Attenuation of bovine herpesvirus type 1 by deletion of its glycoprotein G and tk genes and protection against virulent viral challenge

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\section*{ABSTRACT}

To develop a novel vaccine against infectious bovine rhinotracheitis (IBR), a bovine herpesvirus-1 (BoHV-1) mutant was constructed by deleting the genes for glycoprotein G (gG) and thymidine kinase (tk) through homologous recombination. The resulting sequences for both genes were shown to be correct and a gG expression defect was also confirmed. A parallel study of the BoHV-1 gG\textsuperscript{-}/tk\textsuperscript{-} and gG\textsuperscript{-}/tk\textsuperscript{-} mutants and wild type (wt) in 31 calves was performed at three different doses, 4 \times 10^5 PFU, 4 \times 10^6 PFU and 4 \times 10^7 PFU. Compared to wt BoHV-1, inoculation of BoHV-1 gG\textsuperscript{-}/tk\textsuperscript{-} and gG\textsuperscript{-}/tk\textsuperscript{-} produced no clinical signs and the virus was not reactivated by dexamethasone (dex). Inoculation of BoHV-1 gG\textsuperscript{-}/tk\textsuperscript{-} at the doses of 4 \times 10^5 and 4 \times 10^6 PFU provided full clinical protection for the cattle against wt BoHV-1 challenge at 4 \times 10^7 PFU/calf. Although the mutants were associated with significantly lower levels of serum neutralizing antibody, interferon gamma (IFN-\gamma) and tumor necrosis factor alpha (TNF-\alpha) than wt BoHV-1 on days 3, 5 and 7 after immunization, stimulation of IFN-\beta by BoHV-1 gG\textsuperscript{-}/tk\textsuperscript{-} was significantly higher than that of wt BoHV-1 and gG\textsuperscript{-}/tk\textsuperscript{-} on days 3 and 5. We conclude that BoHV-1 gG\textsuperscript{-}/tk\textsuperscript{-} was attenuated adequately and that it maintains the ability to stimulate immune protection. Therefore, it may be a promising candidate for a marker vaccine against IBR.

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\section*{1. Introduction}

Bovine herpesvirus-1 (BoHV-1) causes infectious bovine rhinotracheitis (IBR). In addition, it can lead to a wide variety of other clinical syndromes such as conjunctivitis, abortion, balanoposthitis and vulvovaginitis [1]. The virus in infected cows may undergo phases of productive infection, latency and reactivation [2]. Vaccination is an effective measure against IBR, but the inactivated and conventional modified live virus vaccines have a number of disadvantages. The inactivated vaccines are usually poor immunogens, while conventional live vaccines lack the serological markers that can differentiate immunization and natural infection [3]. Therefore, in the last decade, the use of gene deletion marker vaccines for the control of BoHV-1 has been investigated widely.

The standard for gene deleted vaccines requires that mutants are attenuated, cannot be reactivated from latency and hold serological markers [3]. Genes that are virulence-related but non-essential for viral viability are the targets to be deleted. Some BoHV-1 genes encoding glycoproteins on the virion envelope that are essential for virus morphogenesis, cell attachment, viral spread and tropism but are not essential for virus existence can be deleted and their proteins absence used as a serological marker [1,4]. For instance, commercially available vaccines that contain a glycoprotein E (gE) deletion or gE\textsuperscript{-} and thymidine kinase (tk) gene double deletion gE\textsuperscript{-}/tk\textsuperscript{-} have been used successfully in eradication programs for IBR in several European countries [5]. However, the recent strategy to improve the marker vaccines for IBR is directed towards the deletion of gene sequences associated with immunosuppression [1].

The glycoprotein gG is a secreted glycoprotein and is conserved in the majority of alpha herpesviruses. It can elicit immunosuppression by functioning as broad-spectrum chemokine binding protein, thereby blocking the interaction of chemokines with specific receptors and glycosaminoglycans [6,7]. Although the gG gene is not essential for viral growth, the gG protein can elicit a strong antibody response in experimental and natural infections, and thus could be exploited as a diagnostic marker [8]. To develop an improved vaccine strain, we have constructed a BoHV-1 mutant with a gG (US4) and tk (UL23) double deletion. The virulence, probability of reactivation and immunogenicity were evaluated in calves by using a mutant with the tk and gE genes deleted as the control. The results
indicated that the gG−/tk− deletion mutant can induce a higher level of interferon beta (IFN-β) on days 3 and 5 post-infection (dpi), and can provide full clinical protection against challenge with wild-type (wt) BoHV-1.

2. Materials and methods

2.1. Virus and cells

The wt BoHV-1 was designated as IBRV HB06 and stored as No. CCTCC V201024 in the Collection Center of Tissue Culture of China (CCTCC) at Wuhan University. It was isolated originally from the nasal swabs of clinically diseased cattle and characterized by this laboratory. The wt BoHV-1 and its recombinants were propagated and titrated respectively in a bovine kidney epithelial cell line (Madin–Darby bovine kidney, MDBK) as described previously [9].

2.2. Construction of recombinant plasmids

The relevant genome sequences were obtained from GenBank (AJ004801). The primers listed in Table S1 in the supplementary data were designed to delete the whole gC gene (1348 bp) and most of the tk gene (650 bp). The flanking upstream and downstream sequences, including parts of US3 and US6, were amplified. HindIII/KpnI and BamHI/EcoRI sites were inserted at the upper and lower arm termini by PCR, respectively (Table S1). Similarly, KpnI and BamHI sites were introduced at the termini of the enhanced green fluorescent protein (egfp) gene expression cassette by PCR.

The resultant gC upper arm, egfp gene, and gG lower arm were cloned sequentially into pcDNA3.1 (+) myc-HisB to obtain pcDNA3.1-ZYP. To knock out the egfp gene, another plasmid containing only the gC upper and lower homologous arms was constructed and designated pcDNA3.1-ZYP-1.

Similarly, the flanking upper and lower sequences of the tk gene, including parts of UL22 and UL24, were amplified, and XbaI/EcoRI and EcoRI/HindIII sites were introduced at the upper and lower arm terminals by PCR. The tk upper arm, egfp gene, and tk lower arm were cloned sequentially into pBluescriptIIISK (+) to obtain the recombinant plasmid pZF08-16. To knock out the egfp gene between the tk homologous arms, plasmid pZF08-16-1 with only the tk homologous arm was constructed.

2.3. Construction of BoHV-1 mutants

In this study, both the single deletion mutant BoHV-1 gG− and the double deletion mutant BoHV-1 gG−/tk− were produced by two rounds of screening (Fig. 1) through forward selection of green fluorescent plaques for BoHV-1 gG−/EGFP+ (Fig. 1C) and gG−/tk−/EGFP+ (Fig. 1D), and backward selection of non-fluorescent plaques for BoHV-1 gG− (Fig. 1C) and gG−/tk− (Fig. 1D).

For the transfection procedure, the linear target DNA fragments, including the homologous gene arms of either the gG gene (pcDNA3.1-ZYP) or the tk gene (pZF08-16), were co-transfected using the calcium phosphate method [10]. The mutants were analyzed subsequently to determine the deletion of the gG and tk genes by Western blot assay of the expression of gG, and by various PCR procedures with the primers (Table S1) specific to different fragments followed by sequencing of the PCR products (Sangon Biotech, Shanghai). The BoHV-1 gG−/tk− mutant was constructed by Dr. Zhengfei Liu in this laboratory using a similar strategy. For the Western blot assay, a specific polyclonal antibody (1:200 dilution) against BoHV-1 gG recombinant protein was prepared by this laboratory [11] and the other steps were executed as reported previously [12].

In addition, the HindIII genome digestion profiles for BoHV-1 gG−/tk− and wt BoHV-1 were compared to confirm the deletion further [13].

2.4. Animals and groups

This study was carried out in strict accordance with the recommendations in the China Regulations for the Administration of Affairs Concerning Experimental Animals 1988 and the Hubei Regulations for the Administration of Affairs Concerning Experimental Animals 2005. The protocol was approved by China Hubei Province Science and Technology Department (Permit Number: SYXX(ER) 2010-0029).

Thirty-one 4-month-old male Holstein calves that were seronegative for BoHV-1 were allotted randomly to four groups as follows. In group 1, four calves were mock-infected with PBS as the blank control. In groups 2, 3 and 4, nine calves were infected by intranasal instillation (INL) with BoHV-1 gG−/tk−, BoHV-1 gG−/tk− and wt BoHV-1 respectively. In each infected group, the nine calves were divided randomly into three sub-groups that were inoculated with 4 × 10⁵ PFU, 4 × 10⁶ PFU or 4 × 10⁷ PFU respectively. Each subgroup was housed in a separate room to avoid mutual interference.
The animals were euthanized at the end of the experiments or when necessary during the experiment.

In addition, six female Japanese white rabbits were purchased from Hubei Center of Disease Control and divided into two groups. The first group was infected by INI at $4 \times 10^7$ PFU/calv with BoHV-1 gG−/tk− and the second group with $4 \times 10^7$ PFU wt BoHV-1.

2.5. Safety testing of the double mutants in animals

2.5.1. Clinical observation

The animals were monitored daily for any abnormal signs, and the rectal temperature of the calves was determined at 10:00 AM for up to 14 dpi.

2.5.2. Nasal/ocular swabs, blood collection and virus detection

Whole blood with and without heparin was collected on days 1, 2, 3, 5, 7, 14, 21 and 28 pi. Nasal swabs were obtained from the calves and rabbits from days 1 to 17 pi at intervals of 2 days and placed in tubes containing 2 ml DMEM with penicillin G (500 IU) and Na streptomycin sulfate (500 μg). All the samples were stored at −70 °C until use. The serum neutralization (SN) titer was determined by a conventional method [13]. Virus quantitation in the nasal swabs was performed by plaque assay on MDBK cells in 24-well cell culture plates. Serum cytokines IFN-γ, IL-4, IFN-β and TNF-α were assayed using commercial enzyme-linked immunosorbent assay (ELISA) kits produced by Applied MABTECH, Sweden, Applied Pierce & Endogen, USA, CUSABIO BIOTECH Co., Ltd, USA and Applied R&D, USA respectively.

2.5.3. Reactivation assay

Three calves from each group, representing different inoculation doses within that group, were selected for the reactivation study. Dexamethasone (dex) at 0.1 mg/kg bodyweight (bwt) was injected intramuscularly into the calves and in six rabbits for 5 consecutive days to reactivate putatively latent virus, according to a previous report [14]. Nasal swabs were collected from the calves and rabbits on days 1, 3, 5 and 7 after injection of dex to detect viral shedding. After clinical observation, the rabbits were euthanized. Their trigeminal ganglia were collected and subjected to viral isolation and PCR detection with the primers 13/14 and 15/16 (Table S1).

2.6. Protection assay

Twenty-eight days following the test intranasal inoculation, all the calves were challenged with the wt BoHV-1 strain at $4 \times 10^7$ PFU/calv by INI. The animals were monitored clinically for up to 21 days by checking the clinical signs and rectal temperature each day. Nasal and ocular swabs were taken daily for 21 days to test for viral shedding. The calves were euthanized subsequently, and the apical lobes of the lungs were collected and fixed in 10% buffered neutral formalin, embedded in paraffin wax, sectioned and subjected to hematoxylin/eosin (H&E) staining for histopathological analysis.

2.7. Bovine IFN-α/β transcription in MDBK cells infected BoHV-1

The expression of bovine IFN-α/β at the mRNA level was evaluated by quantitative real time polymerase chain reaction (qRT-PCR). The MDBK cells, grown in six-well plates, were either mock-infected or infected with 2 PFU/cell of wt BoHV-1 or BoHV-1 gG−/tk−. Total RNA was extracted from the cells at 6 and 12 h post-infection using a rapid high-purity total RNA extraction kit (BioTeke Corporation, China). Relative IFN-α/β mRNA levels were quantified using bovine β-actin as the control gene to normalize the template input. The amplification was performed in 96-well PCR plates (ABI, USA) with 2 μl cDNA and SYBR Green qPCR Master Mix (2×) (ABI, USA) per well in triplicate (ABI 7500 real-time PCR detection system). SYBR green fluorescence was measured over the course of 50 cycles. The comparison of the transcription of different related genes was performed by taking the lowest level of transcription of the gene as 1.

2.8. Statistical analysis

The data were analyzed using analysis of variance (ANOVA). Values of $P<0.05$ and $P<0.01$ were considered to be statistically significant and very significant, respectively.

3. Results

3.1. Identification of the recombinant BoHV-1 gG−/tk− mutant

The three intermediate mutants (BoHV-1 gG−/EGFP+, BoHV-1 gG− and BoHV-1 gG−/tk−/EGFP+) (Fig. 1) were confirmed by
fluorescent screening and PCR detection (data not shown). Regarding the identification of the final construct, BoHV-1 gG−/tk−, PCR was able to amplify a fragment of 1687 bp from the genome, including the whole gG gene, of wt BoHV-1, while it produced only a truncated fragment of 339 bp from the gG−/tk− mutant with the primer set P13/14 (Fig. 2A, Lanes 1 and 3). Similarly, PCR generated a tk gene product of 847 bp from wt BoHV-1 but one of only 207 bp from the gG−/tk− mutant, with the primer set P15/16 (Fig. 2A, Lanes 2 and 4). These findings indicated the successful deletion of the gG and tk genes. The PCR products of 339 bp for the gG gene and 207 bp for the tk gene from the mutant were custom-sequenced and the results showed similarities of 100% to the original sequences.

Western blot assay demonstrated that wt BoHV-1 reacted strongly with rabbit anti-gG polyclonal antibody and produced a band of 65 kDa, which is equal to the molecular mass of gG. However, the BoHV-1 gG−/tk− did not react with the antiserum to gG and no band was developed, indicating the deficiency in gG expression (Fig. 2B).

The genome HindIII digestion profiles of BoHV-1 gG−/tk− and wt BoHV-1 were compared. The gG deletion resulted in truncation of the fragment, which decreased from 8.2 kb in wt BoHV-1 to 6.9 kb in BoHV-1 gG−/tk− as expected. Other fragments did not show this change. This confirmed the correct construction of the mutant further (Fig. 2C).

3.2. Clinical signs

The animals in group 4, inoculated with wt BoHV-1, displayed apparent clinical signs. From day 3 post-inoculation, two calves showed typical nasal and ocular discharge, reddened conjunctivae, anorexia and sluggishness, and one of them showed a cough. All signs persisted for 5 days. However, the groups given the mutant viruses (groups 2 and 3) did not display any abnormal clinical signs. All the animals survived the observation period.

The rectal temperatures of the calves in group 4 were higher than those of the other groups, and a temperature above 40 °C was maintained for 4 days in the subgroup given the highest dose (4 × 10^7 PFU), while in the mutant-inoculated and uninfected control groups, the temperature fluctuated within the normal range from 38.5 to 39.5 °C [15].

3.3. Virus shedding and reactivation

The group 4 calves, inoculated with wt BoHV-1, showed typical periods of infection, with dose-dependent viral shedding time and intensity. The primary infection, as determined by the detection of viral shedding in nasal swabs, persisted for 13 dpi. The peak (10^9 PFU/ml) viral titer was reached at 5 dpi for the subgroups given 4 × 10^7 PFU and 4 × 10^6 PFU (Fig. 3) and at 7 dpi for the subgroup given 4 × 10^5 PFU (data not shown). Subsequently, the viral titers decreased gradually and they were undetectable at 15 dpi.

Comparatively, calves given both BoHV-1 gG−/tk− and gG−/tk− mutants showed significantly less viral shedding and a shorter shedding period than those given wt BoHV-1. The BoHV-1 gE−/tk− was detected at 1 dpi, achieved a peak of 10^8–9 PFU/ml at 3 dpi, and maintained viral shedding until 7 dpi for the 4 × 10^7 PFU subgroup (Fig. 3) and 5 dpi for the 4 × 10^6 PFU subgroup. For the 4 × 10^5 PFU subgroup, BoHV-1 gE−/tk− was only detected at 1 dpi (data not shown).

Interestingly, the BoHV-1 gG−/tk− was shed for even less time than BoHV-1 gE−/tk− (Fig. 3). This mutant could be detected for 5 dpi in the subgroup given the highest dose (4 × 10^7 PFU), was detectable only on the first day in the 4 × 10^6 PFU subgroup, and was undetectable at all times in the 4 × 10^5 PFU subgroup (Fig. 3). However, the virus titers were lower than those in the BoHV-1 gE−/tk− group (Fig. 3).

After withdrawal of dex, the wt BoHV-1 was detected in nasal swabs for 5 days in the 4 × 10^7 PFU and 4 × 10^6 subgroups and for only 3 days in the 4 × 10^5 PFU subgroup. However, neither the BoHV-1 gG−/tk− nor the gE−/tk− mutant could be reactivated by dex injection (Fig. 3A and B).

Similar findings were confirmed in the rabbits. Dex injection only reactivated the wt BoHV-1, resulting in recurrence of viral shedding (Fig. 3C). However, PCR detected the viral DNA successfully for both wt BoHV-1 and the gG−/tk− mutant in the trigeminal ganglia, confirming the latent status (data not shown).
The peripheral blood samples were frozen and thawed three times and inoculated onto MDBK cell monolayers to detect the virus by observing cytopathogenie effect (CPE). Neither wt BoHV-1 nor the two mutants were isolated from the blood.

3.4. Serum neutralizing (SN) antibody titers

The serum samples obtained at 0, 7, 14, 21, and 28 dpi were tested for SN antibody. The highest titers at all time points were induced by wt BoHV-1. For the subgroup given 4 × 10^7 PFU, the titer was 8.26 ± 3.12 at 7 dpi and reached a peak of 1:30.15 at 28 dpi. The gG/ťk' mutant stimulated its highest SN antibody titer of 1:5.89 at 28 dpi, while the gE/ťk' mutant only induced 1:2.3 at 28 dpi. The negative control calves did not produce any titers of SN antibodies. Within each group, the antibody titers increased in a dose- and time-dependent manner. The difference between the titers induced by wt BoHV-1 and the two mutants was significant (P=0.014). Furthermore, the SN antibody response stimulated by the gG/ťk' mutant was stronger than that induced by the gE/ťk' mutant (P=0.04).

3.5. Cytokine production

For the subgroup given the highest dose inoculation, although on the first dpi the levels of IFN-β in the wt BoHV-1 group were higher than those in both the gG/ťk' (P=0.006) and the gE/ťk' mutant group (P=0.004) (Fig. 4C), the gG/ťk' mutant produced the highest level of IFN-β, and a significant difference was maintained between this strain and the wt BoHV-1 group (P=0.022) and between the groups given the mutants (P=0.012) from 3 to 5 dpi. The mean level of IFN-β was 259 ± 51 pg/ml for the gG/ťk' mutant group and 187 ± 31 pg/ml for the gE/ťk' mutant group, while it was 115 ± 26 pg/ml for the wt BoHV-1 group at 5 dpi. At 7 dpi, there was no statistical difference among the three infected groups (Fig. 4C). In contrast to IFN-β production, the concentrations of IFN-γ and TNF-α yielded by wt BoHV-1 were significantly higher than those induced by the gG/ťk' and gE/ťk' mutants. For IFN-γ production, the wt BoHV-1 subgroup given the highest dose (4 × 10^7 PFU) showed 960 ± 64 pg/ml at 1 dpi, which decreased to a low level (577 ± 15 pg/ml) at 3 dpi, but increased again at 5 dpi to a higher peak level (1910 ± 79 pg/ml) at 7 dpi (Fig. 4A). The lower doses (4 × 10^5 and 4 × 10^6) stimulated slow production of IFN-γ at a lower level (data not shown). Similar to IFN-γ, the wt BoHV-1 subgroup was shown to have a significant increase of TNF-α (20.44 ± 110 pg/ml) at 1 dpi, then maintained a low level (<600 ± 70 pg/ml) for 2 days that increased again at 5 dpi and reached a new peak of 41.55 ± 900 pg/ml at 7 dpi (Fig. 4B). The lower doses of inoculated virus stimulated lower levels of TNF-α (data not shown).

Unlike IFN-β production, the overall concentrations of IFN-γ and TNF-α were not different between the two mutants. Compared with the mock inoculated group, all the viral inoculation groups showed significantly higher IFN-γ/β and TNF-α production (P=0.001). However, none of the BoHV-1 strains generated a significant increase of IL-4.

The production of IFN-β and IFN-α by MDBK cells was explored further by qRT-PCR. At 6 hpi, BoHV-1 gG/ťk' infection stimulated 30-fold higher levels of IFN-β transcription than wt BoHV-1 (P=0.001) (Fig. 4D), while at 12 h the difference decreased to 2.97-fold (data not shown). However, IFN-α showed no apparent transcription in this assay.

3.6. Clinical signs in calves after challenge

At 28 dpi, groups 1 (unvaccinated), 2 (BoHV-1 gG/ťk') and 3 (BoHV-1 gE/ťk') were challenged with 4 × 10^7 PFU wt BoHV-1 by INI. No deaths occurred in any of the groups. In the low dose subgroups, one calf in group 2 and two calves in group 3 secreted clear nasal discharge. In addition, only two calves from group 3 had yellowish mucoid ocular discharge. Besides these, calves in the highest dose (4 × 10^7 PFU) subgroup of group 3 experienced fever (>40 °C) for 2 days, but the temperature fluctuations in group 3 and
group 4 were less than 1 °C and were within the normal range between 38.5 °C and 39.5 °C (Fig. 5B). However, all the members of group 1 had severe typical clinical signs (Table 1) and the rectal temperature increased by more than 1 °C (Fig. 5B).

3.7. Virus shedding after challenge

After nasal challenge, wt BoHV-1 could be detected in nasal swabs from each group. Group 1 shed virus for 13 days; however, the virus shedding period in the groups given the mutants was only 7 days for all three doses of BoHV-1 gG−/tk− immunization, 7 days for the 4 × 10^7 PFU dose and 9 days for the lower doses in the BoHV-1 gE−/tk− subgroup (Fig. 5A). The virus titers from the mutant vaccination groups were significantly lower than those in the unvaccinated group (P=0.0052 for wt BoHV-1 vs BoHV-1 gG−/tk−; P=0.02 for wt BoHV-1 vs BoHV-1 gE−/tk−). In addition, only ocular swabs from the unvaccinated group were positive for virus detection.

![Fig. 5. Viral detection in nasal swabs and temperature change after wt BoHV-1 challenge. Nasal swabs of cattles immunized with different mutants were detected by a plaque assay on MDBK cells. (A) Viral shedding in nasal swabs. (B) Temperature change after viral challenge dose. The data represent the averages and standard deviations for each group. *Significant difference (P<0.05) and **very significantly different (P<0.01).](image)

![Fig. 6. Lung tissue histopathology. (A, B, C and D) Represent groups of wt BoHV-1, BoHV-1 gG−/tk−, BoHV-1 gE−/tk−, and the normal tissue group as the negative control, respectively. The normal pulmonary alveoli architecture disappeared and there were a large number of necrotic cells, inflammatory cells in the alveoli and some lymphoid cells infiltrated into the bronchial submucosa for the wt BoHV-1 group (A). The pulmonary alveoli architecture was intact and alveolar space was clean except alveolar walls were slightly thickened in the gG−/tk− (B) and gE−/tk− group (C) compared with the normal tissue structure (D).](image)

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Notes: (/) represents not done.
3.8. Histopathological examination

At 21 days post-challenge, the architecture of the pulmonary alveoli was intact in the gC-/tk- and gE-/tk- groups; the alveolar spaces were clean although the alveolar walls were slightly thickened. In contrast, the normal pulmonary alveoli architecture had disappeared in the unvaccinated group. There were a large number of necrotic and inflammatory cells in the alveoli with some lymphoid cell infiltration in the bronchial submucosa (Fig. 6).

4. Discussion

The evidence presented has demonstrated that we have constructed the double gene deleted mutant BoHV-1 gC-/tk- and gE-/tk- successfully. The cattle artificially infected by BoHV-1 gC-/tk- or the gE-/tk- mutant, did not show any clinical signs at 4 × 10³ PFU. Although the INI mutants survived for some time in the nasal cavity the duration and amount of viral shedding was significantly less for both mutants than for wt BoHV-1. This indicates that the survival ability of BoHV-1 gC-/tk- in vivo was greatly attenuated. These results were supported by the previous finding that single deletion of the tk, gE or gG gene leads to viral attenuation in calves, because both gE and gG are required for virulence [12,13]. The gE protein is required for anterograde neuronal spread in vivo and for neurovirulence [12], while gG is required for viral growth by cell-to-cell infection [6] and it blocks the interaction of chemokines with cellular receptors and glycosaminoglycans (GAGs) [7]. The exact reason why BoHV-1 gC-/tk- was even weaker than the gE-/tk- mutant requires further investigation.

In addition, latency-reactivation is an important mechanism for maintenance of BoHV-1. Although injection of deg reactivated wt BoHV-1, the mutants could not be reactivated by dexamethasone injection although BoHV-1 gC-/tk- could establish latency in the trigeminal ganglia. Therefore, the mutant BoHV-1 gC-/tk- is safe with regard to non-reactivation from latency. The results also lent support to the previous studies that have shown that mutants with deletion of the tk and/or the gE gene could not be reactivated [16,17]. However, the contribution of gC to non-reactivation needs to be explored further.

The nasal challenge with wt BoHV-1 showed that both gC-/tk- and gE-/tk- provided good protection against clinical signs. Although the calves inoculated with the mutants shed virus for some time, the amount and duration were much less than those in calves given wt BoHV-1. The histopathological observation of lung tissue confirmed that both mutants yielded good protection against wt BoHV-1 challenge. These results agreed with a previous study that showed that BoHV-1 with the gE, gG and US2 genes deleted had protective efficacy [18].

To explore the mechanism of immune enhancement related to deletion of BoHV-1 gC, we compared production of IFN-β among the strains. Although a previous report has demonstrated that BoHV-1 infection was able to induce IFN-β production as early as 5 h post-infection, which reached a peak at 72–96 h post-infection and was maintained for up to 8 days after infection [1], we discovered that BoHV-1 gC-/tk- stimulated significantly higher levels of IFN-β than wt BoHV-1 in the early stages of infection. With regard to the viral mechanism for suppression of IFN-β production, it has been reported that the immediate early gene bICP0 can suppress IFN-β promoter activity through the interaction between bICP0 and IRF7 [19]. However, the relationship between gG gene deletion and IFN-β enhancement of production needs to be explored further.

For a better understanding of the protective mechanisms, other immune responses were studied. Although the ability of BoHV-1 gG-/tk- to induce SN antibody was higher than that of BoHV-1 gE-/tk-, both mutants were poor inducers of SN antibody compared with wt BoHV-1. Meanwhile, neither wt BoHV-1 nor the mutants produced significant changes in the Th1-type cytokine IL-4 in calves. However, the levels of IFN-γ, TNF-α and IFN-β in the mutant groups were significantly higher than in the blank controls, although the former two were significantly lower in the mutant groups than in the wt BoHV-1 group. These results agreed with a previous study that live gene-deleted BoHV-1 stimulates Th1-type cellular immunity predominantly [3].

In summary, this study involved the construction of a novel mutant BoHV-1, gC-/tk-. This mutant is efficiently attenuated and provides a good level of protection.

Acknowledgments

This work was supported by Special Fund for Agro-scientific Research in the Public Interest (200803018, 201003060), Special Fund for Modern Agricultural Technology System-Beef Cattle System. We are thankful to Riaz Mustafa for article reading.

Appendix A. Supplementary data


References


