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Adenovirus as Tools in Animal Health

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Abstract

Adenoviruses have long been identified as good candidates for use as viral vectors in gene therapy and as vaccines. These viruses can infect multiple cell types, while in division or in quiescence, and are relatively easy to manipulate so that parts of their genome can be replaced with exogenous genes. Progressive safety improvements in replication-deficient adenoviral vectors have been achieved with the second and third generation, and ending with the gutless adenoviral vectors. Adenoviral vectors are immunogenic and can act as adjuvants. Nonetheless, the potency of human recombinant adenoviral vaccines was below expectations in clinical trials mainly because of the pre-existing adenoviral immunity found in the general population. This drawback can however become advantageous in animal health, as no previous immunity to human adenoviral vectors exists in animals. Other viral vectors viruses are used as vaccine, but adenoviruses remain the most employed and promising recombinant vector in veterinary medicine. In this chapter, we review the generation of adenoviral vectors, the immune response they trigger, and their advantages and disadvantages for veterinary use in terms of safety and efficacy. This chapter also describes how recombinant adenoviral vectors can be integrated as tools for vaccination and immunomodulation in veterinary medicine.

Keywords: adenovirus vectors, vaccines, animal health, immune response

1. Introduction to adenoviral vectors

1.1. Adenovirus

Adenoviruses (Ad) are large (90-100 nm), nonenveloped, not segmented, and linear double-stranded DNA viruses belonging to the viral family *Adenoviridae* that infect a broad range of vertebrate hosts, from fish to humans. They replicate in the nucleus of the infected cells. These viruses have an icosahedral nucleocapsid consisting of three major proteins called hexon (or protein II),

penton base (or protein III), and a nodulated fiber (or protein IV) together with a number of other minor proteins, VI, VIII, IX, IIIa, and Iva2. This capsid contains 26–48 Kbp double-stranded DNA genome (**Figure 1A**), which has a terminal protein (TP) attached to one of its ends. They were first isolated in 1953 from a culture of human adenoid cells, hence their name [1]. Of the more than 100 Ad described since then, 57 infect humans causing conjunctivitis, hemorrhagic cystitis, gastroenteritis, and respiratory diseases. The *Adenoviridae* family contains five genera based on DNA composition and host species: *Aviadenovirus*, *Atadenovirus*, *Mastadenovirus*, *Siadenovirus*, and *Ichtadenovirus* [2]. Within the genera, the viruses are grouped into species, and named from the host followed by letters of the alphabet. For example, the human adenoviruses (HuAd) are classified within the *Mastadenovirus* genus and divided into seven subgroups, from A to G [3, 4]. Classification questions remain, however, unresolved for many nonhuman adenoviruses.

1.2. Adenoviral vectors

Viral vectors are modified viruses used to introduce exogenous DNA into host cells, and their construction uses similar principles. Virus functions can be divided into elements that act in *cis* such as the origins of replication or the encapsidation sequence that must be found in the genome of the viral vector, or act in *trans* such as structural proteins and/or envelope or the machinery necessary for viral replication that do not need to be encoded by the viral genome itself. These *trans* elements can be supplied by stably transfected cells (packaging cells), or through transient transfections with plasmids or helper virus. The general method for viral vector construction consists in substituting the *trans* elements, essential for replication, by the gene of interest. The most popular technique developed for constructing replication-defective (RD) recombinant adenoviral vectors is that described by Dr. F. Graham and known as the “two-plasmid method” (available in commercial kits) [5]. Nonreplicative (defective) particles thus obtained maintain the infectivity of the parental virus, but are unable to produce new infective viral particles, and possess the ability to transfer the therapeutic gene material introduced into their genome. The viruses most commonly used as vectors are poxviruses, retroviruses, and Ad.

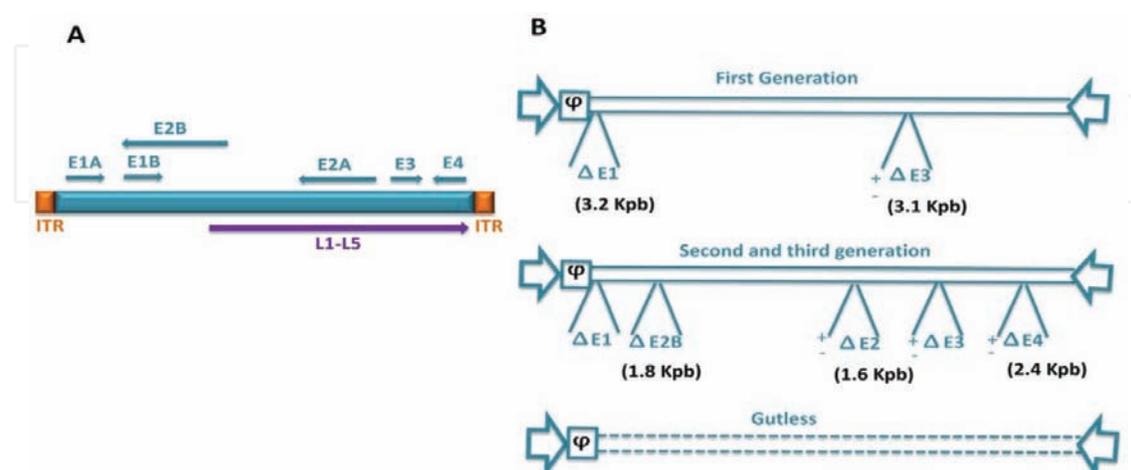


Figure 1. (A) Schematic representation of the adenoviral genome organization. E, early genes; L, late genes; and ITR, inverted terminal repeat sequences. (B) Diagram of the evolution of the different adenoviral vectors. Deletions (Δ) from different areas of the adenoviral genome have improved these vectors in terms of capacity to house an exogenous gene and in terms of safety, avoiding reversions. ψ , cis packaging signal.

Ad possess most of the qualities required to be a successful viral vector. They infect a large variety of mitotic and postmitotic cells replicating episomally without chromosomal integration, thus reducing the risk of insertion mutations and oncogenesis. They have high cloning capacity, high transduction efficiency, and high transgene expression. They are relatively easy to prepare and purify, which permits the obtention of high viral titer with low toxicity. HuAd serotypes 2 and 5 are the best characterized and most used for creating recombinant vectors [6]. The RDAd used as vectors can be divided into three classes, schematized in **Figure 1B** [7, 8], according to the size of the deletions made in their genome, which directly impacts on the size of the exogenous DNA they can harbor.

From a safety point of view, it is preferable to work with replication-defective (RD) Ad [9], and this chapter will mainly focus on RDAd. There are nonetheless several studies that use replication-competent (RC) Ad in veterinary vaccination, for instance to improve mucosal immunity or override maternal-derived immunity [10, 11].

2. Immunogenicity of adenoviral vectors

RDAd vectors induce humoral, cellular, and mucosal protective immune responses in a variety of animal models [12]. They are particularly suited to produce potent cellular immune response to the encoded antigens [13]. Vector innate immunogenicity and antigen expression affect and shape the adaptive immune response triggered by RDAd infection.

Innate immune responses are essential for triggering an effective adaptive response. RDAd activate nucleotide-binding oligomerization domain-like receptor (NLR) and toll-like receptor (TLR) signaling pathways and induce several cytokines such as IL-1, IL-12, IL-6, TNF, and interferon (IFN)- α . Myeloid differentiation protein-88 (MyD88) signaling contributes to the induction of RDAd adaptive immune response since systemic and mucosal immunity was reduced in MyD88-deficient mice after RDAd vaccination [14]. CD8⁺-T cell responses elicited after RDAd vaccination are, however, not dependent on TLRs or IL1-R family member since T-cell responses are not significantly diminished in mice lacking different TLRs, IL-1R, or IL-8R [15]. Type I IFN production and signaling probably participate to transgene immunity. Type I IFN levels correlate with transgene neutralizing antibody titers [16] and IFN- β promoter stimulator-1 (IPS-1) and type I IFN signaling are required for the induction of antigen-specific CD8⁺-T cells in the gut mucosal compartment [17]. Besides TLRs, cells detect cytosolic viral DNA through NLRs, which are at the core of the inflammasome that triggers inflammatory responses producing IL-1 β , IL-18, and IL-6 (reviewed in [18]). NF- κ B-dependent inflammatory gene expression (IL-1 β , IL-6, and MIP-1 β) was significantly reduced in NALP3-deficient mice after RDAd inoculation [19], indicating that the NALP3 inflammasome mediates the innate immune response to RDAd.

The magnitude and quality of the T cell immune response elicited by RDAd is influenced *in vivo* by the vector cellular tropism, which alters the source of cytokines and chemokines produced during vaccination. After intravenous inoculation, Kupffer cells in liver [20] and macrophages in the marginal zone of the spleen [21] are infected by RDAd, whereas after subcutaneous or intramuscular inoculation (the most commonly used vaccination routes), CD11c⁺

dendritic cells (DCs) are transduced in the draining lymph node. The CD11c⁺CD8⁻B220⁻ compartment showed enhanced RDAd uptake and transgene expression [22], but in spite of being less frequently transduced, the CD11c⁺CD8⁺B220⁻ DC subset was more potent at inducing T cell proliferation against the transgene. CD11c⁺ DCs are, therefore, critical for eliciting T cell responses against RDAd-encoded transgenes.

High transgene antigen-specific responses after infection with Ad serotypes, such as HuAd5, are associated with high transgene expression levels *in vivo* [23]. The amount and duration of the antigen expression is thus one of the most relevant parameters that shape the immune response induced by RDAd. In mice, HuAd5 and chimpanzee-derived ChAd3 produce high and persistent antigen expression with low innate immunity activation resulting in strong T cell response induction, whereas RDAds that express less antigen and trigger a robust innate immunity are less potent inducers of T cell responses [23].

Pre-existing vector-specific humoral and cellular immunity limits the duration of transgene expression and is one of the main problems for RDAd uses as vaccines [24]. Vector-specific neutralizing-antibodies suppress the immunogenicity of adenoviral vector vaccines [25]. Although neutralizing antibodies are serotype specific and mainly directed against the hypervariable loops of the viral hexon, non-neutralizing antibodies to more conserved regions of the adenoviral particle cross-react between Ad serotypes [26]. Passive antibody transfer from RDAd-immunized animals to naïve animals demonstrated that adeno-specific neutralizing antibodies reduced the induction of transgene-specific CD8⁺ T cells after homologous challenge. Nonetheless, these neutralizing antibodies change the fate of the CD8⁺ T cells and promote their transition into the memory cell pool [27]. This could be highly relevant for vaccine design, since enhanced CD8⁺ cell expansion to the transgene can be detected when boost inoculation was given with a heterologous RDAd.

It, thus, appears that the balance between immunity to the vector and the transgene defines successful RDAd vaccination strategies. Recognition of the vector is necessary for Ad adjuvancy to take place, while high transgene expression and immunogenicity are also required to drive the immune response toward the antigen of interest.

3. Recombinant adenoviral vectors in veterinary medicine

3.1. Considerations for veterinary vaccines and adenoviral vector vaccines

The use of vaccines to fight animal diseases is one of the most efficient strategies of preventive medicine regarding cost-effect ratio. It helps reduce disease, minimizes long-term healthcare costs, and ultimately reduces inequity in health [28]. Maladies such as rinder pest have been eradicated thanks to vaccine campaigns. Multiple parameters need to be considered for a potential vaccine to become successful, such as its efficacy, safety and immunogenicity, and the possibility of large-scale production at low cost while maintaining genetic stability. Ideally, a vaccine should also be single dose and provide long-term systemic and mucosal immunity [29].

In veterinary medicine, adenoviral vectors that express immunogenic pathogen proteins have been used as vaccine to activate a protective immune response to the pathogen [30, 31]. The use

of HuAd5, most commonly used in human trials, in animal health can be advantageous, as no previous immunity to this adenoviral vector should exist in animals. Recombinant Ad strongly activate the immune system [32] and generate immunity toward both the vector and the expressed transgene. These strong humoral and cell-mediated antigen-specific responses [12, 13] are a prerequisite for a good vaccine candidate that can even preclude for adjuvant need. But it may also present a problem, since immunity to the vector could be generated in vaccinated animals, which would limit efficacy if a second immunization was needed. Several approaches can be undertaken to solve this problem, from using a single inoculation to induce protection, to using heterologous prime-boost systems or using different Ad serotypes for consecutive inoculations [33].

RDAd recombinant vectors can be produced in large scale with a high titer [34] and lyophilized, or produced in thermostabilized forms [35] so that they can be easily stored and transported, even in conditions in which the maintenance of a cold chain can be problematic as in case of distribution to remote locations in hot climate countries. For veterinary medicine, vaccines need to be particularly inexpensive. As part of the One Health strategy, vaccination also offers the added benefit of limiting antibiotic use in animal production, either through direct vaccination effects or by limiting viral diseases that can lead to opportunistic bacterial infections.

3.2. Adenoviral vectors as DIVA vaccines

Most veterinary vaccines do not allow infected-recovered animals to be distinguished from vaccinated animals, the so-called differentiating infected from vaccinated animals (DIVA) approach. DIVA vaccines can be used as control tools for disease outbreaks, limiting animal culling in the eradication process. They, thus, have a great economic importance as they facilitate animal health status monitoring and grant disease-free status more quickly to countries affected by an outbreak. RDAd expressing antigenic proteins are suitable DIVA vaccines as vaccinated animals that only respond to proteins encoded by the vaccine can be differentiated from infected animals that also respond to viral proteins not encoded by the RDAd vaccine. An adenovirus-based vaccine was shown to be successful as foot and mouth disease (FMDV) DIVA vaccine [36]. RDHuAd5 that express peste des petits ruminants virus (PPRV)-F or -H proteins are another example of DIVA veterinary vaccines [37–39]. While vaccinated animals developed antibodies against F and H, infected animals also developed antibodies against N, and due to validated commercially available tests for anti-N and anti-H antibodies, infected animals could be differentiated from vaccinated animals. RDAd-based vaccines appear, thus, particularly suited to implement DIVA strategies.

3.3. Replication-competent vs. replication-defective adenoviral vectors

When Ad are engineered to be RD and express a transgene, most of the immune response they trigger can be biased toward this transgene since transgene expression replaces early adenoviral gene expression, thus limiting adenoviral protein synthesis [24]. Ad can also be engineered to express transgene while remaining replication competent (RC). In these cases, immune responses to the transgene can be enhanced [9, 31, 40], but the immune system is also more prone to react to the vector than in the case of RD vectors since infective lytic cycles occur. This can result in sero-neutralization of the vector over time that limits vaccine

efficacy if booster immunizations are required. Care should also be taken when immunizing immunocompromised individuals with RCAd vectors as vaccine-derived pathology could be induced. Importantly, RCAd could potentially escape the vaccinated host, which limits their application and hinders their approval by legislative bodies. RCAd can nonetheless have applications in veterinary science as demonstrated by the effective campaigns for rabies control in Canada with RC adenoviral vectors expressing the rabies virus glycoprotein delivered to wildlife through baiting [41]. The vaccine was safe in a number of species and showed minimal risk of horizontal transmission [42].

The present chapter will mainly focus on RD adenoviral vectors as veterinary tools since RDAd genetic stability makes them particularly suited for the design of safe and legislatively acceptable vaccines. Despite being one of the most studied recombinant vectors in veterinary medicine, no RDAd vaccine is currently licensed for veterinary use. An RDHuAd5 vector expressing the FMDV P1 region and the 3C^{pro} protease has nonetheless received a conditional US veterinary biological product license. An exhaustive safety study for the issue of a US veterinary biological license product for this vaccine was recently completed [43]. No evidence of reversion to virulence, shedding from vaccinees or presence in milk products was detected indicating that RDAd vaccines are safe and recombinant vaccine particles are unlikely to be found in animal products used for human consumption.

3.4. Human vs. nonhuman adenovirus for veterinary use

HuAd5 vector is the most extensively used adenoviral vector for vaccine design and gene therapy. However, pre-existing adenoviral immunity complicates its use in human therapy since this drastically decreases efficacy [44], but in veterinary medicine, no immunity to HuAd should be present. Indeed pre-existing neutralizing antibodies and cell-mediated immunity to the veterinary specie Ad usually do not cross-react with human adenoviral vectors [45]. This implicates that human adenoviral vectors can trigger strong immune response in the veterinary host. There are nonetheless risks that need assessment prior to commercial release like reversion to virulence. Importantly for livestock animals, it is essential to demonstrate that the recombinant vaccine is absent from the animal products consumed by the human population (e.g., meat and milk) so that veterinary use of RDHuAd vaccines is not perceived as a health risk by legislative bodies and the public in general.

To circumvent pre-existing immunity, nonhuman adenoviral vectors can be used. These are often studied for gene therapy as they improve gene delivery and expression [46], but they could still hold veterinary vaccine applications. For instance, in the cases of zoonosis like Rift Valley fever (RVF) that affect human populations, it could prove advantageous to develop adenoviral-based vaccines on the backbone of nonhuman species to avoid HuAd pre-existing immunity [47]. Since most nonprimate adenoviral vectors produce abortive infections in human cells [48], the risk of virulence reversion and recombinant vector spreading in humans is further minimized. These nonhuman vectors also produce strong immune responses in the veterinary host, although most studies thus far have used RCAd constructs [9, 49, 50]. Nonhuman RCAd could have applications in veterinary vaccination when the Ad itself is pathogenic [51]. Recombinant technology could be used to attenuate pathogenic fowl adenoviruses (FoAd) strains to produce

suitable vaccine strains or FoAd could be manipulated to become vectors that express recombinant immunogenic proteins [52]. Because of the dissemination risks posed by RCAd, RDAd appear nonetheless as the way forward even for nonhuman Ad.

One of the main barriers for the development of nonhuman RDAd vectors is the necessity to construct cell lines capable of complementing the viral genome so that these vaccines can be propagated. The production of RD vectors has nonetheless been achieved for several nonprimate species [48, 53], and RDCaAd2 vectors expressing immunogenic viral subunits have shown potential for vaccination against rabies [54], bluetongue virus (BTV) [55], or FMDV [56]. Because Ad infect a wide range of mammalian cells from different species, these nonhuman RDAd vectors could also be used to circumvent pre-existing immunity. Ultimately, this could help broaden the range of adenoviral vectors available for vaccine design. Understanding nonhuman adenovirus biology and advancing in their manipulation can, therefore, help vaccinologist design novel strategies in veterinary medicine and in human medicine where pre-existing immunity to these vectors will be minimal.

4. Applications of RDAd in veterinary medicine

Typically, RDAd are engineered to express an immunogenic antigen from the pathogen and used as vaccine. However, since RDAd can accommodate fairly large inserts, they can encode for multiple genes and produce virus-like particles. RDAd can also be used to boost adjuvancy in vaccine preparations by expressing cytokines or co-stimulatory molecules, or even impair viral replication by encoding for interfering RNA sequences.

4.1. Antigen-encoding RDAd as vaccines

RDAd encoding for immunogenic determinants showed promising vaccination results in a range of relevant veterinary diseases (**Table 1**). In PPRV, which is the next disease targeted by the World Organization for Animal Health (OIE) for eradication, RDHuAd5 vectors expressing PPRV fusion protein (F) or hemagglutinin (H) induced strong cellular and humoral immunity and protected goats and sheep against virulent challenge [38, 39]. In BTV, immunizations with RDHuAd5 expressing the VP2 and/or VP7 proteins are protected from homologous challenge [57]. RDHuAd5 expressing the FMDV P1 region and the 3C^{pro} protease can protect swine and cattle from the disease [58]. RDAd vaccines can protect multiple mammalian hosts (sheep, goats, and cattle) from Rift Valley fever virus (RVFV) challenge, and induce immunity in camels [47]. RDAd vaccines can also protect across animal classes as an RDHuAd5 vector vaccine expressing the influenza A virus (IAV) H protected chicken from viral challenge [59]. This broad spectrum of hosts makes RDAd vaccines particularly attractive for vaccine design against zoonotic diseases.

The choice of antigen is of prime importance for RDAd vaccine clinical efficiency. The immunogenicity of the transgene influences the immunity triggered to the vector [24, 31]. Strongly, immunogenic transgene products skew the immune response toward these proteins, whereas weakly immunogenic transgene products favor anti-vector immunity that eliminates transduced cells and shortens antigen exposure [60]. For instance, RDAd vaccine expressing only the

Adenovirus*	Disease	Transgene	Model/natural host	Efficacy/findings	References
Antigen encoding					
HuAd5	IAV	HA	Swine	Protection in homol-challenge Partial in heterol-challenge	[94]
HuAd5	IAV	HA	Mouse poultry	Protection Ab + CMI s.c. vaccinated chicken protected	[59]
ChAdY25	RVFV	Gn, Gc	Sheep Cattle Goats Camels	Multispecies protection VNA induction in camels	[47]
HuAd5	PPRV	F, H	Sheep	Protection VNA, Ab production, CMI	[38]
HuAd5	BTV	VP2, VP7	Mouse Sheep	VNA, Ab production CMI and protection	[57]
HuAd5	FMDV	poGMCSF, VP1, VP1 epitopes	Mouse Guinea pigs Swine	Protection	[95]
HuAd5	CSFV	E2 protein	Swine	Complete protection in DNA-Ad prime boost	[67]
VLP encoding					
RC CaAd2	RHDV	VP60	Rabbit	Protection Ab production	[50]
CaAd2	FMDV	P1/3C	Guinea pigs	Ab production and protection	[56]
HuAd5	FMDV	PPV-VP2 expressing FMDV VP1 epitopes	Mouse Swine	Protection VNA production	[96]
HuAd5	FMDV	P1/3Cpro	Swine Cattle	Protection	[58, 62, 63]

Adenovirus*	Disease	Transgene	Model/natural host	Efficacy/findings	References
RNA interference					
HuAd5	FMDV	poIFN- α + poIFN- γ + siRNA against NS proteins	Mouse Guinea pigs Swine	Protection	[79]
HuAd5	FMDV	shRNA	Guinea pigs Swine	Partial protection	[78]
Immunomodulation					
HuAd5	FMDV	poIFN- α + poIFN- γ	Swine	Synergistic protection	[73]
HuAd5	FMDV	poIFN- α	Swine	Protection vs. several FMDV serotypes	[72]
HuAd5	IAV	ovIFN- τ	Mouse	Protection	[74]
HuAd5	Salmonella	poGCSF	Swine	Protection against Salmonella shedding and colonization	[76]
HuAd5	PCV2	poGM-CSF	Swine	Reduced viremia	[81]
HuAd5	PRRSV	poCD40L PCV Capsid protein Gp3 GP5 fusion protein poCD40L	Swine	Partial protection Ab and CMI CD40L improve efficacy	[97]
HuAd5	PRRSV	GP3 GP5 fusion protein, HSP70	Swine	IFN- γ and IL-4 in sera VNA HSP70 improves efficacy	[83]
*All adenoviral vectors are replication deficient unless otherwise stated (i.e., RC).					

Table 1. Examples of adenoviral vector use in veterinary medicine.

FMDV VP1 capsid protein can only induce low levels of neutralizing antibodies [61], whereas RDAd vaccines expressing the complete P1-encoded capsid polypeptide of FMDV and the 3C^{pro} protease can fully protect swine and cattle [58, 62, 63]. Protection was also achieved in animal models with this FMDV antigen formulation expressed in an RDCaAd2 vector instead of the “traditional” RDHuAd5 vector [56], highlighting the efficacy of this antigen construct. The choice of antigen for vaccination should, therefore, be based on the knowledge of host-pathogen interactions and the characterization of the protective immunity that arises during infection.

Typically, RDAd are very effective at triggering cell-mediated immunity, since transduction allows for prolonged presentation of intracellular antigen encoded by the transgene. This can be very useful for vaccine design, and inclusion of genes targeted by cell-mediated immunity could improve immunogenicity [57, 64]. Cell-mediated immunity can target epitopes encoded by conserved genes and thereby recognize infected cells independently of the virus serotype [65]. This could potentially provide some degree of protection against heterologous serotypes [66] in diseases like FMDV, IAV, or BTV in which cross-protection between serotypes is very limited. Inclusion of immunogenic antigens for cell immunity will likely improve RDAd vaccine efficacy.

RDAd vector expressing antigens are nonetheless fully protective in only few cases. Ideally, a veterinary vaccine should consist of a single-dose immunization that provides long-term protection so that costs are maintained low. Some RDAd vaccines can achieve this [58, 66], but experimental vaccination protocols often employ prime-boost strategies for RDAd vaccines to trigger protective immunity. In some cases, prime-boost strategies appear necessary to RDAd vaccine activity [67]. Administration route can also affect RDAd vaccine efficacy [68], and induction of mucosal immunity can be limited. Oral/nasal RDAd administration can nonetheless trigger the mucosal immunity necessary for protection against influenza for instance [66, 69]. RDAd administration route should, therefore, be given careful attention when designing vaccination protocol.

4.2. Immunomodulation through RDAd vectors

Enhancing the immunogenicity of RDAd vaccine candidates so that efficacy is improved is a continuous goal for researchers. This could be achieved through addition of external adjuvant [70], or by making the adenoviral vector encode for immunomodulatory molecules that would favor immune response to the antigen (**Table 1**).

The antiviral activity of the IFN system is well documented [71]. IFNs induce an antiviral state in cells that help the host control viral infections. Systemic administration of recombinant IFNs is nonetheless toxic and too expensive for veterinary medicine. As an alternative, inclusion of IFNs as RDAd transgenes could boost vaccine efficacy and/or provide early protection when highly contagious virus outbreaks occur. Recombinant expression of IFN- α with FMDV VP1 protein or epitopes enhanced the RDAd vaccine activity [61]. IFN-expressing RDAd have nonetheless shown their potential as antiviral agents when administered on their own. RDAd expressing porcine IFN- α can protect against multiple FMDV serotypes [72] and work synergistically with IFN- γ to protect against FMDV challenge [73]. Ovine IFN- τ expression in RDAd demonstrated antiviral efficacy in influenza virus murine model [74]. This ruminant IFN displays many of the antiviral activities of IFN- α in a wide range of mammalian hosts but with reduced toxicity [75]. IFN-expressing RDAd have, therefore, the potential to be used as

off-the-shelf antiviral agents in the early stages of an outbreak in a disease-free country that could control disease spread for highly contagious viral pathogens like FMDV. They can also help bridge the gap in immunity in naïve herds, while the adaptive immune response to the vaccine is being triggered. Cytokine expression by RDAd could also have applications for the treatment of bacterial infections, since for instance, RDAd-expressed porcine G-CSF was successful at reducing *Salmonella* shedding and colonization in challenged pigs [76].

4.3. RNA interference of viral replication and enhanced antigen presentation

RNA interference can be an effective mean to impair viral replication [77], and its delivery through an RDAd vector could be attractive to treat some viral diseases. Expression of small hairpin RNAs specific for the FMDV 3D polymerase and the structural 1D protein could partially protect pigs against challenge [78]. RDAd delivering small interfering RNA, IFN- α , and IFN- γ enhanced anti-FMDV effects and was effective against multiple FMDV serotypes [79]. RNA interference delivered by RDAd could, therefore, be used as a fast-acting antiviral. This strategy could complement the efficacy of IFN-expressing RDAd, as these antiviral effects act through different pathways.

Antigen expression on RDAd can be engineered to promote antigen presentation. This has been achieved for instance by linking the antigen to the invariant chain to promote antigen presentation and thus enhances cell-mediated immunity [80]. Inclusion of GM-CSF or CD40L expression in the RDAd vectors probably favors antigen presentation and improves vaccine effectiveness [81]. Antigen delivery can also be improved by expressing the antigen of interest linked to heat shock proteins. Expression of the HSP70 C-terminal gene linked to the hantavirus glycoprotein Gn can augment cellular and humoral immunity and protects mice from a virulent challenge [82]. Co-expression of HSP70 and PRRSV gp3 and gp5 glycoproteins in an RDAd vector also enhances immunity to the antigens and improves vaccine efficacy [83]. Strategies that boost transgene antigen presentation can, therefore, become a valuable tool to improve RDAd vaccine immunogenicity.

5. Safeties and risks of adenoviral vectors

Different issues such as the oncogenic or mutagenic risk of the modified vector, its origin, its tropism, or its pathogenicity are some of the potential concerns around adenoviral vector use, not only for the host but also for the environment [84]. Adenoviral vectors are classified as Risk Group 2 (RG2) agents, defined as pathogens causing infrequent serious human diseases with available prevention therapies. This group of agents has to be manipulated in a biosafety level 2 containment facility (BSL2) [85]. Gloves, eye, nose, and mouth protection and laboratory coat are required to prevent mucous membrane contact, inhalation of aerosolized droplets, ingestion, or parenteral inoculation.

Ad cause usually mild illnesses, except in immunocompromised individuals. Potential toxicity is documented *in vitro* and in *in vivo* mouse models for the first-generation RDAds, which contain a great proportion of the Ad genome [86]. These vectors also have the risk of reversion to

replication competence because of recombination or complementation between the left terminus end of the vector and the partially overlapping E1 sequence present in HEK293 cell genome or in adenoviral sequences previously acquired by the host (due to the general distribution of the Ad) [87]. Packaging cell lines with nonhomologous sequences with the vector or testing viral vector stocks for RC virus can be employed to reduce this risk [88]. Deletion of the E2A, E2B or E4 regions in the second-generation vectors reduces this risk but complicates the packaging of the recombinant adenoviral particle since specific packaging cells have to be designed to complement the missing adenoviral genome. Obtaining high titer stocks with these systems is more difficult, which often leads to reduced immunogenicity as only lower vaccine doses can be obtained [89]. These issues are even more pronounced with “gutless” adenoviral vectors, which are perfect in terms of safety, but can be problematic in terms of immunogenicity and ease of production.

The route of administration is also relevant in RDAd shedding. Intravenous (or systemic) administration results predominantly in liver adenovirus localization with minimal or no shedding to biologic fluids [6, 90]. When administered subcutaneously or intramuscularly, the point of inoculation should be disinfected to minimize the risk of vector propagation to the environment as leaks can sometimes be detected at the site. Nonetheless, no vector was detected in rodents 72 h after injection in tail swab, and the vectors were cleared from blood within 24 h [90]. As previously mentioned, RDAd vectors do not integrate efficiently into the host cell genome, the transgene expression is only transient and they do not produce infective particles, which inherently improves their biosafety [8]. Adenoviral vector survival in bedding or caging is also reduced compared to parent Ad [8]. Adenoviral vectors can, however, trigger episodes of inflammatory responses. This includes one death after a high dose direct injection of an adenoviral vector into the hepatic artery [91], which produced a fulminant immune reaction probably due to pre-existing vector immunity. It is, however, very difficult to detect vertical or germline transmission of adenovirus vectors in experimental animal models [92].

All measures (autoclave treatments for 30 min at 121°C under 1 atm pressure, 0.5% sodium hypochlorite, 5% phenol, or 2% glutaraldehyde) sufficient to eliminate the peril of adenoviral transmission have to be met to minimize risks (alcohol is not a good decontaminant for Ad), but we must not forget that RDAd do not replicate and should not, unless recombination and complementation occur, be able to shed from inoculated animals, and are thus even less likely to infect another organism. In the case of an RCAd, the risk is reduced to the range and tropism of the Ad; for example, human adenovirus is only known to replicate in two nonhuman species: cotton rat and hamster [93].

It is necessary to deepen in the knowledge of the biodistribution, dissemination, and *in vivo* transgene expression duration of these vectors in veterinary medicine to assess their risk more thoroughly. No standard procedures to monitor these risks exist, and thus, each independent study analyses arbitrarily which biosafety parameters are evaluated.

6. Conclusions and perspectives

In the increasingly globalized world in which we live, animal health is of great importance and the prevention of animal diseases through vaccination is necessary for animal care, food production, food safety, food security, prevention of zoonotic and foodborne infections, reduction of

antibiotic needs, and public health. That vaccination is an integral part of global disease prevention, which can even eradicate diseases is a fact. We have examples of this in both human and animal health with the eradication of smallpox and rinderpest. However, there are still many animal diseases without vaccines or for which treatment needs improvement. Numerous studies constructing, testing, characterizing, optimizing, and identifying adenoviral-based vaccines as optimal against different animal diseases appeared in the last decades. They elicit potent cellular and humoral immunity and can be implemented along DIVA diagnostic tests. RDAd can also be used to deliver immunomodulation to improve disease treatment. Transference to the veterinary market is, however, lagging behind laboratory advances, and no adenoviral vector-based vaccine has yet obtained a veterinary license for systematic use in the field. This nonetheless appears nowadays closer with the recent publication of a positive safety report on an RDHuAd5 FMDV vaccine [43]. Recombinant RDAd reagents could, therefore, have great economic relevance in the future in veterinary medicine. Regulatory committees both in the EU and in the US should favor the approval of these reagents, based on the increasing scientific evidence for their efficacy and safety so that recombinant RDAd can make the leap from laboratory to the field. At the moment, the regulatory bases (EMEA/CVMP/004/04) for the use of adenoviral vector-based vaccines in farms are not well defined, although there are bases established in the EU by the European Medicine Agency (EMA) and its Committee for Veterinary medicinal Products (CVMP) and in the US by the Animal and Plant Health Inspection Service (APHIS) from The United States Department of Agriculture (USDA). A global cooperation between the veterinary industry and governments is needed in the future for adenoviral vector-based vaccines to reach the market.

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Conflict of interest

The authors declare no conflict of interest.

Abbreviations

Ab	antibody
Ad	adenovirus
APHIS	animal and plant health inspection service
atm	atmosphere

BTV	bluetongue virus
Bo	bovine
BSL2	biosafety level 2 containment facility
°C	centigrade
Ca	canine
CDV	canine distemper virus
CMI	cell-mediated immunity
Ch	chimpanzee
CSFV	classical swine fever
CVMP	committee for veterinary medical products
DC	dendritic cells
DNA	deoxyribonucleic acid
DIVA	differentiating infected from vaccinated animals
EMA	European medical agency
EU	European Union
FMDV	foot and mouth disease
Fo	fowl
F	fusion protein
Gn, Gc	glycoproteins
GM-CSF	granulocyte macrophage colony-stimulating factor
HEK	human embryonic kidney
H-HA	hemagglutinin
HV	herpes virus
HSP	heat shock protein
HuAd	human adenovirus
IAV	influenza A virus
IFN	interferon
IL	interleukin
IPS-1	interferon-beta promoter stimulator-1
LRR	leucine-rich-repeat
MIP	macrophage inflammatory protein
MyD88	myeloid differentiation protein 88

N	nucleoprotein
NACHT	neuronal apoptosis inhibitor protein (NAIP), class 2 transcription activator of the MHC (C2TA), heterokaryon incompatibility (HET-E) and telomerase-associated protein 1 (TP1)
NALP3	NACHT, LRR, and PYD domains-containing
NIH	National Institutes of health
NLR	nucleotide-binding oligomerization domain-like receptor
Ov	ovine
PCV	porcine circovirus
PYD	"PYRIN domain," after the <i>pyrin</i> proteins
PPR	peste des petits ruminants
PPRV	peste des petits ruminants virus
Po	porcine
PRRSV	porcine reproductive and respiratory syndrome virus
RHDV	rabbit hemorrhagic disease virus
RD	replication-defective
RC	replication-competent
RVF	Rift Valley fever
RVFV	Rift Valley fever virus
RG2	Risk Group 2
TP	terminal protein
TLR	toll-like receptor
US	United States
USDA	United States Department of Agriculture
VNA	virus neutralizing antibodies
OIE	World Organization for Animal Health

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