

## CONFERENCE PAPER

# Use of adenoviral vectors as veterinary vaccines

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Vaccines are the most effective and inexpensive prophylactic tool in veterinary medicine. Ideally, vaccines should induce a lifelong protective immunity against the target pathogen while not causing clinical or pathological signs of diseases in the vaccinated animals. However, such ideal vaccines are rare in the veterinary field. Many vaccines are either of limited effectiveness or have harmful side effects. In addition, there are still severe diseases with no effective vaccines. A very important criterion for an ideal vaccine in veterinary medicine is low cost; this is especially important in developing countries and even more so for poultry vaccination, where vaccines must sell for a few cents a dose. Traditional approaches include inactivated vaccines, attenuated live vaccines and subunit vaccines. Recently, genetic engineering has been applied to design new, improved vaccines.

Adenovirus vectors are highly efficient for gene transfer in a broad spectrum of cell types and species. Moreover, adenoviruses often induce humoral, mucosal and cellular immune responses to antigens encoded by the inserted foreign genes. Thus, adenoviruses have become a vector of choice for delivery and expression of foreign proteins for vaccination. Consequently, the market requirements for adenovirus vaccines are increasing, creating a need for production methodologies of concentrated vectors with warranted purity and efficacy. This review summarizes recent developments and approaches of adenovirus production and purification as the application of these vectors, including successes and failures in clinical applications to date.

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## Introduction

The history of veterinary vaccine development starts with the well-known story of Louis Pasteur and his rabbit spinal cord vaccine and continues to this day with the demonstration of protection in animals by rabies virus reverse transcriptase DNA plasmid vaccination.<sup>1</sup> In between, in 1947, Frenkel used suspensions of the epithelium obtained from the tongues of recently slaughtered healthy cattle that were maintained *in vitro* and subsequently infected in a manner similar to that used today with baby hamster kidney cells to produce foot and mouth disease virus (FMDV). The Frenkel procedure became the corner stone of vaccine production for many years and paved the way for modern biotechnology as we know it.<sup>2</sup> More recently, recombinant pox viruses have been generated for vaccination against heterologous pathogens, using vaccinia-vectors, expressing the rabies virus glycoprotein and Newcastle disease virus fusion and hemagglutinin (HA) glycoproteins, the first applications of genetically engineered vaccines.<sup>3</sup>

Traditional vaccination involves the use of inactivated, live-attenuated or subunit vaccines. Inactivated, or 'killed', vaccines consist of treated microorganisms that are unable to replicate; however, they do not elicit protein production in the cytosol and hence viral

antigens cannot be presented by MHC class I molecules, thus cytotoxic CD8 T cells are not generated. Live-attenuated vaccines are generally far more potent educing a greater number of relevant effector mechanisms, including cytotoxic CD8 T cells. Nevertheless, these vaccines sometimes have residual pathogenicity,<sup>4</sup> or a pathogenic virus strain may re-emerge by a further series of mutations.<sup>5</sup> Subunit vaccines would be as effective as live whole organisms, inherently safer than vaccines based on whole organisms, but they are not strongly immunogenic, being particularly difficult to obtain MHC class I specific responses.<sup>6–8</sup> The development of vaccines therefore remains an important goal of immunology. Moreover, there remains a need for further vaccines that can reduce the economic impact of disease in production animals.

The ideal vaccine should be 100% efficacious in preventing infection, although this is totally unrealistic. Therefore, the 'realistic' vaccine should provide greater than 90% efficacy in disease prevention within a few days up to a couple of weeks of a single administration. Furthermore, in order to reduce the cost of regular reimmunizations and ensure receipt of a complete vaccination schedule, this protection should be of long duration, and should use a minimal number of doses. Additionally, an 'ideal' vaccine would also stimulate mucosal immunity, since the majority of viruses enter via mucosal surfaces; thus, the 'perfect' vaccine should be designed to be delivered by mucosal routes, that is, intranasal or oral delivery is preferable. Vaccines also

need to be safe and not cause any adverse side reaction, such as immunosuppression or interference with immunity to other vaccines given simultaneously. Finally, the vaccines need to be both genetically and thermally stable. Genetic stability must be present to ensure the absence of reversion of the live vaccine to the virulent organism which might cause disease; and thermal stability is critical since the maintenance of the cold chain is not always guaranteed from manufacturing to delivery. A very important desired characteristic is to have vaccines that can be delivered to animals at a very young age and stimulate immunity in the presence of innate, passive immunity.<sup>9,10</sup> Another very important criterion for an ideal vaccine in veterinary medicine is low cost; this is especially important in developing countries and for poultry vaccines, in which the vaccines must sell for a few cents a dose. Unfortunately, such ideal vaccines are rare in the veterinary field; additionally, there are still severe diseases with no effective vaccines.

The history of heterologous gene expression in adenovirus (Ad) goes back to the discovery of simian virus 40 (SV40) contamination in the 1950s of inactivated Ad strains 1–5 and 7 vaccines during adaptation to growth in rhesus monkey kidney cells; it was observed that the SV40 T-antigen occasionally incorporated into the Ad genome, which led to the realization that Ad could be used to express heterologous genes.<sup>11</sup> Then recognition that purified replication-defective Ads could be propagated on 293 cells without helper viruses paved the way toward intentional production of genetically modified Ads.<sup>12</sup> The popularity of Ad as a recombinant viral vector is largely due to the successful and safe immunization of millions of US military recruits in 1971 with enterically coated Ad4 and Ad7 as a prevention against acute respiratory disease (ARD) outbreaks.<sup>13</sup> Following these first trials, a number of recombinant Ad (rAd) have recently been constructed and tested not only for humans but also for veterinary vaccination.<sup>6,14</sup>

Ads are highly efficacious vaccine carriers with strong immunogenicity. Although the ability of Ad vectors to elicit antigen-specific CD8 and CD4 T cells is well described, little is known about the kinetics or nature of the immune response following Ad immunization.<sup>15,16</sup> However, Yang *et al*<sup>17</sup> observed that Ad vectors have (i) the ability to deliver large amounts of antigen into the lymphoid tissues, (ii) the ability to induce rapid expansion and migration of CD8 T cells throughout the lymphatic system, and (iii) the ability to produce a sustained, high-level CD8 T-cell response, that may explain the strong immunogenicity of these vectors.

The use of rAd as a veterinary vaccine has many advantages: (i) they can infect a wide variety of dividing or nondividing cells; (ii) Ad infections are ubiquitous and are normally without significant or severe clinical symptoms;<sup>18</sup> (iii) they can be administered orally;<sup>19</sup> (iv) Ads have their genome well characterized;<sup>14,20</sup> (v) they can accommodate up to 36 kb of foreign genetic material;<sup>21</sup> (vi) their genome rarely integrates into the host chromosome;<sup>22</sup> (vii) techniques are well established for the construction of rAd vectors;<sup>23</sup> and (viii) they have the ability to replicate at high titers in complementing cell lines.<sup>24</sup>

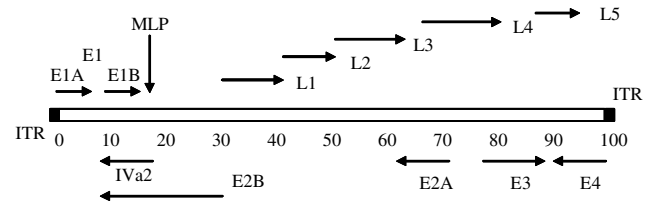


Figure 1 Schematic representation of the adenoviral genome organization.

## Ad biology

Ad is a non-enveloped, icosahedral virus of 60–90 nm with a linear duplex DNA genome of about 36 kb. The genome is divided into early (E) and late (L) genes, expressed, respectively, before and after replication of the viral chromosome (Figure 1). E1 gene products are involved in the control of viral gene transcription, shut-off of cellular proteins and cellular transformation. The E2 gene codes for proteins involved in viral replication, including a DNA-binding protein involved in DNA elongation (E2A) and a DNA polymerase (E2B); E3 gene, dispensable for Ad replication, codes for proteins that interfere with the host immune response against virus infection; finally, the E4 genes are involved in the transition from early to late gene expression, the shut-off of host-cell gene expression, the viral replication and the assembly of the virion (see Russell<sup>18</sup> and Imler<sup>20</sup> reviews). First-generation vectors are usually deleted in their E1 and E3 regions; however, when production is performed on 293 cells, recombination between the left terminus of first-generation Ad vector and partially overlapping E1 sequences in the cellular genome may result in the generation of E1-positive, replication-competent Ad (RCA),<sup>25</sup> a serious safety concern if nonreplicating vectors are desired. Second-generation vectors are replicative-defective Ad, which are further deleted in E2A, E2B or E4, showing reduced immunogenicity and RCA generation, but engineering of stable cell lines that complement these vectors can be cumbersome and lead to poor cell growth and viral titers.<sup>26</sup>

rAd vectors have been developed either as replication-competent, with the expression cassette for the foreign antigen in place of the E3 genes, or as replication-defective viruses, when the antigen expression cassette replaces the E1 genes.<sup>20</sup> Despite the fact that it would be preferable to work with replication-defective virus, different studies have supported the use of RCAs in veterinary vaccination, since the ability of the replication-defective form to induce mucosal immunity and to protect against a respiratory challenge, following immunization, was reported to be limited.<sup>27,28</sup> Moreover, RCA-based vaccines were known to have the potentiality to override maternal-derived immunity.<sup>28,29</sup> Additionally, cattle vaccination with both rAds against herpesvirus-1 showed that the use of the replication-defective form induced lower titers of antibody than the replication-competent form in intratracheal/subcutaneous immunization.<sup>30</sup>

## Species-specific platforms

Currently, human rAd represents one of the most efficient vector systems for delivery of vaccine antigens.

However, the use of human Ad (HAD) as a vaccine delivery system in non-human animals is still limited. Since non-HADs are species specific, the development of animal-specific Ad as a vaccine delivery system would be a logical choice. Thus, Ads isolated from species other than man have generated increasing interest, in particular:

- (i) canine Ad (CAD), which has the ability to replicate and persist locally in the upper respiratory tract of puppies in the face of maternal-derived antibodies when administered via the intranasal route and use both dependent and independent coxsackievirus group B and Ad receptor (CAR) pathways to enter the cells,<sup>28,31</sup>
- (ii) avian Ad, the subject of numerous investigations, has a number of features that make them attractive to the poultry industry: ease of propagation, ease of administration (water, aerosol or injection) and a large range of serotypes that vary in virulence. Chicken embryo lethal orphan (CELO), classified as the type 1 fowl Ad (FAd), is the most studied vector. This virus, like other typical avian Ad, has a much larger genome than mammalian Ad. Despite its large scatter, it has never been associated with serious disease or economical losses in the poultry industry. It can be isolated from healthy chickens and does not induce clinical signs when experimentally inoculated in chickens. Also this virus use both dependent and independent CAR pathways to enter the cells,<sup>31,32</sup>
- (iii) porcine Ad (PAd), which, depending upon serotype, can be grown in the pig kidney, testis, retina, thyroid cells, human kidney, canine melanoma and calf kidney cells, do not require adjuvants for the induction of proper immune responses. Infection is generally sub clinical causing no adverse effects in producing pigs, with PAd also isolated from apparently healthy pigs. PAd is species specific having only been isolated from swine, reducing the possibility of its spread to other animal or man following administration. PAd could induce both systemic and mucosal antibody responses;<sup>33,34</sup> and
- (iv) bovine Ad serotype 3 (BAd3), which, like PAd, do not require adjuvants for the induction of proper immune response, and has been used due to its lack of virulence. Moreover, experimental infections of calves with BAd3 failed to produce either clinical signs or gross lesions, but all animals seroconverted. The cell entry pathway of BAd3 is not well understood.<sup>30,35</sup>

## Applications

### *Adenovirus vaccination in companion animals*

**Feline immunodeficiency virus.** The infection of cats with feline immunodeficiency virus (FIV) results in an immunosuppressive disease that is transmitted by blood and saliva. FIV invades and destroys the monocyte/macrophage system and infects B cells.<sup>36</sup> Different approaches have been made trying to immunize cats against FIV; so far, no effective vaccine has been made. Gonin *et al.*<sup>37</sup> 10 years ago, constructed a replication-defective HAd5, containing the envelope protein ENV

gene of FIV; however, despite the fact that an antibody response to pseudorabies virus in cats showed the potential of rHAd5 vectors to be used in this species, cats injected with  $10^{10.8}$ – $10^{11.8}$  of 50% tissue culture infectious dose (TCID<sub>50</sub>) adjuvanted with montanide ISA 708 (water in nonmineral oil) or with montanide ISA 206 (double water/mineral oil/water) of this rAd did not develop detectable antibody response against ENV. Moreover, it was observed that even if high titers of antibodies against ENV products are induced, they could still be insufficient for protection.<sup>37</sup> Since then, no further work has apparently been performed concerning the use of Ad vectors as a potential vaccine against FIV.

**Canine distemper virus.** Canine distemper virus (CDV) induces fatal diseases including encephalitis with demyelination, diarrhea and respiratory disorders in dogs. Although conventional live modified vaccines are commercially available and widely used in the field, their efficacy is limited in the presence of maternally derived antibodies.<sup>38</sup> Thus, a new and improved CDV vaccine, which could overcome this limitation, would constitute a significant improvement. The construction and characterization of the first replication-competent rCAD2 started recently. The genes that code for CDV HA<sup>28,39</sup> or fusion (F)<sup>28</sup> proteins were inserted in two CAD2s and used as a candidate vaccine in puppies. It was reported that intranasal vaccination with a mixture containing  $10^{5.8}$  TCID<sub>50</sub> of which rCAD2 provided an excellent level of protection in seronegative puppies, inducing almost complete protection. In contrast, intranasal immunization of puppies born to CDV and CAD2 immune dams failed to activate specific and protective immune responses. However, when the same puppies were vaccinated subcutaneously, significant seroconversion and solid protective immunity were triggered. Furthermore, a significant priming of memory responses was evidenced immediately after challenge, this constituting an efficient strategy to overcome both passive and active Ad-specific immunity in the dog.

**Rabies virus.** Rabies still presents a health threat not only to humans but also to dogs and cats, dog being the only important vector for humans, especially in less developed nations where uncontrolled canine rabies often is endemic. A replication-competent and -defective HAd5 expressing a rabies glycoprotein (RG) has been developed, inducing immunity to rabies in rodent, canine, foxes and skunk when given by intramuscular, subcutaneous or intranasal routes with a dose of  $10^8$  TCID<sub>50</sub>/animal. However, oral immunization failed to induce a measurable antibody response to rabies virus (RV) using the same dose.<sup>40,41</sup> Dogs previously vaccinated with commercially available vaccines, immunized with  $10^7$  plaque forming unit (PFU)/animal of a replication-defective HAd5 expressing the RG, have developed higher titers of viral neutralizing antibodies against RV 10 days after vaccination when compared with conventional vaccines under similar conditions. Moreover, the immunization of dogs with the commercial non-Ad vaccines is required yearly or, at best, every 3 years, depending on the type of vaccine. On the other hand, the higher antibody titers obtained with Ad vaccines against RV would reduce the frequency of dog immunization, reducing the costs for pet owners.<sup>42</sup> One

important advantage of this recombinant vaccine is the fact that the immune response to the RG was shown not to be impaired by maternal immunity; thus, this Ad vaccine is highly suitable for neonatal immunization.<sup>43</sup>

#### *Adenovirus vaccination in poultry*

**Avian infectious bronchitis virus.** Infectious bronchitis virus (IBV) is an highly contagious pathogen of poultry causing significant morbidity and mortality. Depending on the strain, IBV can target the respiratory tract, kidney and oviducts, and result in nephritis and reduced egg production. In addition, more than 20 IBV serotypes have been identified worldwide and new serotypic variants have been identified as a result of the widespread use of live attenuated vaccines. Different approaches have been developed in order to generate a more efficacious vaccine against IBV. The expression of S1, a glycoprotein involved in the attachment of cellular receptors, by a vaccinia virus was able to induce virus-neutralizing antibodies to IBV when delivered to mice; however, multiple injections are required to achieve a reasonable degree of protection. Recently, a FAd expressing the S1 of IBV has been developed.<sup>44</sup> A single dose of  $10^6$  TCID<sub>50</sub>/animal was shown to be sufficient to obtain complete protection of chickens at the trachea, the primary site of infection by IBV. Moreover, even in the face of FAV maternal antibodies, a high level of protection was achieved.<sup>44</sup>

**Infectious bursal disease virus.** Infectious bursal disease virus (IBDV) induces an immunosuppressive disease of chickens by destruction of the B lymphocytes. Current vaccination alternatives consist of either live virus or inactivated oil-emulsion vaccines, which induce serum antibody production in breeding hens after natural exposure; they are transferred to the progeny chicks via the yolk sac providing protection for the first critical weeks after hatching.<sup>45</sup> The construction of an rFAd10 containing the VP2 gene from IBDV has been described.<sup>32</sup> This recombinant vaccine was shown to induce an immune response in chickens to VP2 after vaccination with  $10^7$  PFU/animal. Moreover, after challenge with IBDV, intravenously, intraperitoneally, subcutaneously or intramuscularly, vaccinated chickens were protected, although no protection was observed in conjunctival sac vaccinated chickens.<sup>32</sup>

#### *Adenovirus vaccination in swine*

**Classical swine fever virus.** Classical swine fever (CSF), also known as hog cholera, is a serious and contagious viral disease of pigs with a high mortality rate, difficult to control in areas of high pig or wild boar densities, being the most economically important disease of swine in areas of intensive pig farming. For this reason, CSF is included in the A list of infectious diseases of the highest importance for international trade. Thus, it is highly relevant to develop efficacious vaccines for the control of CSF virus (CSFV) in domestic pigs and in wild boar. Prophylactic vaccination is still carried out in many parts of the world.<sup>46</sup> New vaccine developments have included a number of different strategies for delivering the major envelope glycoprotein, E2, against which most neutralizing antibodies are directed.<sup>47</sup> Hammond *et al*,<sup>48</sup> constructed the first rPAd expressing the E2 protein and

showed that a single dose of  $10^7$  TCID<sub>50</sub>/animal in tissue culture supernatant, when administered subcutaneously, is sufficient to completely protect pigs against subcutaneous challenge. Later, the same authors showed that when the challenge is administered orally, only 60% of the animals were protected;<sup>49</sup> more, recently, it was shown that pigs given two oral doses of  $10^6$  TCID<sub>50</sub>/animal of rPAd were completely protected from the disease.<sup>50</sup>

**Pseudorabies virus.** Pseudorabies virus (PrV) is an alpha herpesvirus, which causes the economically important and widespread Aujeszky's disease (AJD) in pigs. PrV is a highly neurotropic virus causing nervous and respiratory complications in pigs, the natural host, and in a variety of other animal species. Vaccination against AJD is widely practised with live attenuated or killed whole virus vaccines. However, neonatal immunization is often limited in the presence of maternally derived antibody, which inhibits the immune response against both vaccines.<sup>51</sup> Recently, the use of rAd vaccines carrying individual PrV genes were constructed as a safe alternative. Glycoproteins gD, gB and gC of PrV were chosen on the basis of their role in eliciting a protective immune response against virus infection. It was shown that piglets vaccinated intramuscularly with  $10^{8.6}$ – $10^{9.6}$  TCID<sub>50</sub>/animal 1 day after birth, with replication-competent HAd5 harbouring these three genes, developed similar neutralizing antibody responses independently of the presence or absence of maternal antibodies and were partially protected against challenge 16 weeks later.<sup>52</sup> For pigs that are slaughtered a few months after birth, one-shot vaccination at birth could provide protection of sufficient duration. Finally, two doses of 2 ml of clarified tissue culture supernatant containing  $10^{5.4}$  TCID<sub>50</sub> of rPAd expressing the PrV gD gene were administered subcutaneously and showed to protect pigs after challenge. Postmortem, gross lesions of pneumonia were found in the lungs of pigs given a single dose of vaccine; the lungs of pigs given two doses were free from disease.<sup>33</sup>

**Foot and mouth disease virus.** Foot and mouth disease (FMD) is a severe, clinically acute, vesicular disease of cloven-hoofed animals including domesticated ruminants, pigs and more than 70 wildlife species. Pigs are recognized as a significant factor in the spread of the disease since a single pig releases as much aerosol virus as 3000 cattle in a short period of time.<sup>53,54</sup> The economic and social impact of FMD can be catastrophic when an outbreak occurs in FMD-free countries populated with immunologically naive animals. The current vaccine is a chemically inactivated preparation of concentrated infected cell culture supernatant. However, FMD-free countries generally prohibit its use because of the lack of an approved diagnostic test that can reliably distinguish vaccinated from infected animals. Moreover, current vaccines can induce a protective response only after approximately 7 days post vaccination; this is a critical issue in disease-free countries, where, in the case of FMD outbreaks, a rapid control in preventing the spread of the disease is crucial (see Doel<sup>2</sup> for a review). Thus, there is a need to develop disease control strategies relying on more rapidly induced protection. Mayer *et al*<sup>55</sup> developed a replication-defective HAd5 vector contain-

ing the capsid polypeptide P1 and the viral 3C protease coding regions, necessary for processing P1 to the capsid proteins VP0, VP3 and VP1, from the FMDV strain A12. They observed that vaccinated swine with  $1 \times 10^8$  PFU/animal in PBS developed antibodies against FMDV structural proteins and an FMDV-specific neutralizing antibody response, which seems to increase slightly by boosting the swine with a second inoculation of  $5 \times 10^8$  PFU/animal in PBS at 4 weeks post initial vaccination.<sup>56</sup> This vaccination protocol was shown to offer a significant degree of protection to the pigs, as five of six pigs were completely protected, while the remaining animal had significantly reduced signs of disease. Later, Moraes *et al*<sup>57</sup> showed that a single dose of  $5 \times 10^9$  PFU/animal in PBS of a replication-defective HA5 expressing the P1 coding region of FMDV strain A24 completely protected pigs against homologous challenge 7, 14 or 42 days after vaccination. Since efficacious vaccination is strain dependent and the infection with one serotype does not confer protection against another, HA5 bicistronic vector vaccines have been developed,<sup>58</sup> decreasing the cost for multivalent adenoviral FMD vaccines. For the construction of these vectors, the P1 capsid coding region for both A24 and O1 strains and the 3C protease coding region of A12 strain were used. However, the neutralizing antibody response after vaccination with  $2.5 \times 10^9$  PFU/animal in PBS of the bicistronic vector was considerably lower than that induced by a commercial FMD vaccine or the monovalent Ad-A24 vaccine.<sup>57</sup> Recently, a new strategy based on the fact that FMDV is highly sensitive to alpha/beta interferon (IFN- $\alpha/\beta$ ) have been developed using a replication-defective HA5.<sup>59,60</sup> This strategy has been shown to completely protect pigs after vaccination with  $10^9$  PFU/animal when challenged 24 h later with virulent FMDV.

**Porcine respiratory and reproductive syndrome virus.** Porcine respiratory and reproductive syndrome virus (PRRSV) is the causative agent of an economically important pig disease, with a worldwide distribution, characterized by reproductive failure in sows and respiratory problems in unweaned and growing pigs. Swine macrophage is the only cell type known to support PRRSV replication, making commercial production impossible. Direct contact between infected and naive pigs is the predominant route of PRRSV transmission. Moreover, pneumonia caused by PRRSV infection is more severe in young pigs compared to adults and may be complicated by concurrent bacterial infection.<sup>61</sup> Gonin *et al*<sup>62</sup> observed that PRRSV-infected pigs present circulating antibodies responsible for viral neutralization mainly directed against GP<sub>5</sub>, an envelope protein. Gagnon *et al*<sup>63</sup> constructed a replication-defective HA5 expressing the GP<sub>5</sub> protein and used this recombinant virus to immunize pigs using two intradermal injections with  $5 \times 10^8$ – $1 \times 10^9$  PFU/animal in a mixture containing 100  $\mu$ l of PBS and 100  $\mu$ l of poloxamer SP1017 at 0.02%. It was observed that following challenge given intranasally 14 days after the booster, pigs produced high antibody titers to GP<sub>5</sub> protein. Moreover, vaccinated pigs presented specific immune memory which, following a subsequent PRRSV infection, resulted in a rapid clonal expansion of memory cells to the neutralizing epitopes of the authentic viral GP<sub>5</sub> protein.

**Transmissible gastroenteritis (Corona) virus.** Transmissible gastroenteritis (Corona) virus (TGEV) infects the enteric and respiratory tissues of newborn piglets resulting in mortalities approaching 100%. The virus infects epithelial cells and, in some cases, lung macrophages.<sup>64</sup> There are several commercially available TGEV vaccines, both inactivated and attenuated; these do not fully protect piglets. Several attempts have been made to develop efficacious recombinant TGEV vaccines.<sup>65</sup> The spike protein (S) was identified as the major inducer of TGEV-neutralizing antibodies and it mediates binding of TGEV to its cellular receptor.<sup>66</sup> Thus, HA5 expressing the S protein was constructed and used to study the induction of antibodies providing protection in swine.<sup>67</sup> It was observed that porcine serum, elicited by  $10^9$  PFU/animal in PBS of this recombinant, when mixed with a lethal dose of virus prior to administration to susceptible pigs, prevented the replication of virulent TGEV administered orally as virus-antibody mixtures and fully protected swine from clinical signs and death. Moreover, the used dose did not produce any clinical symptoms in any of the more than 50 animals inoculated up to 10 weeks after inoculation,<sup>68</sup> suggesting that this vector can be used as a live vaccine in swine without secondary complications associated with the vector. However, a PAd vector could be more effective than HA5. Thus, Tuboly and Nagy<sup>65</sup> constructed a rPAd5 expressing the TGEV protein. It was observed that a single oral dose of  $5 \times 10^6$  PFU/animal of the recombinant virus was sufficient to induce both a systemic and a local humoral immune response.<sup>65</sup> Unfortunately, challenge experiments were not carried out.

**Swine influenza virus.** Swine influenza virus (SIV) is a widespread and important pathogen in species as diverse as poultry, swine, marine mammals and humans. In pigs, influenza can occur either as an enzootic problem in a herd or, more commonly, as explosive outbreaks of ARD. Although rarely fatal, swine influenza can be of substantial economic impact.<sup>69</sup> In addition, there is growing concern for the potential for synergistic infections with influenza and PRRSV. Beyond the impact of influenza for the swine industry, pigs are also very important in the global ecology of influenza A viruses in humans.<sup>70</sup> SIV vaccines that are commercially available are inactivated, whole-virus or subunit vaccines. While these vaccines may decrease the incidence and severity of clinical disease, they do not consistently provide complete protection from virus infection. Two replication-defective HA5 were developed as potential vaccines against SIV: rHA5 expressing the influenza virus H3 HA<sup>71,72</sup> inducing predominantly a subtype-specific humoral immune response;<sup>73</sup> and rHA5 expressing the nucleoprotein (NP),<sup>72</sup> a group-specific stimulating cytotoxic T lymphocytes for crossreactive immunity to all influenza A subtypes.<sup>74</sup> It was observed that the immunization of mice with  $5 \times 10^8$  TCID<sub>50</sub>/animal and pigs with  $2 \times 10^{10}$  TCID<sub>50</sub>/animal in PBS with the first recombinant, described above, developed high levels of virus-specific hemagglutination-inhibition antibody to SIV by 4 weeks post vaccination and the animals were partially protected. On the other hand, pigs vaccinated with  $2 \times 10^{10}$  TCID<sub>50</sub>/ml in PBS of both

recombinant viruses in a mixture were completely protected.

### *Adenovirus vaccination in cattle*

**Bovine viral diarrhoea virus.** Bovine viral diarrhoea virus (BVDV) is responsible for reduced milk production, reduced reproductive performance and growth retardation. In addition, acute infection in adult cattle, congenital defects and increased neonatal mortality are also clinical manifestations of BVDV infection.<sup>75</sup> Finally, it was reported that BVDV may play an indirect role in immunosuppression.<sup>76</sup> Currently, inactivated and modified-live vaccines are used; however, both types of vaccines have significant shortcomings. A rHAD5 expressing the nucleocapsid C protein (p14), which is highly conserved among many different pestiviruses, to which BVDV belongs, was constructed and shown to induce both humoral and cellular immune responses after vaccination with  $10^9$  PFU/animal, in a mouse model.<sup>77</sup> The E2 protein of BVDV, which is a major viral glycoprotein, was also used for mice immunization using the same vector and dose.<sup>78</sup> A strong humoral immune response was detected as the presence of a strong memory response to the E2 protein. Both strategies used an inducer/promoter allowing the generation of rAd in which the production of the transgene may be toxic and permitting the control of the transgene expression *in vitro* and *in vivo*. Finally, a rBAD3 expressing BVDV E2 protein was constructed and shown to induce BVDV E2-specific mucosal and systemic immune responses after intranasal immunization with  $10^7$  PFU/animal of cotton rats.<sup>79</sup> Despite the fact that mice immunized with recombinant fowl pox virus or with a DNA vaccine expressing E2 glycoprotein induced E2-specific immune responses, it was also observed that the neutralization titers were low.<sup>79</sup>

**Bovine parainfluenza virus type 3.** Bovine parainfluenza virus type 3 (bPIV3) has been isolated from normal cattle, cattle showing signs of respiratory disease and aborted fetuses. An intramammary inoculation of bPIV3 resulted in respiratory signs, fever and malaise. Moreover, the milk showed a color change, an increased pH and increased numbers of glandular epithelial cells, neutrophils, lymphocytes and monocytes.<sup>76</sup> Currently, multivalent vaccines containing killed bPIV3 are used. Breker-Klassen *et al*<sup>80</sup> constructed two rHAD5 encoding either the F or the HN protein of bPIV3. It was shown that intranasal vaccination with  $5 \times 10^7$  PFU/animal in PBS of cotton rats produced a strong serological response within 21 days, which increased with the second vaccination. Moreover, F and HN proteins produced by rHAD5 either alone or in combination were sufficient to induce a protective immune response.<sup>80</sup>

**Bovine herpes virus 1.** Bovine herpes virus 1 (BHV-1) causes a variety of diseases in cattle including infectious bovine rhinotracheitis, infectious pustular vulvo-vaginitis, conjunctivitis and occasionally meningo-encephalitis. Live attenuated and killed vaccines are partially effective in reducing the disease incidence and severity.<sup>76</sup> The limitations with the existing vaccines have encouraged the development of alternative, cost-effective vaccination strategies such as recombinant live viral vector-based

vaccines. Mittal *et al*<sup>81</sup> developed the first replication-competent and -defective HAd5 carrying the gD glycoprotein gene from the BHV-1 envelope. They showed that both forms induced immunity in cotton rats after vaccination with  $10^7$  PFU/animal in PBS and no infectious BHV-1 virions were isolated from the trachea of these rats after challenge. The same strategy was used with a rBAD3 and tested in its ability to induce mucosal and systemic immune response in cotton rats<sup>82</sup> and calves<sup>30</sup> with success. More recently, newborn lambs were immunized with  $2 \times 10^9$  PFU/animal of replication-defective HAd5 expressing the gD protein of BHV-1.<sup>83</sup> Both humoral and cell-mediated gD-specific mucosal immune responses were detected in 1–4-day-old lambs after a single immunization and these responses were qualitatively and quantitatively similar to those detected in 5–6 week-old lambs. Moreover, gD-specific maternal antibody did not significantly alter either mucosal or systemic gD-specific immune response. In addition, BHV-1 glycoprotein gC was also tested in its ability to induce protective immunity to BHV-1 in cattle by intranasal administration of  $10^{10}$  PFU/animal using a replication-defective HAd5.<sup>84</sup> However, it was observed that the highest BHV-1 neutralizing antibody titers were obtained with rHAD5 expressing the gD protein followed by the live vaccine.

**Rinderpest virus and peste des petits ruminants virus.** Because of their high mortality and high morbidity rates, rinderpest (RP) and peste des petits ruminants (PPR) are dreaded animal diseases that are drawing back the animal production in many developing countries (Africa and Asia). They affect both domestic and wild ruminants and belong to the list A of the Office International des Epizooties where highly contagious animal diseases with high economic importance are grouped. Currently, attenuated tissue culture vaccines used to control RP and PPR viruses have been successfully used for many years. Being safe and effective, they provide complete and lifelong protection with a single subcutaneous inoculation.<sup>85,86</sup> However, for vaccination programs, the oral route of administration would be more economical. Moreover, the parenteral way of administration means that the current vaccine cannot be used in wildlife. In our laboratory, the production purification, and encapsulation processes for a replication-defective HAd5 expressing two different morbillivirus capsids proteins, the responsible agent of both diseases, is in progress. The aim is the development of heat stable RP/PPR oral vaccines better adapted for mass vaccination in hot climate developing countries. Animal vaccination and challenge testes are ongoing with promising preliminary results.

In summary, according to the numerous reported studies, live human or non-human rAds, either infectious or not, appear to be attractive candidates for vaccination against several animal disease (Table 1). Success situations are clear against most pathogens reported and intermediate successes are apparent against CDV and RP virus (RPV)/PPR virus (PPRV), whereas vaccination against FIV have, thus far, yielded poor or inconclusive results. Nevertheless, this is not a comprehensive picture, since more than 50 viral agents of veterinary importance are known (Table 2).

**Table 1** Host and pathogens for which Ad vaccines have been tested in companion animal, poultry and livestock

Species	Pathogen	Protection	Reference(s)
Feline	FIV	No	37
Canine	CDV	Partial	28,39
	RV	Yes <sup>a</sup>	40–43
Chicken	IBV	Yes	44
	IBDV	Yes	32
Swine	CSFV	Yes	48–50
	PrV	Yes	33,52
	FMDV	Yes	55–60
	PRRSV	Yes	63
	TGEV	Yes	65,67,68
	SIV	Yes	71–73
Bovine	BVDV	Yes <sup>b</sup>	77,79,81
	bPIV3	Yes <sup>b</sup>	80
	BHV-1	Yes	30,81–84
	RPV/PPRV	Partial	Unpublished

<sup>a</sup>Obtained in mice.

<sup>b</sup>Obtained in cotton rats.

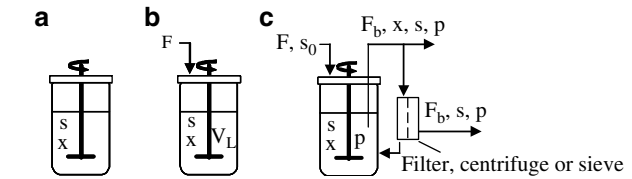
**Table 2** Over 50 viral agents are available in the veterinary vaccine market

Species	Pathogen
Bovine	Coronavirus; foot and mouth disease O, A, C; infectious rhinotracheitis; papillomavirus; parainfluenza 3; respiratory syncytial virus; rotavirus; bovine viral diarrhoea 1 and 2
Porcine	Encephalomyocarditis; influenza; parvovirus; pseudorabies; reproductive and respiratory syndrome; rotavirus; transmissible gastroenteritis; rabies
Equine	Eastern encephalitis; Venezuelan encephalitis; Western encephalitis; herpesvirus 1 and 4; influenza; rabies
Caprine/Ovine	Bluetongue; ecthyma (Orfvirus); rabies
Avian	Encephalomyelitis; fowl pox; hemorrhagic enteritis; infectious bronchitis; infectious bursal disease; laryngotracheitis; marble spleen disease; Marek's disease; newcastle disease; paramyxovirus; pigeon pox; reovirus; tenosynovitis
Feline	Calicivirus; leukaemia; panleukopenia; peritonitis; rabies; rhinotracheitis; lentivirus
Canine	Adenovirus; coronavirus; distemper; hepatitis; measles parainfluenza; parvovirus; rabies

### Ad production and purification as veterinary vaccines

Process design is critical when developing cost-effective veterinary vaccines. The goal of minimizing the number of process steps is a prerequisite if industrial application is intended. In addition, veterinary vaccines do not impose the same final purity requirement as human vaccines, which should reflect on the purification schemes.

The production of Ad vaccines for veterinary applications is mainly based upon the use of continuous cell lines such as 293,<sup>12</sup> MDBK,<sup>28,30</sup> VR1BL, HeLa and A549.<sup>34</sup> During the cell proliferation step, various animal sera



**Figure 2** Bioreactors operation modes: (a) batch; (b) fed-batch; (c) perfusion, *s* = substrate; *x* = cells; *F* = flow of fresh substrate; *F<sub>b</sub>* = bleed, flow of broth with cells out of the bioreactor; *F<sub>p</sub>* = permeate flow, cell free flow of product; *V<sub>L</sub>* = working volume; *p* = product; *S<sub>0</sub>* = substrate in the flow.

are used to enhance cell growth. However, supplementing cell culture media with such components presents several drawbacks like lot to lot variation, potential risk of contamination by viruses, mycoplasma, prions, etc.<sup>87</sup> Furthermore, regulatory authorities in Europe (European Medicines Agency (EMA)) and in the United States (Food and Drug Administration (FDA)) have encouraged biological manufacturers to reduce or eliminate the use of substances of animal origin in their manufacturing processes. Recently, several serum and protein-free culture media became commercially available. Although the use of such defined media is still expensive, the large-scale production of Ad vectors will require the use of this type of culture media to facilitate downstream processing and, in time, decrease the overall process cost.

Production methods differ according to the cell type used: adherent *versus* suspension cell culture. For manufacturing purposes, suspension adapted cell lines are more convenient for production at large scale and different operation modes have been developed for Ad production (Figure 2): (i) batch mode providing the easiest way to proceed as no extra feeding is required and the risk of contamination is lowered given the simplicity of operation; (ii) fed-batch mode, easy to operate and readily scalable, is employed to extend culture lifetime by supplementing limiting nutrients or reducing the accumulation of toxic metabolites; and (iii) perfusion mode, consisting in cell retention at a relatively high concentration inside the bioreactor, while fresh nutrient supply and metabolite removal takes place (see Kamen and Henry<sup>24</sup> for a review).

Methodologies for production of concentrated Ad vectors at low cost are mandatory as the market needs for Ad are increasing. Although Ad has the advantage to be produced at high titers (10<sup>10</sup>–10<sup>11</sup> PFU/ml),<sup>19</sup> to obtain a good immune response in a large proportion of treated animals, particularly for mucosal vaccination, large doses and thus culture volumes are required. Further process development aiming at higher yields of product is clearly necessary. Improvements in volumetric production can be achieved by increasing the cell density at which cells can be infected without lowering the specific yield of the product; however, production of Ad vectors that maintain a high specific yield in batch operation is limited to cell densities in the range of 1 × 10<sup>6</sup> cells/ml; several approaches have been made in order to overcome this so-called 'cell density effect':<sup>88</sup> Garnier et al<sup>89</sup> demonstrated that medium replacement at infection and the addition of glucose at 24 h postinfection

(hpi) together with periodical pH adjustments, allowed a sustained maximum specific productivity at  $1.6 \times 10^6$  cells/ml whereas Nadeau *et al*,<sup>90</sup> further improved this strategy by also adding essential amino acids at 24 hpi thereby stabilizing volumetric productivity at cell densities above  $2 \times 10^6$  and below  $3 \times 10^6$  cells/ml. Such results hint at the existence of substrate limitation and/or byproduct inhibition at high cell densities. At production scale, medium exchange will increase the cost of the final product, which is even more critical when this is to be used in the veterinary field. Perfusion mode operation has been attempted as a means to control the culture environment and remove toxic byproducts;<sup>24,91</sup> nevertheless, the cell-specific productivity could only be maintained by infecting cells at densities up to  $3 \times 10^6$  cells/ml using high perfusions rates of two reactor volumes *per day*, at 2 days postinfection, a very costly proposition.<sup>24</sup> In our laboratory, by understanding kinetics and metabolism of infection, typical cell concentration at infection have already been doubled and further improvements seem possible using a simple and nonexpensive fed-batch mode.

The importance of infection kinetics on Ad production and the significance of variables such as multiplicity of infection (MOI) and harvesting time in process optimization is also mandatory to increase production yields, to avoid rapid depletion of costly and certified master virus banks as well as to ensure that the infection kinetics is reproducible between different production scales. Moreover, the use of low MOI at large scale would be preferential since an intermediate step of virus inoculum production would be avoided.

Finally, veterinary products should be purified with a minimal number of steps and the unit operations employed should be simple and nonexpensive. Traditionally, laboratory purification of rAd was achieved using two rounds of cesium chloride (CsCl) density gradient ultracentrifugation. However, CsCl density gradient method is not scalable. Despite the fact that chromatographic purification is an expensive method, ion exchange, hydrophobic interaction, metal chelate and size-exclusion chromatography have been evaluated for capture and purification of HAd5.<sup>92</sup> Kamen and Henry<sup>24</sup> developed a complex and expensive method for gene therapy application consisting of: (i) harvest of infected cells by continuous centrifugation, (ii) cell lysis by osmotic shock, (iii) DNase treatment with centrifugal/conditioning, (iv) filtration, (v) anion-exchange chromatography, (vi) ultrafiltration/concentration and (vii) size exclusion chromatography. This protocol allows large-scale purification of Ad vectors with purity comparable to the CsCl gradient method; however, it dramatically increases the final product price, well beyond the reach of veterinary utilization. Introgen developed a method consisting of a single ion chromatography run, after concentration/diafiltration and nuclease treatment, with a total recovery of the virus product of 70%.<sup>93</sup> In our laboratory, a protocol to purify HAd5 from the bulk harvested directly from the bioreactor after lysis (without a concentration step) using an ion exchange chromatographic step, ultrafiltration and a final polishing step with gel filtration is in progress with promising preliminary results.

## Perspectives and conclusion

To improve access to the veterinary marketplace, some hurdles still need to be overcome, as efficacy, safety, ease of delivery and cost of production.<sup>94</sup> Some of these factors are dependent from the EMEA and its Committee for Veterinary Medical Products (CVMP) who regulate vaccines developed in the European Union (EU); meanwhile, the United States Department of Agriculture (USDA) and its Animal and Plant Health Inspection Service (APHIS) regulate the development of animal vaccines in the United States (US). Unfortunately, government regulators can often become extremely cautious when confronted with new technology and they not always follow a strictly science-based assessment approach, which should be the only criteria for regulation of products. The regulatory issues governing the use of Ad vaccines on the farm and in the veterinary clinical are not yet well defined, although guidelines on live recombinant vector vaccines for veterinary use are available (EMEA/CVMP/004/04) in EU, while the Rules Governing Medicinal Products in the UE and the code of US Federal Regulations Title 9 (9 CFR) are the main regulatory texts on animal veterinary vaccine production.

The drawbacks of the use of Ad vectors as veterinary vaccine is the rare but possible emergence of E1-containing vectors, due to rare eventual minute homologous recombination events between the viral E1-containing (permissive) mammalian host cell genome and the virus itself, restoring the E1 gene to the viral genome (RCA). To reduce or eliminate the problem of RCA, an E1-transformed human cell line PER.C6 was constructed.<sup>95</sup> PER.C6 was made by transformation of primary human embryonic retinoblasts with limited homology with viral sequences, expected to reduce or eliminate the emergence of RCA during virus amplification.

Up to now, many viruses have been used as viral vectors for delivery of foreign antigens (see Yokoyama *et al*<sup>6</sup> for a review). The development of these recombinant vaccines may be of value in the veterinary field, especially when attenuation of the target pathogen is not possible, when immunity to one or more pathogens must be raised at the same time or when satisfactory quantities of the target pathogen or the live vaccine cannot be produced easily or safely. On the other hand, for their practical use, there might be still some potential problems that must be overcome, for example, safety issues such as reversion of virulence or recombination with field-type viruses spread in the environment and genetic stability. The selection of a good viral vector as recombinant viral vaccine should be determined by the host range of the vector, safety consideration, replication properties, stability, the amount of foreign DNA, which can be inserted and the place where the primary immune reaction is induced by antigens expressed by the vector.

Exciting advances in biotechnology are enabling the development of new vaccines with enormous possibilities that were considered unthinkable. These newer vaccine approaches have benefited from an improved basic understanding of the immunology of antigen presentation and the factors important in sustaining memory immune responses. The continued improvement in our understanding of the nature of protective immunity against various infectious diseases should allow the rational design of vaccines and immunization



strategies that generate protective immune responses against infectious diseases for which no vaccines yet exist.

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