

Recombinant Bovine Adenovirus-3 Co-Expressing Bovine Respiratory Syncytial Virus Glycoprotein G and Truncated Glycoprotein gD of Bovine Herpesvirus-1 Induce Immune Responses in Cotton Rats

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Abstract One of the impediments in the development of safe and cost effective vaccines for veterinary use has been the availability of appropriate delivery vehicle. We have chosen to develop and use bovine adenovirus (BAdV)-3 as vaccine delivery vector in cattle. Here, we describe the construction of recombinant E3 deleted BAdV-3 vectors expressing single vaccine antigen (BAV360; bovine respiratory syncytial virus G) or two vaccine antigens (BAV851; bovine herpesvirus-1 gD and bovine respiratory syncytial virus G). Recombinant proteins expressed by BAV360 or BAV851 were recognized by protein-specific monoclonal antibodies. Moreover, intranasal immunization of cotton rats with BAV360 (expressing a single vaccine antigen) or BAV851 (expressing two vaccine antigens) induced strong antigen-specific immune responses. These results suggest that single replication-competent BAdV-3 expressing vaccine antigens of two economically important respiratory pathogens of calves has potential to act as a feasible approach in the development of economically effective veterinary vaccines for cattle.

Keywords Bovine adenovirus-3 · Bovine herpesvirus-1 · Bovine respiratory syncytial virus · IRES · BHV-1 gD · BRSV G

Introduction

Bovine herpesvirus (BHV)-1 causes a number of clinical manifestations including respiratory disease in cattle [1]. BHV-1 is a member of subfamily alphaherpesvirinae, with a genome of approximately 136 kb [2]. Like other herpes viruses, several glycoproteins are synthesized and incorporated into the BHV-1 envelope [1]. Of these, any of the major glycoproteins (gB, gC, and gD) can induce some degree of immune protection against disease caused by BHV-1 [1]. Since these infections are controlled by vaccinations, efforts have been centered around developing and using vaccines based on live attenuated marker BHV-1 [3] or recombinant forms of glycoproteins gD [4].

Bovine respiratory syncytial virus (BRSV) is a major cause of lower respiratory disease of calves [5]. BRSV is an enveloped pneumovirus and contains a non-segmented single stranded, negative sense RNA molecule of 15,140 bp in length, which encodes 11 proteins [6]. Of these, the envelop-associated fusion glycoprotein F and attachment glycoprotein G represent the main viral antigenic determinants [7]. While scarification of calves with recombinant vaccinia virus expressing F reduces nasopharyngeal excretion of BRSV, the scarification of calves with recombinant vaccinia virus expressing G protein reduces pulmonary BRSV infection [7]. However, induction of resistance to infection may be best achieved by delivering vaccine antigen to respiratory tract using viral vectors, which replicates in the bovine respiratory tract. Earlier, BHV-1, which replicates in bovine respiratory tract

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was used to express synthetic BRSV G [8]. Although recombinant BHV-1 expressing G glycoprotein induced significant resistance to BRSV infection of calves, expression of the G protein of BRSV increased the virulence of BHV-1 vector in animals [9].

We are currently characterizing bovine adenovirus (BAdV)-3 [10–13] with the aim to develop it as an ideal vector for animal vaccination [14–18]. BAdV-3 was chosen because of its low pathogenicity, species specificity, and ability of the virus to grow to high titers in cell culture. Recently, we have constructed replication-competent BAdV-3s expressing vaccine antigens [15, 18] and demonstrated the induction of protective immune responses in calves in the presence of BAdV-3 specific pre-existing antibodies [15, 17].

Since BAdV-3 replicates in respiratory tract of bovine, we constructed and characterized the replication-competent (E3-deleted) BAV-3 expressing BRSV G or BRSV G + BHV-1 gDt (truncated form of gD). In addition, we demonstrate that intranasal immunization of cotton rats with these recombinants induces antigen-specific mucosal and systemic immune responses.

Materials and Methods

Cells and Virus

Madin Darby bovine kidney cells were grown in Eagle's minimum essential medium (MEM) supplemented with 5 % fetal bovine serum (FBS). The wild-type (WBR-1 strain) and recombinant BAdV-3s were propagated in MDBK cells as described [18]. The construction and characterization of BAV302 (E3 deleted BAdV-3) and BAV308 (BAV.E3gDt) expressing secreted form of BHV-1 glycoprotein gD has been described [18]. The P8-2 strain of BHV-1 was propagated and quantified as described earlier [4]. The Iowa strain of BRSV was propagated and quantified as described earlier [19].

Antibodies

Production and characterization of MAbs recognizing BHV-1 gD [20] and BRSV G [21] has been described earlier.

Plasmid Construction

Plasmid pFBAV360

Initially, synthetic G gene (containing codon usage bias toward bovine and potential absence of splice sites) was constructed. A 780 bp *EcoRV*–*HindIII* (blunt end repaired

by T4 polymerase) fragment isolated from plasmid pSPg ligated to *SrfI* digested plasmid pBAV301 to create plasmid pBAV301.G. A recombinant BAdV-3 genome (pFBAV360) containing BRSV G gene inserted in E3 (Fig. 1) was generated by homologous DNA recombination in *E. coli* BJ5183 between *SrfI* digested pFBAV302 and a 6.7 kb *KpnI*–*SpeI* fragment of plasmid pBAV301.g.

Plasmid pFBAV851

A 780 bp fragment isolated by PCR amplification using primers (RB-1, 5'-CCAACCACACCCACCACC-3'; and RB-2, 5'-CTAGTCTAGA TTAGATCT GTGTAGTGG-3') and plasmid pSPg DNA as a template was digested with *XbaI*–*MscI* and ligated to *XbaI*–*MscI* digested plasmid pCITE (Novagen) creating plasmid pCITE.G. A 1.1 kb fragment isolated by PCR amplification using primers (RB-3, 5'-GG TAAGCTCGAGATGGAAGGGCCGAC ATTG-3', rb-4, 5'-GCTGAATTCTAG AGCCTAGCTAGTCC-3') and plasmid pRSV1.3 [22] DNA as a template was digested with *EcoRI* and ligated to *NdeI* (blunt end repaired by T4 polymerase)–*EcoRI* digested pCITE.G to create plasmid pgDt-IRES-G.

Finally, a 2.5 kb *XhoI*–*Sse8387* fragment isolated from plasmid pgDt-IRES-G was end repaired by T4 polymerase and ligated to *SrfI* digested pBAV301 creating plasmid pBAVgDt-G. A recombinant BAdV-3 genome (pFBAV851) containing gDt-IRES-G expression cassette inserted in the E3 region (Fig. 1) was generated by homologous DNA recombination in *E. coli* BJ5183 between *SrfI* digested pFBAV302 and 6.8 kb *SphI* fragment isolated from plasmid pBAVgDt-G.

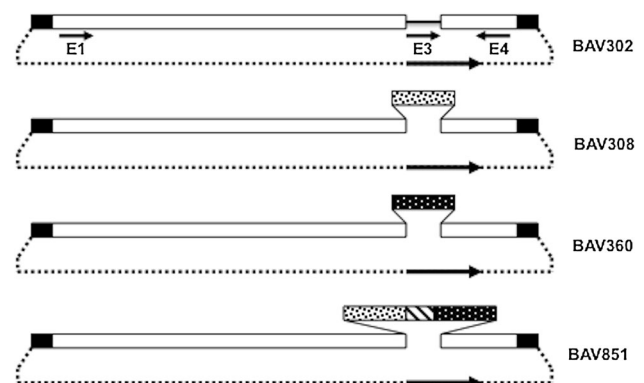


Fig. 1 Schematic representation of full length genomic DNA in plasmids. The origin of DNA sequences of BAdV-3 genome (hollow box), BHV-1 gDt DNA (box filled with black dots) BRSV gD DNA (box filled with white dots) and IRES DNA (box filled with strips) is depicted. Deleted early (E) three region is depicted by line. E1, E3, and E4 regions are marked. Arrows represent direction of transcription

Construction of Recombinant BAdV-3s

VIDO R2 cell monolayers in 60-mm dishes were transfected with 5–10 μg of *Pac*I-digested pFBAV360 or pFBAV851 recombinant plasmid using lipofectin. After 10–15 days of incubation at 37 °C, the transfected cells showing 50 % cytopathic effects were collected and freeze thawed three times, and the recombinant virus purified and propagated on MDBK cells.

Radiolabeling and Immunoprecipitation of Proteins

MDBK cells in 28 cm² wells were infected with 10 pfu or recombinant or wild-type BAdV-3 per cell. After 90 min of adsorption, cells were washed and incubated with MEM containing 2 % FBS. At different times post-infection, the cells were incubated in glucose free Dulbecco's modified Eagle's medium (DMEM) for 60 min before labeling with [³H] glucosamine (100 $\mu\text{Ci}/\text{well}$). After 12 h of labeling, the cells or medium was harvested. Proteins were immunoprecipitated from the medium or cells lysed with modified radioimmunoprecipitation (RIPA) buffer and analyzed by SDS-PAGE as described previously [22].

Animal Inoculation

Four to six-week-old cotton rats of either sex were divided into three groups of five animals each. After inducing anesthesia in the cotton rats with halothane, animals were inoculated twice at day 1 and day 21 by intranasal route with 100 μl of recombinant virus(s). Blood samples were taken at days 0 and 28 after primary inoculation to examine the development of BHV-1 gD specific, BRSV G specific and BAdV-3 specific antibodies by enzyme-linked immunosorbent assays (ELISA) and virus neutralization (VN) assays. At 28 days post-primary inoculation, animals were killed by an overdose of halothane. The lung and nasal secretions were collected separately to monitor the development of BHV-1 gD specific, BRSV G specific, and BAdV-3 specific mucosal IgA antibody response by ELISA.

Antigen-Specific ELISA

The level of antibodies specific for BHV-1 and BRSV in sera, lung, and nasal washes were determined by ELISA. Immunol-2 plates were coated with either purified truncated gD (0.01 $\mu\text{g}/\text{well}$) or BRSV (5 $\mu\text{g}/\text{well}$) and incubated with different dilutions of each sample. Antigen-specific IgG was detected using biotinylated rabbit anti-rat IgG. Antigen-specific IgA was measured by rabbit anti-rat IgA and horse peroxidase-conjugated goat anti-rabbit IgG.

Serum Neutralization Assay

Two-fold serial dilutions of heat-inactivated serum samples were incubated with 100 pfu of BHV-1 or 100 TCID₅₀ of BRSV for 1 h at 37 °C. The virus sample mixture was then plated onto confluent cells in six-well tissue culture plates. After 90 min adsorption, the mixture was removed and the cells were washed twice with PBS and incubated in media with (BHV-1) or without (BRSV) 0.7 % low melting agarose. Titers are expressed as reciprocals of the highest dilution that caused 50 % reduction in the number of plaques (BHV-1) or cytopathic effects (BRSV) relative to control.

Results

Construction of Recombinant BAdV-3 Expressing BRSV G

Initially, we constructed an E3-deleted full length BAdV-3 genomic clone (pFBAV307) containing BRSV original G (G_{ori}) coding sequence inserted in the E3 region in the same transcriptional orientation as E3. Transfection of VIDO R2 cells with *Pac*-I digested pFBAV307 DNA resulted in the development of cytopathic effects. The recombinant virus was named BAV307. Although restriction enzyme analysis of recombinant virion DNA suggested that BAV307 contained the G gene, immunoprecipitation analysis of BAV307 infected cells could not detect expression of recombinant G glycoprotein. Failure to detect G glycoprotein may be due to the aberrant splicing or instability of G glycoprotein transcripts in the nuclei of BAV307 infected cells.

To express optimum levels of G, we constructed a full length BAdV-3 genomic clone (pFBAV360) containing chemically synthesized G (G_{syn}) coding sequence (modified codon usage and removal of internal cryptic splice sites) inserted in E3 region in the same transcriptional orientation as E3. Transfection of VIDO R2 cells with *Pac*I digested plasmid pFBAV360 (Fig. 1) DNA produced cytopathic effects in 3 weeks. The infected cell monolayers were freeze thawed and recombinant virus named BAV360 was propagated in MDBK cells. The viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with *Bam*HI. As expected the 1.8 kb BAV307 DNA fragment was replaced with a 2.6 kb fragment in BAV360 (Fig. 2a).

Construction of Recombinant BAdV-3 Expressing BHV-1 gDt and BRSV G

In order to construct recombinant BAdV-3 expressing two vaccine antigens, initially we constructed a gene cassette

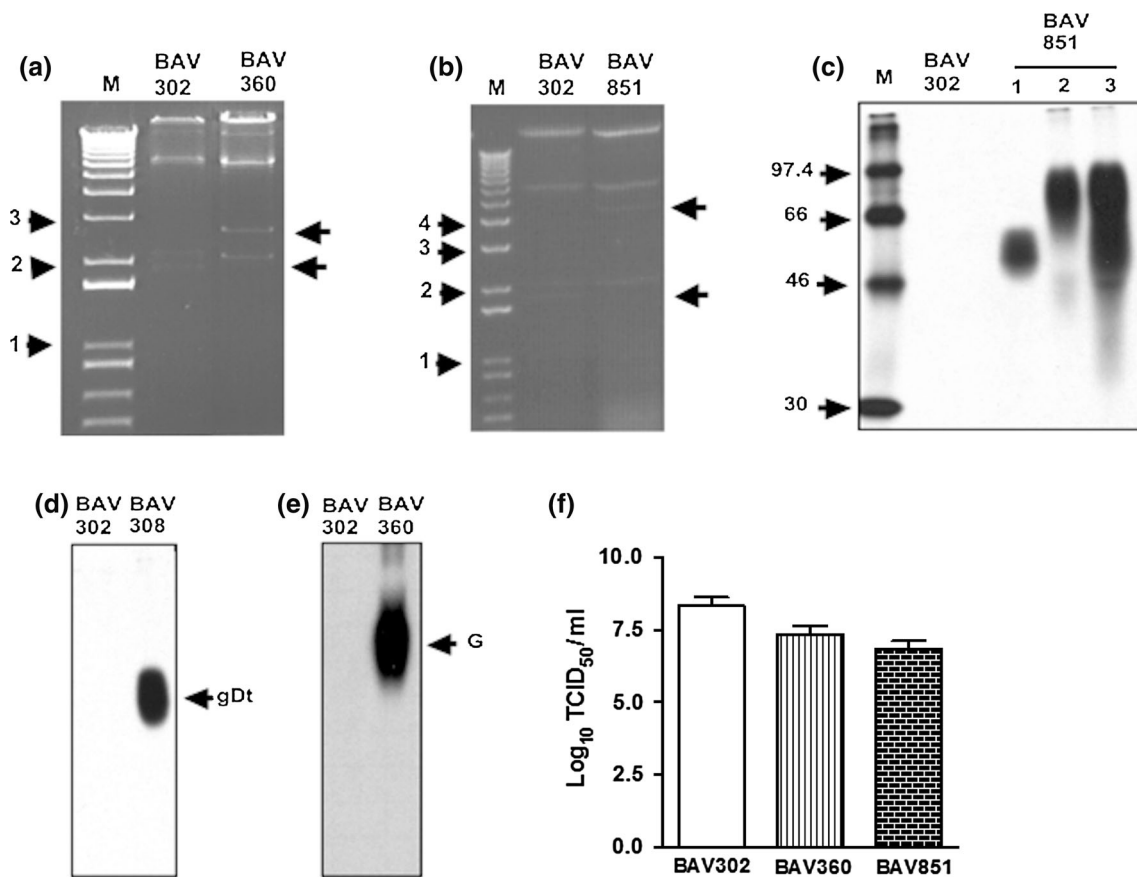


Fig. 2 Analysis of recombinant BAdV-3s. **a, b** Restriction enzyme analysis of recombinant BAdV-3 genome. Viral DNAs were extracted from MDBK cells infected with indicated recombinant BAdV-3 and digested with *Bam*HI. Sizes of marker (M) in kilobases are shown to the left of the panel. The diagnostic bands are indicated by *arrow head* to the right of the panel. **c–e** Immunoprecipitation of recombinant proteins. Proteins from lysates of [³H]-glucosamine-labeled MDBK cells infected with indicated recombinant BAdV-3s were immunoprecipitated with BHV-1 gD specific MABs (panel **c**,

lane 1; panel **d**), BRSV G-specific MAB (panel **c**, lane 2; panel **e**) or mixture of gD plus G-specific MABs (panel **c**, lane 3 and BAV302) and analyzed on 10 % SDS-PAGE under reducing conditions. Molecular size markers (M) in kDa are shown to the left of the panel (**f**). Growth of recombinant BAdV-3s. Near confluent monolayers of MDBK cells were infected with recombinant BAdV-3. At 48 h post-infection, the cells were harvested, freeze thawed and virus was titrated on MDBK cells as described [13]

(gDt-IRES-G) in which gDt and G genes are separated by internal ribosomal entry site (IRES) sequence of endomyocarditis virus (EMC). This will produce a single mRNA in which the 5' gene will be translated by the ribosomal scanning method, while the 3' gene will be translated by the IRES mechanism (afforded by EMC sequence). Using homologous recombination machinery of *E. coli* [23], the gDt-IRES-G gene cassette was inserted in E3 region of pFBAV302 plasmid [18] in the same orientation as E3 creating plasmid pFBAV851 (Fig. 1). The *Pac*I digested plasmid pFBAV851 DNA transfected in VIDO R2 cells produced cytopathic effects in 15 days. Infected monolayers were freeze thawed and recombinant virus named BAV851 was propagated on MDBK cells. The

viral DNA was extracted and analyzed by agarose gel electrophoresis after digestion with *Bam*HI. As expected, the 1.8 kb BAV302 DNA fragment was replaced with a 4.1 kb fragment in BAV851 (Fig. 2b).

Analysis of Expression of Recombinant Vaccine Antigens

To identify the products expressed by recombinant BAdV-3s, MDBK cells were infected with recombinant BAV308, BAV360, or BAV851 and metabolically labeled with [³H] glucosamine for different time periods. To monitor the expression of recombinant proteins, infected cell lysates or supernatants were immunoprecipitated with pool of gD

specific MAbs [20] or G specific MAb 70 [21] and analyzed by SDS-PAGE. As seen in Fig. 2d, anti-gD MAbs recognized a protein of 61 kDa in supernatants of BAV308 infected cells. Similarly, anti-G MAbs recognized a protein of 95 kDa in lysates of BAV360 infected cells (Fig. 2e). No such proteins could be detected in BAV302 infected cells (Fig. 2d, e).

Anti-gD MAbs recognized a protein of 61 kDa in supernatants of BAV851 infected cells (Fig. 2c, lane 1). Similarly, anti-G MAbs recognized a protein of 95 kDa in the lysates of BAV851 infected cells (Fig. 2c, lane 2). As expected, a pool of anti-gD and anti-G MAbs recognized proteins of 61 and 95 kDa in BAV851 infected cells (Fig. 2c, lane 3). No such proteins could be detected in BAV302 infected cells (Fig. 2c).

Growth of Recombinant BAdV-3s

To determine the virus titer, MDBK cells were infected with indicated recombinant BAdV-3 at an MOI of 5 TCID₅₀/cell. At 48 h post-infection, the cells were harvested, freeze thawed and analyzed as described [13, 18]. As seen in Fig. 2f, the titer of BAV360 and BAV851 was 0.5 log and 1.0 log lower, respectively, to BAV302.

Immune Response in Cotton Rats Immunized with Recombinant BAdV-3

Earlier, cotton rats have been used as a small animal model for determining the ability of recombinant BAdV-3s to induce antigen-specific immune responses [16, 18]. To determine the ability of recombinant BAdV-3 to induce BHV-1 gD and/or BRSV-specific immune responses, 4- to 6-week-old cotton rats of either sex were divided into groups were inoculated twice at day 1 and day 21 by intranasal route with 10⁷ plaque forming units (PFU) of BAV302, BAV308, BAV360, or BAV851. Blood samples were taken at days 0 and 28 after primary inoculation to examine the development of BHV-1gD specific and BRSV-specific IgG antibodies by ELISA and neutralizing response by VN assays as described [4, 9, 16]. At 28 days post-primary inoculation, animals were killed by an overdose of halothane. The lung and nasal secretions were collected separately to monitor the development of BHV-1 gD and BRSV-specific mucosal IgA antibody response by ELISA [4, 9, 16]. As seen in Fig. 3, animals immunized with BAV308-induced significant levels of gD specific IgG (panel a) and IgA titers (panel c), or BHV-1 specific neutralization titers (panel b) compared to animals immunized with BAV302. Similarly, animals immunized with BAV360-induced significant levels of gG specific IgG titers (panel a) and IgA titers (panel c) or BRSV-specific neutralizing titers (panel b) compared to animals

immunized with BAV302. Interestingly, animals immunized with BAV851-induced IgG (panel a) and IgA titers (BHV-1 gD and BRSV specific) (panel c), or neutralizing antibody titers (BHV-1 and BRSV specific) (panel b), which were significantly different compared to animals immunized with BAV302.

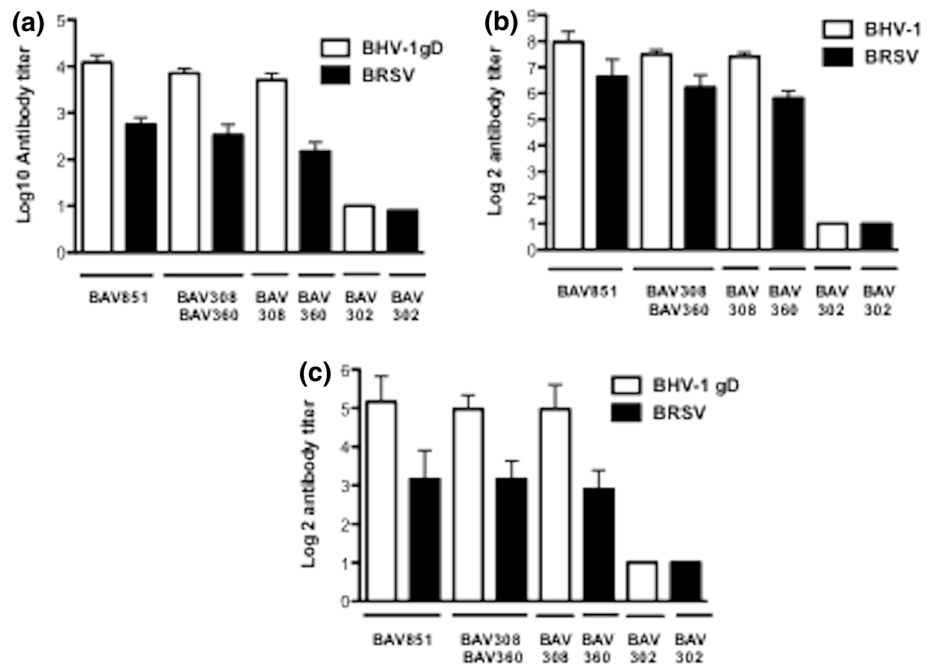
Discussion

Vaccination has proved to be an effective means of controlling respiratory viral diseases in animals. As vaccines developed by conventional means have some inherent disadvantages, efforts have concentrated on developing genetically engineered safe, and efficacious veterinary vaccines. Moreover, since vaccines for veterinary use have to be inexpensive and easy to deliver, we have been developing BAdV-3 vector for delivering vaccine antigens to calves. Earlier, we have described the construction and evaluation of recombinant BAdV-3 expressing BHV-1 gDt [18] as vaccine delivery vector [15]. In this report, we describe the construction of recombinant BAdV-3 (a) expressing BRSV G or (b) co-expressing BRSV G and BHV-1 gDt as individual proteins and tested their ability to induce mucosal and systemic immune responses in cotton rats.

Earlier, several regions of BAdV-3 genome have been used for the insertion of foreign genes [13]. Since E3 deleted BAdV-3 grows to high titers [18], we choose to insert the BRSV G_{ori} gene in E3 region. The insertion of BRSV G_{ori} in the E3 region of BAV302 resulted in the recovery of recombinant virus (BAV307), however, expression of G_{ori} could not be detected in BAV307 infected cells. This could be due to aberrant splicing or instability of G_{ori} transcripts in the nuclei [8] of BAV307 infected cells. To enhance the expression of G protein, the G ORF was resynthesized by changing the nucleotide sequence, which helped to remove the splice-donor consensus sequences and also adjusted the codon bias toward bovine. The recombinant virus BAV360 containing synthetic G inserted in E3 region of BAV302 expressed G protein in appreciable amounts. Similar approach has been used to express bovine viral diarrhea virus E2 antigen in BAdV-3 [16].

Expression of multiple vaccine antigens of the same or different pathogens by a replication-competent adenovirus appears to be attractive approach for developing economical vaccines for use in veterinary medicine. A number of strategies have been used to express multiple antigens from a single replication competent or replication defective HAdV [24–26]. As both BRSV and BHV-1 are involved in the causation of respiratory disease in calves [1, 5], we engineered BAV302 to express BHV-1 gDt and BRSV G

Fig. 3 Antibody response in serum and nasal secretions of cotton rats. Cotton rats were immunized intranasally with indicated recombinant BAdV-3. **a** IgG ELISA titers in sera. **b** Virus neutralization titers in sera. **c** IgA ELISA titers in nasal secretions. Error bars represent the standard deviation of the mean



from a single mRNA using IRES sequences [27] from EMCV. The recombinant BAV851 efficiently expressed gD and G glycoproteins. Although efficiency of IRES dependant second gene expression in a bicistronic construct is usually low [28], we did not observe any difference in the expression of G protein in BAV360 or BAV851 infected cells.

Intranasal immunization of cotton rats with recombinant BAV851 expressing two vaccine antigens (BHV-1 gD and BRSV G) induced antigen-specific immune responses. Interestingly, the magnitude of immune response induced by gD or G expressed by recombinant BAV851 appeared similar to immune response induced by gD expressed by recombinant BAV308 or G expressed by recombinant BAV360. Moreover, the titer of BAV851 expressing two vaccine antigens of different pathogens was 1 log lower than BAV302. Thus, our approach for developing single recombinant BAdV-3 expressing two or more vaccine antigens of economically important respiratory pathogens of calves should help in developing economical vaccines for use in veterinary medicine.

In conclusion, our results demonstrate the feasibility of co-expressing vaccine antigens of BHV-1 gD and BRSV G, two economically important respiratory pathogens of calves in a single BAdV-3 vector and inducing antigen-specific immune responses in cotton rats similar to response induced by two separate recombinant BAdV-3 expressing single vaccine antigen.

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References

1. Tikoo, S. K., Campos, M., & Babiuk, L. A. (1995). Bovine herpesvirus-1 (BHV-1): Biology, pathogenesis and control. *Advances in Virus Research*, 45, 191–223.
2. Schwyzer, M., & Ackermann, M. (1996). Molecular virology of ruminant herpesvirus. *Veterinary Microbiology*, 53, 17–29.
3. Kaashoek, M. J., Moerman, A., Madic, J., Rijsewijk, F. A. M., Quak, J., Gielkens, A. L. J., et al. (1994). A conventionally attenuated glycoprotein gE negative strain of bovine herpesvirus type 1 is an efficacious and safe vaccine. *Vaccine*, 12, 439–444.
4. Van Drunen Little-van den Hurk, S., van Donkersgoed, J., Kowalaski, J., van den Hurk, J. V., Harland, R., Babiuk, L. A., et al. (1994). A subunit gIV vaccine produced by transfected mammalian cells in culture induces mucosal immunity against bovine herpesvirus-1 in cattle. *Vaccine*, 12, 1295–1302.
5. Stott, E. J., Thomas, L. H., Collins, P. A., Crouch, S., Jebbett, J., Smith, G. S., et al. (1980). A survey of virus infections of respiratory tract of cattle and their association with disease. *Journal of Hygiene*, 85, 257–270.
6. Buchholz, U. J., Finke, S., & Conzelmann, K. K. (1999). Generation of bovine respiratory syncytial virus (BRSV) from cDNA: BRSV NS2 is not essential for virus replication in tissue culture, and the human RSV leader region acts as a functional BRSV genome promoter. *Journal of Virology*, 73, 251–259.
7. Taylor, G., Thomas, L. H., Furze, J. M., Cook, R. S., Wyld, S. G., Lerch, R., et al. (1997). Recombinant vaccinia viruses expressing the F, G, or N, but not the M2, protein of bovine respiratory syncytial virus (BRSV) induce resistance to BRSV challenge in the calf and protect against the development of pneumonic lesions. *Journal of General Virology*, 78, 3195–3206.

8. Kuhnle, G., Heinze, A., Schmitt, J., Giesow, K., Taylor, G., Morrison, I., et al. (1998). The class II membrane glycoprotein G of bovine respiratory syncytial virus, expressed from a synthetic open reading frame, is incorporated into virions of recombinant bovine herpesvirus 1. *Journal of Virology*, *72*, 3804–3811.
9. Taylor, G., Rijsewijk, F. A. M., Thomas, L. H., Wyld, S. G., Gaddum, R. M., Cook, R. S., et al. (1998). Resistance to bovine respiratory syncytial virus (BRSV) induced in calves by a recombinant bovine herpesvirus-1 expressing the attachment glycoprotein of BRSV. *Journal of General Virology*, *79*, 1759–1767.
10. Baxi, M. K., Babiuk, L. A., Mehtali, M., & Tikoo, S. K. (1999). Transcription map and expression of bovine herpesvirus-1 glycoprotein D in early region 4 of bovine adenovirus-3. *Virology*, *261*, 143–152.
11. Idamakanti, N., Reddy, P. S., Babiuk, L. A., & Tikoo, S. K. (1999). Transcription mapping and characterization of 284R and 121R proteins produced from early region 3 of bovine adenovirus type 3. *Virology*, *256*, 351–359.
12. Reddy, P. S., Idamakanti, N., Zakhartchouk, A. N., Baxi, M. K., Lee, J. B., Pyne, C., et al. (1998). Nucleotide sequence, genome organization and transcription map of bovine adenovirus type 3. *Journal of Virology*, *72*, 1394–1402.
13. Tikoo, S. K. (2001). Animal adenovirus as a live vaccine vector. *Recent Research Developments in Virology*, *3*, 495–503.
14. Reddy, P. S., Idamakanti, N., Chen, Y., Whale, T., Babiuk, L. A., Mehtali, M., et al. (1999). Replication defective bovine adenovirus type 3 as an expression vector. *Journal of Virology*, *73*, 9137–9144.
15. Zakhartchouk, A. N., Pyne, C., Mutwiri, G. K., Papp, Z., Baca-Estrada, M. E., Griebel, P., et al. (1999). Mucosal immunization of calves with recombinant bovine adenovirus-3: Induction of protective immunity to bovine herpesvirus-1. *Journal of General Virology*, *80*, 1263–1269.
16. Baxi, M. K., Deregt, D., Robertson, J., Babiuk, L. A., Schlapp, T., & Tikoo, S. K. (2000). Recombinant bovine adenovirus type 3 expressing bovine viral diarrhea virus glycoprotein E2 induces an immune response in cotton rats. *Virology*, *278*, 234–243.
17. Pankaj, K., Ayalew, L. E., Godson, D. L., Gaba, A., Babiuk, L. A., & Tikoo, S. K. (2014). Mucosal immunization of calves with recombinant bovine adenovirus-3 co-expressing truncated form of bovine herpesvirus-1 gD ad bovine IL-6. *Vaccine*, *32*, 3300–3306.
18. Zakhartchouk, A. N., Reddy, P. S., Baxi, M., Baca-Estrada, M. E., Mehtali, M., Babiuk, L. A., et al. (1998). Construction and characterization of E3 deleted bovine adenovirus type 3 expressing full length and truncated form of bovine herpesvirus type 1 glycoprotein gD. *Virology*, *250*, 220–229.
19. Mapletoft, J. W., Oumouna, M., Townsend, H. G., Gomis, S., Babiuk, L. A., & van Drunen Littel-van den Hurk, S. (2006). Formulation with CpG oligodeoxynucleotides increases cellular immunity and protection induced by vaccination of calves with formalin-inactivated bovine respiratory syncytial virus. *Virology*, *353*, 316–323.
20. Hughes, G., Babiuk, L. A., & van Drunen van den Hurk, S. (1988). Functional and topographical analysis of epitopes on bovine herpesvirus-1 glycoprotein gIV. *Archives of Virology*, *103*, 47–60.
21. Furze, J., Wertz, G., Lerch, R., & Taylor, G. (1994). Antigenic heterogeneity of the attachment protein of bovine respiratory syncytial virus. *Journal of General Virology*, *75*, 363–370.
22. Tikoo, S. K., Zamb, T. J., & Babiuk, L. A. (1993). Analysis of bovine herpesvirus 1 glycoprotein gIV truncations and deletions expressed by recombinant vaccinia viruses. *Journal of Virology*, *67*, 2103–2109.
23. Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., & Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in *Escherichia coli*. *Journal of Virology*, *70*, 4610–4805.
24. Bauzon, M., Castro, D., Karr, M., Hawkins, L. K., & Hermiston, T. W. (2004). Multiple expression from a replicating adenovirus using native viral promoters. *Molecular Therapy*, *7*, 5226–5534.
25. Ye, W. W., Manson, B. B., Chengalvala, M., Cheng, S. M., Zandle, G., Lubeck, M. D., et al. (1991). Co-expression of hepatitis B virus antigens by a non defective adenovirus vaccine vector. *Archives of Virology*, *118*, 11–27.
26. Zhang, R., & DeGroot, L. J. (2001). An adenoviral vector expressing functional heterogeneous proteins herpes simplex viral thymidine kinase and human interleukin-2 has enhanced in vivo antitumor activity against medullary thyroid carcinoma. *Endocrine-Related Cancer*, *8*, 315–325.
27. Jang, S. K., Krausslich, H. G., Nicklin, M. J., Duke, G. M., Palmenberg, A. C., & Wimmer, E. (1988). A segment of the 5' nontranslated region of encephalomyo-carditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of Virology*, *62*, 2636–2643.
28. Mizuguchi, H., Xu, Z., Ishii-Watabe, A., Uchida, E., & Hayakawa, T. (2000). IRES-dependent second gene expression is significantly lower than cap-dependent first gene expression in a bicistronic vector. *Molecular Therapy*, *1*, 376–382.