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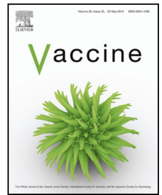

Review

# Bovine adenovirus-3 as a vaccine delivery vehicle

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ABSTRACT

The use of vaccines is an effective and relatively inexpensive means of controlling infectious diseases, which cause heavy economic losses to the livestock industry through animal loss, decreased productivity, treatment expenses and decreased carcass quality. However, some vaccines produced by conventional means are imperfect in many respects including virulence, safety and efficacy. Moreover, there are no vaccines for some animal diseases. Although genetic engineering has provided new ways of producing effective vaccines, the cost of production for veterinary use is a critical criterion for selecting the method of production and delivery of vaccines. The cost effective production and intrinsic ability to enter cells has made adenovirus vectors a highly efficient tool for delivery of vaccine antigens. Moreover, adenoviruses induce both humoral and cellular immune responses to expressed vaccine antigens. Since nonhuman adenoviruses are species specific, the development of animal specific adenoviruses as vaccine delivery vectors is being evaluated. This review summarizes the work related to the development of bovine adenovirus-3 as a vaccine delivery vehicle in animals, particularly cattle.

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## 1. Introduction

Adenoviruses (AdVs) are non-enveloped double stranded DNA viruses with icosahedral capsid symmetry composed of 252 capsomers. The genome size ranges between 24 and 45 kb [1,2]. Although adenoviruses were first identified in the early 1950s from the adenoids of humans with acute respiratory infection [3,4], today, there are over 120 serotypes, isolated from different species, including, mammals, birds, reptiles and fish. While adenoviruses can infect a wide variety of animals, including humans, birds and livestock, they are usually species-specific [2,5,6]. AdVs have been proposed to be clustered in five phylogenitically distinct groups namely; *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, *Siadenovirus* and *Ichtadenovirus*, with each virus group having a distinct and characteristic genome structure [7,8]. The overall genetic organization of members of the *Mastadenovirus* including human adenovirus (HAdV)-5 [9], canine adenovirus (CAdV)-2 [10], Simian adenovirus (SAdV) [11], bovine adenovirus (BAdV)-3 [12], porcine adenovirus (PAdV)-5 [13] and PAdV-3 [14] appears to be conserved. The *Mastadenovirus* genome is divided into five discrete transcriptional units. The early regions E1 and E4 are located at either end of the genome, while E2 and E3 are separate and internal. The central core region constitutes the late region. In addition, there are two delayed early transcriptional units named IVa2 and pIX. Despite this genetic conservation, some regions are highly divergent (based on structure of transcripts and proteins) among different members of *Mastadenoviruses*. In particular, the deduced amino acid sequence of ORFs in the E1, E3 and E4 regions of BAdV-3 show little or no homology to the corresponding proteins encoded by genomes of other members of *Mastadenoviruses* [12]. Since these proteins are involved in virus cell interaction and viral gene expression, variations in these proteins may reflect the diverse host range and pathogenic potential of different members of *Mastadenoviruses.*

In recent years, much attention has been focused on evaluating adenoviruses as viral vectors due to their ability to infect both dividing and non-dividing cells, capacity to package large foreign genes, relative ease to produce high titer recombinants in cell culture [15], elicit strong antigen specific T cell responses and lack of virulence [16,17]. Although recombinant HAdVs have been proven to deliver vaccine antigens to domestic animals [18–21] and birds [22,23], regulatory concerns regarding safety has limited their use in domestic animals. Moreover, species specificity limiting host range, restricted replication in non host species and

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stability of nonhuman adenoviruses has led to the evaluation of animal [CAdV-2 [24], PAdV-3 [25], PAdV-5 [26], BAdV-3 [27,28] and poultry (Fowl adenovirus (FAdV) [29] specific adenoviruses as vaccine delivery vectors. Since BAdV-3 is a natural nonpathogenic virus with restricted host-range, grows to high titers and can be delivered intranasally without affecting the meat quality of food producing animals, BAdV-3 is being evaluated as vaccine delivery vector in animals including cattle.

## 2. Molecular biology of bovine adenovirus-3

BAdV-3 is 75 nm in diameter, non enveloped icosahedral particle containing a double stranded DNA genome. The genome of BAdV-3 is 34, 446 base pairs [12] flanked on either end by 195 bp inverted terminal repeats (ITRs) [30], which play a key role in DNA replication. Unlike other adenoviruses, the ITRs of BAdV-3 are longer and contain a high GC content [12]. Like other adenoviruses, the packaging domain is located in the left end of the viral genome overlapping the transcriptional control region of E1A, but E1A region expression distinctively appears to be essential for packaging in BAdV-3 [31,32]. Though the TATA or CAAT boxes are absent in the regions between the left ITR and upstream of the E1A start codon, the expression of the E1A open reading frame is driven by a promoter located within the ITR [33].

Based on the transcriptional analysis [12,34–37], BAdV-3 genome appears to be organized (Fig. 1) into early, intermediate and late regions [12]. Although genome organization appears similar to other members of *Mastadenoviruses*, the structure and function of some proteins encoded by different regions of BAdV-3 appears to be different [38–44].

## 3. Development of BAdV-3 as a vector for vaccination

### 3.1. Method for isolation of recombinant BAdV-3

Initial attempts to generate recombinant BAdV-3 using homologous recombination in Madin Darby bovine kidney (MDBK) cells co-transfected with purified BAdV-3 genome and a transfer vector containing a deletion in E3 region were inefficient and mostly unsuccessful [45–47] due to low transfection efficiency and inefficient recombination in MDBK cells. However, two improvements namely availability of homologous recombination machinery of *Escherichia coli* [48] and use of bovine retina cells for transfection of restriction enzyme excised modified BAdV-3 genome from plasmids has facilitated the efficient generation of recombinant BAdV-3 [47,49]. Recently, a more efficient and time saving method of generating recombinant BAdV-3 has been reported by transfection of endonuclease *I-SceI* expressing non bovine (cotton rat lung) cells with circular BAdV-3 genomic DNA flanked by *I-SceI* recognition site [50].

### 3.2. Sites for insertion of foreign genes

#### 3.2.1. E1 region

Isolation of replication competent BAdV-3 containing partial deletion of $E1B_{small}$ suggested that this region is not essential for replication of BAdV-3 [51] in fetal bovine retina cells and may be used for foreign gene insertion. However, viable recombinant BAdV-3 with E1A [52–54] or $E1B_{large}$ deletion [33,52] could not be rescued suggesting that these regions are essential for replication of BAdV-3. However, packaging cell lines expressing E1 region of BAdV-3 (FBK-34; MDBK-221; [53]) or HAdV-5 (VIDO R2, FBRT-HE1; BHH3 and BHH8 [53,54] supported the replication of only E1A (541 bp) deleted BAdV-3 (Fig. 2) and not complete E1 region deleted BAdV-3 [52]. Replication-defective BAdV-3s containing insertion of 2.3 kb of foreign DNA in E1A region of E1-E3 deleted BAdV-3 could be isolated only in E1A complementing cell lines [52].

Since replication-defective BAdV-3 vector cannot undergo multiple replication cycle and therefore are less likely to spread to the environment, they are considered safer. One of the major concerns with regard to the use of live viral vectors for vaccination in animals is the release of potential recombinants into the environment. However, since the replication-incompetent recombinants undergo an abortive infection, the level of foreign gene expression is lower than that obtained using replication-competent BAdV-3.

#### 3.2.2. E3 region

The E3 region of BAdV-3 is 1.591 kb. Since E3 region is non essential for replication of adenoviruses [46,55], initial attempts resulted in isolating a viable recombinant BAdV-3 containing deletion of 1.245 kb of E3 region [46]. Although potential insertion capacity of E3 deleted vector is 3 kb [46], the insertion of 2.8 kb foreign DNA [56] has been successful in generating viable replication-competent recombinant BAdV-3. The foreign open reading frames (ORFs) inserted in E3 are efficiently expressed using upstream endogenous (E3/MLP) promoters [28,46,56,57] or exogenous promoters [56,57]. However, insertion of foreign ORFs antiparallel to E3 transcription does not lead to the generation of viable recombinant BAdV-3. Moreover, addition of exogenous consensus sequence for polyadenylation of foreign ORF affects the replication efficiency of recombinant BAdV-3 (Lobanov and Tikoo, unpublished data). Interestingly, efficient expression of a RNA virus required addition of an intron and consensus sequence for polyadenylation upstream and downstream, respectively, of the gene [55]. Since E3 deleted recombinant BAdV-3 are replication competent (Fig. 2), less amount of virus may be required to induce a protective immune response.

#### 3.2.3. E4 region

The first site for insertion of foreign ORF has been identified in a transcriptionally inactive region between start of E4 and right ITR of BAdV-3 [12,34]. Isolation of replication competent BAdV-3 containing insertion of 1.9 kb foreign DNA at nucleotide (34059) suggested that this region is non-essential for BAdV-3 replication (Fig. 2). However, exogenous promoter and consensus sequence for polyadenylation are required for expression of the foreign ORF.

The other sites for potential insertion of foreign ORF have been identified in transcriptionally active E4 region of BAdV-3 [58]. Isolation of replication competent BAdV-3 containing deletion of N-terminus 1.501 kb or C-terminus 1.342 kb (Fig. 2) in E4 region suggested that these regions are not essential for replication of BAdV-3 [58], thus increasing the potential insertion capacity of E3–E4 deleted BAdV-3 to 4.5 kb [58].

## 4. Evaluation of recombinant BAdV-3 as vaccine delivery vector

### 4.1. Recombinant BAdV-3 expressing single antigen

Much of the initial efforts of developing replication-competent BAdV-3 based vectors have focused on non-essential E3 region. Earlier, successful expression of a reporter ORF inserted in partially deleted E3 region of BAdV-3 [45] suggested the feasibility of utilizing E3 region for expression of foreign genes. Thus, subsequent expression of different forms of (BHV)-1 vaccine antigen gD ORF [46], (BVDV) E2 ORF [57] or (BCV) virus HE ORF [55] inserted individually in fully deleted E3 region of BAdV-3 demonstrated the potential of developing BAdV-3 based recombinant vaccines for cattle. These ORFs were selected as earlier reports have suggested the potential of bovine herpesvirus (BHV)-1 glycoprotein gD [59], bovine viral diarrhea virus (BVDV) E2 glycoprotein [60] or bovine

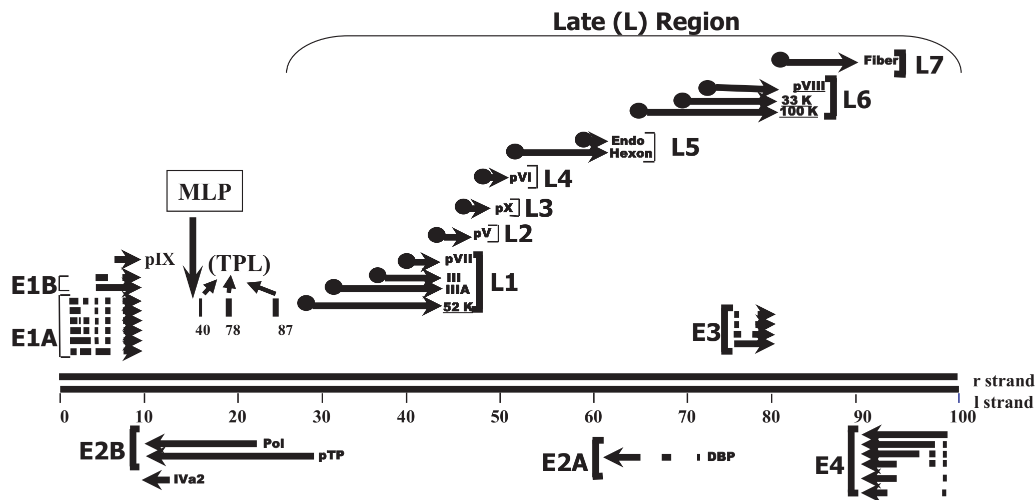


**Fig. 1. Transcriptional map of BAdV-3.** Thick lines represent BAdV-3 genome. Horizontal arrows represent direction of transcription. Right (r) strand; left (l) strand. Early (E). The filled circles represent TPL at 5′-end of each of the late (L) transcript. The numbers below represent the map units (m.u.). The number above represent the total nucleotides in each exon of TPL. The major late promoter (MLP) and tripartite leader (TPL) are depicted (adapted from [12,34–36]).

corona virus (BCV) hemagglutinin ORF [61] to induce protective immune responses in animals and thus act as vaccine antigen.

Reports suggest that cotton rats can serve as small-animal model for investigation of immune responses to BAdV-3 vectored vaccines [62]. The feasibility of using replication-competent recombinant BAdV-3 in inducing antigen specific immune responses in animals was demonstrated by immunizing cotton rats twice intranasally three weeks apart, with recombinant BAdV-3 expressing BHV-1 gD/gDt [46,56] or BVDV E2 protein [57].

One of the concerns with the use of BAdV-3 as a vector has been the presence of varying levels of BAdV-3 vector specific pre-existing antibodies in calves in the field [27,63–66]. To address the concern, the induction of BAdV-3 specific immune response was analyzed in calves containing significant level of pre-existing BAdV-3 specific neutralizing antibodies. The results suggested that intranasal immunization of calves induced significant BAdV-3 specific antibody responses in the presence of BAdV-3 specific neutralizing antibodies [67]. Next, the usefulness of replication-competent BAdV-3 as an effective vaccine delivery vehicle was tested in natural host (calves) containing significant level of pre-existing BAdV-3 specific neutralizing antibodies [27]. We demonstrated the induction of protective immune responses against BHV-1 challenge in calves immunized with recombinant replication-competent BAdV-3 expressing BHV-1 gD/gDt [27]. In this study, calves were immunized twice intranasally, four weeks apart and challenged two weeks later with BHV-1. The immunized

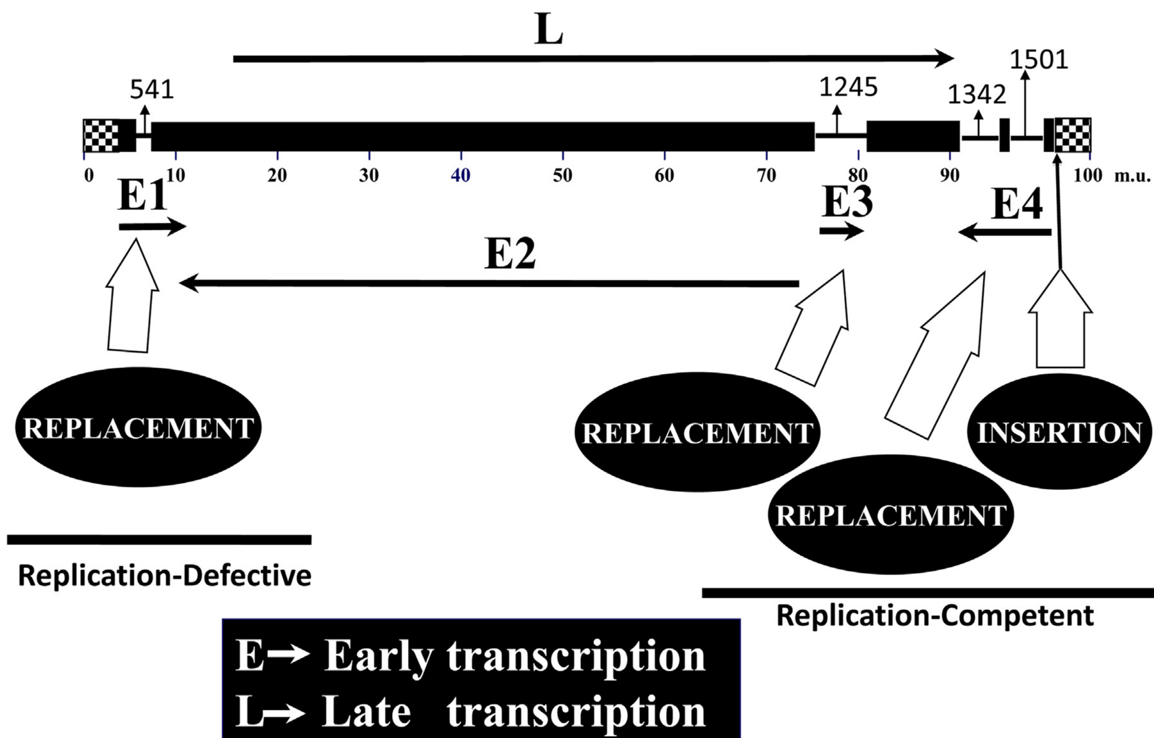


**Fig. 2. Schematic representation of BAdV-3 genome.** BAdV-3 genomic DNA (filled box). Early (E) and late (L) regions are depicted. The numbers above represent the deletion in bps. The numbers below represent the map units (m.u.). The horizontal arrows represent the direction of transcription.

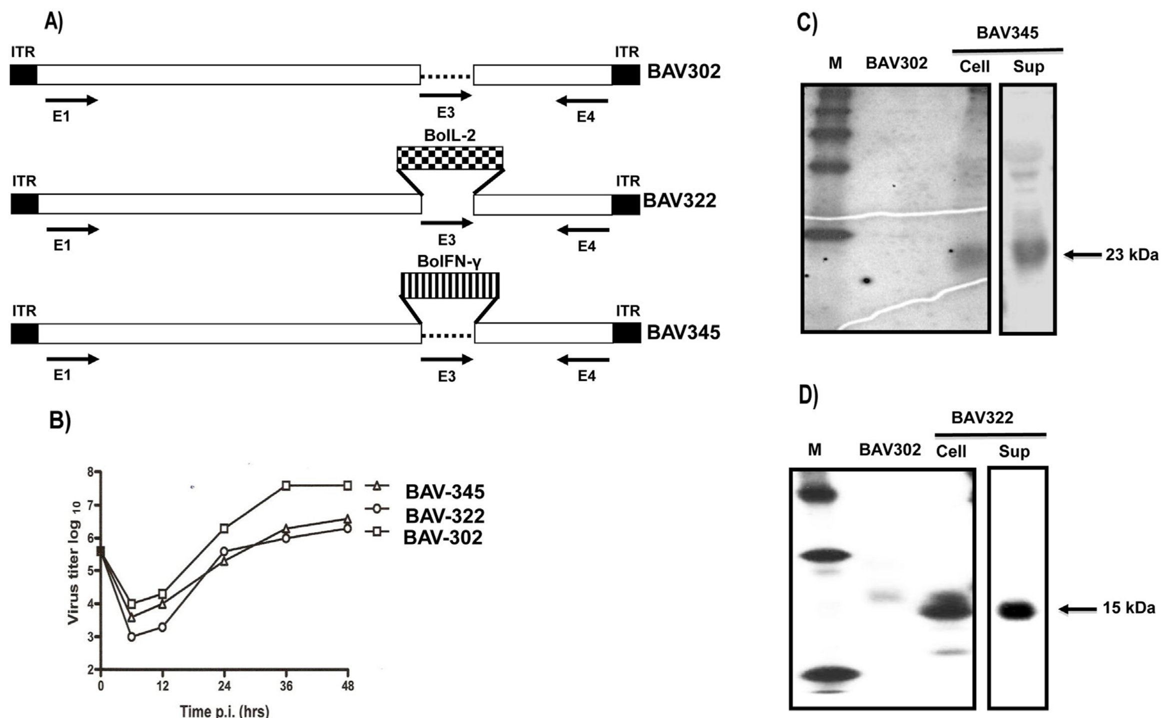


**Fig. 3. Expression of Bovine cytokines.** (A) *Schematic representation of full length genomic DNA in plasmids.* BAdV-3 genome (hollow box; Inverted terminal repeats (ITR). The arrows represent the direction of transcription. Early (E) regions. The name of the recombinant BAdV-3 is depicted on the right of the panel. [Construction of these viruses available on request). (B) *Virus titers.* Confluent monolayers of MDBK cells were infected with indicated BAdV-3. At different times post infection, the cell pellets were freeze–thawed and virus was titrated on MDBK cells. (C and D). *Immunoprecipitation.* Proteins from the lysates of cells (Cell) or media (Sup) of [$^{35}$S] methionine labeled BAV302 (panels C and D), BAV345 (panel C) or BAV322 (panel D) infected MDBK cells were immunoprecipitated with anti-BoIFN-γ MAb 2.2.1 (panel C) or anto-BoIL-2 MAb 4F11 (panel D) and analyzed by 10% SDS-PAGE under reducing conditions.

calves were protected from clinical disease and showed BHV-1 shedding for reduced time [27]. Thus, species-specific vectors can be developed and used to induce mucosal immunity and protection against a disease in a natural host. Moreover, mucosal (intranasal) immunization helps to bypass the effect of vector (BAdV-3) specific pre-existing antibodies on the induction of protective immune responses. In contrast, intratracheal/subcutaneous immunization with replication-competent recombinant BAdV-3 expressing gDt was not as efficient as intranasal immunization in inducing protective immune responses in calves [65].

Despite the requirement of high doses, replication-defective human adenovirus based vectors have been successfully used in inducing protective immune responses in animals [20,68,69,19] and poultry [22,70,71]. In contrast, replication-defective BAdV-3 expressing gDt does not appear efficient in inducing a protective immune response in calves [65]. This could be due to the low amount of replication-defective recombinant used for immunization, which may not be adequate for producing sufficient amount of vaccine antigen [65]. Although the use of high amount of replication-defective BAdV-3 vectors may induce protective immune responses in claves, the cost-effective production of such vaccines may be problematic.

### *4.2. Recombinant BAdV-3 expressing two vaccine antigens*

Since veterinary vaccines have to be safe and cost effective, the development of a recombinant BAdV-3 vectored vaccine providing immunity to more than one pathogen is desirable. Recently, we demonstrated that single recombinant BAdV-3 expressing vaccine antigens from two respiratory pathogens (gDt of BHV-1 and gG of bovine respiratory syncytial virus [BRSV]) of cattle could be isolated [72]. Moreover, two intranasal immunizations, four weeks apart of calves with single recombinant BAdV-3 expressing BHV-1 gDt and BRSV gG induced significant neutralizing antibodies against BHV-1 and BRSV in cotton rats [72]. Thus, the construction of single recombinant BAdV-3 with multiple vaccine antigens is feasible to induce virus specific immunity for protection from multiple pathogens in calves [72].

### *4.3. Recombinant BAdV-3 expressing cytokines*

#### *4.3.1. Cytokines*

Several recent studies in experimental models have demonstrated that it is possible to bias the immune response as required by administration of recombinant cytokines along with vaccine antigens, which has helped to modulate disease progression in animal models [73–77]. Moreover, inoculation of replication-defective HAdV-5-expressing porcine IFN-α, protected pigs from foot-and-mouth disease (FMD) [78], and delayed and reduced disease signs in cattle after challenge with FMD virus [79]. However, despite the availability of different bovine cytokine genes, very few studies have been reported investigating the immunomodulation potential of known bovine (Bo) cytokines in cattle [80–82]. We have demonstrated the feasibility of constructing replication-competent (E3 deleted) recombinant BAdV-3 viruses, expressing bovine BoIFN-γ [80,83] (Fig. 3, panels A–C), BoIL-2 [84] (Fig. 3, panels, A, B and D), or BoIL-6 [28] genes, inserted into E3 region of BAdV-3 (Fig. 3).

#### *4.3.2. Antigen–cytokine*

Required effect of cytokine in modulating immune response is best achieved, when both vaccine antigen and cytokine are synthesized in the same cells. To this end, we demonstrated the feasibility of constructing replication competent (E3 deleted) recombinant BAdV-3 expressing antigen-cytokine chimera (gDt-BoIL-6) or expressed antigen- and cytokine as individual proteins (gDt, BoIL-6) from a single mRNA [28]. In addition, the co-expressed

gDt retained antigenicity and BoIL-6 retained biological activity [28]. Hence, constructing and evaluating single BAdV-3 expressing two antigens (vaccine antigen and cytokine) is possible.

Since gD/gDt expressed by recombinant BAdV-3 did not induce efficient mucosal IgA response required for eliminating BHV-1 shedding in immunized calves, we evaluated the effectiveness of recombinant BAdV-3 co-expressing BHV-1 gDt and BoIL-6 [85] in the immunomodulatory effects of BoIL-6 on induction of BHV-1 gDt specific immune responses in calves [28]. Although co-expression of BoIL-6 did not modulate the gDt specific immune response, the study provided the feasibility of delivering vaccine antigens with other cytokines with potent adjuvant effects to modulate immune responses in cattle.

## 5. Altering tropism of BAdV-3 vaccine vector

Although BAV-3 vector can efficiently deliver vaccine antigens to respiratory mucosal surfaces of cattle, the prospects of oral delivery of BAdV-3 based vaccines should help in reducing the indirect cost (man power and animal handling) associated with vaccination, thus providing additional economic benefits. Though, BAdV-3s potential use in delivering vaccine antigens to enteric mucosal surfaces of cattle has not been successful, it should be possible to develop BAdV-3 vectors which can deliver vaccine antigens to different mucosal surfaces by genetic modification of capsid proteins. This technique can also be applied to deliver vaccine antigens to other animals (e.g. cats, dogs, poultry).

We have tested the feasibility of changing tropism of BAdV-3 by genetic manipulation of major capsid protein fiber and minor capsid protein pIX of BAdV-3. First, the construction of a recombinant BAdV-3 with a chimeric fiber by replacing the knob region of BAdV-3 fiber with the knob region of HAdV-5 fiber alters the tropism of the recombinant BAdV-3 [44]. Second, we have provided proof of principle that BAdV-3 minor capsid protein pIX can be used for stable insertion of longer polypeptides exposed on the surface of BAdV-3 virions [86]. Moreover, incorporation of targeting ligand into the C-terminus of pIX enhanced fiber-knob independent tropism of BAdV-3 [86]. BAdV-3 pIX based targeting is being evaluated in developing improved BAdV-3 vectors for bovine vaccination. Thus, it should be possible to exchange the knob region of BAdV-3 fiber with knob region of an adenovirus fiber with tropism for enteric mucosa of cattle to generate BAdV-3 vectored vaccines that can be delivered orally. Alternatively, it should be possible to develop recombinant BAdV-3 expressing chimeric pIX (pIX containing targeting ligand) recognizing M cells [87–89] in ileal Peyer's patches of enteric mucosa.

## 6. Conclusions

Although advances in recombinant DNA technology have led to the identification of potent vaccine antigens, one of the impediments in the development of effective vaccines has been the appropriate delivery of vaccine. Moreover, the development of new generation of veterinary vaccine need to take into account a number of factors including the cost of production, ease of delivery, safety and efficacy. The development and use of vaccines based on BAdV-3 vector system will be cost effect and safe with no risk of producing disease. In addition, these vaccines can also be used for eradication programs since infected and vaccinated animals can be differentiated. Interestingly, intranasal inoculation of calves with wild-type [67] or replication-competent BAdV-3 [27] causes in apparent infection with negligible (number of animals and duration of virus secretion) excreting of virus. Since BAdV-3 has restrictive host range, [35,44,90], can be isolated from a healthy cattle [64] and causes in apparent infection in calves [27,67], the use of replication-competent BAdV-3 vector based vaccines in cattle may not be a major regulatory concern for use in the field. Earlier reports have suggested the oncogenicity of BAdV-3 in Hamster [91] and rat embryo cells [92]. However, like other adenoviruses, BAdV-3 is not known to induce tumor formation in cattle (its natural host). In fact, certain replication competent human adenoviruses (containing transforming E1 region) are already in different phases of clinical trial for certain cancer vaccination and have been used as vaccines in humans [93].

Based on the very long substantial safety record of replication-competent human adenovirus-4 and 7 vaccines in U.S. military, replication competent adenovirus based vaccines under development are also expected to be safe [93]. The replication-competent adenoviral vectors have a "dose sparing" effect providing an advantage for manufacturing. They also mimic a natural wild-type infection inducing all arms of the immune system including; innate, cellular, humoral and mucosal immunity. Theoretically, replication competent vaccine vectors have the safety of inactivated vaccines and the improved efficacy of live attenuated vaccines [93].

Gomez-Roman et al. demonstrated the detection of vector in the stools of rhesus macaques within a week after vaccination with oral enteric coated replication competent adenovirus-5 based vectors; however, no spread of the virus to the upper respiratory tract was detected and no clinical symptoms of adenovirus induced disease were observed during the 10 weeks of follow up period, proving the safety of the vector and very good tolerance by vaccinated animals [94]. A phase I clinical trial of an oral, replicating adenovirus-4 vector vaccine for H1N1 influenza in humans was also well tolerated and demonstrated to have a good safety profile with low transmissibility [95]. Generally, experimental inoculation of humans or chimpanzees with replication-competent adenovirus vectors has proven to be safe [95,96]. Similarly, replication competent BAdV-3 vector is expected to be safe and tolerable in cattle [27,64,67].

## Conflict of interest statement

The authors have no conflict of interest to declare.

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