adapt to the urban and peri-urban environment and reach higher population densities than in natural sites (12, 20, 21). In urban habitats of Europe, rodents (mice, voles, dormice, squirrels, and rats), hedgehogs, shrews, birds, lizards, and companion animals (dogs and cats), but in peri-urban areas also medium-sized and larger mammals like foxes, roe deer, and wild boars, play the major role as tick-maintenance hosts and reservoirs of tick-borne pathogens (19, 22). Adaptation of wild animals to urban environment can also lead to increased contacts with humans and to increased risk of exposure to zoonotic agents. In addition, animal populations in urban areas can show genetic differentiation from wild populations of the same species. Thus urbanization can alter the biology and population densities of ticks and hosts and may lead to increased transmission of pathogens between vectors and urban-adapted hosts (11, 23). Moreover, urbanization is followed by increased mobility of humans, intensive long-distance trade, and new contacts of humans and companion animals with nature, which may contribute to changing of epidemiological and epizootiological conditions in urban and peri-urban areas (12) (**Figure 1**).

Understanding the ecology of ticks and their association with various hosts in a changing urban and peri-urban European environment is therefore crucial to quantify various parameters necessary for the risk pre-assessment and for the identification of the best public health strategies for tick-borne disease management and prevention. The cascade of events including fluctuations in wildlife community composition and abundance, tick density and emergence, and spread of tick-borne pathogens in various habitat types in Europe are now being modeled as part of the EU FP7 project EDENext[1]. In this review, we focus on *Ixodes ricinus*, one of the principal vectors of pathogens causing arthropod-borne infections in Europe, its associations with hosts and pathogens and risk of infection of humans in urbanized areas.

[1]http://www.edenext.eu

IXODES RICINUS – VECTOR OF MULTIPLE PATHOGENS

Ixodes ricinus (Acari: Ixodidae) is the most widespread tick species in Europe and transmits several viral, bacterial, and protozoan agents of medical and veterinary importance (8, 24–28).

The distribution area of *I. ricinus* has significantly expanded over the past decades. Recently, the species can be found in more northern areas and habitats at higher altitudes than a few decades ago (29–31). Increase in abundance, habitat expansion, including urbanized areas, and prolongation of the questing activity periods of *I. ricinus*, reported in recent years, are attributed to multiple and interacting factors (19, 26, 32). They include changes in land cover and land use due to alterations in agriculture and forestry management, changes in climate, changes in abundance, and distribution of wildlife due to altering wildlife management, and shifts in socioeconomic factors affecting the rate of exposure of humans to infected ticks (25, 26).

Risk factors associated with *I. ricinus* transmissible diseases can be divided generally into: (i) those directly related to climate change (acting on the tick, the host, or their habitats), (ii) those related to changes in the distribution of tick hosts (which may be a direct or indirect effect of human intervention), and (iii) other ecological changes (also commonly influenced by human intervention) (26).

Ixodes ricinus is primarily associated with shrubs and deciduous and mixed forests, with a high abundance of small, medium, and large wild vertebrate hosts. However, as a consequence of changing land use and wildlife management, persistent tick populations and high prevalences of infections with tick-borne pathogens have also been observed in urban and peri-urban sites in many European countries (33–41). *Ixodes ricinus* is a generalist exophilic tick species that is able to feed on over 300 different vertebrate species (42). It has a long-lasting life cycle, involving three active life stages (larvae, nymphs, and adults) that quest and attach to a host and feed on blood for a few days before detachment (parasitic life period) and subsequent molting or laying eggs (females). Each developmental stage requires its specific microhabitat comprising various biotic and abiotic factors. The parasitic on-host life of *I. ricinus* is limited to 3–5 days (larvae), 4–7 days (nymphs), and 7–11 days (females) of feeding on vertebrate hosts, whereas, the non-parasitic off-host life period of all developmental stages can last for several months or years (43). This extremely complex life cycle makes the tick vulnerable to alterations in habitat structure and availability of host animals.

In urban and peri-urban areas, the requirement for high relative humidity (above 80%) for extended periods of time by the off-host stages restricts the occurrence of *I. ricinus* to city parks with litter layers, forest patches, gardens, and cemeteries (22) where the continuous use of water to maintain the vegetation also increases the relative humidity. The other limiting biotic factor for *I. ricinus* in urban environments is the availability of medium-sized and large mammals as hosts of the adults, maintaining persistent and independent tick populations. Shifts in the tick–host associations to, e.g., hedgehogs, foxes, hares, domestic dogs, or cats, due to lack of large mammalian hosts can evoke changes in *I. ricinus*-borne pathogen spectrum, prevalence, and distribution. On the other hand, populations of large animals like deer and wild boar have become more abundant in large city parks and peri-urban areas around European cities, leading to the establishment of tick populations, shift of natural transmission cycles of some pathogens, and increase of the disease risk for humans and domestic animals (19).

VERTEBRATE HOSTS OF TICKS AND TICK-BORNE PATHOGENS IN URBAN AREAS

Terrestrial vertebrate hosts are key players in the epidemiology of tick-borne diseases for at least two reasons. Firstly, they serve as maintenance hosts for ticks as a food resource and secondly, as reservoir hosts they are often responsible for the long-term maintenance of pathogens in both natural and urban habitats. Although many reports exist about the presence of pathogens in various hosts or ticks removed from them, the reservoir capacity for each of the pathogens in many cases remains to be experimentally defined. A reservoir host of tick-borne pathogens must fulfill certain criteria: (i) it must feed infected vector ticks, at least occasionally; (ii) it must take up a critical number of infectious agents during an infectious tick bite; (iii) it must allow the pathogen to multiply and to survive in at least certain parts of its body; and last but not least (iv) the pathogen has to find its way into other feeding ticks (44, 45). For this reason, the simple recording of pathogens (or nucleic acid of them) in a vertebrate host is not sufficient for classifying that host as a reservoir, but only a candidate reservoir when physiological and behavioral features may theoretically support pathogen amplification and transmission to the vector, or a simple carrier host, or a dead end host. Similarly, a higher prevalence in ticks removed from the vertebrate host compared to prevalence in questing ticks is only a good indication that the host is a candidate reservoir. However, to unambiguously prove the reservoir status of a host, xenodiagnostic experiments have to be carried out. They involve feeding of specific pathogen-free tick larvae from a laboratory colony on the tested host and the subsequent analysis of them for pathogens after their molt into the next stage. Unfortunately, for most pathogens and hosts, xenodiagnostic experiments have not been performed so far and the key hosts in the natural (and urban) cycle of tick-borne pathogens remain to be tested. The few exceptions are some species of mice, voles, rats, dormice, squirrels, and shrews (see details in **Table 1**) that had already been proven reservoirs of some tick-borne pathogens.

Urban environments represent many special ecological characters in the complex communities of pathogens, ticks, and hosts. From a public and veterinary health perspective, city parks and peri-urban recreational areas are typical meeting places for humans (their pets) and ticks. Ticks in this respect serve as a bridge for pathogens, connecting reservoir hosts with humans. In addition to the frequent and likely encounter of humans with ticks, vertebrate host communities also differ substantially in many urban habitats compared to natural settings. Some important tick-maintenance and pathogen reservoir hosts (e.g., hedgehogs, squirrels, and songbirds) have no or very few natural enemies within urban environments, thus their populations might reach significantly higher densities compared to natural ones (21, 74). Besides the lack of predators, these urbanized vertebrates can also make use of man-made structures and anthropogenic food resources, like waste and pet food. Hedgehogs are one of the most successful urban adapters reaching up to nine times higher densities in urban areas than in rural areas (74). In Great Britain, red fox density was

Table 1 | Most important mammal hosts of *I. ricinus* and pathogens transmitted by this tick species with urban or peri-urban occurrence.

Order	Species	Associated *I. ricinus* stage	Associated pathogens	Reference
Rodentia	*Apodemus flavicollis*	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 46–50)
	Apodemus sylvaticus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia spielmanii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 46, 48–52)
	Apodemus agrarius	L, N	***Borrelia afzelii*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* ***Babesia microti***	(42, 50, 53)
	Myodes glareolus	L, N	**TBEV** ***Borrelia afzelii*** ***Borrelia burgdorferi* s.s.** ***Borrelia miyamotoi*** ***Cand.* N. mikurensis** *Anaplasma phagocytophilum* *Babesia microti*	(42, 48–50, 54, 55)
	Microtus agrestris	L, N	**TBEV** *Borrelia afzelii* *Babesia microti* *Cand.* N. mikurensis *Anaplasma phagocytophilum*	(42, 49–51, 56)
	Microtus arvalis	L, N	*Cand.* N. mikurensis *Anaplasma phagocytophilum* *Babesia microti*	(53, 55, 56)
	Rattus norvegicus	L, N	***Borrelia afzelii*** ***Borrelia spielmanii***	(46, 57)
	Rattus rattus	L, N	*Borrelia afzelii* *Anaplasma phagocytophilum*	(46, 50, 57)
	Eliomys quercinus	L, N	***Borrelia spielmanii***	(46)
	Muscardinus avellanarius	L, N	***Borrelia spielmanii***	(58)
	Glis glis	L, N	**TBEV** *Borrelia afzelii*	(42, 51)
	Sciurus carolinensis	L, N	***Borrelia afzelii*** *Borrelia burgdorferi* s.s.	(42, 59)
	Sciurus vulgaris	L, N	**TBEV** *Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*	(51, 60, 61)
	Eutamias sibiricus	L, N	*Borrelia burgdorferi* s.s. *Borrelia afzelii* *Borrelia garinii*	(62)

(Continued)

Table 1 | Continued

Order	Species	Associated *I. ricinus* stage	Associated pathogens	Reference
Lagomorpha	*Lepus europaeus*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*	(50, 63)
	Lepus timidus	L, N, A	*Borrelia burgdorferi* s.l.	(63)
Soricomorpha	*Sorex araneus*	L, N	**TBEV** *Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum* *Babesia microti*	(49–51, 63)
	Sorex minutus	L, N	*Borrelia burgdorferi* s.l.	(63)
Erinaceomorpha	*Erinaceus europaeus*	L, N, A	*Borrelia afzelii* *Borrelia spielmanii* *Borrelia bavariensis* *Anaplasma phagocytophilum*	(64–68)
	Erinaceus roumanicus	L, N, A	**TBEV** *Borrelia afzelii* *Borrelia bavariensis* *Anaplasma phagocytophilum* *Cand.* N. mikurensis	(47, 64, 69)
Artiodactyla	*Capreolus capreolus*	L, N, A	*Anaplasma phagocytophilum* *Babesia venatorum*	(70)
	Cervus elaphus	L, N, A	*Anaplasma phagocytophilum*	(71)
	Dama dama	L, N, A	*Anaplasma phagocytophilum*	(71)
Carnivora	*Vulpes vulpes*	L, N, A	*Borrelia burgdorferi* s.l. *Anaplasma phagocytophilum*	(42, 72)
	Meles meles	L, N, A	*Borrelia afzelii* *Borrelia valaisiana*	(73)

Mammal species that are experimentally proven reservoirs for pathogens are in ***bold****. Borrelia burgdorferi s.l. refers to studies with no species identification (genotyping) of the spirochetes. L, larva; N, nymph; A, adult.*

found at least 10-fold higher in cities than in rural areas (75, 76). The tendency to preserve green spaces inside cities is not only a positive aspect to humans but also for many tick-maintenance and reservoir hosts (12). For these urban dwellers, well established and dense shrubbery in parks offers shelter and nest sites. Furthermore, higher temperatures, especially during winter (heat island effect), are highly beneficial (74). All these factors can lead to an unbalanced vertebrate community that easily provides favorable ecological conditions for tick and pathogen cycles.

MAMMALS

Rodents are among the most important maintenance hosts for the subadult stages of *I. ricinus* (77). Furthermore, as pointed out by a recent analysis (78), ecologically widespread, synanthropic species with high density and fast life history such as rodents are often the most competent reservoirs for multi-host pathogens. As a consequence, mice and voles are also known to be important reservoirs for several pathogenic agents like tick-borne encephalitis virus (TBEV), *Borrelia afzelii*, and "*Candidatus* Neoehrlichia mikurensis" (**Table 1**). In addition to well-established rodent populations in cities, the frequent migration of these animals between human dwellings and natural environments can easily bring infected larvae and nymphs of *I. ricinus* into gardens and houses (79). Fluctuations in rodent densities are very important factors of disease risk (24, 80) with different ecological factors affecting rodent population dynamics in different parts of Europe. However, rodent population dynamics are less studied in urban and peri-urban parks than in natural areas. Rodents can harbor different endophilic (nidicolous) tick species (e.g., *Ixodes trianguliceps* and *I. acuminatus*). These do not pose a direct human hazard since they do not feed on humans. Their co-occurrence with *I. ricinus* on the same rodent, however, can lead to an exchange of pathogens among different tick species.

Little is known about the role of rats (*Rattus rattus* and *R. norvegicus*) in the urban maintenance of ticks and tick-borne pathogens. As one of the most efficient urban adapters, despite the control actions usually undertaken, they might be involved in the urban maintenance of Lyme borreliosis (LB) spirochetes (46, 57, 77). Other urbanized rodents, like garden dormice (*Eliomys quercinus*), hazel dormice (*Muscardinus avellanarius*) (46, 58) and hedgehogs (*Erinaceus europaeus* in Western Europe and *E. roumanicus* in Central and Eastern Europe) are also involved in the urban ecology of LB (**Table 1**). Hedgehogs have not only a longer life span compared to rodents but they also have the great

advantage for ticks being able to feed not only larvae and nymphs but also a considerable number of adults (21, 81). Thus, they can easily maintain stable *I. ricinus* populations in urban areas in the long run (64).

In some cases, anthropogenic introduction of mammals into a new area can lead to the emergence of tick-borne pathogens even previously unknown for that region (12). The gray squirrel (*Sciurus carolinensis*) is native to North America, but an invasive species in the UK that has spread across the country and has largely displaced the native red squirrel (*S. vulgaris*). This species is a frequent urban dweller and can be an indirect source of human tick-borne infections since it has been experimentally shown to be reservoir for *B. afzelii* (59). Siberian chipmunks (*Eutamias sibiricus*) appeared as pets in many European countries but soon these rodents were recorded in urban parks of Rome (82, 83), Geneva (84), Brussels, and in and around many other towns (12). Chipmunks seem to be perfect hosts for subadult *I. ricinus* (85). Pisanu et al. (86) showed that these introduced rodents are more heavily infested by *I. ricinus* than native rodents such as the wood mouse (*Apodemus sylvaticus*) and the bank vole (*Myodes glareolus*). It was also found that the introduced rodent is associated with three species of *B. burgdorferi* sensu lato (s.l.), whereas, the native rodents are associated with only one species (62).

Lagomorphs (hares and rabbits) also inhabit anthropogenic landscapes and serve as blood sources for ticks (79). The European hare (*Lepus europaeus*) and the mountain hare (*L. timidus*) were shown to be not only effective tick-maintenance hosts but also reservoirs for *B. burgdorferi* s.l. (63). The European rabbit (*Oryctolagus cuniculus*) belongs to the most invasive mammalian species and its urban populations can harbor a variety of endo- and ectoparasites including *I. ricinus* (87). These lagomorphs can reach high densities and due to their ability to host adult *I. ricinus* as well, they are able to maintain an infective tick population even in urban and suburban areas where large mammals are not necessarily present. This double epidemiological function (tick-maintenance and reservoir host), which makes them key players in urban cycles of tick-borne pathogens is unique for lagomorphs and hedgehogs.

Bats can also carry different stages of *I. ricinus* ticks, thus they can also transport ticks to urban areas (88). Species especially adaptive in human dwellings, e.g., the lesser horseshoe bat (*Rhinolophus hipposideros*), can serve as tick-maintenance hosts but the role of these flying mammals in the pathogen life cycles remains to be clarified (54). Experimental TBEV viremia was shown in the greater mouse-eared bat (*Myotis myotis*), which is also a common urban inhabitant (51).

Among larger mammalian hosts, which can affect the circulation of tick-borne pathogens in peri-urban areas, roe deer (*Capreolus capreolus*), wild boar (*Sus scrofa*), and red foxes (*Vulpes vulpes*) are particularly important, because they can host all three active life stages of *I. ricinus*, and they increasingly live in urbanized areas (89, 90). Studies on roe deer abundance and movements can provide critical information for predicting tick dispersal and TBEV hazard (91, 92). Deer density is also suggested to be related to the LB incidence (31).

Tick density can be influenced by abundance and distribution of roe deer and red deer (*Cervus elaphus*) (93–95). Roe deer and red deer can inhabit a great variety of tick-infested habitats. Roe deer can even occur in some city parks, e.g., in Munich, Germany (70). Furthermore, the ability of deer to migrate more than 100 km carrying a high number of ticks is also known. This may facilitate the spreading of ticks to other areas (95, 96) and therefore potentially also of tick-borne pathogens from one area to another, although for some pathogen such as *Borrelia* spp., these species dilute the infection rate (97).

Wild boar populations have increased in Europe in recent decades and these animals are well adapted to live in urban and suburban forest areas (98). This species can provide a significant contribution to maintaining tick populations, although its role of reservoir of various tick-borne pathogens is only partially known (98, 99).

Foxes inhabit most urban areas across Europe and population increases have been seen in many European countries, e.g., in Great Britain and Switzerland (100, 101). In a recent study, *I. ricinus* was the most frequently detected tick on foxes in Germany, and all stages of this tick species were found on the animals (90). In Romania, *I. ricinus* infested almost 30% of foxes, indicating that these animals may play a significant role in the epidemiology of tick-borne diseases (102).

Urbanization largely concentrates humans in an area as well as a high number of pets (12). Among these, stray dogs represent an especially effective host for ticks, many of which are *I. ricinus* adults (103). They not only roam in large areas connecting natural and urban habitats, but they also get minimal or no treatment against ticks. Although we have limited knowledge on dogs' role in the maintenance of tick-borne human pathogens (104–106), as effective hosts for *I. ricinus* adults they certainly contribute to the size of tick populations within gardens, parks, and sub-urban areas. The estimated 100 million free roaming dogs (owned and stray) living in Europe (107) certainly need to be taken into consideration during urban surveillance and control of ticks and tick-borne diseases.

BIRDS

Birds play an important role in the introduction of ticks and associated pathogens into urban areas (108, 109). Birds, especially ground-feeding song birds, are important maintenance hosts for larval and nymphal stages of *I. ricinus*. Common urban bird species foraging mostly on the ground and low shrub vegetation, such as common blackbird (*Turdus merula*), song thrush (*Turdus philomelos*), and European robin (*Erythacus rubecula*) were shown to be frequently infested with *I. ricinus* (110–112). More specifically, migratory birds have been shown to carry ticks and pathogens to large distances (113). However, the knowledge on the role of migratory birds in favoring the introduction of ticks and pathogens within new sites is so far very limited (114). Furthermore, earlier onset of spring migration and reproduction with more active ground-feeding activity of birds in the period of questing activities of *I. ricinus* larvae and nymphs may represent an additional risk factor for TBEV spread (115, 116). A recent study highlighted that migratory bird species were infested by more ticks than residents, with urbanized birds being the most parasitized (117). Thus in case of cities being close to bird resting or breeding sites (like cities and towns located on river banks) there is a

realistic chance for the introduction and the maintenance of tick-borne pathogens (12). Birds as carriers of infected ticks probably play a role in the geographical spread of pathogens, such as *Rickettsia helvetica*, *Anaplasma phagocytophilum*, *Babesia microti*, and *B. venatorum* (118–120).

LIZARDS

Lizards have long been known as important hosts for ticks capable of feeding large amounts of immature *I. ricinus* (121) and they can often find suitable habitats in cities. In areas inhabited by lizards they can be as important tick-maintenance hosts as rodents (122, 123). Compared to rodents, however, lizards are more suitable hosts for nymphal *I. ricinus* (as shown by a lower larva/nymph ratio) (124–126). Sand lizards (*Lacerta agilis*), common wall lizards (*Podarcis muralis*), and green lizards (*Lacerta viridis*) are the most common species that can contribute to the urban maintenance of *I. ricinus* populations (122, 123, 125).

The role of lizards in the circulation of tick-borne pathogens has been underestimated compared to that of mammals and birds, but they have been proved to be reservoirs of LB spirochetes (122) and might also be involved in the life cycle of other tick-borne pathogens (124). However, experimental and field studies are needed to shed light on this epidemiological issue.

PATHOGENS TRANSMITTED BY *IXODES RICINUS*

Among the pathogens transmitted by *I. ricinus*, the western European TBEV subtype (TBEV-Eur), causing tick-borne encephalitis (TBE) (127) and spirochetes of the *B. burgdorferi* s.l. complex, the causative agents of human LB (128) have the greatest impact on human health. *I. ricinus* can also harbor bacteria of the order Rickettsiales that are of rising medical and veterinary importance. Among them, *Anaplasma phagocytophilum* can lead to granulocytic anaplasmosis in both humans and animals (50); the emerging pathogen "*Candidatus* Neoehrlichia mikurensis" can cause severe febrile illness in immunocompromised patients (129) and fever in humans without any primary disease (130); rickettsiae of the spotted fever group (SFG) (*Rickettsia helvetica*, *R. monacensis*) cause rickettsioses in humans (131). Protozoans of the genus *Babesia*, mainly *B. divergens* and *B. microti*, cause babesiosis in humans, and for *B. venatorum* pathogenicity to humans is suspected (132). The role of *I. ricinus* in transmission of *Bartonella* species (e.g., *B. quintana* and *B. henselae*) causing bartonellosis in humans is suspected (28, 133). *Francisella tularensis*, causing tularemia, and the Q fever agent *Coxiella burnetii* have also been detected in *I. ricinus*, but the role of this tick species in the epidemiology of these diseases is probably not significant (28, 133).

TICK-BORNE ENCEPHALITIS VIRUS

Tick-borne encephalitis is the most important tick-borne arboviral infection of humans in Europe and eastern and central Asia and is caused by the TBEV (Flaviviridae) (134–136). *Ixodes ricinus* is the principal vector for the western European (TBEV-Eur) subtype of the virus (127, 137). TBE is now endemic in 27 European countries (138) and its expansion northward and into higher altitudes has been observed in recent years (137, 139). There is a considerable lack of knowledge in the current fine scale spatial distribution of TBE, including urban areas, thus the risk of infection is still underestimated, especially considering that about two-thirds of human TBE infections are asymptomatic (135).

Incidence of TBE in Europe has been changing in a heterogeneous manner during the last decades, with spatial expansion in some areas and decrease in others (140–142). TBE ecology and epidemiology is expected to be affected considerably by climate change (143) and other drivers like changing in land-use patterns, expansion of forest coverage, increase of abandoned areas, and the creation of new suitable and fragmented landscapes for ticks and hosts within urban areas. Exposure to infected ticks is dependent on several and regionally variable socio-economical factors such as recreational and occupational human activities, public awareness, vaccination coverage, and tourism (26, 94, 144).

The majority of human TBE infections are acquired through bites of infected ticks, more rarely by the alimentary route through consumption of raw milk of infected goats, sheep, or cattle, or unpasteurized dairy products (145–147). As organic markets become more popular, city dwellers also have to be aware of the TBEV infection risk associated with unpasteurized cow and goat milk and milk products.

Tick-borne encephalitis incidence appears to be increasing, including urban areas, partially as a result of improvements in the diagnosis and reporting of TBE cases, but also due to increased exposure of humans to TBE due to outdoor activities. The risk of exposure to TBE was found to be relatively high even in the immediate surroundings of patients' homes, e.g., in the Czech Republic (148), and an enhanced surveillance of TBE cases in Poland revealed that more than 50% of patients resided in urban areas (149).

Tick-borne encephalitis virus circulates mainly in natural sylvatic cycles involving vector ticks and reservoir hosts. However, due to expansion of urban sites to previously natural habitats and penetration of small and large wild animals into urban areas, reservoir hosts for TBEV as well as large tick-maintenance hosts can be present also in urban and peri-urban sites and thus ensure circulation of the virus there (150). Ticks remain infected throughout their life and it is suggested that they are not only vectors, but also long-term reservoirs of the virus (151). Rodents (*A. flavicollis*, *A. sylvaticus*, *M. glareolus*, and *M. arvalis*, see **Table 1**) are important reservoir hosts for TBEV-Eur (152, 153) and probably may maintain the virus in nature through latent persistent infection (154, 155). Co-feeding tick to tick transmission of TBEV, even in the absence of detectable viremia in these rodent species (156), is crucial to explain the focal distribution of the TBE foci and their potential variation over time (157). Experimental TBEV viremia has been demonstrated also in two lizard species (*L. viridis* and *L. agilis*) often occurring in urban areas (51), but field data on their reservoir competence for TBEV are missing. Migratory birds may play an important role in the geographic dispersal of TBEV-infected ticks, which can contribute to the emergence of new foci of disease, including gardens and urban parks, in case abiotic conditions and the vertebrate host spectrum are favorable for the maintenance of the pathogen (158). Among birds, thrushes (*Turdus* spp.) are the most frequently infested with *I. ricinus* ticks and also carry the most frequently infected ticks (159), however, the prevalence of TBEV-infected bird-feeding ticks is relatively low.

Wild and domestic ungulates, carnivores (foxes and dogs), and hares frequently occurring in peri-urban parks and forest patches within urbanized areas, are important actors in the dynamics of TBE, mainly as tick-maintenance hosts and carriers of infected ticks (160–162). Variation in abundance of roe deer was found to considerably affect TBE risk, depending on the threshold densities of tick, rodent, and large vertebrate populations in the area (31, 91, 92, 163). Ungulates probably do not contribute to the amplification of the virus, but may serve as sentinels to identify TBE foci (163, 164).

Accompanying dogs also represent an important risk factor for humans to acquire TBE. They are accidental hosts, but can become ill with TBE. In addition, during walking in natural forest or hunting activities, dogs come in contact with infected ticks and can carry them home or to urban parks, where they may later infest humans (165).

In general, data on TBEV prevalence in tick populations and seroprevalence in reservoir and sentinel hosts in urban areas and on the circulation of various virus strains in Europe are scarce (166–169). Furthermore, our knowledge on the mechanism favoring TBEV persistence and amplification in urban sites is very limited. TBEV infection rate in ticks is usually very low (<1%) (170–172), but can amount up to 15% in microfoci (173). TBEV-positive *I. ricinus* ticks have recently been detected, e.g., in a highly urbanized region in Southern Poland (estimated pool prevalence ranging from 0.19 to 1.11% for positive locations), suggesting the presence of active foci (174). TBEV-infected *Dermacentor reticulatus* adults were also detected in an urban area (Warsaw) in Poland, with higher prevalence (3.12%) than in natural areas. But our knowledge about the importance of this tick species in TBE epidemiology is still limited (175).

Generally, screening of ticks by PCR cannot be recommended for assessment of human TBE risk and alternative methods of environmental TBEV monitoring should be considered, such as serological long-term monitoring of rodents and other wild and domestic animals, which would serve as sentinel species (169).

BORRELIA BURGDORFERI SENSU LATO

In little more than 30 years, Lyme borreliosis (LB), which is caused by the spirochete *B. burgdorferi* s.l., has risen from relative obscurity to become a global public health problem and a prototype of an emerging pathogen (176). During this period, we have accumulated enormous progress in knowledge of its phylogenetic diversity, molecular biology, genetics, host interactions, pathogenicity for humans as well as other vertebrate species, and preventive measures including vaccine development. But relatively little is known about public health consequences of LB in terms of eco-epidemiology issues and risk of acquiring infection in suburban and urban habitats.

Lyme borreliosis is the most abundant tick-borne disease of humans worldwide, though it only occurs in the northern hemisphere. LB occurs in North America (from the Mexican border in the south to the southern Canadian provinces in the north), the whole Europe, parts of North Africa (Maghreb), and northern Asia (Russian Siberia and the Far East, Sakhalin, Japan, China, and Korea). The geographical distribution of LB correlates closely with the range of the principal vectors, ticks of the *I. ricinus* complex (177). LB occurs between approximately 35° and 60°N in Europe, and between 30° and 55°N in North America. In countries at the southern limits of the LB range, its incidence decreases rapidly along the north-to-south gradient (178).

The *B. burgdorferi* s.l. complex now comprises up to 19 *Borrelia* species. Of these, only *B. afzelii*, *B. burgdorferi*, and *B. garinii* are proven agents of localized, disseminated, and chronic manifestations of LB in Europe, whereas, *B. spielmanii* has been detected in early skin disease, and *B. bissettii* and *B. valaisiana* have been detected in samples from single cases of LB (179, 180). The clinical role of *B. lusitaniae* remains to be substantiated.

Principal vectors of *B. burgdorferi* s.l. in Europe, including urban and suburban ecosystems, are two tick species: *I. ricinus* and *I. persulcatus*, the latter only occurring in eastern and north-eastern Europe. Moreover, the occurrence of *I. hexagonus* in the urban environment, due to the presence of suitable hosts, such as hedgehogs, cats, dogs, and foxes in gardens and public parks, could contribute to transmission of LB (65).

The risk of infection is particularly high in deciduous or mixed forest ecosystems or woodlands, along with city parks and urban gardens, especially gardens close to forests (181). The higher risk of contracting LB in the ecotones between forests and arable fields (178) or meadows, although higher densities of infected vector ticks are within forests, is an effect of frequent human presence along the edges of these habitats (182). Also forest fragmentation in suburban areas theoretically poses a greater risk due to enhanced proportion of ecotones (183). Other risks include reforestation (with increased population of forest rodents, but also deer, the principal host of adult vector ticks). For example, in the Czech Republic Zeman and Januska (184) found that LB risk correlated with overall population density of game (red deer, roe deer, mouflon, and wild boar) regardless of rodent abundance. Nevertheless, increased populations of reservoir hosts (forest rodents) usually stimulate the LB incidence.

All activities that increase human contact with ticks present risk for contracting LB, especially recreational (leisure time) activities in forested and urban areas (jogging, berry/mushroom picking, walking, and hiking), seasonal and occasional living by urban residents in country cottages, mowing and clearing of brush around the home in forested areas and gardening. Ownership of pet dogs and cats could also present a relative risk for humans when the pets are frequently parasitized by ticks and the owner tries to remove the ticks (178, 181). Moreover, outdoor employment and work (forestry workers, military personnel in the field, farmers, gardeners, gamekeepers, hunters, and rangers) are at risk. However, in most European countries, occupational exposure generally constitutes only 2% of LB cases (185), whereas, permanent residence in endemic areas with a high prevalence of infectious ticks (e.g., forested peri-urban areas) is a serious risk factor for LB.

Small rodents (*A. sylvaticus*, *A. flavicollis*, and *M. glareolus*) are regarded as the main reservoir hosts of LB pathogens in urban and suburban habitats across Europe (**Table 1**). Garden dormice (*E. quercinus*) (186) and hazel dormice (*M. avellanarius*) are especially competent reservoirs of the human pathogenic *B. spielmanii* (46, 58). Important role in the urban maintenance of *B. spielmanii* and *B. afzelii* could also be played by rats (*R. norvegicus* and *R. rattus*) (46, 57, 187). Other key urban players in the maintenance

of LB spirochetes are hedgehogs (*E. europaeus* and *E. roumanicus*) (64, 65, 188). Red squirrels (*S. vulgaris*) were found to be heavily infested by ticks and feeding ticks showed high prevalence of infection in enzootic areas in Switzerland (60) and might consequently contribute to maintenance of spirochetes also in urban foci.

Dogs and cats are heavily infested with ticks and might act as hosts (probably not reservoirs) or sentinels for LB. The risk of exposure of dogs to numerous vector-borne pathogens has increased, and close relationship with humans in urban areas poses new concerns for human public health (106).

Ground-foraging bird species such as blackbird (*T. merula*), song thrush (*T. philomelos*), robin (*E. rubecula*), and pheasant (*Phasianus colchicus*) play a unique role in the epidemiology of LB and also contribute to the transmission cycle of *B. burgdorferi* s.l. in urban and suburban areas (189–192). Due to their specific immunity (complement system), certain bird species are resistant to some LB spirochetes but susceptible to others (193). They usually carry *B. valaisiana* and *B. garinii* and transmit these spirochetes to ticks. In 1998, two xenodiagnostic studies clearly defined the reservoir role of birds in the epidemiology of LB, one on a passerine bird, the blackbird (190), the other on a gallinaceous species, and the pheasant (194). However, the reservoir competence of other bird species needs to be clarified. A recent study showed that circulation of LB spirochetes is partly maintained by bird-specific tick species, and bridged by *I. ricinus* to other host types (195).

The role of lizards in the maintenance of *B. burgdorferi* s.l. is still controversial, since several lizard species have been shown to possess a complement with borreliacidal activity (196). However, in some areas LB spirochetes are more prevalent in sand lizards (*L. agilis*) and common wall lizards (*P. muralis*) than in rodents (122). The lizard-associated LB spirochete is *B. lusitaniae*, a genospecies previously thought to occur only in Mediterranean and Central Europe (197), but it was shown that it has a far more widespread geographical distribution involving the green lizard (*L. viridis*), the Balkan wall lizard (*Podarcis taurica*), and the sand lizard (*L. agilis*) (123, 125, 126).

We have reviewed the occurrence of *B. burgdorferi* s.l. in host-seeking urban *I. ricinus* ticks across Europe according to the literature (**Table 2**). There are also several additional papers demonstrating the presence of borreliae in ixodid ticks collected in (sub)urban areas (198–202). All accessible data show that borreliae in *I. ricinus* ticks collected in urban parks, gardens, or suburban habitats are prevalent approximately at the same rate as in *I. ricinus* ticks living in forests (203). In urban areas, therefore the risk of contacting LB could be as high as in natural environment.

We should consider that most studies dealing with eco-epidemiology of LB in patients living in urban areas may have limitation, because not always the exact location (or area) where they acquired the vector tick is known. While popular opinion is that outdoor occupations and hiking are risk activities, several studies have implied that infection is often acquired near the home, during gardening and dog walking associated with increased risk (148, 226–228).

ANAPLASMA PHAGOCYTOPHILUM

Anaplasma phagocytophilum is a small, gram-negative obligate intracellular alpha-Proteobacterium and infects neutrophilic, eosinophilic granulocytes, and monocytes of mammals. There, it replicates within a cytoplasmatic, cell-membrane derived vacuole. *A. phagocytophilum* is transmitted by ticks of the *I. ricinus* complex in the Northern hemisphere and in European countries mainly by *I. ricinus* (50).

The bacterium has been known since the last century to cause diseases in domestic ruminants (229) and since the 1960s in horses (230). The first human case was described in the USA in 1994 (231). The causative agents of the diseases were at the time classified into the granulocytic group of the genus *Ehrlichia*, which contained *E. phagocytophila* as agent of tick-borne fever of ruminants, *E. equi* as agent of equine granulocytic ehrlichiosis and the human granulocytic ehrlichiosis (HGE)-agent. In 2001, a reorganization of the order Rickettsiales, based on homologies in the *16S rRNA* gene, reclassified the granulocytic *Ehrlichia*-group as the new bacterial species *A. phagocytophilum* and the respective diseases were then called granulocytic anaplasmosis (232). Clinical cases are also occurring in dogs and cats, then known as canine and feline granulocytic anaplasmosis (233, 234).

After the first cases appeared in the US in the 1990s, human granulocytic anaplasmosis (HGA) has become one of the most important tick-borne diseases in the US, with an incidence in 2010 of 6.1 cases per 1 million inhabitants[2]. The first human case in Europe was described in the 1990s (235), and around 100 cases have been described since then in several European countries, e.g., in Slovenia, Croatia, Czech Republic, Slovakia, Austria, Latvia, the Netherlands, Norway, Poland, Spain, France, and Sweden (236–252). Seroprevalence rates in humans in Europe are around 1–20% and they fluctuate depending on anamnesis, tick exposure, and age of the patients (253).

Mammalian host species (**Table 1**) such as wild ruminants (e.g., roe deer, red deer, fallow deer, but also mountain ungulates), small mammals such as rodents and insectivores, but also foxes, bears, wild boars, birds, and reptiles are infected with *A. phagocytophilum* (50). Prevalence rates in wild ruminant species in Europe are generally high, e.g., ranging in roe deer and red deer from around 12% to over 85% (70, 254–256). On the other hand, prevalence rates in small mammals are from 0% to about 20% (50).

Anaplasma phagocytophilum is detected with varying prevalences in questing *I. ricinus* ticks, and has been found in Europe in nearly 30 countries. The prevalence ranged, for example, in Norway from 0.4 to 17.1%, in Estonia from 3 to 6.5%, in Slovakia from 1.1 to 8.3%, and in Germany from 1.0 to 17.4% [reviewed in Ref. (50)]. So far, transovarial transmission has not been shown in *Ixodes* ticks. As such, for the current state of knowledge, a reservoir host is necessary to keep up the endemic life cycle of *A. phagocytophilum* in nature.

The discrepancy of a high occurrence of *A. phagocytophilum* in ticks and mammals as well as high seroprevalence rates in Europe in contrast to few clinical cases has been explained by the potential underdiagnosing of the disease, or the potential occurrence of less virulent strains in Europe in comparison to the USA. The discrepancy could also be explained by a higher awareness of US physicians to the disease because in the USA it is a notifiable

[2]www.cdc.gov/anaplasmosis

Table 2 | Occurrence of *Borrelia burgdorferi* sensu lato in questing *Ixodes ricinus* ticks in urban and suburban areas in Europe.

Country	City/region (habitat), year	No. of examined ticks	Prevalence[a]	Method	Genomic spp.	Reference
Czech Republic	Prague (U, S)	2,490 N, 143 F, 184 M	2–22%	IFA		(204)
	Prague (U, S) 1994–1997	12,287	3.3–13.3%	IFA		(205)
	Prague 1995–1997	462 N, 173 A	1.9% N, 12.7% A	PCR	Bg 18, Ba 13	(206)
	Brno – outskirts 1988	1,005	3.8% N, 16.4% F, 12.7% M	IFA		(207)
	Brno (U parks) 1992	34 N, 64 F, 65 M	14.7% N, 29.7% F, 30.8% M	DFM		(208)
	Brno-Pisárky (S) 1996–1998	643 N, 123 F, 107 M	10.0% N, 13.8% F, 18.7% M	DFM (and PCR)		(209)
	Brno-Pisárky (S) 2002	243 N, 19 F, 22 M	15.8% N + F + M	DFM (PCR)	Bg 15, Ba 14, Bb 2, Bv 2	(210)
Finland	Helsinki (U, S)	303 N, 189 F, 234 M	32.2% N + F + M	DFM, PCR, BSK	Ba 70%, Bg 25%	(35)
France	Paris (U, S)	360 N, 69 F, 129 M	32% F, 10% N, 20% M	PCR	Ba/Bv 36%, Bg/Bl 60%, Bm 4%*	(211)
Germany	Berlin – West (U, S)	1,414 N, 132 F, 165 M	2.4% N, 9.1% F, 6.1% M (MIR)	BSK		(212)
	Bonn (U, S) 2003	865 N, 241 F, 288 M	17.3% N, 26.6% F, 12.5% M	PCR	Ba 39%, Bg 28%, Bb 16%, Bv 9%	(36)
Hungary	Budapest (parks, forests, and cemeteries) 2013	240 F	40.8%	PCR		(213)
Italy	Imola (U parks) 2006		10.4% N + A	PCR		(214)
Lithuania	Vilnius (city park) 2005	39 A	25%	DFM, PCR	Ba, Bg, Ba + Bg	(215)
The Netherlands	Bijlmerweide (city park) 2000–2002	384 N + F + M	6.8%	PCR	Ba 10, Bb 1, Bv 1	(38)
Poland	Gdansk, Sopot, Gdynia (U, S)	701 N + F + M (164 F, 139 M)	12.4%, 11.6% F, 10.1% M	PCR		(216)
	Szczecin (U, S)	193 N, 22 A	17.7%	DFM		(217)
	Warsaw (U, S), 1996		19.2–31.0%	IFA (PCR)	Bg, Ba, Bv	(218)
	Warsaw (city parks)		6.1%	PCR		(219)
Serbia	Belgrade (U, S) 1996–2005	10,158 N + A	21.9% N + A	DFM (BSK, PCR)	Ba 75%, Bb 22%, Bg 3%	(220)
Slovakia	Bratislava (U, S) 1986–1988	77	7.8%	DFM		(221)
	Košice (U, S) 1991–1995	660 N, 2,904 A	9.2% N, 14.8% A	DFM and IFA		(222)
	Košice, Bardejov (U, S) 2008–2010	670	10.1%	PCR	Ba, Bg, Bv, Bb	(223)
Switzerland	Basel (U, S) 2003	172 N, 35 A	16.4% N + A	PCR		(224)
United Kingdom	London (U parks)	65 F	7.7% F	PCR		(225)

U, urban; S, suburban; Ixodes ricinus: N, nymph; F, female; M, male; A, adult; DFM, dark-field microscopy; IFA, indirect immunofluorescence assay; BSK, cultivation in BSK II medium; Ba, Borrelia afzelii; Bb, B. burgdorferi s.s.; Bg, B. garinii; Bv, B. valaisiana; Bl, B. lusitaniae; Bm, B. miyamotoi; MIR, minimum infection rate.

[a] *Different PCR methods were used that differ in their sensitivity.*

* *No sufficient discrimination between Bg and Bl and between Ba and Bv.*

disease. However, *A. phagocytophilum* shows also genetic heterogeneity and potential differences concerning the potential host tropisms and pathogenicity (118). A potential human pathogenic strain of *A. phagocytophilum* in Europe has been especially suspected to be connected with wild boars. This was confirmed in recent studies (257, 258).

Several studies have investigated the genetic heterogeneity on the basis of several genes such as *16S rRNA*, *groEL* heat-shock protein, major surface protein coding genes, and the *ankA* gene (255, 259–261). Several distinct clusters were found where, in general, strains derived from domestic animals or ruminants clustered together. Roe deer strains often clustered separately from

Table 3 | Occurrence of *Anaplasma phagocytophilum* in questing *Ixodes ricinus* ticks in urban and suburban areas in Europe[a].

Country	City/region (habitat)	No. of ticks posit./examined	Prevalence[b] (%)	Reference
Austria	Graz (RA)	5/518	1	(264)
Czech Republic	Dvur Kralove (U forest)	8/138	5.8	(265)
	Ostrava (U park)	276 (tested in pools)	9.4	(266)
France	Paris (S forests)	2/558	0.7	(211)
Germany	Hamburg (U RA)	51/1,400	3.6	(267)
	Hannover (U RA)	94/2,100	4.5	(268)
	Bavaria (U parks)	500/5,569	9.0	(269)
	Bavaria (U parks)	103/2,862	2.9	(270)
	Bavaria (U parks)	172/2,800	6.1	(271)
	Leipzig (U, S RA)	47/539	8.7	(55)
	Hannover (U RA)	52/1,646	3.2	(272)
Hungary	Budapest (30 sites: U parks, forests, and cemeteries)	21/240	8.8	(213)
Poland	S forests	18/124; 6/46	14.5; 13.0	(273)
Slovakia	Bratislava (U, S forests)	10/248	4	(265)
	Malacky (U park)	4/101	4	(265)
	Košice (U forest)	10/224	4.5	(265)
	Bardejov Poštárka (S forest)	2/75	2.7	(40)
	Košice Adlerova (S forest)	10/261	3.8	(40)
	Jazero (U forest)	5/91	5.5	(40)
	Košice (S forests)	1,075	1.4–5.5	(274)

U, urban; S, suburban; RA, recreational area.

[a] *Negative results not shown,*

[b] *different PCR and real-time PCR methods were used that differ in their sensitivity.*

strains derived from other animals. No evidence was found that wild ruminants are involved in the transmission cycles of potentially pathogenic strains. This was shown again by a recent multi locus sequence typing study (262). However, another study found pathogenic strains associated mostly to ungulates (118).

Furthermore, in a recent large-scale analysis, four *A. phagocytophilum* ecotypes with significantly different host ranges were identified based on *groEL* heat-shock protein gene sequences of various European vertebrate and tick samples (99). So far, all human cases clustered in ecotype I with the broadest host range (including domesticated animals, red deer, wild boar, and urban hedgehogs). Ecotype II was associated with roe deer and some rodents, ecotype III included only rodents. Birds seem to have a different enzootic cycle from all these (ecotype IV). Based on population genetic parameters, ecotype I showed significant expansion, which might have occurred through an increase in either the population of *I. ricinus* ticks, or in the (often urban) vertebrate host species, or in both (99).

Only recently, a HGA case of a German patient has been published having acquired the infection whilst on holidays hiking in Scotland (263). This shows that the risk of contracting this infectious agent can also be in leisure time whilst hiking, or even in the cities whilst being in urban or peri-urban park areas.

In about the last 5 years, considerable research effort has been undertaken in Europe to investigate the epidemiology of *A. phagocytophilum*, especially in urban areas and high prevalences of this pathogen have been found with seasonal and geographic variability. An overview of recent studies investigating questing *I. ricinus* in urban and suburban areas is shown in **Table 3**. However, when considering *A. phagocytophilum* prevalence rates in ticks, the genetic variability has to be taken into account as not all strains may be pathogenic to humans.

CANDIDATUS NEOEHRLICHIA MIKURENSIS

"*Candidatus* Neoehrlichia mikurensis" (*Candidatus* N. mikurensis) is a tick-borne pathogen, which is probably transmitted by *I. ricinus* ticks (24). However, transovarial transmission in this tick species has not been reported yet.

Currently, the genera *Wolbachia*, *Ehrlichia*, *Neorickettsia*, *Aegyptianella*, and *Anaplasma* belong to the rickettsial family Anaplasmataceae (232). Most certainly, the new genus "*Neoehrlichia*" will be included in this family in future. The pathogens of this family are intracellular bacteria transmitted by arthropods and may cause severe diseases in humans and animals. For at least three of the five existing genera within this family (*Anaplasma*, *Ehrlichia*, and *Neorickettsia*) serological cross reactions are not known so far (275). *Candidatus* N. mikurensis is an obligate intracellular gram-negative bacterium, which is characterized by an endothelial cell tropism but it could not be cultivated *in vitro* thus far. Therefore, the status "Candidatus" is still preserved.

A previous study published data on not taxonomically grouped *Ehrlichia* DNA in engorged *I. ricinus* ticks from roe deer in the

Table 4 | Occurrence of *Candidatus* N. mikurensis in questing *Ixodes ricinus* ticks in various habitats in Europe.

Country	No. of sites, habitat	No. of ticks examined	Prevalence[a]	Reference
Austria	U, S, 2002–2003	518	4.2%	(264)
Czech Republic	U, 2010	69	0.4%	(265)
Denmark	Three sites, S, sylvatic, 2011(+tick DNA from archive)	79[a]	3.8%	(285)
France	Two sites, sylvatic	60	1.7%	(282)
Germany	Ten sites, U, S	542	8.1%	(282)
	U, S, 2008–2009	782	24.2–26.6%	(52)
Hungary	Nine sites, 2007	2,004	n.a. 9 of 35 sites positive	(286)
Italy	U, S, 2006–2008	138	10.5%	(287)
The Netherlands	Three sites, sylvatic	180	8.6%	(288)
	Twenty-one sites, U, S, sylvatic, 2006–2010	5,343	5.6%	(289)
The Netherlands/Belgium	n. a., 2006–2010	2,375	7%	(281)
Russia	S, sylvatic, 1997–1998	295	7.1%	(277)
Slovakia	S, sylvatic, 2006	68	2.9%	(290)
	Ten sites, U, S, sylvatic, 2008, 2010	670	2.4%	(40)
	U, S		1.1–4.5%	(265)
Spain	S, 2013	100	2%	(291)
Sweden	Four sites, sylvatic, 2010–2011	949	4.5–11%	(292)
Switzerland	Eleven sites, U, S, 2009–2010	818	6.4%	(293)
	Four sites, U, S, 2009	1,916	3.5–8%	(294)

U, urban; S, suburban.

[a]*Different PCR and real-time PCR methods were used that differ in their sensitivity. n.a., not available.*

Netherlands (276). This pathogen was then named after the senior author as "Schotti-Variant" (276). Similar sequencing results were published for *I. ricinus* and *I. persulcatus* ticks from the Baltics in 2001 (277). Between 1998 and 2001, DNA of a pathogen, suggested to be called *Cand. Ehrlichia walkerii* spp. nov., was found in engorged *I. ricinus* ticks that fed on asymptomatic patients from Italy (278). In 2003, DNA sequences of this new pathogen were detected in DNA extracted from *I. ricinus* ticks from Germany, followed by first investigations on possible reservoir hosts (279). In 2003, a pathogen was found via examination by conventional PCR in three wild rats (*R. norvegicus*) in China. This examination was followed by DNA sequencing of this pathogen, which was then called the "Rattus Variant" (280). In 2004, DNA of this "new" pathogen was found in 7 out of 15 brown rats from a Japanese isle called Mikura (275). The pathogen was passaged in Wistar rats and first investigations on the ultrastructure and the phylogenetic analysis were done, which lead to the currently valid taxonomic denomination "*Candidatus* Neoehrlichia mikurensis." The close genetic similarity of the *16S rRNA* and the *groEL* gene puts *Candidatus* N. mikurensis in the family of Anaplasmataceae.

Candidatus N. mikurensis was found widespread in *I. ricinus* throughout Europe (281, 282). It could be detected in Italy, France, Sweden, Russia, and other European countries (**Table 4**). The prevalences ranged between 1 and 11% but focal areas were found with prevalence rates up to 26.6% (49) (**Table 4**). Furthermore, *Candidatus* N. mikurensis was detected in one out of 126 *I. ricinus* ticks that were collected in Moldavia back in the year 1969 (283) and it was only detected in the genus of *Ixodes* ticks so far (284). Positive ticks were not only found in sylvatic and non-anthropogenic sites but also in urban and peri-urban sites with human influence in Europe (**Table 4**).

Previous studies on potential reservoir hosts revealed that rodents, especially bank voles and yellow-necked mice, but also common voles (*M. arvalis*) were infected at high rates, suggesting a role as reservoir hosts (52, 281, 295, 296), but insectivores were found to be negative for *Candidatus* N. mikurensis thus far (52). Recently, the reservoir role of *Apodemus* mice (*A. flavicollis* and *A. sylvaticus*) and bank voles (*M. glareolus*) has unambiguously been proven in a xenodiagnostic study [(48); **Table 1**]. Urban hedgehogs (*E. roumanicus*) with high density in a Budapest city park were found to be carriers of *Candidatus* N. mikurensis, indicating that non-rodent reservoirs might be also involved in the maintenance of this pathogen, especially in human dwellings (69). Additionally, *Candidatus* N. mikurensis was detected in dogs from Germany and Nigeria (297, 298).

In the past, the detection of *Candidatus* N. mikurensis in rodents and ixodid ticks was an interesting but only incidental

finding without any medical importance (299). In contrast to this assumption, it was recently found in humans (50) with immune deficiency but without being in an occupation group at risk for tick bites over the last decade. *Candidatus* N. mikurensis caused unspecific symptoms such as fever, septicemia, malaise, and weight loss in these patients (300–302). Until October 2012, the first six clinical cases of neoehrlichiosis were the only human cases confirmed by laboratory diagnostic methods. All of these patients suffered from a primary disease, were immunocompromised and came from European countries, such as Germany (301), the Czech Republic (303), Sweden (302), and Switzerland (300). Nevertheless a primary disease is not a necessary precondition to develop neoehrlichiosis as *Candidatus* N. mikurensis could be detected in blood of 7 out of 622 patients from China suffering from fever (130). The authors of these clinical reports emphasize that these seven patients were otherwise healthy and did not suffer from a chronic or immunosuppressive disease. The most recent two human cases were reported in Switzerland, where both patients recovered quickly after a treatment with Doxycycline (294). The data, gained in the last decade, lead to the assumption that *Candidatus* N. mikurensis is an emerging pathogen that might be found by increasing numbers in ticks from sylvatic and urban sites, in small mammals and humans in future (281, 304). Further investigations are needed on the spread, maintenance, and potential reservoir hosts to assess the risk potential of *Candidatus* N. mikurensis.

RICKETTSIAE

Rickettsiae are Gram-negative, obligate, aerobic, intracellular bacterial parasites of eukaryotes that survive freely within the cytosol of the host cell, and belong to the family Rickettsiaceae and order Rickettsiales. Rickettsiae are traditionally subdivided into the typhus and the spotted fever group (SFG). SFG rickettsiae are associated with hard ticks (Ixodidae), with the exception of *Rickettsia akari* (mite-borne) and *R. felis* (flea-borne). Hard ticks can transmit them transstadially and transovarially and serve both as vectors and reservoirs of these pathogens. Vertebrates are suspected to serve as reservoirs of rickettsiae, but they may also be accidental hosts and acquire infection by a tick bite (305). However, in a recent xenodiagnostic experiment infected rodents were not able to transmit *R. helvetica* or *R. monacensis* to *I. ricinus* larvae (48).

In Europe, *R. felis*, *R. typhi*, *R. prowazekii*, *R. akari*, *R. conorii*, *R. slovaca*, *R. sibirica mongolotimonae*, *R. raoultii*, *R. massiliae, R. aeschlimanni*, *R. helvetica*, and *R. monacensis* have been implicated in human diseases or reported as emerging pathogens or isolated from vectors or humans (131, 306–308). Furthermore, the candidate species "*Candidatus* Rickettsia kotlanii," "*Candidatus* Rickettsia barbariae," or "*Candidatus* Rickettsia vini" have been found in ticks in Europe (309–311). Numerous rickettsiae are regularly associated with ticks and have been called symbionts, microsymbionts, or endosymbionts (living in endocellular symbiosis). However, their potential for pathogenicity is still unknown (312).

The presence of tick-borne rickettsiae has been reported from almost all European countries. The current view on geographic distribution of *Rickettsia* species in the world is summarized by Parola et al. (131).

In Europe, *I. ricinus* ticks are known to carry mainly *R. helvetica* and *R. monacensis*. However, *R. massiliae* was also detected in *I. ricinus* ticks (313). The following rickettsial genotypes were detected only by molecular tools in *I. ricinus* ticks collected in Europe: "*Candidatus* R. vini" was proposed as a new *Rickettsia* spp. detected in *I. arboricola* and *I. ricinus* collected from three different bird species in Spain (311), *Rickettsia* spp. strain Davousti, previously found in *Amblyomma tholloni* ticks in Africa, was detected in *Ixodes* spp. collected from migratory birds in Sweden (314),"*Candidatus* Rickettsia moreli" (GenBank accession numbers Y08784 and Y08785) was detected in *I. ricinus* from Spain, and *Rickettsia* spp. clone KVH-02-3H7 (GenBank accession number GQ849216) was detected in *I. ricinus* in the Netherlands (131).

Rickettsia helvetica was first isolated from *I. ricinus* in Switzerland and it was confirmed to be a new member of the SFG rickettsiae in 1993 (315, 316). It has been generally accepted that *I. ricinus* is the main vector and natural reservoir of *R. helvetica.* However, *D. reticulatus* ticks were found to be infected with *R. helvetica* in Croatia (317). *R. helvetica* has been detected in questing and bird-feeding *I. ricinus* ticks in at least 24 European countries (131). The prevalence rates vary from 0.5% in a bird conservation island named Greifswalder Oie in the Baltic Sea to 66% in the Netherlands (318, 319). For example, the highest infection rate of *R. helvetica* in *I. ricinus* from Denmark was found in May, followed by July, August, and October (320). The presence of *R. helvetica* was also confirmed in *I. ricinus* in some urban and peri-urban sites in Slovakia, the Czech Republic, Germany, Portugal, Serbia, and Poland (**Table 5**).

In 1999, *R. helvetica* was associated with chronic perimyocarditis in sudden cardiac death in Sweden (328). This species has been cultivated from a patient with subacute meningitis (329). The hypothetical role of *R. helvetica* as an etiological agent of sarcoidosis could not be confirmed (330). The illness is associated with fever, headache, arthralgia, and myalgias and less frequently with rash and/or an eschar (331, 332).

Rickettsia monacensis was originally isolated as new species from *I. ricinus* collected in a city park in Germany (333). Phylogenetic analyses of the *16S rRNA*, *gltA*, and *rompA* gene sequences demonstrated its close relationship with *Candidatus* Rickettsia spp. IRS3 and *Cand. Rickettsia* sp. IRS4 isolated from *I. ricinus* in north-eastern and south-western Slovakia (334, 335). The prevalence rates of *R. monacensis* in *I. ricinus* ticks vary from 0.5% in Germany to 34.6% in Turkey (322, 336). *R. monacensis* has been detected in *I. ricinus* ticks in at least 18 European countries (131). The presence of *R. monacensis* was also confirmed in *I. ricinus* ticks in some urban and peri-urban sites in Slovakia, the Czech Republic, Germany, Portugal, Serbia, and Poland (**Table 5**). In 2005, *R. monacensis* was identified as a human pathogen in two patients in Spain (in June and September) and latter in one patient in Sardinia, Italy (in April) (337, 338). In addition to fever and flu-like symptoms, the inoculation eschar was identified in an Italian patient, and a generalized rash including the palms and soles was identified in a Spanish patient.

Rickettsia massiliae was originally isolated from *Rhipicephalus sanguineus* ticks collected near Marseille, France, in 1992 and then detected in *R. sanguineus*, *R. turanicus*, *R. pusillus*, *R. bursa*, and *I. ricinus* ticks in France, Greece, Portugal, Switzerland, Spain,

Table 5 | Occurrence of *Rickettsia* spp. in questing *Ixodes ricinus* ticks in various habitats in Europe.

Country	City/region (habitat)	No. examined ticks	Prevalence of *Rickettsia* spp.	Identified species (*n*)	Reference
Czech Republic	Ostrava (U park), 2010	180 N	2.2% (MIR)	14 *Rh,* 6 *Rm*	(266)
		96 A	4.2% (MIR)		
	Proskovice (mixed forest), 2010	1,114 N	3.5% (MIR)		
		83 A	2.5% (MIR)		
France	Paris (S)	360 N, 69 F, 129 M	5.8%	*Rh*	(211)
Germany	Munich, 2006	961 N	1.0%	138 *Rh,* 13 *Rm*	(321)
		1,900 A	7.3%		
	Saarland (RA), 2008–2009	36 N	16.7–47.2%	8 *Rh*	(322)
	Bavaria/Munich (natural alluvial forest), 2008–2009	79 A	21.5%		
	Leipzig/Saxony (coal surface-mining area), 2008–2009	28 N	21.4%		
		100 A	19.0%		
		98 N	8.2–27.6%		
		431 A	9.7%		
	Munich, Regensburg, Ingolstadt, Augsburg, Berg (U parks), 2009–2010	774 L	2.1–9.8%	15 *Rh*, 1 *Rm*	(37)
		1,190 N	6.8%		
		2,495 A	7.5%	77 *Rh,* 4 *Rm*	
		244 L	–		
		742 N	–	180 *Rh,* 8 *Rm*	
		1,142 A	–		
	Munich, Regensburg, Lake Starnberg (U, S)	24 L	2.2–7.5%	29 *Rh,*1 *Rm*	(323)
	Lake Starnberg and Lake Ammersee, pastures	500 N	5.0%		
	Augsburg, forest, 2011	889 A	8.7%		
		140 N	15.7%	9 *Rh*	
		225A	13.3%		
		139 L	2.2–10.1%		
		120 N	17.5%	9 *Rh*	
		79 A	13.9%		
	Hanover (U park), 2010	31 L	16.0%	268 *Rh*	(268)
		1,697 N	25.5%		
		372 A	30.4%		
Poland	Warsaw, national parks and natural areas, 2011	1,147 N 442 A	3.7% (MIR)	38 *Rh*, *Rm*	(41)
			5.9% (MIR)		
Portugal	Alentejo (safari park), 2006–2009	35 A	82.9%	14 *Rh*, 15 *Rm*	(324)
Serbia	Four natural sites, 2 sites (RA), 2007, 2009	26	23.1%	2 *Rh*, 4 *Rm*	(325)
Slovakia	Bratislava (S forest, cemeteries), 2006–2011	445 N	8.3%	61 *Rh*, 3 *Rm*	(326)
		471 A	10.2%		
	Malacky (U park), 2006–2011	59 N	6.8%	10 *Rh,* 3 *Rm*	
		62 A	14.5%		
	Martin (U park), 2006–2011	3 N	0		
		12 A	16.7%		
	Martinské hole Mts (mountain forest), 2006–2011	276 N	5.4%	6 *Rh,* 2 *Rm*	
		482 A	10.0%		
	Vojka nad Dunajom (RA), 2011–2012	2 N	0	30 *Rh,* 3 *Rm*	(327)
		280 A	11.7%		

U, urban; S, suburban; RA, recreational area; Ixodes ricinus: L, larva; N, nymph; A, adult; MIR, minimum infection rate; Rh, R. helvetica; Rm, R. monacensis.

including islands: Sardinia and Sicily (Italy), the Canary Islands (Spain), Cephalonia (Greece), and Cyprus (131). *R. massiliae* was identified in four *I. ricinus* ticks removed from humans at hospitals in Castilla y León, Spain (313). However, to our knowledge, there are no other studies of this species in urban areas.

BABESIA

Ixodes ricinus is the vector of three intraerythrocytic protozoan parasites circulating in Europe and involved in human babesiosis: *B. divergens*, *B. venatorum* (originally designated *Babesia* spp. EU1), and *B. microti*. To date, no other Piroplasmida affecting humans have been reported to be transmitted by this tick species, even though it feeds on a very large spectrum of hosts, which are potentially infected by several parasite species including numerous other *Babesia* species associated to wildlife or domestic animal diseases. However, the list of potential or known tick-borne pathogens is constantly evolving, either due to: (i) the description of *Babesia* species new for science, (ii) the spread of parasite species previously unknown in Europe, or (iii) the discovery of a *Babesia* species previously restricted to animals but now known to be associated with humans. Thus, emergence or re-emergence of tick-borne diseases leads to the development of unknown health risks (339). Therefore, there is a real concern that tick-borne diseases due to parasites will appear in areas previously free of such diseases, and there is a real necessity of an epidemiological surveillance of the parasitic communities hosted, and potentially transmitted by ticks (340).

Although best known as an animal disease, babesiosis is a zoonotic disease, classified as emerging by some authors. Approximately 50 human cases of babesiosis have been reported in Europe, which is probably underestimated because of a large proportion of asymptomatic infections, as suggested by seroprevalence studies (341). Among the *Babesia* species pathogenic for humans, the bovine parasite *B. divergens* is thought to be responsible for most European cases of human babesiosis (342). However, since 2003, cases of human babesiosis have also been attributed to *B. venatorum* in Austria, Italy, and Germany (343, 344) as well as to *B. microti* in a single case in Germany (341). Whilst the clinical signs of human babesiosis are usually limited to splenectomized patients, two human cases (one attributed to *B. divergens*, the other to an unknown origin) have been detected in immunocompetent patients in eastern France (345). It is also noticeable that, as an example, 0.38% of the French population is splenectomized (346). Moreover, the rising number of HIV-positive individuals and the increasing population of immunocompromised humans, especially in urban areas, may therefore lead to boost the number of human babesiosis cases (341). The proportion of the population at risk of *Babesia* infection is thus higher than previously suspected and *Babesia* spp. likely represents real potential agents of an emerging zoonotic disease and needs increased attention and vigilance.

Besides transstadial transmission, transovarial transmission within ticks is characteristic for most *Babesia* spp. (differentiating them from *Theileria* species), which implies that ticks constitute a real parasite reservoir in the field, facilitating the long-term persistence of *Babesia* species in the ecosystem (sometimes over several tick generations) (347). In Europe, infection rates of *Babesia* spp. in ticks are usually rather low, but published values range from 0.9 to 20% (341).

Babesia divergens is a bovine parasite transmitted by *I. ricinus*, and is thought to be responsible for most cases of human babesiosis in Europe (342). This parasite is the most widespread and pathogenic *Babesia* species infecting cattle in northern temperate areas (342). Thus, any urban or peri-urban area where cattle and *I. ricinus* are found is potentially at risk. For example, *B. divergens* has been found in an *I. ricinus* tick collected in an urban park in Germany (37). Recently, the discovery of this parasite in questing *I. ricinus* from a forest area in Eastern France (340), as well as in *I. ricinus* collected from wild cervids in Belgium (348), may suggest that its geographical distribution is increasing, even within forested areas without cattle farms, which would require the existence of reservoir hosts other than cattle. Indeed, it was reported that *B. divergens* is also able to infect ungulates (roe deer, fallow deer, red deer, mouflon, and sheep), splenectomized rats, as well as non-splenectomized reindeer, sheep, and gerbil [see review in Ref. (347)]. Thus, this parasite has been shown to have a wider vertebrate host range than previously thought, leading to a potential risk not only in rural areas but also in peri-urban ones.

Babesia venatorum, implicated in human cases of babesiosis in Europe (343, 344), seems to phylogenetically lie in a sister group with *B. divergens* (343), and some serological cross-reactivity between *B. divergens* and *B. venatorum* has been reported (349). Roe deer were strongly suspected to be the wildlife reservoir of this parasite (350, 351) and its transmission by *I. ricinus* was validated both *in vivo* (351, 352) and *in vitro* (353). In addition, *B. venatorum* has been identified in *I. ricinus* in several European countries including Slovenia (354), Switzerland (355), the Netherlands (356), Poland (357), Italy (358), Belgium (359), Germany (37), and France (211, 351), with prevalence varying from 0.4 to 1.3%, demonstrating a wide geographical spread across the continent. Increasing reports of *B. venatorum* in ticks and wild ruminants make this parasite an excellent candidate for the emergence of a new zoonotic tick-borne disease, in particular in the current context of a growing number of wild hosts such as deer. As roe deer is often found even in suburban or peri-urban parks (if they are connected to more natural or semi-natural areas such as forests or rural areas), *I. ricinus* sampled in such places have already been reported as infected by *B. venatorum* (55, 323). This parasite has been detected in 1.3% of questing *I. ricinus* collected in France in a forest located in the South of Paris metropolitan area in the middle of an urban zone (211). Because of its location and the recreational activities available, this forest is visited by over 3 million people every year, emphasizing the public health risk. Similarly, the first detection of *B. venatorum* in Poland has been reported from ticks collected in an urban area (357), and a later study performed in recreational areas, corresponding to peri-urban forest near Warsaw city, showed also the presence of *B. venatorum* in questing *I. ricinus* (360).

Recent molecular phylogenetic investigations have convincingly established *B. microti* as forming a distinct and early diverging clade relative to other *Babesia* species (including the clade containing *B. divergens* and *B. venatorum*) as well as to *Theileria* species (361–363). *B. microti* is responsible for several hundred cases reported yearly in the USA in both spleen-intact and asplenic patient (132). This rodent parasite is known to be transmitted by *I. ricinus*, and now seems to be widely established in Europe, although only one human case has been reported to date (341). The substantial difference in the human pathogenicity of the North-American and European *B. microti* strains need further studies. It has been identified in *I. ricinus* in several European countries

such as Switzerland (364), Poland (365), Hungary (366) Slovenia (367), Germany (368), the Netherlands (356, 369), Belgium (359), and France (340). Microtine rodents and probably shrews are the reservoirs of *B. microti* (**Table 1**). Infectious tick bites are most likely to occur in deciduous woodland and peri-domestic settings (37, 55, 323). Indeed, this parasite was recovered in questing ticks from a forest in Poland that was qualified as "one of the most popular tourist destinations in Poland," highlighting the risk for humans during recreational activities (360).

NEW OR NEGLECTED TICK-BORNE PATHOGENS: STILL UNKNOWN BACTERIAL, PARASITIC, AND VIRAL SPECIES TO BE DISCOVERED?

Due to advances in molecular biology, new species, strains, or genetic variants of microorganisms are being detected in ticks, resulting in an ever-increasing list of pathogens capable of infecting domesticated animals and humans. Some of them have been linked to human or animal diseases only many years after their first discovery in ticks or animal reservoirs (299). An emblematic example is that of *B. henselae*, the agent of Cat Scratch Disease, known to be transmitted from cat to human by cat scratch (or by fleas). For years, cases of *B. henselae* infection had been described in patients without history of contact with cat without any idea how these people could be infected. By screening pathogens in ticks, *B. henselae* DNA, and RNA were identified in *I. ricinus* (370–373). After many years of debate to know whether *B. henselae* was or was not a tick-borne pathogen, the direct link between tick bites, *B. henselae*, and disease in humans was finally demonstrated (374). Another striking example is the one of *Borrelia miyamotoi.* This *Borrelia* species has been isolated for the first time in Japan in 1995 from *Ixodes* ticks and has been considered as non-pathogenic endogenous tick bacteria until the first human cases of *B. miyamotoi* infection were reported in Russia in 2011 (375). Since then, human infections have been described in the USA and in 2013 in the Netherlands (376–379). In France, *B. miyamotoi* was found to circulate in *I. ricinus* as well as in the bank vole *M. glareolus* (380), and this French genotype was identical to the genotype isolated from a sick person in the Netherlands. These findings have important implications for public health, especially considering that *B. miyamotoi*-positive ticks and rodents were collected from different sites in close proximity to human dwellings. Up to now, no human cases of *B. miyamotoi* infections have been reported in most European countries, however, symptoms caused by *B. miyamotoi* could easily be confused with symptoms caused by other pathogens, which are better known by practitioners, suggesting that surveillance urgently needs to be improved.

A more recent example of neglected pathogens is a new phlebovirus that has been described in humans from northwestern Missouri, USA independently presented to a medical facility with fever, fatigue, diarrhea, thrombocytopenia, and leukopenia, and all had been bitten by ticks 5–7 days before the onset of illness. Electron microscopy revealed viruses consistent with members of the Bunyaviridae family. Next-generation sequencing and phylogenetic analysis identified the viruses as novel members of the phlebovirus genus (381). All these examples demonstrate that new or unexpected tick-borne pathogens are characterized, as soon as they are looked for, in patients bitten by ticks.

CONCLUSION

Tick-borne diseases in urban and peri-urban areas represent a rising hazard for public and animal health in Europe. The rapid global changes that planet Earth is facing, especially due to the human ecological footprint, are also affecting the ecology and epidemiology of infectious diseases, including tick-borne diseases. The *I. ricinus* tick being the principal vector of a plethora of viral, bacterial, and protozoan pathogenic microorganisms is showing adaptations to new habitats and ecological conditions. Persistent and potentially increasing populations of this tick species are present in green areas within European cities. Public parks, small forest patches, gardens, and cemeteries are of increasing interest as they represent places where humans, companion, and domestic animals can encounter ticks and be exposed to infected tick bites. The presence of large vertebrates, that serve as tick-maintenance hosts and find conditions to survive and reproduce in the peri-urban environment, reduces the extinction risk of tick populations. Furthermore, majority of tick-maintenance hosts are ecologically classified as generalist species and in many cases serve as reservoirs of a number of emerging zoonotic pathogens, including those transmitted by *I. ricinus.* The combination of urbanization, climate change, and alterations in land-use patterns along with socio-economic factors (outdoor sports and leisure-time activities, gardening, an increased density of pets, and companion animals near human settlements) act in creating favorable conditions for increasing the exposure of humans to ticks, thus favoring the transmission of tick-borne pathogens in urban and peri-urban areas.

Risk communication campaigns aimed at implementing preventive measures against infectious tick bites in urban and peri-urban habitats therefore deserve particular public health efforts. However, several knowledge gaps and lack of quantitative ecological, epidemiological, and socioecological data limit our ability to provide precise quantitative risk pre-assessment. Therefore, more eco-epidemiological research and surveillance specifically focused on the occurrence of ticks, their infection with pathogenic microorganisms as well as on the presence of tick-maintenance and reservoir vertebrate hosts in urbanized areas is urgently needed. Only a multidisciplinary "One-Health" approach integrating research outputs of specialists from different disciplines (veterinarians, zoologists, ecologists, molecular biologists, epidemiologists, physicians, sociologists, and public health experts, etc.), combined with appropriate outreach and dissemination campaigns, can bring success in making urban and peri-urban areas safer from infection by tick-borne pathogens.

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The heterogeneity, distribution, and environmental associations of *Borrelia burgdorferi* sensu lato, the agent of Lyme borreliosis, in Scotland

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Lyme borreliosis is an emerging infectious human disease caused by the *Borrelia burgdorferi* sensu lato complex of bacteria with reported cases increasing in many areas of Europe and North America. To understand the drivers of disease risk and the distribution of symptoms, which may improve mitigation and diagnostics, here we characterize the genetics, distribution, and environmental associations of *B. burgdorferi* s.l. genospecies across Scotland. In Scotland, reported Lyme borreliosis cases have increased almost 10-fold since 2000 but the distribution of *B. burgdorferi* s.l. is so far unstudied. Using a large survey of over 2200 *Ixodes ricinus* tick samples collected from birds, mammals, and vegetation across 25 sites we identified four genospecies: *Borrelia afzelii* (48%), *Borrelia garinii* (36%), *Borrelia valaisiana* (8%), and *B. burgdorferi* sensu stricto (7%), and one mixed genospecies infection. Surprisingly, 90% of the sequence types were novel and, importantly, up to 14% of samples were mixed intra-genospecies co-infections, suggesting tick co-feeding, feeding on multiple hosts, or multiple infections in hosts. *B. garinii* (hosted by birds) was considerably more genetically diverse than *B. afzelii* (hosted by small mammals), as predicted since there are more species of birds than small mammals and birds can import strains from mainland Europe. Higher proportions of samples contained *B. garinii* and *B. valaisiana* in the west, while *B. afzelii* and *B. garinii* were significantly more associated with mixed/deciduous than with coniferous woodlands. This may relate to the abundance of transmission hosts in different regions and habitats. These data on the genetic heterogeneity within and between *Borrelia* genospecies are a first step to understand pathogen spread and could help explain the distribution of patient symptoms, which may aid local diagnosis. Understanding the environmental associations of the pathogens is critical for rational policy making for disease risk mitigation and land management.

Keywords: *Ixodes ricinus*, ticks, Lyme disease, MLST, PCR, genetics, allele, sequence type

INTRODUCTION

Lyme borreliosis is the most prevalent tick-borne human disease in the northern hemisphere, and is growing in incidence in Europe. For example, Scotland has seen an almost 10-fold increase in reported cases since 2000 (1). The causative agent of Lyme borreliosis is *Borrelia burgdorferi* sensu lato, a complex of related spirochete bacteria comprising a suite of genospecies, which vary in pathogenicity and cause different symptoms. The global distribution of the genospecies differs between continents, for example, *Borrelia afzelii* and *Borrelia garinii* are found only in Europe, *Borrelia carolinensis* is found only in North America, and *B. burgdorferi* sensu stricto is found on both sides of the Atlantic (2).

There are currently at least 18 proposed and confirmed *B. burgdorferi* genospecies globally, which vary in their pathogenicity, reservoir host associations, and geographic distributions within and between countries (3–5). Three genospecies (*B. burgdorferi* s.s., *B. garinii*, and *B. afzelii*) are commonly reported to cause Lyme borreliosis and *B. valaisiana* and *Borrelia lusitaniae* may also be pathogenic (6–10).

Borrelia burgdorferi s.l. is transmitted by *Ixodes* ticks and, in most of Europe, including the UK, the principle vector is *Ixodes ricinus*. *I. ricinus* are generalist ecto-parasites, feeding on most terrestrial vertebrate species. However, each *B. burgdorferi* s.l. genospecies is specialized and associated with a particular host type. *B. garinii* and *B. valaisiana* are commonly found in birds, *B. afzelii* is associated with small mammals, and *B. burgdorferi* s.s. is associated with both birds and small mammals (11–13). We may therefore predict that genospecies prevalence varies with the relative abundance of these host types or, as a proxy, with different habitats that are associated with these hosts. In addition, within host types, some species are more effective at pathogen transmission than others, so we might predict within-genospecies genetic diversity to vary, for example, we might predict that *B. garinii* may have higher genetic diversity than *B. afzelii* because there are many

more species of birds than small mammals. In addition, if genetic variation over space is driven by host movements [e.g., Ref. (14, 15)], we can predict that sequence types within genospecies are more closely related if they occur in closer geographic proximity; we can use spatial genetic variation to infer host movement behavior and in turn this helps us identify how each pathogen spreads over space.

Ixodes ricinus has three active stadia (larvae, nymphs, and adults), each of which requires a single blood meal. Unfed larvae are almost always uninfected because vertical transmission of *B. burgdorferi* s.l. from adult females to larvae is extremely rare (16). Despite the host specificity of each genospecies of *B. burgdorferi* s.l. and the general assumption that each tick stage feeds on only one host, co-infections of both "bird" and "mammal" genospecies can occur within a tick (3), although the frequency of such co-infections is less than expected (17). Such co-infections might suggest that the host specificity of genospecies is not absolute, or a tick stage may occasionally take more than one blood meal (from more than one host type), or through co-feeding [transmission from one tick to another without systemic host infection (18, 19)]. This interesting phenomenon warrants further research and one of our aims is to quantify the frequency and type of mixed infections in our studied *I. ricinus* populations.

The molecular characterization of *B. burgdorferi* genospecies and strains has been revolutionized by multi-loci sequence typing (MLST) and in this study we use the system developed by Margos and others (20) which has been shown to unambiguously delineate genospecies and establish evolutionary and geographic relationships. DNA can be directly amplified by polymerase chain reaction (PCR) from tick extracts and the amplicons sequenced without need for culture. The *B. burgdorferi* MLST website[1] currently documents more than 1500 *B. burgdorferi* strains in the MLST database, currently comprising 572 sequence types from sites in Europe, North America, and Asia.

Previous MLST of studies of *B. burgdorferi* s.l. have been conducted at a continental scale (15, 20, 21) or focused on a single genospecies (20, 22, 23). Our study differs by employing a dense stratified survey to genetically and ecologically characterize the full suite of genospecies at a national scale. Characterizing the variety, distribution, and abundance of sequence types (i.e., the allelic profile) of *B. burgdorferi* s.l. in one country, especially linked to environmental information, will contribute to our understanding of the heterogeneous distribution and prevalence of genospecies, how the pathogens spread, identify the environmental risk factors and will also have implications for patient symptoms and diagnosis. We focused our *B. burgdorferi* s.l. survey on Scotland, where reported cases of Lyme borreliosis have increased almost 10-fold since the turn of the millenium, and where there have been very few previous studies (and no large-scale systematic surveys) of *B. burgdorferi* s.l. genospecies; (24) reported five *B. afzelii* samples and seven *B. burgdorferi* s.s. while (15) genotyped three *B. burgdorferi* samples from one Scottish site and found all to be *B. afzelii.*

This study therefore aimed to examine the phylogenetic population structure of Scottish *B. burgdorferi* s.l. by characterizing the genospecies, sequences types, and alleles and describing their spatial distribution across the country and identifying environmental and regional associations. This was to provide the first large-scale fundamental data on the Lyme borreliosis agents across this country and to gain insight into genetic mixing spatially across Scotland and with other countries (e.g., due to host movements). Furthermore, we were particularly interested in identifying mixed infections (both between genospecies and between sequence types within genospecies) within individual ticks, since this has implications both for patient symptoms and diagnosis and for our understanding of tick feeding behavior. We also aimed to correlate *B. burgdorferi* s.l. genospecies with environmental factors, which is of use in understanding the relationship between host communities and genospecies composition, and in assessing disease risk and mitigation options in different environments.

MATERIALS AND METHODS

FIELD COLLECTION OF TICKS

Of the three life stages, nymphs are thought to pose the greatest risk to humans in terms of transmitting *B. burgdorferi* s.l.: questing larvae very rarely carry the pathogens (16), while adults are much less numerous than nymphs and are much larger and therefore more likely to be noticed and removed quickly. Indeed, (25) estimated that 82% of human tick bites from a forested area in England were from nymphs. Therefore, this study concentrated primarily on *I. ricinus* nymphs. Questing (host-seeking) nymphs were collected during blanket dragging surveys at 25 woodland sites across Scotland in the springs and summers of 2007–2008 [see Ref. (26)]. A 1 m × 1 m square of blanket material was dragged for 10 m and all ticks counted and collected. At least 20 drags were conducted at each site in a semi-random fashion so as to cover the site in a representative way (26). At least 50 nymphs per site were screened for *B. burgdorferi* s.l. (see method below) including at least one nymph from each drag. Woodland was chosen because it is the habitat most often associated with high densities of a variety of tick species, both in Europe and North America [e.g., Ref. (27–30)] and the habitat most associated with acquiring Lyme borreliosis in Scotland (31). A broad geographic spread of sites ensured as much coverage over the country as possible, while collecting ticks from both semi-natural mixed/deciduous and conifer forests ensured that the main woodland habitat types (and therefore by proxy a range of host communities) were sampled. In addition, each site was associated with known cases of Lyme borreliosis (31).

As well as sampling questing nymphs from the 25 woodland sites, ticks were also removed from hosts. Passerine birds were trapped at one of the woodland sites by mist netting, under license issued by the British Trust for Ornithology during the spring and summer of 2008 [see Ref. (32)]. Feeding nymphs and larvae was removed from birds and stored in vials of 70% ethanol. Small mammals (wood mice *Apodemus sylvaticus* and bank voles *Myodes glareolus*) were trapped using longworth live traps, under license from Scottish Natural Heritage, at four of the woodland sites during the spring and summer of 2007. Ticks were removed and stored in vials per animal in 70% ethanol.

TICK POOLING FOR ANALYSIS

Questing *I. ricinus* nymphs collected in 2007 were each homogenized and amplified individually by PCR. However,

[1] www.mlst.net

questing nymphs from 2008 were pooled in groups of five for processing. Our screening of nymphs collected in 2007 determined that the mean infection prevalence of *B. burgdorferi* s.l. was around 5%, therefore, the probability of more than one positive tick occurring in a pool of five was only 2.3%. Pooling was undertaken only for ticks collected at the same site on the same visit (and in the majority of cases from the same 10 m × 1 m survey transect).

Feeding nymphal ticks removed from birds were PCR amplified individually (as feeding nymphs have now fed on two hosts). Feeding larval ticks removed from small mammals were pooled per animal. This was because transovarial transmission is thought to occur at very low frequencies (16) such that any larval ticks collected from one animal should have been exposed to *B. burgdorferi* s.l. present in that particular animal only. Between 2 and 28 larvae were removed from each small mammal and pooled for PCR (nymph ticks from mammals were collected in very small numbers and not assayed).

BORRELIA BURGDORFERI S.L. SCREENING AND MLST

DNA extraction was performed by mechanical destruction of the nymph and larval ticks by ammonia extraction (32, 33). A nested PCR for the 5S-23S rRNA IGS and visualization by agar electrophoresis were used to detect *B. burgdorferi* s. l. (34). A positive control of *B. lusitaniae* (a genospecies not found in northern Europe) and a negative control were used in all PCRs so that it was possible to detect any cross contamination or false positives. MLST, which has been shown to unambiguously delineate genospecies (20), was used to type all positive samples at eight loci (*clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *rplB*, and *uvrA*) (after 20). Positive controls were also typed and comprised *B. afzelii* (strain VS461), *B. garinii* (20047), *B. valaisiana* (VS116), *B. burgdorferi* s.s. (B31), and *B. lusitaniae* (Poti B2). PCR products were sequenced in both directions using an ABI automated DNA sequencer.

Forward and reverse sequences were compared, aligned, and trimmed using Sequence Editor (version 1.0.3, Macintosh computers) and consensus sequences were assigned an allele number. New alleles were submitted to the MLST website[1].

PHYLOGENETIC ANALYSIS

Phylogenetic trees were drawn using MEGA [Version 4.0.2 (35)], which was also used to calculate pair-wise genetic distances and nucleotide differences between sequences. We also used this analysis to test our prediction that *B. garinii* should have greater genetic diversity within Scotland than *B. afzelii* [because there are more birds than small mammal species in Scotland (and Europe) and birds can carry mainland European strains into Scotland].

To test our prediction that samples should be more genetically similar if they are from sites in close proximity (and genetically dissimilar if they are far apart), we used linear regression analysis to examine the relationship between the geographical distance (kilometer) between the collection sites and the molecular diversity in *B. afzelii* and *B. garinii* samples [as defined by the number of single nucleotide polymorphism differences, calculated in MEGA version 4.0.2 (35)]. We compared models that were fit using distance (distance)^2, square root (distance), and log (distance + 1) in order to test for both linear and curvilinear relationships. We chose the best fit based on model outputs of R-squared and F values and residual fits.

To estimate whether we sampled enough ticks to provide a full picture of alleles and sequence types over Scotland we used rarefaction curves. This is a standard method to gage the extent to which sampling achieves saturation coverage (i.e., all types of individual or species in a population are sampled) (36). By plotting the number of samples analyzed against the number of sequence types and alleles found for each of the eight loci, the shape of the curve indicates whether most of the alleles or sequence types have been found (i.e., when the curve plateaus because few additional alleles are being found) or whether there are many more to be discovered (i.e., the curve is still climbing steeply because more alleles are being found). Rarefaction curves were drawn in Analytical Rarefaction 1.3 (University of Georgia), both with data from this study and with *B. burgdorferi* s.s. data taken from Ref. (20).

IDENTIFYING MIXED INFECTIONS

To investigate which novel sequence types found may not be real sequence types but, instead, may be a result of mixed allele infections, we closely examined each allele combination in relation to sequence type using only those samples that were successfully analyzed at eight loci (Table S1 and Figure S1 in Supplementary Material). It was assumed that sequence types that are represented by more than one sample or have previously been added to pubMLST are genuine sequence types. It was also assumed that single locus variants of a sequence type with more than one occurrence were also genuine sequence types. In order to estimate the frequency of intra-genospecies co-infections, we examined *B. afzelii* genotypes because *B. afzelii* was the most frequently found genospecies (and, as the results show, had a very large proportion of novel sequence types).

As well as mixed infections, there are alternative hypotheses for novel and single-occurring sequence types. For example, they could arise as the result of homoplasy (the independent acquisition of the same nucleotide polymorphism in an unrelated lineage due to mutation) or horizontal gene transfer (recombination). We considered homoplasy to be unlikely if there was more than one single nucleotide polymorphism. We tested for horizontal gene transfer by using Clonal Frame (version 1.1) to examine the clonal relationships between sequence types and to estimate the recombination events, which may have disrupted inheritance (37).

ENVIRONMENTAL ASSOCIATIONS

Associations between genospecies and the environmental factors (geographical area, woodland type, and deer abundance) at each tick collection sites were examined. We chose two broad area categories: the Grampian region, which consists of the Cairngorms, Speyside, Deeside, and Moray in the northeastern quarter of Scotland, and all other sites further west of this region (generally characterized by a warmer and wetter climate than Grampian). Woodlands were categorized as either coniferous or mixed/deciduous. All of the coniferous category were commercial plantations generally consisting of Scots pine *Pinus sylvestris* or spruce *Picea spp.* with larch *Larix decidua*, apart from one conifer site, which was semi-natural old-growth Scots pine. The mixed/deciduous woodlands were semi-natural and consisted primarily of mixed birch

Betula spp., rowan *Sorbus aucuparia* and sometimes oak *Quercus spp.,* and beech *Fagus sylvatica* with occasional Scots pine. The index of abundance of deer was the number of groups of roe and red deer dung pellets counted per 10 m × 1 m transect, averaged for each site (see also Ref. (26)).

To statistically test for associations of area and woodland type (categorical variables) with genospecies we used chi-square tests (a separate test each for area and for woodland type). These reveal differences in habitat and area between the proportions of each genospecies that make up the total number of samples tested at each site. To test for associations of deer abundance (continuous variable) with genospecies, we used a generalized linear mixed model including site as a random effect to account for multiple samples per site. Since the response variable was categorical the default model distribution was multinomial with a cumulative logit link. All statistical tests were conducted in SAS Version 9.3.

RESULTS

Of the more than 2200 *I. ricinus* tick samples assayed, 124 tested positive to *B. burgdorferi* s.l. and comprised 87 questing nymphs, 25 nymphs removed from birds, and 12 larvae from small mammals (**Table 1**). Fifty two samples were genotyped at all eight MLST loci and a phylogram of these sequence types was plotted along with most sequence types listed in the MLST database[1] (Figure S1 in Supplementary Material).

There was no overlap (sharing) of alleles between any genospecies (either in our data set or in the pubMLST database), implying that samples could be classified into genospecies level without having to genotype at all eight loci, and this also means that identifying mixed genospecies infections is easy. Seventy five samples were genotyped successfully at fewer loci while still allowing the predominant genospecies to be determined. We were not able to amplify all loci for all genospecies. For example, in *B. valaisiana* isolates, the *clpA* locus failed to amplify in six out of nine samples. This is likely attributable to sequence polymorphisms in the PCR primer oligo nucleotide sequences of these strains.

Table 1 | Genospecies grouping of Scottish isolates by sample type and the number and type of samples processed using MLST at eight loci.

Genospecies	Questing nymphs, *N* (%)	Bird nymphs, *N* (%)	Mammal larvae, *N* (%)
B. afzelii	42 (48)	0	12 (100)
B. garinii	31 (36)	24 (96)	0
B. burgdorferi s. s.	6 (7)	0	0
B. valaisiana	7 (8)	1 (4)	0
B. lusitaniae	0	0	0
B. afzelii + *B. garinii*	1 (1)	0	0
No. samples identifiable to genospecies	87	25	12
No. samples analyzed at 8 loci	40 (38 pools of 5 + 2 individuals)	4 individuals	8 pools of 2–28

Percentages show the proportion of all positive samples represented by the genospecies in question per tick type (questing nymphs, nymphs attached to birds, and larvae attached to small mammals).

As defined by deep branching of phylogenetic trees, non-sharing of alleles and pair-wise genetic differences above the threshold described in Ref. (20) we identified that, of the total 124 positive tick samples from questing nymphs, nymphs from birds and larvae from small mammals, 55 (44%) contained *B. afzelii*, 56 (45%) contained *B. garinii*, 8 (6%) were *B. valaisiana*, and 6 (5%) were *B. burgdorferi* s.s. Of most importance to Lyme borreliosis risk in humans, out of the 87 questing nymph samples 43 (49%) contained *B. afzelii*, 32 (36%) contained *B. garinii*, 7 (8%) were *B. valaisiana*, and 6 (7%) were *B. burgdorferi* s.s. (**Table 1**).

BORRELIA BURGDORFERI S.L. WITHIN-GENOSPECIES DIVERSITY

Of the 52 samples typed at eight loci, there were 35 different sequence types, of which 28 were newly described to the MLST database. The most commonly occurring genospecies was *B. afzelii* and its sequence type ST263 accounted for 8/52 samples (15%). The only other sequence types represented more than once in our study were ST287 (*B. afzelii*), ST168 (*B. afzelii*), ST327 (*B. afzelii*), and ST93 (*B. garinii*). All other sequence types were identified from only single samples in this study, although seven had been identified in previous studies[1]: ST24 (*B. burgdorferi* s.s., found in France), ST82 (*B. garinii*, found in France and England), ST86 (*B. garinii*, found in France, England, Latvia, Russia, Austria, and Italy), ST88 (*B. garinii*, found in France and England), ST93 (*B. garinii*, found in France, Italy, and England), ST168 (*B. afzelii* found in Latvia), and ST205 (*B. valaisiana*, found in England).

The phylogenetic tree (Figure S1 in Supplementary Material) shows a greater variety of alleles, and with more branching, in *B. garinii* than in *B. afzelii*. In addition, the extent of nucleotide divergence at the loci was greatest for *B. garinii,* with *B. garinii* samples typically differing by 40–60 bp across the eight loci compared to *B. afzelii* isolates which typically differed by only 1–15 bp.

Rarefaction analysis (**Figure 1**) indicated that for some of the eight loci, especially for *B. afzelii,* we detected most of the alleles in the population in Scotland (the curves plateau) while for other loci, especially for *B. garinii*, there are still several more alleles to be discovered (the curves are still climbing). For both *B. afzelii* and *B. garinii*, the curves for sequence type diversity did not plateau at all; indeed, for *B. garinii* the sequence type curve almost followed the 45° line representing a new discovery for every sample analyzed.

There was a significant positive relationship between genetic and geographic distances for samples within the same genospecies, i.e., samples were more genetically different if they were collected further apart geographically, although the proportion of variation explained by the models (*R*-squared values) was low (*B. afzelii* $F_{1,407} = 22.9$, $p < 0.001$, $R^2 = 51.0\%$; *B. garinii* $F_{1,209} = 15.6$, $p < 0.0001$, $R^2 = 6.5\%$). The best fit models were those with simply distance (kilometer); those with (distance)^2, square root (distance), or log (distance + 1) had poorer fit of the residuals and lower *R*-squared and *F* values.

MIXED INFECTIONS

There was evidence of inter-genospecies mixed infections in one sample that clearly contained alleles originating from *B. afzelii*

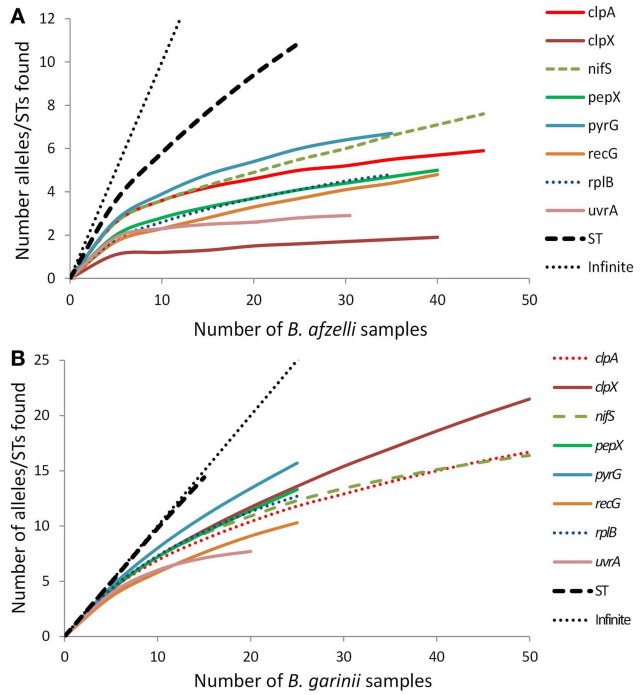

FIGURE 1 | Rarefaction curves for each of the eight *B.* burgdorferi s.l. loci and sequence types (STs) of (A) B. afzelii samples and (B) B. garinii samples analyzed from Scotland. Each graph also includes the 45° line (labeled "infinite") which represents the trajectory that would occur if every new allele or sequence type found was new.

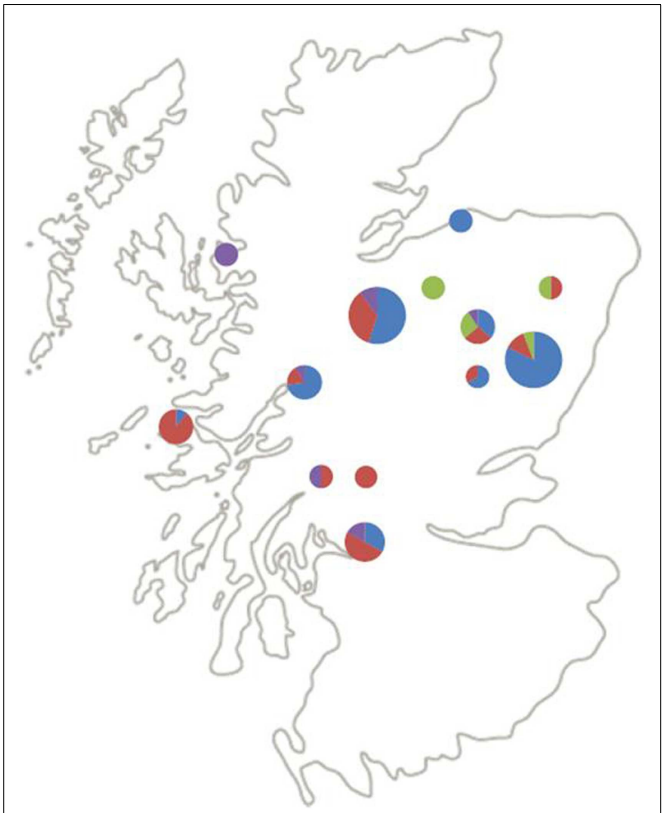

FIGURE 2 | Distribution and relative abundance of *B.* burgdorferi s.l. genospecies across Scotland. Blue = *B. afzelii*, red = *B. garinii*, purple = *B. valaisiana*, green = *B. burgdorferi* s.s. The size of the pie charts indicates the number of samples genotyped at that site (1–5, 6–10, and 11–20 samples for small, medium, and large pie charts, respectively).

and *B. garinii*. This comprises 1.9% of the 52 samples tested across eight loci or 0.8% of the 124 total samples analyzed (including those successful at fewer than eight loci) and 1.1% of the 87 total samples of questing nymphs.

Close examination of the apparent 14 different sequence types found from the 29 *B. afzelii* samples (Table S1 in Supplementary Material) suggested the possible presence of mixed intra-genospecies infections, deduced as follows. Five sequence types occurred more than once in the population (representing 20 samples) and so are plausibly genuine strains, while five sequence types (from five samples) differed at one of the two loci from the above and their different loci were the result of one or two nucleotide differences from the parental genotype so we considered these also to be plausibly genuine strains (for example ST292 has only one SNP difference in one allele from ST263). However, the remaining four sequence types (ST286, ST288, ST295, and ST326) were represented by only a single sample each and comprised either allele combinations found in other common sequence types (e.g., ST263) or two common sequence types plus a single nucleotide mutation in one of the alleles (Figure S1 in Supplementary Material). It can therefore be speculated that out of 29 *B. afzelii* samples, 25 isolates contained genuine sequence types while four apparent sequence types may actually be examples of intra-genospecies co-infection (i.e., harboring alleles from more than one *B. afzelii* sequence type). If so, this would represent a 14% incidence of intra-genospecies co-infection in the Scottish *B. afzelii* population. However, from only 29 samples, the confidence intervals are wide (upper and lower confidence intervals = 30.6 and 5.5%, respectively).

Homoplasy as a mechanism for the apparent novel sequence types seems unlikely since only two of the four appeared to contain a single nucleotide mutation and in only one of the mixed alleles. There was no evidence that horizontal gene transfer (recombination) is the explanation for these apparent sequence types because no recombination events were detected, either using only our Scottish samples or using a combination of Scottish samples and other sequence types listed in the MSLT database.

ENVIRONMENTAL ASSOCIATIONS

Figure 2 shows the geographical distribution of the genospecies of the 87 questing nymphs from the 13 (of the original 25) field sites from which positive *B. burgdorferi* s.l. samples were successfully identified to genospecies. Six sites were coniferous and seven semi-natural mixed or deciduous woodlands. *B. afzelii* and *B. garinii,* the most frequent genospecies, seem to be spread over most of Scotland. *B. afzelii* was detected in 7 of the 13 sites and *B. garinii* in 11 of the 13 sites (**Table 2**). Of the less frequently recorded genospecies, *B. valaisiana* had a broad geographic spread and occurred in 5 of the 13 sites whereas *B. burgdorferi* s.s. was recorded only in 4 sites,

Table 2 | Genospecies of *B. Burgdorferi* s.l. and sample size of positive samples from questing nymphs at each site.

Site	Habitat	Deer index	*B. garinii*	*B. afzelii*	*B. valaisiana*	*B. burgdorferi* s.s.	Total *B. burgdorferi* s.l.
QC	Conifer	0.94	3 (27%)	4 (36%)	1 (9%)	3 (27%)	11
GM	Conifer	0	2 (50%)	0 (0%)	2 (50%)	0 (0%)	4
BM	Conifer	0.61	1 (33%)	2 (67%)	0 (0%)	0 (0%)	3
CB	Conifer	0.35	0 (0%)	3 (100%)	0 (0%)	0 (0%)	3
IR	Conifer	0.08	0 (0%)	0 (0%)	0 (0%)	2 (100%)	2
AP	Conifer	0	0 (0%)	0 (0%)	1 (100%)	0 (0%)	1
DR	Mixed	0.01	7 (35%)	11 (55%)	2 (10%)	0 (0%)	20
FZ	Mixed	0	2 (12%)	14 (82%)	0 (0%)	1 (6%)	17
LA	Mixed	0	2 (18%)	8 (73%)	1 (9%)	0 (0%)	11
DV	Mixed	0.07	8 (89%)	1 (11%)	0 (0%)	0 (0%)	9
SH	Mixed	0	3 (50%)	2 (33%)	1 (17%)	0 (0%)	6
TB	Mixed	0.11	3 (100%)	0 (0%)	0 (0%)	0 (0%)	3
LV	Mixed	0.06	1 (50%)	0 (0%)	0 (0%)	1 (50%)	2
All			32 (35%)	45 (49%)	8 (9%)	7 (8%)	92

Deer index is the number of pellet groups per 10 m × 1 m transect averaged per site.

all in north-east Scotland (although with only six samples from questing nymphs in this study it may yet be recorded elsewhere if more samples are collected in future studies; **Table 2**).

There was a significant difference between genospecies and the two broad area categories ($\chi^2 = 18.8$, df = 3, $p = 0.0003$). *B. garinii* and *B. valaisiana* comprised the highest proportions of samples in the western half of Scotland while *B. burgdorferi* s.s. was most associated with the Grampian region (**Figure 3**). There was also a significant difference between genospecies and woodland type ($\chi^2 = 13.9$, df = 3, $p = 0.0031$). *B. afzelii* and *B. garinii* were more likely to be found in mixed/deciduous than in conifer forests and *B. burgdorferi* s.s., while occurring at only low prevalences, had a tendency to be found more frequently in coniferous forest (**Figure 3**). There was no evidence for a significant association between genospecies and the deer abundance at each site ($F_{1,72} = 0.53$, $p = 0.468$).

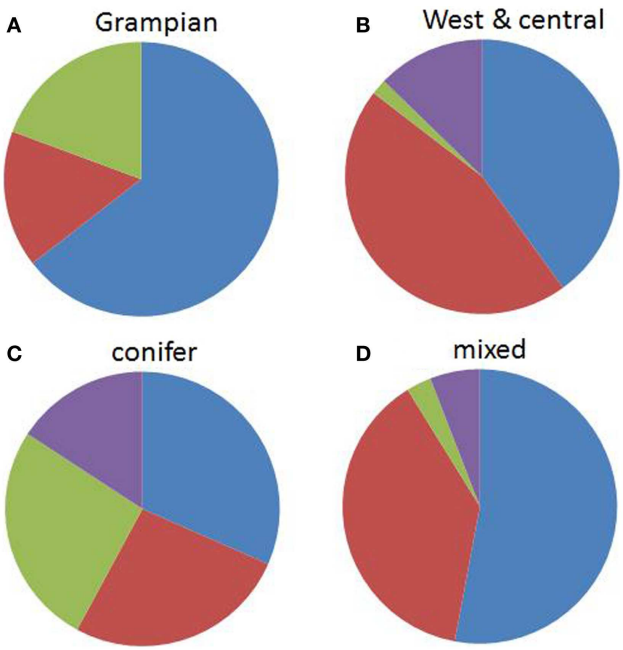

FIGURE 3 | The proportions of *Borrelia burgdorferi* s.l. genospecies by area: (A) west and central Scotland and **(B)** the Grampian Region of Scotland (the Northeast and the Cairngorms) and by habitat: **(C)** conifer forest and **(D)** mixed/deciduous woodlands, averaged over 13 (6 coniferous, 7 mixed/deciduous) sites across Scotland. Blue = *B. afzelii*, red = *B. garinii*, purple = *B. valaisiana*, and green = *B. burgdorferi* s.s.

DISCUSSION

The overall *B. burgdorferi* s.l. prevalence of 5.6% (range 1–14%) in questing nymphs [see also Ref. (26)] is similar to that found in a previous UK study (15) that found 5 and 8% prevalence in 2006–2007 and 2008–2009, respectively (range 0–12%) in ticks collected from sites mainly in England. This is a lower prevalence than that found in many countries in continental Europe [e.g., Ref. (3, 15)].

Of the *B. burgdorferi* s.l. positive questing nymphs, 49% were *B. afzelii*, 36% were *B. garinii*, 8% were *B. valaisiana*, and 7% were *B. burgdorferi* s.s. The finding that the most common genospecies in Scotland is *B. afzelii* [see also Ref. (24) which found that almost half of its 12 positive *I. ricinus* from the Scottish highlands carried *B. afzelii*] while *B. valaisiana* is relatively rare is interesting, because a quite different pattern has been found in England where *B. afzelii* is less common while *B. garinii* and *B. valaisiana* (the two bird-associated genospecies) seem to be predominant [e.g., Ref. (38)], although Ref. (15) found *B. afzelii* at around half of their English sites. The reasons for any difference in genospecies predominance between Scotland and the rest of the British Isles warrants further

research. The abundance of *B. afzelii* in Scotland is more similar to the situation in continental Europe where it is also common and often the dominant genospecies [e.g., Ref. (3, 39, 40)].

Corroborating current knowledge of genospecies specificity [e.g., Ref. (38)], we found that the sole genospecies found in larval ticks removed from small mammals (wood mice and bank voles) was *B. afzelii* while, as expected, *B. garinii* was the dominant genospecies found in nymphs removed from passerine birds (and one *B. valaisiana* sample from a bird).

WITHIN-GENOSPECIES DIVERSITY

As we predicted from the concept that the spatial distribution of genetic relatedness is driven by local host movements, for both *B. afzelii* and *B. garinii*, we found a positive relationship between geographical distance (kilometer separating field sites) and genetic distance (single nucleotide polymorphism differences) between pairs of samples within genospecies, i.e., alleles and sequence types are more different the further apart they are geographically. However, the most abundant strain type (*B. afzelii* ST263) occurs widely across Scotland (up to 110 km in this study), and could perhaps, therefore, be an ancestral strain. *B. garinii* was considerably more genetically diverse than *B. afzelii* (that was dominated by ST263). This is consistent with our predictions based on higher number of bird species than small mammal species and the higher frequency of non-local alleles being brought into Scotland from other countries by migrating birds. We found that all (apart from one, previously identified from Germany) of the *B. afzelii* sequence types we identified from Scotland were novel. This helps corroborate the proposal of Ref. (15) (stemming from previous limited data on Scottish *Borrelia burgdorferi* s.l.) that there should be a clear distinction between English and Scottish *B. afzelii* due to limited movement of small mammals between England and Scotland [see Ref. (41)]. In contrast to *B. afzelii*, several *B. garinii* sequence types that we identified in Scotland had previously been identified from France and England, suggestive of greater host (bird) movement between these countries. A high degree of diversity in *B. garinii* samples has also been noted in Ref. (42), among others, who attributed this to their bird host's large migration ranges.

MIXED INFECTIONS

The identification of mixed samples can be influenced by the extent of horizontal gene transfer events, which will decrease the observed clonality of the species. In the case of *B. burgdorferi* s.l., however, there is no evidence to support horizontal gene transfer of chromosomal genes and, certainly between genospecies, the deep branching, and absence of pan-alleles argues against horizontal gene transfer. We found only one sample clearly exhibiting alleles from two genospecies, which is most likely to be a result of mixed-genospecies infections within a tick. This is a frequency of 1–2% (depending on whether the total number of 124 samples is used or only those 52 samples analyzed at all eight loci or including only questing nymphs). This is much lower than many other areas: a meta-analysis in Ref. (3) of over 100 articles citing the infection prevalences in 112,579 questing ticks from 24 countries across Europe found that the occurrence of inter-genospecies co-infection in Ixodid nymphs was, overall, 12.1%, while as many as 64% of questing nymphs were co-infected in Denmark (43).

Assuming that transovarial transmission of *B. burgdorferi* is absent or rare such that unfed larvae are uninfected (16, 44) outlined three potential mechanisms for an unfed nymph to be co-infected with more than one genospecies: (i) through transmission by co-feeding (feeding in close proximity to a tick infected with a different genospecies to that in the host); (ii) through being unable to complete a full blood meal from one host (e.g., dislodged before repletion) and so feeding from a second host containing a different *B. burgdorferi* s.l. genospecies; or (iii) through feeding from a single host that carried more than one genospecies, perhaps because of a compromised immune system. Most mixed infections in Europe are *B. garinii* mixed with *B. valaisiana* (3) (i.e., the two genospecies associated with birds). It is noteworthy, therefore, that our two mixed genospecies samples contained alleles from *B. afzelii* and *B. garinii*, the small mammal- and the bird-associated genospecies respectively. Therefore, it is unlikely that our mixed infection resulted from a host infected with both genospecies. However, it could have resulted from the tick having an incomplete feed on a *B. afzelii* infected small mammal followed by a further feed on a *B. garinii* infected bird (or *vice versa*). It could also have potentially resulted from co-feeding: the tick attached to a bird or small mammal infected with one pathogen while feeding in close proximity to a nymph that was infected with the second pathogen.

While mixed infections of different genospecies are relatively easy to identify in samples and are now well documented [e.g., Ref. (3, 15)], it may be more likely for a tick to be co-infected with different strains of the same genospecies (each with a different sequence type), although it is much less easy to identify. This phenomenon has been previously reported: 39% of adult *Ixodes scapularis* ticks from North America were infected with more than one genotype of *B. burgdorferi* s.s. (45). From close examination of the novel sequence types we found, we estimated that the incidence of intra-genospecies co-infection is around 14% in the Scottish *B. afzelii* population. We also considered the alternative explanations for these four apparent sequence types. The allele combinations ruled out vertical inheritance. Homoplasy is unlikely since several of the shared alleles harbor more than one single nucleotide polymorphism compared to the putative ancestral strain in several cases. In addition, it has been reported as an unlikely event in the *B. burgdorferi* s.l. genome and nearly non-existent in the chromosome [reviewed in Ref. (46)]. At an environmental scale, there is no evidence of horizontal gene transfer in the IGS locus, but the *ospC* locus showed evidence of intragenic recombination (47). It would appear that recombination events are possible within the *B. burgdorferi* s.l. genome, but they are rare and limited to particular genes (e.g., *ospC*). *ospC* may be subject to recombination in a way that other genes are not due to its role in the immune system (unlike the selectively neutral housekeeping genes of MLST) (48).

Our finding of commonly occurring mixed within-genospecies co-infections is important also because it suggests that the MLST database may inadvertently contain examples of mixed intra-genospecies co-infection. Detecting and excluding these samples could be overcome only by culturing colonies from ticks and selecting individual colonies or by using detection methods, which can pick up multiple genospecies in a sample. Unless samples in the MLST database contain sequence types from individual colony

cultures (the majority are not, as they are from field collected whole ticks), then the database may contain many of these intra-genospecies mixed sequence types and should therefore be treated with caution.

More work is required to examine this phenomenon of mixed infections, for example by the cultural separation of samples with mixed chromatograms or forensic methods such as designing PCR probes to identify individual genospecies or even specific alleles. This may also help answer whether intra-genospecies co-infection is more common within certain genospecies. It perhaps would be more likely for intra-genospecies co-infection to occur in *B. garinii*, as there is more diversity of allele numbers.

ENVIRONMENTAL ASSOCIATIONS

Borrelia afzelii was found relatively evenly throughout Scotland whereas *B. burgdorferi* s.s. was found only around the Grampian region of north east Scotland (albeit detected in only nine samples). *B. garinii* and *B. valaisiana* were widely spread across Scotland although, statistically, they occurred at higher proportions in the warmer and wetter western areas of Scotland. These distributional differences are likely to be associated with differences in relative host abundances, which are generally driven by habitat which in turn is affected by both climate and anthropogenic land management goals. Four of the six (67%) Grampian sites were coniferous, compared with two of the seven (29%) western sites but within each of our two very basic woodland categories are many sub-categories of habitat such as lowland deciduous, upland birch, Atlantic oak, juniper scrub, ancient pine, and commercial plantation. While the number of sites we originally sampled was 25, many more would be needed to statistically test genospecies associations with finer habitat categories. However, using our two basic woodland types, *B. garinii* and *B. afzelii* more likely to be found in deciduous forests while higher proportions of *B. burgdorferi* s.s. were found in coniferous forest. This is likely to reflect the differences in relative abundance of host types between habitats: *B. garinii* and *B. afzelii* are associated with birds and small mammals, respectively, and semi-natural mixed or deciduous woodlands are generally associated with higher abundance and biodiversity of both birds and small mammals than are conifer plantations. However, it is unclear why *B. burgdorferi* s.s. should occur more frequently in coniferous woodlands, since previous studies suggest that the key reservoir hosts for *B. burgdorferi* s.s. are, as for *B. afzelii*, small mammals [e.g., Ref. (49, 50)]. Given the low number of *B. burgdorferi* s.s. positive samples we found in Scotland, this could be a statistical artifact, but further work is required to identify the key reservoir host for *B. burgdorferi* s.s. in Scotland and its relative abundance between habitats. Given that the spatial distribution of *B. burgdorferi* s.s. seems to be restricted to the Grampian Region and Speyside, and there may be an association with coniferous forests, we can speculate that red squirrels *Sciurus vulgaris* (that are also more abundant in conifer forests in this region than many other parts of Scotland) could be important *B. burgdorferi* s.s. in Scotland. Indeed, *B. burgdorferi* s.s. is prevalent in red squirrels in Switzerland and red squirrels can transmit *B. burgdorferi* s.s. to feeding ticks (51). Similarly, associations have been found between *B. burgdorferi* s.s. and western gray squirrels *Sciurus griseus* in California (52).

CONCLUSION

This large-scale intensive analysis of more than 2000 *I. ricinus* tick samples from over 1200 10 m × 1 m transect surveys, and from birds and small mammals, at 25 sites has provided the first comprehensive analysis of the *B. burgdorferi* s.l. genospecies present in Scotland. That the most prevalent genospecies was *B. afzelii* was surprising as it has been postulated to be rare in the United Kingdom (2, 15). We found much lower inter-genospecies co-infections (1%) than found in other countries but, importantly, we found frequent intra-genospecies co-infections (14% of *B. afzelii*), suggesting co-feeding ticks, ticks feeding on multiple hosts, or multiple infections within hosts. Our findings that genetic and geographic distances are positively correlated and the differences in intra-genospecies genetic diversity can help us understand how, and from where, each pathogen spreads spatially over time. We speculate that red squirrels may be an important reservoir host for *B. burgdorferi* s.s. in northeastern Scotland, from circumstantial evidence of its regional and habitat associations, as well as previous evidence from Switzerland. The association between some genospecies and geographic area could be useful to practitioners in diagnostics, since each genospecies varies in the symptoms caused, especially if future work can determine the pathogenicity of different local strains. By examining the spatial patterns of genospecies and strain types in many countries, and linking this to their pathogenicity, it may become possible to understand the heterogeneous spatial distributions of genospecies, disease risk, and patient symptoms across more globally.

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SUPPLEMENTARY MATERIAL

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Circulating strains of *Brucella abortus* in cattle in Santo Domingo de los Tsáchilas Province – Ecuador

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The Province of Santo Domingo de los Tsáchilas in Ecuador represents the largest informal cattle market. Because of its strategic position, cattle movement is very high and therefore we selected this region, to determine the strain variation of *Brucella* sp. Part of the study aimed at the isolation, biotyping, and genotyping of *Brucella* species from milk and supra-mammary lymph nodes of sero-positive bovines, using selective Farrell medium, biochemical assays, and IS711-PCR, AMOS-PCR, and HOOF-Prints techniques. In total, 656 animals from 12 sero-positive dairy herds and from the provincial slaughterhouse were diagnosed by Rose Bengal and Wright's Slow Agglutination test with EDTA. Amongst these animals, 50 animals were sero-positive for brucellosis. Twenty-five lymph nodes and 25 milk samples from each group of positive reactors were transferred to culture medium. Isolation was possible from 4 (16%) lymph nodes and 9 (36%) milk samples; out of these, 10 isolates were diagnosed as *Brucella* sp. All four isolates of lymphatic tissue corresponded to *Brucella abortus* biotype 1, confirmed as field strains by molecular analysis. Milk isolations, showed biochemically a more dispersed pattern in which *B. abortus* biotypes 1 and 4 were found; yet four samples gave a pattern similar to *B. abortus* biotype 2; however, only biotypes 1 and 4 were confirmed by molecular analysis. The concordance between biochemical and molecular diagnostic tests reached 76.9%.

Keywords: *Bovine brucellosis*, *Brucella abortus*, Ecuador, *Brucella abortus* biotype 1, *Brucella abortus* biotype 4, VNTR

INTRODUCTION

Brucellosis is a widespread zoonotic disease, affecting cattle, sheep, goats, pigs, and humans (1). From a total of nine species of *Brucella* reported so far, four species are zoonoses: *Brucella abortus*, *B. canis*, *B. melitensis*, and *B. suis* which have been typically related to cattle, dogs, sheep goats, and pigs, respectively. Other species such as *B. microti*, *B. neotomae*, *B. ovis*, *B. pinipedialis*, and *B. inopinata* are supposed to be host specific (2, 3).

In cattle, the main symptoms associated with brucellosis include abortion and poor health in newborn calves. Epididymitis and infertility have been also reported in bulls (4, 5). In Ecuador, annual losses due to brucellosis in cattle are estimated to be around 5.5 million USD due to abortions, reduced milk yield, and mortality (6). In addition, in several municipalities in Ecuador, the presence of brucellosis in humans has been directly related to its presence in the cattle population (7), with, so far, only, *B. abortus* as the causative agent of human brucellosis (8, 9), contrary to neighboring Colombia and Peru, were in addition to *B. abortus*, *B. melitensis*, and *B. suis* have equally been reported in man (8, 10).

Determining the strain variability of *Brucella* can be helpful to understand the geographical and epidemiological dispersion of the disease as shown in the United States where molecular techniques have been used to evaluate strain diversity of *B. abortus* to define foci of transmission between cattle and wildlife, i.e., elk and bison, and also to identify infections related to the use of vaccines (11). In northern Ecuador, previous studies have reported *B. abortus* biotype 1 and 4 in human samples (9, 12), yet the diversity of *Brucella* sp. in cattle has not been investigated previously.

The livestock market in Santo Domingo de los Tsáchilas province is the largest in the country because of its strategic geographical location (13). This cattle market is very informal, facilitating the movement and exchange of animals and meat to large cities. It is also an important center for the trade of animals from the dairy areas of the Sierra region to different areas in the coastal region for fattening bull calves, as such it is hardly surprising that many of the outbreaks of foot-and-mouth disease started in this region (13). Thus, the sanitary condition of animals in this region might offer a reflection of the health status of cattle from different zones of the country. In this context, and given the zoonotic risk related to cattle brucellosis, the evaluation of the disease prevalence supported by a study of strain variability in cattle

passing through this region will be an important epidemiological tool, including the assessment of the importance of food-borne brucellosis.

MATERIALS AND METHODS

STUDY DESIGN

The study area was located at Santo Domingo de los Tsáchilas Province (0.14°: −0.70°N, −78.73°: −79.62°E). In total, 656 blood samples were collected from 12 sero-positive dairy farms, previously identified during a large-scale national survey (data not published) and at the provincial abattoir between May and June 2013. Samples were analyzed by Rose Bengal plate (RB) and Wright's Slow Agglutination Test with EDTA (SAT-EDTA). Equally, milk and supra-mammary lymph nodes were carefully sampled avoiding contamination and stored at 4°C until screening by RB and/or SAT-EDTA. Samples from positive reactors were processed for bacterial growth in the specific growth medium.

SEROLOGICAL TESTS

All blood samples were tested by Rose Bengal (Bengatest antigen® 4% v/v suspension) and Wright's SAT-EDTA (antigen SAW®, Synbiotics ASAW code). For RB, the slightest trace of agglutination was considered as positive. For SAT-EDTA, 100 μl of antigen was added to a doubling serum dilution from 1/12.5 up to 1/25.600. Data were recorded as international agglutination units (international units per milliliter) with values equal or greater than 30 IU/ml, corresponding to a transparency of 25% of a 1/25 dilution, considered as a positive reactions as described by Godfroid and Boelaert (14).

MICROBIOLOGICAL ISOLATION

In a microbiology laboratory (biosafety type III), lymph nodes were macerated using the Stomacher®, milk samples were centrifuged at 3000 *g* for 10 min. Both macerated nodes and cream were tested for bacterial growth in selective Farrell medium [Columbia blood agar base CM0331 (Oxoid) + horse serum (reference: 16050-130 Gibco) + modified *Brucella* Selective Supplement SR0083A (Oxoid)]. Cultures were kept at 37°C and 5% CO_2 for 5 days (15). Then, isolates were transferred to agar base [Columbia blood agar base CM0331 (Oxoid)] to obtain distinct *Brucella* sp. colonies. Finally, part of the colonies was used for DNA extraction and another part was stored at −70°C for further analysis.

BIOTYPING AND MOLECULAR IDENTIFICATION

Isolated colonies were biotypified by macroscopic observation and biochemical assays, i.e., urease, catalase, oxidase, and hydrogen sulfide production. Additionally, bacterial cultures were grown on media with stained safranin, thionin, and fuchsin at different concentrations, and tested for agglutination with Anti-A and Anti-M mono-specific sera (15).

For molecular identification, genomic DNA was extracted according to Marmur and Kirby [phenol–chloroform–isoamyl alcohol (16)]. DNA amplification was performed using protocols IS711-PCR and AMOS-PCR as described by Ref. (17, 18) to identify genera and species, respectively. Primers for DNA amplification are presented in **Table 1**. Each PCR-reaction had a final volume of 20 μl. Master mix was made with 1 U/45 μl of Taq Polymerase, 1X buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM

Table 1 | Primers used in the study.

Primer (name)	5′-3′ Sequence	
Primer sequence for IS711-PCR for genus identification		
IS6501 3′	GAT AGA AGG CTT GAA GCT TGC GGA C	
IS6501 5′	ACG CCG GTG TAT GGG AAA GGC TTT T	
Primer sequence for conventional AMOS-PCR for species identification		
B. abortus-specific primer	GAC GAA CGG AAT TTT TCC AAT CCC	
B. melitensis-specific primer	AAA TCG CGT CCT TGC TGG TCT GA	
B. ovis-specific primer	CGG GTT CTG GCA CCA TCG TCG	
B. suis-specific primer	GCG CGG TTT TCT GAA GGT TCA GG	
IS711-specific primer	TGC CGA TCA CTT AAG GGC CTT CAT	
Primer sequence for "HOOF-prints" biotype		**Primer (reverse)**
Locus-1	GGT GAT TGC CGC GTG GTT CCG TTG AAT GAG	REV-3
Locus-2	CCC GCA TGA TCC GCG AAC AGC TGG ATG	REV-1
Locus-3	CAG GCG CTT GAG GAT GAG GCG GCA G	REV-3
Locus-4	GCA GAA TTT TCG AGG CAT TCG GCG ATG	REV-3
Locus-5	GTG CTC CAG GGC GCC GGG AGG TAT GTT TAG	REV-3
Locus-6	GCC GCA GGA AAG CAG GCG ATC TGG AGA TTA TC	REV-3
Locus-7	CAG AGC CGT CGG TGG TTA CTT GAG TAG GGC AG	REV-1
Locus-8	GTG GGA AGC GTT ATC CTT TAA CGG GAG TAA GGG	REV-1
REV-1	GGG GAG TAT GTT TTG GTT GCG CAT GAC CGC	–
REV-3	GGG GGC ART ARG GCA GTA TGT TAA GGG AAT AGG G[a]	–

[a] *R = A to G.*

of each primer, and approximately 10 ng of DNA. To characterize the *Brucella* biotype, the "HOOF-Print" technique was used as described by Bricker et al. (19) and Bricker and Ewalt (20) for eight *loci*; all VNTR were amplified separately using primers described in **Table 1**; each PCR-reaction had a final volume of 15 µl and the master mix was composed with 0.6 U of Taq Polymerase, 1X buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.2 mM of each primer, and approximately 10 ng of DNA.

DATA ANALYSIS

The proportions of isolation of *Brucella* sp. were contrasted by Fisher exact test with 5% statistical significance. Additionally, an estimation of the test concordance was measured in terms of positive and negative agreements over the total isolations. Data were analyzed in "R" software version 3.1.0.

RESULTS

SEROLOGY

Out of 656 blood samples, 50 were sero-positive, i.e., 25 were from the slaughterhouse and 25 were from sero-positive dairy farms of Santo Domingo.

MICROBIOLOGICAL ISOLATION

Twenty-five milk and 25 lymph node samples were processed and isolated in a specific microbiological medium. The bacterial growth of *Brucella* spp. was evidenced in nine (36%) and four cases (16%), respectively. No statistical difference was found between the types of sample used for the isolation (p-value = 0.1085); yet isolation from milk appeared to be better than from tissues.

BIO-TYPIFICATION

Table 2 shows the biochemical features of the microbiological isolations from sero-positive animals and from those where *Brucella* was isolated (milk or supra-mammary lymph nodes). Out of nine milk isolations, six were biochemically compatible with *B. abortus* biotype and three were "not determined" isolations (ND, samples: 8, 10, and 13) because they did not present urease activity, nor growth in CO_2 and no H_2S production. Isolations from lymphatic nodes (samples 1–4) were also biochemically compatible with *B. abortus*. In total, nine isolates were sensitive to inhibition by basic fuchsin, four were insensitive but agglutinated with anti-A sera. Nine isolates agglutinated with anti-A sera (i.e., samples 1–5, 6, 7, 9, and sample 11) and only one agglutinated with anti-M sera (sample 12) hence corresponding to

Table 3 | Genotyping of *Brucella* spp. from isolates of milk and lymph nodes collected in Santo Domingo de los Tsáchilas province.

Sample no.	Code	PCR[a]-IS711	AMOS[b]-PCR	VNTR[c]
1	1482	+	*B. abortus*	*Bvar1*
2	1483	+	*B. abortus*	*Bvar1*
3	1550	+	*B. abortus*	*Bvar1*
4	1552	+	*B. abortus*	*Bvar1*
5	1476	+	*B. abortus*	*Bvar1*
6	1285	+	*B. abortus*	*Bvar1*
7	1286	+	*B. abortus*	*Bvar1*
8	1294	+	*B. abortus*	*Bvar1*
9	1301	+	*B. abortus*	*Bvar1*
10	1302	+	*B. abortus*	*Bvar1*
11	1306	+	*B. abortus*	*Bvar1*
12	1307	+	*B. abortus*	*Bvar4*
13	1308	+	*B. abortus*	*Bvar1*

[a] *PCR, polymerase chain reaction.*

[b] *AMOS-PCR, PCR for detection of B. abortus, B. melitensis, B. ovis, and B. suis.*

[c] *VNTR, variable number of tandem repeat.*

Table 2 | Differential characters of *B. abortus* and biotypes isolated from milk and lymph nodes collected in Santo Domingo de los Tsáchilas province.

Sample no.	Code	Source	Activity					Growth on dye media			Agglutination in mono-specific sera		Biotype
			Oxidase	Catalase	Urease	CO_2	H_2S	Fuchsin	Safranin	Thionin 20 µg	Anti-A	Anti-M	
1	1482	Lymph node	+	+	+	+	+	+	−	−	+	−	*Bvar1*
2	1483	Lymph node	+	+	+	+	+	+	+	−	+	−	*Bvar1*
3	1550	Lymph node	+	+	+	+	+	+	+	−	+	−	*Bvar1*
4	1552	Lymph node	+	+	+	+	+	+	+	−	+	−	*Bvar1*
5	1476	Milk	+	+	+	+	+	+	+	−	+	−	*Bvar1*
6	1285	Milk	+	+	+	+	+	−	−	−	+	−	*Bvar2*
7	1286	Milk	+	+	+	+	+	−	−	−	+	−	*Bvar2*
8	1294	Milk	+	+	−	−	−	+	+	+	−	−	ND
9	1301	Milk	+	+	+	+	+	−	−	−	+	−	*Bvar2*
10	1302	Milk	+	+	−	−	−	+	+	+	−	−	ND
11	1306	Milk	+	+	+	+	+	−	−	−	+	−	*Bvar2*
12	1307	Milk	+	+	+	+	+	+	+	−	−	+	*Bvar4*
13	1308	Milk	+	−	−	−	+	+	+	+	−	−	ND

ND, not determined.

Table 4 | HOOF-Prints: results of alleles configuration to identify *Brucella abortus* biotypes from isolates of milk collected in Santo Domingo Province in Ecuador.

Sample[a]	Code	Locus-1	Locus-2	Locus-3	Locus-4	Locus-5	Locus-6	Locus-7	Locus-8	Biotype
6	1285	4	3	6	6	5	6	3	2	Bvar1
7	1286	4	3	6	6	5	6	3	2	Bvar1
9	1301	4	3	6	6	5	6	3	2	Bvar1
11	1306	4	3	6	6	5	6	3	2	Bvar1
12	1307	7	4	5	3	2	2	7	2	Bvar4

[a]*Samples shown in this table correspond to samples that were different from B. abortus Bvar1 in biotyping; i.e., Biotype 2 and 4.*

B. abortus biotype 4. As described by Corbel and Brinley Morgan (21), Mayfield et al. (22), and Rodríguez Torres et al. (23), growth in basic fuchsin medium and agglutination with anti-A sera, is indicative for *B. abortus* biotype 1; however, lack of bacterial growth in basic fuchsin and agglutination with anti-A sera is indicative for *B. abortus* biotype 2. Yet, as shown in **Table 2**, by molecular analysis, all isolates were *B. abortus* biotype 1. All milk isolates were identified as *B. abortus* biotypes 1 and 4.

MOLECULAR IDENTIFICATION

In total, 13 isolates corresponded to *B. abortus* identified by IS711 and AMOS-PCR (**Table 3**). The "HOOF-Prints" protocol allows biotype classification, as such VNTR markers evidenced the presence of *B. abortus* biotype 1 in 12 out of 13 isolates. All these isolates were field strains and were different from vaccine strains S19 and RB51, as confirmed by conventional AMOS-PCR. Furthermore, one isolate, from a milk sample, was confirmed to be *B. abortus* biotype 4 (Sample 12). The allelic diversity found in *Brucella* isolates from Santo Domingo Province is given in **Table 4**. Molecular patterns found are similar to biotype 1 and 4, reported by Bricker et al. (19). Samples, biochemically found as biotype 2 (samples 6, 7, 9, and 11), were confirmed as *B. abortus* biotype 1 whilst sample 12 was corroborated as biotype 4.

On the other hand, the concordance of biochemical and molecular tests estimated a proportion of coincidences of 76.92%.

DISCUSSION

This study demonstrated the presence of bovine brucellosis in the province of Santo Domingo de los Tsáchilas province.

Biochemical tests used for biotyping isolates allowed the identification of *B. abortus* biotypes 1, 2, and 4, biotypes which have been previously reported in human populations in Ecuador using biochemical and molecular techniques (9, 12). Samples 6, 7, 9, and 11, were biochemically identified as *B. abortus* biotype 2, yet as *B. abortus* biotype 1 by HOOF-Prints protocol, which is highly sensitive test (11, 19). It is known that the biochemical tests are of limited use for identifying biotypes, since the biochemical response depends on environmental conditions during the preparation of media and reagents and the amount and time for growth of the strains (24–26). In addition, the intraspecific *Brucella* molecular variability could have caused this biochemical response (21–23, 27). However, further studies are suggested to confirm or reject the presence of *B. abortus* biotype 2 in Ecuador or that the biochemical results are due to a genetic adaptation of *B. abortus* biovar 1.

Molecular tests indicated that all strains described in this study were field strains and not vaccine-type strains; as for *B. abortus* biotype 1 field strains, in spite of being genetically similar to vaccine strains, the former do not grow in thionin (2 μg/ml) in a culture medium.

The presence of *B. abortus* biotype 4 as previously reported in humans by Ron-Román et al. (9), was confirmed in this study. The biochemical characteristics of *B. abortus* biotype 4 differ from *B. abortus* biotype 1 and 2 because the former is agglutinated by anti-A instead of anti-M sera. In the same way, the allelic configuration allowed differentiating between biotypes 1 and 4 in HOOF-Prints technics.

The type strains of all classical *Brucella* species and biovars were surveyed to assess the discriminating power of microsatellite fingerprint technique. This technique was used to assess the level of divergence amongst and within populations of naturally infected cattle and wildlife (19, 20, 28, 29).

In this survey, both *B. abortus* biotype 1 and 4 were reported as described by Ron-Román et al. (9, 12) in humans from northern Ecuador. The presence of the two biotypes (1 and 4) in animals in Santo Domingo province shows that due to intensive cattle movement, the presence of several biotypes is possible. Finally, the study findings suggest that microbiological isolation of *Brucella* spp. is more successful from milk samples (44%) than from lymph nodes in slaughter cattle (16%).

In conclusion, the strain diversity of *B. abortus* was assessed in a region with intensive cattle movement and *B. abortus* biotypes 1 and 4 were found; although, some isolations of *B. abortus* biotype 1 presented phenotypic variability according to biochemical tests. These findings were correlated with results found in humans in northern Ecuador. Further research is needed to study intra-species variability and to investigate the possibility of other biotypes and *Brucella* species present in the tropical regions of Ecuador.

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Challenges posed by tick-borne rickettsiae: eco-epidemiology and public health implications

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Rickettsiae are obligately intracellular bacteria that are transmitted to vertebrates by a variety of arthropod vectors, primarily by fleas and ticks. Once transmitted or experimentally inoculated into susceptible mammals, some rickettsiae may cause febrile illness of different morbidity and mortality, and which can manifest with different types of exhanthems in humans. However, most rickettsiae circulate in diverse sylvatic or peridomestic reservoirs without having obvious impacts on their vertebrate hosts or affecting humans. We have analyzed the key features of tick-borne maintenance of rickettsiae, which may provide a deeper basis for understanding those complex invertebrate interactions and strategies that have permitted survival and circulation of divergent rickettsiae in nature. Rickettsiae are found in association with a wide range of hard and soft ticks, which feed on very different species of large and small animals. Maintenance of rickettsiae in these vector systems is driven by both vertical and horizontal transmission strategies, but some species of *Rickettsia* are also known to cause detrimental effects on their arthropod vectors. Contrary to common belief, the role of vertebrate animal hosts in maintenance of rickettsiae is very incompletely understood. Some clearly play only the essential role of providing a blood meal to the tick while other hosts may supply crucial supplemental functions for effective agent transmission by the vectors. This review summarizes the importance of some recent findings with known and new vectors that afford an improved understanding of the eco-epidemiology of rickettsiae; the public health implications of that information for rickettsial diseases are also described. Special attention is paid to the co-circulation of different species and genotypes of rickettsiae within the same endemic areas and how these observations may influence, correctly or incorrectly, trends, and conclusions drawn from the surveillance of rickettsial diseases in humans.

Keywords: *Rickettsia*, spotted fever rickettsioses, ticks, co-feeding transmission, transovarial maintenance, acquisition feeding, eco-epidemiology, molecular epidemiology

INTRODUCTION

Rickettsiae are obligately intracellular bacteria with complex life cycles that are dependent upon certain animals, mostly vertebrate mammals, and also include reptiles and birds, and diverse arthropods for their survival. Among them, arguably, the tick-borne agents are the best studied group. These certainly are the most important group as regards veterinary, wildlife, and human diseases because they transmit most of the large spotted fever group of rickettsiae (SFGR) that cause disease in humans. These currently include over 25 formally recognized species and an ever growing number of unnamed and non-cultivated genotypes, which are still poorly characterized (1). However, some of these new rickettsiae have proven to be causes of emerging human diseases; they are often first recognized from their associations with different animals and their ectoparasites and only later detected in clinical specimens and associated with specific diseases. Most frequently, the detection of new rickettsiae has occurred by detailed examination of different species of ticks so this distribution may well be biased by that methodology, rather than reflecting the true abundance and distribution of rickettsial lineages in nature (2).

The majority of tick-borne rickettsiae belong to the core classic spotted fever group (3). This bias may, in fact, just reflect the focus of medical and veterinary studies on ticks and the use of classic procedures and molecular procedures that work well only with this subgroup, rather than the diversity of rickettsiae found in ticks, let alone the full range of arthropods known to harbor rickettsiae. Historically, SFGR were referred to as the agents of a group of endemic rickettsioses, implying their focal distribution and limited associations with specific ecological settings (4). The advent of molecular tools and their commonplace application to investigation of associations of rickettsiae with their animal and invertebrate hosts has significantly changed this picture and our understanding of these variable associations. In 1982, Nyven Marchette wrote that the ecological relationships of rickettsiae for the most part are known or at least amenable to investigation (5). In his extended monograph, Dr. Marchette thoroughly summarized the facts about known or reported associations of spotted fever group rickettsiae with different tick species but sadly, many aspects of these associations still remain unexplored more than 40 years later (6), possibly due in part to overreliance on tools developed in the "molecular era."

Typically rickettsioses are described as zoonotic diseases, although, the term "zoonosis" is rather inaccurate and loosely used in this context compared to its primary definition as a disease that normally exists in animals but can infect humans. Indeed, two contemporary unresolved issues regarding rickettsioses and public health are highlighted with this problematic usage: (a) How large a role do animal hosts play in the life cycle of rickettsiae, aside from their essential role as a blood source for their tick hosts? Do true zoonotic rickettsial infections really occur? and (b) What routes of infection of animals and humans are most important in the acquisition of spotted fever rickettsioses? Is the feeding and salivating tick or the infected tick itself all that is important in transmission? – Are infected excreta of ticks important source of infection and do these infectious powders originate directly from the animal hosts of ticks post feeding, from a tick contaminated environment or only on a host during the acquisition of the blood meal?

Here, we evaluate and reflect on the contemporary knowledge and understanding of *Rickettsia*–host and *Rickettsia*–vector interactions in the greater context of recent findings on invertebrate immunity, microbial communities of arthropods, and especially on vector associations with various primary and secondary endosymbionts. We attempt to identify key gaps in our understanding of the eco-epidemiology of SFG rickettsioses and their importance for understanding the epidemiology of human diseases caused by these microorganisms. Finally, we discuss how these varied biological associations in ticks may influence outbreaks of rickettsial infections, their implications for epidemiological investigations, and provide relevant public health recommendations.

To paraphrase George Santayana and take his advice (7), we will first review current dogma and then revisit the conclusions of the pre-molecular era in order to suggest what is still needed from contemporary investigations. In particular, we examine the gaps in the ways that our increasingly powerful contemporary molecular tools are being used to address these questions.

RECENT SHIFTS IN DOGMA ABOUT THE EPIDEMIOLOGY OF CLASSIC RICKETTSIAL TICK-BORNE DISEASES

The incidence of tick-borne rickettsial diseases is currently going through its second pronounced increase in the last 40 years. Since 1970s, four endemic rickettsioses Rocky Mountain spotted fever (RMSF), Mediterranean spotted fever (MSF), North Asian tick typhus (NATT), and Queensland tick typhus (QTT), have been on a continuous increase (4). The incidence of Japanese spotted fever has also increased steadily since its discovery in the mid-1980s (8). It is possible that other tick-borne rickettsial infections have shown similar increases but only these more common and severe diseases have much useful, albeit based on the limits imposed by contemporary views, disease surveillance information. Ecological factors, particularly those driven by climate change, surveillance methodologies, and human population increases and behavioral changes (recreation, association with nature) may all be contributing factors to this phenomenon (9, 10). Elevated attention to this increase and the advent and adaptation of new molecular tools used for field and laboratory studies in the 1990s, complemented by increased funding support for these studies has opened Pandora's Box. The discovery and description of novel nosological entities caused by previously unknown spotted fever group rickettsiae has continued unabated since then (1, 11). Consequently, to a degree, the traditional views of tick-borne rickettsioses as endemic diseases with largely focal distributions and limited host and geographic ranges, predetermined seasonality and defined tick associations became obsolete or at least very incomplete (12). This expansion of our awareness about the existence of other rickettsial agents with varied clinical and epidemiological attributes has been thoroughly reviewed but it has presented new challenges to the medical and public health communities (1). This paradigm shift is due to the fact that numerous rickettsiae of unknown to variable degrees of pathogenicity for humans co-circulate in overlapping geographic regions and may even be found in the same tick species. The details of these findings and those vector and geographic associations have been reviewed and summarized by several authors (1, 11).

Within the limits of current knowledge, RMSF is endemic throughout the Americas and MSF is endemic through southern Europe and Africa to the Asian subcontinent. These are still the most prevalent spotted fever rickettsioses requiring medical attention and stand out for their morbidity and mortality. Thus, these are still the priority agents for surveillance of reportable rickettsial diseases in the corresponding countries where they are found. At least eight human rickettsial pathogens circulate in ticks in different and often overlapping parts of Eurasia (including *R. conorii*, *R. massiliae*, *R. slovaca*, *R. raoultii*, *R. sibirica*, *R. mongolotimonae*, *R. helvetica*, *R. rioja*, and possibly others), at least four in Africa (*R. africae*, *R. conorii*, *R. massiliae*, and *R. aeschlimanni*), four in Australia (*R. australis*, *R. honei*, and *R. honei* subsp. *marmionii* and possibly *R. gravesii*), and several in the Americas (*R. rickettsii*, *R. parkeri*, and *R. massiliae*). *Rickettsia amblyommii* and its closely relatives are highly prevalent SFG rickettsiae in the aggressive human-biting tick *Amblyomma americanum* in the USA and *Amblyomma* spp. in Central and South America; their pathogenicity for humans is widely speculated but not yet clearly demonstrated (1, 13–15). Some rickettsioses are known to cause only a handful of cases but it remains to be determined whether their impact will always be small because of their low pathogenicity or low vector carriage or transmission potential or these numbers are just a reflection of their being discovered only recently and diagnostic assays are still insufficiently specific to determine which agent is causing an infection. However, even low pathogenicity agents may contribute to the apparent overall increased incidence of rickettsial diseases in the world because cross-reactive serological tests are still the primary means for diagnosing rickettsioses. Many of these "cases" may, in fact, reflect an unrelated exposure to a tick bearing a *Rickettsia* agent that caused an immune response rather than the occurrence of a rickettsiosis. This fact led to the change in national reporting of RMSF cases in the United States to their classification as spotted fever rickettsioses. Consequently, a central issue for public health remains: whether or how our greatly expanded knowledge on the current temporal and spatial distribution of rickettsial agents will have any effect on medical practice and the diagnosis of rickettsioses. This is particularly true for underdeveloped regions where rickettsioses may occur in the same locations as high impact diseases such as malaria, leptospirosis, arboviral infections, and other diseases presenting initially with a fever, headache, and/or rash – the most prevalent manifestations of rickettsioses. It is more likely that a better understanding of the complexity of the eco-epidemiology of rickettsial agents

with shared vectors and animal hosts will be useful primarily for improved modeling of the life cycles of these agents, for improving our surveillance and outbreak response tools for rickettsioses, and for establishing cost-effective targeted control efforts for the primary problematic agents or their vectors. Whether this long-range strategy will be of greater benefit to society by reducing the disease burden from nature than educational efforts to ensure proper clinical recognition of the affected individuals, or by developing much better therapeutic regimens based on bactericidal antibiotics to reduce the burden of hospitalization, long-term sequelae, or fatal infections is currently unknown.

BASIC CONCEPTS ABOUT RICKETTSIAE AND RICKETTSIAL ECOLOGY

Two basic concepts about tick-borne rickettsial ecology originated in the seminal observations made by Ricketts early in the twentieth century who hypothesized that the agent of RMSF, *R. rickettsii*, is maintained in nature in a continuous cycle between infected ticks and one or several of the host animals parasitized by *Dermacentor andersoni* (16, 17); he also speculated that hereditary transmission of *R. rickettsii* in ticks might occur on a limited scale (18). At that time, he conducted limited laboratory experiments and established the susceptibility to *R. rickettsii* of several small animals, including the ground squirrel, rock-squirrel, chipmunk, and woodchuck. He was able to demonstrate that ticks feeding on these hosts did acquire the infectious agent and could subsequently transmit it to guinea pigs. These findings led Ricketts to the hypothesis that new lines of infected ticks (2 years are required for a complete life cycle for this tick) were started each season by simultaneous feeding of different stages of infected and uninfected ticks on susceptible host animals, primarily rodents, rabbits, and hares. However, the extent to which infections persist in the host animal, the relative importance of different hosts for tick maintenance – especially for transmission and acquisition of *R. rickettsii* by different stages of ticks, and the means and efficiency of transfer of rickettsiae from an infected to an uninfected tick were all uncertain and are still open questions today. Thus, these remain as unknown or semi-quantitative variables in modern attempts to model the dynamics of this maintenance-transmission system (19, 20).

Subsequently, many different animal species have been qualitatively associated with maintenance and circulation of *R. rickettsii* in nature either by direct isolation of rickettsiae from tissues or by demonstrating their seroconversion by different immunoassays following needle or tick inoculation of the agent or exposures in nature [reviewed in Ref. (21, 22)]. Some major questions remain unanswered from this work (**Table 1**): (1) do these animals experience clinical disease or rickettsemia or any agent replication during rickettsial infection; do these reservoirs serve as sources of outbreaks of human disease, and which are the most important; (2) do all of these animals serve as sources of infection for various ticks vectoring RMSF to humans and other animals, and (3) do these animals serve primarily as hosts for feeding of infected and non-infected ticks and a site for facilitating exchange of the pathogen between different ticks and tick life stages or are they otherwise dead end hosts like humans? Similarly, whether the known different tick vectors of RMSF, *D. variabilis*, *D. andersoni*, *Amblyomma* tick species, *Haemaphysalis leporispalustris*, and *Rhipicephalus sanguineus* differ markedly in their abilities and specific mechanisms used to sustain rickettsial populations in nature is still unknown. However, *R. rickettsii* itself has clearly diverged genetically in association with these different vector species and geographic regions (23–25).

Some of these questions were independently investigated by numerous Russian investigators studying the ecology of NATT since the middle of 1930s in the vast territories of eastern and western Siberia and from eastern Altai to Primorye and the Far East of Russia (41). Like Ricketts they observed transovarial and transtadial transmission of *R. sibirica* but by different *Dermacentor* species (chiefly. *D. silvarum* and *D. nuttalli*) and by *Haemaphysalis* sp. ticks whose life cycle in turn depends upon the availability of different host animals. As one outcome of those studies, *R. sibirica* was isolated from a variety of wild rodents, including voles, susliks, lemmings, chipmunks, hamsters, striped field mice, Norway rats, house mice, and hares (41). At the time, it was postulated that under favorable climatic-landscape conditions that support specific biocenotic systems of tick vectors and host animals, natural foci of tick-borne rickettsiae can exist for many generations of ticks and host animals independent of man (42). As a part of the long-term studies conducted by Kulagin and several other investigators such as Shapiro and Korshunova et al., it was also suggested that despite being a primary animal host for larval and nymphal ticks vectoring *R. sibirica*, wild rodents are unlikely to be primary players in the maintenance of the rickettsiae [cited in Ref. (41)]. These observations were based on testing of blood and tissue samples and the conclusion that the wild rodents develop only transient rickettsiaemia and most of them did not carry rickettsiae in the spring, which is the interepidemic period for NATT. While the primary role of the tick in long-term maintenance of the agent rather than host animal reservoirs seems better established for *R. sibirica* (43), the other quantitative and qualitative questions posed above for *R. rickettsii* and other spotted fever group rickettsiae are also still unanswered for *R. sibirica*.

At present, RMSF is recognized as the most malignant of known tick-borne rickettsioses while NATT generally manifests as a relatively mild illness (44). Furthermore, *in vitro* study has also indicated a different pathogenic potential and associated capacity to cause injury among different strains of *R. rickettsii* and most of these cause much greater cellular injury than *R. sibirica* (45–47). These characteristics may also determine the rates and outcomes of rickettsial interactions with the tick vectors and animal hosts and thus determine the natural fluctuations of those cycles and persistence of the agents in the environment. Consequently, given the variables involved, it is not surprising that the incidental contact of man with these cycles can vary greatly from year to year and thus the number of cases that occur each year in a given area can fluctuate wildly.

ASSOCIATIONS OF TICK-BORNE RICKETTSIAE WITH WILD ANIMALS AND THEIR SYLVATIC CYCLES

The first isolate of *R. rickettsii* from a naturally infected animal in North America, was not made until 1954, when Gould and Miesse recovered a mild strain from the tissues of a meadow-mouse (*Microtus pennsylvanicus*) in Virginia (26). Another strain was

Table 1 | Variable effects of *Rickettsia* observed in different host animals.

Rickettsia species, isolate	Associated tick species	Animal species	Observed effects in the source animal	Records of isolations (source)	Reference
R. rickettsii, *Microtus* agent B14009[a]	*D. variabilis*[b]	Meadow mouse, *Microtus pennsylvanicus*	None (apparently healthy)	Yes (brain, spleen, and liver)	(26)
R. rickettsii, Mp23, Mp40, Pit1	*D. variabilis*[b]	Wild mice, *Peromyscus leucopus*, *Pitymys pinetorum*	Tissue persistence; seroconversion	Yes (liver and spleen)	(27)
R. rickettsii, Di6	*D. variabilis*[b]	Opossum, *Didelphis marsupials virginiana*	Tissue persistence; low level seroconversion	Yes (liver and spleen)	(27)
R. rickettsii, Rab1	*D. variabilis*[b]	Eastern cottontail rabbit, *Sylvagus floridans*	Tissue persistence; seroconversion	Yes (liver and spleen)	(27, 28)
R. rickettsii, Si7	*D. variabilis*[b]	Cotton rat, *Sigmodon hispidus*	Tissue persistence; low level seroconversion	Yes (liver and spleen)	(27)
R. rickettsii, Sheila Smith	N/A	Cotton rat, *Sigmodon hispidus*	Tissue persistence; short-term rickettsiemia; low level seroconversion	Yes (blood)[b]	(29)
R. rickettsii, Sawtooth	*D. andersoni*	Snowshoe hare, *Lepus americanus*	Rickettsiemia (exp)	Yes (xenodiagnosis)	(22, 30)
R. rickettsii, Sawtooth	*D. andersoni*	Golden-mantled ground squirrel, *Citellus lateralis tescorum*	Rickettsiemia (exp)	Yes (xenodiagnosis)	(22, 30)
R. rickettsii, Sawtooth	*D. andersoni*	Chipmunks, *Eutamias amoenus*	Rickettsiemia (exp)	Not reported	(22, 30)
R. rickettsii, Sawtooth	*D. andersoni*	Columbian ground squirrel, *Urocitellus columbianus*	Rickettsiemia (exp)	Yes (xenodiagnosis)	(22, 30)
R. rickettsii, Sawtooth	*D. andersoni*	Meadow mice, *Microtus* spp.	Rickettsiemia (exp)	Yes (xenodiagnosis)	(22, 30)
R. rickettsii, Sawtooth	*D. andersoni*	Bushy-tailed woodrat, *Neotoma cinerea*	Seroconversion	No	(22, 30)
R. rickettsii, Taiaçu	*A. cajennense*	Capybara, *Hydrochoreus hydrochaeris*	Seroconversion; rickettsiemia (exp) afebrile	Yes (xenodiagnosis)	(31)
R. rickettsii, ITU	*A. cajennense*	Capybara, *Hydrochoreus hydrochaeris*	Seroconversion	No	(32)
R. rickettsii, Taiaçu	*A. cajennense*	Opossum, *Didelphis aurita*	Rickettsiemia (exp) asymptomatic; no macro or micro pathological abnormalities	Yes (xenodiagnosis)	(33)
R. rickettsii, Taiaçu	*Rh. sanguineus*	Dog, *Canis familiaris*	Rickettsiemia (exp)	Yes (xenodiagnosis)	(34)
R. rickettsii, Sawtooth	*Rh. sanguineus* (presumably North America)	Dog, *Canis familiaris*	Rickettsiemia (exp); seroconversion	Yes (cell culture)	(35)
R. rickettsii, Wachsmuth	*Rh. sanguineus* (presumably North America)	Dog, *Canis familiaris*	Rickettsiemia (exp); seroconversion	Yes (xenodiagnoses)	(35)
R. rhipicephali, 3-7-♀6	*Rh. sanguineus* (presumably North America)	Dog, *Canis familiaris*	Seroconversion	No	(35)

(Continued)

Table 1 | Continued

Rickettsia species, isolate	Associated tick species	Animal species	Observed effects in the source animal	Records of isolations (source)	Reference
R. montanensis	*Rh. sanguineus* (presumably North America)	Dog, *Canis familiaris*	Seroconversion	Not tested	(35)
R. parkeri	*A. maculatum*	Cattle	Seroconversion	No	(36)
R. parkeri, Portsmouth	*A. maculatum*	Cotton rat, *Sigmodon hispidus*	Short-term rickettsiemia; seroconversion	Yes, re-isolation	(37)
R. parkeri, Portsmouth	*A. maculatum*	Northern bobwhite quail, *Colinus virginianus*	Seroconversion	No	(37)
R. conorii, Malish	*Rh. sanguineus*	Dog, *Canis familiaris*	Rickettsiemia (transient); febrile illness; seroconversion	Yes (xenodiagnoses)	(38, 39)
		Hare, rabbit	Asymptomatic rickettsiemia; seroconversion	No	(40)

[a] *Name of the isolate is based on the description found in the original publication, although the identification of this isolate as* R. rickettsii *is very presumptive based on the biological characteristics included in the publication (26). It may, in fact, be* Rickettsia montanensis *but the isolate is no longer available.*

[b] *Tick species is indicated based on the known circulation of* D. variabilis *in the area where isolates were obtained.*

exp., experimental animals under laboratory conditions.

recovered from the liver of a naturally infected cottontail rabbit (*Sylvilagus floridanus*) trapped in the same state in 1961 (28). Subsequently, isolates of *R. rickettsii* were made from spleen and liver tissues of wild mice (*Peromyscus leucopus*, *Pitymys pinetorum*), cotton rats (*Sigmodon hispidus*), a golden-mantled ground squirrel (*Citellus lateralis tescorum*), and from chipmunks (*Eutamias amoenus*) trapped in Virginia and Western Montana (27, 30). Several small animals, mainly wild rodents, were sources of *R. sibirica* isolates obtained across a large territory known for the endemicity of NATT (41).

Despite these existing field observations the question remains unanswered whether all these animals or only certain species represent efficient sources for infection of ticks and how long they can provide that function after acquiring the agent. For example, bushy-tailed woodrats (*Neotoma c. cinerea*) were consistently negative for *R. rickettsii* by isolation, although they originated from the Bitterroot Valley of Western Montana where RMSF is highly endemic (22). To address this difference, Burgdorfer et al. (22) performed quantitative analyses of susceptibility to virulent *R. rickettsii* Sawtooth for various species of small animals and evaluated their role as possible sources for infecting larval *D. andersoni* (22). The data obtained by this extensive and important study are illustrative of typical experiments but it emphasizes the limitations of laboratory experimentation. Whether the rickettsial challenge dosage vastly exceeds that delivered by low levels of repeated tick infestations and its impact where the animal may already be immune to rickettsiae, or what responses occur to infestations with single infected ticks, are important quantitative issues. Similarly, the role of tick effectors in facilitating or ablating an infection relative to intradermal, subcutaneous, intramuscular, and intraperitoneal inoculation of cultured agent is also a concern (48). It must be remembered that ticks filter large volumes of blood during their feeding so that assays of rickettsial content in samples of host blood from a single time point may not accurately reflect the amount that ticks may acquire. Wild caught animals were exposed to nymphal *D. andersoni* infected with *R. rickettsii* Sawtooth, a guinea pig virulent tick strain originating from the west side of the Bitterroot Valley (22). Columbian ground squirrels and chipmunks developed rickettsiaemias that appeared on the third or fourth day and lasted for 6–7 days with a maximum of 9 days in one chipmunk. The largest concentrations of rickettsiae in both species of rodent were found on day 6 or 7, when blood dilutions of 10^{-3} still produced infections in guinea pigs. For comparison, rickettsiaemias were observed in guinea pigs that were fed on by the same number of infected ticks as were used with the Columbian ground squirrels and chipmunks. The concentration of rickettsiae in the blood of guinea pigs was much higher, with a prolonged period of at least 6 days in which 100 or more infectious doses per 0.5 ml of blood were present. The golden-mantled ground squirrels experienced rickettsiaemia with maximum titers of at least 10^3 for a relatively short period of time. In snowshoe hares, rickettsiaemia lasted as long as 5 days but the concentrations of rickettsiae were much lower and rarely exceeded 10 infectious guinea pig doses. Least susceptible were bushy-tailed woodrats, in which rickettsiae could be demonstrated only following attachment of hundreds of infected ticks. In meadow mice, rickettsiae circulated for as long as 6–8 days in concentrations that, in some specimens, reached at least 1000 infectious doses per 0.5 ml of blood. Subsequent experiments were conducted with Columbian and golden-mantled ground squirrels, meadow mice, and snowshoe hares. The results indicated that naive *D. andersoni* larvae that fed on these hosts during peak rickettsiaemia invariably exhibited high infection rates while those that fed during the initial or final stages ingested rickettsiae insufficient in

numbers to establish permanent infection of tick tissues. Accordingly, meadow mice, Columbian ground squirrels, chipmunks, and golden-mantled ground squirrels must be considered highly efficient sources of infection, at least for those ticks that feed during the periods when large quantities of rickettsiae are present in the blood. In meadow mice, which appeared to be the most susceptible, rickettsiae circulated in concentrations of 10^2–10^3 guinea pig infectious doses for as long as 4 days. Similar titers, although for 1 or 2 days only, were detected in chipmunks and Columbian and golden-mantled ground squirrels. These studies demonstrated that hares do respond to infectious tick bites with rickettsiaemias that in general are much milder than those observed in ground squirrels, chipmunks, or meadow mice. However, for at least 1 or 2 days, infectious titers may reach the level necessary to infect 50% or more of larval *D. andersoni*. Despite these limitations, isolates of *R. rickettsii* were recovered from the blood of a snowshoe hare (*Lepus americanus*) (30). Finally, the bushy-tailed woodrat, a common host of immature *D. andersoni*, was the only species of animal that did not circulate rickettsiae in the blood following attachment of infected ticks, suggesting that this rodent is not susceptible to spotted fever group rickettsiae and is of no significance for infecting ticks in nature.

Although, the natural reservoir of *R. conorii* is not yet fully demonstrated, Rovery et al. suggested that rabbits could be rickettsemic without developing severe disease, so wild rabbits (*Oryctolagus cuniculus*) might be a reservoir for *R. conorii conorii* and could play a role in the transmission of *R. conorii conorii* in the French Mediterranean (40). These observations and other previous publications suggest that rabbits and hares may play a significant role in the circulation of rickettsial pathogens in nature. To some extent distribution of human cases of RMSF in the western USA coincided with distribution of Nuttall's cottontail (*Sylvilagus nuttallii*). This animal is an important host of the larval and nymphal stages of *D. andersoni* and *H. leporispalustris*. Furthermore, there are significant overlaps in the geographic ranges of *D. variabilis* and the eastern cottontail rabbit (*S. floridans*), *D. occidentalis* and the Pacific Coast brush rabbit (*S. bachmani*) and *D. parumapertus* and the black-tailed jack rabbit (*Lepus californicus*). *Sylvilagus* is one of the few animal genera that is present in both North and South America, and it may have had a role in the introduction of *R. rickettsii* and other rickettsiae into South America or vice versa (5, 41). Similarly, according to Lyskovtsev, the European hare (*Lepus europeus* Pallas) was among the animals known to develop sufficient rickettsiemia to recover an isolate of *R. sibirica* (41). There is also experimental evidence suggesting a role for hares in the circulation of *R. slovaca* (49).

Many other species of wild animals have been implicated in rickettsial maintenance but solely in the context of their roles as blood meal hosts for different tick species. These animals can certainly differ in their susceptibility to rickettsial infection, whether they develop clinical disease or just subclinical infection, whether the infection is persistent or sterile immunity occurs, and the extent of their immunity to subsequent reinfection. Body size might also be important as the number of ticks that may attach can increase substantially as one progress from small to medium size to large hosts (50). Opossums have been implicated in maintenance of *R. rickettsii* in both North and South America (27, 33). Different species of opossums only develop an inapparent infection, whether in nature or after experimental inoculation, but they can sustain at least 3–4 weeks of rickettsiaemia demonstrable by tick acquisition feeding and direct isolation of rickettsiae persisting in their tissues (27, 33). Similar observations were made for the capybara (*Hydrochoerus hydrochaeris*), a large rodent, which is a primary animal host for *Amblyomma cajennense* in Brazil (31, 32).

THE ROLE OF DOGS AND OTHER PERIDOMESTIC ANIMALS IN TRANSMISSION AND MAINTENANCE OF SPOTTED FEVER GROUP RICKETTSIAE

Dogs are viewed as important sentinel animals for rickettsial disease agents since they can suffer clinical illness following infection with *R. rickettsii* and *R. conorii* (51). Infected dogs can present with fever, lethargy, vomiting, and anorexia, and may develop other symptoms and manifestations similar to those of the disease in humans, including ocular lesions, bleeding disorders, joint pain, and neurologic abnormalities. The factors resulting in clinical infection may include breed, other underlying health conditions, and very likely the dosage and degree of infestation of the dogs and protection arising from previous exposure to immunizing levels of ticks and rickettsial agents of low pathogenicity. Overt rickettsial diseases in dogs have been confirmed by PCR and sequencing of rickettsial DNA and seroconversion (52–54). However, many dogs are only subclinically infected and do not exhibit these severe manifestations even if they may seroconvert (55–57).

Acute MSF and RMSF in dogs are accompanied by rickettsiemia detectable between days 2 and 12 after inoculation using cell culture isolation or PCR (54), though asymptomatic dogs may remain infectious for ticks for as long as 30 days (38). These illnesses are followed by complete clearance and development of antirickettsial IgG; its persistence and titers depend on the number of inoculated rickettsiae (35, 57). In contrast, dogs infected with *R. montanensis*, a widely distributed SFGR of unknown pathogenicity found in *D. variabilis*, remain asymptomatic (58). However, such exposure is usually sufficient to elicit a cross-protective immune response to subsequent inoculation with *R. rickettsii* (58). Antibody responses in dogs infected with *R. rickettsii* show a similar pattern of reactivity to *R. rickettsii*, *R. montanensis*, *R. rhipicephali*, and *R. bellii* (59). However, treatment with tetracycline causes significant delays in serologic responses of infected dogs to heterologous rickettsial species (59); as in humans, untreated canine rickettsial infections may result in fatalities (60). Several case reports in the literature describe diagnosis of RMSF in dogs associated with and, in some cases, leading to identification of the infection in people in the same household or vicinity (61, 62). Rickettsiae are transmitted by ticks, rather than directly from one infected dog to another. Manual removal of engorged ticks from dogs has been identified as a potential risk factor for human infection, which can occur by self-inoculation of the pathogen onto mucous membranes by contaminated fingers as has been shown with *R. conorii* and *R. conorii caspiae* (63, 64).

Dogs also play an important role as biological hosts of several tick species, which can transmit rickettsiae to humans and other dogs (51). Once infested, dogs serve to increase the infected tick populations present in close association with human habitats, and can introduce infected ticks into the peridomestic

environment (65). In the case of *R. conorii*, laboratory experiments demonstrated that dogs are a competent reservoir for this rickettsia (38); whether dogs become rickettsemic with every human or guinea pig-pathogenic *Rickettsia* is not known. In an experimental setting, beagles subcutaneously inoculated with *R. japonica* did not produce rickettsiemia or clinical symptoms of infection (56). Dogs with different genetic backgrounds appear to differ in their susceptibility to rickettsiae and *R. conorii* infection in particular (38).

In the new world, the association of *Rh. sanguineus* with dogs acquired new importance and attention after the rediscovery of a sustained transmission cycle of *R. rickettsii* by this tick in arid regions of North America. The first site was discovered by recognition of atypical foci of RMSF in eastern Arizona well outside the distribution of *Dermacentor* sp. ticks (66). Similar foci were subsequently discovered in Brazil and several sites in Mexico (67, 68). The genotype of *Rickettsia rickettsii* circulating in AZ has a unique genotype that differs from those of *R. rickettsii* in Mexico and Brazil (23, 68). Furthermore, the brown dog ticks in Brazil and Mexico associated with those outbreaks differed from those in the Arizona outbreak (68). In either region, dogs are considered to be an amplifier of rickettsial prevalence through co-feeding transmission by the infected ticks. Surprisingly, *R. rickettsii* from South America does not cause any substantial mortality in the infected brown dog ticks and exhibits more efficient transstadial and transovarial transmission than that occurring in USA (34). These outcomes are quite different from the interactions of *R. rickettsii* and *Dermacentor* ticks in Northern America that are discussed below (69).

Another human pathogen found in *Rh. sanguineus* is *R. massiliae* (70). It was originally described in *Rhipicephalus turanicus* from France, but has since been identified in several other countries including the new world (1). One genotype of *R. massiliae*, known as Bar29, has been shown to infect humans (70, 71). The USA genotype AZT80 (of Bar29 genotype) of *R. massiliae* was implicated as a cause of canine illness in California; however, those associations could not be confirmed beyond serological observations (72). Additional work is needed to define the importance of *R. massiliae* in human and animal health. Likewise, further work will be required to validate observations associated with the natural exposure of dogs to various SFG rickettsiae through tick bites (73).

Other species of spotted fever group rickettsiae have been identified in ticks that bite both dogs and humans so that dogs may play some role in their eco-epidemiology. In most cases, only laboratory evidence for canine susceptibility to infection has been obtained and their role as a source of human infection is less certain. These studies are most advanced with *Rickettsia parkeri*, which is long known from tick surveys but which has only recently been recognized as an emerging pathogen of humans in USA and in Uruguay, Argentina and Brazil (1, 73–75). In Brazil, dogs are commonly infested with *A. ovale* and *A. aureolatum*, which frequently carry the *R. parkeri*-like Atlantic Rain Forest *Rickettsia* (73). Similar agents have been found in other *Amblyomma* species from birds (*A. calcaratum*), capybara (*A. dubitatum*), anteaters (*A. nodosum*), marsh deer (*A. triste*), dogs (*A. tigrinum*), and *A. maculatum* from dogs, horses, and cattle in Peru (76–81). Whether all these variants can cause human disease is unknown but the large number of tick species containing *R. parkeri*-like agents and their diversity of hosts suggests that understanding their maintenance and transmission will be challenging. However, the *A. triste* agent could be maintained with high efficiency for five generations of ticks on rabbits by transovarial and transstadial passage; this agent is thought to be a primary human disease agent in the southern countries of South America and it is very similar to North and South American strains from *A. maculatum* (80). However, the causation and significance of the apparent bimodal distribution of agent load in the *A. triste* ticks is unclear. Cattle have been recognized as hosts for *A. maculatum* for many years in USA (36). Laboratory studies conducted in Mississippi indicate that upon exposure to *R. parkeri* by injection or by feeding *R. parkeri*-infected *A. maculatum* calves seroconvert to *R. parkeri* antigen, but only a few animals develop short-lasting rickettsiemia (36). In a parallel field study, cattle were not rickettsiemic, suggesting that they only play a critical role in tick feeding and vagility, and thus in maintenance of *R. parkeri* by vertical and transtadial transmission. However, the ticks also appear to stimulate rickettsial growth in host tissues during engorgement (48). Migrating birds may have an important role in dissemination of *R parkeri* and other agents present in ticks including their importation between the continents (82).

The role of cats in the eco-epidemiology of tick-borne SFG rickettsioses has received much less attention than evaluations in dogs because cats are less frequently infested with ticks. Typically <10% of free ranging cats have ticks, but some may be infested with large numbers of ticks (83). Moreover, serological findings of rickettsial exposures in cats must also be carefully interpreted due to possible cross-reactivity with the much more widely prevalent flea-borne agent, *R. felis*, found commonly in cat fleas and frequently in other flea species (84). Detection of DNA from *R. massiliae* and other unidentified other core spotted fever group rickettsiae in ticks on cats indicates that cats may serve as an important peridomestic source of infection in some situations (85).

The importance of African ruminants in natural cycles of many tick-borne agents like those causing heartwater and bovine anaplasmosis has been very well documented. Less clear is the role of domestic stock in maintenance of rickettsial agents in peridomestic settings where they might directly cause human disease. Molecular surveillance data reported by Mutai et al. (86) implied that substantial and comparable numbers of Kenyan domestic animals develop rickettsiemia (based on quantitative PCR detection of the conserved rickettsial 17 kDa protein gene fragment): 16.3% in cattle and 15.1% in sheep, but only 7.1% in goats, which were also less frequently infested with ticks (86). However, because the ticks were collected directly from the animals, one cannot know if they were transmitting or acquiring rickettsial agents. Four known human pathogenic species were detected: *R. africae* was detected in 93% of PCR positive ticks (seven species, four genera including *Amblyomma*, *Hyalomma*, *Rhipicephalus*, and *Boophilus*), while *R. mongolitimonae*, *R. aeschlimannii*, and *R. conorii israelensis* were found infrequently in addition to several other less-well known genotypes of *Rickettsia*. In Israeli, *Hyalomma* ticks from camels as well as several camel bloods were infected with *R. aeschlimannii*; *R. africae* was also detected in all four species tested (87). Similarly, in Egypt, *Hyalomma dromedary*, *H. impeltatum*, and *H. marginatum*

marginatum collected from camels in some areas had high rates of rickettsial infection with *R. africae* (over 57% of *H. dromedary*) and *R. aeshlimannii* (over 73% of *H. impeltatum*) (88). Determining the frequency which these ticks bite humans as well as their interactions with and dependence on other potential wildlife reservoirs of these rickettsiae will be required to determine their significance for public health.

EFFECTS OF SPOTTED FEVER GROUP RICKETTSIAE ON TICKS

Hard ticks (Ixodidae) are the primary vectors of spotted fever group rickettsiae and they develop through three discrete life stages (**Figure 1**). However, recently soft ticks (Argasidae) have also been found to harbor SFG rickettsiae of unknown pathogenicity (89); they typically feed rapidly and thus do not stay attached for long periods and do not have a scutellum or the pronounced morphological differentiation found after molting from larvae to nymphs to adults as occurs in hard ticks. The natural life span of nonnidiculous ticks and the survival and expansion of tick populations depends on the degree of blood satiation and environmental factors, particularly, temperature and humidity and habitat types and host abundance; therefore, changes in annual seasonal conditions appears to be more important for the success of some temperate zone ticks species than for others (90–94).

Ticks can have one host, two host, or three host life cycles. Two-host ticks feed as larvae and nymphs on the same host. Following detachment and dropping to the ground or leaf litter after blood engorgement on a host animal, the fertilized female deposits eggs in sheltered places in crevices of the soil surface or grass. The fertility of individual females depends on the tick species and degree of engorgement, so the numbers of eggs laid vary from 3000 to 8000 per female as estimated for *Dermacentor* ticks [cited in Ref. (41)]. Larvae hatch between day 4 and 82 after oviposition and quest very close to the original egg mass; they molt into nymphs after they have obtained a blood meal. Larvae and nymphs of *Dermacentor*, *Haemaphysalis*, and *Amblyomma* sp. ticks feed on small mammals, insectivores, rodents, small carnivores, and birds. *Haemaphysalis* spp. also parasitize wild birds. Engorged nymphs detach from the hosts and molt into adult ticks in 11–25 days. Adult ticks parasitize large wild and domestic animals. In contrast, one host ticks like *Rhipicephalus* sp. and *Amblyomma albipictus* differ from many other Ixodidae by feeding on a single animal species; their different stages can be found frequently at the same time on an infested host. Nymphs and adults will attach and feed on animals long enough to transmit rickettsiae if infected (95). Alternatively, as has been mentioned previously, larvae and nymphs can become infected by feeding on a rickettsiemic animal, and frequently and most efficiently nymphs and adults can become infected through co-feeding transmission of agents from other tick stages (96, 97). Aggregation of ticks on hosts appears to be essential for keeping the infection rates of ticks at environmentally sustainable levels (98). This mechanism can operate in the face of host immunity so it is a very important mechanism for rickettsial maintenance in nature, especially when ticks are abundant (39, 99). Humans and other hosts can become accidental victims of exposure to rickettsia-infected ticks, and can suffer from febrile disease or become rickettsiemic and infect feeding ticks but this acquisition route probably does not contribute significantly to the maintenance of rickettsiae in nature compared to transovarial, transtadial, and co-feeding mechanisms.

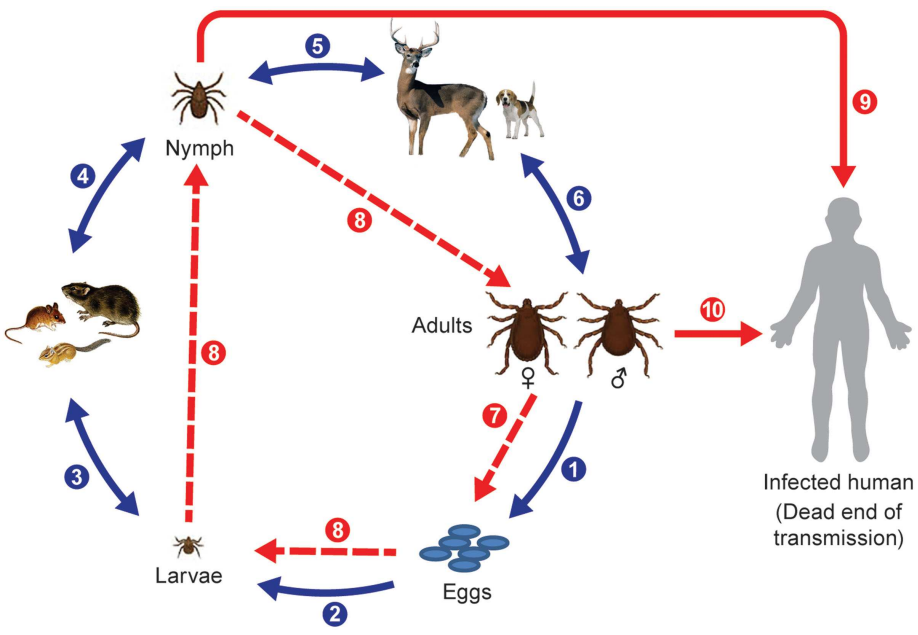

FIGURE 1 | Life cycle of Ixodid ticks and natural transmission of rickettsiae. Blue arrows indicate main steps of tick natural cycle: (1) oviposition by engorged female; (2) eggs hatched into larvae; (3) larvae feed on small animals; (4) engorged larvae hatch into nymphs; (5) nymphs feed on large or small animals; and (6) nymphs molt into adult ticks that feed on large animals or bite humans. Broken red arrows indicate transovarial (7) and transstadial transmission (8) of rickettsiae, and solid red arrows indicate transmission of rickettsiae to humans through a bite of a nymph (9) or an adult tick (10).

For most SFGR, the transovarial-transtadial pathways appear to be the essential mechanisms for their maintenance in the environment because this occurs independently of tick density and can cause significant expansions in the infected tick populations just from the progeny of single ticks; however, the molecular mechanism(s) of pathogen host tolerance and potential mutualism are not at all well understood even if many pathogen genomes are completely sequenced. The host–agent interactions (or relationships) apparently do vary substantially among rickettsiae with differing pathogenic potential toward humans and animals, possibly accounting for the large numbers of species of ticks and hosts that harbor agents and for their variable responses to the presence of those agents. The diversity of interactions is certainly very clear experimentally with respect to the effects of different agents on the ticks themselves. Two very different scenarios are evident but their relative importance and degree of parallel or synergistic occurrence varies with the agent and the tick host. In the first extreme, transovarial transmission is regarded as the sole mechanism of maintenance of the rickettsial agent in a given population of ticks. This is most evident for the rickettsial "obligate endosymbionts," which have not been cultivated outside of ticks except in continuous tick cell lines (100). Rates of 100% infection are achieved in species like *I. scapularis* and *I. pacificus* but the agent appears not to be transmissible to other hosts. The second extreme is where the *Rickettsia* agent can have variable effects on the tick host and is generally acquired by uninfected ticks from persistently infected vertebrate hosts or must be acquired by co-feeding from ticks, which have already acquired those agents. In *D. andersoni* ticks infested with *R. rickettsii*, the rate of transovarial transmission was estimated to be from 35 to 100% by mild or massively infected females, respectively (101, 102) while it appears to be close to 100% in *Amblyomma americanum* with *R. amblyommii* (14). Independently, this mechanism was established and recognized as a significant part of transmission and natural maintenance of *R. sibirica* in *D. nuttalli* by S. M. Kulagin [cited in Ref. (41)] and *R. conorii* (103). Subsequently, inheritance of a pathogen was found to depend on multiple variables associated with the experimental conditions (104). The importance of these factors on natural maintenance of *R. rickettsii* is less clear. In fact, a pronounced detrimental effect of the Sawtooth strain of *R. rickettsii* on survival and oviposition rates of *D. andersoni* females as well as their fecundity was observed in experiments conducted with laboratory reared ticks (69). Similarly, AZ-type highly virulent isolates of *R. rickettsii* appear to have a detrimental effect on *Rh. sanguineus* circulating in Arizona, which was determined by detection of the decreased prevalence of *R. rickettsii* in larvae and nymphs developed from eggs laid by infected females (unpublished personal observation) and variable rates of transovarial transmission and filial infection rates with *R. rickettsii* were seen between infected *A. cajennense* and *A. aureolatum* (105). Interestingly, this is not a unique association since *R. conorii* Malish exhibited similar effects on *Rh. sanguineus* ticks (106–109). However, these observations on adverse effects of highly virulent spotted fever group rickettsiae are probably not universal and depend on many yet to be identified variables (**Table 2**). No substantial mortality difference was observed between uninfected *Rh. sanguineus* or following infection with a Brazilian strain of *R. rickettsii* (34); similarly, there was minimal cost due to the acquisition of *R. massiliae* by *Rh. turanicus* (110). While the subspecies *R. conorii israelensis* and *R. conorii* Malish are closely related, their biological differences in terms of their effects on tick survival and ability to maintain the agent transovarially and transtadially were dramatically different (108, 109). Observations with *H. leporispalustris* ticks infected with *R. rickettsii* Taiaçu strain suggested an increased fitness of the infected ticks (111), a result analogous to the positive role of endosymbionts in many arthropod hosts. In general, it appears that the rate of transovarial transmission and extent of damage caused in a particular tick-host system depends upon the particular strain and species of *Rickettsia* being used and how well it propagates in the ovary of a particular tick.

Transovarial maintenance of *R. peacockii* (East Side Agent) within the maternal lineage of *D. andersoni* has been hypothesized to result in a reduced probability of acquisition and transovarial transmission of *R. rickettsii* (104, 117) – the so-called interference phenomenon between these microorganisms although the data supporting the hypothesis have been criticized (104). The molecular mechanism(s) of this interaction is unknown, but in the experiments that served as a base for this hypothesis the ticks whose ovarial tissues and deposited eggs contained *R. peacockii* acquired *R. rickettsii* less efficiently from guinea pigs than uninfected ticks. The ovaries containing *R. peacockii* also excluded *R. rickettsii*, which could grow in other tissues in the tick. It appears, however, that infected ticks may acquire a second and even a third rickettsia through a bloodmeal (118, 119). Exclusion of *R. rickettsii* by *D. andersoni* previously infected with either *R. rhipicephali* or *R. montanensis* also appears to occur (117). Capillary fed *D. variabilis* infected with either *R. rhipicephali* or *R. montanensis* demonstrated mutual exclusion and lack of transovarial transmission of the superinfecting rickettsia (116). These processes may be regulated by differential expression of tissue-specific, and in some cases *Rickettsia* species-specific, selected tick immune genes as demonstrated in experiments evaluating the response of *D. variabilis* to *R. amblyommii* and *R. montanensis*, respectively, using an *ex vivo* tick organ model (120). The ecological relevance of these incomplete experiments is suggested by the limited number of cases of RMSF, which occur in areas where *R. peacockii* is prevalent compared to similar geographic areas where it is much less prevalent. However, whether this observation is applicable to other ticks and rickettsial agents and whether the fundamental mechanisms involved are conserved will need substantial study. The presence of *R. bellii* in *Amblyomma dubitatum* appeared to reduce the acquisition of *R. rickettsii* from rickettsemic animals but transmission still occurred in some ticks (121).

MODELING OF TICK-BORNE RICKETTSIAL DISEASES

Prediction of the transmission dynamics of zoonotic and vector-borne diseases that are associated with wildlife present many challenges due to the absence or insufficient characterization of wildlife host species, pathogens, and vectors in many locations. The null case for tick-borne rickettsioses is simple in that the vectors themselves have an essential role(s) since transmission can only rarely occur in their absence. In other words, direct transmission from infected animal reservoirs, the definition of a zoonosis, is actually rare or lacking altogether in the absence of vectors.

Table 2 | Effects of spotted fever group rickettsiae on their tick vectors.

***Rickettsia* species, isolate**	**Tick species (origin)**	**Effects**	**Reference**
R. rickettsii, Sawtooth	*D. andersoni* (Montana)	↓ Larval and nymphal molting ↓ Female feeding success ↓ Oviposition ↓ Reduced transmission of rickettsiae	(69)
R. rickettsii, Como-96	*D. andersoni* (Montana)	↓ Larval and nymphal molting ↓ Female feeding success ↓ Reduced transmission of rickettsiae	(69)
R. rickettsii, Wachsmuth	*D. andersoni* (Montana)	↓ Larval and nymphal molting ↓ Female feeding success ↓ Reduced transmission of rickettsiae	(69)
R. rickettsii, Taiaçu	*A. aureolatum* (Atibaia, Saõ Paulo)	↓ Larval and nymphal molting ↓ Oviposition	(112)
R. rickettsii, Taiaçu	*A. cajennense*	↓ Transovarial transmission ↓ Reproductive performance	(105)
R. rickettsii, Taiaçu	*Rh. sanguineus* (Seropédica, Rio de Janeiro)	Low filial infection rate (<50%) Low larva infection rate (7.8–8.3%)	(34)
R. rickettsii, Taiaçu	*Haemaphysalis leporispalustris*	↑ Biological performance	(111)
R. peacockii, Skalkaho	*D. andersoni* (Montana)	No effects	(69)
R. conorii, Malish (VR163)	*Rh. sanguineus* (Thailand)	Detrimental effect	(107)
R. conorii conorii (strain not identified)	*Rh. sanguineus* (Southern France)	↓ Molting success ↓ Longevity of nymphs ↓ Infection rate in survived ticks ↑ Mortality	(113)
R. conorii conorii, Ghazonet	*Rh. sanguineus* (Algeria)	No detrimental effect observed 100% Transovarial transmission 99% Filial infection rate	(114)
R. conorii conorii, Malish (VR163)	*Rh. sanguineus* (North American and Mediterranean colonies)	Significant effect observed	(108)
R. conorii israelensis, ISTT-CDC1	*Rh. sanguineus* (North American and Mediterranean colonies)	No significant effect observed	(108, 109)
R. massiliae, Bar 29	*Rh. turanicus* (Corsica)	No detrimental effects observed	(110)
Rickettsia africae, ESF 2500-1	*Amblyomma variegatum*, (Ivory Cost)	No detrimental effects observed 100% Transovarial transmission 93.4% Filial infection rate	(115)
R. montanensis (strain not identified)	*D. variabilis* (Old Dominion University colony)	No detrimental effects observed	(116)
R. montanensis, M/5-6	*D. andersoni* (Montana)	↓ Rate of transovarial transmission	(69)
R. rhipicephali (strain not identified)	*D. variabilis* (Old Dominion University colony)	↓ Egg mass weight ↓ Rate of transovarial transmission	(116)

Similarly, the incidence of human rickettsioses is also nearly zero when their lifestyles and activities do not bring individuals into any contact with animals or vectors. However, the range of variables to consider and measure beyond these two extremes makes useful modeling a daunting task.

Several aspects of the potential impact of ticks on human health have been evaluated in recent years. Particularly, satellite data coupled with sophisticated Geographic Information Systems (GIS) have permitted the evaluation of the relationship of various parameters such as occurrence and distribution data on ticks to defined

ecological niches and animal host ranges, and the extrapolation of climate change data to future risk assessments (122). The basic starting concept is that each species of tick and its animal hosts are found within specific ranges of environmental variables (temperature, humidity, ecotypes), which support their reproduction and individual survival (123), so that the existing climate and environmental parameters associated with an agent define a set of conditions necessary for the predicted existence of a particular population in a very specific small area or averaged over a much larger region (122). The geographical range of a tick population depends on many parameters ranging from the life cycle of the tick, abundance of its hosts, and anthropogenic influences on vegetation, land use, and host displacement (124, 125). The Mediterranean region is expected to experience the greatest changes in risk of tick-borne infections in Europe due to predicted increases in average temperature and decreases in average rainfall. The climatic changes are predicted to affect the distribution of several tick species including expansion of ranges for *Rh. turanicus* and *Hyalomma marginatum marginatum*, and retreats for *D. marginatus* and *Rh. bursa*, which will be displaced toward higher latitudes (122, 126). Climate-based modeling conducted for human biting *D. andersoni* indicated a shift toward peak abundances of *D. andersoni* adults occurring in sheltered northern/eastern exposures, rather than in drier and hotter southern/western exposures (127). Modeling of the impacts of the changes in the climatic conditions in France on the activity and distribution of *Rh. sanguineus* confirmed empirical observations of the northward migration trend of this cosmopolitan tick, which carries many human and veterinary pathogens (128). Historic changes in the geographical distributions of ticks within different parts of their ranges have already been observed for several species of Ixodid ticks such as *Ixodes ricinus* in northern European countries (129) or *Amblyomma americanum* in USA (92). In this regard, meteorological data and weather forecasts appear to be useful for predicting the activity and density of ticks, particularly as was implemented for predicting infections transmitted across Europe by *I. ricinus*, *D. reticulatus*, *Rh. sanguineus*, and the flea *Ctenocephalides felis* (130). Maps were constructed (www.FleaTickRisk.com) that use current meteorological data for weekly predictions of ectoparasite activity in different areas. The activity index of the previous week is used as the criterion for estimating the risk of tick infestation and associated transmission of several tick-borne pathogens, including *Rickettsia* for the coming week. The data supplied by the model are used as an epidemiological tool by veterinarians and other healthcare professionals to improve the advice they provide to pet owners, but this approach may serve as a good model for developing similar efforts to forecast the risk of human tick-borne diseases.

At a finer scale, measurement of the annual changes in the population size and density of wildlife hosts and different contributions of various host species to tick success may also help to predict the persistence and transmission frequency of a given tick-borne pathogen, and thus its potential for spillover at the interface of human and wildlife habitats (131). Because of the complexity of the variables, only a few attempts have been made to model local aspects of tick-borne rickettsioses as opposed to the wider impact of meteorological factors on the distribution of the host ticks. An attempt to understand the spatial concordance between RMSF incidence and the habitat probability of its main vector *D. variabilis* is perhaps the best example of the value and limits of this approach (132). The latter study specifically focused on defining these habitat associations only in parts of Texas, but it was clearly limited by the small amount of data available at the site including insufficient tick sampling, unsophisticated diagnosis of human SFG rickettioses, an ineffective reporting system for RMSF, and anthropogenic inferences due to movement of people and various economic and agriculture activities, which directly affected tick habitats during the study period.

Earlier models of *R. rickettsii* transmission in *D. andersoni* and *D. variabilis* had been done without our current understanding of both the biology of rickettsia-tick interactions and the contemporary view of the molecular epidemiology and biogeography of RMSF (4, 24). Consequently, the original predictions derived in those studies do not meet current expectations but could serve as the basis for an updated model. Early modeling of *R. rickettsii* transmission in *D. andersoni* (19) analyzed only the role of vertical transmission in maintaining *R. rickettsii* infection and its potential effect on the size of the vector population, particularly the cumulative effect of a pathogen load passed down through consecutive generations based on a varying 10–100% transmission rate (102). The load of *Rickettsia* in the population would increase with each successive generation if it were predominantly vertically acquired; its accumulation might then eventually become a factor in controlling the tick population size and indirectly affect the survival of the host because of the damage caused by this organism.

Limited later modeling attempts, which considered additional variables for both vector and pathogen, established the following predictions. Simulated *in silico* predictions based on estimated relationships between the rate of transmission and the density of ticks determined that approximately 252 adult *D. variabilis* per ha are required to sustain transmission of *R. rickettsii* (133). These calculations were based on the assumption that a maximum of 98% of engorged immature nymphs of *D. variabilis* survive to the adult stage (134), and took into consideration the various effects of biotic and environmental variables including weather, host density and their habitat, infectivity levels of ticks and mammals, as well as the fecundity of infected ticks and the efficiency of transovarial transmission of rickettsiae (133). The authors emphasized the deviations between reported and simulated cases in Maryland and Oklahoma (133), but were unaware of the predominant presence of *R. montanensis* in *D. variabilis* in Maryland and elsewhere in USA (135, 136). The same estimates of tick densities were used to test if the occurrence of RMSF can be predicted based only on the presence of particular mammalian species as well as the relative abundance of important host species and their effect on the adult *D. variabilis* population size (137).

Cooksey et al. defined the RMSF transmission threshold as the density of ticks at which the yearly rate of increase of rickettsiae to humans is at an equilibrium level or 1.00 (133). It was estimated that for the RMSF potential of 1.61 to occur required the availability of 102 immature *D. variabilis* per 0.4 Ha, which with a 98% survival rate results in 252 adult ticks in the area (133, 137). The most accurate estimates were predicted for *D. variabilis* infesting raccoons and opossums with infestation rates ranging from

0 to 17; three sites were predicted to have a RMSF potential of >1.61. These estimates were then the state of the art based on the existing modeling approaches and the available understanding of RMSF ecology and epidemiology; the current information based on molecular epidemiology data makes those estimates very questionable. The state of Tennessee is ranked among the areas with the highest reported rate of RMSF and highest morbidity and mortality (138); however, field studies and large scale testing of ticks failed to even demonstrate the presence of *R. rickettsii* in any of the associated territories (139). Instead, only a high prevalence of *R. montanensis* was detected.

For *D. variabilis*, the presence and numbers of immature ticks seem to be the most important determinants defining the dynamics of various species of *Rickettsia*. Thus, it seems crucial to evaluate the actual burden of ticks on the host animal populations to accurately measure this variable. To address these questions, Dallas et al. (124) evaluated the association of nymphal and larval *D. variabilis* with its primary mammal host, *P. leucopus* and computed the tick burden in relation to other host variables, including mass, sex, and habitat (124). Consistent with other rodent-tick systems, this study demonstrated that the burden of immature *D. variabilis* is positively associated with male mice of higher body mass captured in the field habitat (124). This correlation is likely due to the higher probability of males to encounter ticks due to their larger home range and higher susceptibility to tick infestations because of their higher surface area.

Static models of tick-borne diseases are obviously limited due to their cross-sectional character and limited assumptions based on measurable parameters. Dynamic models would appear to encompass more of the essential information required to predict and evaluate other parameters like invasion of tick species with their associated tick-borne pathogens as might occur with exotic ticks on birds (82). While focused on transmission of the agent of human monocytic ehrlichiosis, *Ehrlichia chaffeensis*, which must be acquired by ticks every generation since it is not maintained transovarially, a broader agent-based model for tick–borne disease(s) was recently reported (20). It evaluates the interactions between ticks and their hosts as well as the transmission of tick-borne disease between two populations. The applicability of this model to rickettsial diseases has not been tested. However, the model predicts a significantly lower prevalence of ehrlichiae in both ticks and their hosts compared to predictions made with similar data using other models.

CRITICAL QUESTIONS AND FUTURE DIRECTIONS

As we have discussed, the distribution and prevalence of tick-borne pathogens and the diseases they transmit are strongly influenced by many factors, including changes in broad geographic and local climatic parameters, variations in land use, changing human activities, and animal behaviors that may cause disruption of ecosystems. The accuracy of the estimates of human disease depend greatly upon the adequacy of contemporary medical and public health practices including the extent and efficiency of surveillance efforts and the prevalence and specificity and quality of diagnostic practices in clinical practice. Changes in landscape ecology may result in the pronounced expansion of the number of ticks or of their biological hosts, with a consequent increased risk for human or animal health. In contrast, human activity can cause pronounced habitat fragmentation and associated alterations in the movement of hosts carrying ticks. Those movements may also affect the dynamics of disease transmission due to alterations in the biodiversity of the hosts, vectors, and pathogens present in "island" habitats. In the context of rickettsial diseases, those juxtaposed changes have resulted frequently in the detection and description of emerging and reemerging rickettsioses both in endemic settings and globally (1), increased recognition of travel associated rickettsioses (140), discovery of previously unknown pathogenic agents (141), and the belated recognition of the overlapping circulation of several rather unrelated bacterial agents that, nonetheless, may share the same tick vectors and influence the transmission of those pathogenic for vertebrates (142).

In recent years, because of enhanced concerns over global warming, much attention has been paid to the effects of climate change on arthropods. While it is clear that many tick-borne diseases are experiencing apparent increases, which factor is the key causative variable associated with this increase is less clear. In USA, northward and western expansion of the distribution of *A. americanum* and inland distribution of populations of *A. maculatum* has created new areas for exposure to the several tick-borne diseases transmitted by these ticks (92, 143). Similarly, the continued northward movement of *Rh. sanguineus* in Mediterranean countries and presence at higher elevations of *Dermacentor* ticks in European countries (122) has increased concerns for transmission of diseases in those areas. However, while straightforward, the environmental sampling of ticks and screening for different pathogens is necessarily very sporadic and lacks an associated systematic assessment of tick density and whether different animal reservoirs and changes in habitat may have become important in those sites. This combination of factors has made it very difficult to model the risk of infection under the influence of novel or unquantified variable host factors, including the diversity of small and large animal hosts, their varying competence as reservoirs for pathogens, and the interplay between known and novel undescribed pathogens. Although this information might be interpreted in the context of fairly well understood spatial and temporal patterns of tick distributions, the microspatial factors, and seasonal fluctuations can greatly influence the focal presence and geography of ticks and their interaction with multiple animal hosts and their associated microbiota.

Ticks do not simply serve as an environment for rickettsial propagation and maintenance through the germ line. Instead, species of *Rickettsia* exhibit a continuum of interactions with their arthropod hosts. While some may act as the prototypical vertically maintained endosymbiont with potential benefit to the tick, others are opportunistic pathogens or transient commensals employing vertical and horizontal transmission mechanisms to varying degrees but without obvious or yet known effect on the tick, and fortunately for tick populations, only a select few cause major detrimental effects on their tick hosts. However, the molecular factors that tip the balance between these life styles for different species, and even strains of *Rickettsia*, are not yet understood even though the genome sequences of many of these isolates have been obtained. The rapid use of other advanced molecular tools in microbiome surveys have further revealed the

complexity of the microbial communities associated with different tick species. These can include other bacteria, viruses, protozooans, and fungi, each with their own biology, and potential effects on host–pathogen interactions and diverse interactions with the vectors that harbor them (144–146). The quantitative ratio of rickettsial pathogens to the total microbial community in ticks may vary significantly depending on the tick species, its life stage, and the sex of the adult (147). Much needs to be learned about the functional, and potentially genetic, interactions between different microbes in ticks and the effects of the quantity of different microbial taxa on the tick. Whether intrinsic symbiont populations play a role in the selective acquisition, transmission, and expression of virulence factors by rickettsial pathogens needs further investigation. Whether those interactions can be exploited for control of the vector or for blocking acquisition of the pathogens are important practical and public health issues. Both systematic environmental sampling to assess the consistency of certain components of the bacterial community and state-of-the-art laboratory manipulations will be required to dissect both their natural ecological importance and their potential practical applications (148).

One of the most profound developments of the last decade in the eco-epidemiology of tick-borne rickettsioses has been its increasing numerical impact on public health. Sporadic disease certainly results from the complex and multifaceted interactions occurring between human and reservoirs of infected ticks sustained by wildlife. However, increasingly larger outbreaks of some tick-borne diseases are mediated directly through domestic and companion animals, which harbor large populations of infected ticks. One of the best examples is the persisting foci of *R. rickettsii* associated with *Rh. sanguineus* and peridomestic dogs in eastern Arizona and sites in Mexico, which serve both as hosts for feeding the ticks, the "reservoir" for these rickettsiae, and the site for co-feeding transmission of the agent (66, 68). Although the exact origin of the agents causing these sustained outbreaks is not fully understood, they arose independently because of the difference in the genotype of both the tick and the *Rickettsia*. The foci also embody good examples of how human-aided movement and maintenance of dogs can sustain the propagation of their ticks and their rickettsiae and the potential for their rapid spread into new environments. Even sustained efforts to decrease tick populations with acaricides were unsuccessful while elimination of the dog host immediately reduced transmission.

Other Ixodid ticks, which have adapted to two or three vertebrate host cycles, have significant opportunities for expanding the range of some rickettsioses to very large territories when the infected ticks migrate with passerine birds as immatures (149). Increasing numbers of the reports have described the presence of known and novel rickettsiae and other tick-borne pathogens in ticks collected from migratory birds (82, 150). Birds can serve not only as a larval tick host and vehicle for their migration for very long distances but can also establish persisting rickettsiemia [cited in Ref. (41)] that provides an infected host for different ticks at the end of a migration. In this manner, birds can serve as a highly mobile dispersive large effective reservoir for rickettsial pathogens. Establishment of new endemic foci at sites during the migration routes does require an optimal combination of climate and ecological factors and the availability of susceptible ticks and competent animal hosts to facilitate further dispersal and maintenance of the rickettsiae and for human exposure to these agents. Although many of these interactions have been postulated, systematic and quantitative assessment of these importations need to be conducted as well as broadened studies of the vector competence of these ticks and the susceptibility of potential native animal hosts to the imported pathogens.

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Zoonotic malaria – global overview and research and policy needs

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The four main *Plasmodium* species that cause human malaria, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, and *Plasmodium ovale*, are transmitted between humans by mosquito vectors belonging to the genus *Anopheles*. It has recently become evident that *Plasmodium knowlesi*, a parasite that typically infects forest macaque monkeys, can be transmitted by anophelines to cause malaria in humans in Southeast Asia. *Plasmodium knowlesi* infections are frequently misdiagnosed microscopically as *P. malariae*. Direct human to human transmission of *P. knowlesi* by anophelines has not yet been established to occur in nature. Knowlesi malaria must therefore be presently considered a zoonotic disease. Polymerase chain reaction is now the definitive method for differentiating *P. knowlesi* from *P. malariae* and other human malaria parasites. The origin of *P. falciparum* and *P. vivax* in African apes are examples of ancient zoonoses that may be continuing at the present time with at least *P. vivax*, and possibly *P. malariae* and *P. ovale*. Other non-human primate malaria species, e.g., *Plasmodium cynomolgi* in Southeast Asia and *Plasmodium brasilianum* and *Plasmodium simium* in South America, can be transmitted to humans by mosquito vectors further emphasizing the potential for continuing zoonoses. The potential for zoonosis is influenced by human habitation and behavior as well as the adaptive capabilities of parasites and vectors. There is insufficient knowledge of the bionomics of *Anopheles* vector populations relevant to the cross-species transfer of malaria parasites and the real extent of malaria zoonoses. Appropriate strategies, based on more research, need to be developed for the prevention, diagnosis, and treatment of zoonotic malaria.

Keywords: African apes, *Anopheles* vectors, human malaria, malaria control, malaria transmission, non-human primate malaria, *Plasmodium*, zoonosis

INTRODUCTION

The history of the many discoveries made over a period of more than 100 years showing that human malaria was caused by a protozoan parasite that is transmitted by mosquito vectors has been extensively documented [Ref. (1) and references therein]. It is now known that infections with four species of malaria parasites, viz. *Plasmodium falciparum*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium vivax*, transmitted between humans by mosquito vectors of the genus *Anopheles* are primarily responsible for human malaria. The World Health Organization in its latest malaria report (2) estimated that about 207 million persons had clinical disease and 627,000 died worldwide from malaria in 2012. Human malaria is endemic in many parts of sub-Saharan Africa, South and Southeast Asia, and Central and South America (3, 4). About 3.4 billion people in the world are at risk of malaria, although the risk is relatively low outside Africa and South/Southeast Asia. *P. falciparum*, the dominant parasite in Africa (3), is responsible for 92% of the deaths that occur mostly among children living in sub-Saharan Africa. *P. vivax*, a common human malaria parasite in much of Asia and South America (4), is regarded to cause serious disease but rarely death. However, there are indications that severe vivax malaria and attendant fatalities may have increased in Asia-Pacific and South America over the past 5 years (5). *P. malariae* and *P. ovale* are the two other rarer human malaria parasites.

Approximately 250 species of *Plasmodium* are presently believed to parasitize mammals, birds, and reptiles. All are transmitted by insect vectors. More than 30 species of *Plasmodium* have been reported in non-human primates, including apes, gibbons, and New and Old World monkeys. All primate malarias are believed to be transmitted only by *Anopheles* mosquitoes.

Some monkey malaria parasites have been experimentally transmitted to humans in the past through the bites of infected mosquitoes. They include *Plasmodium cynomolgi* (6–8), *Plasmodium knowlesi* (9, 10), and *Plasmodium inui* (11) from Old World monkeys, *Plasmodium brasilianum* (12) and *Plasmodium simium* (13) from New World monkeys, and *Plasmodium schwetzi* (now regarded to be either *P. vivax* or *P. ovale*-like parasites) from chimpanzees (14). However, recent discoveries, made possible by the use of polymerase chain reaction (PCR) and high throughput DNA sequencing, now establish that human malaria can also be a zoonotic disease.

PLASMODIUM KNOWLESI ZOONOSIS IN ASIA

Natural human infection with *P. knowlesi*, a common parasite of mainly macaque (*Macaca* species) monkeys in Southeast Asia, has been known to be possible for half a century (15). The more recent discovery of a focus of human infections in the Kapit division of the Sarawak state of Malaysian Borneo, however, showed that

P. knowlesi zoonosis was more prevalent than previously suspected (16). The infections in Kapit were misdiagnosed by routine microscopy of blood films as being caused mainly by *P. malariae*. This is because the late blood stages of *P. knowlesi* and *P. malariae* are morphologically very similar. The subsequent retrospective use of *P. knowlesi*-specific primers for PCR amplification and DNA sequencing of the genes for the small subunit ribosomal RNA (*ssrRNA*) and circumsporozoite protein (*csp*) was essential for correct species identification in Kapit (16). Human *P. knowlesi* infections were subsequently demonstrated in Pahang in peninsular Malaysia and in Sabah in Malaysian Borneo by species-specific PCR (17). A more recent retrospective *ssrRNA*-based PCR analysis of malaria blood samples showed that human infection with *P. knowlesi* is in fact widespread in peninsular Malaysia as well as the states of Sarawak and Sabah in Malaysian Borneo, accounting for 96% of all malaria cases in one peninsular hospital (18). Erroneous microscopic identification of *P. knowlesi* as a human malaria parasite in blood films was reported to be common in peninsular Malaysia (18) as was the case in Malaysian Borneo (16, 17). Mixed infections of *P. knowlesi* and human malaria parasites in patients have been observed in both peninsular Malaysia and Malaysian Borneo (17, 18). The use of DNA-based identification methods has since documented human *P. knowlesi* infections in much of Southeast Asia, ranging from the Andaman and Nicobar islands of India in the West to the Philippines in the East [Ref. (19) and references therein].

Plasmodium knowlesi is normally a parasite of *Macaca fascicularis* (the long-tailed or crab-eating macaque), *Macaca nemestrina* (the pig-tailed macaque), *Trachypithecus obscuras* (dusky leaf monkey or spectacled langur), and *Presbytis melalophus* (banded leaf monkey or brown langur) [Ref. (19) and references therein]. The main documented vectors are members of the *Anopheles leucosphyrus* group, some of which have been reported to feed equally well on humans and monkeys [Ref. (19) and references therein, Ref. (20)]. Within the leucosphyrus group, *Anopheles balabacensis* is a particularly efficient vector of simian *P. knowlesi* in Malaysia as is *Anopheles dirus* in Vietnam [reviewed in Ref. (9, 20, 21)]. A map of the likely range of *P. knowlesi* based on the distribution of host monkey species and the relevant anopheline vectors, suggests that a potential for *P. knowlesi* zoonosis exists across large tracts of the populous South and Southeast Asian region (19). Because microscopy and rapid diagnostic tests commonly employed to diagnose malaria lack the requisite specificity, it is possible that many more patients with *P. knowlesi* malaria may have been unknowingly misdiagnosed in South and South East Asia.

Experimental *P. knowlesi* inoculations have been used in the past to treat neurosyphilis (9). The parasite has a 24 h asexual cycle in blood and therefore produces a quotidian fever that can potentially differentiate it from the 72 h quartan fever of *P. malariae* infections in humans. However, the clinical symptoms of naturally acquired *P. knowlesi* infections in humans are often not very specific and the infections can sometimes prove severe and fatal with high parasitemias [Ref. (9) and references therein]. Mixed infections of *P. knowlesi* with human malaria parasites are not uncommon in patients (17, 18, 21) and human infection with *P. knowlesi* in some sites in Vietnam has been reported to be asymptomatic (21). This contrasts with the milder symptoms and lower parasitemias observed with *P. knowlesi* in its natural monkey hosts (9). More extensive investigations on the clinical features and treatments of *P. knowlesi* infections in humans are warranted by the increasing realization that such infections are more common than previously reported in countries like Malaysia (18). From the treatment point of view, it is relevant to note that *P. knowlesi* strains from patients in Malaysian Borneo were reported to be sensitive to artemisinins, moderately but variably sensitive to chloroquine, and less sensitive to mefloquine (22). It is not clear whether the less than desirable sensitivity of *P. knowlesi* to chloroquine and mefloquine is a result of drug selection in humans and/or the intrinsic biology of the parasite, and this merits further investigation.

Despite the experimental transfer of *P. knowlesi* infections to humans and documented natural infections in Southeast Asia, the direct transfer of *P. knowlesi* from one infected person to another uninfected human by mosquito vectors has yet to be unequivocally demonstrated to occur in nature. Rather, human infections appear to be acquired from monkeys when people venture into forests. Epidemics of *P. knowlesi* malaria have not been reported and clustering of infections was not observed at Kapit, Sarawak in Malaysian Borneo (16). However, *P. knowlesi* is able to form mature gametocytes in the blood of infected persons (23, 24). *P. knowlesi* sporozoites are also detectable in the salivary glands of *An. dirus*, a human malaria vector in Vietnam, commonly as mixed infections with *P. vivax* and *P. falciparum* sporozoites (21). The existing data therefore raise the possibility that human to human transmission of *P. knowlesi* may occur in areas with a high density of *P. knowlesi* zoonosis. However, there is presently no evidence to firmly establish that such transmission takes place, and if it does occur, the possible rates of knowlesi malaria transmission between humans and its epidemiological significance need to be investigated.

PLASMODIUM CYNOMOLGI ZOONOSIS IN ASIA

The experimental infection of humans with *P. cynomolgi*, commonly a parasite of macaque monkeys in Asia, through the bites of infected *Anopheles freeborni* mosquitoes was demonstrated half a century ago (6). However, the first natural infection of a human was only reported very recently in peninsular Malaysia (25). Microscopic examination of the blood film from this patient suggested infection with *P. vivax*. *P. cynomolgi* blood stages are morphologically very similar to those of *P. vivax*. A common nested PCR test (26) yielded an amplified fragment of *ssrRNA* whose size was characteristic of *P. vivax*. A more discriminating PCR followed by DNA sequencing of *ssrRNA*, however, showed conclusively that the parasite causing the infection was in fact *P. cynomolgi* (25). The clinical presentation of this patient was mild with 24 h cyclic chills rather than the expected tertian fever resulting from the 48 h asexual blood stage cycle of *P. cynomolgi*. The patient lived adjacent to a small forest where macaques were present. The suspected mosquito vector in this case was *Anopheles cracens*, belonging to the *An. leucosphyrus* group of mosquitoes, and a known vector of the simian malaria parasites *P. cynomolgi* and *P. inui* (25). This case suggests that other instances of human infections with *P. cynomolgi* in South and Southeast Asia may have been similarly misdiagnosed as *P. vivax* infections by microscopy.

PLASMODIUM SIMIUM AND *PLASMODIUM BRASILIANUM* AS ZOONOSES IN THE NEW WORLD

Plasmodium simium is a parasite of the platyrrhine monkeys of South America. The New World platyrrhine monkeys diverged from catarrhine monkeys of the Old World about 30–35 million years ago (27). Sequence analysis of genes coding for the merozoite surface protein-1 (*msp1*) and cytochrome *b* (*cytb*) and the *ssrRNA* and *csp* genes from worldwide isolates of *P. vivax* and a limited number of *P. simium* isolates from South America showed that the two parasites were very closely related (28). It is unlikely that *P. vivax* originated in the New World from *P. simium* because there is now evidence to show that *P. vivax* in humans arose from related parasites in African apes (29). This conclusion is supported by the greater genetic diversity of *P. vivax* worldwide compared to *P. simium*, which is found only in South America (28). It is therefore more likely that *P. simium* arose from *P. vivax* that was transferred to platyrrhine monkeys by infections prevalent in settlers from the Old World in the post-Columbus era. At least two such cross-species transfers must have taken place as two different alleles of the *P. vivax csp* are also found in *P. simium* (28).

Natural infection of a human by *P. simium* was reported from Brazil in 1966 (13). A volunteer collecting mosquitoes by human landing catches in a forest became infected with the parasite, which was identified by careful microscopic examination of blood films to be *P. simium*. The infection could be syringe-transferred from his blood to a platyrrhine squirrel monkey (*Saimiri* spp.) It was suspected that platyrrhine howler monkeys (*Alouatta fusca*) were the original source of the infection, and *Anopheles cruzi* the vector, in this instance of zoonosis. It was suggested that human *P. simium* infections contracted in forests may often be misdiagnosed as *P. vivax* during routine microscopy due to the presence of similar patterns of stippling or Schuffner's dots in infected red blood cells, and because many microscopists might be unaware of the possibility of simian malaria parasites infecting humans (13).

Plasmodium brasilianum is a parasite of many species of platyrrhine monkeys in South and Central America. Sequence analysis of *csp*, *msp1*, and *ssrRNA* of *P. malariae* and *P. brasilianum* showed that the two parasites were very closely related but the likely direction of a cross-species transfer could not be established due to the limited number of parasite isolate sequences available for analysis (28). There is now evidence to suggest that parasites closely related to *P. malariae* are found in African apes (30, 31). If it can be shown that *P. malariae* in man, like *P. falciparum* (30) and *P. vivax* (29), also arose through cross-species transfer from African apes in Africa, it would appear plausible that *P. brasilianum* in platyrrhines is a result of the cross-species transfer of *P. malariae* brought to the New World by settlers in the post-Columbus era.

Anopheles freeborni mosquitoes infected by feeding on a platyrrhine spider monkey, *Ateles geoffroyi geoffroyi*, from Panama carrying *P. brasilianum*, have been shown to transmit the parasite through biting to five human volunteers (12). This raises the possibility that the transmission of *P. brasilianum* from platyrrhine monkey hosts to humans occurs naturally in South and Central America with the resulting human infections being diagnosed as *P. malariae* by microscopy and other diagnostic tests. Since very few gene sequences are available for the two species, and the existing ones show little difference between them, establishing zoonosis through DNA-based diagnostics is difficult at the present time. It also seems likely that *P. malariae* and *P. brasilianum* are in fact variants of the same parasite species that are able to infect both humans and platyrrhine monkeys in the New World.

PLASMODIUM FALCIPARUM AS AN ANCIENT ZOONOSIS IN AFRICA

It was believed for some time, based on limited DNA sequencing, that the closest relative to human *P. falciparum* was the chimpanzee (*Pan troglodytes*) parasite *Plasmodium reichenowi* (32). The origin of *P. falciparum* was, however, recently established through sequencing a number of genes from parasite DNA isolated from fecal samples of wild apes in Africa (30). DNA sequence analysis showed that parasites more closely related to *P. falciparum* than *P. reichenowi* are prevalent in western gorillas (*Gorilla gorilla*) in West and Central African forests. Analysis of the sequences of the mitochondrial *cytb* showed that the *P. falciparum*-related ape parasites could be phylogenetically classified into six host-specific clades (30) tentatively named as *P. reichenowi*, *Plasmodium gaboni*, and *Plasmodium billicollinsi* in chimpanzees and *Plasmodium praefalciparum*, *Plasmodium adleri*, and *Plasmodium blacklocki* in western gorillas (33). All known human *P. falciparum cytb* sequences formed a distinct lineage within the gorilla-specific *P. praefalciparum* clade (30). Analysis of the apicoplast caseinolytic protease C (*clpC*) and nuclear lactate dehydrogenase (*ldh*) genes, and the entire mitochondrial genome, yielded a phylogenetic relationship compatible to that obtained with *cytb*. Therefore, *P. falciparum* most probably arose through the cross-species transfer of an ancestral *P. praefalciparum*-like parasite from gorillas that then successfully adapted itself to humans (30). *Anopheles moucheti*, a known human malaria vector that is found in forested areas of Central Africa, has been shown to carry *P. praefalciparum* in Gabon (34). Inoculation of *P. praefalciparum* into humans is therefore very likely to be a continuing process in West and Central Africa but productive infections in potentially exposed humans in Cameroon were not detected and are therefore likely to be extremely rare (35). However, more studies involving other African sites are needed to further clarify this aspect.

Apes are not readily infected with *P. falciparum* and in order to obtain high parasitemia, chimpanzees have to be splenectomized (36). Platyrrhine owl (*Aotus* spp.) and squirrel (*Saimiri* spp.) monkeys are also infectible with *P. falciparum*, but a period of adaptation in splenectomized animals is required (36). It has been proposed that the adaptation and host specificity of *P. falciparum* for humans is a result of the specific interaction of a *P. falciparum* protein ligand PfEBA175 with *N*-acetylneuraminic acid on human erythrocyte glycophorin A (37). However, more recent evidence suggests that the specificity may be due to the interaction between PfRh5, an essential erythrocyte invasion ligand found in the rhoptry organelles of the parasite, and the corresponding human erythrocyte receptor termed basigin (38). Basigin or CD 147 is responsible for the Ok blood group phenotype. PfRh5 does not bind gorilla basigin and only binds weakly to chimpanzee basigin and these properties have been suggested to cause the host restriction of *P. falciparum* to humans (38).

Analysis of single nucleotide polymorphisms (SNPs) in two *P. falciparum* genes for common housekeeping proteins (P type Ca^{2+}-ATPase, *serca*, and adenylosuccinate lyase, *adsl*), from parasite isolates in different parts of the Old World showed that genetic diversity in *P. falciparum* decreased significantly with the distance from sub-Saharan Africa (39). This finding may be interpreted as being compatible with a hypothesis that the carriage of *P. falciparum* from an origin in sub-Saharan Africa to the Old World was a result of the migration of modern humans (*Homo sapiens*) and/or ancestral *Homo* species to whom *P. falciparum* had become adapted.

PLASMODIUM VIVAX AS AN ANCIENT AND CONTINUING ZOONOSIS IN AFRICA

Sequence analysis of mitochondrial DNA from ape fecal and monkey blood samples in tropical sub-Saharan African forests also show that *Plasmodium* parasites genetically very similar to human *P. vivax* are common in wild chimpanzees (*P. troglodytes*) and western (*G. gorilla*) and eastern gorillas (*Gorilla beringei*) but not bonobos (*Pan paniscus*) or monkeys (29). Human *P. vivax* sequences from different parts of the world formed a distinct lineage within the ape sequences but the data suggest that the ape and human parasites belong to a single species, *P. vivax*. Analysis of the apicoplast *clpC* and four nuclear genes (*ldh*, *asl* coding for adenylosuccinate lyase, β-*tub* coding for β tubulin and *crk-2* coding for cell division cycle 2-related kinase) also produced results that were consistent with this conclusion (29).

Plasmodium vivax uses the Duffy blood group antigen molecule on red blood cells as the main receptor for binding and subsequent invasion. There are three major alleles for the antigen, viz. FY*A and FY*B, which differ by a single amino acid, and FY*O that gives rise to the absence of the receptor and the Fy(a-b-) serological phenotype in homozygous individuals. The Fy(a-b-) phenotype has been commonly associated with near complete resistance to *P. vivax* in most of Africa (40). The FY*O allele is estimated to have been selected in Africa approximately 33,000 years ago, i.e., after the initial migration of *H. sapiens* out of Africa, probably because of the protection provided against vivax malaria (41). However, approximately 5% of West Africans and a greater proportion of East Africans remain Duffy antigen positive and there is also evidence that *P. vivax* has evolved to use receptors other than the Duffy blood group antigen in East and West Africa (42–44).

The greater similarity of human *P. vivax* to ape *P. vivax* than human *P. falciparum* to gorilla *P. praefalciparum*, suggests that there may be continuing cross-species exchange of *P. vivax* between humans and apes in tropical Africa. Zoonosis was recently confirmed by sequence analysis of parasite DNA from a Caucasian European traveler, who contracted *P. vivax*-like malaria while working in the Central African Republic, which showed that the parasite had SNPs characteristic of ape and not human *P. vivax* (45). *P. vivax* with SNPs reportedly characteristic of human *P. vivax* has been identified in African apes confirming that the reverse transfer is also possible (45). *Anopheles moucheti* and *Anopheles vinckei* have been shown to carry ape-like *P. vivax* in Gabon and are potential vectors for zoonotic transmission to humans in or near forests (34, 45). The Fy(a-b-) phenotype or the absence of the Duffy antigen in a majority of the West and Central Africans would, however, protect against zoonotic *P. vivax* but such protection will not apply to the minority of Duffy antigen positive Africans and non-native populations or infection with *P. vivax* strains that can use alternative red blood cell receptors for invasion. Recent evidence suggests that Duffy antigen negative persons in Congo have antibodies specific for *P. vivax* asexual blood stage antigens (46) and that blood stage *P. vivax* infections can be detected by PCR in Duffy antigen positive and negative asymptomatic individuals in Cameroon (47). These recent observations are consistent with a proposal that Duffy antigen positive apes are a reservoir of *P. vivax* in West and Central Africa for transmission to humans to produce mild infections and elicit antibodies (48). More studies are clearly needed to determine the nature and extent of *P. vivax* zoonosis in Africa.

PLASMODIUM MALARIAE AND *PLASMODIUM OVALE* AS POSSIBLE ANCIENT AND CONTINUING ZOONOSES IN AFRICA

Studies in the 1940s by Rhodain suggested that a chimpanzee (*P. troglodytes*) parasite *Plasmodium rhodaini* was very similar, if not identical, to the human quartan malaria parasite *P. malariae*, with both parasites being readily transferable between humans and chimpanzees [reviewed in Ref. (49)]. Analysis of a limited number of *cytb* and *ssrRNA* sequences available now suggests that both *P. malariae* and *P. ovale* have closely related counterparts in wild chimpanzees in tropical Africa (30, 31). A larger number of *P. malariae* and *P. ovale* and ape sequences are needed for detailed analyses for determining the likely ancestral parasites of the two human species. Presently available data are, however, compatible with a hypothesis that human *P. malariae* and *P. ovale*, like *P. falciparum* and *P. vivax*, originated by cross-species transfer from African apes and then spread worldwide. Testing this hypothesis would require DNA sequences from a greater number of human *P. malariae* and *P. ovale* isolates worldwide and isolates of related parasites from wild African apes.

UNDER-DIAGNOSIS OF ZOONOTIC INFECTIONS IN HUMANS

The continuing difficulty in correctly identifying *P. knowlesi* in Southeast Asia, because of its similarity to *P. malariae* in blood films as discussed in Section "*Plasmodium knowlesi* Zoonosis in Asia" above, illustrates the potential for under-diagnosis of other forms of zoonotic malaria in many parts of the world. Microscopists working in hospitals and health centers in malaria-endemic countries where zoonoses may occur are not likely to differentiate between human parasites and closely related zoonotic parasites when routinely screening blood films. Antibody-based rapid diagnostic kits suitable for detecting *P. knowlesi* in blood with acceptable specificity and sensitivity are not presently available (50). It has also been suggested that presumptive drug treatment of malaria-like symptoms, and the lack of resistance to common anti-malarial drugs in *P. praefalciparum*, can rapidly cure zoonotic infections so that such infections may therefore go undetected in sub-Saharan Africa (33). Zoonotic malaria species may also develop low parasitemias in humans, so that their presence may be masked by coinfection with more virulent human species like *P. falciparum*. This has been observed for *P. knowlesi* in Vietnam (21). The availability of SNPs specific for ape parasites (e.g., for

Table 1 | Documented ongoing malaria zoonoses.

Plasmodium species	Location	Reference
P. knowlesi	Southeast Asia	(15–21)
P. cynomolgi	Malaysia	(25)
P. vivax	West Africa	(45)
P. vivax/*P. simium*	South America	(13)

P. praefalciparum and ape *P. vivax*) will facilitate identification of zoonotic infections as already demonstrated for *P. vivax* (45). However, ape malarial parasites related to *P. falciparum* including *P. praefalciparum* were not detected in humans who are likely to have been inoculated with such parasites by mosquito vectors in remote rural areas of Cameroon (35). Such DNA-based tests are not available for routine identification of zoonotic malaria parasites in malaria-endemic countries. They have also not yet been developed for *P. malariae* and *P. ovale* in Africa and may not be applicable in South America for differentiating *P. simium* from *P. vivax* and *P. brasilianum* from *P. malariae*.

Of the many *Plasmodium* species known to parasitize non-human primates, only a few species have been shown to be naturally transmitted to humans at the present time (**Table 1**). Although experimentally transmissible to humans (11), natural human infection with *P. inui* has not yet been documented. The absence of suitable diagnostic methods may explain the failure to detect *P. inui*, and perhaps many other non-human primate malaria parasite species, in humans. However, there are several possible causes that can prevent such other non-human primate *Plasmodium* species from naturally infecting humans. These include the inability to infect and form sporozoites in mosquito vectors that feed on humans, susceptibility to human innate immune responses, absence of compatible parasite ligands and human erythrocyte and hepatocyte receptors that facilitate productive invasion, and existence of intracellular hurdles that bar development even if host cell invasion were to be successfully achieved.

CHANGES IN HUMAN HABITATION AND ECOLOGY AS WELL AS ADAPTIVE CHANGES IN PARASITES AND VECTORS MAY FACILITATE THE EMERGENCE OF NEW HUMAN MALARIA PARASITES FROM NON-HUMAN PRIMATES

Increasing populations coupled with a demand for more agricultural land and timber is leading to extensive deforestation in malaria-endemic countries of the tropics. Humans are also venturing into forested areas in greater numbers for mining, lumbering, and recreation, and more people are beginning to live close to forests. Wild ape populations are also diminishing in Africa as are wild monkey populations throughout the world (51). Anopheline vectors that are responsible for sylvatic transmission of malaria parasites are therefore likely to be faced with decreasing numbers of monkey and ape hosts and increasing numbers of potential human hosts. Examples of anopheline malaria vectors becoming more anthropophilic over the course of a few years as a result of a shortage of animal hosts have been long documented (52). Mosquito vectors are generally highly versatile in adapting to environmental changes (53). Therefore, it is likely that ongoing changes in human habitation patterns and ecology as well as vector adaptation will lead to an increasing probability of humans becoming inoculated with monkey and ape malaria parasites and vice versa. In rare cases, such cross-species transfer may result in a non-human primate *Plasmodium* species varying sufficiently to adapt itself to human–human transmission, as was the likely case for the ancestor of *P. falciparum*. There is also the rare possibility that human malaria parasites may undergo genetic recombination with closely related ape and monkey parasites to yield new parasite strains that are more virulent to humans. Malaria parasites are haploid in their primate hosts, and meiosis and zygote formation takes place in the mid gut of mosquitoes. Genetic recombination between different *P. falciparum* strains occurs in mosquitoes (54). A mosquito vector infected with gametocytes of two closely related human and non-human primate parasites at the same time can theoretically serve as a vehicle for similar genetic recombination. Such a mix of gametocytes can be acquired through rapid sequential blood meals on infected humans and non-human primates in forested areas or in the case of *P. vivax* be acquired from a co-infected human or non-human primate. *P. vivax* may be particularly prone to such a recombination process since it appears to be readily able to switch between apes and humans in Africa as discussed in Section "*Plasmodium vivax* as an Ancient and Continuing Zoonosis in Africa" and between platyrrhine monkeys and humans in South America as discussed in Section "*Plasmodium simium* and *Plasmodium brasilianum* as Zoonoses in the New World." An epidemiologically pertinent but molecularly distant example from another pathogen is the swine influenza variant 2009 A (H1N1) that caused the 2009 influenza pandemic. It contained many genetic re-assortments with common swine influenza virus genes that resulted greater pathogenicity to humans (55). Genetic recombination is, however, likely to occur much less frequently with malaria parasites than influenza viruses because of the much greater complexity associated with the parasite sexual recombination process, but the possibility nevertheless needs to be borne in mind.

RESEARCH AND POLICY NEEDS

Not enough is presently known about the bionomics of anopheline vectors that transmit ape and monkey malaria parasites that are closely related to the human ones. This is more so in tropical Africa and South America than Asia. Details of their feeding preference for human and non-human primate hosts, resting habits, dispersal distances after blood feeding, insecticide sensitivities, and ongoing adaptation to ecological changes are essential for evaluating the potential role of such vectors in zoonoses. Conversely, there is a need to determine the extent to which the more anthropophilic vectors of human malaria feed on apes and monkeys and transmit malaria parasites.

There is also a need for more studies on the sensitivities of zoonotic parasites to common anti-malarial drugs used to treat human malaria and the clinical features of the different types of zoonotic malarias in humans.

The importance of investigating possible zoonoses in communities living in close proximity to forests and people venturing into forests needs to be understood by public health personnel responsible for malaria detection and control. They need accessible resources, such as a collaborating laboratory, to identify

suspected cases of zoonotic malaria with DNA-based techniques. Development, if possible, of non-radioactive DNA probes and loop-mediated isothermal amplification of DNA (LAMP)-based diagnostic techniques (56) that can be used for detecting zoonotic species in endemic country laboratories will be particularly helpful in this regard.

Zoonoses, particularly the existence of sylvatic reservoirs of *P. vivax* and *P. malariae* in South America and Africa, can compromise malaria control and eradication efforts. This needs to be recognized by the public health authorities responsible for malaria control. In a broader context, the agencies responsible for health and environmental planning in the tropics need to be aware of the present and likely future changes in levels of exposure to zoonotic malaria and develop appropriate mitigating and preventive strategies.

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Larvicidal, repellent, and irritant potential of the seed-derived essential oil of *Apium graveolens* against dengue vector, *Aedes aegypti* L. (Diptera: Culicidae)

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Aedes aegypti L. is one of the primary disease vectors spreading various dreadful diseases throughout the world, specifically over tropics and subtropics. Keeping in view the adverse effects of chemical insecticides-based intervention measures, the eco-friendly and bio-degradable essential oil extracted from the seeds of celery, *Apium graveolens* were investigated for its efficacy against *Ae. aegypti*. Larvicidal bioassay carried out with the seed oil against early fourth instars of *Ae. aegypti* caused an LC_{50} and LC_{90} values of 16.10 and 29.08 ppm, respectively, after an exposure to 24 h. The cidal effect of the celery seed oil augmented by 1.2-fold; after an exposure to 48 h; revealing an LC_{50} value of 13.22 ppm. Interestingly, the seed oil did not cause immediate larval mortality, suggesting a delayed toxicity against the larval stage. Present investigations also revealed remarkable effective repellency of the oil leading to 100% protection till 165 min as compared to control that did not result in any repellency against adult *Ae. aegypti*. Interestingly, only one bite was recorded in the 165th-min after which only two bites were scored until 180 min of exposure of the adult mosquitoes to the oil. An exciting observation was that the knocked-down effect in adults exposed to 10% oil-impregnated papers. The contact irritancy assays with paper impregnated with 1% celery seed oil caused first flight only after 4 s resulting in an average of 63.66 flights during 15 min of exposure revealing the relative irritability of 26.97. The qualitative phytochemical analysis of the seed oil showed the presence of flavonoids, lactones, and terpenoids as the major constituents suggesting their probable role in the toxicity. Our results confirmed that celery seed essential oil can be used as an efficient larvicide and repellent against *Ae. aegypti*. The identification of the bioactive components, their mode of action, and studying effects on non-target organisms and the environment would help in devising mosquito-management strategies.

Keywords: essential oil, larvicide, repellent, % protection, irritancy

INTRODUCTION

Aedes aegypti is considered as one of the major disease vectors that spread several dreadful diseases such as dengue fever, Chikungunya, and yellow fever worldwide specifically tropical and subtropical countries. In India, dengue fever is gradually becoming the most important public health problem. Every year, the number of reported dengue cases is rising. In 2013, a total of 75,454 dengue cases were reported in India, which led to 167 deaths (1). Earlier, in 2002, Pancharoen et al. (2) have reported the rising incidences of more severe forms of dengue, i.e., dengue hemorrhagic fever and dengue shock syndrome with unusual manifestations such as central nervous system involvement. The reports of World Health Organization (3) also reveal that about 40% of the world's population is at risk of dengue. It has been suggested that the major approach to control mosquito-borne diseases should include either targeting the mosquito larvae at breeding sites or by killing/repelling the adult mosquitoes (4).

Till today, chemical insecticides have been used on a large scale to control mosquitoes at both larval and adult stage. However, the adverse effects posed by these synthetic insecticides such as non-degradability, environmental pollution, toxicity to non-target population, and the developing resistance in mosquitoes have increased during the last five decades. These adversities have insisted on the need of formulating alternate mosquito control strategies. Botanicals have always attracted researchers as an environment-friendly, safe, and low-cost alternative to chemical insecticides (5). Several reports are available, which confirm the larvicidal and repellent efficacy of plant extracts or essential oils against different species of mosquitoes without posing toxicity hazards to humans (6–10).

Essential oils, the natural volatile substances obtained from various plants, have been exploited commercially in pharmaceuticals, as flavoring agents in foods, for aroma in fragrant products, and as insecticides. The essential oils have been primarily investigated for their antibacterial, antifungal, and insecticidal activities (11–13). However, they have received great attention as probable bioactive insecticides displaying a wide-spectrum activity, low mammalian toxicity, and rapid bio-degradability.

Apium graveolens, commonly called celery, is an aromatic herb. The essential oil, extracted from celery fresh dried seeds through

steam distillation, is used in several products of medical importance. Reports are available on the potential use of celery leaf stalks and seeds as popular aromatic herbs and spices (14, 15). Researchers have confirmed that certain bioactive components isolated from the crude alcoholic and hexane extracts of *Apium graveolens* seeds possess nematocidal activity against *Caenorhabditis elegans* and *Panagrellus redivivus*, antifungal activity against *Candida albicans*, *C. kruseii*, and *C. parapsilosis*, bactericidal activity against *Helicobacter pylori*, and mosquiticidal effects against *Ae. aegypti* fourth instars (16). However, much literature is not available on the larvicidal and repellent activities of the essential oil derived from the *Apium* seeds against *Ae. aegypti*. Hence, the present investigations were carried out to assess the larvicidal and the repellent potential of the celery seed essential oil against an Indian strain of *Ae. aegypti*. The present study may provide useful information on the bioactive component from native plant source, which could help in the development of new mosquito control agent.

MATERIALS AND METHODS

REARING OF *AE. AEGYPTI*

The present investigations were carried out on the dengue fever mosquito, *Ae. aegypti*, which were collected from ponds located in Delhi, India, and surroundings. The colony of mosquito was maintained in an insectary under controlled conditions of 28 ± 1°C, 80 ± 5% RH, and 14:10 L/D photoperiod (17). Adults were kept in cloth cages and provided with freshly soaked deseeded raisins as food. A wet cotton swab was kept on the top of cage to provide water. Blood meals at regular intervals were provided to female adults for maturation of egg follicles by keeping restrained albino rats in the cages. An enamel bowl lined with Whatman filter paper was kept in the cage for egg laying. The collected eggs were allowed to hatch in trays filled with de-chlorinated tap water. Freshly hatched larvae were fed upon a 1:3 ratio mixture of yeast powder and grinded dog biscuits. The water was changed every day to prevent the formation of any scum, and larvae were provided with fresh food. The pupae formed were collected in bowls and kept in the cages for adult emergence.

LARVICIDAL BIOASSAY

The larvicidal bioassay was performed on the early fourth instars of *Ae. aegypti* following the WHO protocol with slight modifications (17). The 99.9% pure essential oil, extracted from seeds of *A. graveolens*, was obtained from M/s Auroville, Puducherry, India. Ethanol was used as the solvent to prepare the graded series of celery seed oil for bioassays. Bioassay was carried out on 20 early fourth instars of *Ae. aegypti*; taken in plastic bowls containing 99 mL of distilled water; which were then transferred to a glass jar containing 100 mL of distilled water and 1 mL of the particular concentration of oil. Each dilution had four replicates for statistical significance. Control bioassays were performed replacing the oil–ethanol solution with ethanol alone.

During the bioassays, the larvae were not provided with any food. The larval mortality was determined by observing the movement of the larvae after 24 h of treatment by touching them gently with the help of a glass rod. The larvae without any sign of movements were considered dead, while those, which moved a little but did not show any kind of swimming movement were considered moribund. The moribund larvae unable to revive were considered dead. The experimental set up was kept undisturbed for next 24 h and mortality counts were recorded again to evaluate the delayed toxicity of the essential oil.

STATISTICAL ANALYSIS OF DATA

The larvicidal bioassays with more than 20% mortality in control tests and more than 20% pupae formed were discarded and carried out again. The control mortality ranging between 5 and 20% was corrected using Abbott's formula (18).

$$\mathrm{CM} = \frac{\mathrm{T} - \mathrm{C}}{100 - \mathrm{C}} \times 100$$

where, CM is the corrected mortality, *T* is the % mortality observed in experimental tests, and *C* is the % mortality in control tests. The larvicidal data were subjected to probit regression analysis based on generalized linear model using computerized SPSS 18.0 Program. The regression analysis models the normal distribution of the relationship between response (% mortality) and dose (concentration) as a linear model via a link function by the transformation of % mortality in probit values. The LC_{50} and LC_{90} values with 95% fiducial limit were calculated in each bioassay to measure the difference between the test samples. Other statistical parameters, such as regression coefficient and standard error were also calculated. The fitted model is assessed by statistics for heterogeneity, which follow a chi-square distribution.

BEHAVIORAL STUDIES IN OIL-TREATED *AE. AEGYPTI* LARVAE

During each larvicidal bioassay, the larvae were monitored carefully for behavioral modifications, if any, caused by extract-mediated disruption of biological functions. The behavioral observations included wriggling speed, horizontal movements, vertical movements, aggregation behavior, and knockdown of the larvae during treatment. The larval behavior was recorded and photographed with Canon Power Shot SX50HS. Similar observations were made in controls for comparison with treated larvae.

ADULT REPELLENCY BIOASSAY

The repellent potential of the celery seed oil was evaluated against adult *Ae. aegypti* using human-bait technique. Five human volunteers, non-allergic to mosquito bites, were invited from different institutes and a consent letter regarding the experiment was taken from each of them. The letters were deposited in the institute for reference, if needed. Starved and 3–10 days old females of *Ae. aegypti* were released in groups of 25 into separate laboratory cages (45 cm × 45 cm × 40 cm) for the investigations. During repellency bioassays, the arms of the human volunteers were thoroughly cleaned, washed with neutral soap without any fragrance, thoroughly rinsed with distilled water, and allowed to dry for 10 min before the application of extracts. A square of 5 cm × 5 cm size was marked on each forearm of human volunteers using a permanent marker. One forearm of all the volunteers was used for repellency bioassay and approximately 0.1 mL of the essential seed oil of *A. graveolens* was applied to the marked area. The other forearm of each volunteer was considered control and the marked area was

applied with ethanol. The rest of the area of each forearm was covered by a paper sleeve thus leaving only the marked area open. Both the control and treated arms were introduced simultaneously into the cage. Any attempt of inserting the stylets by a female mosquito was considered a bite. The numbers of bites occurred in 3 min were scored every 15 min, for 3 h; from 11:00 to 14:00 hours.

Protection time was recorded as the time elapsed between the application of essential oil and the time of a confirmed bite. The tests, with none of the adult *Ae. aegypti* landing on the control arm or attempting to bite, were discarded. These tests were carried out again with a fresh batch of adults to ensure that failure to bite was due to repellence potential of oil and not because of the mosquitoes being pre-disposed to get a blood meal. Three replicates of each experiment were carried out. Each replicate was conducted in separate cages and with different volunteers to negate the effect of skin variability on repellency, if any.

The percent protection from the mosquito bite provided by the oil was calculated by using the following formula:

$$\% \text{ Protection} = \frac{\text{Number of bites on the control arm} - \text{Number of bites on the treated arm}}{\text{Number of bites on the control arm}} \times 100$$

CONTACT IRRITANCY ASSAY

The contact irritancy assay was performed on 3-day-old non-blood females of *Ae. aegypti*. The oil-impregnated papers were prepared with Whatman filter paper no. 1, which were cut out in circles of 8 cm diameter. The papers were impregnated with 10 and 1% of celery seed essential oil and then allowed to shade-dry. The completely dried paper was placed on a glass plate, and a perspex funnel with a hole on the top was kept inverted over the impregnated paper. Single female adult was released in the funnel and per-conditioned for 3 min. Thereafter, the time at which first flight was taken was recorded. The experiment was continued for 15 min and the total number of flights undertaken by each female adult was scored. Three replicates were carried out for each treatment. Parallel control tests were performed with papers impregnated with acetone. The relative irritability caused by the seed oil was calculated with respect to control by the following formula:

$$\text{RI (Relative Irritability)} = \frac{\text{Mean number of take-offs stimulated by the oil}}{\text{Mean number of take-offs stimulated by control}}$$

PHYTOCHEMICAL ANALYSIS

The essential oil extracted from *A. graveolens* seeds was analyzed for the presence of phytochemical components using standard procedures as illustrated by Harborne (19). The qualitative biochemical assays were performed to identify the presence of secondary metabolites; alkaloids, carbohydrates, flavonoids, phenolic compounds, phlobatannins, saponins, tannins, and terpenoids.

RESULTS

The potential of essential oil extracted from the seeds of celery plant, *A. graveolens* was evaluated as larvicidal and repellent agent against dengue vector, *Ae. aegypti*. The investigations validated the significant potential of essential oil as the probable agent for the control and management of rising *Ae. aegypti* population.

LARVICIDAL BIOASSAY

The 24 h exposure of early fourth instars of *Ae. aegypti* to the essential seed oil of *A. graveolens* resulted in quite low LC_{50} and LC_{90} values of 16.10 and 29.08 ppm, respectively (**Table 1**). The toxicity potential of the oil increased by 1.2-fold ($LC_{50} = 13.22$ ppm) on exposure of the larvae to the oil for another 24 h. The bioassay did not cause the formation of pupa or larval–pupal intermediates resulting in complete mortality of the larvae. The control treatments did not cause any mortality till 48 h (**Table 1**).

BEHAVIORAL STUDIES IN OIL-TREATED *AE. AEGYPTI* LARVAE

The larvae were scrutinized carefully during the treatment period for any behavioral modifications. The observations revealed that *A. graveolens* oil did not cause immediate or quick mortality. Initial exposure of the larvae to the essential oil did not affect the larvae and all larvae were found moving normally and exhibited a typical appearance. The restlessness in the larval behavior was noticed after 10 min of treatment. The principal lethal effects of the seed oil were observed after approximately 20–25 min of treatment in the form of incapability of rising to the water surface, body tremors, and convulsions. The symptoms of paralysis were clearly visible in 20% of the larvae after an hour leading to death of these larvae. Continued exposure of the larvae to oil caused mortality in approximately 50% larvae after 5–6 h, and the death of most of the larvae was observed within 10 h.

ADULT REPELLENCY BIOASSAY

Investigations conducted on the repellency potential of celery seed oil against adults *Ae. aegypti* revealed it as a promising and notable repellent. The oil resulted in 100% protection against bites by female *Ae. aegypti* in the first 150 min as compared to the ethanol that did not cause any repellency against mosquito bites (**Table 2**). When the experiment was continued for next 15 min, only one bite was recorded as compared to the nine bites scored on the control arm. It clearly reveals the reduced protection from 100 to 88.8% in the 165th-min. The percent protection to adult *Aedes* further decreased to 77% with two bites recorded after 180 min of exposure to the oil (**Table 2**). It is significant to note that direct application of the *A. graveolens* oil did not induce any dermal irritation during the experiment as well as afterward.

Table 1 | Larvicidal activity of the essential oil derived from *Apium graveolens* seeds against early fourth instars of *Aedes aegypti*.

	Larvicidal activity		Regression coefficient ± SE	χ^2 (df)	*p* Value
	LC_{50}	LC_{90}			
Exposure to 24 h	16.10 (11.93–22.08)	29.08 (21.40–64.61)	4.99 ± 1.36	0.79 (3)	0.8519
Exposure to 48 h	13.22 (9.80–18.70)	28.14 (19.64–60.78)	3.90 ± 0.87	1.27 (3)	0.7363

Table 2 | Percent repellency and percent protection to the bites of *Aedes aegypti* after application of the essential oil of *Apium graveolens* on the arms of human volunteers.

Time (min)	Mean no. of mosquito bites		% Repellency after oil application	% Protection after oil application
	Control	Celery seed oil		
15	3.0	0.0	100.0	100.0
30	5.67	0.0	100.0	100.0
45	3.33	0.0	100.0	100.0
60	3.0	0.0	100.0	100.0
75	7.67	0.0	100.0	100.0
90	8.33	0.0	100.0	100.0
105	7.33	0.0	100.0	100.0
120	5.67	0.0	96.0	82.4
135	6.33	0.0	100.0	100.0
150	7.67	0.0	100.0	100.0
165	9.00	1.0	96.0	88.8
180	8.67	2.0	92.0	76.9

CONTACT IRRITANCY ASSAY

A significant elicit response was observed in the adults of *Ae. aegypti* when subjected to contact irritancy assays. The exposure to 10% oil led to complete knockdown of adults when released for acclimatization in the funnel for 3 min. Conversely, exposure to 1% seed oil caused first flight of the female adult after a mean time of 4 s (**Table 3**). The average total of 63.66 take-offs were observed after 15 min of exposure to 1% oil as compared to only 2.36 flights when exposed to ethanol-impregnated paper resulting in the relative irritability of 26.97.

Table 3 | Response of 3-day-old adult females of *Aedes aegypti* in the contact irritancy assays to celery seed essential oil-impregnated papers.

Impregnated paper	Mean time lapse before first take-off (min)	Mean number of take-offs for females (in 15 min)	Relative irritability
Control	7.05 ± 0.72[a]	2.36 ± 0.72	1.0
10% oil	Knockdown	–	–
1% oil	0.04 ± 1.66	63.66 ± 0.66	26.97

[a] *Mean ± SEM.*

Table 4 | Phytochemical screening of the essential oil of the seeds of *Apium graveolens*.

S. No.	Plant constituents	Celery seed essential oil
1	Alkaloid	–
2	Carbohydrates	–
3	Saponins	–
4	Phenolic compounds	–
5	Tannins	–
6	Flavonoids	+
7	Terpenoids	+
8	Phlobatannins	–
9	Lactones	+

PHYTOCHEMICAL ANALYSIS

The qualitative biochemical analysis for the secondary metabolites present in the essential oil of *A. graveolens* seeds revealed the presence of terpenoids, lactones, and flavonoids as the main constituents. Other tested components were not detected in the essential seed oil of *A. graveolens* (**Table 4**).

DISCUSSION

Mosquito-borne diseases are increasing each year in tropical and sub-tropical countries. Since many decades, chemical insecticides have been used to combat the mosquito menace. However, the continued and frequent use of these insecticides has caused adverse effects, including toxicity to non-target organisms and humans, environment pollution, and increased development of resistance in the mosquito population. Botanicals have now become favorite agents among researchers as suitable alternatives to the toxic chemical insecticides. A few reports are available regarding the larvicidal and repellent potency of essential oils, volatiles extracted from different plants, against *Ae. aegypti* (20–22).

Keeping these in view, present studies were conducted to assess the probable role of celery seed oil as larvicidal, repellent, and irritancy agent for the control and management of *Ae. aegypti* population. Our investigations on early fourth instars of *Ae. aegypti* showed that 24 h of exposure to the oil resulted in an LC_{50} and LC_{90} value of 16.10 and 29.08 ppm, respectively ($p > 0.05$). It was also revealed that when the larvae were exposed to the oil for 48 h, the toxicity potential of the oil rose by 1.2-fold. However, keeping in view the large population of *Ae. aegypti* and enormous heterogeneity in their population; the chi-square distribution and insignificant p values obtained in our investigations suggest bioassays with more random selection of larvae and increased number of replicates to confirm the larvicidal potential of celery seed oil. Similar larvicidal activity of the ethanol-extracted *A. graveolens* was reported by Choochote et al. (23) against a Thailand strain of *Ae. aegypti,* the fourth instars exhibiting LD_{50} and LD_{95} values of 81.0 and 176.8 mg/L, respectively. The significant larvicidal activity of the volatile oils of *A. graveolens* has also been reported by Pitasawat et al. (24) against the two mosquito species, *Ae. aegypti* and *Anopheles stephensi* after an exposure to 24 h.

Several researchers have evaluated the larvicidal potential of various other essential oils against mosquitoes. The excellent larvicidal efficiency of the essential oil extracted from *Mentha piperita* revealing an LC_{50} and LC_{90} value of 111.9 and 295.18 ppm, respectively after 24 h of exposure has been reported against dengue vector (8). They also showed that the toxicity of the peppermint oil increased by 11.8% on exposure to the oil for 48 h. Similarly, Warikoo et al. (25) observed essential oil isolated from *Pinus longifolia* as the efficient larvicidal agent against *Ae. aegypti.* On exposure to commercially available pine oil, the early fourth instar larvae showed an LC_{50} value of 0.330 mg L^{-1} and an LC_{90} value of 1.118 mg L^{-1}. Recently, Liu et al. (10) established the

larvicidal potential of essential oil derived from the roots of *Toddalia asiatica* and the constituents isolated from the oil against *Ae. albopictus*. The essential oil extracted from the leaves of *Feronia limonia* showed remarkable larvicidal activity against *An. stephensi* with LC_{50} value of 15.03 ppm after 24 h, while against *Ae. aegypti* and *Cx. quinquefasciatus*, the LC_{50} values reported were 11.59 and 22.49 ppm, respectively (26). Lee (27) evaluated the larvicidal activity of essential oils derived from 11 aromatic medicinal plants against early fourth instars of *Ae. aegypti* and reported 100% mortality on exposure to all oils at 100 ppm. Cheng et al. (28) investigated the larvicidal potential of the essential oils from the leaves of eight provenances of indigenous cinnamon (*Cinnamomum osmophloeum* Kaneh.) and reported the excellent inhibitory effect of the essential oils of cinnamaldehyde type and cinnamaldehyde/cinnamyl acetate type against the IV instars of *Ae. aegypti*. Choochote et al. (23) have observed significant larvicidal activity of the volatile oils of *Curcuma aromatica* against the fourth instars of *Ae. aegypti* with an LC_{50} value of 36.30 ppm. Pushpanathan et al. (29) reported the LC_{50} values of 50.78 ppm when the essential oil extracted from *Zingiber officinalis* was tested against *Cx. quinquefasciatus*. Tiwary et al. (30) tested the essential oil of *Zanthoxylum armatum* against three species of mosquitoes and reported *Cx. quinquefasciatus* to be the most susceptible against oil with an LC_{50} value of 49 ppm followed by *Ae. aegypti* and *An. stephensi* with LC_{50} values in the range of 54–58 ppm.

Present study demonstrated the delayed toxicity of *A. graveolens* oil against early fourth instars of *Ae. aegypti* instead of quick larval mortality. Similarly, Choochote et al. (23) suggested the delayed larval killing effect of the ethanol-extracted celery seed oil *A. graveolens* against *Ae. aegypti*. The larvae treated with celery seed essential oil exhibited excitation and aggressive vertical and horizontal movements. These symptoms suggest the probable impact of oil on the neuro-muscular co-ordination in chemical synapses. These findings are in conformity with few earlier studies (17, 23, 31).

The exposure of the adults *Ae. aegypti* to celery seed oil established the promising and remarkable repellency potential. The oil provided 100% protection to human volunteers in the first 150 min followed by only one to two bites in the next 30 min of exposure. Likewise, the crude seed extract of celery has been reported to exhibit repellent activity against *Ae. aegypti* with ED_{50} and ED_{95} values of 2.03 and 28.12 mg/cm^2, respectively, providing the biting protection time of 3 h on application at a concentration of 25 g% (23). The mosquito repellent potential of celery, *A. graveolens*, has also been compared with commercial repellents by Tuetun et al. (32). Kumar et al. (8) reported the repellent properties of essential oil extracted from *M. piperita* against adults *Ae. aegypti* with 100% protection till 150 min after which only one to two bites were recorded during the next 30 min, as compared to eight to nine bites on the control arm. The skin repellent tests performed at 1.0, 2.0, 3.0, and 4.0 mg/cm^2 with essential oil extracted from *Z. officinalis* gave 100% protection against *Cx. quinquefasciatus* up to 120 min (29). The repellent activities of the essential oil of *Cinnamomum zeylanicum*, *Z. officinale*, and *Rosmarinus officinalis* have been also reported by Prajapati et al. (33) against *An. stephensi, Ae. aegypti,* and *Cx. quinquefasciatus*.

In the present investigations, the contact irritancy assays with the celery seed oil resulted in a significant elicit response in the adults of *Ae. aegypti*. Exposure to 1% oil led to average number of 63.66 take-offs in 15 min with first flight after a mean time of 4 s. The behavioral modifications in the mosquitoes through chemical actions in contact irritancy may diminish population that may ultimately reduce disease transmission (34). Exposure of the female adults of *Ae. aegypti* to the crude leaf extracts of *Parthenium hysterophorus* showed a similar significant repellency behavior (9). Nour et al. (35) suggested the utilization of 0.1% essential oils of *Ocimum basilicum* as promising natural repellents against *Anopheles* mosquito.

Studies have shown that secondary metabolites; steroids, alkaloids, terpenoids, saponins, phenolics, essential oil, etc., of plants play a major role in the mosquito control being associated with a wide range of bioefficacy. The phytochemical analysis of the essential oil of *A. graveolens* seeds showed the presence of terpenoids, lactones, and flavonoids as the main constituents. Fazal and Singla (36) have reported D-limonene (80%) as the prime constituent in celery seed oils. They have also reported selinene, various sesquiterpene alcohols, b-elemne, linalool, *N*-butylphthalide, sedanenolide, and sedanonic anhydride as the other secondary metabolites in celery seed oil. However, the larvicidal and repellency potential of the essential celery seed oil reported in the present study need to be further investigated for identification of the compound responsible. It is further suggested that the efficacy observed may be because of the individual or the synergistic effects of various compounds present in them, identified or unidentified.

The potential role of *A. graveolens* seed essential oil as larvicide and repellent has been investigated against *Ae. aegypti*. Nevertheless, this is an explorative evaluation and the studies have suggested the potential bioefficacy of celery seed oil. However, keeping in view the heterogeneous and large population of *Ae. aegypti*, further complex investigations with more random selection of larvae and increased number of replicates are needed to ascertain the efficacy of celery seed oil against *Ae. aegypti*. Moreover, the identification of bioactive components present in the oil and understanding their mode of action is essential for its use as mosquito control agent. Field trials are recommended before the use of *A. graveolens* seed oil as an anti-mosquito natural, environment-friendly product in the mosquito-management program.

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New records of mosquitoes (Diptera: Culicidae) from Bolívar State in South Eastern Venezuela, with 27 new species for the state and 5 of them new in the country

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This is the first part of a series of studies related to mosquito ecological and biogeographic aspects. A total of 69 mosquito species (Diptera: Culicidae) was collected in 16 localities sampled in the Gran Sabana Municipality, Canaima National Park, and Venezuela. Twenty-seven mosquito species are recorded for the first time from Bolívar State, Venezuela. Five of them species are reported for the first time in Venezuela: *Anopheles malefactor* Dyar and Knab (1907); *Chagasia bonneae* Root (1927); *Chagasia ablusa* Harbach (2009); *Culex anduzei* Lane (1944), and *Uranotaenia leucoptera* Theobald (1907). Their medical importance is commented, and ecological and epidemiological aspects are discussed. A checklist of the mosquito species reported in the Gran Sabana County is given.

Keywords: mosquitoes, vectors, inventory, emerging diseases, Bolívar State, Gran Sabana, Venezuela

INTRODUCTION

The occupation of Amazonia in Brazil and Venezuela, has formed part of the integration processes that both governments are implementing in their common border, which makes evident that both governments are aware of the importance that the Amazonia has in our present world (1). Diseases such as malaria, dengue, Chikungunya, Yellow Fever, Mayaro virus, West Nile virus, and several emerging and reemerging arboviruses, which are responsible for millions of cases of sickness and death among people living in the tropical regions, continue to be of great concern to the World Health Organization authorities in our present world (2). Dengue is an important arbovirus that affects humans; it is transmitted by *Aedes aegypti* (Linnaeus) and *Aedes albopictus* Skuse. *A. aegypti* and *A. albopictus* species have been involved in Dengue transmission in the Manaus rural areas, Brazilian Amazon (3). *A. albopictus* a secondary dengue vector in Asia, has spread to America and Europe largely, due to the international trade of used tires (a typical larval habitat), timber, and other goods such as "lucky bamboo" (a decorative house plant that is marketed worldwide). This species has a wide geographical distribution; it is particularly resistant, and can survive in both rural and urban environments. Mosquito's eggs are highly resistant and can remain viable throughout dry season, and can survive in cold temperature regions of Europe (3). *A. albopictus* and *A. terrens* (Walker) species are two potential Yellow Fever vectors in jungle. These species utilize a wide variety of natural larval microhabitats, such as tree-holes, bamboo internodes, and artificial containers and may be found in the same natural environments as *Haemagogus* species (1). *Haemagogus* species have been involved in Sylvain yellow fever transmission in Venezuela (4). Human malaria, is one of the most serious parasitic diseases in tropical ecosystems, it is caused by parasites of the genus *Plasmodium* (Apicomplexa: Plasmodidae) and transmitted among human hosts by bites of the infected *Anopheles* female (1). Five parasite species cause malaria in humans. *Plasmodium falciparum* and *P. vivax* are the two most common. *P. falciparum* is the most dangerous, with the highest rates of mortality (1).

In 2012, Venezuela reported the highest recorded incidence of malaria in its history with 51,264 cases. In Bolívar State, incidence cases increased to 44,180 (86.2% of the country); with three counties (Sifontes, Gran Sabana, and Cedeño) in "epidemic" and two counties (Piar and Sucre) in "alarm" status (5). *Anopheles darlingi* Root has been considered as human malaria's principal vector in South America. In Amazonas and Bolívar states, it is responsible for 90% of malaria cases reported in Venezuela (1, 6).

Mosquito borne diseases such as malaria, dengue, Venezuelan Equine Encephalitis (VEE), West Nile virus and others equine encephalitis, Mayaro, or Chikungunya are zoonoses with increasing incidence in the current decade in tropical and temperate countries. These diseases emerge as a consequence of changes made to terrains that often increase the natural and artificial mosquito larval habitats. Many of these mosquito species are of public health importance. Mosquito's population increases result in a risk increased of tropical diseases transmission (1). There are many factors that can accelerate the emergence of zoonoses, such as environmental changes, habitat modifications, variations of human and animal demography, deterioration of strategies of vector control, or changes in pathogen genetics (1). Efforts to control such species that transmit emerging diseases have primarily been concentrated on the use of synthetic insecticides. Unfortunately, this has resulted in the appearance of physiological mosquito resistance, toxicity problems to human, environmental contamination, ecological imbalance, and economic burden (1). Such problems have created the need to look for alternative, environmentally friendly control mechanisms, based on those found in nature.

These include the essential oils from plants, some of which have been used by people for medicinal purposes; also biological control (e.g., *Bacillus sphaericus* against *Anopheles*) and biochemical control with synthetics juvenile hormones (7, 8).

It is well known that the rate of species lost worldwide surpasses that by which taxonomic knowledge is increased (9). Thus, it is necessary to intensify studies focusing on diversity and from areas with an acceptable level of conservation (e.g., Canaima National Park, Venezuela). The Gran Sabana Region (Canaima National Park) is an undulating plain grass-dominated upland savanna covering close to 18,000 km^2, with altitudes ranging from 750 to 1,450 m (10). Most of the Gran Sabana uplands have a humid submontane climate, with average annual temperatures ranging between 18 and 24°C, average annual rainfall between 2,000 and 3,000 mm, and a short dry season occurring from December to March. This area is drained by tributaries of the Orinoco River (Venezuelan part of the igneous metamorphic Guyana Shield), most of them black-water Rivers, with very acidic and low mineral waters (10). The ecological studies in the Gran Sabana are relatively scarce, especially for short and long-term evaluations on possible changes induced by human activities (e.g., climatic change). Additionally, ecological studies on richness and distribution at regional levels are few. It is well known that Culicidae larvae are dependent on habitat characteristics and that they are sensitive to biotic factors, as predators and also to several abiotic factors (pH, temperature, dissolved oxygen, salinity, and conductivity).

The present article, refers to the finding of 69 species and 17 genera of mosquito (Diptera: Culicidae), collected in the Gran Sabana county, Bolívar State, Venezuela. Twenty-seven mosquito species are recorded for the first time from Bolívar State, and five species are reported for the first time in the country.

MATERIALS AND METHODS

STUDY AREA

The study area is located in the southern of Gran Sabana Municipality, Canaima National Park, a natural protected area in southeastern Venezuela, Amazonian Region that borders Brazil and Guyana (**Figures 1–3**). This study is based on material collected in indigenous territory (Pemón ethnic group) of Gran Sabana, Canaima National Park, located in Bolívar State, and roughly occupies the same area as the Gran Sabana region (11). The park was established on 12 June 1962. It is the second largest park in the country, after Parima-Tapirapecó in Amazonas State, and sixth biggest national park in the world (1). About 65% of the park is occupied by plateaus of rock called tepuis, which are a kind of plateau of 1.7–1.8 billion years old. The oldest Roraima sandstone is estimated to be 1.6 billion years old. These ancient mountains of the Guiana Shield (**Figure 1**) in the Amazonian Region (12) constitute a unique biological environment and are of great geological interest, which makes them one of the oldest formations in the world (11).

FIGURE 1 | Geographic situation of the Guiana Highlands in Venezuela.

FIGURE 2 | Relative situations of the Bolivar State in Venezuela.

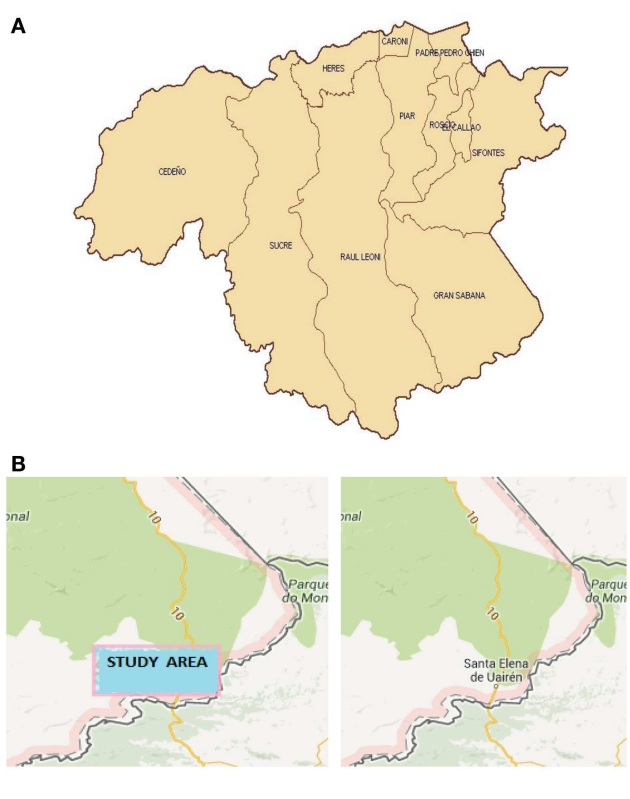

FIGURE 3 | (A) Relative situation of the municipalities in the Bolivar State; and **(B)** situations of the study area and Santa Elena of Uairén.

The park is home to indigenous Pemón Indians (Pemón ethnic group), part of the Carib linguistic group (11). Pemón Indians have an intimate relationship with the tepuis, and believe they are the home of the "Mawari" spirits. Most transport within the park is done by light plane, from airstrips built by various Capuchin missions, or by foot and canoe. Pemón indigenous have developed some basic and luxurious camps, which are mainly visited by tourists from around the world and Venezuelan tourists. In 1994, Canaima National Park was named a World Heritage Site by UNESCO, as a natural reserve that has abrupt relief special and unique around the world, "The Tepuis," The park includes the entire watershed of the right bank of the Caroní River, and two of the highest waterfalls in the world, the Angel Falls and the Kukenán Falls and plenty of waterfalls of lower altitude (11).

Field surveys of mosquito adults (human bait) and mosquito larvae sampling, were carried out in the Gran Sabana County. The annual mean temperature is 22°C (18–24°C); the total annual rainfall is 1,500–5,700 mm, with annual average rainfall between 2,000 and 3,000 mm, with altitudes ranging from 750 to 1,450 m and annual mean of 205 days with rain per year, and a very short dry season occurring from December to March (10). The estimated population is 39,000, mainly concentrated in the capital, Santa Elena of Uairén (**Figures 2** and **3**).

Sixteen localities and four rivers were sampled for mosquitoes in the Gran Sabana County near the common border Brazil–Venezuela, between August 2011 and November 2013. These sites were selected as representative areas of the Gran Sabana, Venezuelan Canaima National Park. All localities are at altitudes >600 m. These altitudes are variables and can range from 600 to 1,450 m. These localities and rivers are: Santa Elena de Uairén, Waramasén, Manak-Krú, Maurak, Colinas de la Laguna, Altamira, San Antonio, Kinok-Pon-Parú, La Primavera, Chiricayén, Chiririka, Uaiparú, Betania, Kamaiwa, El Paraíso, El Paují, Chiririka, Uairén, Uaiparú, and Kukenán rivers.

COLLECTION AND IDENTIFICATION OF MOSQUITOES

A sampling program of mosquito larvae was carried out between August 2011 and November 2013 by visiting the study area periodically (dry and rainy season every year). Mosquito samples were taken with a standard dipper. The natural aquatic habitats (not-Phytotelmata) were *a priori* classified into four categories: lagoons, streams, rivers, and freshwater herbaceous swamps. At each breeding site, 30 dips for mosquito larvae samples were made. Most of the mosquito collections were made in tropical humid forests and edges of streams, rivers, and herbaceous swamps (morichales) of savanna areas in Gran Sabana County. Additionally, mosquito larvae were collected in artificial and natural containers. In Phytotelmata, larvae were collected by extracting water with a plastic pipettes from tree-holes, cut bamboo internodes, leaf axils of bromeliads, foliar axils of Araceae, fallen leaves of Musaceae, fallen palm spates, especially the "Moriches" palms (*Mauritia flexuosa*), and from floral bracts of Heliconiaceae. Immature mosquitoes were collected from 40 samples (water-holding structures) per plant species per locality. Collected immature specimens (IV instars larvae) from half the samples (20 samples) were preserved in ethanol (90%) for identification purposes. Specimens (larvae and pupae) from the rest of samples (50%) were pooled, transported, and reared in the field laboratory to obtain the associated specimens. The species presents, were recognized on the basis of correlated anatomical features in associated life stages. Sampling to adult collections on human landing catches (**Figures 4A,B**) were carried out in the same localities and rivers. The taxonomic determination of the specimens was based on direct observation of morphological characters, through a stereoscopic microscope (adults) and transmitted light microscope (larvae), using several taxonomic keys and descriptions and re-descriptions of species. The abbreviations employed for mosquito genera and subgenera are those proposed by Reinert (13).

All diagnostic and differential characters were confirmed by using several taxonomic keys and using descriptions and re-descriptions of species (4, 14–28, 29). All immature and adults specimens are deposited at the Collection of Center for Endemic Disease studies, located in Las Delicias, Maracay, Venezuela.

RESULTS

A list of mosquito taxa of the Gran Sabana Municipality is presented. A total of 69 mosquito species (Diptera: Culicidae) and 17 mosquito genera from the indigenous territory (Pemón ethnic group) are reported (**Table 1**). A total of 19 species of anophelines were collected; 17 of them belonging to genus *Anopheles*

FIGURE 4 | (A) Adult collections on human landing catch during the day. **(B)** Adult collections on human landing catch in streams edges during the nights.

Table 1 | Inventory of Culicidae from Gran Sabana Municipality, Venezuela.

Culicidae: 1. Subfamily Anophelinae

1.1 *Anopheles* (Human Malaria vectors)
Anopheles (Nyssorhynchus) triannulatus Neiva and Pinto (1922)
Anopheles (Nyssorhynchus) brasiliensis Chagas (1907)
Anopheles (Nyssorhynchus) marajoara Galvao and Damasceno (1942)
Anopheles (Nyssorhynchus) nuneztovari Gabaldon (1940)
Anopheles (Nyssorhynchus) argyritarsis Robineau-Desvoidy (1827)
Anopheles (Nyssorhynchus) darlingi Root (1926)
Anopheles (Nyssorhynchus) oswaldoi Peryass (1922)
Anopheles (Nyssorhynchus) strodei Root (1926)
Anopheles (Nyssorhynchus) rangeli Cova-García and López (1940)
Anopheles (Lophopodomyia) squamifemur Antunes (1937)
Anopheles (Anopheles) matogrosensis Lutz and Neiva (1911)
Anopheles (Anopheles) peryassui Dyar and Knab (1908)
Anopheles (Anopheles) punctimacula Dyar and Knab (1906)
Anopheles (Anopheles) eiseni Coquillet (1902)
Anopheles (Anopheles) malefactor Dyar and Knab (1907)[b]
Anopheles (Stethomyia) nimbus Theobald (1902)[a]
Anopheles (Kertezsia) cruzii Dyar and Knab (1908)[a]

1.2. *Chagasia*
Chagasia bonneae Root (1927)[b]
Chagasia ablusa Harbach (2009)[b]

Culicidae: 2. Subfamily Culicinae

2.1. *Culex* (VEE vectors and West Nile virus vectors)
Culex (Phenacomyia) corniger Theobald (1903)
Culex (Culex) quinquefasciatus Say (1823)
Culex (Culex) brevispinosus Bonne-Wester and Bonne (1920)
Culex (Culex) coronator Dyar and Knab (1906)
Culex (Culex) nigripalpus Theobald (1901)
Culex (Culex) pinarocampa Dyar and Knab (1908)[a]
Culex (Melanoconion) dunni Dyar (1918) (VEE vector)[a]
Culex (Melanoconion) educator Dyar and Knab (1906)
Culex (Melanoconion) spissipes Theobald (1903) (VEE vector)
Culex (Melanoconion) mistura Komp and Rozeboom (1951)
Culex (Carrollia) urichii Coquillet (1906)
Culex (Carrollia) anduzei Lane (1944)[b]
Culex (Lutzia) bigoti Bellardi (1862) (predators)[a]

2.2. *Aedes* (Dengue, Mayaro, Chikungunya, VEE, and Yellow Fever vectors)
Aedes (Stegomyia) aegypti Linnaeus (1762) (Dengue, Chikungunya, Mayaro, and Yellow Fever vector)
Aedes (Finlaya) terrens Walker (1856) (potential Yellow Fever vector)
Aedes (Ochlerotatus) scapularis Rondani (1848) (EEV vector)
Aedes (Ochlerotatus) serratus Theobald (1901) (EEV vector)
Aedes (Ochlerotatus) fulvus Wiedemann (1828) (EEV vector)
Aedes (Ochlerotatus) angustivittatus Dyar and Knab (1907) (EEV vector)[a]

2.3. *Psorophora* (VEE vectors)
Psorophora (Janthinosoma) cyanescens Coquillet (1902)
Psorophora (Janthinosoma) albipes Theobald (1907)
Psorophora (Janthinosoma) ferox Von Humboldt (1819)
Psorophora (Psorophora) ciliata Fabricius (1794)[a]
Psorophora (Psorophora) lineata Von Humboldt (1819)
Psorophora (Grabhamia) cingulata Fabricius (1805)

2.4. *Mansonia* (VEE vectors)
Mansonia (Mansonia) pseudotitillans Theobald (1901)[a]
Mansonia (Mansonia) titillans Walker (1848)[a]

2.5. *Coquilletidia* (VEE vectors and West Nile virus vectors)
Coquilletidia (Rhynchotaenia) juxtamansonia Chagas (1907)[a]
Coquilletidia (Rhynchotaenia) venezuelensis Theobald (1912)[a]
Coquilletidia (Rhynchotaenia) nigricans Coquillet (1904)[a]

2.6. *Haemagogus* (Mayaro and Sylvain Yellow Fever vectors)
Haemagogus anastasionis Dyar (1921)[a]
Haemagogus janthinomys Dyar (1921)[a]
Haemagogus celeste Dyar and Núñez-Tovar (1927)

2.7. *Uranotaenia* (Avían Malaria vectors)
Uranotaenia (Uranotaenia) typhlosomata Dyar and Knab (1907)[a]
Uranotaenia (Uranotaenia) calosomata Dyar and Knab (1907)[a]
Uranotaenia (Uranotaenia) geometrica Theobald (1901)
Uranotaenia (Uranotaenia) pulcherrima Arribalzaga (1891)
Uranotaenia (Uranotaenia) nataliae Arribalzaga (1891)[a]
Uranotaenia (*Uranotaenia) leucoptera* Theobald (1907)[b]
Uranotaenia (Uranotaenia) lowii Theobald (1901)[a]

2.8. *Aedeomyia* (Avían Malaria vectors)
Aedeomyia (Aedeomyia) squamipennis Arribalzaga (1878)

Culicidae: Subfamily Culicinae: 3. Tribe: Sabethini

3.1. *Sabethes* (Mayaro and Sylvain Yellow Fever vectors)
Sabethes purpureus Theobald (1907)

3. 2. *Limatus* (potential arbovirus vectors)
Limatus asulleptus Theobald (1903)[a]
Limatus durhami Theobald (1901)

3.3. *Wyeomyia* (potential arbovirus vectors)
Wyeomyia (Wyeomyia) celaenocephala Dyar and Knab (1906)[a]

3.4. *Runchomyia* (facultative predators)
Runchomyia (Ctenogoeldia) frontosa Theobald (1903)

3.5. *Johnbelkinia* (facultative predators, arbovirus vectors)
Johnbelkinia ulopus Dyar and Knab (1906)[a]

3.6. *Trichoprosopon* (potential arbovirus vectors)
Trichoprosopon digitatum Rondani (1848)

Culicidae: Toxorhynchitinae (agents biological control: predators)

4.1. *Toxorhynchites* (predators)
Toxorhynchites (Lynchiella) theobaldi Dyar and Knab (1906)
Toxorhynchites (Lynchiella) haemorroidalis Fabricius (1787)[a]

[a] *New for the State,*
[b] *New for the country.*

Meigen and 2 species belonging to genus *Chagasia* Cruz. Additionally, 50 species of culicines were collected, belonging to 14 genera of *Culicinae* and one genus (*Toxorhynchites*) of *Toxorhynchitinae* (**Table 1**).

Special attention should be placed in the new species records for Bolívar State and for Venezuela. A total of 27 mosquito species are recorded for the first time in Bolívar State. Five of them are new for the country (**Tables 1** and **2**) and they namely: *Anopheles (Anopheles) malefactor* Dyar and Knab (1907); *Chagasia bonneae*

Table 2 | (A) GPS collection coordinates in localities with new records; (B) GPS collection coordinates in localities with new records.

Localities and coordinates	Species records
(A)	
SANTA ELENA	
4°36′07″ 61°06′34″	*Aedes angustivittatus, Culex anduzei*
4°32′53″ 61°08′30″	*Uranotaenia nataliae, Uranotaenia lowii*
4°36′41″ 61°06′22″	*Uranotaenia calosomata, Uranotaenia typhlosomata*
4°35′49″ 61°06′59″	*Haemagogus janthinomys, Sabethes purpureus*
4°36′01″ 61°06′52″	*Coquilletidia (Rhynchotaenia) venezuelensis*
WARAMASEN	
4°33′26″ 61°16′59″	*Limatus asulleptus*
4°34′17″ 61°14′45″	*Johnbelkinia ulopus*
4°33′25″ 61°16′58″	*Wyeomyia (Wyeomyia) celaenocephala*
4°33′36″ 61°16′29″	*Anopheles (Anopheles) malefactor*
4°33′39″ 61°16′29″	*Uranotaenia (Uranotaenia) leucoptera*
4°33′38″ 61°16′28″	*Chagasia bonneae and Chagasia ablusa*
4°33′43″ 61°16′32″	*Culex (Melanoconion) dunni, Culex (Lutzia) bigoti*
EL PAUJÍ	
4°28′32″ 61°35′34″	*Anopheles (Kertezsia) cruzii*
4°31′52″ 61°37′26″	*Wyeomyia (Wyeomyia) celaenocephala*
EL PARAÍSO	
4°26′53″ 61°41′65″	*Anopheles (Kertezsia) cruzii*
4°26′53″ 61°41′65″	*Wyeomyia (Wyeomyia) celaenocephala*
KINOK-PON	
4°33′31″ 61°12′47″	*Uranotaenia (Uranotaenia) leucoptera*
4°33′37″ 61°12′42″	*Uranotaenia (Uranotaenia) nataliae*
WAIPARU	
4°31′52″ 61°37′26″	*Anopheles (Nyssorhynchus) darlingi*
MANAKRU	
4°36′39″ 61°07′20″	*Toxorhynchites (Lynchiella) haemorroidalis*
4°36′24″ 61°07′11″	*Toxorhynchites (Lynchiella) theobaldi*
4°36′28″ 61°07′10″	*Coquilletidia (Rhynchotaenia) juxtamansonia*
(B)	
CHIRICAYEN	
4°39′39″ 61°20′30″	*Chagasia bonneae and Chagasia ablusa*
4°40′10″ 61°20′36″	
4°42′11″ 61°19′48″	
4°43′03″ 61°19′18″	
MAURAK	
4°33′46″ 61°10′46″	*Toxorhynchites (Lynchiella) haemorroidalis*
4°33′55″ 61°12′37″	*Coquilletidia (Rhynchotaenia) venezuelensis*
4°35′11″ 61°10′50″	*Chagasia bonneae and Chagasia ablusa*
BETANIA	
4°39′33″ 61°22′59″	*Mansonia (Mansonia) titillans, Culex bigoti*
4°39′29″ 61°23′11″	*Psorophora (Psorophora) ciliata*
4°39′30″ 61°22′52″	*Mansonia (Mansonia) pseudotitillans*
4°39′25″ 61°22′47″	*Coquilletidia (Rhynchotaenia) juxtamansonia*
4°39′57″ 61°23′22″	*Coquilletidia (Rhynchotaenia) nigricans*
SAN ANTONIO	
4°31′14″ 61°07′14″	*Psorophora (Psorophora) ciliata*
4°31′12″ 61°07′12″	*Coquilletidia (Rhynchotaenia) juxtamansonia*
4°31′13″ 61°07′08″	*Mansonia (Mansonia) pseudotitillans*
4°31′15″ 61°07′04″	*Mansonia (Mansonia) titillans*
4°31′17″ 61°06′56″	*Coquilletidia (Rhynchotaenia) nigricans*
4°31′16″ 61°06′54″	*Coquilletidia (Rhynchotaenia) venezuelensis*
CHIRIRICA	
4°34′36″ 61°06′34″	*Culex (Carrollia) anduzei, Wyeomyia celaenocephala*
4°34′36″ 61°06′58″	*Haemagogus anastasionis, Culex (Lutzia) bigoti*
4°34′49″ 61°06′59″	*Haemagogus janthinomys*
4°34′49″ 61°11′43″	*Culex (Melanoconion) dunni*

(Continued)

Root (1927); *Chagasia ablusa* Harbach (2009); *Culex* (*Carrollia*) *anduzei* Lane (1944), and *Uranotaenia leucoptera* Theobald (1907).

The twenty-seven mosquito species records are namely:

1. *Anopheles cruzii* Dyar and Knab (1908).
2. *Anopheles malefactor* Dyar and Knab (1907).
3. *Anopheles nimbus* Theobald (1902).
4. *Chagasia bonneae* Root (1927).
5. *Chagasia ablusa* Harbach (2009).
6. *Culex pinarocampa* Dyar and Knab (1908).
7. *Culex anduzei* Lane (1944).
8. *Culex bigoti* Bellardi (1862).
9. *Culex dunni* Dyar (1918).
10. *Aedes angustivittatus* Dyar and Knab (1907).
11. *Mansonia titillans* Walker (1848).
12. *Mansonia pseudotitillans* Theobald (1901).
13. *Coquilletidia juxtamansonia* Chagas (1907).
14. *Coquilletidia nigricans* Coquillet (1904).
15. *Coquilletidia venezuelensis* Theobald (1912).
16. *Uranotaenia typhlosomata* Dyar and Knab (1907).
17. *Uranotaenia calosomata* Dyar and Knab (1907).
18. *Uranotaenia nataliae* Arribalzaga (1891).
19. *Uranotaenia leucoptera* Theobald (1907).
20. *Uranotaenia lowii* Theobald (1901).
21. *Psorophora ciliata* Fabricius (1794).
22. *Haemagogus anastasionis* Dyar (1921)
23. *Haemagogus janthinomys* Dyar (1921)
24. *Limatus asulleptus* Theobald (1903).
25. *Wyeomyia celaenocephala* Dyar and Knab (1906).
26. *Johnbelkinia ulopus* Dyar and Knab (1906).
27. *Toxorhynchites haemorroidalis haemorroidalis* Fabricius (1787).

DISCUSSION

The last revision of Anophelini Tribe (Diptera: Culicidae) in Venezuela (30) reported the total of 42 species, belonging to 2 genera: *Chagasia* (1 species) and *Anopheles* (41 species). In the present study, additional records are presented. The Anophelini species: *A. malefactor*, *Chagasia bonneae*, and *Chagasia ablusa* are three new records for Venezuela (**Tables 1** and **2**). These species are not potential malaria vectors. Adults and immature specimens were identified, according to keys proposed by Wilkerson (26)

and Harbach and Howard (20). Larval specimens of the three species were collected in Waramasén (**Figure 5**) and reared to obtain associated specimens; larval habitats of the three species are streams edges, especially sites with algae and partial shade (**Figure 5**). The same larval habitats for *A. malefactor* and *Anopheles punctimacula* Dyar and Knab (1906) were found by Wilkerson (26). The geographical distribution of *A. malefactor* was restricted to Panamá and northwestern Colombia (26). With this new record (*A. malefactor*) their geographical distribution in Central and South America now includes Panamá, Colombia, and Venezuela. Literature records indicate that *A. punctimacula* was found naturally infected with malaria parasites in Panamá and Colombia (26). However, *A. malefactor* is not a potential malaria vector in South America (26).

The species *Anopheles (Kertezsia) cruzii* Dyar and Knab (1908) and *Anopheles (Stethomyia) nimbus* Theobald (1902) are reported for the first time in Bolívar State (**Table 1**). Larvae of *A. nimbus* were collected in river edges of Chiririka River (**Figure 6**) especially in habitats with algae and partial shade. This species does not transmit diseases of medical importance to man. Adults were not collected. Larvae were reared to obtain the associated specimens. *A. nimbus* was identified, according to key proposed by Navarro (22). Females of *A. cruzii* are very aggressive crepuscular biters and they are potential malaria vectors (31); their larvae live in water of leaf axils of bromeliads (31). Adult females were captured on human landing catches, in the forest in El Paraíso and El Paují, between 16:30 and 18:45 h. Immature specimens were not collected. Diagnostic and differential characters of *A. cruzii* were confirmed in adults specimens, using descriptions of Wilkerson and Peyton in basis to 11 females collected in Iguape, Brazil (28). *Anopheles darlingi* Root (Major malaria vector in the southern Bolívar State) is reported for the first time in the Gran Sabana Municipality; also reports for the first time in South America, the two highest mosquito records for *A. darlingi.* These altitude records are: Colinas de la Laguna, with 870 MSL and Santa Elena, with 893 MSL. Larvae of *A. darlingi* were only collected in shaded pools in the forest. When the water level dropped during the dry season, pools formed in or near the river bed (Uaiparú and Uairén rivers). These localities with *A. darlingi* larval habitats are at altitudes >600 m. These altitudes can range from 600 to 1,200 m. The lower altitude record for *A. darlingi* in the Gran Sabana was found in Uaiparú River, with 628 m, and the highest altitude record was found in Santa Elena, near of the Uairén River (**Figures 6** and **7**) with 893 m. The same larval habitats of *A. darlingi* were found in Suriname by Rozendaal (32).

In this article, we also report for the first time in Venezuela, the presence of *Culex (Carrollia) anduzei.* With this new record, their geographical distribution in South America now includes Brazil and Venezuela. Immature specimens were identified, according to keys proposed by Valencia (25). *C. anduzei* females do not transmit diseases of medical importance to man (25). Larvae were collected in plastic and metallic artificial containers in Chiririka (**Figure 6**), El Paraíso, El Paují, and Santa Elena (**Figure 7**). Adult specimens of *C. anduzei* were not captured. Additionally, we report for the

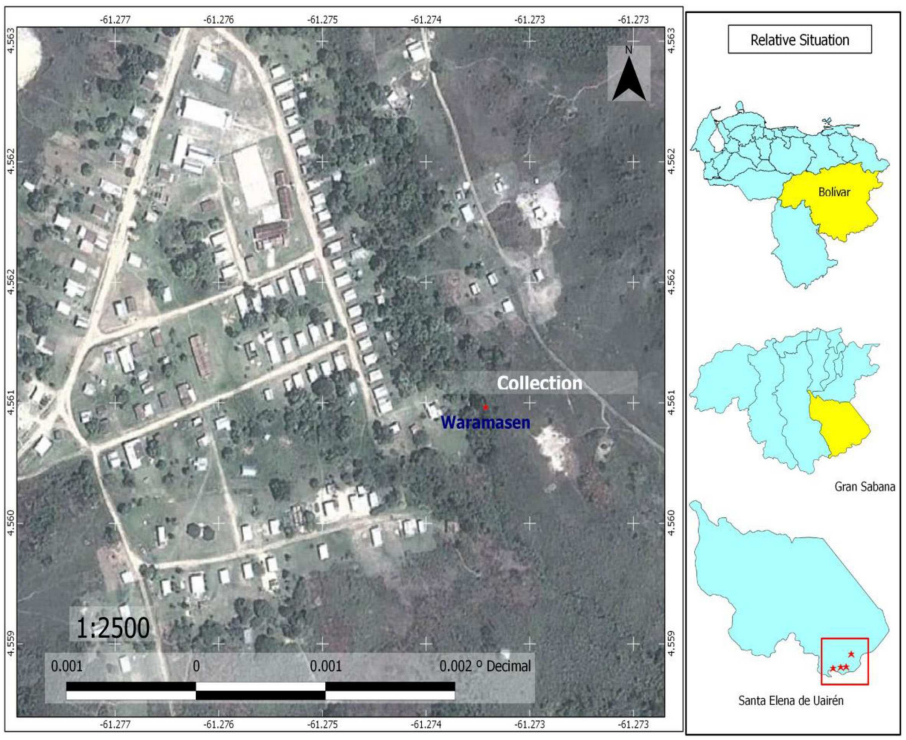

FIGURE 5 | Map of Waramasén, showing sites where larvae of *Culex bigoti, Chagasia bonneae, Chagasia ablusa, Uranotaenia leucoptera,* and *Anopheles malefactor* were collected for the first time; and showing sites where adults of *Johnbelkinia ulopus, Limatus asulleptus, Wyeomyia celaenocephala,* and *Culex dunni* were captured for the first time.

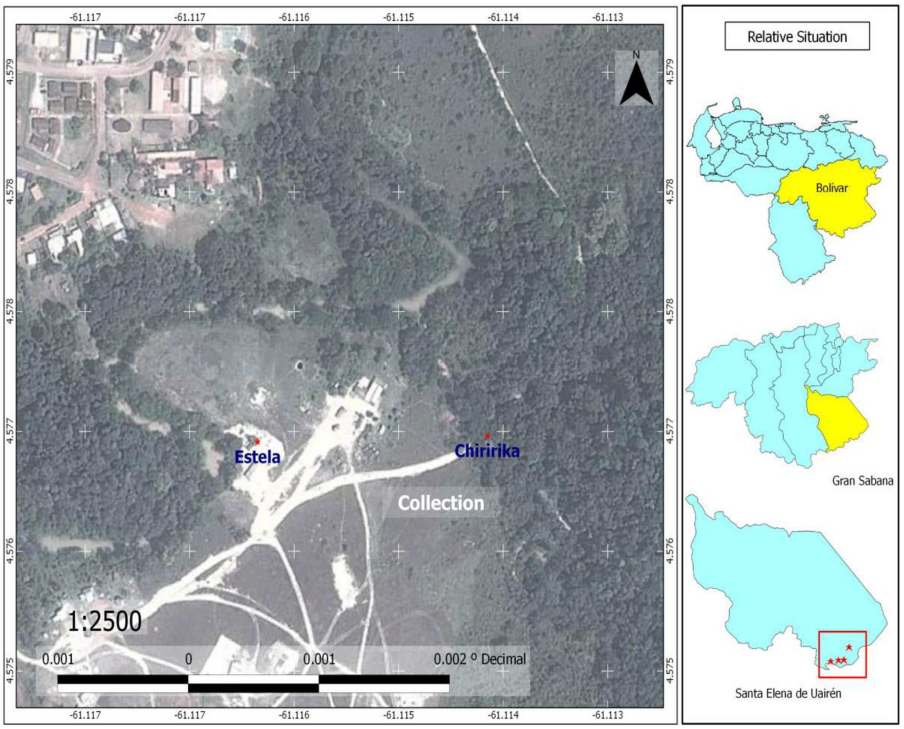

FIGURE 6 | Map of Chiririka, showing sites where adults of *Haemagogus anastasionis, Sabethes purpureus,* and *Wyeomyia celaenocephala* were captured for the first time; and showing sites where larvae of *Anopheles nimbus* (Chiririka River) and *Culex anduzei* (the Estela house) were collected for the first time in Bolívar State.

first time the presence of *Culex* (*Lutzia*) *bigoti* Bellardi in Bolívar State. This species does not transmit diseases of medical importance to man. Larval specimens were collected in Waramasén and were found in artificial containers (tires, and plastic and metallic containers). It is well known that *Cx. bigoti* larvae are predators in several artificial containers (tires, tin cans, old paint cans, plastic and metallic containers). In Paraná State, Brazil, larval specimens of *C. bigoti* were found in tires, cans, and cut bamboo (33). In Venezuela, this species was collected in artificial containers (tires and plastic containers) but, was not collected in epiphytic and terrestrial bromeliads (12, 34). *Toxorhynchites* (*Lynchiella*) *theobaldi* Dyar and Knab (1906) and *Toxorhynchites* (*Lynchiella*) *haemorroidalis* Fabricius (1787) were collected in tires and tree-holes; they are a potential predators used for mosquito control. A long-term investigation in Florida (USA), demonstrated a reduction in tree-holes, mosquitoes, attributable to predation by *Toxorhynchites rutilus* (35).

Additionally, *Culex dunni* Dyar (1918); *Aedes* (*Ochlerotatus*) *angustivittatus* Dyar and Knab (1907); and *Psorophora* (*Psorophora*) *ciliata* Fabricius (1794) are reported for the first time in Bolívar State. These three species are potential vectors of the VEE (36–38). In addition, we reports three new species of the genus *Coquilletidia* and two new species of *Mansonia* for Bolívar State; they are namely: *Mansonia titillans* Walker (1848), *Mansonia pseudotitillans* Theobald (1901), *Coquilletidia juxtamansonia* Chagas (1907), *Coquilletidia venezuelensis* Theobald (1912), and *Coquilletidia nigricans* Coquillet (1904). The two species of the genus *Mansonia* are potential vectors of the epizootic cycle of the VEE (36) and *Coquilletidia venezuelensis* is a potential vector of enzootic cycle of the West Nile virus in Venezuela (39).

The genus *Haemagogus* Williston includes mosquitoes with diurnal activity and immature habitats on Phytotelmata (tree-holes and cut bamboo internodes). Adult females bite in forests during the day (40). *Haemagogus* species have been involved in yellow fever transmission, a virus circulating in forest areas in Latin America among arboreal primates and marsupials by means of mosquito bite (40). The genus comprises 28 species; 9 of them are present in Venezuela (4). The presence of *Haemagogus anastasionis* Dyar (1921), *Haemagogus janthinomys* Dyar (1921), and *Haemagogus celeste* Núñez-Tovar (1927) was detected in forest areas of Chiririka and Santa Elena (**Figures 6** and **7**). Adult specimens were identified, according to photographical key proposed by Liria and Navarro (4). These three species were captured on human landing catches in the forest, between 16:30 and 18:45 h, near of the Uairén and Chiririka rivers edges (**Figures 6** and **7**). Immature specimens were not collected. *H. anastasionis* and *H. janthinomys* species are two potential vectors of jungle yellow fever and jungle cycle of Mayaro virus in Venezuela (4); and both are new species records for the Gran Sabana and the Bolívar State. With these two new records, the genus *Haemagogus* in Bolívar State now includes six species: *H. celeste* Dyar & Núñez-Tovar, *H. equinus* Theobald, *H. leucocelaenus* (Dyar & Shannon),

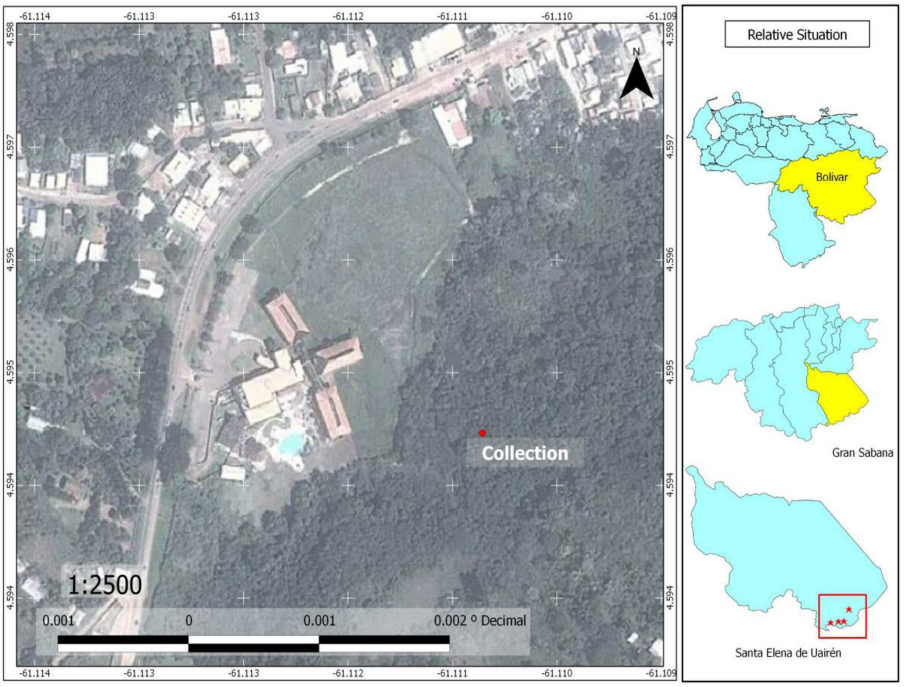

FIGURE 7 | Map of Santa Elena of Uairén, showing sites where adults specimens of *Culex (Culex) pinarocampa* and *Haemagogus janthinomys* were captured, near of the Uairén River; and showing sites where larvae of *Anopheles darlingi* were captured in pools formed near of the Uairén River bed.

H. albomaculatus Theobald, *H. anastasionis* Dyar, and *H. janthinomys* Dyar. The species *Sabethes purpureus* Theobald (1907) was collected on human landing catches near of the Uairén River edge (**Figure 7**). This species is also a potential vector of sylvatic yellow fever and jungle cycle of Mayaro virus. The Mayaro alpha virus produces non-specific, sub-lethal disease symptoms, often confused with dengue, but with symptoms of arthralgias (arthrosis) that can cause incapacitating disability (40). The Mayaro virus shows a great plasticity in vertebrate host infection, whereas high specificity in the mosquitoes of Culicidae family, vectors in the jungle cycle. Risk factors of infection are associated with forest areas of northern South America and the rainy season (40). The enzootic cycle is similar to the jungle cycle of yellow fever, which involves *Haemagogus* mosquitoes and monkeys as reservoirs (40). However, the involvement of others secondary vectors (e.g., *Sabethes*) and others hosts may be important in spread of the virus. Humans may have high levels of viremia and efficient experimental transmission has been demonstrated in *Aedes aegypti*, *Aedes albopictus*, and *Aedes scapularis*, suggesting a significant risk to public health in urban, rural, and domestic locations close to enzootic foci of Mayaro virus (40). In addition, we collected adults of *A. aegypti* and *A. scapularis* Rondani (1848) in urban and periurban areas of Santa Elena, Waramasén, Chiririka and Maurak, representing a significant risk to the inhabitant's populations of theses localities. *A. albopictus* specimens were not collected in the Gran Sabana.

Aedes aegypti and *A. albopictus* species have been involved in dengue transmission in the Manaus rural areas, Brazilian Amazon (3). The presence of *A. albopictus* in the Brazilian Amazon represents a potential risk of transmission of Chikungunya and Mayaro virus in urban, rural, domestic, and wild environments (1). The occurrence of its larvae and pupae, breeding in the same containers with other domestic species, associated with several sources of blood meal available in urban, rural, and wild environments, reveal its gradual establishment in the indoor of households and its potential involvement in the transmission of zoonotic pathogens to humans. The ability that their eggs may remain viable in nature for long periods of diapauses and the demonstrated transovarial transmission occurrence of several arboviruses has raised the need to expand the strategies directed toward combating *A. albopictus* in Vector Control Programs in South American (1). In Venezuela, *A. albopictus* was detected for the first time in 2009 (41), suggesting a significant risk to public health in urban, rural, domestic, and wild environments (1). Dormancy of the egg stage (and drought resistance) is considered to be a reproductive strategy for the long-term survival of multivoltine mosquitoes that develop in temporary habitats, such as tree-holes and other natural water containers that are subject to water fluctuations (42). Egg diapause involves a long stable arrest of hatching, even when environmental conditions are favorable for hatching (42).

Additionally, in this article we also report for the first time three new records of *Sabethini* Tribe mosquitoes for the Bolívar State (**Table 1**); and they are: *Wyeomyia celaenocephala* Dyar and Knab (1906), *Limatus asulleptus* Theobald (1903), and *Johnbelkinia ulopus* Dyar and Knab (1906). Adult females were collected on human landing catch during the day in Waramasén and Chiririka (**Figures 5** and **6**). These three species are potential arboviruses vectors in Venezuela (43) and *J. ulopus* larvae are facultative predators (43).

Species of the genus *Runchomyia* (**Table 1**) does not transmit diseases of medical importance to man; their larvae live in leaf axils of epiphytic and terrestrial bromeliads and in floral bracts (19). In 1986, the species *Runchomyia frontosa* Theobald was reported in carnivorous bromeliads from the Gran Sabana, Venezuela. The immature mosquitoes were collected living in the fluids held by the carnivorous bromeliad *Brocchinia reducta* Baker (44). The larvae of *Runchomyia frontosa* is a facultative predator, filter feeding, or consuming large prey that it captures with its enlarged maxillae in carnivorous bromeliads (44). Adult females of *Runchomyia frontosa* were collected on human landing catch during the day in El Paraíso and Waramasén (**Table 1**). This species does not transmit diseases of medical importance to man (19).

In addition we report, five new records of the genus *Uranotaenia* in Bolívar State, they are: *Uranotaenia typhlosomata* Dyar and Knab (1907); *Uranotaenia calosomata* Dyar and Knab (1907); *Uranotaenia nataliae* Arribalzaga (1891); *Uranotaenia leucoptera* Theobald (1907); and *Uranotaenia lowii* Theobald (1901). Species of genus *Uranotaenia* were found mainly in ground-water habitats, including springs, stream margins, herbaceous swamps, and temporary pools with vegetation (19). Some species have been found in tree-holes, plant axils, and artificial containers. Females of some species are known to feed on frogs, birds, and mammals, but are normally not attracted to humans (19). They are avian malaria vectors in Venezuela. In the Llanos of Venezuela, was found a high endemicity of avian malaria (45, 46). Immature specimens were collected in lagoons and herbaceous swamps (Morichales) in Santa Elena de Uairén, Waramasén, Maurak, Colinas de la Laguna, Altamira, San Antonio, Kinok-Pon-Parú, La Primavera, Chiricayén, Manak-Krú, Maurak, and Uaiparú. *Uranotaenia leucoptera* was collected only in tree-holes in Waramasén and Manak-Krú. This species is recorded for the first time from Venezuela. This study also extends the geographical distributions of *Uranotaenia leucoptera* in South America to Venezuela.

These findings shows the importance of further studies related to mosquito's geographical distribution, ecological aspects, arbovirus detection, epidemiological surveillance, and possible epidemiological link with emerging and reemerging arboviruses in the common border of Brazil and Venezuela. The entomological surveillance has an important role among the preventive measures against emerging diseases transmitted by insects, particularly by mosquitoes.

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The Australian public is still vulnerable to emerging virulent strains of West Nile virus

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The mosquito-borne West Nile virus (WNV) is responsible for outbreaks of viral encephalitis in humans and horses with particularly virulent strains causing recent outbreaks in Eastern Europe, the Middle East, and North America. In Australia, a strain of WNV, Kunjin (WNV_{KUN}), is endemic in the north and infection with this virus is generally asymptomatic. However, in early 2011, following extensive flooding, an unprecedented outbreak of WNV_{KUN} encephalitis in horses occurred in South-Eastern Australia, resulting in more than 1,000 cases and a mortality of 10–15%. Despite widespread evidence of equine infections, there was only a single mild human case reported during this outbreak. To understand why clinical disease was seen in horses without similar observations in the human population, a serosurvey was conducted using blood donor samples from areas where equine cases were reported to assess level of flavivirus exposure. The seroprevalence to WNV_{KUN} in humans was low before the outbreak (0.7%), and no significant increase was demonstrated after the outbreak period (0.6%). Due to unusual epidemiological features during this outbreak, a serosurvey was also conducted in rabbits, a potential reservoir host. Out of 675 animals, sampled across Australia between April 2011 and November 2012, 86 (12.7%) were seropositive for WNV_{KUN}, with the highest prevalence during February of 2012 (28/145; 19.3%). As this is the first serological survey for WNV_{KUN} in Australian feral rabbits, it remains to be determined whether wild rabbits are able to develop a high enough viremia to actively participate in WNV transmission in Australia. However, they may constitute a sentinel species for arbovirus activity, and this is the focus of on-going studies. Collectively, this study provides little evidence of human exposure to WNV_{KUN} during the 2011 outbreak and indicates that the Australian population remains susceptible to the emergence of virulent strains of WNV.

Keywords: West Nile virus, equine encephalitis, seroprevalence, humans, rabbits

INTRODUCTION

Flaviviruses are a group of medically important arboviruses causing large disease outbreaks around the world with approximately 50 million cases per year. Mosquito-borne flaviviruses in the Japanese encephalitis virus (JEV) serogroup, including JEV, West Nile virus (WNV), and Murray Valley encephalitis virus (MVEV), cause severe, potentially fatal neurological disease in humans, horses, and some avian species. WNV has traditionally been associated with outbreaks of viral encephalitis in Europe and Africa (1). In 1999, WNV appeared for the first time in the USA, associated with an outbreak of a fatal or debilitating disease in humans and equines, and extremely high levels of morbidity and mortality in several species of native birds in New York (2, 3). Since its introduction into the USA, WNV has caused more than 16,196 human cases of neuroinvasive disease and more than 1549 deaths in the USA alone and spread to most parts of North, Central, and South America via mosquito-bird transmission cycles (4).

The Kunjin strain of WNV (WNV_{KUN}) is a closely related virus from Australia. Although WNV_{KUN} was initially considered a separate species in the flavivirus genus, studies by our laboratory and collaborators revealed that it shared a high degree of antigenic and genetic homology to WNV strains, justifying re-classification of the virus as a subtype of WNV (5–7). Until recently, the relatively benign WNV_{KUN}, had only been associated with a few cases of non-fatal encephalitis in humans and a small number of equine cases since it was first isolated in 1960 (8). However, in early 2011 an unprecedented outbreak of equine encephalitis occurred in South-East Australia, causing mortality of 10–15% of horses infected (9). Upon isolation, a new equine virulent strain of WNV_{KUN} was confirmed, being the first strain to cause a major outbreak in Australia (9). Symptoms of equine infection with this strain included ataxia, muscle paralysis and tremors, changes in temperament, incoordination, and general weakness, which are consistent with clinical signs of the

equine WNV encephalitis caused by virulent North American (WNV_{NY99}) and European strains (10, 11). Notably, this new WNV strain, named $WNV_{NSW2011}$, arose in regions of Southern Australia where WNV_{KUNV} had not been observed previously, including coastal and inland cities of New South Wales (NSW) (9).

Abundant rainfall in the latter half of 2010 and extending into the first quarter of 2011 led to extensive flooding of inland areas of NSW. This unforeseen event provided ideal climatic conditions for breeding of freshwater mosquito populations, which recorded a sixfold increase in number compared to the previous season (12). This prolific population growth of the primary vector for WNV_{KUN} is presumed to have spurred the major outbreak in 2011 (13, 14). Nevertheless, only a small number of human infections were recorded during the time period of the equine encephalitis epidemic. Perhaps even more curiously, a large number of the equine cases occurred east of the Great Dividing Range in the much dryer coastal regions where no flooding was experienced during the same period and mosquito populations were low (14). This prompted us to consider epidemiological factors other than a bird–mosquito–human transmission chain. One animal species that occurs in abundance in the main affected regions is the rabbit (*Oryctolagus cuniculus*). Feral rabbits have previously been shown to sustain a brief viremia sufficient to transmit to mosquitoes when experimentally infected with MVEV (15). Similarly, Eastern cottontail rabbits (*Sylvilagus floridanus*) develop a viremia sufficient for mosquito transmission when experimentally infected with WNV_{NY99} (16), and preliminary work in our group had shown that domestic rabbits (*O. cuniculus*) can become infected with $WNV_{NSW2011}$ (17).

We therefore carried out a serological survey on humans from east-coast regions of NSW with high incidences of equine encephalitis cases, using plasma samples obtained from the Australian Red Cross Blood Service (Blood Service), to assess human exposure during the outbreak and evaluate the on-going risk of virulent strains of WNV_{KUN}. In addition, we tested feral rabbits sampled by the NSW Department of Primary Industries (NSW DPI) from two areas in NSW and one area in all other states during and up to 1.5 years after the equine epidemic. Collectively, our data suggest that an overwhelming proportion of the Australian human population remains susceptible to the emergence of virulent strains of WNV, and that feral rabbits may represent a possible reservoir, at least in some areas of South-Eastern Australia.

MATERIALS AND METHODS

HUMAN PLASMA SAMPLES

Plasma samples were acquired from healthy Australian blood donors after routine infectious disease testing was complete. Samples were selected from donors residing in eastern NSW coinciding with locations of $WNV_{NSW2011}$ equine infection [**Figure 1**; see also Figure 3 in Ref. (14)], with samples collected from November 2009 to November 2010 ($n = 148$) forming the pre-2011 sample group and those from September 2013 ($n = 168$) forming the post-2011 sample group. Samples in the pre-2011 group were collected into EDTA plasma preparation tubes [Becton, Dickson and Company (BD) Biosciences, San Diego, USA], while samples collected in the post-2011 group were collected into EDTA tubes (BD Vaccutainer® Whole Blood Collection tube). All samples were centrifuged and plasma aliquots archived at −30°C. Donor demographic data were obtained for each sample (age, gender, suburb, and postcode). This study was carried out under approval from the Blood Service Human Research Ethics Committee.

RABBIT SERUM SAMPLES

Serum samples derived from rabbits captured or shot as part of surveys conducted by NSW DPI in all states of Australia were made available for testing. Collections were performed in two central areas of NSW (Euchareena and Oaky Creek in the central tablelands), and one area in each of the other states and the Northern Territory (near Hattah-Kulkyne National Park in Victoria; near Coorong in South Australia; south-east Queensland; South-West Western Australia; **Figure 2**) during seven time periods between April 2011 (Autumn) and November 2012 (Spring). A total of 675 samples were available for testing. The age of the rabbits was determined by lens dry weight measurements as described previously (18).

EPITOPE BLOCKING ELISA

Virus-specific antibodies to WNV_{KUN} or MVEV were detected in human plasma and rabbit sera using a blocking ELISA (bELISA) (19) as detailed in Prow et al. (20). Seropositivity is defined as inhibition of the binding of virus-specific monoclonal antibodies (mAb) by more than 30%. All samples were initially screened for flavivirus antibodies using the anti-flaviviral E protein mAb 4G2 as the competing antibody (20). All flavivirus positive samples were then tested for WNV_{KUN} (both human and rabbit samples) and MVEV (human samples only) using the same bELISA protocols with the mAbs 3.1112G and 10C6, respectively. Horse sera previously tested to be positive and negative for the virus of interest (20) were employed as controls for the bELISA on human plasma, while serum from naïve SPF-bred New Zealand White rabbits and rabbits experimentally infected with $WNV_{NSW2011}$ (kindly provided by W. Suen, University of Queensland) were used as negative and positive controls for the bELISA involving rabbit sera. Controls consisting of no sera and no antigen (coating buffer only) were also included in each bELISA.

VIRUS NEUTRALIZATION ASSAY

Samples positive in the bELISA were tested for neutralizing activity against WNV_{KUN} (rabbit and human samples) and MVEV (human samples only) in a microtiter serum neutralization assays, using MVEV (strain 1–51) or WNV_{KUN} (strain MRM16) as previously described in detail (20).

STATISTICAL ANALYSIS

The proportion seropositive and 95% CI was calculated. For both human and rabbit data, seropositivity was first compared across age group, location (state/region), sex (human only), and time period in univariate analyses using the chi-squared test or a univariate logistic regression. Where a significant relationship with seropositivity was observed for two or more variables, these variables were entered into a logistic regression model [with seropositivity (reactive or non-reactive) as the dependent variable

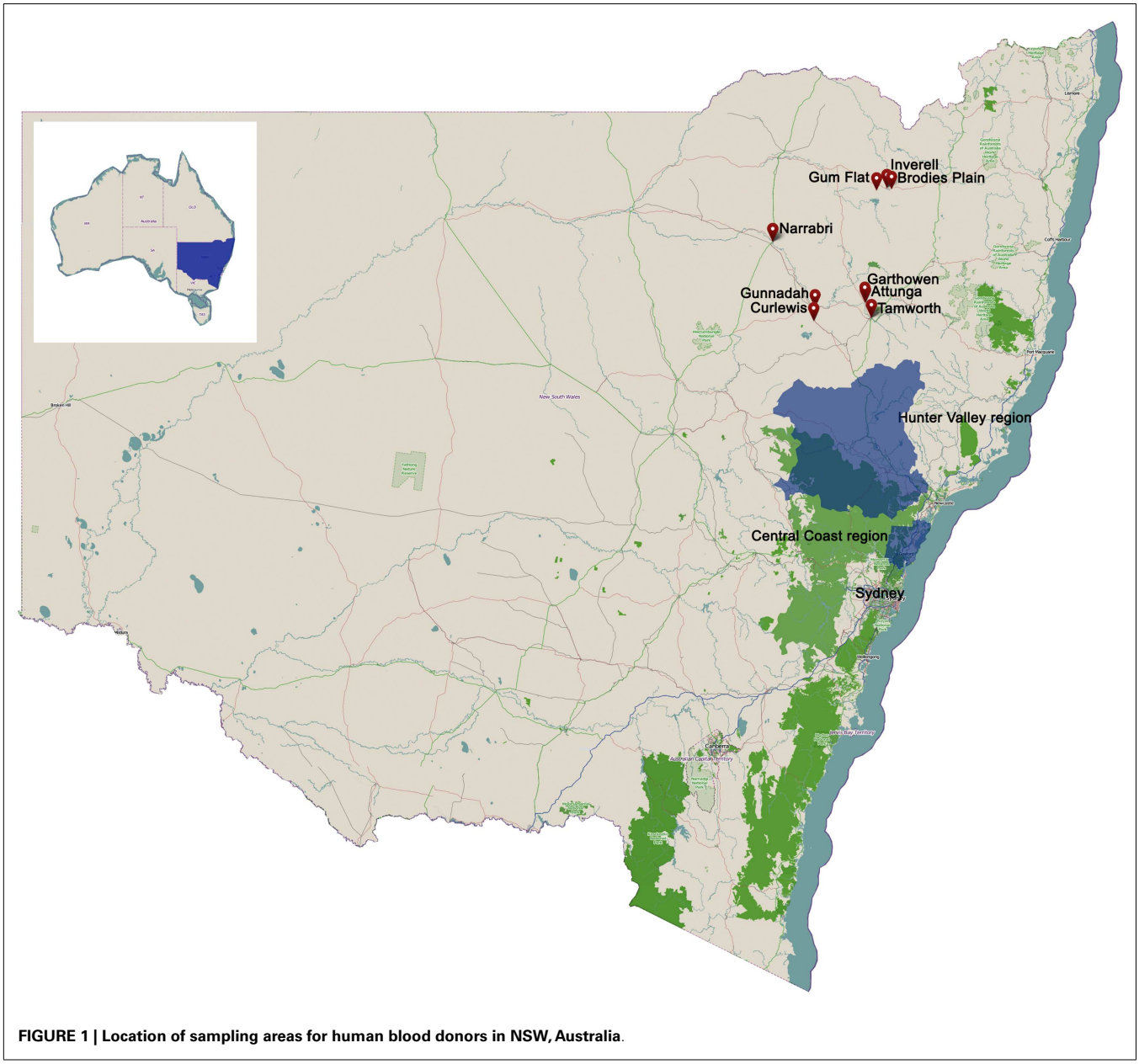

FIGURE 1 | Location of sampling areas for human blood donors in NSW, Australia.

(non-reactive as the reference) and the other variables as factors]. Microsoft Excel (Microsoft Pty. Ltd., North Ryde, NSW, Australia) and the Statistical Package for the Social Sciences (SPSS; IBM Australia Ltd., St. Leonards, NSW, Australia) were used for data management and analyses.

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RESULTS

PROFILE OF THE HUMAN STUDY POPULATIONS

A total of 316 individuals were included in this study, consisting of 148 in the pre-2011 cohort and 168 in the post-2011 cohort (**Table 1**). Just over half (52.2%) of the samples were from male donors. The median age of donors was 43 years (IQR 26–56) for the pre-2011 sample group and 51 years (IQR 40.5–61) for the post-2011 sample group, with a slightly skewed distribution toward older donors in the post-2011 sample group. The majority of samples were collected from regional NSW, west of the Great Dividing Range (**Table 1**).

SEROPREVALENCE OF FLAVIVIRUSES, WNV_{KUN}, AND MVEV IN HUMAN BEINGS IN COASTAL NSW

Flavivirus total antibody was detected in 15 of the 148 samples in the pre-2011 group (10.1%), while 13 of 168 (7.7%) samples were observed to be seropositive for flavivirus antibody in the post-2011 group (**Table 2**). Seroprevalence of WNV_{KUN} total antibody in the pre-2011 samples was 0.7% with just one sample testing seropositive, which is similar to the seroprevalence observed in the post-2011 samples (0.6%) where again, just one sample was observed to be seropositive to WNV_{KUN} (**Table 2**). These two WNV_{KUN}

seropositive samples were both from males, with ages of 68 and 65, respectively, both of whom were residents of Tamworth in the North West Slopes subregion of regional NSW. Both WNV_{KUN} seropositive samples were also tested in the neutralization assay and confirmed to have neutralizing antibodies against WNV_{KUN} with titers of 40 and 80, respectively. None of the total flavivirus seropositive samples tested positive for antibodies against MVEV.

No significant difference was observed between pre- and post-2011 time points in either total flavivirus seropositivity ($p = 0.454$) or in WNV_{KUN} seropositivity ($p = 0.928$). Overall, the proportion of males seropositive for total flavivirus antibody was significantly higher than females ($p = 0.001$); however, age group ($p = 0.146$) and region ($p = 0.889$) did not significantly influence seropositivity (**Table 3**).

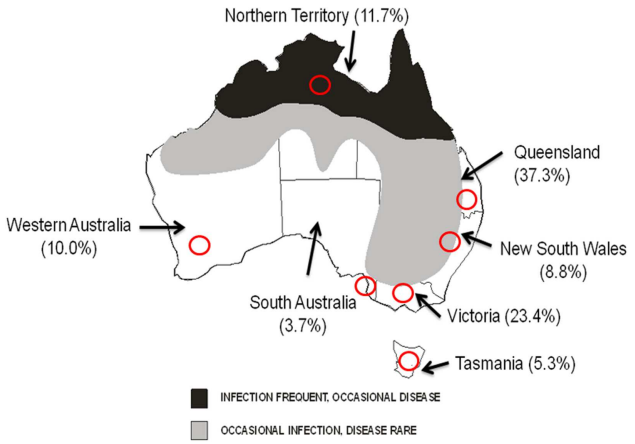

FIGURE 2 | Known distribution of WNV_{KUN} in Australia and seroprevalence in Australian rabbits. States and Territories are shown. Red circles indicate approximate areas of rabbit sampling. Numbers in brackets represents overall seroprevalence of antibodies to WNV_{KUN} in rabbits during 2011–2012.

SEROPREVALENCE OF FLAVIVIRUSES IN RABBITS

Out of a total of 675 rabbits sampled between April 2011 (Southern Hemisphere Autumn) and November 2012 (Southern Hemisphere Spring) 86 animals (12.7%) had antibodies specific for WNV_{KUN} as determined in the 3.111G bELISA (**Table 4**). Of these, 28 (32.5% of bELISA positive) rabbits had WNV_{KUN} neutralizing antibodies with titers varying from 20 to 160, as determined in the microneutralization assay (data not shown). By univariate analysis, there was a significant association between state ($p < 0.001$) and time period ($p = 0.050$), while age had no apparent effect ($p = 0.876$). By multivariate logistic regression, both state ($p < 0.001$) and time period ($p = 0.018$) were still associated with seropositivity (**Table 5**). Specifically, the seroprevalence was higher in the states of Queensland and Victoria compared to Tasmania, and also in summer 2012 and winter 2012 compared to spring 2012 (**Tables 4** and **5**; **Figures 2** and **3**).

Table 1 | Characteristics of the human study population.

Time point/region	Number of samples	Age group						Male (%)
		≤24	25–34	35–44	45–54	55–64	≥65	
PRE-2011								
Hunter valley/Central Coast/Sydney	16	8	1	2	1	3	1	38
Regional NSW	132	26	20	23	21	22	20	52
Total	148	34	21	25	22	25	21	50
POST-2011								
Hunter valley/Central Coast/Sydney	32	4	1	5	11	8	3	59
Regional NSW	136	16	11	20	31	35	23	53
Total	168	20	12	25	42	43	26	54
TOTAL								
Hunter valley/Central Coast/Sydney	48	12	2	7	12	11	4	52
Regional NSW	268	42	31	43	52	57	43	52
Total	316	54	33	50	64	68	47	52

Table 2 | Flavivirus seroprevalence in blood donors from eastern NSW collected in the months prior to and soon after the equine 2011 WNVKUN epidemic.

Time point	Number tested	Total flavivirus		WNV_{KUN}	
		Positive	% (95% CI)	Positive	% (95% CI)
Pre-2011	148	15	10.1 (5.27–15.00)	1	0.7 (0.00–2.00)
Post-2011	168	13	7.8 (3.70–11.78)	1	0.6 (0.00–1.76)
Overall	316	28	8.9 (5.73–11.99)	2	0.6 (0.00–1.51)

Table 3 | Breakdown of total flavivirus seropositivity by sex, age group, and region.

Variable	Number tested	Total flavivirus seropositivity		Univariate analysis	
		n	% (95% CI)	Odds ratio (95% CI)	*p*-Value
Time period					
Pre-2011	148	15	10.1 (5.27–15.00)	†	–
Post-2011	168	13	7.8 (3.70–11.78)	1.345 (0.618–2.928)	0.456
Sex					
Female	151	5	3.3 (0.46–6.17)	†	–
Male	165	23	13.9 (8.65–19.22)	4.730 (1.750–12.784)	0.002
Age group (years)					**0.242**
<25	54	1	1.8 (0.00–5.45)	†	–
25–34	33	3	9.1 (0.00–18.90)	5.30 (0.528–53.237)	0.157
35–44	50	8	16.0 (5.84–26.16)	10.095 (1.214–83.930)	0.032
45–54	64	7	10.9 (3.29–18.58)	6.509 (0.775–54.683)	0.085
55–64	68	7	10.3 (3.07–17.52)	6.082 (0.725–51.044)	0.096
>65	47	2	4.3 (0.00–10.03)	2.356 (0.207–26.840)	0.490
Region group					**0.889**
Hunter valley/Central coast/Sydney	48	4	8.3 (0.51–16.15)	†	–
Regional NSW	268	24	9.0 (5.54–12.37)	0.924 (0.306–2.794)	0.889

† *Not applicable.*

Table 4 | KUN seropositivity in Australian rabbits, from April 2011 to November 2012.

	n Tested	KUN seropositive	
		n	% (95% CI)
Total	675	86	12.74 (10.23–15.26)
TIME PERIOD			
Autumn 2011	86	9	10.5 (4.00–16.93)
Winter 2011	82	10	12.2 (5.11–19.28)
Spring 2011	148	18	12.2 (6.90–17.43)
Summer 2012	145	28	19.3 (12.89–25.74)
Autumn 2012	86	4	4.6 (0.20–9.10)
Winter 2012	66	11	16.7 (7.68–25.66)
Spring 2012	62	6	9.7 (2.32–17.04)
STATE			
South Australia	109	4	3.7 (0.14–7.20)
Queensland	59	22	37.3 (24.95–49.63)
New South Wales	294	26	8.8 (5.60–12.09)
Northern Territory	60	7	11.7 (3.54–19.79)
Tasmania	19	1	5.3 (0.00–15.30)
Victoria	94	22	23.4 (14.84–31.96)
Western Australia	40	4	10.0 (0.70–19.30)
AGE (MONTHS)			
<3	53	8	15.1 (5.46–24.73)
3–5.9	84	7	8.3 (2.42–14.24)
6–8.9	108	14	13.0 (6.63–19.30)
9–11.9	79	9	11.4 (4.39–18.40)
12–14.9	56	8	14.3 (5.12–23.45)
≥15	278	37	13.3 (9.32–17.30)
N/A	17	3	–

Table 5 | Multivariate logistic regression analysis: influence of factors on KUN seropositivity in Australian rabbits.

Variable	Multivariate logistic regression analysis		
	Odds ratio	95% CI	*p*-Value
TIME PERIOD (REFERENCE GROUP: SPRING 2012)			
Autumn 2011	0.554	0.178–1.721	0.307
Winter 2011	0.635	0.204–1.984	0.435
Spring 2011	0.608	0.219–1.688	0.340
Summer 2012	0.344	0.127–0.928	0.035
Autumn 2012	1.610	0.414–6.258	0.492
Winter 2012	0.275	0.090–0.842	0.024
STATE (REFERENCE GROUP: TASMANIA)			
South Australia	1.025	0.104–10.147	0.983
Queensland	0.060	0.007–0.500	0.009
New South Wales	0.363	0.044–2.975	0.345
Northern Territory	0.297	0.033–2.655	0.278
Victoria	0.105	0.013–0.868	0.037
Western Australia	0.256	0.026–2.559	0.246

DISCUSSION

West Nile virus is an on-going global public health concern, causing large outbreaks in the USA, Europe, and more recently Australia. Following extensive flooding across Eastern Australia in 2011 promoting ideal conditions for mosquito breeding, an unprecedented outbreak of equine encephalitis occurred, leading to the isolation of the first virulent strain of WNV in Australia. However, a number of unusual epidemiological features associated with this outbreak, including the lack of severe human cases,

previously unseen transmission of WNV_{KUN} in coastal regions and a number of cases reported outside the areas affected by heavy rainfall, instigated further studies to try to explain the 2011 outbreak.

Historically, WNV_{KUN} has been associated with only mild symptoms in humans and horses. The emergence of the $WNV_{NSW2011}$ strain suggested the virus may have undergone changes and evolved to become more virulent and able to cause fatal encephalitis in horses. However, since no severe human cases were seen during the outbreak, this study aimed to determine whether the lack of human involvement in the outbreak was simply due to pre-existing antibody against WNV_{KUN}. The very low seroprevalence of WNV_{KUN} in humans observed in samples collected both before (0.7%) and after (0.6%) the 2011 outbreak of equine flavivirus encephalitis suggest that, in contrast to what was seen during the WNV incursion into the USA, humans residing in easternmost NSW were not exposed to the new $WNV_{NSW2011}$. However, these seroprevalences are considerably lower than seen in previous serosurveys conducted in the Murray Valley region of NSW between 1991 and 2011, where WNV_{KUN} seroprevalences had been observed to range between 2.2 and 2.5% in the human population (21, 22). Higher WNV_{KUN} seroprevalence rates, ranging from 2.1 to 3.1%, have also been observed in Victorian residents (21, 23). It is possible that our results are biased because of the small sample size relative to previous studies and the fact that we studied a seemingly health conscious subset of the population, i.e., blood donors, who may take measures to limit their exposure to mosquitoes and thus arbovirus disease in accordance with Public Health recommendations. This suggestion is also supported by the absence of antibodies to MVEV despite 16 confirmed cases of MVEV in humans during 2011, as reported by the National Notifiable Diseases Network (12). However, it should be noted that the coastal regions of NSW are not typically affected by flavivirus diseases. Rather, MVEV tends to occur in the Murray Valley basin and occurrence is governed by rainfall and the resulting migration of birds. Previous VIC-based studies observed a MVEV seroprevalence of 3.7% ($n = 2{,}783$) in 1991–1992 and 2.2% ($n = 1115$) in 2011 (21, 23).

Another factor governing transmission of arboviruses is vector competence, which is the intrinsic ability for an arthropod vector to become infected with and transmit an arbovirus (24). Studies are currently being undertaken to determine whether the NSW2011 strain of WNV_{KUN} exhibits increased fitness in the primary vector, *Cx. annulirostris*, compared to non-pathogenic strains (van den Hurk et al., personal communication). If the NSW2011 strain is transmitted more efficiently by mosquitoes, then increased vector competence of *Cx. annulirostris* may have contributed to the equine epidemic, making it even more intriguing that corresponding increases in human exposures and clinical cases were not seen.

A large scale serological survey was conducted in the NSW equine population in late 2011 following cessation of the equine epidemic (25). This study had two notable findings, firstly that the overall seroprevalence of WNV_{KUN} in horses was low (3.9% of 1054 horses across the state) and lower than in previous studies. Secondly, almost all the seropositive horses came from far-western

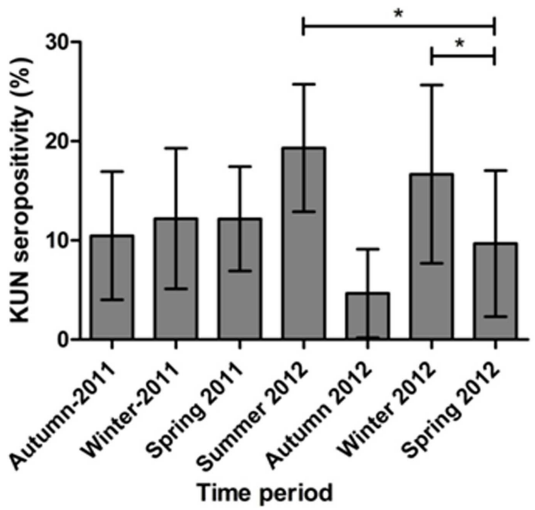

FIGURE 3 | WNV_{KUN} seroprevalence in feral rabbits in Australia sampled at the peak (Autumn 2011) and end (Winter 2011) of the equine encephalitis epidemic (Autumn 2011) and over the following 15 months. Bars represent the proportion of animals seropositive at each time period, with the bars representing the 95% confidence intervals. Asterisks represent a significant difference ($p < 0.05$) compared to spring 2012.

districts of NSW (25), while the majority of clinical cases were seen in the eastern half of NSW (14). This also suggests that infection with this new strain of WNV_{KUN} was associated with very high morbidity in the equine population.

In considering the distribution of equine encephalitis cases in the 2011 epidemic (14), we hypothesized that animal species other than birds might be involved in the spread of WNV_{KUN} along the eastern coastal areas of NSW. Notably, this area largely avoided the flooding events of early 2011 and dry conditions prevailed in the region during the summer and autumn of 2011. One species with wide distribution across Australia is the feral rabbit (www.invasiveanimals.com). Rabbits have been shown experimentally to develop a viremia in the absence of clinical signs following infection with WNV (16, 17). Interestingly, the present study revealed a higher seroprevalence in rabbits caught in Queensland and Victoria, compared to NSW. However, this finding may be biased due to the sampling areas, with the NSW sampling area being west of the main region for equine cases [(14); **Figure 1**]. In contrast, the high seroprevalence in Victorian rabbits is in concordance with the equine epidemic extending into this state, and the fact that the rabbit sampling area (Hattah-Kulkyne National Park) was within the region with many equine cases. The findings for Queensland are particularly notable, as this state avoided the equine encephalitis epidemic despite major flooding (14) and despite the presence of a susceptible equine population (20). The lack of correlation between equine clinical cases and seroprevalence in rabbits in some areas may be taken to suggest that rabbits have no role in the transmission cycle. Nevertheless, the finding that seroprevalence in this species was independent of age may

suggest that they could be used as sentinels for arbovirus activity in a particular region. As the rabbit is a pest species in Australia and various programs are in place to control them, culled animals could be sampled for serological surveys prior to destruction and in this way inform Public Health authorities about potential arbovirus activity in a particular region.

WNV is estimated to have infected approximately four million humans in North America, causing over 37,000 clinical infections and 1443 deaths between 1999 and 2012 (4). On-going surveillance of currently circulating WNV strains in North America has indicated that the virus is continually changing with at least three different genotypes identified to date. Given that North American and Australian strains of WNV are very close genetically, sharing ~99% amino acid identity, and all WNV strains share a common transmission cycle, the possibility of emerging virulent strains of WNV in Australia, able to induce severe human disease, remains a definite possibility. In light of the observations from this study, the Australian population is still vulnerable to these emerging virulent strains, as very few people appear to have levels of WNV_{KUN}-specific antibodies sufficient to afford protective immunity.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Natalie A. Prow, Helen M. Faddy, Roy A. Hall, Helle Bielefeldt-Ohmann. Performed the experiments: Natalie A. Prow, Elise K. Hewlett, Flaminia Coiacetto, Wenqi Wang, Tarnya Cox. Analyzed the data: Helen M. Faddy, Natalie A. Prow, Tarnya Cox, Helle Bielefeldt-Ohmann. Drafted the manuscript: Natalie A. Prow, Helen M. Faddy, Helle Bielefeldt-Ohmann. Revisions and final approval of the submitted version: all authors.

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Emergence of West Nile virus lineage 2 in Europe: a review on the introduction and spread of a mosquito-borne disease

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West Nile virus (WNV) is transmitted by mosquitoes and causes fever and encephalitis in humans, equines, and occasionally wild birds. The virus was first isolated in sub-Saharan Africa where it is endemic. WNV lineage 1 has been responsible for repeated disease outbreaks in the countries of the Mediterranean basin over the past 50 years. This lineage was also introduced into North America in 1999 causing widespread human, equine, and avian mortality. WNV lineage 2, the first WNV lineage to be isolated, was believed to be restricted to sub-Saharan Africa causing a relatively mild fever in humans. However, in 2004, an investigation in Hungary of a case of encephalitis in a wild goshawk (*Accipiter gentiles*) resulted in the isolation of WNV lineage 2. During the summer of 2004, and in subsequent years, the virus appeared to spread locally throughout Hungary and into neighboring Austria. Subsequently, WNV lineage 2 emerged in Greece in 2010 and in Italy in 2011, involving outbreaks on the Italian mainland and Sardinia. Further spread through the Balkan countries is also suspected. Whole genome sequencing has confirmed that the virus responsible for the outbreaks in Greece and Italy was almost identical to that isolated in Hungary. However, unlike the outbreaks in Hungary, the burden of disease in Mediterranean countries has fallen upon the human population with numerous cases of West Nile fever and a relatively higher mortality rate than in previous outbreaks. The emergence of WNV lineage 2 in Europe, its over-wintering and subsequent spread over large distances illustrates the repeated threat of emerging mosquito-borne diseases. This article will review the emergence of WNV lineage 2 in Europe; consider the pathways for virus spread and the public health implications for the continent.

Keywords: West Nile virus, lineage, emergence, encephalitis, Europe

INTRODUCTION

In recent years, arthropod-borne viruses have shown an increasing ability to spread beyond the areas, which had been considered to be their established geographic ranges. A number of factors are driving this process including bird migration, increasing global trade, and the movement of vector species (1). This range expansion threatens public and livestock health. Examples of viruses that have emerged in Europe and which are pathogenic for livestock include bluetongue virus (2) and most recently Schmallenberg virus (3). Such disease outbreaks incur both economic and animal health costs that threaten the livestock industry. Other emerging viruses are zoonotic, and the repeated emergence of West Nile virus (WNV) in Europe is a particular example of one such range expansion (4). The ability to detect and respond to emerging disease outbreaks, through rapid pathogen testing and host-specific serological assays, is a key component for disease response. This review considers the emergence of WNV lineage 2 in Europe as an example of the threat to public and livestock health from emerging zoonoses.

West Nile virus is classified within the genus *Flavivirus*, family *Flaviviridae*, and is phylogenetically and antigenically related to Japanese encephalitis virus. The virus was first isolated from the blood of a woman suffering a febrile illness in the West Nile district of Uganda (5). This first isolate is now believed to belong to lineage 2 suggesting its early zoonotic potential. Subsequent studies made further isolations of WNV from human sera in Egypt (6), and from birds and mosquitoes (7). This established that mosquitoes were the likely virus vector and through blood-feeding on birds, the virus was maintained in an endemic cycle. Early phylogenetic studies (discussed in more detail in subsequent sections) demonstrated that there are two major lineages, both present in Africa (8). Subsequent events including the emergence of WNV in North America, its spread throughout the western hemisphere, and repeated outbreaks in Europe suggest that WNV has the largest distribution of any arthropod-borne virus. In Africa, WNV is endemic and widely distributed. Human cases have been sporadic, but environmental conditions favoring mosquitoes, such as high diurnal temperatures or frequent rainfall, have led to large epidemics (9). One such

episode in the Karoo region of South Africa in 1974 involved tens of thousands of human infections. Currently, approximately 5–15 human cases are confirmed each year in South Africa, however, only a small proportion of cases undergo laboratory investigation, so this may be a considerable underestimate of the actual number of infections (9). In horses and birds, serology studies have demonstrated a high seroprevalence of WNV infection in southern Africa (10, 11).

Annual late-summer outbreaks of WNV are now a regular occurrence in European countries that border the Mediterranean Sea and the virus is now considered endemic in some regions (12, 13). Most outbreaks have been identified as WNV lineage 1 and were closely related to outbreaks in Israel and North America (14). This lineage has also been responsible for deaths in humans, horses, and avian species. Strikingly, in Africa, where lineage 2 predominates, relatively few cases of neurological disease are reported, whereas in North America and Europe, numerous human and equine cases have occurred, leading to the suggestion that lineage 1 strains had increased pathogenicity, while lineage 2 strains were of low virulence (9). However, experimental studies in mice (15) and case reports (16, 17), have demonstrated that both WNV lineages have the ability to cause zoonotic disease, with the potential for fatal neuroinvasive disease. This has been realized fully with repeated outbreaks of West Nile fever in Greece since 2010, caused by WNV lineage 2, resulting in hundreds of human cases of West Nile neurological disease (WNND) (18).

EMERGENCE OF WNV LINEAGE 2 IN EUROPE

The first WNV isolated in 1937 in Uganda has since been shown to be a lineage 2 isolate, and for many years this lineage was believed to be restricted to sub-Saharan Africa (5). Until the early 2000s, WNV infections beyond Africa, including its emergence in North America, and Kunjin virus in Australia, were caused by viruses within lineage 1 (19). This included a number of outbreaks in Europe and countries around the Mediterranean Basin (12). Outbreaks of West Nile disease were recorded in Algeria (1994), Morocco and Romania (1996), Tunisia (1997), Italy (1998), Israel and Russia (1999), and France (2000). Detailed phylogenetic analysis of viruses isolated from these outbreaks suggested that those around the western Mediterranean were caused by a single strain, referred to as the WMed subtype, and that this was a single introduction of virus that overwintered over a number of years (20). It was conjectured that this sub-lineage was transferred between Mediterranean countries by viremic birds, leading to the initiation of new outbreaks. A second closely related sub-lineage included viruses isolated from Romania and Russia, and a more divergent sub-lineage was responsible for outbreaks in Israel and North America. Each sub-lineage likely represents a separate introduction of WNV into Europe from Africa.

West Nile lineage 2 was first detected in Europe in 2004 with its isolation from the brain of a goshawk (*Accipiter gentiles*) in Hungary (21). A human case of WNV lineage 2 infection was retrospectively confirmed to have occurred in Russia in the same year (22). Subsequent surveillance between 2004 and 2009 of dead birds of prey, especially in goshawks, led to repeated isolations of the virus across Hungary (23). The species nests and thrives in areas of deciduous and coniferous forests and so would be targeted by ornithophilic *Culex pipiens* complex mosquitoes, the main transmitters of WNV in Europe. The prevalence of infection in the northern goshawk could also result from oral transmission as this species preys on other birds, particularly pigeons. Local spread resulted in WNV infection of raptors in Austria (24). Subsequent outbreaks have occurred in a number of other European countries including Austria, Greece, Romania, Serbia, and Italy (**Table 1**; **Figure 1**).

The outbreak in Greece has been particularly severe. The virus was first detected in 2010 (32) in Northern Greece and developed, in contrast to the emergence in Hungary, into a large human epidemic. The majority of cases were reported west of the city of Thessaloniki, between the rivers Axios and Aliakmonas. Retrospective serology suggests that this virus, or a related one, had been circulating in Greece for some years prior to the first human cases of WN fever (WNF) (33). However, this was at a low level, <1% seropositivity, and was not accompanied by reports of disease. The first case in 2010 occurred in early July and incidence peaked in mid-August. The last cases occurred in early October. In total, 262 patients were recorded, with 65 classified as West Nile fever and 197 suffered neurological disease, of which 33 died (34). Age profiling demonstrated that the elderly were most at risk of disease, particularly those over 70 years of age, and risk was further increased if the individual had an existing medical condition such as hypertension, heart disease, or diabetes. Subsequent surveillance isolated WNV lineage 2 from mosquitoes (35), giving rise to the Nea Santa-Greece 2010 strain, and from wild resident birds (36). Epidemics of WNV lineage 2 have occurred during the late summer in Greece in both 2011 and 2012. Further sequencing of viruses detected in humans has confirmed that the same virus strain is present in both humans and wildlife and has been present in each subsequent year, suggesting endemic persistence in Greece (37). This strain appears to cause disease in humans and wild avian species with relatively few confirmed reports of disease in equine species.

West Nile lineage 2 emerged in Italy in 2011, the year after the first detection in Greece. The first reported case occurred in a man in his late 50s in the coastal town of Ancona on the Adriatic Sea (27). The patient reported symptoms of malaise and

Table 1 | Information on confirmed outbreaks of WNV lineage 2 in Europe between 2004 and 2013.

Country	Year	Species affected	Reference
Russia	2004	Human	(22)
Hungary	2004–2008	Wild birds, sheep, horses, human	(21, 23)
Austria	2008	Wild birds	(24)
Greece	2010	Human, wild birds, mosquitoes	(25, 26)
Romania	2010	Human	
Russia	2011	Human	(22)
Italy	2011	Human, wild birds, mosquitoes	(27, 28)
Italy (Sardinia)	2012	Human	(29–31)
Serbia	2012	Human	
Italy	2013	Human	

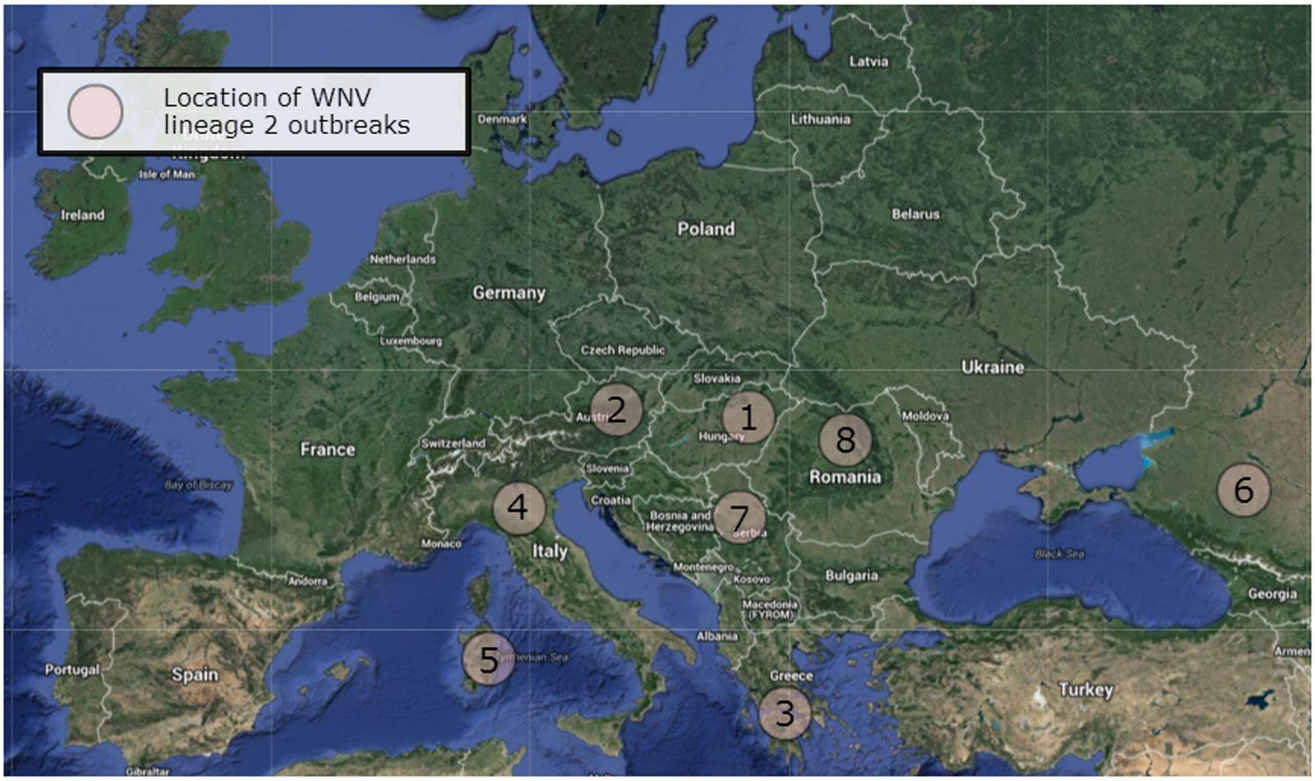

FIGURE 1 | West Nile virus lineage 2 outbreaks in Europe. The outbreaks occurred in Hungary (1), Austria (2), Greece (3), Italy (4), Sardinia (5), Russia (6), Serbia (7), and Romania (8). Map data: Google maps.

fever early in September, and was admitted to hospital. With the patient reporting no history of travel outside of Italy, this was considered an autochthonous case. Shortly after this, six cases of neurological disease due to WNV lineage 2 were reported in Sardinia between September and October, 2011 (28). Mosquito surveillance for WNV detected lineage 2 in *Cx. pipiens* mosquito pools and in a collared dove (*Streptopelia decaocto*) in northern Italy, where lineage 1 has been endemic since 2008 (38). Detections of WNV lineage 2 in Italy have continued in subsequent years, and have included further human cases (29, 31). As in Greece, instances of WNV infection in horses in Italy are rare.

West Nile fever was detected in humans in Romania in 2010 and Serbia in 2012. In Romania 57 cases were reported, 54 with neuroinvasive disease (26). In Serbia, 58 patients were confirmed infected (30). Of these, 52 developed neuroinvasive disease and 9 died. Virus isolation and phylogenetic analysis confirmed that both outbreaks were due to WNV lineage 2 (26, 39).

There is currently no vaccine against WNV licensed for human use, and although WNV lineage 2 has not been detected in countries in northern Europe, travelers to affected areas during the periods of vector activity are at risk of infection. This was illustrated by a human case of WNV infection in a 73 years-old Belgian woman who was visiting Greece in the summer of 2012 (40). Following her return to Belgium, samples of serum and cerebrospinal fluid were both positive for WNV IgM and IgG. The serum sample, taken 29 days after development of fever, was positive for WNV by real-time RT-PCR. A 116 base pair sequence derived from the amplicon was highly suggestive of the presence of WNV lineage 2.

Bird migration has been considered one of the major drivers for translocation of WNV (41–43). The emergence of WNV lineage 2 in Hungary followed by dissemination, both locally and to countries to the south could have resulted from translocation through infected bird movements. Two of the regions where the virus has emerged are dominated by wetland areas, namely the Po Delta in north-east Italy and the Aliakmonas Delta in northern Greece (44). Such areas attract migrating birds moving north from Africa and then returning south again from breeding grounds in central Europe. These areas are also associated with abundant populations of *Cx. pipiens* complex mosquitoes. Surveillance in these areas has detected WNV in pools of *Cx. pipiens*, and to a lesser extent *Cx. modestus* (45). This could drive the spread of WNV to indigenous bird species and eventually lead to spill-over infection in humans and horses. This coalescence of events, bird migration, landing periods in wetland areas, and peak vector abundance are needed to stimulate emergence, hence human cases tend to occur in late summer. The repeated emergence of WNV lineage 2 over subsequent summers suggests that over-wintering is occurring, supported mainly by phylogenetic evidence (20), although this is by no means conclusive as local re-introductions could give the same result. If over-wintering is occurring then this would likely be through infected adult females, the primary means of survival of *Culex* mosquitoes from 1 year to the next. The prevalence of

virus within the population would gradually increase through the subsequent summer, although always remaining at low levels relative to the total population of mosquitoes, but triggering spill-over infection in humans during late summer.

DISEASE CAUSED BY WNV LINEAGE 2 IN EUROPE

West Nile virus infection is typically asymptomatic. However, a febrile self-limiting illness is reported in around 20% of infected humans and is associated with headaches, myalgia, nausea, vomiting, and chills. A papular rash is reported in some cases, but generally symptoms resolve within 7 days (46–48). In approximately 1% of cases, WNV will enter the central nervous system, infecting neurons and cause neuroinvasive disease (49, 50). Neurological forms are varied and can include encephalitis, meningitis, meningoencephalitis, or acute flaccid paralysis (49). Symptoms are exacerbated by old age and immunosuppression. A follow-up study from the 2010 Greece epidemic reported anorexia, muscle weakness, memory loss, and depression to be the most common sequelae in a group of elderly patients who suffered from WNND. Only 31.8% (7/22) patients recovered fully (48–50).

Equines have been reported to be susceptible to lineage 2 strains, with an increased risk of developing WNND. Clinical signs in horses include ataxia, weakened limbs, paresis, complete paralysis, seizures, chewing, partial blindness, and jaundice/hepatitis (16).

Lineage 2 has been reported with varying mortality across the affected regions. The reasons for this may be linked to the previously characterized threonine 249 to proline (T249P) substitution within the NS3 gene, which was present in WNV isolates responsible for the Greek outbreak but not the Hungarian or Italian isolates (16, 51). However, an Italian strain identical to the Nea Santa-Greece 2010 strain (with T249P) was reported from the Veneto region of Italy, although there has been an absence of human disease as had been observed in Greece (25, 52). The Romanian outbreak of WNV lineage 2 in 2010 had an 8.8% fatality rate with 57 cases of WNND compared to a previous outbreak of WNV lineage 1 in 1996, which had a 4.4% fatality rate.

PHYLOGENY OF WNV LINEAGE 2 AND STRAIN VARIATION

West Nile virus has a single-stranded RNA genome of approximately 11 kb in length. The genome encodes three structural proteins and seven non-structural proteins. A large number of complete WNV genomes have now been sequenced and can be used for phylogenetic comparison with emerging viruses. This has greatly assisted in the investigation of the likely origins of WNV emergence. The 2004 lineage 2 WNV strain was isolated from a wild goshawk (*Accipiter gentiles*) in Hungary, and the genomic sequence of this isolate demonstrated closest homology with a group of southern African strains (53). The introduction of WNV lineage 2 into the wetlands of Hungary could have occurred through migratory birds that had become infected in Africa and remained sufficiently viremic during migration to infect mosquitoes in Europe on arrival (23). However, the goshawk is not considered a migratory bird, suggesting that this African lineage 2 strain must have been transmitted by local mosquitoes prior to detection in 2004.

Genetic characterization of the lineage 2 strain detected in Greece confirmed that it was most closely related to the strain that had previously emerged in Hungary (25). Similarly, strains detected in Serbia in 2012 were most closely related to strains previously identified in Greece, Hungary, and Italy (39). The strains detected in Italy in 2013 show closest homology with lineage 2 strains isolated in Italy in 2011 and Austria in 2008 (27). These observations suggest that the spread of lineage 2 throughout Europe was due to spread of the 2004 Hungarian strain, rather than from separate incursions via migratory birds from Africa. Migratory birds could also have played a role in the spread of lineage 2 WNV in Europe during the southerly migration. During autumn, turtle doves (*Streptopelia turtur*) migrate from breeding areas in central Europe to wintering areas in Africa, and serological studies on individual birds on their arrival in resting areas of Greece suggest that they were exposed to WNV lineage 2 in the area of origin in central Europe, and may constitute a candidate for introduction of lineage 2 strains from central Europe to Greece (37).

The mutation T249P within non-structural protein 3 (NS3) of Greek lineage 2 strains is similar to that observed in neuroinvasive lineage 1 isolates (51), while analysis of Italian strains isolated in 2013 has identified a number of amino acid substitutions including E638K and D831G within the NS5 protein (51). **Figure 2** highlights the relationship between lineage 2 strains detected in Europe, and the southern African strains from which they may have originated. The isolates used for phylogenetic analysis are detailed in **Table 2**. The figure demonstrates the close homology between strains from Italy, Austria, Greece, Serbia, and Hungary (marked by a bracket), with clear divergence between these strains and strains from Africa and Russia, suggesting that lineage 2 WNV might now circulate in a separate enzootic cycle within Europe. A number of these viruses

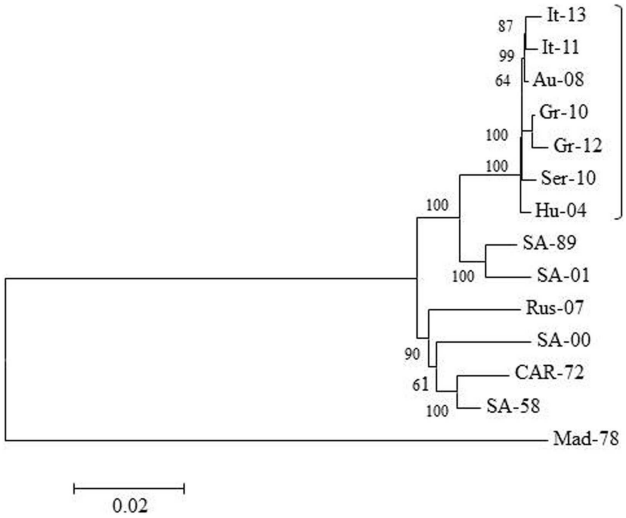

FIGURE 2 | Phylogeny of WNV lineage 2 in Europe and Africa. The neighbor-joining tree was generated from an alignment of complete WNV genomes (10,350 base pairs) using MEGA 5 software. Each sequence identifier represents the country of origin and the year of isolation. Further details on each virus are provided in **Table 2**. Bootstrap values are shown at key nodes and were derived from 1000 replicates. The divergent virus MAD-78 is used as an outgroup.

Table 2 | Details of WNV lineage 2 sequences used for phylogenetic analysis in Figure 2.

Sequence ID	GenBank accession no.	Original ID	Species	Country	Year
Hu-04	DQ116961	Goshawk-Hungary/04	*Accipiter gentilis*	Hungary	2004
Gr-10	HQ537483	Nea Santa-Greece 2010	*Culex pipiens*	Greece	2010
Gr-12	KF179639	Greece/2012/Kavala.39.1	Human	Greece	2012
It-13	KF588365	Italy/2013/Rovigo/32.1	Human	Italy	2013
It-11	JN858070	Italy/2011/AN-2	Human	Italy	2011
Au-08	KF179640	Austria/2008-gh	*Accipiter gentilis*	Austria	2008
Ser-10	KC496016	Novi Sad-2010	*Culex pipiens*	Serbia	2010
Rus-07	FJ425721	Reb_VLG_07_H	Human	Russia	2007
SA-89	EF429197	SPU116/89	Human	South Africa	1989
SA-01	EF429198	SA93/01	Human	South Africa	2001
CAR-72	DQ318020	ArB3573/82	*Culex tigripes*	Central African Republic	1972
SA-58	EF429200	H442	Human	South Africa	1958
SA-00	EF429199	SA381/00	Human	South Africa	2000
Mad-78	DQ176636	Madagascar-AnMg798	*Coracopsis vasa*	Madagascar	1978

were derived from human cases, confirming the zoonotic nature of these outbreaks.

MOSQUITO SPECIES ASSOCIATED WITH DISEASE TRANSMISSION: THE CASE FOR EMERGENCE IN THE UNITED KINGDOM

Outbreaks of WNV are associated with abundant populations of mosquitoes, which can occur as a result of flooding and subsequent dry and warm weather, or formation of suitable larval breeding habitats (54). Although WNV has been isolated from over 40 species of mosquitoes, the principal mosquitoes involved in WNV transmission belong to the genus *Culex*, particularly species in the *Cx. pipiens* complex (55, 56). Two species are formally recognized in the complex, *Cx. pipiens* (northern, temperate regions) and *Cx. quinquefasciatus* (southern, tropical regions) (57). In the UK, the subgenus *Culex* is represented by the nominotypical *Cx. pipiens* (58), which include two forms, *Cx. pipiens* f. *pipiens* and *Cx. pipiens* f. *molestus*. In addition, *Cx. torrentium* and *Cx. europaeus* are also recorded in the UK (59). These species are fairly common and widespread, with the exception of *Cx. europaeus*, for which few records are available. A further species, *Cx. modestus*, was also believed to be rare in the UK (59). However, recent inventories of UK mosquito fauna have revealed that populations of *Cx. modestus* are well established and commonly found in the North Kent marshes (60, 61).

The occurrence and abundance of potential vector species are a prerequisite for enzootic transmission of mosquito-borne viruses in the UK. Thirty-four species of mosquitoes have been recorded in the British Isles, of which nine species have been implicated in WNV transmission elsewhere (62). Thirteen species are likely to be bridge vectors as they readily bite both birds and humans. In Britain, the ecology of, and the potential risk of WNV transmission by, mosquito species have been detailed by several authors (62–64).

Cx. pipiens sensu stricto (*s.s.*) bite both humans and birds; the two forms within this species are morphologically indistinguishable, but they are physiologically different. *Cx. pipiens* f. *pipiens* is mostly ornithophagic and rarely bite humans, the immature stages are found in permanent water, it overwinters in the adult stage and is multivoltine (62). By contrast, *Cx. pipiens* f. *molestus* is highly anthropophilic (though it may bite birds), the immature stages live underground (e.g., flooded basements, sewer tunnels, underground railway systems), and all life stages occur throughout the year (63). The females can be nuisance biters in winter. Medlock et al. (64) stated that the form *molestus* might pose a threat for WNV transmission in suburban and rural areas. Where both forms are sympatric, hybridization can result (65, 66). This leads to increased numbers of mammophilic mosquitoes that can affect transmission of WNV (67). In the UK, the increased use of water containers in private gardens has also been cited as a possible factor that could lead to increases in mosquito abundance in urban areas that in turn could lead to more nuisance biting and increase the risk of WNV maintenance should it be introduced (68).

Golding et al. (60) showed that *Cx. modestus* accounted for 73% of all mosquito species from all sites sampled in the county of Kent and was collected in strong association with *Anopheles maculipennis sensu lato (s.l.)*. Although the risk of WNV transmission to humans in the UK is still low due to limited human exposure to bridge vectors, the risk for transmission was higher in Kent because of the presence of other bridge vector species such as *Cx. pipiens s.l.* and both migratory and resident birds. The authors highlighted the potential risk for horses, which are often grazed in this part of country. At present, screening of specimens of *Cx. modestus* for WNV and other flaviviruses such as Usutu virus is being undertaken by cross-governmental groups in the UK under a "One Health" initiative.

INVASIVE MOSQUITO SPECIES

The increase in human population, expansion of trading routes and tourism, deforestation, and climate change are some of the factors that have facilitated the rapid dispersal of vector species into new geographical areas (69). Recent emergence of bluetongue virus and outbreaks of WN fever and Chikungunya fever in Europe are just a few examples of the risk of exotic vector-borne pathogens being transported to a new region (69).

For invasive mosquitoes in Europe, six exotic species have been identified (*Aedes aegypti, Ae. albopictus, Ae. atropalpus, Ae. japonicus, Ae. koreicus, and Ae. triseriatus*). These have mainly been imported through the international trade in used tires and Lucky bamboo, although public and/or private ground transport have also been implicated. Of these, *Ae. albopictus* presents the greatest threat. Female *Ae. albopictus* lay desiccation-resistant eggs above the surface of the water in tree holes, tires, or other water-holding containers (70). Its ability to breed in artificial containers has facilitated its spread in recent decades along major transportation routes. Currently, *Ae. albopictus* is considered one of the top 100 invasive species of mosquitoes worldwide (71).

Aedes albopictus is a known vector of CHIKV, and laboratory infectious studies have shown that this species is a competent vector of WNV (72). It has been recorded in 20 European countries including the Netherlands and Belgium (its northernmost latitude) (73), although it has not been recorded in the UK. Surveillance for this and other invasive mosquito species should be a priority as their presence would radically change the risk of virus emergence.

CONCLUSION

West Nile virus lineage 2 was introduced into Europe in 2004 and subsequently emerged in a number of countries. It has overwintered in these countries and in the case of Italy is co-circulating with WNV lineage 1 and Usutu virus. Migratory birds are likely to be the main vehicle of movement for the virus, both as a source of introduction and the means by which it has spread within Europe. However, there is little direct evidence to support this. The disease manifestations appear to vary in different countries. In Hungary, raptors appear to be affected while in Greece the main burden of disease has fallen on the human population. The reasons for this are not clear, but the variation could be caused by mutations within particular virus strains that persist in different locations. The abundance and composition of mosquito populations, particularly *Culex* species, are critical for the spread of disease. Further study of factors such as mosquito distribution, host biting preference, and species hybridization will improve understanding of WNV persistence and assessment of the risk to human populations.

Following detection of WNV, there is a need to implement public health measures to protect at risk populations, particularly the elderly. This includes measures to reduce mosquito biting such as destruction of larval habitats and applications of larvicides. Measures that protect the individual include the application of mosquito repellents and clothing that reduces the exposure of bare skin. Currently, there is no human vaccine available. The risk of further spread of WNV lineage 2 to countries around the Mediterranean Sea is high. Countries in northern Europe appear to be at lower risk due to reduced mosquito abundance and lower winter temperatures. However, constant vigilance is needed to monitor for any change in environmental or ecological conditions that could make the introduction and persistence of WNV in northerly latitudes possible.

AUTHOR CONTRIBUTIONS

Nicholas Johnson conceived the idea for this review. Luis M. Hernández-Triana, Claire Jeffries, Karen Mansfield, George Carnell, Anthony Fooks, and Nicholas Johnson prepared the manuscript. All authors read and approved the final manuscript.

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Recent outbreaks of Rift Valley fever in East Africa and the Middle East

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Rift Valley fever (RVF) is an important neglected, emerging, mosquito-borne disease with severe negative impact on human and animal health. Mosquitoes in the *Aedes* genus have been considered as the reservoir, as well as vectors, since their transovarially infected eggs withstand desiccation and larvae hatch when in contact with water. However, different mosquito species serve as epizootic/epidemic vectors of RVF, creating a complex epidemiologic pattern in East Africa. The recent RVF outbreaks in Somalia (2006–2007), Kenya (2006–2007), Tanzania (2007), and Sudan (2007–2008) showed extension to districts, which were not involved before. These outbreaks also demonstrated the changing epidemiology of the disease from being originally associated with livestock, to a seemingly highly virulent form infecting humans and causing considerably high-fatality rates. The amount of rainfall is considered to be the main factor initiating RVF outbreaks. The interaction between rainfall and local environment, i.e., type of soil, livestock, and human determine the space-time clustering of RVF outbreaks. Contact with animals or their products was the most dominant risk factor to transfer the infection to humans. Uncontrolled movement of livestock during an outbreak is responsible for introducing RVF to new areas. For example, the virus that caused the Saudi Arabia outbreak in 2000 was found to be the same strain that caused the 1997–98 outbreaks in East Africa. A strategy that involves active surveillance with effective case management and diagnosis for humans and identifying target areas for animal vaccination, restriction on animal movements outside the affected areas, identifying breeding sites, and targeted intensive mosquito control programs has been shown to succeed in limiting the effect of RVF outbreak and curb the spread of the disease from the onset.

Keywords: RVFV outbreaks, *Aedes* mosquitoes, rainfall, East Africa

INTRODUCTION

Rift Valley fever (RVF) is an important neglected, emerging, mosquito-borne disease with severe negative economic impact as it affects human and animal health. The disease is caused by RVF virus (RVFV) an acute febrile arbovirus in the *Phlebovirus* genus and Bunyaviridae family. The disease was first characterized by Daubney et al. (1) while working at the Veterinary Research Laboratory at Kabete in Kenya. An earlier report by Stordy (2) had described a similar disease syndrome, which may well have been RVF, it was described as an acute and highly fatal disease in the Rift Valley in exotic wool sheep, which had been imported into East Africa from Europe (1, 3). These European stock species were more severely affected than native African stock. The disease remained a veterinary concern in East Africa until a major outbreak occurred in Egypt in 1977. A second outbreak outside East Africa occurred in 2000 when RVF moved into Saudi Arabia and Yemen in the Arabian Peninsula (4). This was the first time the disease was being detected outside of Africa – where it had been confined so far – becoming a threat to the Middle East.

From the most recent outbreaks that occurred in Kenya, Somalia, Tanzania in 2007 (5, 6), and Sudan in 2008 and 2010 (7, 8), RVF appears to have great potential for spreading into new areas and with huge impact on human and animal health. This calls for an integrated approach between different governmental sectors and organizations within and between countries and regions to address both human and animal health. Limited information is available on the evolution of RVF between East Africa and Middle East. In order to highlight the urgent need of establishing a health system for controlling RVF in the region, this review article aims to gather experiences and highlight basic information on the ecological aspects, epidemiological, and risk factors associated with the distribution of recent outbreaks in East Africa and Middle East.

TRANSMISSION AND IMPACT

The virus is known to infect a range of animal hosts including sheep, cattle, goats, camels, buffaloes, and others. The incubation period in animals is between 1 and 6 days in general, 1 and 3 days in sheep, and only about 12 and 36 h in lambs (9). Sheep and to a lesser extent cattle were the principle disease hosts in both East and southern Africa (3). Sheep seemed to be the most susceptible animal as it was noted that RVF caused high rates of abortions during pregnancy and high mortalities among newborns (10, 11). Lambs can die before they acquire passive immunity and mortality and abortion rates among old sheep range from 5 to 100% (9). Infections can therefore cause severe disease and result in significant economic losses. For example, the 2007 outbreak was the most widespread affecting livestock in 11 regions in Tanzania and Kenya. A total of 16,973 cattle, 20,193 goats, and 12,124 sheep died of the disease, with spontaneous abortions reported in 15,726 cattle, 19,199 goats, and 11,085 sheep (12, 13). Considering the wide-ranging impacts of the disease on the livestock sector and other segments of the economy, the 2007 RVF outbreak in Kenya alone induced losses of over Ksh 2.1 billion (US$32 million) on the Kenyan economy (14). The overall economic loss in East Africa is estimated to exceed $60 million because of disruption in trade from these recent epizootics between 2006 and 2007 (15). In Saudi Arabia, during the outbreak of 2000, it was estimated that around 40,000 animals including sheep, goats, camels, and cattle died whereas 8,000–10,000 of them aborted (16). The outbreaks of 2007 in Sudan led to bans in livestock trade between Saudi Arabia and Sudan, resulting in vast economic impact on the animal market in the two countries (17).

Infection by RVF usually spreads among livestock first through mosquitoes bites. In addition, the infection can also be transmitted vertically between animals (18) (**Figure 1**). From domestic animals, the virus is transmitted to humans mainly through direct contact with blood, excreta, meat, or secretions of infected animals, consumption of raw milk (19–21), and in few cases, transmission through mosquito bites that belong to the genera *Anopheles*, *Aedes*, and *Culex* seems to occur (22, 23) (**Figure 1**). Symptoms of RVF in humans vary from a flu-like syndrome to encephalitic, ocular, or hemorrhagic syndrome. The case fatality rate of the hemorrhagic syndrome form can be as high as 50% (24). The most severe outbreaks of 1997–1998 and 2006–2007 in Tanzania, Kenya, and Somalia caused 478 human deaths in 1998 and 309 in 2007 (25–27). The outbreak of 2000 resulted in 883 human cases with 124 deaths (case fatality rate, 14%) in Saudi Arabia (28) and 1,328 human cases, with 166 deaths in neighboring north western Yemen (29–31). In Sudan, the outbreak of 2007 resulted in 698 cases, including 222 deaths (32, 33).

MOSQUITO VECTORS

Mosquitoes in the *Aedes* genus have been considered the primary maintenance host and source of RVFV that initiate disease outbreaks (34, 35). RVFV is known to be carried in the eggs of *Aedes* mosquitoes, which can survive for several years in the dried mud (36). On flooding, *Aedes* mosquito species play an important role in initiation of infection and virus circulation. The survival of RVFV during inter epizootics was believed to depend on transovarial transmission of the virus in flood water by *Aedes* mosquitoes (37). Other mosquitoes in the *Culex* and *Anopheles* genus are thought to be important in amplification of virus activity during outbreaks. The virus has also been detected in phlebotomine sand flies, *Culicoides* midges, and *Amblyomma* tick species although these infections are thought not to play an important role in the life cycle of the virus or in disease outbreak settings (38–42). In the laboratory, the RVFV was also transmitted trophically or mechanically by other hematophagous flies but field relevance of these transmission routes are still unclear (43, 44).

The important RVF vectors in East Africa include *Aedes mcintoshi*, *Aedes ochraeus*, *Culex pipiens*, *Aedes dalzieli*, and *Aedes vexans* (45). Records also indicated that *A. mcintoshi* is the main vector for RVF in Kenya (41, 42, 46). Investigation on RVFV by reverse transcription-polymerase chain reaction (RT-PCR) during the recent outbreak of 2006/2007 in Kenya showed that 77 out of 3,003 pools representing 10 species, from 4 affected districts, tested positive for RVFV, including *A. mcintoshi*/*circumluteolus* (26 pools), *Aedes ochraceus* (23 pools), *Mansonia uniformis* (15 pools); *Cx. poicilipes*, *Culex bitaeniorhynchus* (3 pools each); *Anopheles squamosus*, *Mansonia africana* (2 pools each); *Culex quinquefasciatus*, *Culex univittatus*, *Aedes pembaensis* (1 pool each). *A. pembaensis*, *Cx. univittatus*, and *Cx. bitaeniorhynchus* were for the first time observed positive for the virus (42). The observation of infected *A. ochraceus* in Garissa, Kenya, represents a new RVFV-vector association in East Africa. *A. ochraceus* is a known vector of RVFV in West Africa (39), along with *A. vexans arabiensis* and *A. dalzieli*. *A. vexans arabiensis* is also a vector of RVFV in Saudi Arabia (40, 44) and although the species has not been documented in Kenya, it has been found in neighboring Somalia and Sudan (47, 48) (**Table 1**).

It has been suggested that different mosquito species serve as epizootic/epidemic vectors of RVFV in diverse ecologies, creating a complex epidemiological pattern in East Africa (42). *A. aegypti* has been found naturally infected with RVFV and seemed to be the main source of the infection during the outbreak of 2007 in Sudan. During this outbreak, RVFV was successfully detected by RT-PCR in larvae, male and females of *An. arabiensis*, *An. cousteni*, *Cx. pipiens* complex, *Cx. poicilipes*, and *A. aegypti* (**Table 1**). The infections were considered as a precursor for viral circulation in these species (incriminated in dissemination or acquired the virus in its mid gut only) (21). The detection of RVFV in male and larval stages indicated transovarial (vertical) transmission of the virus within these mosquito species. It may also show possible venereal RVFV transmission when a male is infected vertically and then infects the female during mating (21).

Laboratory established colonies of *A. aegypti* from Tahiti exhibited the highest infection rates of RVFV when compared with other potential vectors in the Mediterranean region (56). *A. aegypti* has also demonstrated infection and transmission rates of the nonstructural proteins (NSs) deletion virus similar to wild-type virus, but dissemination rates were significantly reduced (35). *Cx. pipiens* was incriminated as the main RVF vector in Egypt based on field isolates and also in Maghreb and South Africa based on laboratory experiments (57–59).

OCCURRENCE OF RVF OUTBREAKS

The RVF has demonstrated capacity for emerging in new territories or for re-emerging after long periods of silence. Since the

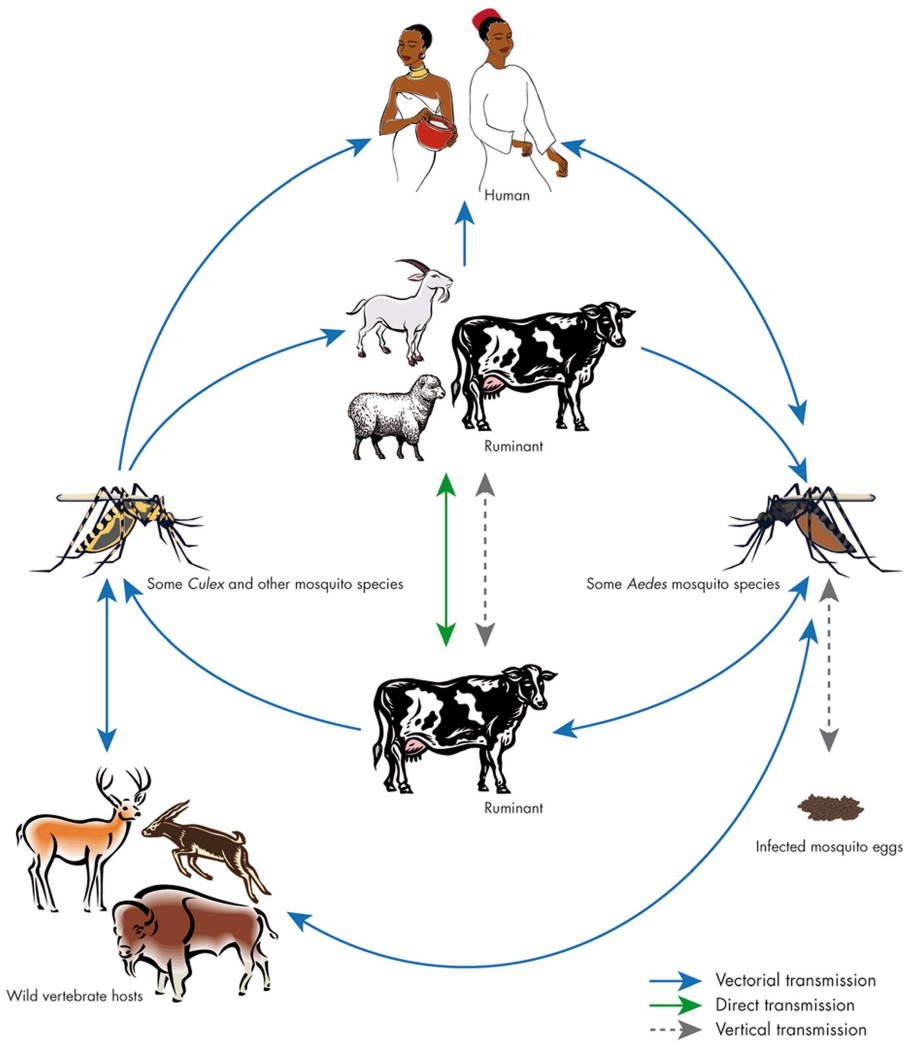

FIGURE 1 | Cycle of Rift Valley fever. The virus can be maintained in an enzootic cycle involving *Aedes* mosquitoes, which are able to transmit the virus vertically to their offspring. Epizootic outbreaks are often linked with unusual rains or warm seasons, favoring the hatching of infected *Aedes* eggs that are then able to initiate the virus circulation. Subsequently, large numbers of secondary vectors belonging to the *Culex* genus could be infected and induce the emergence of epidemic/epizootic outbreaks. Transmission to humans occurs through direct contact with high-virus loads when aborted fetuses are manipulated. Source: Balenghien et al. (34), with permission from Thomas Balenghien, CIRAD, UMR Contrôle des maladies, F-34398 Montpellier, France.

first outbreak in 1915, epizootics occurred periodically in Kenya until the disease was recognized in South Africa in 1951 (60), when humans became ill after handling dead and infected animals (3). Further, RVF outbreaks have been confirmed in most sub-Saharan countries (61) moving through the Rift Valley from Kenya to Tanzania, Zimbabwe, Zambia, and subsequently, RVF outbreak was first recorded in 1987 in West Africa in Senegal and Mauritania (3). RVF spread northwards through the Nile Valley into Southern Sudan and to White Nile state in Sudan where the first outbreak was identified in 1973. The disease then spread among other neighboring states within the country (**Figure 2**) and up to the Egyptian delta where a major epidemic with 20,000–200,000 clinical illnesses and 600 deaths was reported in 1977 (3, 62, 63). The disease also spread from continental Africa to Madagascar in 1991 (64–67) and to the Arabian Peninsula in Saudi Arabia and Yemen in 2000 (4). The recent RVF outbreaks in East Africa in Somalia (2006–2007) (5), Kenya (2006–2007) (6), Tanzania (2007) (5), and Sudan (2007–2008) (7) showed the changing epidemiology of the disease from being originally associated with livestock to infecting humans considerably and resulting in high-fatality rates (7).

Sindato and others investigated the spatial and temporal pattern of RVF outbreaks in Tanzania over the past 80 years (68). All RVF outbreaks reported during 1930–2007 were found to occur between December and June. Expansion of the disease into new geographical areas from the original documented outbreaks was observed. For example, between 1930 and 1957 only <1% of the districts in Tanzania were repeatedly involved in the outbreaks (**Figure 3**). The 1977–1978 outbreak wave had involved 3.33%

Table 1 | Mosquito species incriminated in the transmission of RVFV during the outbreaks recorded in East Africa and the Middle East.

Year of outbreak	Affected country	Collected mosquitoes	Reference
1997–1998 and 2006	Kenya	*Culex zombaensis, Culex poicilipes, Culex bitaeniorhynchus, Culex quinquefasciatus, Culex univittatus, Anopheles coustani, Anopheles squamosus, Aedes mcintoshi, Aedes ochraceus, Aedes pembaensis Mansonia africana, M. uniformis*	(42, 49)
1997–1998 and 2007	Tanzania	*Aedes mcintoshi*	(6)
1997–1998	Eastern Africa	*Culex theileri*	(50)
1977	Egypt	*Culex pipiens*	(51, 52)
2000	Kingdom Saudi Arabia	*Culex pipiens, Aedes vexans arabiensis, Ae. Vittatus, Ae. (Stegomyia) nilineatus, Aedes vexans arabiensis*, and *Culex triteniorynchus*	(30, 40, 53–55)
2000	Yemen	Not defined	(30, 54, 55)
2007–2008	Sudan	*Cx pipiens, Cx. Poicilipes, An. arabiensis, An. coustani, Ae. aegypti*	(5, 21, 49)
1997–1998 and 2006–2007		Not defined	(6)

districts. A relatively larger outbreak wave in 1997–1998 involved 7.70% of the districts and the widespread outbreak in 2006–2007 involved humans and domestic ruminants in 39.17% of the districts in the country (**Figure 4**). However, despite this expansion into districts, which were not involved before, RVF outbreaks still show significant spatio-temporal clustering in eastern Rift Valley during the last 80 years in Tanzania (68). The space-time clustering of livestock and human cases showed a tendency to spread from the north to east-central and western parts of the country (**Figures 3** and **4**). Uncontrolled livestock movement has been suggested as being responsible for the geographical expansion and the cumulative effect of the amount of rainfall was considered the main cause of the outbreaks (68–72). It has been suggested that the bimodal rainfall pattern experienced in this ecosystem provides an environment for *Aedes* mosquito species to emerge in large numbers at the onset of the rainy season, and therefore, resulting in extensive biting rates and transmission of the virus in animals and humans (68).

It is well established that RVFV outbreaks occur predominantly after unusual flooding events. *Aedes* mosquito species are seen as reservoir, as well as vectors, since their transovarially infected eggs withstand desiccation and larvae emerge when the eggs get into contact with water (37, 73). Transovarial transmission is assumed as the mechanism of virus persistence between epizootic events. After flooding, the infected *Aedes* mosquito eggs will hatch in the persisting water collections, and develop into infective adult mosquitoes. A study in the Ferlo region of Senegal in 2003 observed that when the rainy season began with heavy rains, the temporary ponds that serve as the breeding sites for mosquitoes were flooded to their maximum level immediately. As a result, *A. vexans arabiensis* populations were found to be abundant at the very beginning of the season, when the majority of eggs in quiescence were flooded (74). The effect of flood water on *Aedes* breeding habitats has also been studied artificially in central Kenya by sequentially flooding such habitats to determine the numbers of mosquito eggs hatching during each flooding. The authors documented that approximately 90% of the larvae sampled during four flooding events emerged during the initial one (75). This probably explains why excessive rainfall can result in high density of initial infected population. This hypothesis was supported by the study from Senegal, which found that female mosquitoes hatching from eggs laid during the previous year quickly laid eggs on the pond's wet soil (74). The study also observed that during rainless periods lasting longer than 7 days, the time needed for embryogenesis, these new eggs undergo dormancy as the water level goes down. Once, the rains fall again, large numbers of new eggs hatch resulting into an increase in population, and thereby suggesting that several generations of infected adults can exist during the same rainy season. This dynamic has been seen to also maximize the virus' chance to persist from one year to another in high-stock population, thus, facilitating endemisation of RVFV that is then amplified through feeding of infected adult female mosquitoes on wild and domestic ungulates and may reach epizootic and epidemic dimensions (42, 74).

This dynamic can be observed from the 2007 RVF outbreak in Gezira State, Sudan (**Figure 5**), when satellite monitoring (June–September, 2007) showed that most of the central Sudan could be unusually subjected to heavy rainfall (76). Accordingly, a RVF risk warning has been generated for central and southern Sudan. Indeed, the predicted unusually heavy rains occurred during July–August and resulted in severe floods (77). In September, suspected human RVF cases were reported (78). The first cases appeared in southern areas of Algabalain locality in White Nile state. The first symptoms among the suspected cases were hemorrhage and fever with rapid death. All reported cases in the beginning of the outbreak were scattered and did not reach any health facilities (21). First human index case was confirmed on 8–14 October, 2007 (76). The RVF outbreak in Sudan came to public attention on October 18, 2007 when the Federal Ministry of Health (FMoH) Sudan asked the WHO to assist in the investigation and control of a suspected hemorrhagic fever. The WHO and FMoH teams started investigations in the White Nile state, central Sudan, on October 24, and on the basis of initial results, an outbreak of RVF was declared on October 28, and more help was requested for control measures (79). An announcement was made regarding RVF in animals (80) when the outbreak reached its peak in humans by early November (**Figure 5**). At the end of the outbreak as of late December 2007

FIGURE 2 | Sudan map shows states with confirmed Rift Valley fever cases are in boldface during 2007 and 2010 outbreaks. Source: Aradaib et al. (8), with permission from Stuart T. Nichol, Centers for Disease Control and Prevention, Atlanta, GA, USA.

to January 2008, a cumulative total of 698 cases, including 222 deaths, was reported from six states (Gezira, Kassala, Khartoum, River Nile, Sennar, and White Nile), yielding an overall CFR of 31.8%. This RVF outbreak was the first one reported in humans and connected directly to heavy rainfall, flooding, and increase in mosquito breeding sites in Sudan (81).

The RVF outbreak of 2007 in Sudan not only validated the association between abnormal rainfall and RVF outbreak but also prediction of RVF outbreak and early warning signs from satellite monitoring. This also showed that the wave of RVF outbreak is likely to end as the water pools due to rainfall and warm temperatures faded-out (68). This is indeed the case as there is only one short-rainy season in Sudan, which ends in October and then the winter season begins at the end of November and runs up to late February. The presumed link between extraordinary flooding events and RVF outbreaks was also well validated, among others, by a successful prediction of the 2007 outbreak in Somalia, Kenya, and northern Tanzania, using climate modeling (82). In fact, each of the seven documented moderate or large RVF outbreaks that have occurred in East Africa over the last 60 years have been associated with El Niño Southern Oscillation (ENSO) associated with above normal and widespread rainfall (83). This association of RVF with

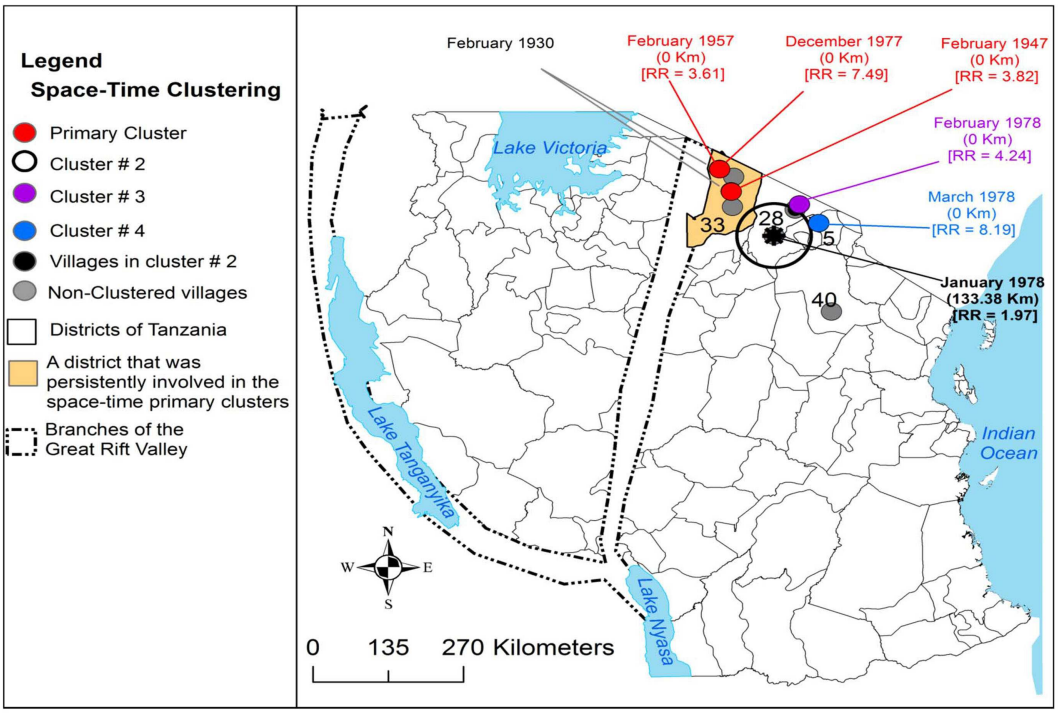

FIGURE 3 | Distribution of village-level space-time clusters of RVF cases from 1947 to 1978. The authors set model parameters for maximum spatial and temporal window sizes and that such cluster could include a maximum of 50% of all cases. They indicated there were no clusters detected in 1930, from 1947 to 1978 three primary clusters were persistently detected in Ngorongoro district, each involving one village. An asterisk represents the center of cluster that involved more than one village; relative risk for each cluster is displayed (RR) along with the buffer (circle) size in kilometers (km). Source: Sindato et al. (68), with permission from Calvin Sindato, National Institute for Medical Research, Tabora, Tanzania.

excessive rainfall and flooding was also observed in other countries outside the African continent in Arabian Peninsula, i.e., the outbreak of 2000 in Yemen (84).

Interestingly, all RVF outbreaks in Sudan originated in White Nile State where the first RVFV was identified in 1973 as the cause of an extensive epizootic (10), then moved northward through the White Nile river valley to Khartoum in 1976 (85) and extended to the neighboring states of Gezira, Sennar, and Kassla states during 2007 outbreak (**Figure 1**). In the White Nile State, the river Nile has a very wide basin, which floods annually between June and September, resulting in wide wetland along the valley of the river from the border of Southern Sudan up to Khartoum. It is important to mention that the five states are located in the Central Clay Plain soil of the Sudan, which extends from west of Kassala through Gezira, Khartoum, White Nile up to southern Kurdufan. This type of soil and topography when flooded create large shallow wetlands similar to what is known as "*dambo*," which is often shown as suitable breeding habitats for *Aedes* mosquitoes in central, southern, and eastern Africa. This suggested that local environment is very important and is directly linked to RVF outbreak. Significant association was observed between RVF outbreaks from 1930 to 2007 and clay and loam soil textures in the eastern Rift Valley ecosystem of Tanzania where clustering of RVF outbreaks were persistently and predominantly detected (68). Clay soil rather than sandy soil texture supports long-period retention of water contributing to flooding and wetness of habitat suitable for breeding and survival of *Aedes* mosquito vectors. This suggests that while rainfall might be the major determinant for the onset and switch-off of an outbreak, it is unlikely that it is the only factor responsible for the spread and clustering of RVF cases. A causal association between local environmental factors, livestock density and movement, encroachment of mosquitoes into new areas, and occurrence of RVF has been suggested in previous studies (20, 86, 87).

RISK FACTORS DURING RVF OUTBREAK

It is generally accepted that during the 2007 outbreak in Sudan, animal contact was the most dominant risk factor followed by animal products and mosquito bites (78). This is supported by the fact that the 2010 outbreak was first characterized by abortions in ewes followed by infections in persons with histories of contact with aborted fetal material (8). Contact with RVFV-infected animals such as consuming or handling products from sick animals, touching an aborted animal fetus, or being a herdsperson has been documented as the most important risk factor for severe infection during the 2007 outbreak in Kenya (76). A similar result was observed during the previous RVF outbreak of 1997–1998 in northern Kenya (11). These findings are consistent with those from another study from Sudan stating that most of the animals such as sheep, cattle, goats, and camels stay very close to their owners' houses at night (21, 88).

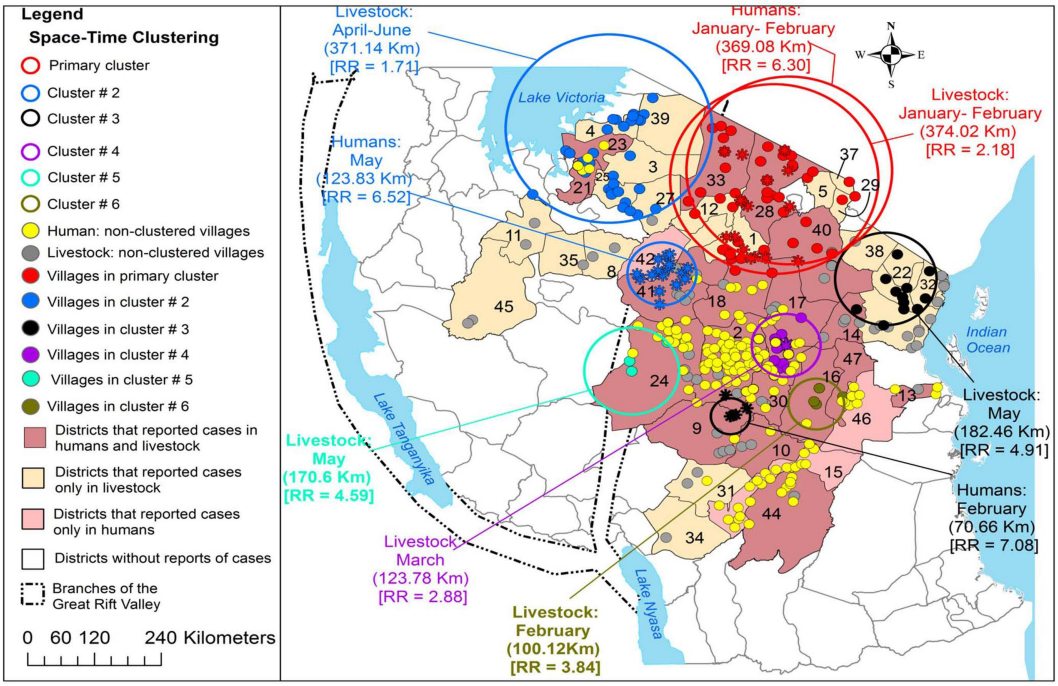

FIGURE 4 | Distribution of village-level space-time clusters of RVF cases in humans and domestic ruminants. The authors set model parameters for maximum spatial and temporal window sizes and that such cluster could include a maximum of 50% of all cases. They conducted the analysis of clustering of cases separately for humans and domestic ruminants during the 2006/2007 outbreak wave. Between January and February 2007, there was an overlap of livestock and human primary clusters in the same location. Asterisks correspond to villages that were included within human space-time clusters; relative risk for each cluster is displayed (RR) along with the buffer (circle) size in kilometers (km). Source: Sindato et al. (68), with permission from Calvin Sindato, National Institute for Medical Research, Tabora, Tanzania.

Despite the fact that there was no evidence for horizontal transmission between humans in Sudan or elsewhere, risk from infected pregnant women through vertical transmission can occur. During the 2007 outbreak in Sudan, a 29-year-old pregnant woman presented in early labor with symptoms suggestive of RVF and delivered a baby weighing 3.2 kg with skin rash, palpable liver, and spleen. Two samples from the mother and neonate were screened and found to be positive for RVF-IgM (89). This case demonstrated that RVF can be vertically transmitted in human. A similar case was also reported before in Saudi Arabia, during the 2000 outbreak (90). These are consistent with the claims recently made about the burden of emerging zoonotic infectious disease among women in general and pregnant women, in particular (91).

Movement of animals during an outbreak can be a serious risk factor. Complete genome sequences from RVFV strains detected during the 2007 and 2010 outbreaks in Sudan suggested multiple introductions of RVFV into Sudan as part of sweeping epizootics from eastern Africa (8). All RVFV strains observed grouped into Kenya-1 or Kenya-2 sub lineages, which defined the eastern Africa outbreak in 2006–2008 (92). The sequencing also suggested that an earlier common ancestor from 1996 coinciding with the 1997–1998 outbreaks in the horn of Africa. The Kenya-2 sub lineage is now known to be widely distributed in Tanzania and Sudan (8, 92, 93). The movement of animals from southern states in White Nile to northern ones in Gezira, Khartoum, and Kassala for marketing was most likely responsible for the geographical expansion of the virus in central and eastern Sudan (**Figure 2**). Identical or nearly identical sequences of the virus strains were identified for different states and years, Khartoum in 2007 and Gezira in 2010, as well as Khartoum and West Nile in 2007. These sequences indicate recent movement of the virus in this region and support the necessity and utility of surveillance systems for recognizing when and where a large epidemic is imminent (8).

SURVEILLANCE AND CONTROL OF RVF OUTBREAK: THE EXAMPLE OF SAUDI ARABIA IN 2000

Saudi Arabia and Yemen experienced a huge RVF outbreak in the year 2000 (29, 30, 54, 55). It was the first outbreak in Middle East outside its endemic areas in Africa. The outbreak in Saudi Arabia is suggested to have been from eastern Africa by importation of infected animals (40), similar to the suggested route of introduction of RVFV into Egypt in 1977 from Sudan (94). The virus causing the Saudi Arabia outbreak belonged to the same strain that caused the 1997–1998 outbreaks in East Africa (95).

After the outbreak was declared, a team was established in collaboration between the Ministries of Health, Agriculture, and Water, and the Ministry of Municipalities and international organizations such as CDC, WHO, and National Institute of Virology, South Africa, to control the outbreak (30, 54, 96). A strategy called "One Health" was then implemented by Saudi Arabia targeting

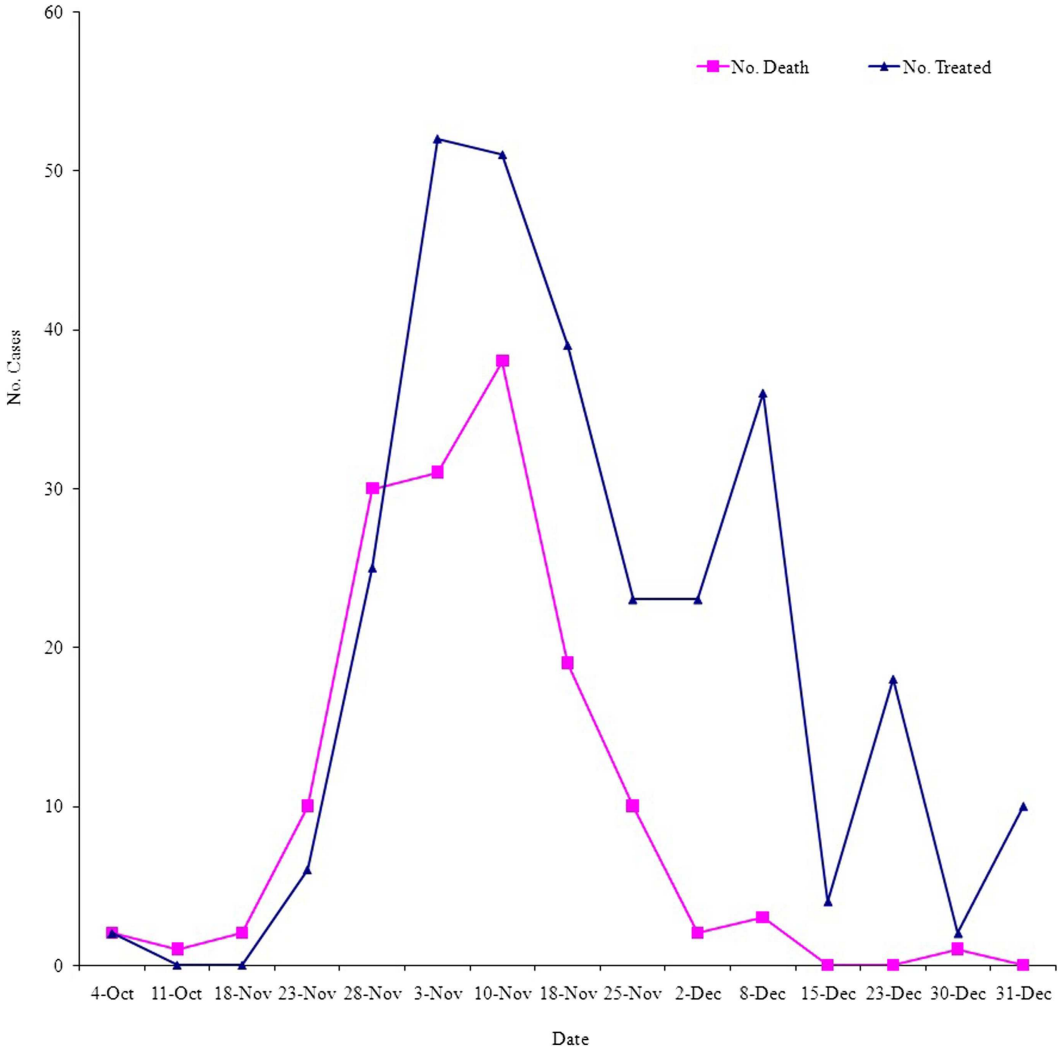

FIGURE 5 | Cases (No. received the treatment) and deaths from RVF over the period of the outbreak in Gezira State, Sudan, September–December 2007. Source: Epidemiology Unit, Ministry of Health, Gezira State.

both the animal and human hosts (17). The urgent integrated control measures that were implemented by this strategy during the outbreak included the following activities: (1) disposal of dead animals in an appropriate manner, (2) active surveillance surveys to detect cases of RVF among humans and animals to locate target areas for animal vaccination, and (3) apply a vaccination campaign that started in October 2000 (16, 30, 31, 54, 96). Around 1,200,000 doses of the vaccine were reported to be imported into Saudi Arabia and the campaign continued in 2001 with more than 10 million ruminants being vaccinated (31). These activities were accompanied by (4) a restriction on animal movements outside the affected areas and a ban on animal imports from RVF-enzootic countries (29). (5) For effective case management, detailed case definition was developed, training sessions on how to manage the suspected cases clinically was implemented, two well-prepared laboratories (one in the affected regions and the other in the capital of the country) for diagnosis of RVFV antibodies in suspected cases were also provided by the Saudi Ministry of Health (29, 54, 96). (6) Epidemiological investigation was also performed to identify risk factors (30). (7) In addition, an entomological study to search for the mosquito breeding grounds (30) was followed by an intensive mosquito control program with spraying (54, 97). This strategy succeeded to limit the effect of the outbreak and curb the disease from spreading to other areas. Since 2000, only sporadic cases have been recorded in Saudi Arabia and only in the same regions where the original outbreak was reported (16). Later investigation on this strategy concluded that "One Health" approach is the best option to mitigate outbreaks of RVF. Collaboration between veterinary, health, and environmental authorities both at national and regional levels is needed to control RVF outbreak (17).

CONCLUSION AND PERSPECTIVE

From the foregoing narrative, we can conclude that RVF causes huge health and economic losses signified by the number of human

deaths and high mortality and abortion rates in livestock. It is also clear that whereas RVF was previously restricted to specific areas in sub-Saharan Africa, the disease seems to be spreading into new territories beyond the traditional foci as evidenced by outbreaks in the Arabian Peninsula. The epidemiology of RVF is complex and transmission involves multiple mosquito vector species. A multiplicity of factors shapes the epidemiology of RVF. Key among these is rainfall and flooding, soil types, contact with animals, breeding sites, and availability and movement of livestock. Epizootics are interspaced with long periods of quiescence.

It is our considered view that repeated outbreaks could be forestalled with adequate sensitization of the policy makers. It is also clear that with enhanced coordination among stakeholders, e.g., Ministries of Health and Livestock, researchers, and local communities it is possible to better handle future outbreaks. Such coordination of stakeholders seems to have worked effectively in managing the outbreak in Saudi Arabia. Other regions such as eastern Africa that has borne the brunt of previous outbreaks should learn from the Saudi experience. In light of improved warning signs derived from satellite imagery and mapping, governments should come up with clear strategies and action plans for preparedness and handling of future outbreaks. Such strategies should include strong surveillance systems, adequate and well trained personnel, among others.

AUTHOR CONTRIBUTIONS

Yousif E. Himeidan suggested the topic, framed, drafted, and wrote up the manuscript. Mostafa M. Mahgoub collected the data on patients from Ministry of Health, Gezira State. Eliningaya J. Kweka, El Amin El Rayah, and Johnson O. Ouma drafted and reviewed the manuscript. All authors read and approved the final version.

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Comparative study of the pathological effects of western equine encephalomyelitis virus in four strains of *Culex tarsalis* Coquillett (Diptera: Culicidae)

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Early reports suggested that mosquito cells infected with arboviruses remain viable and undamaged. However, more recent experimental evidence suggests that arboviral infection of mosquito tissues might indeed result in pathological changes, with potential implications for vector survival and virus transmission. Here, we compare the pathological effects of western equine encephalomyelitis virus (WEEV) infection in four strains of *Culex tarsalis* previously reported to differ in their competence as WEEV vectors. Pathological effects were observed in cells of the midgut epithelium, salivary glands, and eggs. Cell rounding and sloughing of midgut epithelial cells was associated with those strains reported to be the least susceptible to WEEV infection, whereas midgut necrosis and vacuolation upon infection were associated with strains showing higher susceptibility. Although pathological effects were sporadically observed in infected salivary glands, further studies are required to evaluate their impact on vector competence. Additionally, the potential implications of observed *C. tarsalis* egg infection with WEEV are discussed.

Keywords: arbovirus, *Culex tarsalis*, mosquito, pathology, vector competence, western equine encephalomyelitis

INTRODUCTION

Transmission of a mosquito-borne virus to a vertebrate host requires mosquito ingestion of a viremic blood meal, subsequent infection of the mosquito's midgut cells, spread to tissues within the hemocoel, and finally infection of the salivary glands. Following completion of this "extrinsic incubation period," virions must be released into the saliva and injected into a new host during a subsequent blood meal. Previous investigations have described benign, non-pathological, and chronic viral infections resulting in continuous virus production throughout the lives of infected mosquitoes (1–3). However, pathological effects have also been observed both *in vivo* (4–10) and *in vitro* (11). Weaver et al. (6) reported pathological changes, including cell sloughing and tissue necrosis in *Culex tarsalis* that fed on viremic blood meals containing western equine encephalomyelitis virus (WEEV; Togaviridae, *Alphavirus*). Furthermore, it has been proposed that the effects exerted by viral infection in the mosquito can influence vectorial capacity (12).

The temporal dynamics of WEEV infection and associated variations in transmission have been described for four strains of *C. tarsalis* that differed in their susceptibility to WEEV (13, 14). Here, we describe varying tissue pathology associated with WEEV infection in these four strains and discuss the potential influence of these variations on vector competence.

MATERIALS AND METHODS

MOSQUITO REARING AND INFECTION

Mosquito rearing and handling methods used in this study have been previously described (13). Briefly, larvae were reared at 22–24°C, with a 16 h light:8 h darkness photoperiod, and were fed ground alfalfa pellets and AquaMax® (Purina Mills, LLC; St. Louis, MO, USA). Adults were maintained under a similar photoperiod at 26°C and were provided a 10% sucrose solution *ad libitum*.

Four strains of *C. tarsalis* were used in the current study: (a) WEEV resistant (WR), (b) high viremia producer (HVP), (c) Coachella Valley (COAV), and (d) Kern National Wildlife Refuge (KNWR). The WR and HVP strains were selected for refractoriness or high susceptibility, respectively, to infection with WEEV (the HVP strain was derived from the original WEEV susceptible –WS- strain) (15, 16) and have been maintained at the University of California Arbovirus Field Station since the mid 1980s. In preparation for this study, the HVP and WR strains were reselected for several generations by examining the susceptibility of single families (13). The COAV and KNWR strains were collected in California's Riverside and Kern counties, respectively, and had been maintained as unselected colonies for 2 years prior to this study.

For viral infections, we used the WEEV strain BFS1703, which was isolated from *C. tarsalis* collected in Kern County, California, in July 1953 (17) and has been widely used for evaluating the competence of *C. tarsalis* to transmit WEEV (15, 18, 19). Virus was passaged twice in suckling mice and once in Vero cell culture prior to the beginning of the study.

The method used to infect mosquitoes has been previously described (13). Briefly, three to five day-old mated females were starved for 18 h and then allowed to engorge on viremic blood via an artificial membrane feeder (13, 20). Blood solutions contained *ca.* 3 or 5 $\log_{10}$ plaque forming units (PFU) of WEEV per 0.1 ml of chicken blood containing 14.3 freeze dried

USP units of sodium heparin per milliliter (Becton-Dickson, Franklin Lakes, NJ, USA). Hereafter, these viral doses will be designated as '3-log' and '5-log'. The 5-log dose was comparable to viremias produced by competent avian hosts that were able to infect most competent vectors, whereas the 3-log dose was similar to that produced by a less competent host, but still able to infect highly susceptible mosquito hosts such as the HVP strain. Doses below this were insufficient to infect most mosquitoes (21).

For uninfected controls, mosquitoes were allowed to feed on virus-free blood by the same method. Fully engorged females were transferred to an incubator maintained at 26°C and 18 h light:6 h darkness photoperiod, and provided with 10% sucrose solution that was changed daily.

IMMUNOCYTOCHEMISTRY

For each strain, two uninfected controls and five individuals fed on each viral dose were collected at days 1, 2, 3, 4, 7, 14, and 21 post-infectious blood meal (DPI). These mosquitoes were immobilized on wet ice, killed and fixed by injection of 10% buffered formalin (pH 7.5), and stored in 100% ETOH until further processed. Subsequently, these specimens were dehydrated, cleared, and infiltrated with paraffin as previously described (22, 23), embedded in paraffin blocks, cut into 10 μm thick serial longitudinal sections using an American Optical® 820 Spencer™ microtome (American Optical Co., New York, NY, USA), mounted on microscope slides, and stored at 4°C until used for immuno-staining.

Mounted sections were immuno-stained as previously described (14). Briefly, the avidin–biotin-peroxidase complex (ABC) technique was applied, using a 1/1,600 dilution of mouse anti-WEEV ascites fluid as the primary antibody, and the horse-anti-mouse Vectastain Elite® ABC kit (Vector laboratories, Burlingame, CA, USA) for detection, following the manufacturer's protocols. Stained sections were examined for the presence of viral antigens – as evidenced by the rusty-brown color generated by the ABC technique (**Figure 1**) (22)– as well as for the presence of any pathological changes, using a Nikon® Optiphot™ compound microscope (Nikon Instruments Inc., Melville, NY, USA) equipped with a digital Spot RT™ camera (Diagnostic Instruments, Sterling Heights, MI, USA).

DATA ANALYSIS

Statistical analyses were performed using the SPSS® software package version 13.0 for windows (SPSS Inc; Chicago, IL, USA). Chi-Square tests were used when comparing overall frequencies (i.e., time groups pooled together) between 3- and 5-log groups in each strain. If no significant differences ($P > 0.05$) between the dose groups were found within a strain, dose groups were pooled for further analysis; otherwise, each dose group was analyzed separately.

Analyses of differences among strains were performed using Kruskal–Wallis (K–W) tests because frequency data were not normally distributed for at least one strain in each one of the parameters studied. If K–W tests indicated significant ($P < 0.05$) differences among strains, *post hoc* analysis was performed by applying Chi-Square tests to all pair-wise combinations of strains. To maintain an overall alpha level of 0.05, a Bonferroni correction was applied to the *post hoc* testing.

RESULTS

Pathological changes in infected individuals were consistently observed in the midgut epithelium (**Figures 1–3**) and eggs (**Figure 4**). Additionally, atypical cellular morphology was sporadically observed in the salivary glands of infected individuals (**Figure 5**); however, the consistency of salivary acini often caused them to be detached from the slides during the washes required for immunocytochemical staining, making it impossible for us to obtain consistent data across all experimental groups for this particular tissue. Therefore, only the pathological changes observed in the midgut and eggs will be further reported and discussed.

MIDGUT PATHOLOGY

Three types of pathological changes were found in infected midguts: vacuolation, necrosis, and cell rounding and sloughing (CRS, **Table 1** and **Figure 6**).

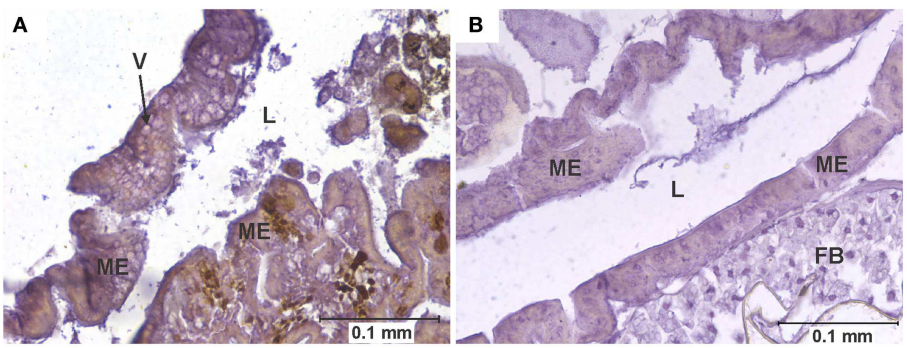

FIGURE 1 | Vacuolation of midgut epithelium. **(A)** Section of the posterior midgut of a specimen of the COAV strain, 3 days after ingesting a blood meal containing 5-log PFU of WEEV per 0.1 ml blood. Notice the extensive formation of vacuoles in the cytoplasm. Rusty-brown staining is indicative of a positive immunocytochemical reaction, denoting the presence of WEEV antigen in the tissue. **(B)** Comparable section of the posterior midgut in an uninfected control. FB, fat body; L, midgut lumen; ME, midgut epithelium; V, vacuole.

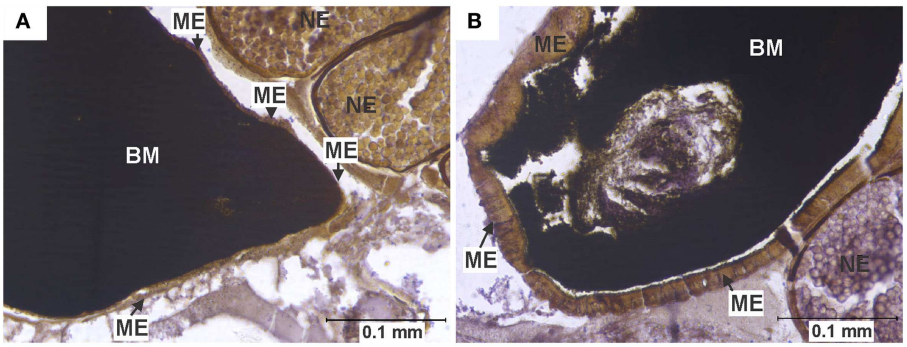

FIGURE 2 | Midgut epithelium necrosis. **(A)** Section of the posterior midgut of a specimen of the HVP strain, 2 days after ingesting a blood meal containing 5-log PFU of WEEV per 0.1 ml blood. Notice how the epithelium has become necrotic, being reduced to a very thin band, with neither discernible cell boundaries nor traces of cytoplasm or organelles. **(B)** Comparable section of posterior midgut in an uninfected control, where no necrosis is observed. BM, blood meal (in the midgut lumen); ME, midgut epithelium; NE, non-infected egg.

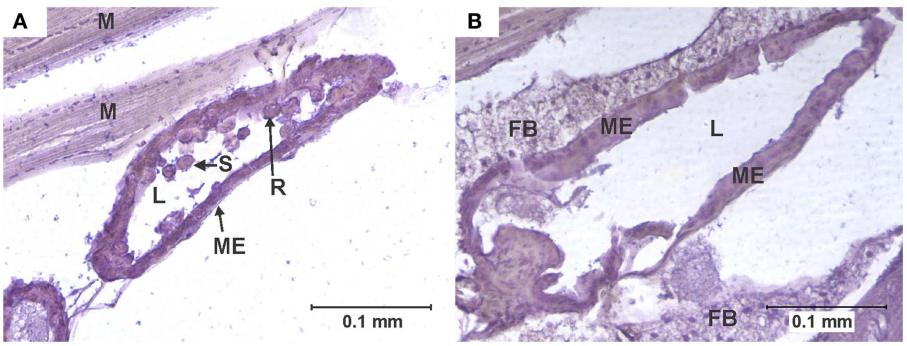

FIGURE 3 | Cell rounding and sloughing (CRS). **(A)** Anterior midgut section of a specimen of the KNWR strain, 14 days after ingesting a blood meal containing 3-log PFU of WEEV per 0.1 ml blood. Notice how several epithelial cells have sloughed-off into the lumen, and some rounded cells protrude from the tissue. **(B)** Comparable section of anterior midgut in an uninfected control, where no CRS is observed. FB, Fat body; L, midgut lumen; M, skeletal muscle; ME, midgut epithelium; R, rounded epithelial cell; S, sloughed epithelial cell.

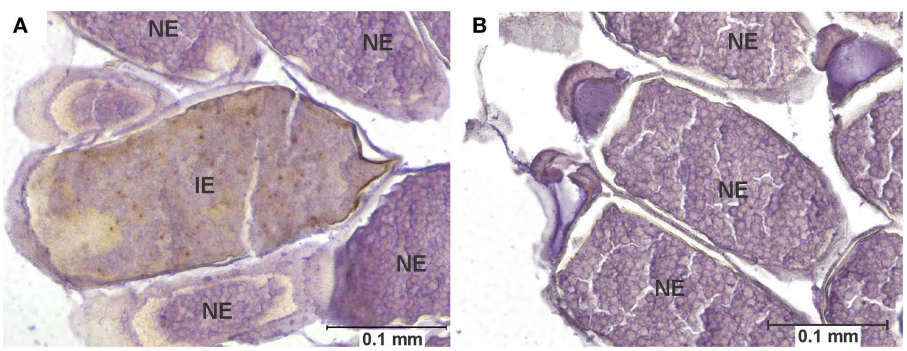

FIGURE 4 | Egg infection and pathology. **(A)** Longitudinal section of the abdomen of specimen of the COAV strain, 21 days after ingesting a blood meal containing 5-log PFU of WEEV per 0.1 ml blood. Rusty-brown staining is indicative of a positive immunocytochemical reaction, denoting the presence of WEEV antigen in the tissue. Notice the smooth yolk texture observed in infected egg, in contrast with the uniformly granular texture of yolk observed in the neighboring uninfected eggs. **(B)** Comparable section of the abdomen of a specimen of the COAV strain, showing only normal, uninfected eggs. IE, infected egg; UE, uninfected egg.

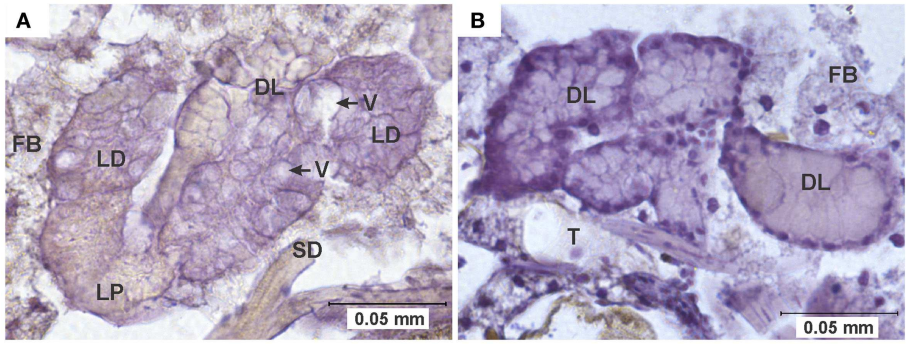

FIGURE 5 | Pathology in salivary glands. **(A)** Salivary gland of a specimen of the HVP strain, 7 days after ingesting a blood meal containing 5-log PFU of WEEV per 0.1 ml blood. Notice the extensive cytoplasmic vacuolation of acinar cells, and the absence of staining indicative of viral antigen. **(B)** Comparable section of a normal salivary gland from an uninfected control. DL, distal lobe; FB, fat body; LD, lateral-distal lobe; LP, lateral-proximal lobe; SD, salivary duct; T, trachea; V, vacuole.

Table 1 | Overall frequencies of the different types of pathology found in the midgut of four different strains of *C. tarsalis* infected with WEEV.

	HVP			WR			COAV			KNWR		
	Control ($n = 14$)	**3 log ($n = 35$)**	**5 log ($n = 35$)**	**Control ($n = 14$)**	**3 log ($n = 35$)**	**5 log ($n = 35$)**	**Control ($n = 10$)**	**3 log ($n = 35$)**	**5 log ($n = 34$)**	**Control ($n = 14$)**	**3 log ($n = 34$)**	**5 log ($n = 35$)**
CRS (%)	0	14	14	29	17	20	10	31	18	0	15	17
Vacuolization (%)	0	23	31	0	6	0	0	6	21	0	6	9
Necrosis (%)	0	0	9	0	0	0	0	11	15	0	0	0

COAV, Coachella Valley; CRS, cell rounding and sloughing; HVP, high viremia producer; KNWR, Kern National Wildlife Refuge; WR, WEEV resistant.

VACUOLATION

Arbovirus replication has been associated with the intense proliferation of intracellular vacuoles 0.3–2 μm in diameter, which are thought to be major sites of viral nucleic acid replication and virion assembly (24–26). In our study, specimens were recorded as presenting vacuolation when abundant vacuoles of the appropriate size were observed in the cytoplasm of midgut cells (**Figure 1**). This type of pathology was observed in all strains in the 3-log group, and in all but the WR in the 5-log group (**Figure 6**). In agreement with previous studies (24), no vacuolation was observed in uninfected controls.

Although vacuolation was observed as early as one DPI (COAV, 5-log group; **Figure 6**), there was no particular trend in the frequency of specimens showing vacuolation over time. Furthermore, no significant differences ($P > 0.05$) were found between dose groups in the overall frequency of individuals presenting vacuolation in any strain. There were, however, significant differences between strains ($X^2 = 21.23$; df = 3; $P < 0.001$), with the HVP presenting vacuolation at significantly higher frequencies than both the WR ($X^2 = 16.19$; df = 1; $P < 0.001$) and the KNWR ($X^2 = 9.63$; df = 1; $P = 0.002$).

NECROSIS

In close agreement with reports by Weaver et al. (6), midgut tissue necrosis was evident as a gross degeneration of cellular integrity, to the point where midgut tissue was reduced to a thin layer consisting almost exclusively of basal lamina and traces of plasma membrane (**Figure 2**).

In experimentally infected specimens, necrosis was found only in the HVP (5-log group) and COAV (both dose groups) strains, consistently at two DPI (**Figure 6**). In these strains, no significant differences ($P > 0.05$) in the frequency of specimens showing necrosis were found between dose groups. Additionally, the overall frequency of specimens showing necrosis was not significantly different between the HVP and COAV strains ($P > 0.05$).

No signs of necrosis were observed in uninfected controls of any strain.

CELL ROUNDING AND SLOUGHING

An individual was recorded as presenting CRS when midgut epithelial cells were observed to be either completely detached from the midgut epithelium or clearly protruding into the midgut lumen (6, 27) (**Figure 3**).

Among infected mosquitoes, CRS was observed in all strains and dose groups (**Figure 6**). Statistical analysis failed to reveal significant differences ($P > 0.05$) in the overall frequency of CRS both between dose groups within each strain, or between strains. Interestingly, CRS was also observed in uninfected controls of the WR and COAV strains (**Figure 6**). No significant differences ($P > 0.05$) were found between the WR and COAV strains relative to the frequency of individuals presenting CRS in infected or uninfected control groups.

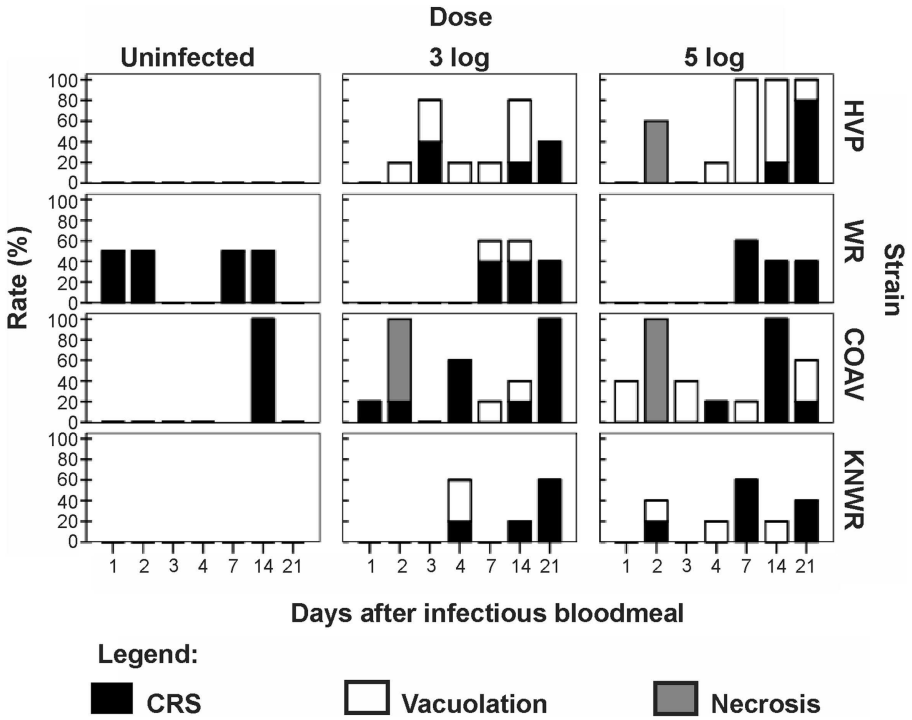

FIGURE 6 | Frequency of pathological changes in the midgut of *C. tarsalis*. Cell rounding and sloughing was the only pathology found in uninfected individuals. Signs of necrosis were only found in HVP and COAV strains, consistently at 48 h after infection. COAV, Coachella Valley; CRS, cell rounding and sloughing; HVP, high viremia producer; KNWR, Kern National Wildlife Refuge; WR, WEEV resistant.

CRS was not seen in uninfected controls in the HVP and KNWR strains.

EGG PATHOLOGY

Eggs were interpreted as displaying pathology when all of the following characteristics were seen together: (a) positive immunocytochemical staining, indicative of the presence of viral antigen, (b) an unusually 'smooth' yolk texture in contrast with the granular texture of the yolk in normal eggs, and (c) distortion of the chorion (**Figure 4**).

Among infected mosquitoes, the HVP strain showed signs of egg pathology in both the 3- and 5-log groups. The COAV and KNWR strains showed signs of egg pathology only in the 5-log group. No evidence of egg pathology was observed in the WR strain, or in uninfected controls of any strain (**Figure 7** and **Table 2**).

From a total of six females presenting egg pathology among all infected mosquitoes, five (83%) had been incubated for >14 DPI and one specimen (17%) had been incubated for two DPI (**Figure 7**). Statistical analysis revealed no significant differences ($P > 0.05$) between dose groups or strains in the frequency of infected individuals showing egg pathology.

DISCUSSION

Pathological changes were observed in the midgut, salivary glands, and eggs of WEEV-infected mosquitoes. These changes, which included CRS, vacuolation, necrosis, and egg yolk smoothing, were consistent with other reports of pathological effects of arboviruses in mosquito vectors (4, 6–9, 28) and contribute to a growing body of evidence that challenges the traditional belief that the impact of arboviral infection on mosquito cells is negligible (1–3).

The fact that CRS was observed in uninfected controls as well as infected mosquitoes is consistent with the notion proposed by Okuda et al. (27) that mosquitoes can regularly replace midgut epithelial cells following a blood meal, thus eliminating cells damaged by the toxic by-products of blood digestion. Others have suggested that viral infection triggers a high frequency of apoptosis and CRS in midgut cells, probably as a mechanism that modulates the viral load in this important tissue (6, 10, 29). Interestingly, uninfected controls of the WR strain presented the second highest frequency of CRS in our study (**Table 1**). Such high intrinsic turnover rate of midgut epithelial cells may enable the WR strain to eliminate and replace infected cells before they become significant foci of viral multiplication. This mechanism could, at least in part, account for the WR strain's refractoriness to WEEV infection.

Alternatively, CRS observed in specimens that did not receive an infective blood meal could be due to infection of our experimental strains with mosquito-specific viruses, which have been reportedly found in wild populations of *C. tarsalis* from various geographic locations (30).

Vacuolization and necrosis were observed only among individuals that received an infectious blood meal, suggesting that these types of pathological changes are closely associated with viral infection. Vacuolation has been reported as a result of

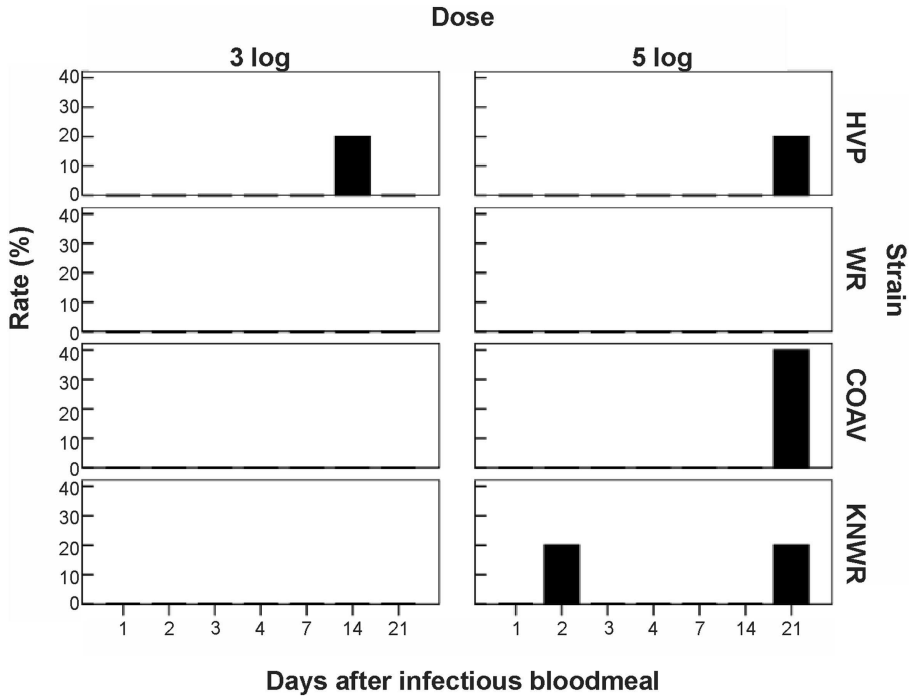

FIGURE 7 | Frequency of virus-induced pathology in eggs of *C. tarsalis*. No significant differences ($P > 0.05$) in the number of individuals presenting pathological eggs were found either between dose groups or between strains. COAV, Coachella Valley; HVP, high viremia producer; KNWR, Kern National Wildlife Refuge; WR, WEEV resistant.

Table 2 | Overall frequencies of individuals presenting egg pathology associated with WEEV infection.

	HVP		WR		COAV		KNWR	
	3 log ($n = 35$)	5 log ($n = 35$)	3 log ($n = 35$)	5 log ($n = 35$)	3 log ($n = 35$)	5 log ($n = 34$)	3 log ($n = 34$)	5 log ($n = 35$)
Frequency (%)	3	3	0	0	0	6	0	6

COAV, Coachella Valley; HVP, high viremia producer; KNWR, Kern National Wildlife Refuge; WR, WEEV resistant.

arthropod-borne virus replication in infected cells (24, 25), which is consistent with our observation that the highest vacuolation rates were found in the HVP strain, and the lowest in the WR strain (**Table 1** and **Figure 6**). Furthermore, the significantly lower vacuolation rates observed in the KNWR strain compared to the HVP strain (**Table 1**) suggest a lower intensity of viral replication in the former strain, and are therefore consistent with reports that found the KNWR strain to be relatively refractory to WEEV infection (13, 14). It is interesting to note that vacuolation and necrosis were in some instances observed in tissues showing no evidence of WEEV antigen presence, as indicated by the absence of immunostaining (**Figure 5**). This suggests that either tissues, which had been initially infected eventually managed to clear the virus (but the pathological effect persisted), or that these tissues are infected at levels below the detection threshold of the immuno-staining methods used. Alternatively, as was the case with CRS, we cannot rule out the possibility that these pathological changes are related to the unintentional infection of our experimental strains with mosquito-specific viruses (30).

Earlier studies by Kramer et al. (31) indicated that resistance to WEEV infection in *C. tarsalis* mosquitoes of the WR strain was intimately linked to a mesenteronal barrier, because direct injection of virus into the hemocoel resulted in infection rates and viral titers comparable to those observed in highly susceptible strains. Furthermore, it has been proposed that ultrastructural alterations of the midgut, such as those caused by the ingestion of a blood meal, can be associated with increased susceptibility to viral infection (15, 32). Therefore, it is plausible that a disruption of the mesenteronal barrier caused by an infectious blood meal (for example, the midgut tissue necrosis observed in our study) would increase the odds of virus dissemination into the hemocoel, and subsequent infection of the salivary glands. Consistent with this idea, the HVP and COAV strains (which do display midgut necrosis following an infectious blood meal) have been observed

to reach higher dissemination and salivary gland infection rates than the WR and the KNWR strains (14). In close agreement with observations by Weaver et al. (6), evidence of midgut necrosis disappeared by 72 h post-infectious blood meal, suggesting that this tissue has the ability to quickly recover from widespread virus-induced pathological changes.

Infected eggs displaying pathological changes were observed in three of the four strains used in this study (HVP, COAV, and KNWR) suggesting that WEEV infection in *C. tarsalis* eggs is not uncommon, even in unselected geographic strains of relatively recent colonization such as the COAV and KNWR. Interestingly, five out of six (83%) females presenting egg pathology had been incubated for at least 14 DPI, forcing them to retain eggs in their bodies for much longer than they would under natural conditions (<5 days post-blood meal). Although we cannot rule out the possibility that the egg pathology observed in these females is a response to the aforementioned forced retention of eggs, the positive immuno-staining observed in all eggs recorded as "displaying signs of pathology" indicates that they were indeed infected with WEEV. Therefore, it seems plausible that the pathological changes observed in these eggs are related to WEEV infection.

Additionally, it is worth mentioning that the only strain presenting egg infection in the low dosage (3-log) group was the HVP strain, which is characterized by developing unusually high WEEV titers (16). In contrast, the only strain that showed no egg infection was WR, which is characterized by its ability to maintain low WEEV titers (16). These observations suggest that a viral titer threshold must be reached in the infected mosquito before egg infection – and associated pathology – can occur.

Evidence of *C. tarsalis* egg infection with WEEV has been reported (33); however, subsequent field and laboratory studies have failed to produce evidence of transovarial transmission of WEEV in *C. tarsalis* (18, 34). Furthermore, although vertical transmission of WEEV in field-collected *Aedes dorsalis* has been reported once in the past (35), extensive efforts have failed to replicate this phenomenon in the laboratory (18, 36). Taken together, these data suggest that vertical transmission of WEEV in mosquitoes is a rather rare event.

In our study, all eggs found to be positive for WEEV antigen presented clear signs of pathological changes in their yolk and chorion, which probably rendered these eggs non-viable. Therefore, our data support the notion that *C. tarsalis* does not normally transmit WEEV transovarially (18, 34). Nevertheless, these infected eggs were surrounded by apparently healthy, viable eggs (**Figure 4**), suggesting that they could be oviposited as part of otherwise normal egg rafts. This sporadic occurrence of non-viable, virus-laden eggs may explain why Thomas (33) was able to isolate virus from *C. tarsalis* egg rafts deposited by orally infected females, but could not conclusively demonstrate transovarial transmission of WEEV. Interestingly, a similar phenomenon has been observed in *C. tarsalis* infected with West Nile virus (WNV); although egg infection is frequent, trans-generational transmission occurs only rarely (W.K. Reisen, personal communication).

Romoser et al. (28), referring to the infection of *A. mcintoshi* eggs with Rift Valley fever virus, hypothesized that the oviposition of virus-laden eggs might have important epidemiological consequences, as it represents a mechanism by which viral particles could be deposited directly in the aquatic environment inhabited by mosquito larvae, which could eventually ingest these virions. The ingestion of infective viral particles during larval stages has been reported to result in transstadially transmitted infections, producing adult mosquitoes that are able to transmit virus to new hosts when they blood feed (37). Our observations of WEEV infection in *C. tarsalis* eggs are consistent with the hypothesis proposed by Romoser and his collaborators, and suggest that this process could take place in at least some mosquito/virus systems, therefore potentially playing a role in the environmental persistence of vector-borne viruses.

As noted in the results, difficulties in salivary gland tissue preparation and immuno-staining precluded a systematic assessment of salivary gland pathology at this time. However, several cases of atypical cellular morphology (vacuolation) were observed in the salivary glands of infected individuals (**Figure 5**). Salivary gland pathology associated with arbovirus infection has been found in other studies (7–9, 28, 38). Girard et al. (8, 9) have suggested that WNV-induced damage to either salivary glands or ganglia controlling salivation in *Culex pipiens and Culex quinquefasciatus* might result in reduced volumes of saliva being expectorated, which would in turn cause a reduction in feeding efficiency and the viral load injected into new hosts. Furthermore, it has been reported that long-term arboviral infections result in progressive declines in transmission rates and/or the volume of virus expectorated by infected mosquitoes (9, 13, 19). Although the instances of salivary gland pathology we observed in this study are consistent with the idea of the progressive decline of transmission rates due to damage to salivary cells, more research is needed to establish the exact role of salivary gland pathology in vector competence.

AUTHOR CONTRIBUTIONS

Marco V. Neira, Farida Mahmood, William K. Reisen, Calvin B. L. James, and William S. Romoser designed the experiments. Farida Mahmood reared mosquitoes, performed experimental infections and fixed specimens. Marco V. Neira and William S. Romoser performed immunocytochemical staining, microscopical analysis, data analysis, and wrote the manuscript. All authors reviewed, edited and approved the manuscript.

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The importance of veterinary policy in preventing the emergence and re-emergence of zoonotic disease: examining the case of human African trypanosomiasis in Uganda

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Rapid changes in human behavior, resource utilization, and other extrinsic environmental factors continue to threaten the current distribution of several endemic and historically neglected zoonoses in many developing regions worldwide. There are numerous examples of zoonotic diseases which have circulated within relatively localized geographical areas for some time, before emerging into new regions as a result of changing human, environmental, or behavioral dynamics. While the world's focus is currently on the Ebola virus gaining momentum in western Africa, another pertinent example of this phenomenon is zoonotic human African trypanosomiasis (HAT), endemic to south and eastern Africa, and spread via infected cattle. In recent years, the ongoing northwards spread of this disease in the country has posed a serious public health threat to the human population of Uganda, increasing the pressure on both individual families and government services to control the disease. Moreover, the emergence of HAT into new areas of Uganda in recent years exemplifies the important role of veterinary policy in mitigating the severe human health and economic impacts of zoonotic disease. The systemic challenges surrounding the development and enforcement of veterinary policy described here are similar across sub-Saharan Africa, highlighting the necessity to consider and support zoonotic disease control in broader human and animal health systems strengthening and associated development programs on the continent.

Keywords: veterinary services, neglected zoonotic diseases, public health, human African trypanosomiasis, Uganda, veterinary policy

INTRODUCTION

Human African trypanosomiasis (HAT or sleeping sickness) is classified by the World Health Organization (WHO) as a neglected zoonotic disease (NZD). Endemic across sub-Saharan Africa, HAT is transmitted to human beings through bites from the *Glossina* species of tsetse fly. Although not a novel or recently emerging disease, zoonotic HAT has been described as the "avian flu of its time" (1), associated with devastating epidemics and emergence into previously naive areas, as recently observed in Uganda (2). HAT is therefore an interesting case study through which to examine the impact of veterinary policy in mitigating the emergence and re-emergence of zoonoses into new regions; a topical issue given the unfolding Ebola crisis in parts of western Africa and other "worrying developments (*that*) show it might be time to reassess the old ideas about the distribution of these (*neglected tropical*) diseases" (3). The first purpose of this paper is to provide a short review of the history of HAT in Uganda, in order to contextualize the subsequent exploration of the factors that impede or facilitate the refinement and enforcement of veterinary policies in the country. Finally, the discussion of available options and recommendations to prevent further emergence and re-emergence of HAT in the country can be applied to zoonotic diseases more generally across the continent.

There are two forms of HAT, their geographic boundaries roughly separated by the Rift Valley. The acute, zoonotic *Trypanosoma brucei rhodesiense* form is found in eastern and southern Africa, and the chronic, non-zoonotic *Trypanosoma brucei gambiense* form occurs throughout western Africa. Animal African trypanosomiasis (AAT or "nagana") is the corresponding syndrome in livestock, caused by various species of trypanosome also transmitted by the *Glossina* tsetse fly. Tsetse and trypanosomiasis control and eradication programs in Africa have a long history, dating from colonial times when European powers were concerned with human epidemics and the loss of animal productivity associated with the disease (4). Control efforts were largely successful up until the 1960s; however, human cases have been rising steadily since independence, with 50–60 million people across the continent currently exposed to the bite of a tsetse fly (5). Despite "political will at the highest levels" to resurrect collective action for the control of human and AAT in Africa, many feel that there is still a long way to go before the disease regains the attention it deserves and reduces to the pre-1960s level (6, 7). The intersectoral

approach required for the control of tsetse and trypanosomiasis "lies at the heart of African rural development"; shown to benefit the livelihoods of the rural poor both directly through improved health and increased nutritional outputs of livestock and indirectly through improved agricultural productivity and subsequent food security via provision of livestock outputs such as draught power and manure (5, 8).

Sharing symptoms with malaria and HIV/AIDs, HAT is often misdiagnosed or underreported by health authorities, with estimations that of the 300,000 new cases of HAT every year, only 30,000–40,000 are recorded because of issues with accessibility and quality of health services, particularly in rural areas (5). With vague first stage symptoms including recurring fever, joint pain, and nausea, the diagnosis of second stage disease is even more difficult in the absence of trained professionals, given the requirement for the trypanosome parasite to be detected in a sample of cerebrospinal fluid. Once diagnosis is made, the treatment (particularly for *T. br. rhodesiense*) is severe, with death, resulting in around 10% of cases. Without treatment, however, patients infected with both the acute and chronic forms of HAT will likely die. The current gaps in funding and technology for HAT diagnosis and treatment are a telling indicator of its "neglected" status; in the case of *T. br. rhodesiense*, no new drugs have been developed for over 60 years.

"A SENSE OF URGENCY": RECENT EMERGENCE OF ACUTE HUMAN AFRICAN TRYPANOSOMIASIS IN CENTRAL AND NORTHERN UGANDA

Uganda is presently the only country to harbor foci of both the acute and chronic forms of HAT, with a focus of *T. br. gambiense* in the West Nile region to the northwest, and *T. br. rhodesiense* endemic across the southeast region of Busoga. The initial emergence of *T. br. rhodesiense* HAT in Uganda was thought to have occurred as a result of European invasion along the Congo River to Lake Victoria, with *T. br. gambiense* entering the country as a result of human migration from central to western Africa (9). Although tsetse flies have been in Uganda for "thousands of years," the public health impact and subsequent academic interest in the disease started during the early twentieth century, when it was estimated a third of Uganda's population died of acute HAT in burgeoning epidemics (9).

Progression in molecular technologies toward the end of the twentieth century established the domestic cattle population as the most significant animal reservoir in Uganda, essential for the maintenance of *T. br. rhodesiense* within human populations (10–12). Major HAT epidemics in Uganda's history have been associated with large cattle losses; over a million Ugandan cattle died in the 1890s, Rinderpest outbreaks across the country, resulting in large tracts of overgrown grazing land conducive to escalating tsetse infestations. It is thought that the tsetse flies "ran short" of cattle to feed on during this time, reverting to human blood meals, which perpetuated the spread of disease during this period (9). More recently, northwards spread of HAT has been associated with conflict and lord's resistance army (LRA) insurgence from the 1980s; people fled their homes, taking their livestock with them, which led to overgrowth of tsetse territory; "By 1990, there were only roughly one thousand cattle left in Soroti district as most of the cattle had died because of diseases, been rustled or eaten by the armed groups in the different conflicts . . . growth of forests as a result of the war attracted the tsetse flies . . . sleeping sickness was rampant" (9).

Recent net migration of human beings and livestock back into previously overgrown areas has been attributed to this northwards spread of disease during the latter part of the 1990s (11, 13, 14). Mass rural development programs, funded by both the Ugandan government and foreign donors, were implemented upon the return of civic stability to several districts in central and northern Uganda during the 1990s. A major re-stocking exercise commenced, promoting the return of cattle and other livestock back into the area to assist the resumption of agro-pastoral activities. By 2005, however, there was a public health crisis in Uganda; molecular technologies indicated acute *T. br. rhodesiense* HAT had spread into eight new districts in as many years, with only 150 km separating the acute and chronic foci of human disease (2). The spread of disease is thought to be largely attributed to cattle movement from infected *T. br. rhodesiense* areas in the southeast of the country to previously free areas further north. It is feared that overlap of the two disease foci will spark a public health nightmare; given the parasites are morphologically similar on blood and cerebrospinal fluid smears, the only way to determine the appropriate therapeutic treatment is to know which geographical area the human patient comes from. Furthermore, the clinical effects of mixed infection, and the optimal treatment protocol, are currently unknown.

METHODOLOGY

Combination qualitative research consisting of semi-structured interviews (SSIs), focus group discussions (FGDs), and observation techniques was recently undertaken in Uganda, in order to understand current veterinary policies and their level of enforcement, particularly regarding pre-movement treatment of livestock in HAT endemic areas. Key informant interviews ($n = 13$) were conducted with ministerial representatives and policy officers in the Ministries of Health and Agriculture at both the central and district government levels. In addition, field visits were undertaken in Soroti and Serere Districts in north-central Uganda, where HAT cases had recently emerged. Observations at local livestock markets complemented FGDs ($n = 16$) with farmers and local animal health officers. All key informant interviews were conducted in English, with the FGDs conducted in a mixture of Ateso and English, facilitated by a translator. The resulting transcripts were entered into Microsoft Word 2010 and manually coded according to themes, ideas, and opinions in order to develop the ensuing narratives presented in this manuscript.

RESULTS

A major objective of this research was to identify and analyze the current livestock movement and disease control policies that exist in Uganda, and subsequently their level of enforcement through direct observations and data collection at the district level. The major policy concerning zoonotic disease control within animal reservoirs in Uganda is the Animal Disease Act (1918), which describes the requirements for addressing outbreaks of notifiable zoonoses such as rabies, anthrax, and trypanosomiasis (15). A separate Veterinary Public Health Act exists to promote

meat inspection and milk hygiene for the control of food-borne zoonoses such as brucellosis and bovine tuberculosis. Both acts are in need of updating, however, at present "*these are the policies that are followed as no new ones have been written post-independence*" (Informant interview, government sector).

Specifically concerning livestock movement, the official policy within the 1918 Animal Disease Act states animals cannot be moved into new areas "without clearance from veterinary officers" (15). These livestock movement restrictions were further revised after the research linking cattle movement and spread of human disease was published, resulting in new policy requiring pre-movement trypanocidal treatment of all cattle in endemic regions (16). The current procedure that should therefore be followed when cattle move from HAT endemic areas in the country's southeast is contained within Section 18 of Uganda's Animal Disease Act entitled *Rules for Infected Areas*, with particular reference to the following items:

> Item 1: no stock or carcass shall be moved in or from any such area without the written permission of the commissioner of livestock and entomology or the veterinary officer or inspecting officer in charge of the area.
>
> Item 6: no person shall leave any such area without having complied with such precautions for preventing the spread of disease as may be required by the veterinary officer or inspecting officer in charge of the area.

A second objective of the ongoing HAT research in Uganda is to establish the extent to which this existing pre-movement treatment policy is currently enforced at district livestock markets in Uganda, particularly given its importance in decreasing disease into new areas and across international borders. When asked whether they have witnessed government veterinarians treating animals at the market places, the majority of farmers indicated this was done "*from time to time*," however, some were suspicious of veterinarians under-dosing "*as the dose is very small*" (Focus Group Participants, Serere and Soroti Districts, Uganda). Most respondents signified they had to pay for treatment (between 1000 and 5000 USh) and received certificates; "*there is a doctor there, after you buy the cow, they tell us that they are treating for a certain disease, and it is a must; we cannot go without them treating our cow, it is under order*" (Focus Group Participant, Serere District, Uganda). When probed, few individuals knew what treatment their animals were receiving, justifying their lack of knowledge as "*the government vets are trying to reduce the diseases; they themselves know what they are treating for*" (Focus Group Participant, Soroti District, Uganda). Although this may be true to some extent, the current lack of community sensitization to HAT severity and its transmission is an issue; if farmers are not aware of the linkages between their cattle and *T. br. rhodesiense*, they will not see the importance for continuous post-treatment spraying, and could be further at risk of contracting the disease from their cattle in endemic areas.

A further interesting observation was made regarding the strict enforcement of livestock movement restrictions during an outbreak of Foot and Mouth Disease (FMD) in the area at the time of research (Anna L. Okello, field observations). In this way, it seems that despite the human and financial resource issues in the wider veterinary systems in Uganda, enforcement of veterinary policy is indeed possible where political motivation exists. Despite the reasons for quarantine being largely understood and respected by farmers, it was evidently placing a strain on livelihoods in the area; focus group accounts of meager cash flows as a result of livestock market closures were echoed by a private veterinary service provider in the district; "*(the quarantine) has been devastating for families and this time it has collided with going back to school – parents have no money for school fees or books, and this has been reflected in the low school attendance*" (Key informant interview, Serere District, Uganda). Some farmers explained how the lack of available animals for dowry effectively ceased marriages in the district; "*nobody is getting married – we have been told to hold off the marriage until the quarantine lifts*" (Focus Group Participant, Soroti District, Uganda).

DISCUSSION

A major driver of the recent northwards movement of *T. br. rhodesiense* is the ongoing northern migration of cattle from endemic regions in the southeast, particularly since the opening up of the central and northern regions after decades of conflict (11, 13, 14). While this case study focuses on HAT, it must be remembered that this is not the only potentially fatal zoonotic disease to emerge or re-emerge as a result of infected animals entering regions where it has been previously controlled; brucellosis, rabies, porcine cysticercosis, and bovine tuberculosis are all spread through poor enforcement of veterinary policies regarding abattoir inspection, inadequate attention to disease control, and unregulated regional trade. It is for this reason that understanding the current levels of enforcement of veterinary policy, and the bottlenecks regarding this, is important.

In Uganda, a commonly cited reason for the delayed revision of the outdated veterinary policy concerns the lack of evidence – particularly prevalence data – for disease prioritization; "*you need to provide information on what the problem is, the nature of transmission, its economic and public health importance – then you can bring the stakeholders on board for their views*" (Key informant interview). For the majority of neglected tropical diseases, however, there is often difficulty in securing funds for prevalence studies in the first place; "*as much as you don't want a political crisis, we need the data for justification of spending . . . We are all fighting for meagre resources*" (Key informant interview).

Second, limited financial and political focus on veterinary services across much of sub-Saharan Africa has resulted in severe systemic sectoral weaknesses. For this reason, while the policy may exist, the enforcement does not, as was observed in Uganda; "*we have a very broad Animal Disease Act, which is the major policy document that directs disease control in the country, and it has provisions for most of those things. The current problem is with the implementation*" (Informant interview, government sector). Poor adherence to national policy advising pre-movement treatment of cattle with trypanocidal drugs prior to their removal from *rhodesiense*-endemic areas in the southeast of Uganda has therefore likely played a role in the northwards spread of disease in recent years. Some have also indicated the insufficient technical input

into the re-stocking movement, and its highly politicized nature – "*the re-stocking deal was done in the office of the Prime Minister*" (Informant interview, government sector) – may have inadvertently exacerbated the issue even further. This lack of opportunity for technocrats to input into the policy process has been well documented in sub-Saharan Africa (17).

At the ground level, there was further evidence that enforcement of current veterinary policy differed largely according to geographical location, likely reflecting individual commitment and available veterinary resources in that particular district. For example, while Kaberamaido and Serere Districts appeared to enforce stricter protocols surrounding pre-treatment movement of cattle, others did not; "*treatment occurred long ago but not now, we've not seen anything the past few years*" (Focus group participant, Soroti District, Uganda). The requirement for livestock movement policy enforcement is even more urgent given the propensity for farmers to prioritize cattle sales when they know international buyers and donor re-stocking programs are in operation; "*when the traders arrive from Sudan, (we) get good prices. They're taken by truck to Sudan, far away. And also the NGOs and government (for re-stocking); they give good prices, the community knows when to sell through word of mouth*" (Focus Group, Serere District, Uganda). In general, the documented field observations and farmer experiences support recently published concerns that "(Ugandan veterinary policy) implementation continues to be very patchy, with some traders and even some NGOs wholly bypassing controls" (18).

The other major observations concern the limited community level advocacy regarding the dangers of *T. br. rhodesiense* infected cattle, and the need for enabling policies that promote the required preventative animal husbandry at the farm level. In human beings, HAT's similar clinical presentation to higher profile diseases such as malaria and HIV contributes to poor public awareness; "*people are dying from sleeping sickness, but they were thinking it was HIV/AIDS, because that thing can also make you become very thin, so some people were just left to die like that*" (Informant interview Serere District, Uganda 2011). Similarly for livestock, while the various animal-specific *Trypanosome* species result in a clinically ill animal, *T. br rhodesiense* does not have any clinical impact, thus, resulting in even less desire for farmers to spend money treating what they essentially view as a "healthy" animal. The current evidence indicates that farmers prioritize tick control, given the visibility of these parasites on stock and the known impact of tick-borne diseases such as East Coast Fever (ECF). While dual-preparations that act on both ticks and tsetse flies are registered for livestock in Uganda, they are more expensive and less readily available, so smallholder farmers are less inclined to use them. Most farmers interviewed purchase the cheaper tick-only preparations that have no impact on *Trypanosome* transmission, consistent with previous findings by Bardosh et al. (19). Improving the supply and economic justification from both the human and animal health perspective for why farmers should use tsetse-tick preparations would be an important way forwards for the Ugandan veterinary sector, and one that is recommended by the authors.

Finally, it is interesting to note that the Animal Disease Act can be enforced for trade diseases such as FMD, despite in this case the socioeconomic impact of quarantine appearing arguably greater than the disease itself, given the limited access of smallholder farmers to formal livestock markets. Considering "pro-eradication" policies for the control of trade diseases such as FMD are able to remain strong in this way, it is vital that the same attention is turned to zoonoses control in endemic countries, to prevent further emergence into naive areas or across international borders.

Through documenting the recent observations of HAT control in Uganda, we have highlighted the role of the veterinary sector in zoonoses prevention, presenting a case for greater economic and institutional focus on the African veterinary sector as a whole. However, long distances, poor infrastructure, and limited cash flow/disposable income all impact the provision and utilization of quality human and animal health services across much of sub-Saharan Africa. This ultimately affects disease reporting, impacting the level of attention placed on a disease issue by policy makers (20). Furthermore, even where policy actors do accept the need for zoonoses control, the issues of policy development and enforcement are compounded where multiple sectors are involved, further impeding the timely adoption and implementation of innovative policy processes for zoonoses control (17). It is for this reason that veterinary sector improvements need to consider the various external economic and developmental bottlenecks to policy development, enforcement, and control that could impact disease control more generally on the continent.

CONCLUSION

There are several ongoing policy issues regarding livestock movement and disease control in Uganda, stemming from a variety of social, economic, and political factors. Up to date, enforceable veterinary policy has the potential to significantly contribute to the economic development of a country via joint impacts on human and animal disease control, improved production outputs that impact regional markets, and access of the human population to improved nutrition. Despite this, the importance of veterinary policy is often underestimated by the health and development sectors alike, particularly regarding its role in the prevention of emerging and re-emerging zoonotic disease, as described by this case study.

Weak enforcement of a veterinary policy directive that simultaneously controls a fatal human disease and improves livestock productivity questions the type of evidence, advocacy, and capacity required to ensure zoonoses control efforts in animal reservoirs are realized. We need to overcome this disabling triad of capacity, advocacy, and evidence in order to move beyond our relatively limited understanding of zoonoses transmission – and its implications in changing agricultural contexts – toward a fuller, holistic, and more responsive veterinary system of analysis, intervention, and control. Awareness of the potentially harmful human diseases carried by domestic animals and wildlife is vitally important, not only just for the communities that face daily exposure to them but also for the policy actors and key decision-makers that play a role in preventing the emergence and spread of zoonoses at the crossroads of human health, agriculture, and development.

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Chagas' disease: an emergent urban zoonosis. The Caracas Valley (Venezuela) as an epidemiological model

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The unprecedented emergence of important public health and veterinary zoonoses is usually a result of exponential population growth and globalization of human activities. I characterized Chagas' disease as an emergent zoonosis in the Caracas Valley (Venezuela) due to the following findings: the presence of reservoirs (*Didelphis marsupialis*, *Rattus rattus*) and vectors (*Panstrongylus geniculatus*, *Panstrongylus rufotuberculatus*) infected with *Trypanosoma cruzi* in urbanized or marginalized areas; the elevated contact between *P. geniculatus* and human beings detected by parasitological and molecular examinations of triatomine feces demonstrated the possibility of transmission risks; a study of outbreaks of urban Chagas' disease reported the first proven case of oral transmission of *T. cruzi* to human beings; the risk of transmission of glandular metacyclic stages from marsupials by experimental ocular and oral instillation; mice genitalia infected with *T. cruzi* contaminated blood resulted in the formation of amastigotes very close to the lumen suggesting that there may be a possibility of infection via their release into the urine and thence to the exterior; the ubiquitous histotropism and histopathology of *T. cruzi* was demonstrated using a mouse model; the presence of experimental *T. cruzi* pseudocysts in adipose, bone-cartilage, and eye tissue indicated a potential risk for transplants. Socio-sanitary programs that include improvements in housing, vector control, and access to medical treatment, as well as strategies aimed at combating social inequalities, poverty, and underdevelopment should be undertaken in those areas where zoonoses are most prevalent. Disciplines, such as Ecology, Epidemiology, Medical Entomology, Human and Veterinary Medicine, Environmental Studies, Public Health, Social and Political Studies, Immunology, Microbiology, and Pharmacology could all provide important contributions that aim to reduce the occurrence of factors governing the spread of emergent diseases.

Keywords: Chagas' disease, emerging urban zoonosis, Caracas Valley (Venezuela)

INTRODUCTION

American trypanosomiasis (Chagas' disease), a metaxenic zoonosis caused by *Trypanosoma (Schizotrypanum) cruzi* Chagas, 1909, is endemic to Neotropical and Nearctic regions (Salt Lake City, 41° N in USA/56° S in the argentine Patagonia and northern Chile as well as the Caribbean Islands). A total of 8 million people in 21 countries are infected by the disease with mortality rates of approximately 10,000 per year. This parasitosis is found in particular landscapes inhabited by mammal reservoirs and insect vectors (Hemiptera, Reduviidae, Triatominae), which together make up the wild cycle, the most primitive zoonotic transmission dynamic, in which the parasite travels between more than 200 species of mammals from seven orders and about 140 species of insects in 15 genera (1, 2).

The most ancient reservoirs in this cycle are the Eutherian (Cingulata, Dasypodidae: armadillo); Rodentia (Echimyidae); and Metatherian (Didelphimorphia, Didelphidae: opossum) (3, 4). These synanthropic mammals have migrated from their natural ecosystems to human communities, while at the same time, human beings have encroached onto their habitats. In both cases, *T. cruzi* infects individuals giving rise to the so-called peridomestic and domestic Chagas' disease cycles. This accidental anthropozoonosis may develop in rural as well as urban areas and is conditioned by both environmental and social elements (1, 5). In Venezuela, *T. cruzi* circulates between 39 species in the Marsupiala, Edentata = Xenartra, Chiroptera, Carnivora, Rodentia, Primates, and Lagomorpha and is transmitted by 22 insect vectors. The main vector is *Rhodnius prolixus* due to its broad distribution (over nearly three quarters of the total land area of Venezuela) and its high domiciliation (6–8).

Trypanosoma cruzi is a hemoflagellate (Protozoa, Kinetoplastida, Trypanosomatidae) with two developmental stages: extracellular bloodstream trypomastigotes and intracellular tissue amastigotes. In vectors, both forms are extracellular: epimastigotes that develop in the lumen of the midgut, and metacyclic trypanosomes found in the rectal part of the hindgut. The latter of these two forms is the infective stage, and transmission of the parasite primarily occurs when contaminated feces evacuated by the parasitized insect come into contact with healthy mucosa or the damaged skin of the host. In contrast, in the African trypanosomes and the Neotropical *Trypanosoma rangeli*, the parasites initially develop in the gastrointestinal tract of the insect vector

and from there migrate by tropism to the salivary glands where the infective stages develop before being transmitted mechanically when the insect bites a host. In both cases, the *Trypanosoma* species cause zoonotic parasitosis in human beings (9, 10).

American trypanosomiasis discovered in Brazil by Chagas (11), was demonstrated in Venezuelan human beings by Tejera (12), and Pifano (13) described the characteristics of the environmental niches of this zoonosis as well as the features of the principal bioregions where it develops.

DEFINITION OF EMERGING INFECTIOUS DISEASES AND ZOONOSES

The emergence and spread of smallpox during the "discovery" of America in the 16th century, which attacked vulnerable non-immune indigenous populations and the sudden appearance of the great pandemics: bubonic plague, cholera, typhus, syphilis, and leprosy in Europe during both the Middle Ages and the Modern period are all examples of emerging diseases. These were triggered by massive migrations from rural to more-developed areas resulting in overcrowding and social changes, which together with a lack of hygiene exposed populations to transmissible agents (14).

Among the many, and often divergent, definitions of the concept of emergent infectious diseases, two appear to be the most appropriate as they are based on the dynamics of several elements that act synergistically to produce the diseases. The first of these is "Infectious diseases are said to be emergent when qualitatively unexpected phenomena resulting from local interactions appear; this tends to occur suddenly" (14). The other is that emerging infectious diseases are infections that have newly appeared in a population, or already exist but whose incidence is rapidly expanding over a geographical, host or vector range, or have been reported in new populations (15–18). Thus, every infectious disease is emergent until it reaches endemic status. These broad definitions encompass a range of human diseases all of which represent a significant threat to public health.

Zoonoses are infectious diseases that are transmitted between their natural hosts and human beings (19).

PURPOSE OF THE REVIEW

The principal determining factors that make up the web of causation of the infectious diseases can be summarized as follows: biological (infectivity and pathogenicity) and molecular properties of the pathogens; characteristics of the vertebrate hosts; vector competence and migration, anthropogenic factors causing significant damage to ecosystems resulting in the alteration or destruction of mammal hosts and insect vectors habitats as well as changes in their behavior; and decrease in the funding allocated to health authorities for their surveillance, prevention, and control. While a few infectious diseases have been eradicated (smallpox) or controlled (dracunculiasis, measles, polio), it is probable that most of the rest of them will not be, at least in the short term, because the causal factors for their emergence remain. This being so and faced with the fact that the recurrence rate will not only continue but also increase, new approaches and tools must be found and implemented in order to at least prevent the establishment of these diseases (10, 15–20). This article was stimulated by these considerations.

The studies based in the Caracas Valley will be compared with reports of emergent diseases produced by zoonotic agents from other countries. The Caracas Valley was selected because it is still classified by the health authorities in Venezuela as "not endemic" and is, therefore, are not included in the Venezuelan National Control Programme (21).

CHARACTERISTICS OF THE CARACAS VALLEY

Caracas, the capital city of Venezuela, lies in a depression within the coastal mountain range in north-central Venezuela. The city is located at 960 m.a.s.l. and comprised five municipalities and two federal entities: the Capital district (Libertador municipality) and Miranda state[] (Chacao, Baruta, El Hatillo, and Sucre municipalities). All these municipalities together make up the Caracas Metropolitan district (10° 23′ 18″–10° 34′ 00″ N; 66° 51′ 30″–67° W 10′35″) (**Figure 1**). The Caracas valley has an altitude of 870–1043 m.a.s.l., average temperature 22°C, and annual rainfall of 870 mm; the natural vegetation is pre-montane forest. The population density of Caracas has been increasing exponentially due to steady migration from other parts of Venezuela, as well as neighboring countries, and is now fully urbanized. This migration has resulted in a population with minimum hygiene standard as well as poor socioeconomic conditions. Much of this population is housed in urban slums, located on the banks of the numerous rivers that cross the city as well as in the neighboring savannas and mountains forming a regrettable belt of misery. The slums adjoin built-up medium and high value residential or commercial areas and similar situations have developed in other areas along the valley, all of which have profoundly modified the natural environment. The Caracas Valley is, thus, an excellent example of the progressive modification of an ecosystem, where an increasing human population has invaded a region already populated by Chagas' disease insect vectors and mammal hosts (21, 22).

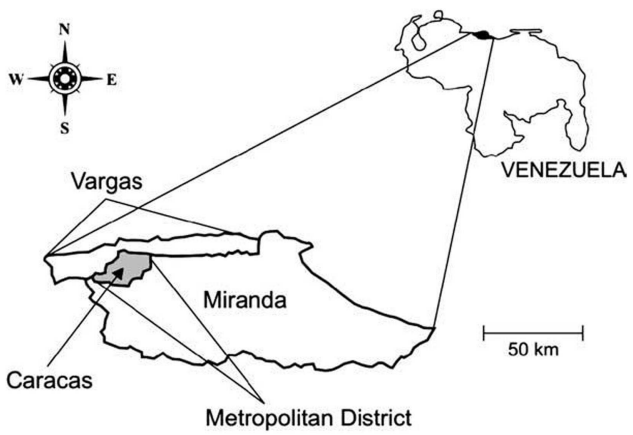

FIGURE 1 | Map of Venezuela showing the relative location of the area of the Metropolitan District.

Table 1 | Histopathology in organs of white mice infected with isolates of *Trypanosoma cruzi* from urban *Panstrongylus geniculatus*.

Organ	Observations	Isolates			
		VP1	VP2	VP5	VP7
Heart	Diffuse myocarditis	XXX	XXX	XXX	
	Pancarditis		XXX		
	Myocyte destruction	XX	XXX	XXX	
	Abundance of amastigote and trypomastigote nests	XXX	XXX	XX	XX
	Fibroblast proliferation			X	
	Histiolymphomonocytic inflammatory infiltrate	XX	XXX	XX	XX
	Ganglionitis and periganglionitis	X			
	Neural edema and destruction	X			
Skeletal muscle	Histiolymphocytic myositis	XXX	X	XXX	XXX
	Abundance of amastigote and trypomastigote nests	XX	XX	X	XX
	Myocyte destruction	X		XXX	XX
	Fibroblast proliferation		X	X	
	Neuritis, perineuritis, perivascularitis	X			X
Duodenum	Inflammation of smooth muscle only, with:	X		X	X
	Abundance of small parasite nests	X	X	XX	
	Parasitization of myoenteric plexi	X		X	
Colon	Diffuse inflammation of smooth muscle only,	X	X	X	X
	With: abundance of small parasite nests	X	X	XX	X
	Parasitization of myoenteric plexi	X	X	XX	X
Liver	Scanty discrete inflammatory foci in parenchyma	X	X		X
	Foci of parenchymal necrosis			X	X
	Amastigote nests in gall bladder smooth muscle		X		X
Spleen	Hyperemia in red pulp		X	X	
	Scanty amastigotes in fixed macrophages of sinusoids	X			X
Pancreas	Amastigotes in acinous cells, Islets of Langerhans, and connective tissue	X	XX	XX	X
Lung	Small amastigote nests in peribronchial smooth muscle or arteriole walls	X	X	X	X
	Discrete inflammatory foci	X			X
Brain	Scanty parasite nests in microglial cells	X			
	Scanty parasite nests in white matter	X	X	X	X
	Parasite nests in cerebellum				XX
	Discrete inflammatory foci		X		
Bone marrow	Scanty amastigotes in fixed macrophages		X		

X, moderate; XX, marked; XXX, intense.

CHRONOLOGICAL DESCRIPTION OF INVESTIGATIONS INTO THE CHAGAS' DISEASE WEB OF CAUSATION IN THE CARACAS VALLEY THAT DEMONSTRATE ITS STATUS AS AN EMERGING URBAN ZOONOSIS

The research undertaken by Pifano (22) was the first to demonstrate the presence of Chagas' disease in the Caracas Valley. This was done by identifying the components of the transmission cycle as follows: reservoirs (*Didelphis marsupialis*) and vectors (*Panstrongylus geniculatus*) captured in slums and housing estates were found to be infected by *T. cruzi* by examining fresh blood samples and smears stained with Giemsa, hemoculture, xenodiagnoses, and histopathology of opossums, as well as the examination of vector fecal samples.

The author suggested that the omnivorous/predatory diet of these marsupials, which feed on the reservoirs and vectors of *T. cruzi*, increases the probability of their becoming infected by this heteroxenous parasite. This, together with their synanthropic nature has enabled them to easily adapt to anthropically modified environments frequently invading areas inhabited by human beings. Furthermore, vectors showed positive, by immunodiffusion, for human and animal blood.

The tissue ubiquity and the pathology caused by *T. cruzi* obtained from *D. marsupialis* were determined in this host and experimental NMRI mice (23–26) (**Table 1**).

Deane et al. (27) described a unique property of *D. marsupialis*, whereby the *T. cruzi* morphotypes typically found in the intestines

of its vectors, were also observed in the anal scent glands of this species. This property has been reported from wild *D. marsupialis* and *Didelphis albiventris* in both Brazil and Venezuela (28–30). In addition, the experimental colonization of *D. marsupialis* and *Lutreolina crassicaudata* glands was produced by s.c. injection of bug urine and fecal material (31–33).

Urdaneta-Morales et al. (34, 35) described this *T. cruzi* luminal cycle from naturally infected *D. marsupialis* captured in highly urbanized areas within the Caracas Valley. Numerous epimastigotes of different sizes, most undergoing binary division giving rise to rosettes, intermediate forms, and metacyclic trypomastigotes infective to mammals, were observed. These glands, thus, provide a favorable environment for the development of the *T. cruzi* vector cycle.

Furthermore, glandular material inoculated s.c., i.p., or instilled orally and ocularly in healthy opossums and white mice caused parasitemias with the pleomorphic forms typical of the parasites as well as intracellular parasitism with the multiplication of amastigotes in cardiac and skeletal muscle (**Figure 2**). Glandular metacyclics from LIT (liver infusion tryptose) cultures inoculated into these mammals gave the same results. Infection caused 100% mortality in the mice, whereas all the opossums survived. Nymphs of *R. prolixus* fed on both hosts showed infective metacyclics in the feces (34, 35).

Herrera and Urdaneta-Morales (36) captured 37 *Rattus rattus*, one *R. norvegicus*, and nine *Mus musculus* in urbanized areas (Colinas de Bello Monte, Los Chorros, El Cafetal, Las Acacias, San Román, Parque del Este and Caricuao) in Caracas. Of these, conventional examinations of fresh blood and smears stained with Giemsa, and xenodiagnosis revealed the presence of *T. cruzi* stages in two *R. rattus* individuals (**Figure 3**). Tissue sections from *R. rattus* showed numerous pseudocysts with amastigotes in the heart as well as moderate parasitism of the skeletal muscle and smooth muscle of the duodenum. All mice inoculated with xenodiagnosis' bug fecal material showed a moderate pattern of tissue tropism in the same organs as in well as in the colon and pancreas (**Figure 4**). *Trypanosoma (Herpetosma) lewisi* was also detected in infected rats, but not in the other rodents examined.

Reyes-Lugo and Rodríguez-Acosta (37) found *P. geniculatus* colonizing the interior of a well-built house in an area of transition between cloud forest and humid montane woodland in the mountainous region of Hoyo de la Puerta (Miranda state) on the outskirts of Caracas. A total of 20 *P. geniculatus* specimens in all stages of development gorged with blood were shown to be infected by *T. cruzi*. The authors also found *R. rattus* in several tunnels connecting the inside floor of the house with the outdoors, in which several *P. geniculatus* individuals and their eggs were found. The authors suggested that human activities have led to the disappearance of the natural habitats of this triatomine and with them its food sources, thus favoring its domiciliation. This situation has been described by Reyes-Lugo (38) in a further eight localities in the center-north of Venezuela, including the Caracas Valley.

Xenotransplantations have demonstrated zoonotic infections produced by viruses, bacteria, protozoa, fungi, and helminths, thus showing their importance as risk factors for these diseases (20). Using a mouse model, the possibility of the transfer of *T. cruzi* was determined in organs often used during these procedures. Isolates from *D. marsupialis* and *R. rattus* captured in Caracas were inoculated in adipose, bone-cartilage, and eye tissue, observing the intracellular presence of the parasite in all cases (39–41) (**Figures 5–7**). This constitutes an alternative transmission pathway, whereby natural *T. cruzi* intracellular multiplication could be enhanced in immunosuppressed hosts. Isolates of *T. cruzi* stages found in the eye tissue of mice produced an electrophoretic band pattern that identified the parasite as TcI (ZI).

Based on several references cited by Zeledón (42) regarding the presence of *T. cruzi* trypomastigotes in opossum (*D. marsupialis)* and mouse urine, together with the suggestion of Dias (43) that the presence of these stages in the chagasic female's menstrual blood

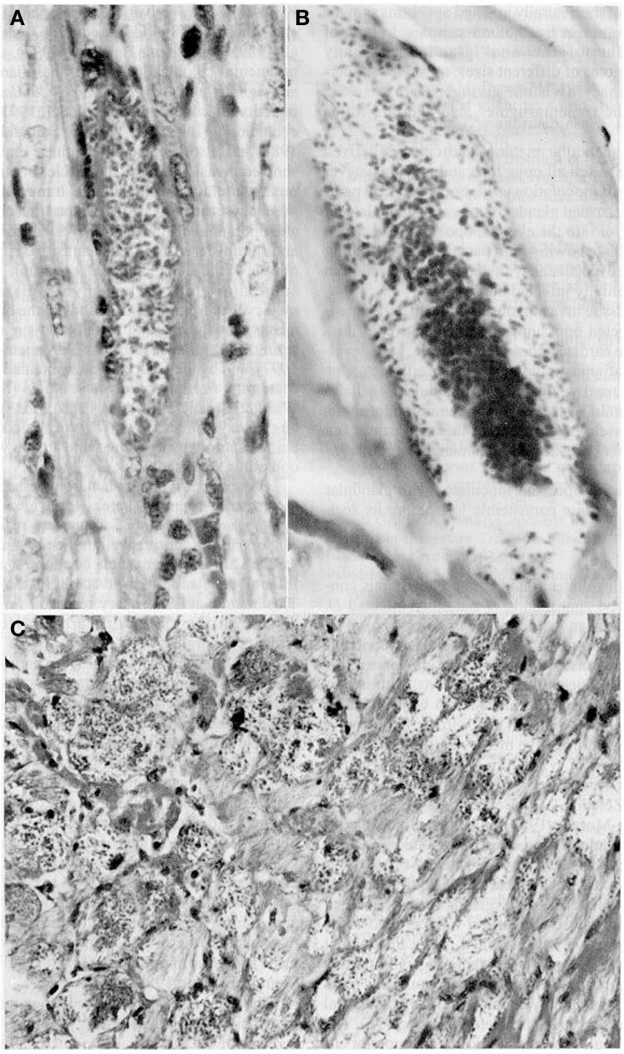

FIGURE 2 | Tissue sections showing pseudocysts containing amastigotes (H-E). **(A)** Heart of *Didelphis marsupialis* infected orally by glandular material cultured in LIT medium (400×; **(B)** muscle layer of anal gland of opossum, infected as above (1000×); **(C)** heart of mouse infected intraperitoneally by glandular material from a naturally infected opossum (400×).

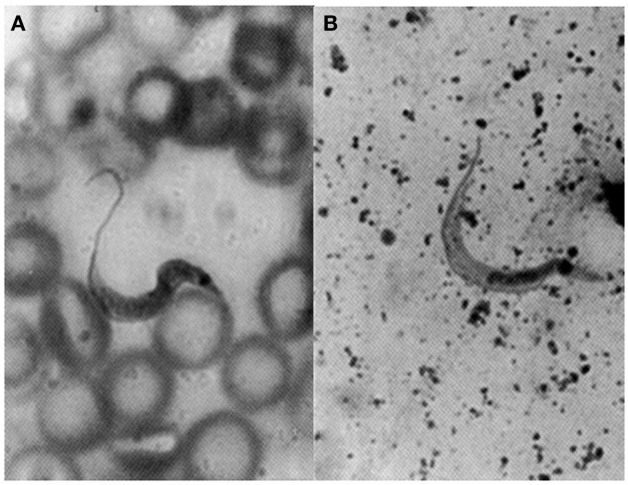

FIGURE 3 | Flagellate stages of *Trypanosoma cruzi*: (A) stout bloodstream trypomastigote from naturally infected *Rattus rattus* (Giemsa, 1400×); **(B)** metacyclic trypomastigote from feces of *Rhodnius prolixus* used for xenodiagnosis of naturally infected *R. rattus* (Giemsa, 1400×).

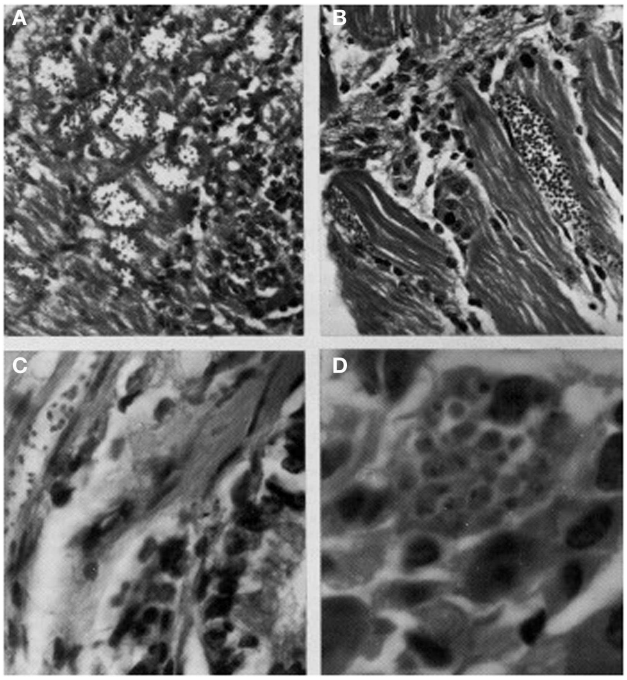

FIGURE 4 | Histological sections showing pseudocysts of *Trypanosoma cruzi* with amastigotes in (A) cardiac tissue of naturally infected *Rattus rattus* (H-E; 560×); (B) skeletal muscle of naturally infected *R. rattus* (H-E, 560×); **(C)** smooth muscle fiber from the colon of an experimentally infected mouse (H-E, 960×); **(D)** acinar cell of pancreas of experimentally infected mouse (H-E, 1400×).

could enable transmission through coitus, Herrera and Urdaneta-Morales (44) carried out the following investigation: the blood of mice infected with *T. cruzi* isolates from *R. rattus* was instilled into the urinary meatus (females) and penis (males) of healthy mice, and a *T. cruzi* isolate from a human being was inoculated into the scrotum. The genital instillations resulted in the invasion of the heart, skeletal muscle, duodenum, pancreas, sternum, ovary, testis, and vas deferens. In addition, scrotal inoculation caused the invasion of the liver, spleen, lung, kidney, urinary bladder, and seminal vesicle (mucosa close to the lumen) (**Figure 8**).

Carrasco et al. (21) established that there is a high risk of the transmission of Chagas' disease in the Caracas Valley due to the contact between *P. geniculatus* and human beings. Natural infection by *T. cruzi* was determined by examinations of fresh and stained stools. In addition, the random amplification of polymorphic DNA for parasite identification and group typing, and a dot-ELISA test to identify the gut content of the triatomine bugs showed that 66% of the 88 triatomines studied were infected by TcI, of which 60% reacted positively to human antiserum. The relationship between the percentages of bugs with fecal contents reactive to human antiserum and those reactive to all the antisera used was 98%, while approximately 41% of the bugs fed on human beings were infected by *T. cruzi*.

The first proven cases of the transmission of *T. cruzi* to human beings occurred in urbanized areas within the Caracas Valley (Chacao municipality and Antímano parish). These outbreaks represent two of the most numerically important cases of orally acquired Chagas disease in Latin America to date. A total of 124 patients in the acute phase of infection were characterized by their clinical symptoms, electrocardiograms, immunoenzymatic tests, indirect hemagglutination, and PCR. Examinations of blood, hemoculture, and inoculation in a mouse model were also performed. Polluted guava juice was statistically shown to be the only source of infection and the person who prepared the juice was infected with *T. cruzi*. The presence of infected *P. geniculatus* and *R. rattus* was confirmed in the slum where this individual lived (45, 46).

The capture of two specimens of *Panstrongylus rufotuberculatus* infected with *T. cruzi* inside houses in the town of El Hatillo, close to Caracas (originally a forested area but now completely urbanized), was reported by Zavala-Jaspe et al. (47). One of the isolates was characterized by the following: parasitological examinations of the intestinal contents, infection of a mouse model, hemoculture, xenodiagnoses, and *in vivo* and *in vitro* metacyclogenesis producing morphotypes characteristic of the parasite; infection by *T. cruzi* was confirmed by PCR.

All of these chronologically described results constitute important epidemiological risks for public health in this capital city.

DYNAMIC OF EMERGENT DISEASES: TRANSMISSION PATHWAYS-DISEASE TRANSMISSIONS

Until the end of the 80s, it was thought that emergent diseases had been eradicated or that they were limited to underdeveloped areas. This was due to the fact that the biomedical control programs were abandoned, which resulted in a huge spate of emerging diseases. The situation was aggravated by the failure to isolate or identify the pathogens responsible, coupled with the difficulty of characterizing the symptoms due to their (in the majority of cases) extensive pre-patency and subclinical nature. Research into the global occurrence of important outbreaks led to the conceptualization of the

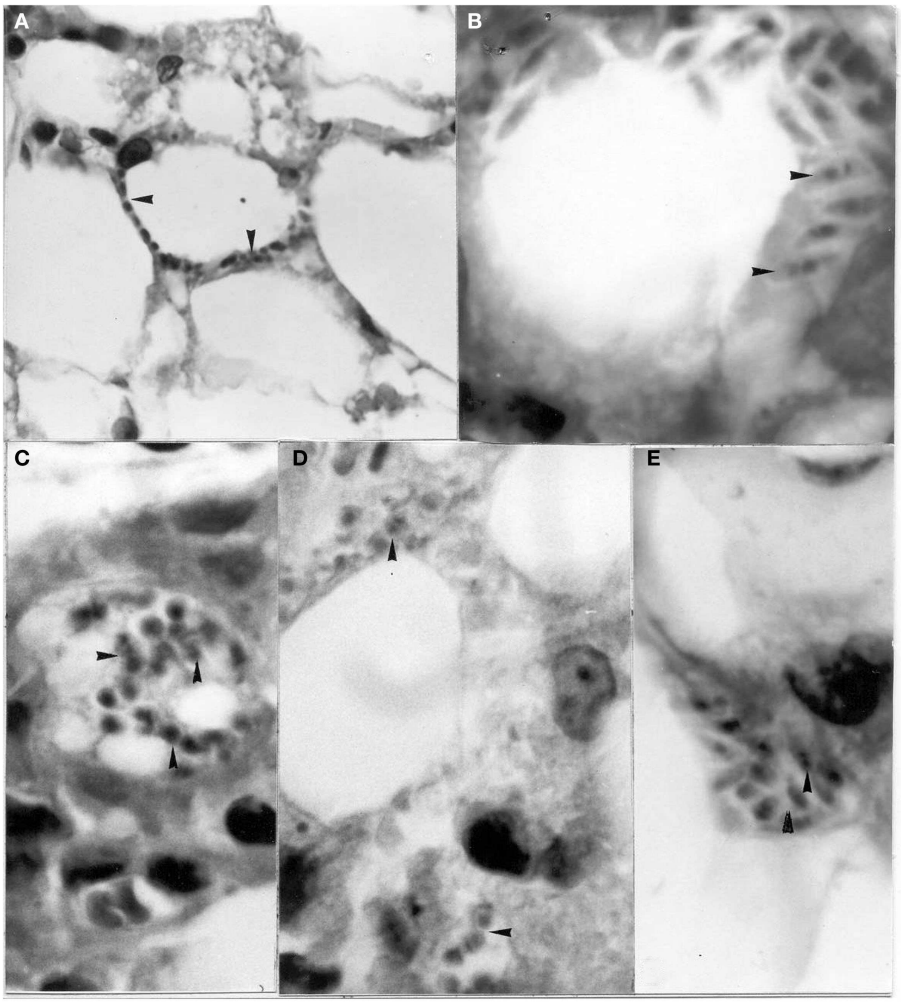

FIGURE 5 | Amastigotes and intermediate stages (arrows) of *Trypanosoma cruzi* in (A,B) perifery cytoplasm of an adipocye; **(C)** cytoplasm of immature adipocite (preadipocite); **(D)** intercellular substance in connective adipose tissue; **(E)** parasitized macrophage located between uninfected adipocytes (H-E; 1400×).

causes of the emergence of these diseases (15, 19). In both excellent reviews of the many risk factors that influence the emergence of infectious diseases according to their pathogens, an estimated 75% of these diseases, principally viral, bacterial and parasitic, and possibly vector-borne, have emerged over the past two decades from a wildlife source.

Given that each reservoir and vector species occupies a specific ecological niche and shows a particular behavior pattern, the physical and biological environments should be maintained in a dynamic equilibrium with human populations and society in order to prevent the emergence and spread of new diseases. It is important to realize that the emergence of these diseases is most likely to occur after changes to the components of this dynamic equilibrium. Consequently, zoonoses (including the parasitic ones) and particularly emergent zoonoses have sparked interest in the international scientific community. This is due to their increasing importance to human and animal health as they cause high indices of morbidity and mortality in both rural and urban areas and in endemic and non-endemic areas, with huge repercussions for the economies and health of the countries affected (5, 48–52).

Human beings, in their struggle for survival, have profoundly modified the natural environment, adapting it to their needs as they colonize different landscapes and habitats, all of which provide natural niches for pathogens responsible for zoonotic infections. These can derive from, and become emergent in, many different environments: wild, rural, regional-urban, and even global-urban. These niches house an enormous number of vertebrates and invertebrates that, in turn, host a huge variety of macro- and micro-pathogens; the risks of transmission to human beings are obvious (5).

Anthropogenic changes (environmental changes, deforestation, reforestation, road construction, urban growth, trade, translocation, and keeping of exotic pet, ecotourism, consumption of contaminated water or raw or undercooked food in order to retain its flavor and nutrients) are those that have proven to cause the

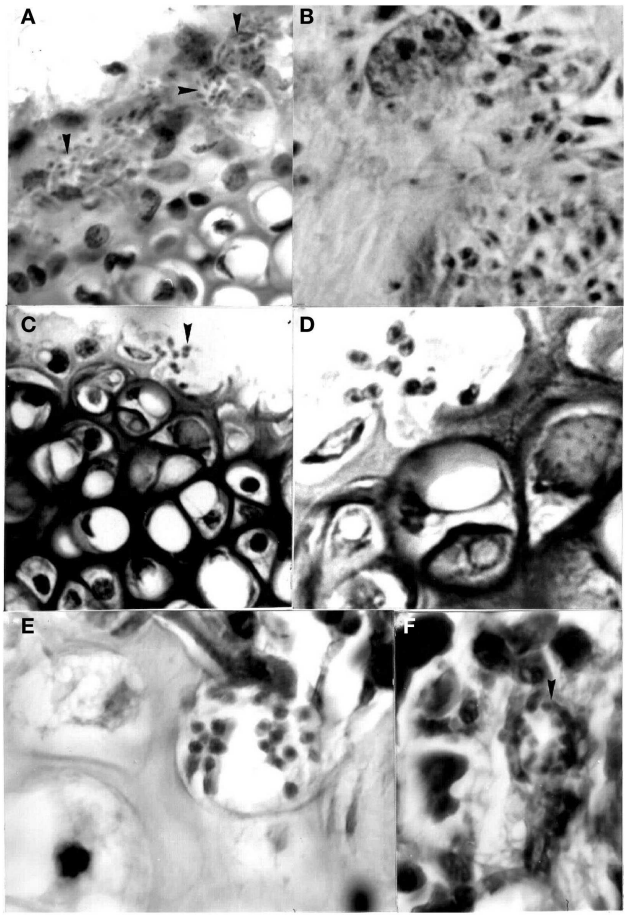

FIGURE 6 | Pseudocysts with stages (arrows) of *Trypanosoma cruzi* in sternum of mice experimentally infected with different isolates. **(A)** Perichondrium with nests showing amastigotes and intermediate stages in chondroblasts (400×; HE); **(B)** same stages in stroma of perichondrium and in the cytoplasm of a macrophage (1000×; HE); **(C)** and **(D)** two broken chondrocytes with several amastigotes and one flagellated stage (400× and 1000×, respectively; HE); **(E)** several amastigotes in a osteocyte (1000×; HE); **(F)** pseudocyst with amastigotes in the bone marrow (1000×; HE).

most stress on the balance between the physical and biological environments intrinsic to the pathogen-host-vector association.

The key factor for the unprecedented levels of these emergent diseases is the synergism between two circumstances, the globalization of human activities with the exponential increase in the population growth rate causing progressive deterioration of the relationship between human and environmental health, and therefore modification and destruction of the natural niches of reservoirs, vectors, and pathogens. Unprecedented increases in the incidence of infectious emergent diseases and the extinction of species with the resulting loss of biodiversity are examples of this. Immune pressure and the abuse in antibiotic use also cause highly frequent changes in pathogens that are expressed as mutations in their virulence, pathogenicity, and genetic structure. Another important characteristic worthy of mention is the generalist nature of the pathogens that affect human beings: those that are capable of infecting multiple hosts, especially when they belong to more than one order, are highly likely to infect human beings. An excellent example of this type of pathogen is *T. cruzi* that, as already mentioned, parasitizes species of mammals from 7 orders and triatomines from 15 genera (10, 15–20, 48–61).

NEGLECTED ZOONOSES

The critical situation regarding infectious diseases that persists in many indigenous populations in several continents must be highlighted. These zoonoses have a higher prevalence in regions between latitudes 35°N and 35°S, with altitudes below 2200 m and temperatures ranging from 15°C to 40°C. These populations are some of the world's poorest, most anonymous and ignored. They have been subjugated by foreign powers that "discover" them, conquer and enslave them; they have suffered unjust discrimination and political and economic exclusion. Multinational corporations have, among many other things, denied them their rights to education, their sources of cultural and physical survival, their traditional knowledges, the ownership of some of the most biologically diverse territories in the world thus producing local or global extinctions of species, and their languages, in short, their ethnic cultural identity.

Emerging diseases are considered as "neglected" because the huge investments resulting from The Millennium Declaration, adopted by the United Nations in September of the year 2000 and applied at a large scale to projects for the prevention and/or control of HIV/AIDS, malaria and tuberculosis (the "big three"), have not up until now been used for combatting them. Many scientists, institutes for health and pharmaceutical companies continue to ignore and exclude them. The argument put forward for this is that there is no reliable information about the health burdens of these "low hanging fruit" populations. This lack of knowledge has led to a reduction or elimination of funding and research within these marginalized communities. All this, in spite of the fact that millions of people from Sub-Saharan Africa, Asia, Latin America, and the Caribbean continue to suffer diseases at least as (if not more) serious as the three mentioned (62–68).

PROGRAMS FOR THE PREVENTION AND CONTROL OF ZOONOSE

The great diversity of risk factors that produce the emergence, re-emergence and, in many cases, persistence of zoonoses caused by pathogens that are already known, as well as those that have only recently been characterized, or have undergone changes in their bio-ecological characteristics in poor communities are particularly notable in countries governed by strong socio-politico-religious groups, as is the case for Africa, Asia, and also Latin American and Caribbean countries whose populations are indigenous or of African descent.

By other hand, most of the alterations in the behavior, cognitive or psychosocial patterns of the indigenous populations infected with zoonoses are, in general, similar even between communities from different continents, it is appropriate to mention them here. The *per se* knowledge that they are ill, or if they are told that they have a disease of this nature, produces symptoms that begin with slight depression, before developing into anxiety, stigma, and

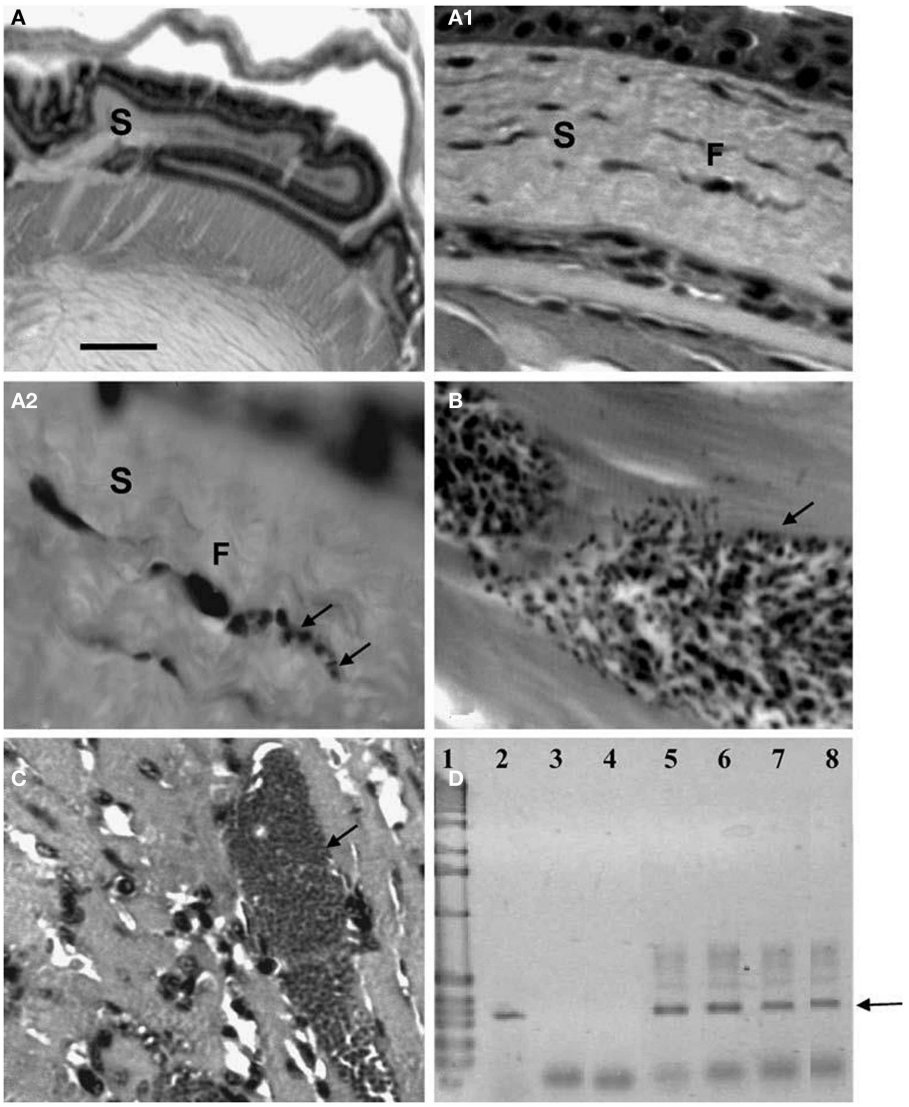

FIGURE 7 | Histological and molecular parasitism in NMRI mice experimentally infected with different isolates and strains of *T. cruzi*. **(A – A2)**. Sequence of microphotographs with amplification of a nest of amastigotes in a fibroblast (F; arrows; 40×, 400×, and 1000×, respectively) of corneal stroma (S); **(B)** trypomastigote nest in thigh skeletal muscle (arrow); **(C)** amastigote nest in heart muscle (arrow); **(D)** amplification of the 330-bp fragment from the conserved regions of kDNA (arrow) extracted from ocular tissues of experimentally infected NMRI mice in 2.5% agarose gel electrophoresis (ethidium bromide stain): Lane 1 1-kb ladder molecular marker (Gibco BRL Life Technologies), lane 2 nude *T. cruzi* DNA, lane 3 negative PCR control, lane 4 MRAT/VE/1996/CO22 isolate, lane 5 MHOM/BR/1950/Y strain, lane 6 MHOM/VE/1970/EP isolate, lane 7 MDID/BR/1999/M1 isolate, and lane 8 MDID/VE/1995/CO79 isolate.

alexithymia. Their concern that the disease will worsen, the impossibility of finding a cure and their fear that they will infect others, distances them from their family circle and friends, while the weakening of their immunological systems only serves to worsen their condition (68–70).

Currently (63–65), a comprehensive framework model is being applied in some continents with promising results. This model attempts to control some diseases and reduce poverty with a view to returning the basic rights of these populations (equality, ethical treatment, and health) stolen from them. According to specialists, a key strategy for minimizing the deficiencies in funding is to try to increase the commitment of the family and local community so that they themselves help to prevent, control, and even eliminate zoonoses through feasible and economic methods. Some examples of such community-based approaches are Community-Led Total Sanitation (CLTS), which has completely eliminated open defecation resulting in a massive reduction in the incidence of enteric diseases; controls in China for reducing the rate of transmission of *Schistosoma japonicum* from infected bovines and human beings to snails; management of sleeping sickness in Uganda caused by *T. rhodesiense* by chemotherapy of bovine reservoirs en masse and the use of insecticides for the control of the insect vectors.

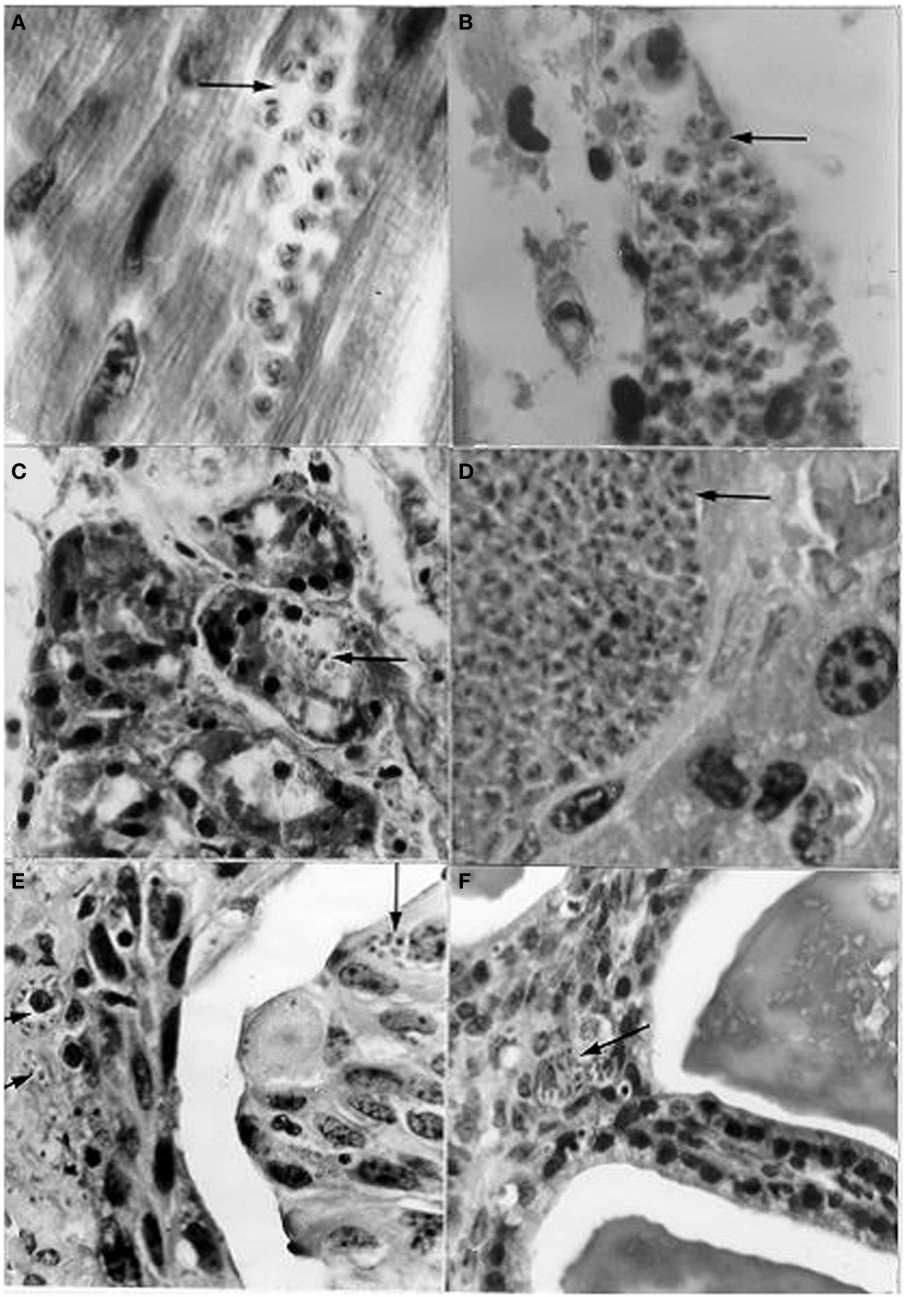

FIGURE 8 | Histological sections from albino mice intravaginally instilled with a strain of *Trypanosoma cruzi* from *Rattus rattus,* and scrotally inoculated with a strain from a human patient, showing nests (arrows) with amastigotes in (A) cardiac tissue; (B) skeletal muscle; **(C)** pancreas (acinus); **(D)** liver; **(E)** urinary bladder (epithelium very close to the lumen and *lamina propria*); **(F)** seminal vesicle (mucosa close to the lumen). [**(A,B,D)**: I400×; **(C,E,F)**: 950×; H-E].

Molyneux and Malecela (66) drew up a set of macro-priorities and recommendations with the idea of clarifying these objectives as listed below (**Table 2**). These authors emphasize the scarce knowledge that clinicians and policy makers, general speaking, have when they diagnose for example a zoonosis with no specific symptoms such as Brucellosis, Leptospirosis, Rickettsiosis, or Q fever, as Malaria, as well as their ignorance of the possibility that zoonotic infections can, in the long term, cause cancer (Trematodiases) or Neurocysticercosis. All this as a consequence of the absence of microbiological and molecular tools in laboratories and hospitals, due to the financial limitations they suffer; further evidence of the sad neglect these populations face.

With this in mind, strategies that should be developed include primary health education in conjunction with the health sectors

Table 2 | Macro research priorities identified by DRG6 (Disease Reference Group – WHO UNDP World Bank Special Programme).

1. There is a need to develop a comprehensive methodology for calculating the societal burden of disease attributable to zoonoses recognizing that a high proportion of the population of rural (and often urban) populations in least-developed countries depends on livestock.
2. More studies are required to generate data on the costs, cost-benefits, and cost effectiveness of interventions for endemic zoonoses. Such studies should also incorporate the economic effect of animal disease as an indirect contributor to poverty through its impact on nutrition, loss of meat and milk products, and livestock as a capital asset.
3. There is a need for operational and systems research to identify reasons for the limited communication and interaction between the key sectors – health, agriculture, and livestock – particularly in countries where a large proportion of the population is dependent on livestock.
4. There is a need to evaluate effective community-based approaches and interventions for zoonotic diseases, drawing on the experience and success of initiatives for water and sanitation improvements, mass drug delivery, and community-based health care.
5. Experiences from separate initiatives in different geographic and epidemiological settings need to be evaluated to ensure that such experiences are amplified and synergized, with potential for integration between programs.
6. Investing in systems for the collection of reliable data on disease/infection incidence and prevalence from both veterinary and medical sectors is recognized as a priority, both for the measurement of disease burden and the evaluation of control measures.
7. Investment in endemic zoonoses in least-developed countries would provide multiple benefits not only by improving the health and livelihoods of marginalized communities but also by reducing threats and enhancing the response capacity for emerging zoonoses that pose a threat to the global community.
8. Effective lessons are often best learned by the implementation of strategies (such as the onchocerciasis control program), with research to evaluate factors leading to success measured by effectiveness and cost-effectiveness embedded within program implementation.
9. As endemic zoonoses disproportionately affect impoverished and marginalized populations, investments need to be specifically targeted to overcome barriers to health care in these communities, including isolation, population movement or migration, social or political unrest, and conflict.

of competent indigenous organizations; the translation of books into indigenous languages, training for indigenous health workers, teachers and community leaders with an emphasis on teaching about the main risk factors, and the time of year these are most prevalent; constant entomological and serological monitoring, after training, by community leaders; the implementation of vector control methods, such as indoor residual spraying pyrethroid and improving housing; safe preparation of food for its immediate consumption or transport; safe water for drinking and basic sanitary conditions and the monitoring and treatment of infected individuals. In addition, surveillance and the use of drugs and vaccines, research in natural products; clinical and epidemiologic research; use of electron microscopy, genomics/bioinformatics, and applied biotechnology should be available to ensure healthier lives. All these aspects should occur within the context of environmental sustainability, and all are necessary in the regions where most of the zoonoses are found. There is an urgent need for a concerted effort by disciplines such Ecology, Epidemiology, Medical Entomology, Human and Veterinary Medicine, Public Health, Environmental Studies, Immunology, Microbiology, Pharmacology, Social and Political Studies, and Anthropology (62, 69–77).

We list the following pathogens, many of them emergent–reemergents with the potential to cause neglected pandemic zoonoses, based on the definitions of emergent diseases already given in this review: Human immunodeficiency virus (VIH), Ebola virus, Dengue virus, Simian immunodeficiency virus, Hanta virus, Hendra virus, Nipah virus, Menangle virus, West Nile virus, Avian influenza A H5N1 virus, Monkeypox virus, SARS virus, Rift Valley fever virus, Junin virus (Argentine hemorrhagic fever), Cercopithecine herpesvirus (Meningoencephalitis virus), Australian bat lyssavirus, *Rickettsia africae (*African tick bite fever), *Ehrlichia canis* (Leukocytic Rickettsia, human being, dogs), *Vibrio cholerae* (cholera), *Borrelia burgdorferi* (Lyme borreliosis), *Leptospira interrogans* (Icteric leptospirosis), *Treponema pallidum* (Syphilis), *Mycobacterium tuberculosis* (tuberculosis), *M. ulcerans* (Buruli ulcer), *M. leprae* (leprosy), *Chlamydia trachomatis* (human blinding trachoma; sexual disease), *C. psittaci* and *C. pecorum* (birds, mammals), *Clostridium difficile* and *Staphylococcus aureus* (antibiotics resistant), *Brucella suis* (wild boar brucellosis), *Salmonella* sp. (serotype enteritidis), *Coxiella burnetii* (Q fever), *Campylobacter jejuni,* and *Listeria monocytogenes* (gastroenteritis by contaminated food), *Cryptosporidium* spp. (waterborne disease human being, animals), *Escherichia coli* O157:H7 (enterohemorragic strain, Toxic shock syndrome by contaminated food; verotoxine production), *Leishmania* (*Leishmania)* spp. (muco-cutaneous and visceral leishmaniasis), *T. cruzi* (Chagas' disease), African *Trypanosoma, Plasmodium* spp. (Malaria); *Ascaris* spp., *Necator americanus*, *Ancylostoma* spp., *Trichuris* spp. (whipworms), *Strongyloides* spp. (Geohelminths, digestive, pulmonary system infections), *Dracunculus* spp. (Guinea worm disease; human being, animals), *Onchocerca* spp. (river blindness), *Wuchereria bancrofti* (lymphatic filariasis, "elephantiasis") (Nematelminthes infections); *Schistosoma* spp. (urinary and intestinal schistosomiasis or bilharzia), *Clonorchis sinensis* (liver and bile duct cancer), *Opisthorchis viverrini* (cholangiocarcinoma: gall bladder cancer), *Fasciola hepatica* (liver fluke disease, human beings, ungulates), *Paragonimus westermani* (acute lungs infection) (Platyhelminthes, Trematoda infections); *Tenia solium* (Neurocysticercosis) (Platyhelminthes, Cestoda infection); and *Sarcoptes scabiei* (mite, scabies) among others (13, 15, 16, 52–54, 57, 60). This list of emergent–reemergent pathogens should, like any other, be periodically revised and updated given the dynamics of the factors that determine changes in its transmission pathways.

Following on from this, it is appropriate to discuss the following considerations as a consequence of the permanent rise in industrialization and urbanization in Latin America and the Caribbean Region, the transmission of several infectious diseases, has increased dramatically (4, 5, 20, 55, 64, 69, 78–83). Its diagnosis, however, can be delayed for several months depending on the technique used. This obviously delays treatment causing the patient's condition to worsen. The fact that *T. cruzi* and its vectors can remain viable in food over long periods of time only exaggerates this problem. Nevertheless, these situations can be averted with the use of newly developed molecular tools, which, together with reference datasets, have shortened the diagnosis time to only a few days (45, 46, 84). It is to be hoped that in the future, some of the risk factors of these diseases will be at least qualitatively and quantitatively reduced, if not eliminated. Several of these measures could seem utopic because of the small attention they have traditionally received, but it would be a mistake not to mention them. The current healthcare system, although insufficient, should at least be used to improve the situation of the poorest communities while other tools are being developed. The need for investigation into the use of drugs to combat the infections, while minimizing the risks of the evolution of pathogen resistance to them, is obvious (85).

The investigations undertaken in the Caracas Valley, presented and discussed using a bio-eco-epidemiological approach, show that Chagas' disease is most certainly an emerging urban zoonosis and underline the high risks of infection for human beings and their domestic animals.

The following is a summary of the most important epidemiological aspects of the public health situation regarding the transmission of Chagas' disease by *D. marsupialis* in the Caracas Valley, which taken together create conditions of permanent risk from zoonotic infection by *T. cruzi* in areas with or without the presence of vectors: the adaption of *D. marsupialis* to city environments, from slums to economically wealthy urbanized zones; the capacity this marsupial has to infect mammals with metacyclic trypomastigotes that develop in the lumen of the anal scent glands; the violent ejection of fluid from the scent glands in response to threat and the proximity of these glands to the rectum and the urogenital organs, thus providing a means by which the feces and urine can become contaminated with the glandular fluid containing the metacyclics, leading to the possibility of infection through the consumption of spoiled food and drink.

The finding that contact between *T. cruzi* bloodstream trypomastigotes and genital mucosa can produce blood and tissue infections through the formation of pseudocysts with amastigote stages close to the lumen of the urinary bladder and seminal vesicle suggests an alternative possibility for the transmission of this zoonosis; when the cysts rupture, they could liberate the parasites either into the genital secretions or urine and thus to the exterior.

The fact that *R. rattus* may also act as a reservoir for *T. cruzi* and the evolution of the adaption of *P. geniculatus* and *P. rufotuberculatus* to human dwellings increase and geographically widen the risks for the transmission of Chagas' disease in the Caracas Valley.

The infection of adipose, bone-cartilage, and eye tissues by *T. cruzi* demonstrates their role as important micro-reservoirs of this parasitic flagellate and reveals yet another transmission pathway during organ transplants in immunosuppressed patients.

This review was initially undertaken in response to the risks of transmission brought about by the close contact between *D. marsupialis*, *P. geniculatus*, and human beings along the whole of the Caracas Valley, and the report (for the first time) of 124 acute cases of proven *T. cruzi* transmission to inhabitants in a totally urbanized sector of this capital city, as well as important results from other investigations carried out in areas encompassing the poorest slums to zones of high economic value. The permanent and high levels of industrialization and the migration of people from endemic regions of Venezuela and other countries can only further increase the probability of the emergence of this zoonosis.

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Trypanosoma cruzi, the causal agent of Chagas disease: boundaries between wild and domestic cycles in Venezuela

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Trypanosoma cruzi the etiological agent of American Trypanosomiasis or Chagas disease (ChD) is transmitted by triatomines vectors between mammals including man. *T. cruzi* has existed for *circa* 150 Ma in the Americas and nearly 10 million people are currently infected. The overlap between wild and domestic ecotopes where *T. cruzi* circulates is increasing. Host–parasite interactions have been determined by infection patterns in these cycles, all under natural or laboratorial conditions. This mini-review describes specific parasite niches, such as plant communities or biological corridors between domestic and wild landscapes, in order to help identify risk factors for ChD and define the boundaries between wild and domestic transmission cycles, with an emphasis on research undertaken in Venezuela.

Keywords: *Trypanosoma cruzi*, domestic cycle, wild cycle, Chagas disease, Venezuela

INTRODUCTION

Parasites and their hosts form part of trophic webs and may be considered bioindicators of climate changes and anthropogenic impacts (1). American trypanosomiasis (AT) or Chagas disease (ChD) is a complex parasitosis caused by *Trypanosoma cruzi* (Kinetoplastida, Trypanosomatidae), which can be dispersed by enzootic or anthroponotic routes in trophic webs, which involve several mammals groups including human beings (**Figure 1**). So, this parasite affects currently, until 10 million people and as such can be considered a re-emerging public health problem especially in Venezuela (2, 3).

The *T. cruzi* life cycle begins when vectors (Hemiptera, Reduviidae, Triatominae) expel feces or urine with infective metacyclic trypomastigotes which then come into contact with mammals via intact mucous or skin abrasions. The trypomastigotes pass into the bloodstream and invade a wide range of tissues where they differentiate into amastigotes, epimastigote, and trypomastigotes once again. The latter are re-released into the bloodstream and can be imbibed by another or the same vector, which pass into the intestine and transform, once more, into metacyclic, performing a vectorial transmission (3).

Recent outbreaks of oral transmission in Brazil and other Latin American countries, including Venezuela, emphasize the importance of this alternative route in enzootic and zoonotic cycles (4).

Trypanosoma cruzi has been grouped into six discrete typing units (DTU): *T. cruzi* I (TcI) to *T. cruzi* VI (TcVI). The TcI–TcVI classification is, however, a relatively recent nomenclature and the associations of the different genotypes with particular hosts, ecotopes, or transmission cycles remains under debate (5).

Trypanosoma cruzi has existed in the Americas for *circa* 150 Ma and has been in contact with Amerindians for 15,000 years. The genome of this parasite in mummies from the American Pacific (7,500 BC to 1,500 AC) indicates a pre-Columbian origin thus breaking the myth of its establishment as a product of recent colonialism (6).

The host–parasite associations and risk factors associated with ChD is being described in recent studies of specific niches such as mammal caves, plant communities, and biological corridors between domestic and wild ecotopes, in order to widen the understanding of the boundaries between wild and domestic *T. cruzi* cycles in Venezuela.

VECTOR–PARASITE: PATTERNS OF WILD AND DOMESTIC *TRYPANOSOMA CRUZI* CYCLES

Triatomines are eclectic in their ecological niches: they are found from 42°N to 46°S and between 400 and 1200 m.a.s.l. (7). A total of 140 species are grouped into 8 tribes and 15 genera. *Triatoma maculata*, *Rhodnius prolixus*, and *Panstrongylus geniculatus* are the most important in Venezuela by their frequency of infection by *T. cruzi* and their association with domestic and peridomestic ecotopes in economically depressed rural areas (8).

Rhodnius prolixus in Venezuela, is predominantly intradomiciliary with a high-reproduction rates, a voracious blood intake and fast defecation time, all of which are attributes of a primary vector. In the wild, this triatomine is predominantly found in palms with synanthropic vertebrates providing the blood source (9, 10). *T. maculata* is found in palms, dry trees, wooden fences, and bird nests near human dwellings. Its domiciliation, in function to phenotypic and genotypic discrimination according to its ecotopes, guarantee the previous consideration about its presence a risk factor for parasite transmission in Brazil, Colombia, and Venezuela (11–13).

Coconut palms (*Coccus nucifera*) is a suitable triatominae ecotope in peridomicile environments in north-eastern Venezuela, as was corroborated by the presence of 242 *R. prolixus* and 144 *T.*

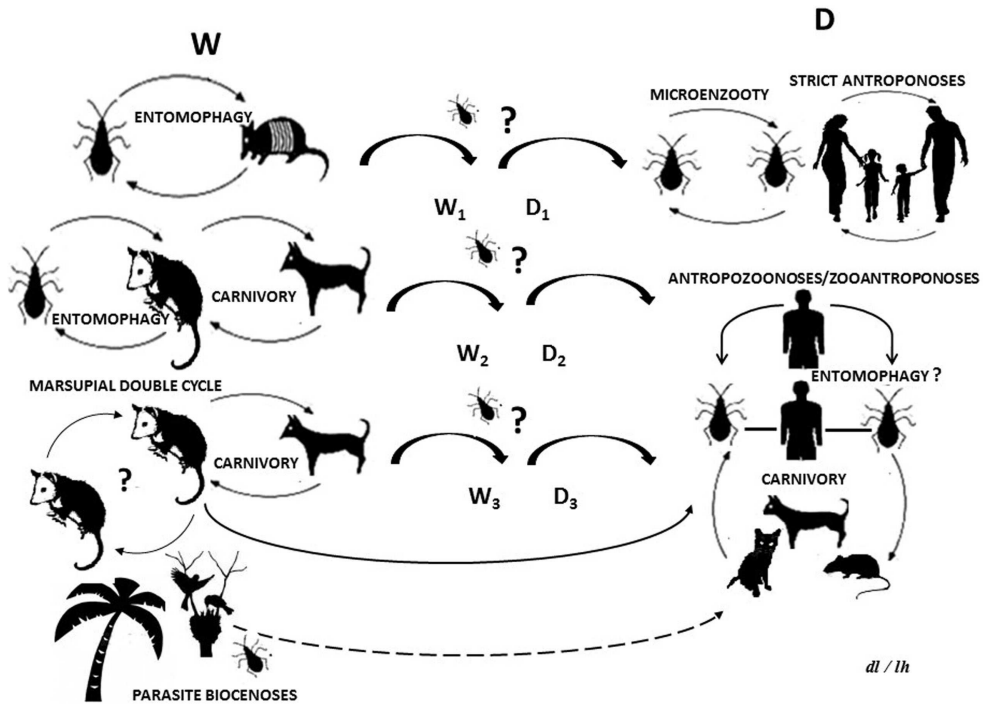

FIGURE 1 | Life cycle of *Trypanosoma cruzi*: (W) wild cycle with enzootic parasite circulation between wild and synanthropic mammals and other biotic components. (D) Domestic cycle with parasites circulating as a zooanthroponosis, anthropozoonosis, strict anthroponosis, or micro-enzooty. (W_n) Wild sub-cycles (D_n) domestic sub-cycles. Question marks indicate uncertainty as regards parasite circulation patterns or processes. The dotted line indicates a hypothetical route.

maculata adults in 14 coconut palms just 5 m away from human habitations. PCR amplification of the D7 divergent domain of the 24S rRNA genes; the non-transcribed spacer of mini-exon genes and the size-variable domain of the 18S rRNA genes confirmed that 98% of the *R. prolixus* and 70% of the *T. maculata* individuals were infected by *T. cruzi*–TcI. Exploration of coconut and its derivatives in industry and ethno-botany could pose a risk by exposing human beings to contaminated triatomines fluids (14).

Other triatomines vector species can acts as boundaries between wild and domestic environments. Examples are *Eratyrus mucronatus* and *Panstrongylus rufotuberculatus* found in palms, tree holes, and the dens of animal reservoirs, in Bolivia, Colombia, and Venezuela (15, 16). *P. rufotuberculatus* is a widely dispersed triatomine; in Venezuela could be monitored in peridomiciles in Anzoátegui state when wildlife fauna was affected or natural enemies were altered. Inoculation of the intestinal content of these insects in murine model, shown invasion of chondral tissue, brain, and kidney, revealing novel clinical aspects to be considered in relation to ChD (16).

Other triatomine species is *P. geniculatus*, which has been associated to infection of rodents and marsupials in rural or domestic ecotopes. The loss of its natural niches has also promoted its avid penetration in human dwellings. This is particularly worrying as this insect has been imputed as parasite font in cases of oral transmission of ChD in Caracas, and other cities in Venezuela. The modification of reservoir niches by climate change or the human exploitation of landscapes favors its peridomestic and domestic colonization (8, 17, 18).

The recent report of K-DNA and satellite DNA of *T. cruzi* in the intestines of *P. geniculatus* from sites along the Orinoco River, near Amerindian settlements, was associated with records of this species in neighboring countries, which could constitute evidence of biological corridors of the parasite with potential impacts on indigenous populations (19).

RESERVOIR–PARASITE: THE INTERACTION OF WILD MAMMALS, HUMAN BEINGS, AND DOMESTIC ANIMALS

Up until now, 180 species have been identified as reservoirs included in Artiodactyla, Carnivora, Cingulata, Chiroptera, Didelphimorphia, Lagomorpha, Perissodactyla, Pilosa, Primates (including man), and Rodentia orders (18).

Trypanosoma cruzi is considered as euryxenic according to the range of reservoirs it inhabits and eurytopic as regards the different organs it infects. Alternative transmission routes have also been reported in order to fluctuations in reservoir subpopulations, which could explain the plasticity of this zoonosis and urban outbreaks. The parasite may be orally transmitted via ingestion of infected triatomines, contaminated food, blood, or viscera from reservoirs (18, 20).

Studies of the distribution patterns of *T. cruzi* genotypes should consider ecological peculiarities since that genetic diversity has on the outcome of zoonosis or human disease (20). *T cruzi* Z3 in the southern Amazon (*Trichomys* rodent–*T. cruzi* complex in Brazil)

and *T. cruzi* TcIII in the northern Amazon (*Dasypus–T. cruzi* complex in Venezuela) provide instances of the expansion of these wild genotypes into urban cycles (21, 22).

Particularly, *Dasypus novemcinctus* form part of an ancient enzootic *T. cruzi* cycle in the touristically important north-eastern region of Venezuela. These mammals act as TcIII reservoirs, as has been shown by the PCR amplification of the intergenic region of HSP60 genes for *T. cruzi* and the restriction digest of PCR products by *Eco*RV. The interaction of *Dasypus novemcinctus* with human beings, domestic animals, and peridomestic triatomines in this region, may be an important risk factor (22).

The ubiquity of *T. cruzi* in mammal reservoirs and its effect on host fitness represents an element that has been scarcely studied. Parasite isolates from *D. marsupialis*, *R. prolixus*, and *T. maculata* from rural and urban areas of Venezuela have yielded 10^5 flagellates/ml of blood in mice models, producing 80% mortality with neurological symptoms such as ataxia, paralysis, and sphincter relaxation. Alterations as meningo-encephalitis, edema of the neuropil and parasitism near vascular system could facilitate the hematological dispersion of the parasites. These neurological disorders could alter the behavior of mammals toward predators thus modifying parasite transmission in trophic web (23, 24).

CONCLUSION

Parasitism implicates energy movement among organisms, affecting the interactions and robustness of some trophic webs. *T. cruzi* is a clonal parasite with wild and domestic cycles, some author has proposed that vector–mammal interaction and saturation vector feeding rates, depend on mammal density when the vector/mammal ratio is low and vector density when this ratio is high (25). The number of infected mammals is conditioned by their relative abundance, which thus influences their availability as a blood source for triatomines.

New incursion of some vectors or mammals reservoirs species in *T. cruzi* life cycle, is important in the epidemiology of AT and ChD. The potential trophic web can include ingestion of insects, contaminated food, or host carnivorous behavior, which could be the primary route for *T. cruzi* transmission in some wild cycles (**Figure 1**). Synanthropic mammals and vectors are not excluded from this, thus, providing a way by which the wild and domestic cycles could be crossed (26–28).

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Ultrastructural study on tissue alterations caused by trypanosomatids in experimental murine infections

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The ultrastructural study in different tissues of mice experimentally infected with isolates of *Trypanosoma evansi*, *Trypanosoma cruzi*, and *Leishmania mexicana* reveals changes in cardiac myocytes, skeletal muscle fibers, and hepatic, adrenal, kidney, and spleen cells. Some of these changes were cytoarchitectural and others consisted of necrosis. Alterations in the microvasculature were also found. The mononuclear cell infiltrate included neutrophils, eosinophils, and macrophages. This work shows that diverse mice tissues are important target for trypanosomatids.

Keywords: pathology, ultrastructure, murine tissues, experimental infections, trypanosomatids

INTRODUCTION

At the present time, there is a vast literature concerning the effects of protozoan parasites in the ultrastructure of mammalian tissues. These works include horse and mouse skeletal muscles infected by *Trypanosoma evansi* (1, 2) and *Toxoplasma gondii* (3, 4), human skeletal and mouse cardiac muscles by *Trypanosoma cruzi* (5, 6), avian skeletal muscle by *Plasmodium cathemerium* (7), and human skeletal muscle by *Plasmodium falciparum* (8). Alterations in the mouse adrenal gland and liver were provoked by *Trypanosoma evansi* (9, 10) and *Plasmodium berghei* (11, 12). In this context, it would be interesting to know if structural changes observed are similar in all studied species. Furthermore, with this investigation we intend to perform a systematic work on the ultrastructure of alterations in experimental murine infections by some trypanosomatids. This could help to understand better the biology of trypanosomatids in vertebrate host.

MATERIALS AND METHODS

EXPERIMENTAL INFECTIONS

For experimental infections, Balb/c mice were used. They were divided into 3 groups of 10 mice each one for the three used species (*Trypanosoma cruzi*, *Trypanosoma evansi*, and *Leishmania mexicana*), and one additional 10-mice group was used as a control. Animals were infected by intraperitoneal (i.p.) route with inocula consisting of 10 parasites/g of animal body weight and uninfected mice were kept as controls. Three mice from each group were randomly selected after prepatent period and killed under anesthesia during peaks of infection. Then tissue samples were removed and processed for transmission electron microscopy. The experimental procedures were approved by the ethical committee of the Sciences Faculty at the Central University of Venezuela, and the work was conducted in agreement with the regulatory standards.

TRANSMISSION ELECTRON MICROSCOPIC STUDY

Tissue samples were fixed with Karnovsky's solution, in phosphate buffer at pH 7.4 and 320 mOsm, post-fixed in 1% O_sO_4, and embedded in epon resin. Sections were cut with a diamond knife in a Porter-Blum MT-2B ultramicrotome and stained with uranyl acetate and lead citrate. Ultrathin sections were observed in a Jeol JEM – 1011 transmission electron microscope, at an accelerating voltage of 80 kV.

RESULTS

Pathological reactions in experimentally infected mice with diverse trypanosomatids showed some common characteristics in studied tissues of different replicates for each experimental group. Cardiac myocytes in *T. evansi* (**Figure 1A**) and skeletal muscle fibers in *T. evansi* and *L. mexicana* (**Figure 1B**) parasitized animals exhibited atrophy. In cardiac myocyte sections, myofibrillar disorganization and myofilament loss were seen (**Figure 1A**). Skeletal muscle fibers from mice infected with *T. evansi* showed segmental necrosis. In these areas (**Figure 1C**), mitochondrial paracrystalline debris was located next to contractile masses. As it is showed in **Figure 2A**, in *T. cruzi* similarly to the case of infection with *T. evansi*, liver hepatocytes showed an increment of lipid droplets, depletion in glycogen content, and decrease of microvilli in Disse's space. Sinusoid endothelial cells were widened with scarce pinocytotic vesicles. Hepatocyte debris was observed in some sinusoids, suggesting parenchymatous cell necrosis (**Figure 2B**). In mice infected by different trypanosomatids, adrenal cortical cells alterations were represented by lack of cytoarchitectural relations between mitochondria and smooth endoplasmic reticulum (SER), swelling of SER elements, decrease of mitochondrial cristae, widened nuclear envelop, change of electron density in cell cytoplasm, and presence of lysosomes (**Figures 3A,B**). Intracellular erythrocytes were observed in the infection with *T. cruzi* (**Figure 3B**), while *T. evansi*

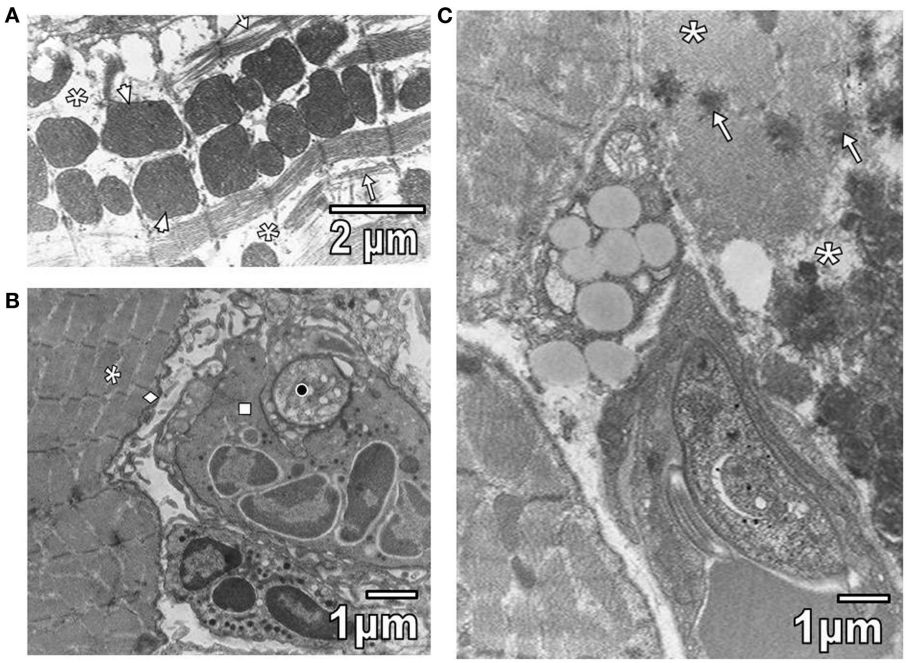

FIGURE 1 | (A) This section shows widened intermyofibrillar spaces (asterisks) of cardiac myocytes in *T. evansi* extensively occupied by mitochondria (arrowheads) and disorganized sarcomeres (star). **(B)** Section of skeletal muscle from a mouse parasitized with *L. mexicana* is shown. Intermyofibrillar (asterisk) and subsarcolemmal (rhombus) spaces are slightly widened. Observe a parasite (black circle) inside of neutrophil (square). **(C)** Section of skeletal muscle from a mouse parasitized by *T. evansi* is shown. Note areas of segmental necrosis (asterisks) showing mitochondrial debris with paracrystalline inclusions (arrows).

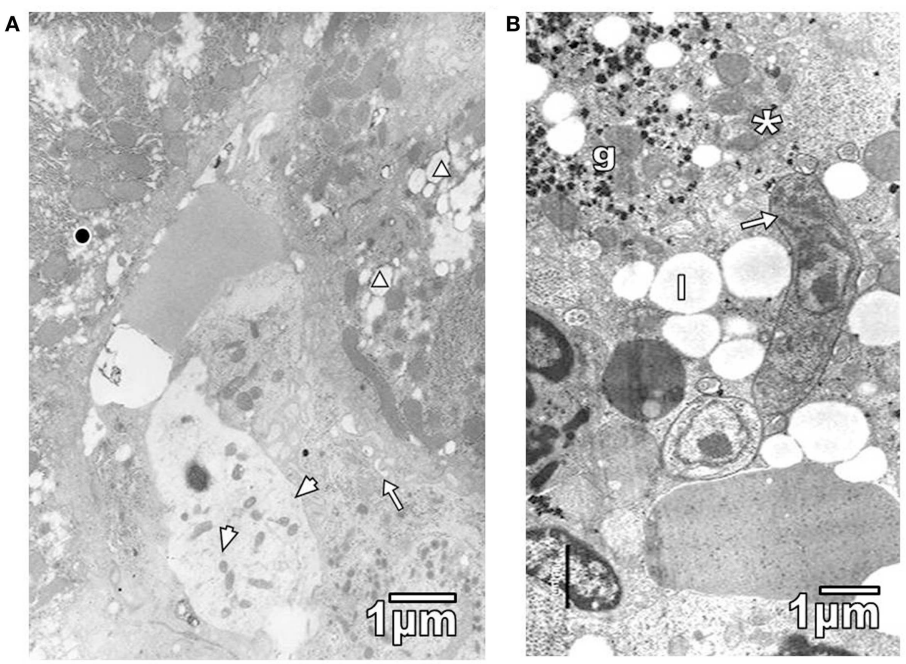

FIGURE 2 | (A) Section of hepatic parenchymatous cells from a mouse parasitized with *T. cruzi* is shown. Observe lipid droplets (triangle), glycogen depleted areas (black circle), some microvilli in Disse space (arrow), and widened sinusoid endothelial cells with few pinocytic vesicles (arrowheads). **(B)** Sinusoid lumen showing *T. evansi* parasite (arrow) and hepatocyte debris, including glycogen particles (g), mitochondria (asterisk), and lipid droplets (l).

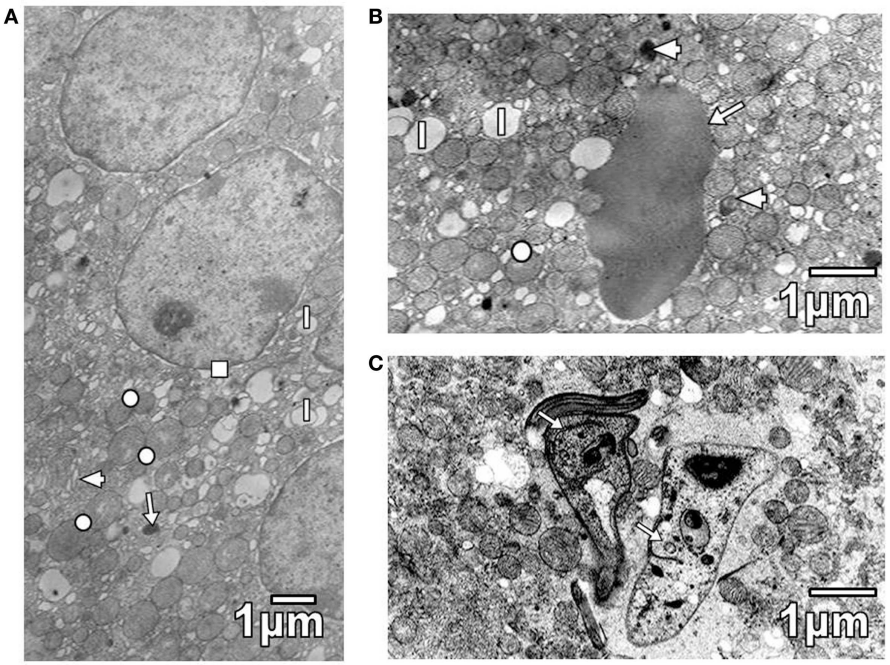

FIGURE 3 | (A) Section of cortical cells shows swollen elements of SER (arrowhead), some mitochondria with decreased cristae (circle), lipid droplets (l), lysosomes (arrow), and a widened nuclear envelop (square). **(B)** Section of a cortical cell from *T. cruzi* parasitized mouse, showing mitochondria with variable number of cristae (circle), lipid droplets (l), and lysosomes (arrowheads). Note the presence of an erythrocyte (arrow). **(C)** Section of a cortical cell from a mouse parasitized with *T. evansi* is shown. Observe the presence of parasites (arrows).

parasites were seen in cortical cells of parasitized mice (**Figure 3C**). Additionally, capillary fenestrae were widened.

Kidney convoluted tubules were observed with thickened basement membrane, disorganization of interdigitations, and significant decrease of their number; in some areas was noted swelling of rough endoplasmic reticulum (RER) and mitochondrial cristae (**Figure 4A**). As it is seen in **Figure 4A**, the capillary endothelial cell cytoplasm also presented swollen RER and mitochondria. Spleen ultrastructure was studied in *T. evansi*-infected mice. Tissular disorganization, fibrosis, and apoptotic bodies (**Figure 4B**), as well as necrosis were observed. The inflammatory infiltrate consisted of mononuclear cells, such as neutrophils, eosinophils, and macrophages (**Figures 1B** and **5A,B**). Trypanosomatids were found in extracellular spaces and inside of mononuclear cells (**Figures 1B** and **5A,B**).

DISCUSSION

Infection with several trypanosomatids leads to a rapidly lethal disease in different strains of mice. According to various authors [for review Ref. (13)], living and dead trypanosomes produce a number of biologically active substances, which are involved in the etiology of lesion. As it has been shown in mice infected with *T. cruzi*, the acute infection is characterized by a severe immune depression (14). Immunosuppresion also occurs in Leishmaniasis (15). The mechanism of action of released molecules by *T. cruzi* and *Leishmania* sp. could suggest a role as regulatory activating and inhibiting factors of host immune cells (14, 16, 17). The role of IFN-γ during *T. cruzi* infection was demonstrated when IFN-α and IFN-γ receptor KO mice showed higher rates of parasitemia and mortality (18). Infected IFN-γ KO mice showed increase in cellular infiltrates in heart and skeletal muscles and reduced survival (18–20).

A number of reports have documented the role of NO in host defense against pathogens. In the case of *T. cruzi*, experimental infection induces NO production and suggests that IFN-γ and TNF-α are involved in the phenomenon (21, 22). Recently, it was reported that in *L. amazonensis* infected mice, pravastatin increased the phagocytosis mediated by complement and immunoglobulin receptors, and induced a rise of nitric oxide production by macrophages, allowing these cells to kill ingested leishmania organisms, with reduction of the overproduction of tumor necrosis factor (23). Other experiments have shown that IFN-γ and TNF-α-mediated activation of macrophages leads to increased production of NO, which in turn suppresses T cell activation. NO and oxygen radicals release from locally activated macrophages and stimulated endothelial or tissue cells, have been implicated as the final mediators in cytokine-induced pathology in malaria (24, 25).

Our investigation in murine experimental infection with trypanosomatids is in line with a degree of striated muscle alterations, which varied from slight to severe, during the pathogenesis and development of disease. The ultrastructural pathology data are similar to previous results concerning cardiac myocytes in hamsters and mice experimentally infected with *T. cruzi* (6, 26), "derrengadera" by *T. evansi* in wild horse skeletal muscle fibers (1), and in mice parasitized with *T. gondii* (3). In advanced Chagas'

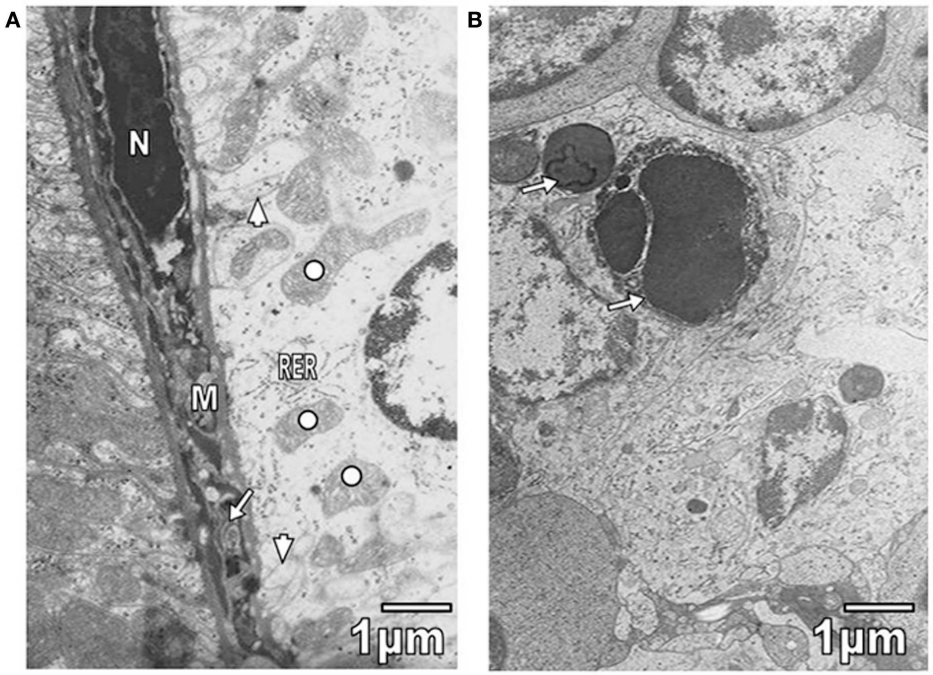

FIGURE 4 | (A) Section from proximal convolute tubule of a mouse parasitized by *T. evansi* is shown. Observe portions of interdigitations (arrowheads), swelling of RER cisternae, and mitochondrial cristae (open circle). In the capillary, swollen RER (arrow), mitochondria (M), and hyperchromatic nucleus (N) are seen. **(B)** Section of spleen from parasitized mouse with *T. evansi* is shown. Note the presence of apoptotic bodies (arrows).

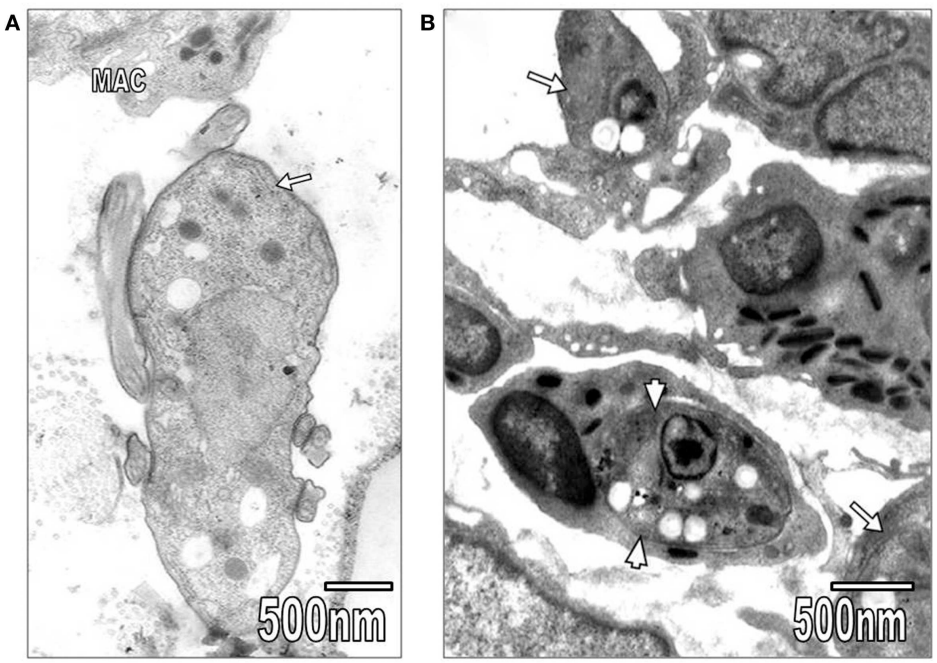

FIGURE 5 | (A) In this section, *T. evansi* (arrow) and a macrophage (MAC) are seen. **(B)** Section showing *L. mexicana* organisms inside of an eosinophil (arrowheads) and a macrophage (arrows).

disease patients, capillary damage also has been reported in skeletal muscle (5).

These results in relation to changes in liver hepatocytes of mice infected by *T. cruzi* and *T. evansi* were in some aspects similar to those described in liver of mice parasitized with *P. berghei* (27), including an increment of lipid droplets and depletion of glycogen particles, simultaneously with a decrease of microvilli in the Disse's space. Also, necrotic hepatocytes and a thickening of endothelial cell cytoplasm were found in both cases. In adrenal cortex of mice infected with *P. berghei* (11), erythrocytes were observed inside cortical cell cytoplasm, as we also observed in the infection with *T. cruzi* and the report by Rodríguez-Acosta et al. (28) in cortex of adrenal gland in mice injected with a lethal dose fifty (LD_{50}) of bee venom. In the investigation of Pulido-Méndez et al. (11), parasites were not seen inside of cortical cells. On the contrary, in *T. evansi* infected cortical cells contained trypanosomes as it was described by Rossi et al. (9), and in the present work.

The ultrastructural pathological changes as those described in hepatocytes and adrenal cortical cells also were found in kidney of mice in *Plasmodium berghei* infection (12), including swelling of some organelles and disorganization of interdigitations and decrease of their number in some areas. Interestingly, loss of interdigitations and tubular vacuolization were also described in convoluted proximal tubules of mice intraperitoneally injected with a lethal dose fifty LD_{50} of *Apis mellifera* (29) in association with swelling of endothelial cell mitochondria and RER as in the present work.

Besides the splenic changes caused by action of parasite could be related to a possible capability for particular proteolytic secretions (9), due to a *T. evansi* induced hepatic alteration since a liver deterioration can rise the portal pressure. Indeed, advanced hepatomegaly increases the portal flux causing the blood to flow through collateral systems via portal and cava veins (30). The portal flux increments are determined by vasodilatation of the splanchnic tissue (stomach, intestine, pancreas, and spleen) admitting consequently an augmentation in the blood flux arriving to the organs (31). The first ultrastructural indication of damage is through a considerable amount of splenic debris. According to Jain (32), the presence of such remains is derived from erythrophagocytosis and cell debris phagocytosis occurring in the infected spleen.

The mononuclear cell infiltrate consisted of neutrophils, eosinophils, and macrophages. Macrophages and eosinophils were reported by Tonino et al. (3) in mice infected by *T. gondii*. Similarly, macrophages were reported by Quiñones Mateu et al. (1) in horses parasitized by *T. evansi* and in mice infected by *P. berghei* (12). In our investigation, we did not observe mastocytes as described in the infection with *T. gondii* (3) and lymphocytes as reported in mice infected by *P. berghei* (12). Our ultrastructural study demonstrates that several tissues of mice are certainly targets for trypanosomatids. Moreover, the murine model is very useful for pathological studies in trypanosomiasis using transmission electron microscopy.

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Genetic and morphometric variability of *Triatoma sordida* (Hemiptera: Reduviidae) from the eastern and western regions of Paraguay

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Triatoma sordida is widely distributed throughout the Chaco and the Eastern Region of Paraguay. It is associated to palm trees and artificial ecotopes located in peridomestic environments. The aim of this work was to determine genetic and morphometric variability and feeding behavior among population of *T. sordida* captured in domicile and peridomicile areas of Paraguay. Feeding contents and levels of genetic and morphometric variation were determined in 124 *T. sordida* from domicile and peridomicile populations of San Pedro and Paraguarí departments of the Eastern Region and Boquerón and Presidente Hayes departments of the Western region using Double Diffusion Gel, random amplified polymorphic DNA (RAPD), and head and wings morphometry. Morphometric analysis revealed isolation of populations by geographic region and larger size in triatomine populations from the Western Region. RAPD showed no specific patterns for domicile and peridomicile populations. The estimator of diversity (F_{ST}; 0.08) and high gene flow obtained (N_m; 5.7) did not allow the establishment of genetic differentiation within the same region. The blood meal source showed that poultry feeding was 38% of host preferences, and human blood was the second feeding preference (24%) in the insects from the Eastern Region while poultry feeding was predominant in those from the Western Region (30%). This work showed homogeneity between *T. sordida* populations of the same region and between domicile and peridomicile. The genetic diversity was determined among *T. sordida* populations of both geographical regions suggesting differentiation associated to eco-geographical isolation by distance. It is important to notice that pattern feedings were different between the two regions. Further studies should be focused on how phenetic and genetic variations could be related to the adaptation capacity of these triatomine populations to domicile, increasing their vector potentiality in the transmission of Chagas disease.

Keywords: *Triatoma sordida*, Chagas disease, RAPD, morphometric analysis, feeding content

INTRODUCTION

The subfamily Triatominae (Hemiptera: Reduviidae) includes over 144 species of strictly hematophagous insects, considered potential vectors of *Trypanosoma cruzi* among mammals. However, not all of them are epidemiologically important (1–4). In Paraguay, 11 species of triatomines have been registered and from them *Triatoma infestans* (5) and *T. sordida* (6) have been found naturally infected with *T. cruzi* (7, 8).

In the Southern Cone countries, the most important hematophagous vector involved in the transmission of Chagas disease is *T. infestans*. *T. sordida* of wild origin seems to have been disseminated from Brazilian plateaus toward the south, and now is found in Argentina, Bolivia, Paraguay, and Uruguay where it occupies extensive geographical areas but generally in small populations of individuals (9, 10). *T. sordida* is considered as a ubiquitous species with high ecological potential living in various ecotopes and feeding from different sources. This insect could withstand large environmental changes that cause the disappearance of his competitors and could widen its ecotopes to dead and dry trees (11). However, these ecotopes usually do not offer feeding sources, stimulating its dispersion to peridomiciles and domiciles and there is ever-greater contact inside and around houses with species other than *T. infestans* that were not very important for vector transmission in the past because they used to be found only in natural ecotopes, as *Triatoma sordida*. Their epidemiological importance regarding vector transmission of Chagas disease is still low, but they may become a bigger problem if they become domesticated, thereby occupying the empty place left by *T. infestans* (12, 13).

On the other hand, the sympatry with *T. infestans* in domicile and peridomicile is known as well as the diversity of the ecotopes it occupies and the difficulty this has meant for its control.

T. sordida is associated with re-infestation sources of dwellings treated with insecticides and currently is considered a potential vector of Chagas disease (11, 14–16).

Morphometric and molecular analyses are important tools that provide evidence of the population structure of insect vectors. Enzymatic and genetic studies performed on this triatomine species have confirmed the variability of loci in two groups (17–19). *T. sordida* group 2 seems to be restricted to the Chaco and group 1 is widely distributed in Bolivia and Brazil (18, 20). Besides, the genetic distances between both populations led to infer the hypothesis of recent cryptic speciation (21).

The study based on morphometric analysis and the molecular patterns of random amplified polymorphic DNA (RAPD) in *T. brasiliensis* have reported the existence of a common relationship between wild and domiciliary populations (22). Similarity, the gene-flow index and reduced genetic divergence found between different populations of *Triatoma rubida* support sub-specific designation for this species (23). In relation to *T. sordida*, low levels of genetic variation among populations of southeastern Brazil have been reported through the analysis of 28 allozyme loci. None of these loci presented significant differences between any pair of populations, and only two showed polymorphism, accounting for low levels of heterozygosity (10). Similarly, the genetic study of *T. sordida* from different ecotopes of Paraguay revealed low genetic diversity levels suggesting that extra-household populations could represent an important epidemiological link to maintain the transmission of trypanosomatids (24).

Thus, triatomines studies based on diverse molecular markers have been used to clarify phylogenetic and evolutionary relationships between species, apart from inferring divergences and population structure, as it has been demonstrated previously in others triatomines, where wide polymorphism between discrete populations suggest the existence of a species complex (25, 26). These facts suggest an increment of the epidemiological significance of vectors considered secondary (12, 22). In the case of *T. sordida*, the polymorphism levels and its implication in the infestation of dwellings in endemic areas for Chagas disease in Paraguay are still unknown. The objective of the present work was to determine the feeding behavior and genetic and morphometric variability among population of *T. sordida* captured in domicile and peridomicile of the two geographical regions of Paraguay. Finally, this study contributes to improve surveillance strategies embracing this potential vector.

MATERIALS AND METHODS

STUDY SITES, BUG COLLECTION, AND PARASITOLOGICAL SEARCH

The Eastern Region is humid, sub-tropical, composed by valleys, small hills, and wooded areas. The average annual temperature is 24.3°C and the average annual rainfall is between 1000 and 1600 mm. (27). The Western or Chaco Region is characterized by extreme temperatures ranging from 45°C in spring and summer to 27°C in winter with annual minimum rainfall of 100–900 mm. (28). Both regions are separated by an important ecological barrier, the Paraguay River.

The specimens were collected by manual capture in poultry house, stables, and pigsties of peridomicile and intra-domicile areas of San Pedro (SP) and Paraguarí (PA) departments of the Eastern Region of Paraguay; Boquerón (BO) and Presidente Hayes (PH) departments of the Western Region (**Figure 1**). All triatomines were maintained alive and classified previously as *T. sordida* according to Lent and Wygodzinsky (29).

FIGURE 1 | Geographical localization of the study area. The green dots show the capture places in the different departments of the Republic of Paraguay. Departments: Boquerón (BO), Presidente Hayes (PH), Paraguarí (PA), and San Pedro.

One hundred twenty-four specimens were analyzed: 63 males and 61 females (**Table 1**). All insects were studied by morphometric analysis and half of them were studied by molecular methods. Parasitological search was also carried out microscopically in all insects by the direct observation of their feces and morphological identification performed after staining with Giemsa at 400× in an Olympus microscope in order to identify trypanosomatids. Characteristic morphological features of *T. cruzi* were identified as described by Hoare (30).

MORPHOMETRIC ANALYSES

Head and wings were selected according to protocols previously described (31–33). In the head, seven homologous points were selected (**Figures 2A,B**), while six distances measurements between the points of intersection of the veins were used for wings (**Figure 2C**). All measurements were made in duplicate by the same researcher and the images were captured using a lucid camera connected to an Olympus stereoscopic microscope DF Plan 1×.

The matrixes were tabulated by sex and population. The sexual dimorphism and Guillaumin profile were determined to obtain information about the general size of a group respect to other (34, 35). The principal components analysis (PCA) was carried out using covariance matrix from which a factorial map was constructed to show the differences in size and shape among sexes and populations (36). The free-allometry analysis for shape differences was performed after the discriminant analysis (DA) made

Table 1 | Distribution of *T. sordida* captured in different localities of the Paraguayan regions.

Localities	Paraguayan region	Latitude	Longitude	Number of *T. sordida* evaluated		
				Males	Females	Total
Cerro Guy (PA)	Eastern	25°41′39.95″	57°10′51.96″	16	20	36
San Pedro (SP)		24°05′19.39″	57°04′35.47″	15	11	26
Gral. Bruguez (PH)		24°45′13.31″	58°49′37.29″	17	16	33
Galilea (BO)	Western	22°35′ 00″	59°55′ 59.90″	15	14	29
				63	61	124

Departments: Boquerón (BO), Presidente Hayes (PH), Paraguarí (PA), and San Pedro (SP).

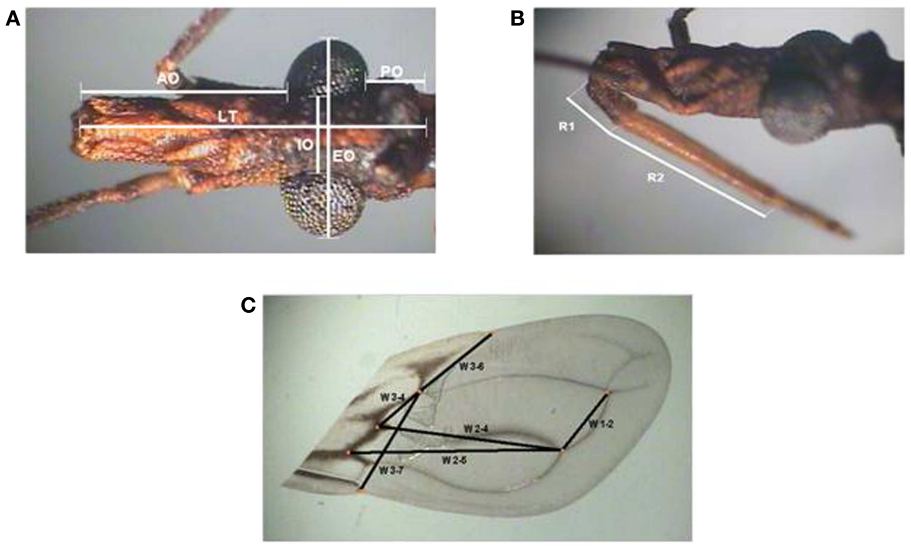

FIGURE 2 | Diagram of head and wings measurements used for the morphometry of *Triatoma sordida* is shown. **(A)** Dorsal (left) and **(B)** lateral (right) views of the head indicating morphometric measurements taken. AO, anteocular distance; PO, post-ocular distance; LT, total length; IO, inner distance between eyes; EO, outer distance between eyes; R1, length of first rostral segment; R2, length of second rostral segment. **(C)** Dorsal view of right wing with measurements taken for the distance between the following points: W1–2 between point 1 and 2, W2–4 between point 2 and 4, W2–5 between point 2 and 5, W3–6 between points 3 and 6, W3–4 between points 3 and 4, and W3–7 between points 3 and 7.

on the set of common principal components (CPC), excepting the first common principal component (CPC1), according to a protocol described by Dujardin et al. (37). For this, it was indispensable to check the compatibility with the model of CPC using a Chi-square goodness-of-fit test (X^2). All parameters were calculated using the JMP 4.0.0 (38) and NTSYSp.c version 2.10p (39) statistical packages.

FEEDING SOURCE ANALYSES

Extraction of blood content

The intestinal content of 62 adult specimens (29 males and 33 females) was extracted; 34 of them were from the Eastern Region (PA) and 28 from the Western Region (Pte. Hayes). In order to do this, a section was made in the front third of the abdomen of the specimens. When the content volume was insufficient, the complete promesenteron was transferred to a vial (40). Each vial had the same blood sample with 180 μL of 4% saline solution and 20 μL of 10% crystal violet and the mixture was maintained at 4°C for 24 h (41).

Determination of the feeding source

The Gel Double Diffusion method was carried out in a glass slide (7.5 cm × 5 cm) using 3.5 mL of 1.3% agar (I.D. Oxoid Agar) diluted in veronal hydrochloride buffer (pH 8.6). This preparation was maintained in a humid chamber for 24 h (40, 42). In the agar, there was a central hole that was filled up with the specific antiserum and six peripheral holes, five containing the diluted antigen (blood sample from different triatomines) and one with saline solution (negative control).

The antisera used for the identification of the feeding source was against human, poultry (chicken), dog, cat, goat, mice, and guinea pig blood and were also put in contact with the intestinal content of the triatomines searching for the corresponding antigen. All the antisera were prepared and tested previously in the

Laboratory of General Ecology of the University of Buenos Aires, Argentina.

RANDOM AMPLIFIED POLYMORPHIC DNA

The extraction of DNA was carried out according to the protocol of Promega Wizard Genomic Purification Kit, USA (43) in five legs of each insect (25).

The amplification reaction was performed according to the protocol of Williams et al. (44) modified by Carrasco et al. (45). The DNA of 62 specimens: PH (8 males, 8 females), BO (7 males, 8 females), PA (8 males, 8 females), SP (8 males, 7 females) was amplified with four primers to distinguish triatomine species of the same genus or identify affinities between species. The primers were: A_1 (5′-TCACGATGCA-3′), A_2 (5′-GAAACGGGTG-3′), L_4 (5′-GTGGATGCGA-3′), and L_5 (5′-AAGAGCCCGT-3′). The PCR was set up as follows: a final volume of 25 μL PCR mixture that contained 0.25 mM dNTPs (Pharmacy Biotechnology, Sweden), 10 pmol of primers, 1.0 unit of *Taq* polymerase (Gibco Life Technology), 5 ng of DNA template in a buffer with 2 mM MgCl; 50 mM KCl, 10 mM Tris–HCl, pH 8.8 was used; each amplification included a DNA-free negative control. The visualization of the products was obtained using 2.5% ultra-pure agarose gel electrophoresis stained with ethidium bromide. The bands obtained were digitalized by KODAK 1D (Kodak Digital Science) software.

DATA ANALYSIS

The binary matrix was built using the specimens that generated better band reproducibility and intensity. For the analysis, it was assumed that the *T. sordida* populations were in Hardy–Weinberg equilibrium and that there were no selection processes favoring any particular genotype. All loci were entered in a binary matrix and a similarity index was obtained from this matrix (46) in order to build a UPGMA (unweighted pair group method of arithmetic mean) dendogram. The genetic distance was based on Nei (47) and the index of genetic differentiation (F_{ST}) was determined according to Nei and Chakraborty (48), which is used to examine the level of genetic divergence among sub-populations and provides an estimation of the genetic flow (N_m). These parameters were analyzed using the software POPGENE (version 1.31) (49).

RESULTS

PARASITOLOGICAL ASSAYS

Feces of 124 insects were analyzed looking for *T. cruzi*. None of them showed natural *T. cruzi* infection, confirming in this occasion the low infection rates of *T. sordida* specimens in both regions (data not shown).

MORPHOMETRIC ASSAYS

Size analysis

The Guillaumin profile allowed, in general, determining that individuals from the Western Region were larger than those from the Eastern Region. The sexual dimorphism of the analyzed structures (W2–5, W3–6, W3–7, W2–4, W1–2, W3–4, AO, R_2, R_1, EO) was significant ($p = 0.01$–0.0001), excepting the post-ocular (PO) distance of the head ($p = 0.08$). The Bonferroni correction showed that the characters of the wings differentiated the sexual dimorphism better than those of the head.

The size analysis using the principal components showed that, although females were consistently larger than males, the populations from BO department had significantly larger wings and heads than those from the other studied localities (**Figure 3**). The significance of the size differences among insects of different departments was determined by Kruskal–Wallis non-parametric ANOVA test, using the mean of each group separately ($p = 0.0001$) for head and ($p = 0.001$) for wings.

Conformation and shape analysis

The DA for isometry-free variables evidenced the significant separation of the triatomine populations of the two geographical regions, better reflected by females according to the values of Wilks lambda = 036, $p = 0.0003$ (head) and Wilks lambda = 042, $p = 0.0001$ (wings). With the elimination of the allometric size, only the head variables were compatible with the CPC (common principal component) model (x: 33.57, $p = 0.2982$ in females and x: 36.29, $p = 0.1987$ in males). Through the DA, the specimens were correctly classified into their respective groups with considerable concordance (Kappa between 0.66 and 0.83). This canonical variation analysis also showed the isolation of PA population for both sexes (**Figure 4**).

FEEDING SOURCE ANALYSES

Figure 5 shows the percentages of *T. sordida* intestinal content that reacted with different vertebrate hosts. The preferred feeding source was varied and included the finding of blood from pets, poultry, and even rodents. The most frequent feeding source of the specimens collected inside and around the houses was poultry blood (hen or chicken): 30% for Gral; Bruguez community of Western Region and 38% for Cerro Guy community of Eastern Region. In the latter, the most frequent second blood source was human (24%) followed by cat and dog blood. In the Western Region, the second frequent blood source was multiple blood (feeding on several animals) where the most common blood mixtures were poultry-human, poultry-rat, and poultry-dog-cat.

MOLECULAR ANALYSIS

The RAPD profiles were complex (**Figure 6A**), and the size variation of amplified fragments ranged from 200 to 2500 bp. A total of 98 polymorphic loci generated by four primers were selected for comparative analysis according to their intensity, resolution, and reproducibility. The remarkable polymorphism showed patterns of different bands, but specific patterns were neither observed for insects collected in intra-domicile/peridomicile environments nor for the different departments. The similarity matrix was calculated in accordance with the degree of paired band between each pair of individuals (46) from which the UPGMA tree was generated (**Figure 6B**). On the other hand, the grouping of the means of allelic frequency showed two particular groups, corresponding to specimens from Western (BO and PH) and Eastern (SP and PA) Regions. The Nei's genetic distance (1978) indicated a larger separation between the populations separated by larger geographic distance (approximately 520 km), i.e., between BO and PA. The analysis with the POPGENE software showed an estimate of the genetic diversity value (Gst) of 0.08 while the migration index value (N_m) was 5.7 individuals per generation.

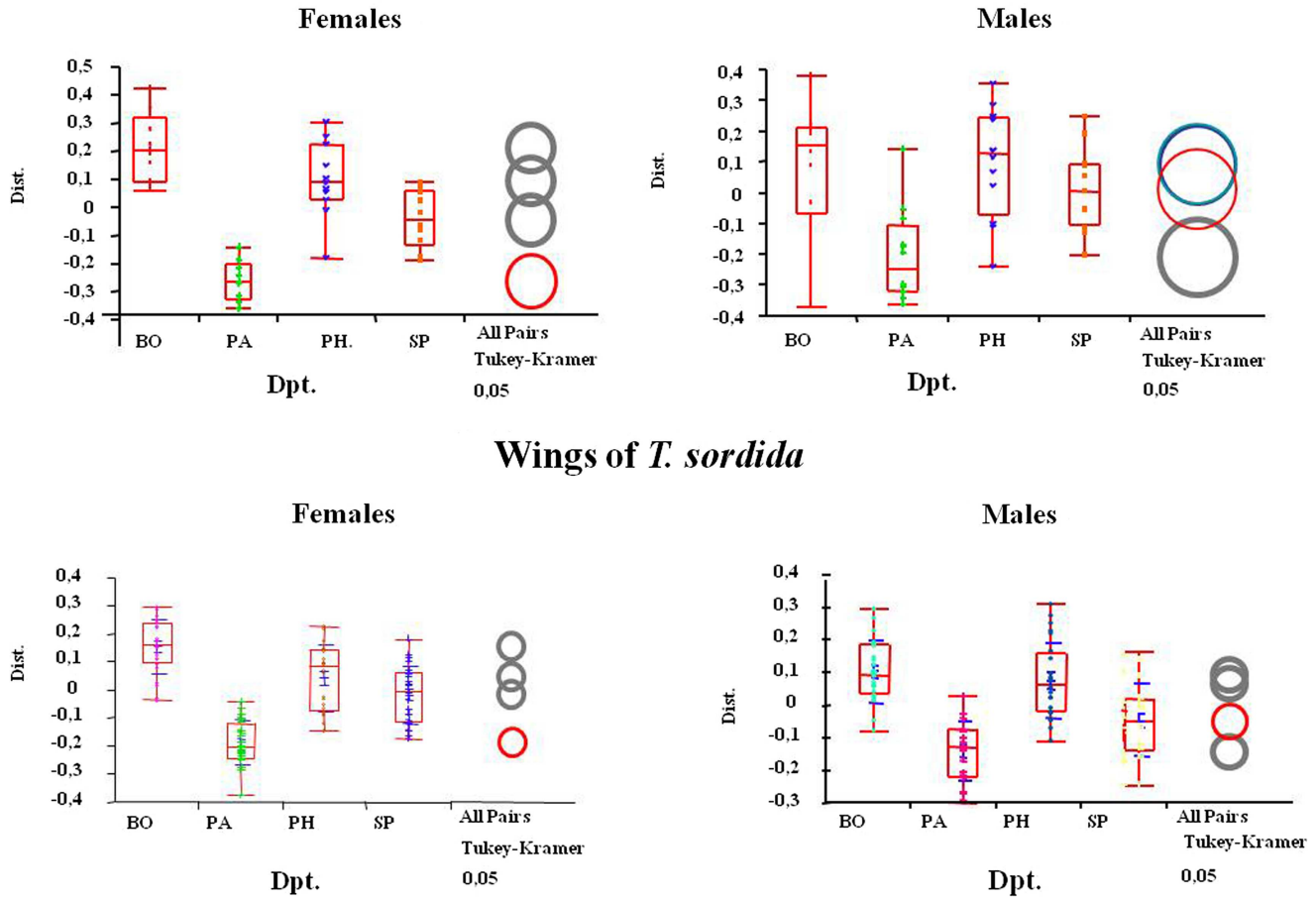

FIGURE 3 | Size differences between geographical populations (from the indicated departments) in heads (up) and wings (below) of *T. sordida* males and females are shown. The arithmetic mean can be observed as a horizontal line that divides the boxes in two. The ends of the boxes correspond to 25th and 75th quantiles while the vertical lines show the maximum and minimum values of the distribution. BO, Boqueron Department; PA, Paraguari Department; PH, Presidente Hayes department; SP: San Pedro Department.

DISCUSSION

This is the first report of *T. sordida* invading and trying to colonize houses in both Paraguayan regions. These areas have been intensively sprayed and a tendency of invasion has been observed when residual insecticide activity ends. Previous reports demonstrated very low rates of domiciliary colonization of *T. sordida* in Argentina and Brazil (11, 15), but the scenario is quite different in some areas of Bolivia (50).

We have explored the intraspecific relationships among *T. sordida* populations from different endemic areas for Chagas disease in Paraguay, considering the limited information on their behavior and based on reports about their wide dispersion and high peridomicile infestation (51). A previous study referred that human blood is the second more important feeding source of *T. sordida* in endemic areas of Paraguay (52), which suggests an increment in the transmission risk of the parasite without the necessity of establishing colonies in rural dwellings. In this study, we still observed human blood as the second feeding source in *T. sordida* from the Eastern Region but insects captured at the Western Region mainly showed a peridomestic pattern feeding where blood from animals, including sylvatic ones, was detected. Although triatomines were not found positive for *T. cruzi* infection, this new scenario should be taken into consideration in locations where *T. sordida* is frequently found inside the houses where *T. infestans* is absent.

Both morphometric and molecular analyses were carried out to determine the genetic structure of triatomines in order to generate useful information to establish more effective strategies for vector surveillance, incorporating information on their phenotypical variations and sexual dimorphism, excluding changes caused by environmental factors (53) that were corroborated by RAPDs techniques.

Our study did not show any differences in the sexual dimorphism of specimens from peridomicile and domicile, suggesting a continuous exchange with the sylvatic triatomine populations and no transition from sylvatic to domestic habitats or a domiciliation

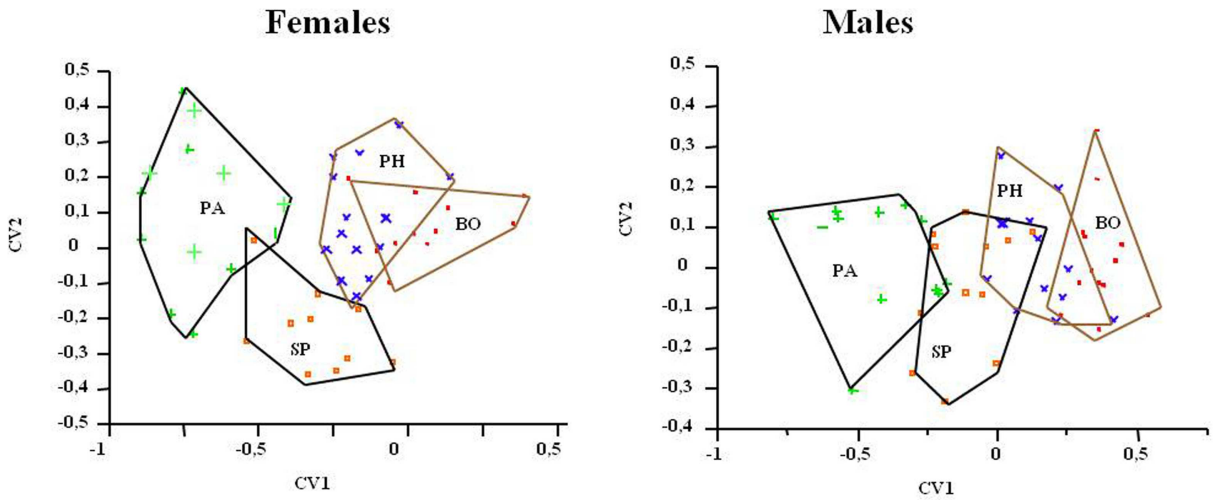

FIGURE 4 | Discriminant analysis (allometry-free), derivate of variables of *T. sordida* heads in four different populations. The polygons contain the specimens of each *T. sordida* population. BO, Boquerón Department; PA, Paraguarí Department; PH, Presidente Hayes Department; SP, San Pedro Department.

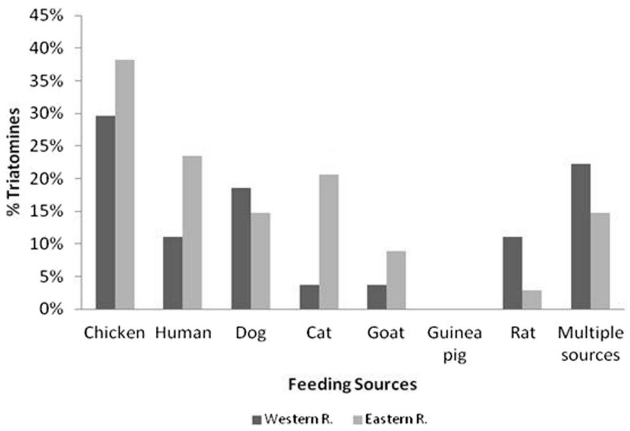

FIGURE 5 | Frequency of blood ingestion for *T. sordida* in the Eastern and Western Regions is shown. The results of most common vertebrates are included. In the category "Multiple Sources," the specimens with blood ingestion from several vertebrates are included.

process and adaptation to new habitats were found as it was demonstrated for *T. infestans* and *Panstrongylus geniculatus* (53–55). That is to say that in spite of the frequent finding of *T. sordida* species in the domicile, specific changes related to domiciliation process were not observed, suggesting their temporary presence in dwellings, which was already reported by other authors (56). According to Jaramillo et al. (57), the size of triatomines can be modified in response to environmental changes, thus the significant variation shown by the multivariate analysis of the isometric size in triatomines from Western and Eastern Regions corroborates the influence of micro-environmental conditions, like fourrage disposition in peridomiciles, which leads to the permanency of the insects in such ecotopes. The triatomines from PA department presented significant morphometric variations in relation to the other populations, suggesting a recent adaptation to peridomiciliary ecotopes. Although Chaco populations have conditions for colonization, the triatomines remain in sylvatic ecotopes that are more unstable (58–60). On the other hand, the decrease of size in PA can be attributed to the less favorable modified environments due to the big density of insects and the competition for the nutritional source available in that ecotope (61). The migration of triatomines to peridomicile is produced in response to agricultural habits and destruction of natural forests for anthropic action, even more if we consider that the PA department has been subjected to frequent modifications (agricultural area) or faced control interventions with insecticides. This leads to the dispersion of triatomines and the later adaptation to "new ecotopes"; or simply this adaptation is compatible with the hypothesis of restricted migrations if such ecotopes have enough food sources available (7, 62). According to Dujardin et al. (61), this adaptation to different ecotopes (ecological pressure) is the main mechanism that drives the speciation in the sub family Triatominae. It is important to notice that Chagas Disease Control Program in Paraguay recently showed more frequent domiciliary infestation of *T. sordida* in several localities of the PA department during the monitoring man/hour search carried out by its technical personnel.

With the elimination of the allometric changes, the DA evidenced the separation of the type morphologies of each region, which can be associated with environmental differences, geographical distances, or the intervention of genetic factors. The significant discrimination observed between the populations of PA and BO suggests a separation due to the distance among both in agreement with what was reported in a previous study made with sensilla patterns between populations of *R. prolixus* of the Andean area and oriental plains of Colombia (63). On the other hand, the observed overlapping of the factorial map among populations of the same region leads us to think of a process of passive migration.

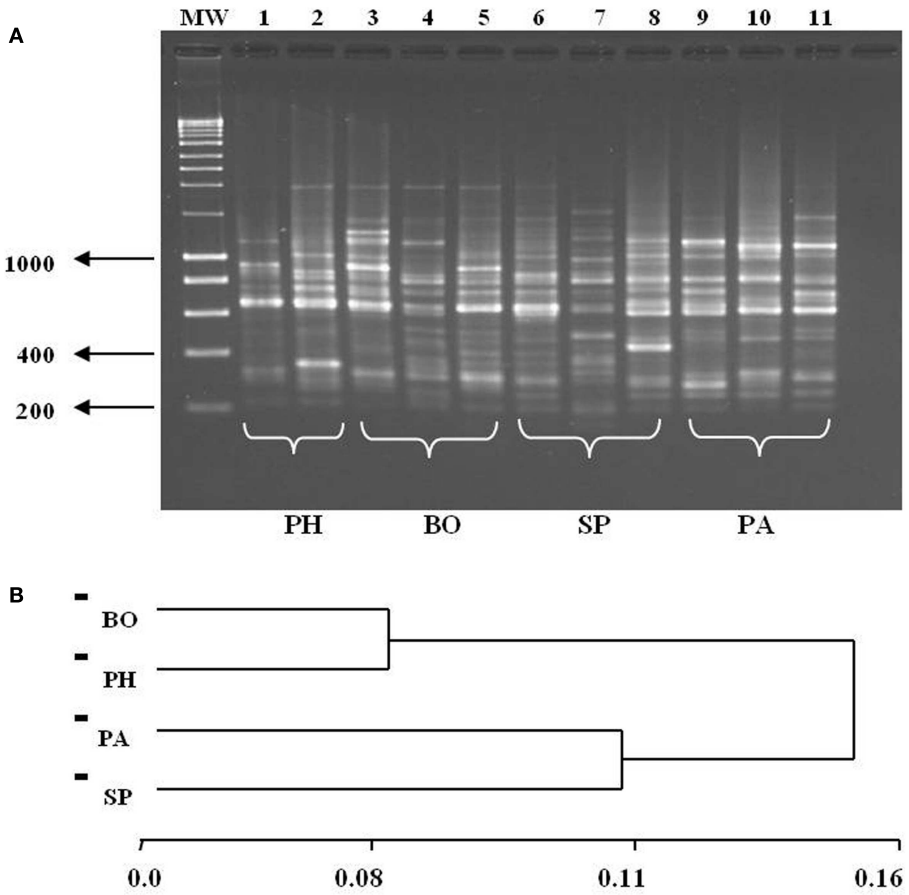

FIGURE 6 | (A) RAPD profiles with DNA extracted from different *T. sordida* populations using the primer A2. Lanes 1–2: Pte. Hayes (PH), Lanes 3–5: Boquerón (BO), Lanes 6–8: San Pedro (SP), Lanes 9–11: Paraguarí (PA), CN, negative control. PM, Hyperladder I Bioline. **(B)** Dendrogram of grouping four *T. sordida* populations by genetic distance based on Nei's genetic distance. BO, Boquerón; PH, Pte. Hayes; PA, Paraguarí; SP, San Pedro.

The genetic variability can be a consequence of the metric differences observed in specimens from different habitats, i.e., that the metric characteristics are almost exclusively under environmental control and the genetic variations could be the result of the contribution of genetic and environmental features (64). The RAPD method showed genetic structuring between *T. sordida* populations of both geographical regions and the genetic similarity was bigger among populations of the same region, suggesting the existence of a constant gene flow among them. This seems logical but such grouping may be reflecting recent events with few codon changes caused by the adaptation process of sylvatic populations to artificial ecotopes.

The similarity analysis shown by the F_{ST} index suggests an exchange among insect populations from neighboring departments that gets reduced among regions. Therefore, the F_{ST} estimator shows little genetic differentiation and according to Nei's classification (1973), this fact seems logical as they are insects of the same species. However, we suppose that the separation between regions and the morphobiometric differences of PA populations could be related to the genetic changes caused by local selective pressures. The grouping observed in the dendrogram could be associated with epidemiological differences in their respective origin focuses, considering that the Western Region is an area with high pressure of triatomine infestation (16, 58). However, to confirm this we suggest increasing the study of *T. sordida* populations in Paraguay and the use of more sensitive molecular markers to compare these findings with cryptic speciation groups previously described (18, 34).

The dendrogram is similar to the result previously obtained for *T. infestans* populations that demonstrated allelic differences among neighboring localities, which increase among populations more distant from each other (65). It has been suggested that the genetic isolation by geographical distance greatly contributes to the genetic variability of triatomine populations caused by the passive dispersal of the insects in association with human migrations, resulting in the founder effects and subsequent genetic drift (33). The migration index obtained in this study suggests the mobility of *T. sordida* between neighboring populations and the results presented suggest a genetic homogeneity between *T. sordida* from the same region, which is due to the permanent genetic flow between neighboring populations. However, the observed heterogeneity between specimens from Western and Eastern Regions could be

associated with the big distances and even with the presence of the Paraguay River as a geographical barrier, which would be in agreement with the separation obtained with the morphometric analyses, i.e., that the differences between PA and BO involves a differentiation process, possibly associated with the eco-geographic isolation by distance and absence of genetic flow. Feeding behavior also confirm differences in these two populations, while triatomines from the Western Regions showed a poultry feeding profile, patterns of the insects from Eastern regions were associated with poultry feeding and human blood feeding profiles in triatomines captured in domicile. Mixed feeding showed an intense mobilization behavior of these triatomines between peridomicile and domicile areas.

It is important to notice that the genetic analysis has shown intraspecific divergences that allow us to think of possible gene variations involved in the shape expression, although we could not discard the possibility that the separation reflected in the dendrogram is influenced by the variation of allelic frequencies of the individuals. In this case, new questions arise and further studies will be required with models of population genetics to obtain better markers related to the infestation risk of potentials triatomines, mainly as a consequence of the control of *T. infestans* in Paraguay as other secondary vector species such as *T. sordida* are more frequently detected in the studied region.

Further studies should be focused on how phenetic and genetic variations could be related to the adaptation capacity of these triatomine populations to domicile, increasing their vector potentiality in the transmission of Chagas disease.

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Triatoma maculata, the vector of *Trypanosoma cruzi*, in Venezuela. Phenotypic and genotypic variability as potential indicator of vector displacement into the domestic habitat

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Triatoma maculata is a wild vector of *Trypanosoma cruzi,* the causative agent of Chagas disease; its incursion in the domestic habitat is scant. In order to establish the possible domestic habitat of *T. maculata*, we evaluated wing variability and polymorphism of genotypic markers in subpopulations of *T. maculata* that live in different habitats in Venezuela. As markers, we used the *mtCyt* b gene, previously apply to evaluate population genetic structure in triatomine species, and the β-tubulin gene region, a marker employed to study genetic variability in *Leishmania* subgenera. Adults of *T. maculata* were captured in the period 2012–2013 at domestic, peridomestic (PD), and wild areas of towns in the Venezuelan states of Anzoátegui, Bolívar, Portuguesa, Monagas, Nueva Esparta, and Sucre. The phenotypic analysis was conducted through the determination of the isometric size and conformation of the left wing of each insect (492 individuals), using the MorphoJ program. Results reveal that insects of the domestic habitat showed significant reductions in wing size and variations in anatomical characteristics associated with flying, in relation to the PD and wild habitats. The largest variability was found in Anzoátegui and Monagas. The genotypic variability was assessed by *in silico* sequence comparison of the molecular markers and PCR-RFLP assays, demonstrating a marked polymorphism for the markers in insects of the domestic habitat in comparison with the other habitats. The highest polymorphism was found for the β-tubulin marker with enzymes *Bam*HI and *Kpn*I. Additionally, the infection rate by *T. cruzi* was higher in Monagas and Sucre (26.8 and 37.0%, respectively), while in domestic habitats the infestation rate was highest in Anzoátegui (22.3%). Results suggest domestic habitat colonization by *T. maculata* that in epidemiological terms, coupled with the presence in this habitat of nymphs of the vector, represents a high risk of transmission of Chagas disease.

Keywords: *Triatoma maculata, Trypanosoma cruzi,* vector, Chagas disease, epidemiology, architecture of wings, molecular markers, RFLP-PCR

INTRODUCTION

Triatomines (Hemiptera, Reduviidae, Triatominae) are blood-sucking insects that act as vectors of tripanosomatids such as *Trypanosoma rangeli* and *T. cruzi* (Kinetoplastida, Trypanosomatidae), the latter being the causal agent of American trypanosomiasis or Chagas disease. This is one of the parasitic diseases of great medical importance in the Neotropics. Chagas disease remains a public health problem in America, being distributed from the central-southern region of the United States to Southern Argentina and Chile; patients with this disease have been found in Canada and some European countries (1, 2).

Transmission of Chagas disease in Venezuela and elsewhere in South America has been traditionally associated with the domestic (D) and peridomestic (PD) environments in rural areas with poor socioeconomic conditions and high presence of vectors. However, colonization by triatomines such as *Triatoma maculata* and *Panstrongylus geniculatus* of D environments has increased, whereas before these triatomines had been mostly associated with PD or wild (S) habitats (3). In Venezuela, *T. maculata* is found in most of the states comprising the country, with the exception of Táchira and Delta Amacuro. Distribution is established from 0 to 1,500 m of altitude, with natural habitats such as palms, dry trees, fences and bird nests, and rates of infection with *T. cruzi* lower than those recorded for *Rhodnius prolixus*. Apparently, as a result of anthropogenic changes, the characteristic habitats of *T. maculata* have changed the insect becoming domestic (4).

The domiciliation of triatomines seems to be an event that can sometimes lead to the simplification of genotypic and phenotypic characteristics, which can be adaptive to macro- and microclimatic variations and reduction of wildlife mammals that serve as blood source, among others factors. These factors favor the dispersion and increase triatomine populations in anthropogenic niches (5, 6). There is suggestive evidence of a recent increase in adaptive capacity of *T. maculata* in populated areas, hence the importance of studying this vector (7).

The taxonomic position of triatomines has been revised through phenotypic studies using various methodologies, such as analysis of biochemical markers, e.g., isoenzymes, or morphometric techniques, e.g., variability analysis of the size and shape of anatomical structures (8–12), and genotypic assessment methods of polymorphism of genetic markers, e.g., the mitochondrial cytochrome *b* (mt*Cyt* b) sequence (13–15) and the ribosomal spacer region (ITS-2), among others. All of these methodologies have shown interspecific variability in different triatomines species and have been used to evaluate population genetic structure. The geometric morphometry analysis, which allowed differentiating domestic and wild insects of medical importance such as mosquitoes, and ontogenetic studies of triatomine populations have been useful in discriminating vectors, which cannot be identified by morphological or molecular variability studies (16–18).

The combined use of phenotypic methods such as geometric morphometry analysis and assessment methods such as genetic polymorphism of molecular markers would be useful in the evaluation of vector populations related to Chagas disease and the establishment of appropriate interventions for disease control. Analysis of changes in the wings, supported by the study of molecular markers such as mt*Cyt* b, has been used in Colombia in the differentiation of species of *Rhodnius* (14). In Venezuela, comparative studies, both phenotypic and genotypic, on vectors of Chagas disease are scarce; one of these studies suggests a polymorphism related to the geographical origin of the specimens in the restriction patterns of the mt*Cyt* b gene in *T. maculata* (4).

This work assesses the dispersion of this vector throughout S, PD, and D ecotopes in several Venezuelan states using both the phenotypic and genotypic approaches, in specimens of *T. maculata* captured in different regions of Venezuela. The phenotypic approach includes a geometric morphometry study to establish wing variability; the genotypic variability was evaluated through the polymorphism of the molecular markers *mtCyt* b and the β-tubulin genes region. Previously, the β-tubulin marker has been used to establish genetic variability between *Leishmania* subgenera.

MATERIALS AND METHODS

STUDY AREA AND INSECTS

Field work was conducted in the endemic Venezuelan states Anzoátegui, Sucre, and Monagas (east of the country); Nueva Esparta (northeast, Margarita island), Bolívar (south), and Portuguesa (west). Sampling was carried out following the method proposed by Schofield (19). Specimens of *T. maculata* were collected through a direct search by personnel previously trained; sampling was conducted twice/year in each region and the capture effort occurred at 5 h/man by day or night for 5-day visit to each region. A total of 26 locations distributed in these states were visited to collect, in periods of high and low precipitation, specimens of *T. maculata*, directly from ecotopes defined following previous criteria. In D habitat, attention was put on internal walls of the houses, rooms, roofs, furniture, and in the ceiling; external domiciles or PD habitat (30 m around the exterior walls of the house), the area explored included farmyards, henhouses, and wood piles; and in S habitat (removed 30 m from the PD habitat), the area explored covered palm trees, tree holes, cave, and crops (20, 21). A total of 492 adult insects, male (M) and female (F), were collected and used in this study. Three indices were calculated; the colonization index was determined as CI (%) = 100 × total numbers of houses presenting nymphs/total number of houses with adults; the dispersion as DI (%) = 100 × number of locations with adults/number of locations studied, and infection index as II (%) = 100 × number of adults infected with *T. cruzi*/total number of adults captured. Specimens were dissected and their intestinal contents and/or hemolymph examined under the microscope for the presence of *Trypanosoma*; samples of the intestinal contents were used to evaluate the presence of *T. cruzi* and *T. rangeli* by means of a PCR assay (22). After dissection, the collected specimens were preserved in 70% alcohol and stored at −20°C for further analysis. Colonies from each location were established in the laboratory for morphometry analysis.

GEOMETRIC MORPHOMETRY ANALYSIS: METRIC DATA, SHAPE, AND SIZE VARIATION

For each individual, only the left wing was examined and included in the analysis. The wings were mounted between microscopy slides and cover-slips and photographed using a digital camera Leica S6D. Nine landmarks (**Figure 1A**) were identified in each wing according to previous references (18, 23); the geometric coordinates of each landmark were digitized and shape variables (partial warps) were obtained using *tpsDig* Version 2 (24). Data were submitted to a discriminant analysis to examine differences in wing shape between male (M) and female (F) and statistical significant was evaluate by the Wilk's lambda statistics. For comparison of wing size between genders and among ecotopes within each gender, we used the isometric estimator centroid size (CS) derived from coordinate data (10, 15, 23). Statistical analysis was carried out using the MorphoJ software package for geometric morphometric (25). Landmark coordinate (x, y) configurations were registered and aligned using the Procrustes analysis and covariance analysis was implemented with proportions of re-classified groups and MANOVA. Then, wing shape variable and the CS were analyzed using the principal component analysis. For this analysis, M and F were processed separately due to the sexual size dimorphism of Triatominae. The relationship between shape and size was explored by a regression analysis. The significance of wing conformation due to landmark variation was established using the canonical variate analysis.

DNA EXTRACTION AND MOLECULAR MARKER AMPLIFICATION

Total genomic DNA was extracted from all six legs of each specimen using the Wizard Genomic kit (Promega, Madison, WI, USA. Cat. No. A1620). Purity and integrity of the DNA were determined by agarose gel electrophoresis. The same procedure was used to

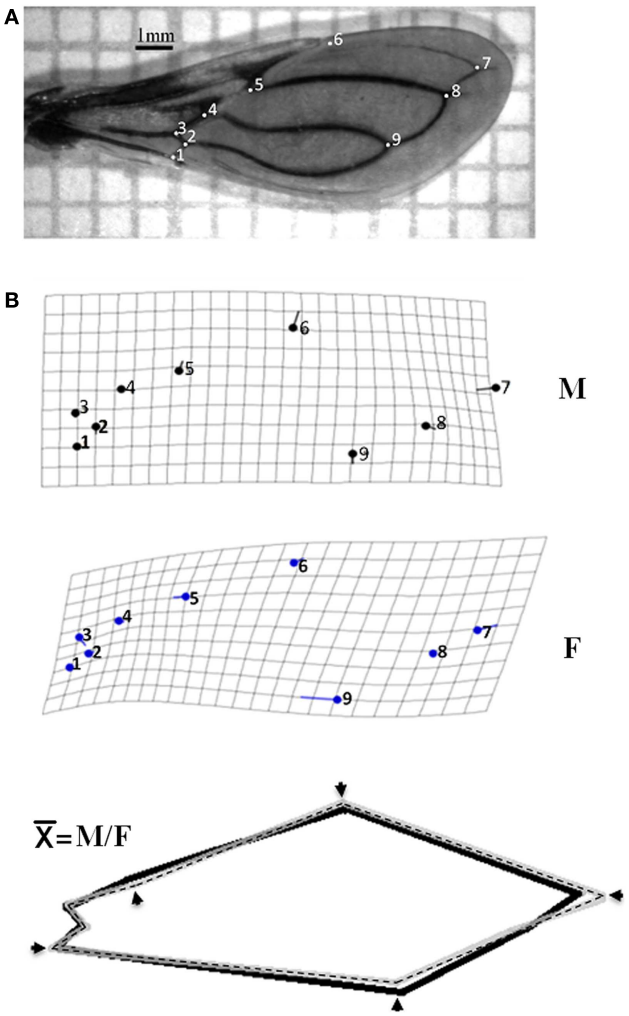

FIGURE 1 | Differences in wing shape between genders of *Triatoma maculata* from Venezuela. **(A)** Landmark points type I identified in wing of *T. maculata*. Numbering of points (PAR 1–9) refer to the arrangement followed to obtain the coordinates using *tps* Dig 2.0. **(B)** Differences in wing shape architecture of *T. maculata*. Differences in wing shape between male (M) and female (F) of *T. maculata* are represented by grids deformation and variation between homologous landmark (solid circles). After superposition to the homologous consensus ($\bar{X}$) between M (solid lane) and F (dashed lane), the differences in wing shape are represented by incongruence between homologous landmarks (arrows).

isolate the DNA from triatomine intestinal content. The extracted genomic DNA was resuspended in TE buffer (10 mM Tris, pH 7.4 and 1 mM EDTA), and stored at 4°C for further analysis.

For direct molecular identification of *T. cruzi* and *T. rangeli*, DNA isolated from the intestinal content of insects was used to amplify the variable region of the minicircles of kinetoplast DNA (kDNA) and the non-transcribed spacer region of the mini-exon (26, 27).

Two markers, previously reported as fit to evaluate genetic diversity in different organisms, were used in *T. maculata*. First, the mt*Cyt* b gene apply to evaluate population genetic structure in different triatomine species (28, 29), and second, the β-tubulin gene region, a marker employ to study genetic variability in *Leishmania* subgenera (30, 31).

The β-tubulin primers were designed by multiplex alignment of similar genes available in the NCBI Genbank from *Triatoma tibiamaculata* (KC249297), *T. infestans* (JK33877), *T. braziliensis* (EC917343), *Rhodnius prolixus* (FG544591), *Aedes aegypti* (XM00165064), and *Drosophila melanogaster* (AE0135994), and after bioinformatics sequence analysis, the primers sequences TubTmf and TubTmr were selected (**Table 1**).

Markers were amplified under standard polymerase chain reaction (PCR) assays as described previously; the reaction was carried out in a final volume of 25 μl containing 12.5 μl cocktail of PCR mix 2X (GoTaq Master Mix, Promega, Madison, WI, USA. Cat.# M7122), 0.4 μmol primers (stock 100 μM) and 5 ng total genomic DNA; the PCR reaction was performed in an MJ Research PTC-200 thermocycler.

PCR-RFLP OF THE β-TUBULIN GENE MARKER

The PCR fragment of the β-tubulin gene marker from specimens of different ecotopes was partially sequenced using the *Sequence Navigator* version 1.0.1 (Perkin Elmer Applied Biosystem) and the *in silico* restriction map was established (NETcutter version II). The endonucleases *Bam*HI and *Kpn*I (Life Technology) were selected for double digestion of the β-tubulin-PCR product following the manufacturer's instructions, and the digested DNA fragments fractionated by agarose gel electrophoresis.

ELECTROPHORESIS

The purity and integrity of the DNA were determined by electrophoresis in 0.6% agarose gel at 80 V for 1 h in TBE buffer (90 mM Tris-HCl, pH 8.0; 90 mM boric acid; 2.5 mM EDTA). The PCR products were analyzed by electrophoresis on 1.5% agarose gel in TBE buffer and the RFLP products were subjected to electrophoresis in 3% agarose gel in TBE buffer. After electrophoresis, the gel was stained with ethidium bromide, visualized with UV illumination, and recorded on a gel documentation system.

GENETIC DIVERSITY

The relationships, genetic differentiation, among the pattern fragments of the RFLP analysis of the β-tubulin marker (presence or absence of fragments) from different states and ecotopes were estimated using the NJ algorithm and the tree is based on a Kimura 2-parameter distance matrix (32, 33). Statistical support for branches in the NJ tree was assessed by the bootstrap method with 1,000 replicates. The analysis was conducted using the software MEGA V.4 (34).

RESULTS

PHENOTYPIC VARIABILITY

Colonization and infection of T. maculata with T. cruzi

Of the 492 specimens of *T. macula*, 49.2% were M; the insects were collected from six Venezuelan states, whose distribution by location showed a majority of these specimens distributed in the PD (67.07%) and domestic (26%) ecotopes, followed to a lesser extent by the wild ecotopes (7.3%). On average, 20.73% of these

Table 1 | Molecular markers, primers, and PCR assay conditions.

Marker	Primers 5′–3′	PCR assay cycles	Fragment size (pb)	Reference
mt*Cyt* b	***CYT BF* 135** GGACAAATATCATGAGGAGCAACAG ***CYT BR* 135** ATTACTCCTCCTAGCTTATTAGGAATTG	94°C, 5′ 94°C, 30′′ 55°C, 30′′]27Cy 72°C, 30′′ 72°C, 5′.	600	(28, 29)
β-tubulin	***TUB TMr*** GACACGCAGCGCTTGCGCACTCGT ***TUB TMf*** CCCGTCCTGCCTCGCCTGC	94°C, 5′ 94°C, 1′ 64°C, 1′]5Cy 72°C, 1′ 94°C, 1′ 60°C, 1′]30Cy 72°C, 1′ 72°C, 5′.	980	(31)

Cy = number of cycles; ′ = min; ′′ = s.

Table 2 | Geographical origin and ecotope of *Triatoma maculata*.

State[a] (total/F/M)	Location	Coordinates		Ecotopes			CI (%)	II (%)
		N	W	D	PD	S		
Anzoátegui (284/134/150)	Pico de Neverí	09°75′	065°02′	0	13	0	22.3	18.4
	El Enial	09°79′	065°02′	0	33	4		
	San José de las Margaritas del Llano	09°79′	065°02′	33	94	5		
	Los Ranchos	10°23′	064°60′	29	40	0		
	Guastrantal	10°11′	064°59′	0	23	7		
	Mundo nuevo	09°30′	064°35′	1	2	0		
Monagas (86/52/34)	Caripito	09°98′	063°49′	9	4	8	13.81	26.8
	Aragua de Maturín	09°77′	063°15′	6	29	3		
	Musu	09°61′	063°08′	8	5	5		
	La Planchada	09°93′	063°40′	0	7	0		
Portuguesa (63/37/26)	Jabillal	09°42′	069°18′	10	18	3	5.47	19.1
	Las panelas	08°58′	069°58′	6	20	6		
Sucre (29/15/14)	La Sabana	10°18′	064°21′	0	3	1	1.76	37.9
	Guayabal	10°13′	064°42′	2	4	3		
	La Piscina	10°42′	064°19′	2	6	1		
	UDO Cumana	10°46′	064°14′	0	4	3		
Bolívar (16/7/9)	Caruachi	08°35′	062°54′	0	2	0	8.2	18.7
	Guasipati	08°30′	062°64′	3	3	0		
	Tocoma	08°18′	062°84′	0	4	0		
	Gran sabana	08°13′	062°74′	0	3	0		
	La laguna	08°00′	062°64′	0	1	0		
Nueva Esparta (8/3/5)	Porlamar	10°95′	063°88′	0	2	0	0	12.5
	Roble	11°06′	063°84′	0	2	0		
	Fuentidueño	10°90′	063°96′	0	1	0		
	La sierra	10°99′	063°91′	0	3	0		

[a]*Venezuela states.*

N, total number of insects; F, female; M, male; D, domestic; PD, peridomestic; S, wild; CI, colonization Index; II, infection index.

specimens were positive for *T. cruzi* according to the specific kDNA and mini-exon-PCR assays (**Table 2**). The higher CI found in Anzoátegui and Monagas states in relation to the other states coupled to a high II to *T. cruzi* in these two states suggests a higher rate of household colonization and showed the importance of *T. maculata* as a vector.

Size and shape variation

Average size of the membrane region of the wing was 1,984 mm for F and 1,786 mm for M. The discriminate function for gender re-classified wings, 75% for M (M 30/40) and 82% for F (F 33/40); this function in turn contained 94% of gender variance, showing significant differences in the formation of the wing according to gender (Wilk's lambda: 0.543 and 0.754 for M and F respectively; $p < 0.001$). When analyzing the intraspecific allometric effect (degree of deformation of the wing), using size (component 1) as the independent variable and wing conformation (component 2) as the dependent variable, the contribution of these components was 49.5% of the variation. The differences in conformation based on the deformation of grids made by discriminating analysis disclosed that the M has a lower degree of variation compared with F, and a clear sexual dimorphism (**Figure 1B**). The mean wing deformation ($\bar{X}$), obtained through the overlapping of gender-related wing deformation grids, showed changes in the landmark points; the decrease in wing size was observed in at least one of the landmarks (PAR 1–9) and in some cases by the loss of anatomical landmarks (**Figure 1B**). The same changes of the landmark points were found after the third filial generation in colonies from the same location established in the laboratory.

The formation of wing architecture based on the variation of CS in each ecotope (**Figure 2**) evidenced an association between PD and S insects with no significant difference between them ($p = 0.0392$ for PD and $p = 0.0382$ for S), whereas significant differences ($p = 0.0051$) were observed when comparison was carried out between PD and S specimens together with insects collected in D, regardless of gender (**Figure 2**).

The variation in wing size and conformation allowed the grouping of three states (**Figure 3**). The results showed clusters of M and F of *T. maculata* captured in S and PD ecotopes in Anzoátegui, Monagas, and Portuguesa. Interestingly, higher variation in the consensus tendency was found in both genders in specimens captured in D ecotopes of Anzoátegui and Monagas (**Figure 3**, squares a and b).

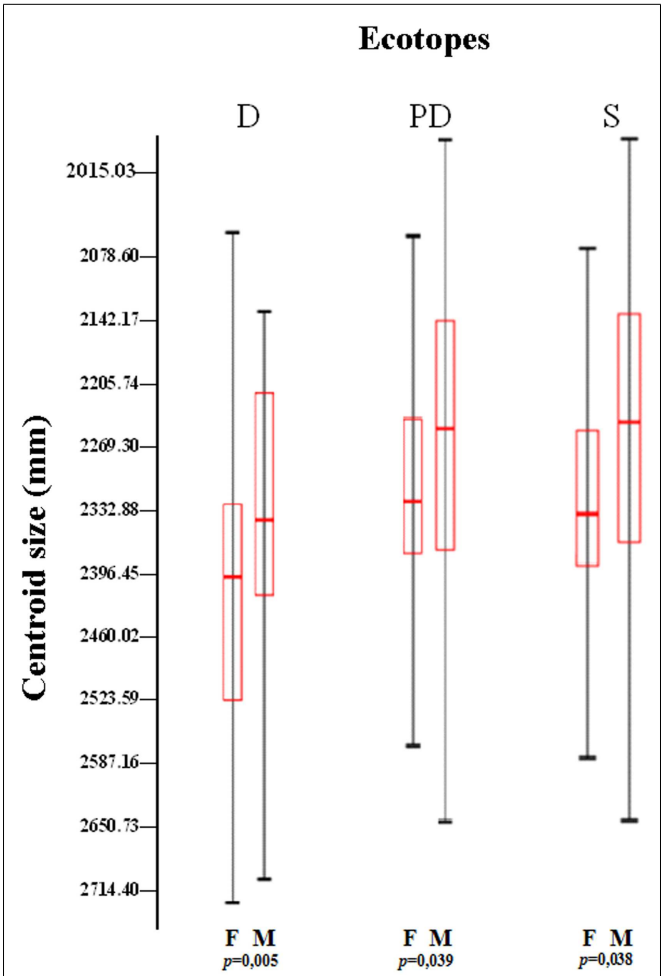

FIGURE 2 | Variation of the centroid size among ecotopes and gender of *Triatoma maculata* from Venezuela. The boxes show the isometric size differences of wings between ecotopes (D, domestic; PD, peridomestic; and S, wild) and sex (M, male and F, female), from different states and locations. Each box shows the mean (horizontal line inside the box), standard deviation (vertical line). The number of individuals was M, 242 (D, 29; PD, 172; and S, 43) and F, 250 (D, 24; PD, 158; and S, 63). *p*: statistical significant differences.

GENETIC DIVERSITY

Mini-exon analysis

Twenty percent of specimens were identified as positive for *T. cruzi* infection and 1.2% presented a co-infection with *T. rangeli* as demonstrated by a PCR assay for the mini-exon. The lineage of *T. cruzi* circulating in all states was identified as TcI after amplification of a band of 200 bp from the non-transcribed region of the mini-exon (results not shown).

Variability of molecular markers

Genomic DNA from specimens of *T. maculata* representative of each state, regardless of location or ecotope, was evaluated by amplification of mt*Cyt* b and the β-tubulin gene region (**Table 1**; **Figure 4**). The results showed a unique PCR product of 600 bp for mt*Cyt* b (**Figure 4A**), which was common in size among specimens of different locations of different states and also among the ecotopes of these locations; the low variability of this product, as determined by partial sequencing, did not allow to establish differences based on mt*Cyt* b between specimens (results non-shown).

In contrast, a unique fragment of 980 bp was generated by the amplification of the β-tubulin marker (**Figure 4B**); the partial sequencing of this fragment showed differences between states and ecotopes, suggesting genetic variability in populations of *T. maculata*.

In order to establish the variability of the β-tubulin gene marker, the PCR product from specimens of different states and ecotopes was sequenced and *in silico* restriction fragment maps were established and used to identify the restriction enzymes to be used to evaluate genetic differences among specimens from different ecotopes using RFLP analysis. The results revealed a partial common pattern for double digestion with *Bam*HI-*Kpn*I, with quantitative and qualitative differences among specimens of the majority of states, represented for bands of 980 (**a**), 620 (**b**), 450 (**c**), and 300 bp (**d**) (**Figure 4C**). This pattern was independent of location

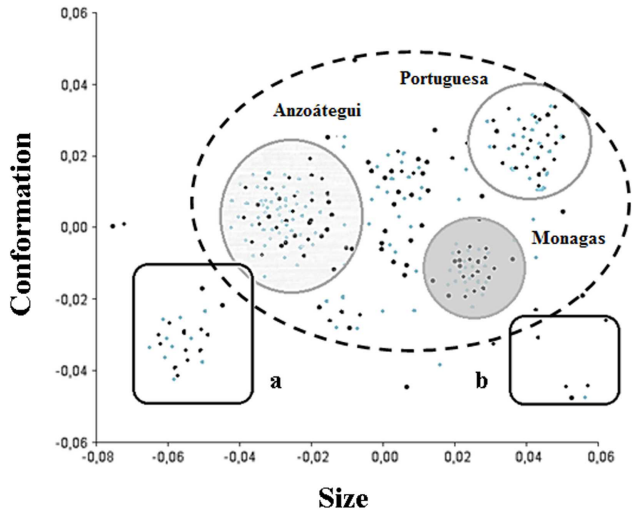

FIGURE 3 | Phenotypic variability of *Triatoma maculata*. Diagram of factorial data of the main components of wing architecture of *T. maculata*. The components size and conformation make the higher contribution (70%) in wing variability. The dashed line circle represents the standard group, whereas the solid line ones represent groupings by confidence ellipses (95%) of male (black points) and female (gray points) specimens from Anzoátegui, Monagas, and Portuguese states. The boxes represent the grouping of M and F specimens that showed the greatest differences in wing size and shape when compared to the consensus configuration.

or ecotope, the exception being Portuguesa state, where differences were found between ecotopes, since in this location only band (**c**) was present in the S pattern. In addition, other individual fragments were observed among the different ecotopes in all states; however, differences between ecotopes of the same state were evident, e.g., Anzoátegui, Sucre, Monagas, and Portuguesa (**Figure 4C**, lanes 1–9 and 16–18), as well as between the same ecotope when different states were compared (**Figure 4C**, lanes 1, 4, 7, and 16).

The PCR-RFLP patterns obtained for the region of β-tubulin (**Figure 4C**) were evaluated according to the presence or absence of bands to establish comparative marker variability among different states and particularly among ecotopes. Anzoátegui and Monagas states had the largest differences in pattern by ecotopes (genetic differentiation index, Fst 0.476, $p = 0.019$). Anzoátegui state showed a 60% similarity between PD and S ecotopes, and the remaining 40% was due to D ecotope. The rest of the states revealed similar groupings in their genetic profiles (**Figure 4C**). This suggests that the restriction patterns obtained could be an alternative for intraspecific differentiation of *T. maculata* associated with different ecotopes. Comparative analysis of data from Anzoátegui and Monagas states showed that about 60% of the restriction fragments are common regardless of ecotope. Maximum Parsimony analysis with 1,000 replicates, statistically supported by bootstrapping, generating a similar clustering among specimens of S and PD ecotopes, which would indicate similarity, while the genetic pattern of D differs, causing it to clump as a synapomorphic group (**Figure 5**), separated according to ecotopes.

DISCUSSION

The present study demonstrated for the first time domiciliary adaptation processes of *T. maculata* in several Venezuelan states, using two approaches, phenotypic as the geometric morphometry of wing, and genotypic as the variability of the β-tubulin molecular marker. This, together with the presence of nymphs in houses and a high rate of infection with *T. cruzi* of specimens captured inside the home indicates that there is increased risk factor in the transmission of Chagas disease in Venezuela. Our results showed that there is discrimination of *T. maculata* according to its ecotopes, since specimens from S and PD ecotopes were more similar between them in wing architecture and variability of the β-tubulin marker in comparison with that from D ecotope. Previous studies suggest that *T. maculata* is contributing to increased risk of transmission of *T. cruzi* in the human population from various regions of Venezuela, particularly in the north-eastern region where specimens of this species showed a high percentage of infestation and a high rate of infection with *T. cruzi* (35).

Our results demonstrate the utility of geometric morphometry study of wing architecture to establish sexual dimorphism, phenotypic variability, and the association of these variables to different ecotopes of *T. maculata*. Thus, this is a robust tool to determine intraspecific differences possibly related to the geographical distribution in the macro- and microenvironment.

It has been demonstrated that Chagas disease vectors traditionally considered exclusively S have the possibility to change their behavior and colonize D habitat, with a high risk in the epidemiology of the disease (36). The association with particular habitats or ecotopes of different phenotypical and genotypical characters in triatomines has proved important in vector identification, dispersion, and colonization properties and in general in the epidemiology and control of Chagas disease in South America (14, 37–39).

Different molecular strategies have been used to study genetic diversity in populations of triatomine vectors, such as variability of the mt*Cyt* b gene, microsatellites, and random amplified polymorphic DNA (RAPD) (14, 28, 37, 40). Previous studies on the genetic variability of *T. maculata* in Venezuela using mt*Cyt* b partially enabled to infer the occurrence of different haplotypes for populations in Anzoátegui and Portuguesa (4). In our study, mt*Cyt* b presented the lowest level of variability, and these variations failed to discriminate *T. maculata* by ecotopes.

The polymorphism for β-tubulin marker in specimens of *T. maculata* generated evident variations between ecotopes of different locations of the Venezuelan states. This was particularly seven haplotypes for samples collected in homes and five haplotypes for copies of PD and wild ecotopes. The correspondence observed between phenotypic and genotypic grouping indicates that the joint application of both approaches is a robust tool for the study of vector domiciliation.

This is the first time that the molecular marker of β-tubulin was used for evaluation of the genetic variability in *T. maculata*, the evaluation of this sequence being important in establishing it for use as a valuable tool in the genetic evaluation of triatomine population.

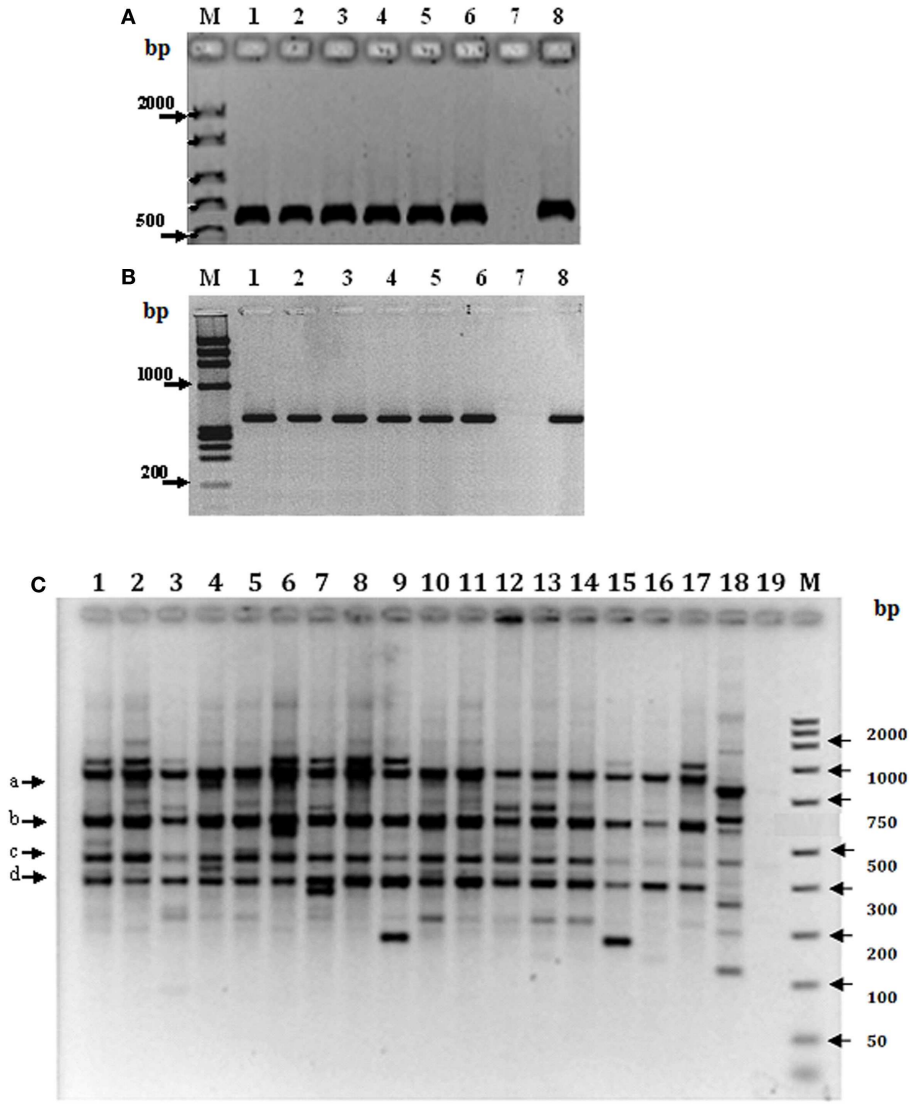

FIGURE 4 | Genetic variability of *Triatoma maculata* from Venezuela. Amplification products of *mtCyt* b **(A)** and the β-tubulin gene region (**B**). Lanes 1–6 in **(A,B)**, Venezuelan states: Anzoátegui, Monagas, Sucre, Bolívar, Portuguesa, Nueva Esparta; lane 7, negative control without DNA and lane 8, DNA from a laboratory specimen. **(C)** RFLP analysis of the PCR product of the β-tubulin marker representative of different states, locations and ecotopes. Lanes 1–3: Anzoátegui, Los Ranchos, lane 1 D; lane 2, PD; and Guastrantal, lane 3 S; lanes 4–6: Sucre, Guayabal, lane 4 D; lane 5 PD; and La Piscina, lane 6 S; lanes 7–9: Monagas, Caripito, lane 7 D; lane 8 PD; and lane 9 S; lanes 10–12: Bolívar, Guasipati, lane 10 D; lanes 11 and 12 PD; lane 13–15: Nueva Esparta, La Sierra, lanes 13 and 14 PD; and lane 15 S; lanes 16–18: Portuguesa, Las Panelas, lane 16 D; lane 17 PD; and lane 18 S. Lane 19: negative control (no DNA). M: 100 bp (GIBCO BRL) as molecular marker.

CONCLUSION

This work presents for the first time the relationship between phenotypic and genotypic approaches in the discrimination of *T. maculata*, a vector of Chagas disease, according to its ecotopes in Venezuela. Wing architecture and variability of the β-tubulin DNA region, the variables used to differentiate *T. maculata* populations, showed that specimens from S and PD ecotopes were more similar between them than those captured in the D ecotope which, together with the presence of nymphs in the houses and a high rate of infection with *T. cruzi* of D specimens, increases the risk factor in the transmission of Chagas disease in Venezuela. Eradication of the vector in the domestic ecotopes followed by vigilance of re-infection will be important in reducing transmission of Chagas disease; however, integrative research is necessary to understand the vector population structure, domiciliation, and parasitic transmission.

AUTHOR CONTRIBUTIONS

Leidi Herrera (Coordinator), Roberto García-Alzate, Antonio Morocoima, and Rafael Matías Reyes-Lugo, designed the field work. Roberto García-Alzate, Daisy Lozano-Arias, Leidi Herrera, and Alexis Mendoza-León performed analyses. Roberto

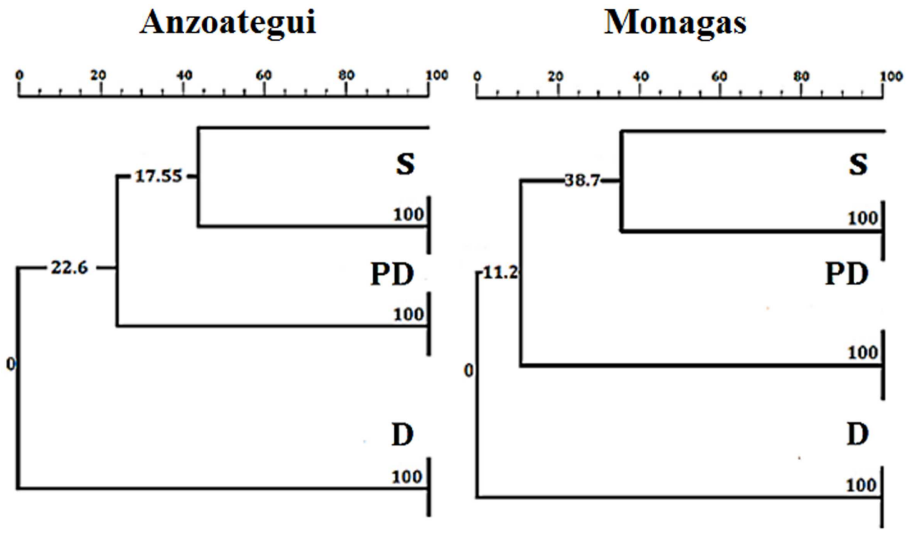

FIGURE 5 | Neighbor-joining tree based on Kimura 2-parameters of RFLP fragments of the β-tubulin marker of *Triatoma maculata*. The numbers on the branches indicate boostrap support. D, PD, and S: domestic, peridomestic, and wild ecotopes. The same tree as for Monagas was found for Sucre, Bolívar, Nueva Esparta, and Portuguesa states.

García-Alzate, Daisy Lozano-Arias, and Alexis Mendoza-León analyzed the data and prepared the manuscript. All authors read and approved the final manuscript.

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First record of *Triatoma maculata* (Erichson, 1848) (Hemiptera: Reduviidae: Triatomini) in the municipality of Riohacha, La Guajira – Colombia

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Introduction: Knowledge of vector insect species, their habitat, and geographical distribution is crucial for determining the risk of transmission of the etiological agents that cause disease in humans, which allows defining strategies for prevention, surveillance, and control in line with the characteristics of each area.

Objective: To determine the presence and public health importance of vectors of Chagas disease in the indigenous settlements of Marbacella and El Horno of the Wayúu ethnic group in the municipality of Riohacha, La Guajira, Colombia.

Materials and Methods: From active search, installation and inspection of biosensors, and occasional catches, Hemiptera: Reduviidae: Triatomini were collected intra and in the peridomicile housing of the indigenous settlements of El Horno and Marbacella of the Wayúu ethnic group. Indices of intra and peridomestic infestation, colonization, density, dispersion, and natural infection with *Trypanosoma cruzi* Chagas, 1909 were calculated.

Results: 79.6% ($n = 90$) of the specimens were collected in the peridomicile and 20.3% ($n = 23$) in the intradomicile, all corresponding to *Triatoma maculata* (Erichson, 1848). The natural infection indices with *T. cruzi* accounted for 43.5% for Marbacella and 36% for El Horno.

Conclusion: This is the first reported capture of individuals of *T. maculata*, considered a secondary vector of Chagas disease in Colombia, naturally infected with *T. cruzi* in the municipality of Riohacha expanding the geographical distribution of the species in the department of La Guajira.

Keywords: Chagas disease, vector indices, Wayúu ethnicity, Triatomini

INTRODUCTION

Chagas disease is a parasitic disease caused by *Trypanosoma cruzi* Chagas, 1909, mainly transmitted by blood-sucking insects belonging to the subfamily Triatominae (Hemiptera) (1). It is endemic in 21 countries in the Americas, and it is considered that between 18 and 20 million people are infected and 100 million are at risk of acquiring infection (2). American trypanosomiasis in Colombia is considered a public health problem because it is estimated that about eight million people are exposed to disease transmission and between 700,000 and 1,200,000 are infected with the parasite (3, 4).

In Latin America, the transmission of *T. cruzi* particularly occurs in rural areas, where triatomines have adapted to human habitat due to bioecological, political, socio-economic, and cultural factors. *Rhodnius prolixus* Stal, 1859, *Triatoma dimidiata* Latreille, 1811 and *Triatoma infestans* Klug, 1834, have represented, in terms of parasitological, epidemiological, and public health, the most important vectors in the transmission of Chagas disease (5, 6). In Colombia, *R. prolixus* and *T. dimidiata* are considered as major vectors because they are widely distributed and have high indices of house infestation, colonization, and natural infection with *T. cruzi* (4, 7, 8). However, in areas where these species are not domiciled but have had outbreaks of the disease, studies have suggested the participation of secondary vectors such as *T. maculata* (9–11), *Rhodnius pallescens* Barber, 1932 (12), *Eratyrus cuspidatus* Stal, 1859 (13) and *Panstrongylus geniculatus* Latreille, 1811 (14). *P. geniculatus* was indicted in a major acute outbreak of Chagas disease in the Colombian Orinoco in 2014 (15).

In the municipality of Riohacha, La Guajira Department were recorded *R. prolixus*, *T. maculata*, and *T. dimidiata* with house infestation indices of 12.28% and naturally infected with *Trypanosoma rangeli* Tejera, 1920 and *T. cruzi*, respectively, with the *R. prolixus* (79.27%) and *T. maculata* (23.37%) populations being the most abundant in intradomiciliary environments, suggesting that this area of the country has optimal biological and ecological

factors for the presence of important epidemiological triatomine bugs in human habitats (16). Because 44.9% of the human population in this municipality is indigenous (17), mainly of the Wayúu ethnic group, there are characteristics and socio-cultural practices in rural areas, which combined with the above could increase the level of risk of vectorial transmission of *T. cruzi* in the municipality of Riohacha.

In this context, the aim of this study was to determine the presence and public health importance of vectors of Chagas disease in the indigenous settlements of Marbacella and El Horno of the Wayúu ethnic group in the municipality of Riohacha, La Guajira, Colombia.

MATERIALS AND METHODS

STUDY AREA

The municipality of Riohacha, the capital of the department of La Guajira, is located on the Colombian Caribbean coast (**Figure 1**). The locations correspond to the indigenous settlements of El Horno (11°30′16.35″N and 72°59′21.31″W) and Marbacella (11°30′24.5″N and 72°59′09.7″W) situated at 7.18 km from the Riohacha city center (**Figure 1**). The indigenous settlements are inhabited by about 300 members of the Wayúu ethnic group who are mainly engaged in fishing, making crafts, and grazing goats. The area corresponds to Tropical Dry Forest (Bs-T) (18, 19) with preferentially herbaceous vegetation, an average annual temperature of 28.2°C (minimum 23.4°C, maximum 33.2°C), relative humidity ranging between 59.3 and 77.5%, and an annual bimodal rainfall regime, with the first rainy season between the months of April and June and another more representative period in the months of September and October, with rainfall between 700 and 2,000 mm (20–23).

ENTOMOLOGICAL MATERIAL

Three entomological surveys were performed during dry season (May, June and July 2013) and one during rainy season (September–October 2013) to search for feces, exuviae, eggs, immature, and adult forms of Hemiptera: Reduviidae: Triatomini, associated with the dwellings in the indigenous settlements of Marbacella and El Horno in Riohacha. Each sampling included

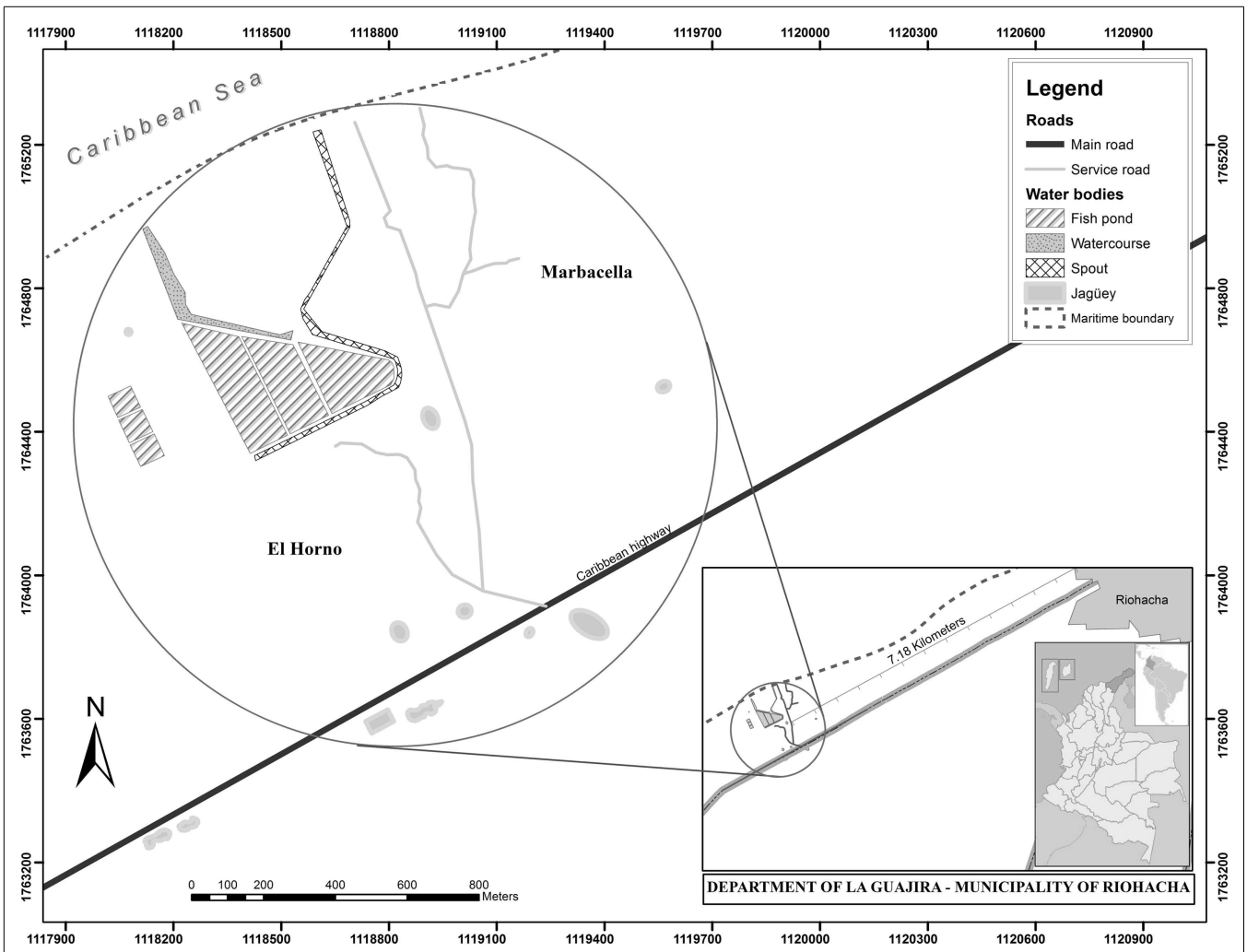

FIGURE 1 | Geographical location of El Horno and Marbacella settlements, Riohacha, La Guajira, Colombia is shown.

direct search in each house following the National Protocols of Entomological Surveillance (4); setting of Maria sensor type traps (24); and training people in the community for triatomine recognition.

The search was performed directly inside the houses examining the furniture, appliances, clusters of objects, cracks in walls and other places with favorable conditions for the presence of these insects, and around the homes inspecting kitchens, henhouses, pens, woodpiles, shady arbors, and animal nests (4, 24). Maria sensor type traps ($n = 20$) were installed at a height of 1.50 m from the floor, on the inside walls of randomly selected houses at each sampling, participation workshops were held for residents of communities to recognize the epidemiologically relevant triatomine species reported for the area and the invitation was to actively search and deliver entomological material collected. For this, full-size photographs of insects were used as well as entomological material from colony, pin mounted, and properly labeled. Biosafety standards for handling insects were emphasized and latex gloves, masks, entomological tweezers, and labeled bottles for data collection were delivered. In all cases, the insects were collected in plastic vials labeled with the catch information for each sampling method and were transported to the Laboratory of Entomology – Genetics Area of Economic Interest Insects, Faculty of Agricultural Science, Universidad Nacional de Colombia.

The taxonomic identification of immature stages and adults was based on morphological characters, including male genital specimens (6). The determination of the natural infection with *T. cruzi* was performed by optical microscopy inspection in 0.9% saline of the content of the distal portion of the insect gut. Also parasitic DNA detection was performed by polymerase chain reaction (PCR) amplification of a satellite nuclear region with *cruzi1* primers (5′ASTCGGCTGATCGTTTTCGA3 ′) and *cruzi2* (5′AATTCCTCCAAGCAGCGGATA3′) from abdomen of representative *T. maculata* specimens of each home. PCR was performed by initial denaturation of 94°C for 5 min followed by 40 cycles of 94°C for 1 min, 64°C for 30 s, and 72°C for 1 min. The PCR products were analyzed by electrophoresis in 2% agarose gels stained with GelRed (Biotum) (25).

For each indigenous settlement the following indices were calculated: intra and peridomestic infestation; colonization; infection; dispersion; and density, all in accordance with the guidelines of the Pan American Health Organization (PAHO) (26).

ETHICAL ISSUES

The project was approved by the Research Ethics Corporate Committee of the Fundación Santa Fe de Bogotá. The insect collection was conducted by officials from the Health Office of the department of La Guajira; researchers at the Fundación Santa Fe de Bogota, and Universidad Nacional de Colombia, as well as by citizens of the community who were trained to perform these activities and signed an informed consent where they were explained the objectives, risks, and benefits of the activity. The heads of households where the direct search for triatomines and installation of Maria sensors were conducted were informed of the purpose of the study, identifying their benefits and risks and were asked to consent to the development of the activities. The results obtained were shared with the Health Office of the department and the community of the indigenous settlements of El Horno and Marbacella of the Wayúu community of Riohacha, La Guajira.

RESULTS

A total of 68 homes out of 75 (90.7%) were inspected due to the reluctance of the inspections by some heads of household. Direct search and occasional catches evidenced the presence of Hemiptera: Reduviidae: Triatomini in 16 homes using an accumulated capture effort of 80 man-hours during dry season and 16 man-hours in the rainy season (**Figures 2** and **3**). Thirty-five adults, 63 nymphs, and 14 nymph exuviae were collected in various development stages and a wing, all from *T. maculata* (**Table 1**).

Out of 113 collected specimens, 79.6% ($n = 90$) were collected in the peridomicile and 20.3% ($n = 23$) in the intradomicile (**Figures 2** and **3**), in both cases mainly related to poultry rest and shelter areas and nests. No flagellar parasitic forms to *T. cruzi* were observed in the specimens where inspection by light microscopy ($n = 16$) was performed. However, in 19 of the 32 specimens of *T. maculata* analyzed by molecular biology techniques, a band of 166 bp for the nuclear satellite DNA of *T. cruzi* (**Figure 4**) was seen. **Table 2** shows the vector indices calculated for each indigenous settlement from the data collected.

DISCUSSION

T. maculata is one of the species of the *Triatoma* genus with the greatest geographical distribution in Colombia, being recorded in 11 departments located in the regions of the Orinoco plains and the Caribbean plains (4, 7). The department of La Guajira registers *T. maculata* in the municipalities of Maicao, Barrancas, El Molino, Fonseca, Hato Nuevo, Maicao, San Juan, Urbilla, and Villanueva (7, 8, 16). The results of this study extend and update the geographic distribution of the species in the department, reporting it for the first time in the municipality of Riohacha.

T. maculata was the only species recorded in all samples taken in the indigenous settlements of Marbacella and El Horno, a finding that is consistent with that reported in other studies where the predominance of this species is observed in regions with temperatures ranging between 22 and 25°C, rainfall between 1,500 and 2,000 mm annually and vegetation consisting mostly of scrub and thorn (9, 10, 27, 28).

Overall, *T. maculata* showed greater abundance in peridomestic spaces associated with henhouses, pets rest areas, and shelters, with infestation indices identical for both indigenous settlements. This biological preference continues in different geographical locations in Latin America (6, 10, 16, 27, 29–31), which is explained due to the preference for ornithophilic-type eating habits that could be conditioning their biological behavior (32). However, the population density (0.8 for Marbacella and 4.2 for El Horno) and dispersion (21.6 and 33.3, respectively) of the species in this area may be associated with processes of high intraspecific competition due to the search for blood supply and the degree of anthropic disturbance (33), revealing a significant population dynamics resulting in the mobility of the species into different environments.

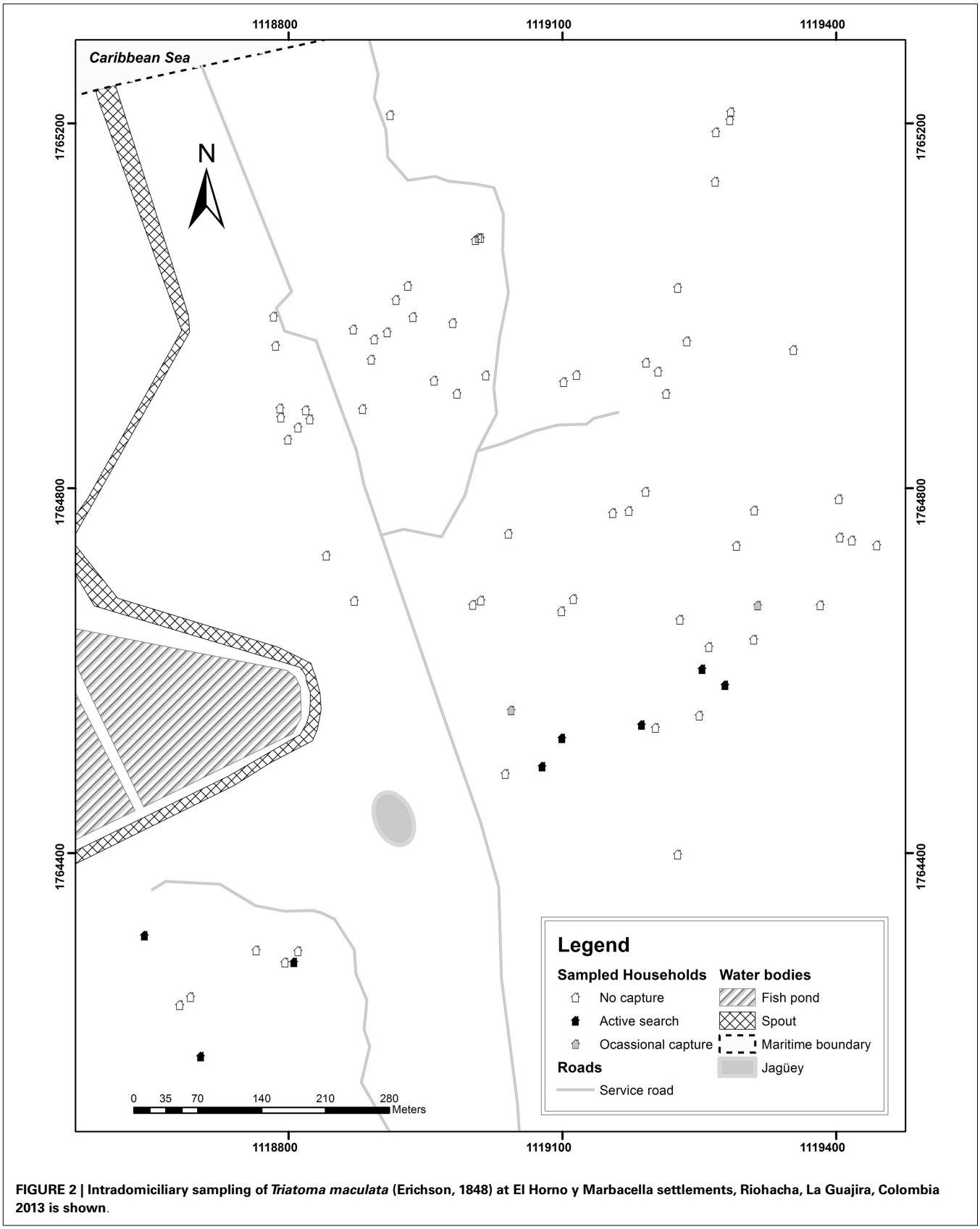

FIGURE 2 | Intradomiciliary sampling of *Triatoma maculata* (Erichson, 1848) at El Horno y Marbacella settlements, Riohacha, La Guajira, Colombia 2013 is shown.

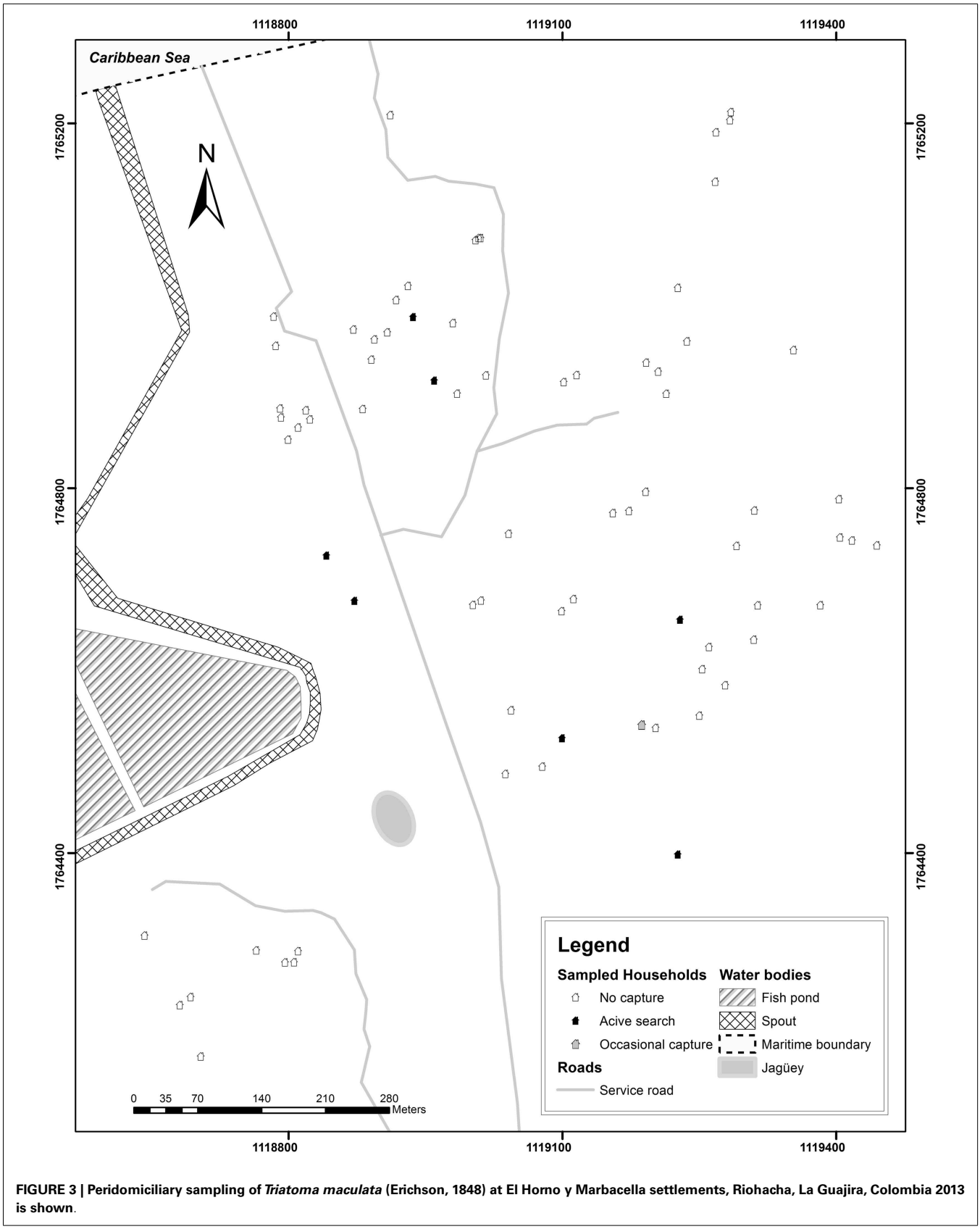

FIGURE 3 | Peridomiciliary sampling of *Triatoma maculata* (Erichson, 1848) at El Horno y Marbacella settlements, Riohacha, La Guajira, Colombia 2013 is shown.

Table 1 | Sampling of *Triatoma maculata* (Erichson, 1848) Reduviidae: Triatomini using different trapping methods of Marbacella and El Horno settlements, Riohacha, La Guajira, Colombia 2013.

Capture method	Positive housing	Marbacella										El Horno									
		Indoor					Outdoor					Indoor					Outdoor				
		A			*N*	*R*	*A*			*N*	*R*	*A*			*N*	*R*	*A*			*N*	*R*
		♂	♀	i			♂	♀	i			♂	♀	i			♂	♀	i		
Active search	14	1	0	4	1	2	6	6	0	7	2	2	1	0	0	6	1	0	1	55	5
Occasional capture	2	4	2	0	0	0	4	3	0	0	0	0	0	0	0	0	0	0	0	0	0
Maria biosensors	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

A, adults; i, indeterminate sex; N, nymphs; R, exuviae of nymphs and adult triatomines bugs vestiges.

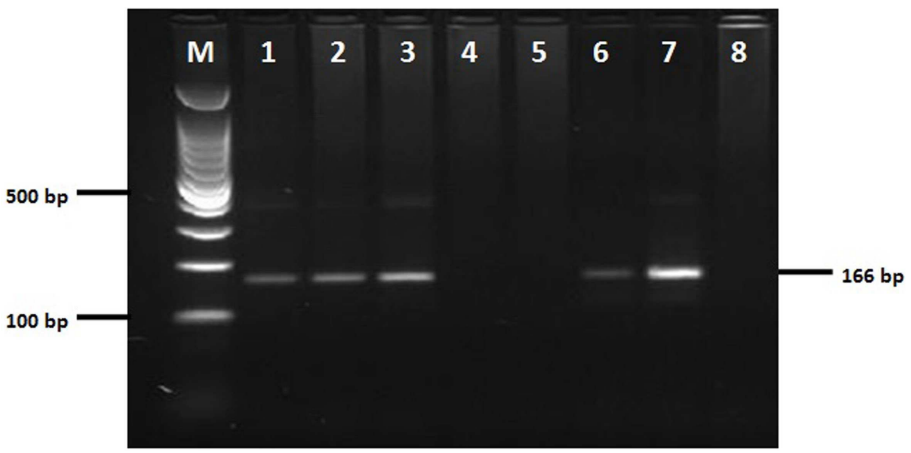

FIGURE 4 | Detection of DNA of *Trypanosoma cruzi* in insects *Triatoma maculata* by amplification of *T. cruzi* nuclear repetitive region-specific. Lanes 1–8 are amplification products of tandem repeat satellite region from *T. cruzi* (166 bp). Lanes 1–3: positive samples of *Triatoma maculata* (166 bp). Lanes 4–5: negative samples of *Triatoma maculata*. Lanes 6–7: positive controls of *T. cruzi* (166 bp) Lane 8: negative control. Electrophoresis on a 2% agarose gel visualized by staining with gel red 100-bp weight marker.

Table 2 | Vector indices of *Triatoma maculata* (Erichson, 1848) Reduviidae: Triatomini in Marbacella and El Horno settlements, Riohacha, La Guajira, Colombia.

Settlements	Housing inspected	Positive housing	Collection insects						II (%)	PI (%)	HC (%)	VI (%)	VD (%)	DI
			Indoors			Outdoors								
			N	A	R	N	A	R						
Marbacella	51	11	1	11	2	7	19	2	13.7	11.8	9.1	43.5	21.6	0.8
El Horno	17	5	0	3	6	55	2	5	17.6	11.8	20	36	33.3	4.2

A, adults; N, nymphs; R, exuviae of nymphs and adult triatomines bugs vestiges; II, intradomiciliary infestation indice; PI, peridomiciliary infestation indice; HC, household colonization indice; VI, vector infection indice; VD, vector dispersion indice; DI, vector density indice.

In Colombia, *T. maculata* was found naturally infected by *T. cruzi* with high infection indices between 50 and 72%; intradomiciliar infestation indices between 13 and 20%, and 38.3% colonization in the municipalities of Talaigua Nuevo and Mompox, department of Bolívar (9, 10). These studies demonstrate the ability of *T. maculata* to infest and colonize stable artificial ecotopes as human habitat and potential efficiency as a vector of Chagas disease.

In this study, exuviae, nymphs, and adults of *T. maculata* were found within households of both indigenous settlements, with colonization indices of 9.1% for Marbacella and 20% for El Horno, which could be suggesting infestation and colonization processes inside the housing. In addition, indices of natural infection with *T. cruzi* between 36 and 43% are reported, which is an important risk factor for the inhabitants of the Wayúu ethnic group in both communities. In contrast to these results, in San Miguel and Xaguas parishes in the Lara State, Venezuela, albeit with similar indices of infestation and colonization, low indices of *T. cruzi* infection were recorded, so that the epidemiological significance of *T. maculata* depends on the environmental, ecological, and socio-cultural factors characteristic of each geographical area (27, 28, 34).

In domiciled triatomines, a relationship is established between housing characteristics and the type of construction material, distribution of peridomestic annexes and finishes of ceilings, walls and floors, as well as socio-cultural practices of its inhabitants with respect to the population distribution and development of triatomine species in homes (35–37). This is of particular importance in both indigenous settlements because due to the physical characteristics of housing (mainly bahareque walls and sand floor) and the use of intradomiciliary spaces as places of poultry refuge and nesting, intrusion, and colonization processes may be assisted by passive transport factors and optimal conditions for the biological development of the species (36, 37).

Ignorance of the local health authorities and communities about the presence and levels of infestation, colonization, and natural *T. cruzi* infection with *T. maculata* and the risk it poses to the people, particularly children, makes it important to strengthen entomological surveillance in the area, together with participation strategies and community action to establish early warning systems for the recognition of the different stages of the insect and the early diagnosis of domiciliation processes of *T. maculata.*

With an aim to the interruption of intradomiciliar transmission of *T. cruzi* with triatomine, Colombia has defined control schemes based on the application of synthetic chemical insecticides in areas of infestation (4). It is important to understand the ecological, biological, and socio-cultural context where vector transmission is occurring and involve affected communities so that strategies for prevention, monitoring, and effective and sustainable control are jointly constructed. The ecobiosocial approach through transdisciplinary research, systems thinking, community participation, and environmental sustainability proves useful for the design, implementation, and evaluation of control strategies appropriate to the ecological, social, and cultural context (38–40). It is suggested to extend these studies in other indigenous settlements, as well as in the urban area of Riohacha because it is possible to find housing conditions and natural ecotopes with appropriate habitats described for this species, which, together with the significant human density in this capital, constitutes a significant risk for Chagas disease. Although in the present study the active search by technical personnel and the community was the most effective method to found exuviae, nymphs, and adults of triatomine, it is important to include in entomological surveillance other strategies based on biosensors (24, 41) and fumigant canisters (41–43) to improve the sensibility of results and its impact in public health policies. In particular, the fumigant canister could be used both to get an idea about infestation indices and to control the insects simultaneously. Nevertheless, it is necessary to inform the community about the risks, benefits, and consequences of the use of insecticide in the fumigant canister.

In conclusion, this is the first report of *T. maculata* naturally infected with *T. cruzi* in the municipality of Riohacha and its geographic distribution in the department of La Guajira has expanded. *T. maculata* was found mainly associated with peridomestic spaces (chicken coops and pens), although indices of intradomiciliar infestation, colonization, and natural infection by *T. cruzi* were found, posing a risk to the Wayúu community of El Horno and Marbacella indigenous settlements. The ethnic and cultural importance of the Wayúu community and the burden that the disease involves in terms of disability and reduced life expectancy (4), makes it necessary for the results to be contextualized as a serious public health problem.

AUTHORS CONTRIBUTION

Edith Natalia Gómez-Melendro: development of field and laboratory component. Participation in the preparation of the scientific paper. Diana Carolina Hernández-Castro: development laboratory component and participation in the development of the scientific paper. Catalina Gonzalez-Uribe: principal investigator and participation in the development of the scientific paper. Helena Brochero: principal investigator, coordination of the entomological component and participation in the development of the scientific paper.

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Epidemiological study on sand flies in an endemic focus of cutaneous leishmaniasis, Bushehr city, southwestern Iran

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Cutaneous leishmaniasis is the most important health problem in the city of Bushehr, southwestern Iran. The objective of the study was to determine some ecological aspects of sand flies in the city during 2010–2011. Sand flies were collected monthly from outdoors and indoors by sticky traps at four selected districts of the city. They were also dissected and examined by nested-PCR for identification of the parasite during August–September of 2011. A total of 1234 adult sand flies were collected and 6 species including 3 of Genus *Phlebotomus* and 3 of Genus *Sergentomyia* were identified. Four species including *P. papatasi* (3.98%), *P. sergenti* (1.14%), *S. tiberiadis* (87.18%), and *S. baghdadis* (7.7%) were found indoors. Six species including *P. papatasi* (3.47%), *P. sergenti* (3.17%), *P. alexandri* (0.1%), *S. tiberiadis* (77.74%), *S. baghdadis* (15.41%), and one female of *S. clydei* (0.11%) were collected from outdoors. Sand flies started to appear from March and disappear at the end of January. There was only one peak in the density curve in July. The study revealed that *S. tiberiadis* and *S. baghdadis* could enter indoors which 89 and 81.8% of them were found blood-fed, respectively. Moreover, *P. papatasi*, *S. tiberiadis*, and *S. baghdadis* were active indoors and outdoors in most months of the year. Nested-PCR of *P. papatasi* females was positive against kinetoplast DNA of *L. major* and *L. turanica* and also mixed natural infections were found by *L. gerbilli* and *L. turanica*. Moreover, mixed infections by *L. major* and *L. turanica* were observed in this species. *Sergentomyia clydei* and *S. tiberiadis* were found to be negative to any DNA of *Leishmania* species. *Phlebotomus sergenti* females were found infected with DNA of *L. turanica* and this is the first report of natural infection and detection of the parasite from this sand fly species in worldwide.

Keywords: epidemiology, Iranian sand flies, *Phlebotomus sergenti, Phlebotomus papatasi, Leishmania turanica, Leishmania major*

INTRODUCTION

There is a long history of Cutaneous Leishmaniasis (CL) in Iran. The oldest traditional medical book has been written by an Iranian scientist, Avicenna (IbnSina, born in 980, died in 1037), which was completed in 1025, about 1000 years ago. It is called Qanun (The Laws of Medicine) and it was used as a textbook until eighteenth century in the universities of European and Islamic countries. In this book, Avicenna has mentioned on cutaneous lesions of his patients, which was called Khyroonieh, with long duration and the treatment of the ulcers had been difficult and resistant to different drugs, the clinical signs of the ulcers were imagined to be CL (1). The impact of the disease on human health in this part of middle-east was not really recognized until 1940s, since then Iranian leishmaniasis has been the subject of an epidemiological program directed by Ansari, Hadjian, Mofidi, Pooya, Mesghali, and Nadim (2), constitutes an increasing public health problem in the country.

Cutaneous leishmaniasis is endemic in two forms in Iran, Anthroponotic Cutaneous Leishmaniasis (ACL) and Zoonotic Cutaneous Leishmaniasis (ZCL). About 20,000 cases of leishmaniasis are reported annually, which 80% of them are ZCL, 0.5% Visceral Leishmaniasis (VL), and the rest is ACL. *Phlebotomine* sand flies of Iran have been studied since 1930 by a limited number of Iranian and foreign entomologists such as Adler, Theodor, and Lourie but Mesghali was the first Iranian to conduct basic studies on sand flies in this country (3).

Cutaneous leishmaniasis has been epidemic during the years 1988, 1997, and 2008 in the city of Bushehr (Health center of Bushehr province, unpublished data). The causative agents of the disease are *Leishmania major* and *Leishmania tropica*. In some parts of the city, *Tatera indica* is the main reservoir host and *Nesokia indica* as the secondary reservoir. The prevalence of scar was 5.9% among the inhabitants and for ulcer it was <0.5% in 2010 (4). Bushehr is one of the most important free trade industrial zones

of the country and the Bushehr Nuclear Power Plant, which is unique in terms of its technology in the Middle East is located 12 km, southeast of the city along the Persian Gulf so lots of people travel around and some make several trips in a year for business.

Bushehr, like most of other Iranian cities, has expanded quickly over recent years. Mass emigration to the city from other parts of the province and urbanization of peripheral with poor facilities and sanitation, construction of buildings nearby rodent colonies, increase of non-endemic people in south Pars Project, Bushehr Military Complex, and the presence of Bushehr Nuclear Power Plant are the main reasons of occurrence of CL in the city. If the disease does not receive considerable attention by the health authorities, it may spread into other parts of the country, which are free from CL. However, the entomological studies on sand flies have not been carried out in the city yet and there is no accurate data on vector(s) of the disease.

The objective of this study was to determine some ecological aspects of sand flies in the city of Bushehr during 2010–2011, as an initial step in the development of effective strategies for the control of leishmaniasis in the city.

MATERIALS AND METHODS

STUDY AREA

The city of Bushehr located in a plain running along the coastal region on the Persian Gulf coast of southwestern Iran and is the administrative center of its province.

Field studies were carried out during 2010–2011, in the city of Bushehr (Latitude: 28°55′ 30″ N, Longitude 50°50′ 17″ E, altitude: 5 m above sea level) (**Figure 1**). The city had a population of 221,016 in 2011, while this was 133,753 in 1991 with an increase about twofolds in the last two decades. The area has a hot desert climate though it does receive more rainfall than most cities on the Persian Gulf. The rain is confined to the period from November to May, when temperature is pleasantly mild and is extremely erratic. The long summer from April to October is brutally hot, humid, and completely rainless. In 2010, the maximum and minimum mean monthly temperature was 39 and 12.1°C in August and February, respectively, and the total annual rainfall was 4.29 mm with a minimum of 0.1 mm in May and 2.45 mm in February. The minimum mean monthly relative humidity was 58% (December) and the maximum was 74% in January (Bushehr Meteorological Organization, unpublished data).

SAND FLY SAMPLING AND MONITORING

To obtain enough data, four infected districts of the city were selected, called Chaharmahalleh in the north, Sangi in the center, Tangak in the south, and Imamzadeh in the southwest. Sand flies were collected monthly from fixed places of indoors (bedrooms, sitting rooms, toilets, bathrooms, store rooms, hallways) and outdoors (rodent burrows, base of walls, and cracks in it in the yards) fixed places, using 30 sticky traps (castor oil coated white papers 20 cm × 35 cm) from the beginning (March) to the end of active season (January). Collected sand flies were stored in 70% ethanol. For species identification, sand flies were mounted in Puri's medium, produced at the medical entomology department (5), and identified after 24 h using the keys of Theodor and Mesghali (6). In case of molecular studies, sticky traps were used to collect sand flies at the end of transmission season from indoors and outdoors in September 2011, stored in 96% ethanol at −20°C until examination and all fed and gravid females were tested individually by nested-PCR for identification of *Leishmania* parasite.

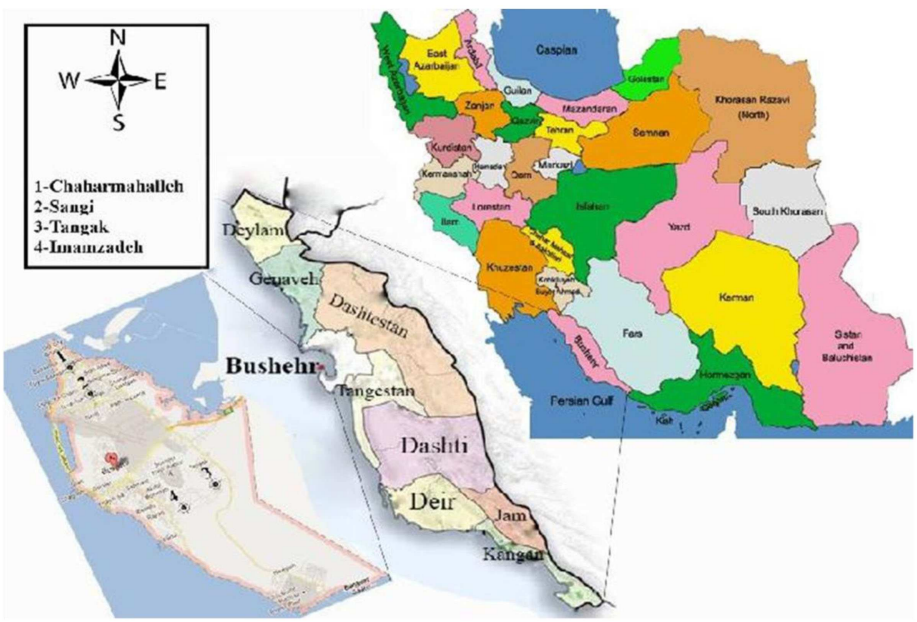

FIGURE 1 | Map of the city of Bushehr, showing the geographical location and study sites.

DNA EXTRACTION

The middle parts of female sand flies (including thorax and abdomen) were used; the samples were washed with absolute ethanol and after drying washed three times in cold sterile phosphate-buffered saline (PBS; pH 7.2). Before submitting the sandflies to the DNA extraction procedure, they were subjected to 13 freeze/thaw cycles, using liquid nitrogen and boiling water and sampler tips or pestle, to disrupt tissue and treated as described for the tissue samples (4). Genomic DNA was extracted and purified using Qiagen extraction Kit (Qiagen, 69504) according to the manufacturer's manual with the minor modification of increasing incubation time to 5 min to increase the yield of DNA in the final step. DNA was stored at −20°C until analysis. The concentration of extracted DNA was measured spectrophotometrically by NanoDrop (Thermo Fisher Scientific, USA).

MOLECULAR ASSAYS

Primer design for amplification of ITS2

Primers designed previously and used to amplify a 230 bp product in *L. major*, a 206 bp in *L. gerbili*, and a 141 bp in *L. turanica* across the internal transcribed spacer 2 [Akhavan et al. (7)]. The external primers, Leish out F (5'-AAA CTC CTC TCT GGT GCT TGC-3') and Leish out R (5'-AAA CAA AGG TTG TCG GGG G-3'), and internal primers, Leish in F (5'-AAT TCA ACT TCG CGT TGG CC-3'), and Leish in R (5'-CCT CTC TTT TTT CTC TGT GC-3') were selected to distinguish among the parasite species in a nested-PCR system (7).

Nested-PCR

We used nested-PCR to identify the *Leishmania* species. Conditions and parameters for PCR were as previously described with the minor modification (8). All samples were tested in 25 µl amplification reaction mixtures with 12.5 µl of the master mix (Taq DNA polymerase, 2× Master Mix Red, Amplicon, Germany), 1.8 µl of primers, 10.7 µl H_2O, and 1 µl of template DNA. The first-round PCR was performed based on the following conditions: initial denaturation at 95°C for 5 min; followed by 35 cycles including denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 45 s; and a final extension at 72°C for 5 min. The second-round (nested) PCR was performed as the same first-round exception for annealing at 58°C for 30 s. At the end, 10 µl of the reaction mix was analyzed by 2.5% agarose gel electrophoresis.

Additionally, for all PCR reactions, one negative control without DNA and one positive control with standard DNA were included to confirm the results of two rounds of nested-PCR. The PCR products of the negative and positive controls of the first-round PCR were used as negative and positive controls in the second round, respectively. Finally, 10 µl of the PCR products were loaded on 2.5% (W/V) agarose gels, and stained with ethidium bromide to visualize by electrophoresis. Initially, ITS-PCR was confirmed with standard DNA of reference strains *L. major* (MRHO/IR/75/ER), *L. gerbilli* (MRHO/CN/60/GER BILLI), and *L. turanica* (MRHO/SU/1983/MARZ-051) as positive controls and distilled water were used as negative controls (7, 8).

PCR-RFLP analysis

PCR products (20 µl) were digested with *Mnl*I 2 µl at 37°C for 4 h without prior purification using conditions recommended by the supplier (Fermentas Life Sciences, Germany). The restriction fragments were subjected to electrophoresis in 3% agarose gel containing ethidium bromide for 3 h at 65 V and visualized on a UV transilluminator.

RESULTS

SAND FLY SPECIES

A total of 1234 adult sand flies, 882 from outdoors and 352 from indoor resting places were collected and identified. The following four species were found indoors: *P. papatasi* (3.98%), *P. sergenti* (1.14%), *S. tiberiadis* (87.18%), and *S. baghdadis* (7.7%). From outdoors, six species including *P. papatasi* (3.47%), *P. sergenti* (3.17%), *P. alexandri* (0.1%), *S. tiberiadis* (77.74%), *S. baghdadis* (15.41%), and *S. clydei* (0.11%) were collected (**Figures 2** and **3**). The study revealed that *S. tiberiadis* and *S. baghdadis* could enter indoors, which 89 and 81.8% of them were found blood-fed, respectively.

The sand flies started to appear in April and disappeared at the end of January. There was only one peak in the density curve in July (**Figure 4**). Moreover *P. papatasi*, *S. tiberiadis*, and *S. baghdadis* were active indoors and outdoors in most months of the year. Sand flies were active 10 months in the city and the decrease of sand fly density at the end of January was most probably due to the rains. No sand fly was found in the city during February and March due to cold weather.

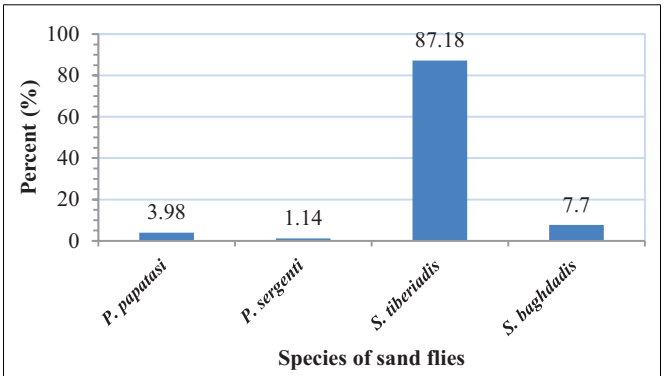

FIGURE 2 | Fauna and percent of collected sand flies from indoors, Bushehr city, Iran.

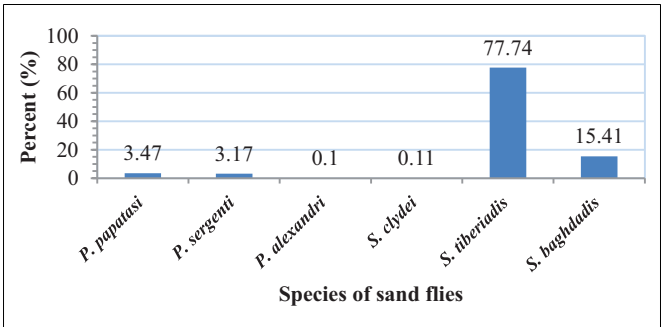

FIGURE 3 | Fauna and percent of collected sand flies from outdoors, Bushehr city, Iran.

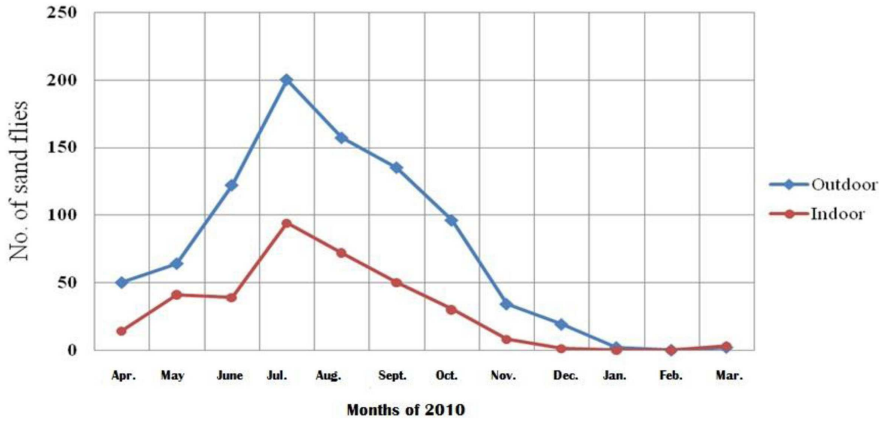

FIGURE 4 | Monthly prevalence of sand flies in the city of Bushehr, Iran, 2010–2011.

Table 1 | Natural *Leishmania* infection rate (%) of sand flies by nested-PCR in the city of Bushehr, Iran, September 2011.

Capture site	Species	No. of examined	Leishmania infection rate (%)			
			L. major	***L. turanica***	***L. gerbilli* + *L. turanica***	***L. major* + *L. turanica***
Outdoors	*P. papatasi*	4	25 (1/4)	50 (2/4)	–	–
	P. sergenti	3	–	66.6 (2/3)	–	–
Indoors	*P. papatasi*	6	–	66.7 (4/6)	16.7 (1/6)	–
	P. sergenti	2	–	–	–	–
	S. tiberiadis	1	–	–	–	–
Rodent burrows (outdoors)	*P. papatasi*	2	–	–	–	50 (1/2)
	S. tiberiadis	1	–	–	–	–
	S. clydei	1	–	–	–	–

Figures in parentheses are numbers of positive/no. of examined specimens and figures out of parentheses are percent of positive.

LEISHMANIA INFECTION OF SAND FLIES

Twenty individual female specimens including four species of *P. papatasi*, *P. sergenti*, *S. tiberiadis*, and *S. clydei* were tested against *Leishmania* parasite DNA. *Leishmania* DNA was found in 11 (55%) out of 20 specimens. **Table 1** shows the natural *Leishmania* infection rate of sand flies by nested-PCR and **Figures 5–7** show the patterns of ITS-PCR for sand flies. One out of four female *P. papatasi* from outdoors and four of six from indoors were found to be infected by *L. major* and two of four from outdoors *by L. turanica*, which produced species-specific bands of 231 and 141 bp, respectively. Mixed natural infections with *L. gerbilli* and *L. turanica* were also observed in 16.7% of *P. papatasi* from indoors. In rodent burrows, mixed infections of both *L. major* and *L. turanica* was found in one of two of this sand fly species.

Two out of three female *P. segenti* from outdoors were found to be infected by *L. turanica* and produced a PCR band of 141 bp. One of the infected *P. sergenti* was gravid and the other was semi-gravid and both of them were collected by sticky traps near dwellings.

DISCUSSION

In the present study, three *Phlebotomus* and two *Sergentomyia* species were identified for the first time in the city of Bushehr. *Phlebotomus papatasi*, *P. sergenti*, and *P. alexandri* have medical importance because of their proven or probable roles as vectors of parasites causing human leishmaniasis in the Old world (9). *Phlebotomus papatasi* and *P. sergenti* are known to feed readily on humans (3, 10).

Anthroponotic cutaneous leishmaniasis caused by *Leishmania tropica* represents a serious medical problem in several countries in the Middle-East region, including Iran. *Phlebotomus sergenti* is one of the proven vectors of *L. tropica* in some of these countries (11–14). This species is considered to be the probable vector of ACL in 14 endemic foci located in eight provinces of Iran (3). In the present study, *P. sergenti* represented 3.17 and 1.1% of all *Phlebotomus* caught from outdoors and indoors, respectively. It was active 4 months (April, May, July, August) in indoors and 7 months in outdoors and has been caught from all infected districts except Sangi district located in the center of the city. *Phlebotomus sergenti*

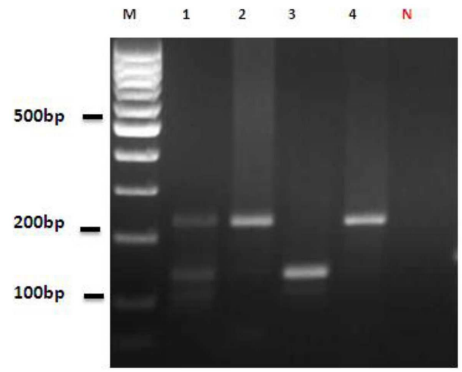

FIGURE 5 | Nested-PCR amplification of DNA extracted from infected sand flies and reference strains. Lane M, 100 bp DNA ladder (Fermentas); Lane 1, mixed infection of *Leishmania gerbilli* and *Leishmania turanica* detected from *Phlebotomus papatasi*; Lanes 2 and 4, reference strains, *Leishmania gerbilli*; Lane 3: reference strain, *Leishmania turanica*; Lane N, negative control (distilled water).

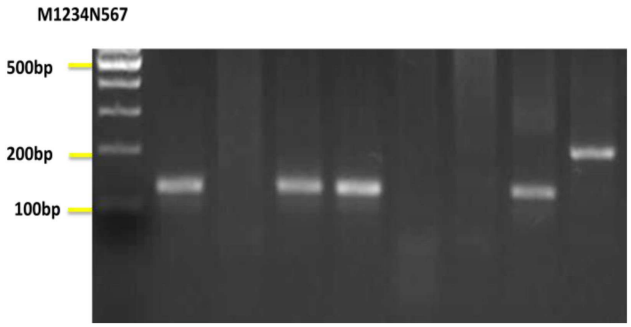

FIGURE 6 | Nested-PCR amplification of DNA extracted from sand flies. Lane M, 100 bp DNA ladder; Lanes 1,4,6 *Leishmania turanica* detected from *Phlebotomus papatasi*; Lane 3, *Leishmania turanica* detected from *Phlebotomus sergenti*; Lane 7, *Leishmania major* detected from *Phlebotomus papatasi*; Lane N, negative control; Lanes 2,5 negative samples.

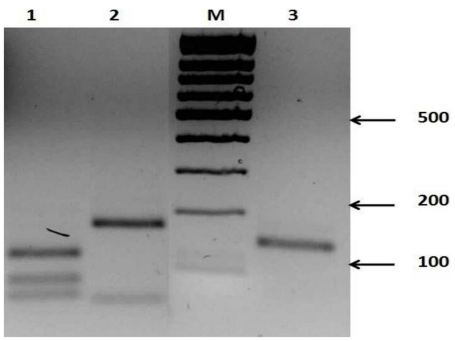

FIGURE 7 | Restriction products of nested-PCR amplicons in three species of *Leishmania* after digestion with MnlI. Lane M, 100 bp DNA ladder; Lane 1, *Leishmania major*; Lane 2, *Leishmania gerbilli*; Lane 3, *Leishmania turanica*.

is rarely found indoors and its density is very low as 4 male specimens of this species were caught but 28 of these flies were collected from outdoors during August–October in the city. Whether we could not find natural infection of females of this sand fly species by *L. tropica*, it has been recorded from the cities of Esfahan in the center and Shiraz in the south by molecular and monoclonal diagnostic antibody tests (3, 15). *Phlebotomus sergenti* has a wide distribution in the country and extends beyond the distribution of *L. tropica.*

Phlebotomus alexandri is widely distributed in Palaearctic region, but it is never common (6). It is usually considered as a mountain species (16–18) although it occurs in some low land areas as well (17). It is thermophilic and moderately hydrophilic species and aggressive to human beings (19). The present results indicate that this species is a new record in this coastal area of Bushehr, Iran. It is suspected vector of *L. infantum* and *L. major* in the provinces of Fars and Khuzestan, southern Iran (3).

Sergentomyia tiberiadis is a thermophilic and xerophilos species, usually in low and dry rocky mountains of southern Afghanistan, but absent in humid areas. In Iran, it is found in eight provinces, five in the south and the others in the center, northwest, and northeast. Regarding its restricted distribution to Pakistan, Afghanistan, and Iran and specially its presence in human residences in the areas of CL, vectoral role of this species needs to be investigated. *Sergentomyia baghdadis*is distributed in Iraq, Iran, Pakistan, and southern Afghanistan, it is thermophilic and hydrophilic species of plains, sometimes numerous in human dwellings and rodent burrows. Whether it is considered as a possible vector of reptilian leishmaniasis, its feeding habits and relation to CL should be investigated as well.

In the current study, we found DNA of *L. major* and *L. turanica* and mixed infection *of L. gerbilli* and *L. turanica* in *P. papatasi*, which is agreement with the findings by Strelkova et al. in 1996 and Parvizi and Ready in 2008 (20, 21) indicate the possible transmission of both *L. major* and *L. turanica* by *P. papatasi.* This sand fly species is considered as the vector of *L. major* to humans in the city of Bushehr. *Sergentomyia clydei* and *S. tiberiadis* were found to be negative for *Leishmania.*

Phlebotomus sergenti was found naturally infected by *L. turanica* near dwellings and according to our knowledge this is the first report of infection of this sand fly species by *L. turanica* in worldwide. Natural infection of *P. sergenti* was found in an experimental study by Chajbullinova and her colleagues in 2012; they showed that in *P. sergenti*, *L. turanica* promastigotes were present only on the defecation of blood meal remnants (22). In a study in China by Li-Ren and colleagues in 1995, *P. mongolensis* and *P. anderjevi* have been found naturally infected in the field and also in experimental studies (23). In Turkmenistan and Uzbekistan, Strelkova and her colleagues detected *L. turanica* from *P. papatasi, P. anderjevi, P. caucasicus, P. mongolensis, P. alexandri*, and *S. clydei* in natural foci of ZCL (20). In Iran, *L. turanica* has also been detected from *P. papatasi* in central and north of the country and also from *P. caucasicus* in the northwest (3). Reports of *L. turanica* from other *Paraphlebotomus* species suggest that vector competence for *Leishmania* may differ between members of this subgenus. However, further studies are needed to clarify the role of these vectors in the

circulation of *L. turanica*. This *Leishmania* species has also been detected repeatedly from *R. opimus* in Iran, Uzbekistan, China, Kazakhstan, and Mongolia. It has also been detected from *T. indica* and *Rattus norvegicus* in the city of Bushehr and also from *Nesokia indica* in southwest of the country (4).

In 2014, two isolates from two patients in northeast of Iran were examined by Nested-PCR-RFLP and sequenced several times. They were identified as *L. turanica* haplotype TurkHo3, which was previously isolated and identified from sand flies and rodents in this region (24). It can be approved as causative agents of ZCL by more extensive sampling and followed by standardized molecular diagnosis. Specific entomological and epidemiological studies including monitoring annual fluctuations of *P. papatasi* and *P. sergenti*, different aspects on sand fly ecology in the infected districts of the city, drawing attention to the diagnosis and treatment of *Leishmania* infections, follow up studies on more of *Leishmania* species from *P. sergenti* in the area are necessary in order to reach a better understanding of the interaction between *L. major* and *L. turanica* are recommended. The management of organic waste, controlled urbanization, and improvement of sanitary condition in the suburbs would reduce to a significant degree the density of sand fly vectors.

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