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Enhanced immunogenicity of a BoHV-1 gG-/tk- vaccine

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ABSTRACT

Bovine herpesvirus type 1 (BoHV-1) is a widespread respiratory infection that significantly impacts cattle health worldwide. To address this issue in China, we previously developed a novel double gene-deleted vaccine targeting gG and tk. In this study, we further evaluated the efficacy of this vaccine by challenging vaccinated cattle with a prevalent wild-type BoHV-1 strain and comparing its effectiveness against a commercially available inactivated BoHV-1 vaccine. Post-immunization, all cattle maintained normal rectal temperatures and exhibited no respiratory symptoms. Cattle receiving the gene-deleted vaccine showed a significant increase in the expression of immune markers IFN-γ and TNF-α. Following exposure to wild-type BoHV-1, all immunized groups produced high levels of neutralizing antibodies and specific gB antibodies. Notably, virus shedding was significantly lower in the vaccinated groups compared to the non-immune challenge group. Histological analysis of lung tissues revealed that vaccinated calves had more intact lung structure than their unimmunized counterparts after the challenge. Additionally, the gG−/tk- gene-deleted vaccine demonstrated a higher protective rate based on the average scores of clinical symptoms and lung lesions. Overall, the BoHV-1 gG−/tk- gene-deleted vaccine outperformed the other vaccines tested. This study confirms that the gene-deleted vaccine provides robust protection and superior immunogenicity compared to existing inactivated vaccines, underscoring its potential for future market application.

1. Introduction

Bovine herpesvirus-1 (BoHV-1) significantly impacts the health and productivity of cattle and buffalo, posing a serious threat to the livestock industry [1]. The pathogen leads to severe diseases such as infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/balanoposthitis (IPV/IBP), which can result in increased morbidity and mortality among infected animals [2]. Furthermore, BoHV-1 causes immunosuppression, heightening the risk of secondary bacterial infections, which can lead to bovine respiratory disease (BRD) and potentially result in fatal outcomes [3,4]. The economic ramifications of BoHV-1 are considerable, with the global cattle industry incurring losses exceeding $3 billion annually [5]. In Mexico alone, economic losses attributed to abortions linked to BoHV-1 infection are estimated between $937 and $1100 per aborted cow [6].

BoHV-1 is classified within the Varicellavirus genus, part of the Herpesviridae family and the Alphaherpesvirinae subfamily [7]. The virus has a genome size of approximately 135–140 kb, encoding around 70 proteins that play essential roles in its pathogenesis [8]. A notable protein, glycoprotein (gG), functions as a conserved secreted glycoprotein that binds to chemokines, thereby preventing their interaction with specific receptors and glycosaminoglycans, this mechanism contributes to the immunosuppressive effects of the virus [9]. Additionally, the virus encoded thymidine kinase (TK), which, although not required for replication, is crucial for establishing neural latency. Mutant strains lacking the TK gene show significantly reduced neuropathogenicity [10].

Current strategies to prevent and control BoHV-1 primarily hinge on vaccination and decontamination. While several vaccines are available, the effectiveness varies substantially. In North America, both inactivated

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vaccines and modified live vaccines (MLVs) are utilized to manage BoHV-1-related diseases [11,12]. In Europe, the use of marker vaccination is mandatory, allowing for differentiation between vaccinated cattle and those infected with wild-type virus strains [13]. In contrast, vaccination remains the primary method of control in China; however, several available inactivated vaccines fail to elicit robust cellular immune responses, necessitating multiple doses to achieve adequate levels of neutralizing antibodies.

In response to this need, our lab previously constructed the BoHV-1 gG−/tk- vaccine and conducted preliminary evaluations of its potential immune response in cattle. Further results from rabbit studies indicated that this vaccine effectively protects against BoHV-1 infection [14]. In this study, we aims to further evaluate the efficacy of this novel vaccine in cattle by challenging vaccinated animals with a prevalent wild-type BoHV-1 strain found in China and comparing its efficacy against a commercially available inactivated BoHV-1 vaccine. By examining the interactions of key viral proteins and the resulting immune response, this comprehensive evaluation seeks to provide valuable insights into the market potential of this innovative BoHV-1 gG−/tk- gene-deleted vaccine.

2. Materials and methods

2.1. Cells and viruses

The wild-type strain of BoHV-1 was isolated from clinical samples and designated BoHV-1 HB06 (GenBank accession number: AJ004801.1). This strain is maintained at the National Key Laboratory of Agricultural Microbiology. For the study, Madin-Darby bovine kidney (MDBK) cells were obtained from the China Institute of Veterinary Drug Control.

2.2. Culture of BoHV-1

BoHV-1 strains HB06 and gG−/tk- were cultured as previously described [15]. Briefly, the viruses were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum. The cultures were maintained using MDBK cells at 37 °C in a 5 % CO_2 incubator.

2.3. Animal experiments

A total of 20 Holstein dairy cows, aged two to four months and seronegative for BoHV-1, Pasteurella, *Mycoplasma bovis* and *Mannheimia haemolytica*, were purchased from pasture and randomly divided into four groups. To prevent cross-infection, all cattle were housed in isolation.

The gene-deleted vaccine was reconstituted in 2 mL of saline solution, all 1 mL of the vaccine (1.0×10^6 $TCID_{50}$) was administered into each nasal cavity of the cattle in the immunization group using a 2 mL syringe. The mock group received 2 mL of PBS via the same method.

For the inactivated vaccine group, 2 mL of the commercial inactivated vaccine was injected intramuscularly into the neck of each cow, following the manufacturer's instructions. A booster vaccination was administered 21 days after the initial dose.

At 28 days post-immunization, all groups, except the blank control group, were challenged with 4.0×10^7 $TCID_{50}$ BoHV-1 HB06 strain (Table 1).

Nasal swabs were collected daily for 28 days following both immunization and challenge. Blood samples were taken weekly until the experiment conducted, with additional samples collected on day 0, 1, and 3 post- immunization for further analysis. All samples were stored at −80 °C. Animals were euthanized at the end of the experiment.

Table 1
Animal immunization and challenge information.

Group	Vaccination strain and dose*	Challenge strain and dose
1	1.0×10^6 $TCID_{50}$ BoHV-1 gG−/tk-	4.0×10^7 $TCID_{50}$ BoHV-1 HB06
2	Commercial inactivated vaccine	4.0×10^7 $TCID_{50}$ BoHV-1 HB06
3	DMEM medium	4.0×10^7 $TCID_{50}$ BoHV-1 HB06
4	Blank control	

* Dissolve the BoHV-1 gG−/tk- gene-deleted vaccine in 2 mL of saline solution. The vaccines were dropped to the nasal cavity using a 2 mL syringe, each nasal cavity was inoculated with 1 mL of vaccine. The commercial inactivated vaccine was administered was injected intramuscularly in the neck according to the instructions.

2.4. Clinical evaluation and sample collection

Clinical signs, including rectal temperature, mental and respiratory status, nasal and ocular discharge, nasal mucosa condition and cough condition were continuously monitored throughout the experiment. To measure rectal temperature, a thermometer was inserted approximately 12–15 cm into the rectum until the reading stabilized, and the average value was recorded.

Nasal swabs were collected daily for 28 days following both immunization and challenge. The swabs were vortexed thoroughly in tubes containing 1 mL of sterile PBS, filtered through a 0.45-μm filter, and stored at −20 °C for subsequent real-time PCR (RT-PCR) analysis.

Blood samples were collected weekly for antibody and cytokine detection until the end of the experiment.

Lungs samples were obtained 28 days post-challenge. Tissue specimens were fixed in 4 % paraformaldehyde for 48 h and subsequently embedded in paraffin. Sections were stained with hematoxylin-eosin for histopathological examination.

2.5. Virus shedding

To quantify the shedding of BoHV-1 following the challenge, DNA was extracted from nasal swabs collected for RT-PCR analysis targeting the envelope glycoprotein B (*gB*) gene of BoHV-1. The primer and probe sequences of gB gene are as follows: gB-F: AGCACCTTTGTGGACCTAA, gB-R: GCTGTATCTCGCTGTAGTCG, gB-P: CCGCGAGTTCTTGCCGCTAGAAGTGT (5′ → 3′). The RT-PCR program consisted of the following conditions: 95 °C for 30 s; 95 °C for 10 s, 60 °C for 20 s, 40 cycles; 95 °C for 15 s, 60 °C for 20 s, 95 °C for 15 s. The number of copies of the viral shedding is calculated as follows: The number of copies (copies/mL) = [6.02×10^{23} × plasmid concentration (g/mL) × 10^{-9}] / (Number of plasmid bases × 660).

2.6. Neutralization assay

The serum samples were inactivated by heating at 56 °C for 30 min and then serially diluted in a 96-well cell culture plate. Four replications were done for each sample at each dilution. The diluted serum samples were incubated with 100 $TCID_{50}$ of BoHV-1 HB06 virus at 37 °C in a 5 % CO_2 incubator for 1 h. Subsequently, the serum-virus mixture was transferred to a 96-well cell culture plate containing MDBK cells and cultured at 37 °C in a 5 % CO_2 incubator for three days.

The neutralizing antibody titers, defined as the highest serum dilutions that inhibit BoHV-1 infection, were calculated using the Reed-Muench method.

2.7. Detection of ELISA antibodies

Commercial ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) were used to assess changes in serum antibody levels against BoHV-1 gB as well as alterations in serum cytokines, including IFN-γ and TNF-α.

2.8. Calculation of protective efficacy

The clinical symptoms of the cattle were scored daily after the BoHV-1 challenge, and the gross pathological changes of the lungs were scored at the end of the experiment. The immunoprotective efficacy of the BoHV-1 gG−/tk- gene-deleted vaccine following challenged with BoHV-1 HB06 was calculated using the following equation:

$$PE = \frac{(S_{PC} - S_{NC}) - (S_{VAC} - S_{NC})}{(S_{PC} - S_{NC})} \times 100\%$$

S_{PC}, S_{VAC}, and S_{NC} represent the mean of clinical symptom scores and lung pathological changes observed in the non-immune challenge group, vaccinated challenge group and control group, respectively. **Table S1 and table S2** describe the scoring criteria for clinical symptoms as well as gross pathological changes in the lungs, respectively.

2.9. Statistical analysis

Student's *t*-test and one-way ANOVA was employed using GraphPad Prism 9.5.1 to identify significant differences among the groups. The criteria for statistical significance were established at the following *p*-value thresholds: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Additionally, error bars represent the standard error of the mean.

3. Results

3.1. Clinical signs

Throughout the observation period, all animals in the immunized groups maintained normal rectal temperatures and exhibited stable mental statuses, with no significant respiratory issues. The only exception was the cattle vaccinated with the inactivated vaccine, which showed a slight elevation in rectal temperature for one day following the initial immunization, as well as a temporary increase after the booster dose (Fig. 1A).

In contrast, cattle in the non-immune challenge group experienced elevated rectal temperatures exceeding 39.5 °C from day 2 to 6 post-challenge with BoHV-1. Additionally, four animals in that non-immune challenge group exhibited symptoms such as nasal and ocular secretions, coughing, and typical nasal mucosal bleeding (Fig. 1B).

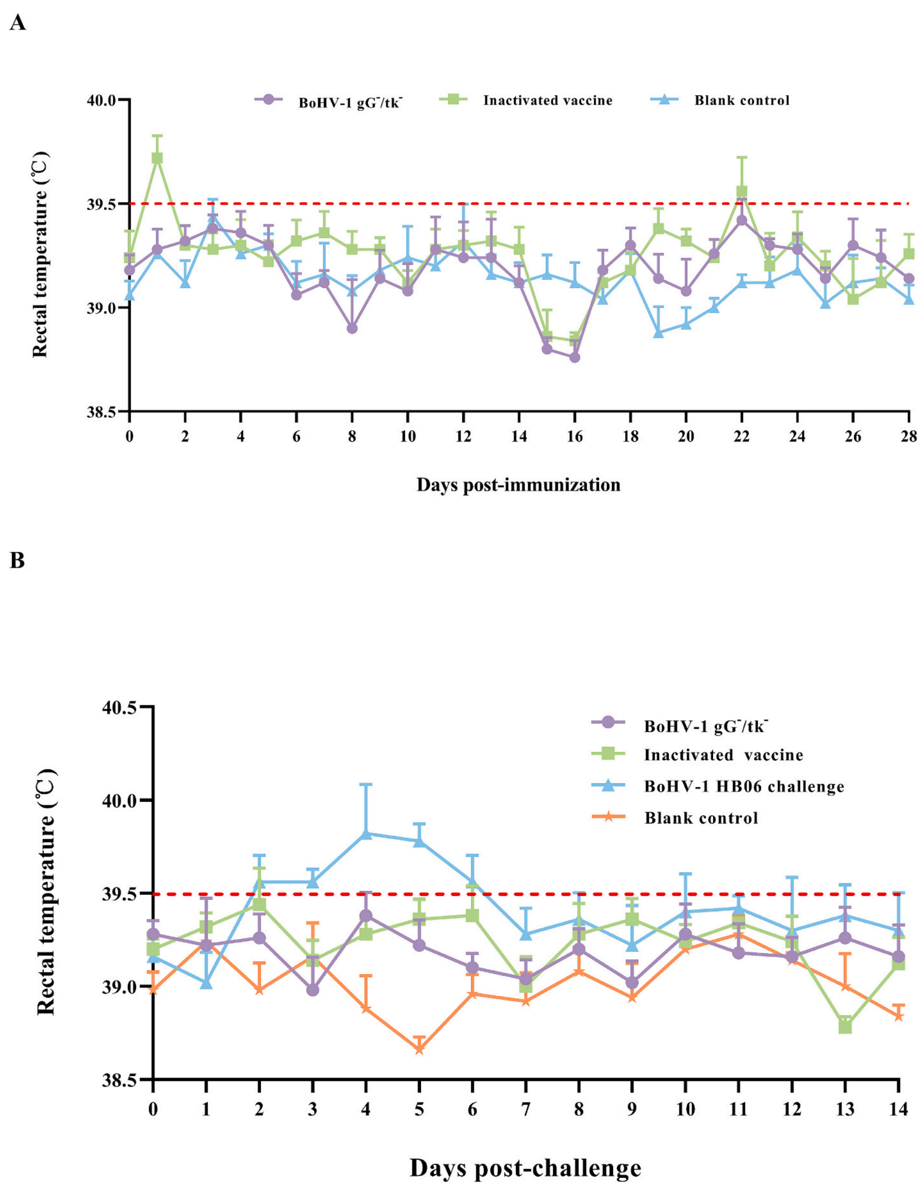

Fig. 1. Rectal temperature changes after **(A)** vaccination and **(B)** BoHV-1 HB06 challenge. The red line represents the upper threshold of normal rectal temperature. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. BoHV-1 gene-deleted vaccine elicits elevation of cytokines

Following vaccination, we assessed the expression levels of IFN-γ and TNF-α. The gene-deleted vaccine group exhibited a sharp increase in IFN-γ levels, significantly surpassing those of the inactivated vaccine group during the immunization period ($p < 0.05$) (Fig. 2A). Notably, the peak IFN-γ level reached 212.8 pg/mL in the gene-deleted vaccine group three days post inoculation.

Simultaneously, TNF-α levels showed a gradual increase in the gene-deleted vaccine group. Throughout the observation period, TNF-α levels in this group remained significantly higher than those in the inactivated vaccine group ($p < 0.01$) (Fig. 2B). These indicate that the gene-deleted vaccine effectively stimulates a robust cellular immune response.

3.3. Antibody response

3.3.1. BoHV-1 neutralizing antibody response

To assess the levels of BoHV-1 neutralizing antibodies, a serum neutralization assay was conducted. The gene-deleted vaccine group demonstrated detectable production of neutralizing antibodies (1:8.4) by day 7 post-immunization. During the first 14 days, a significant difference in neutralizing antibody levels was observed between the gene-deleted and inactivated vaccine groups ($p < 0.01$). Prior to the inactivated vaccine group's booster vaccination at 21 days, the neutralizing antibody titer in the gene-deleted vaccine group increased steadily and remained consistently higher than those in the inactivated vaccine group (Fig. 3A).

Both the gene-deleted and inactivated vaccine groups generate a robust humoral immune response when challenged with BoHV-1 HB06, with the inactivated vaccine group exhibiting superior performance. Notably, the neutralizing antibodies produced by both immunized groups were significantly higher than those in the unimmunized challenge group at 7 and 14 days post challenge ($p < 0.05$) (Fig. 3A).

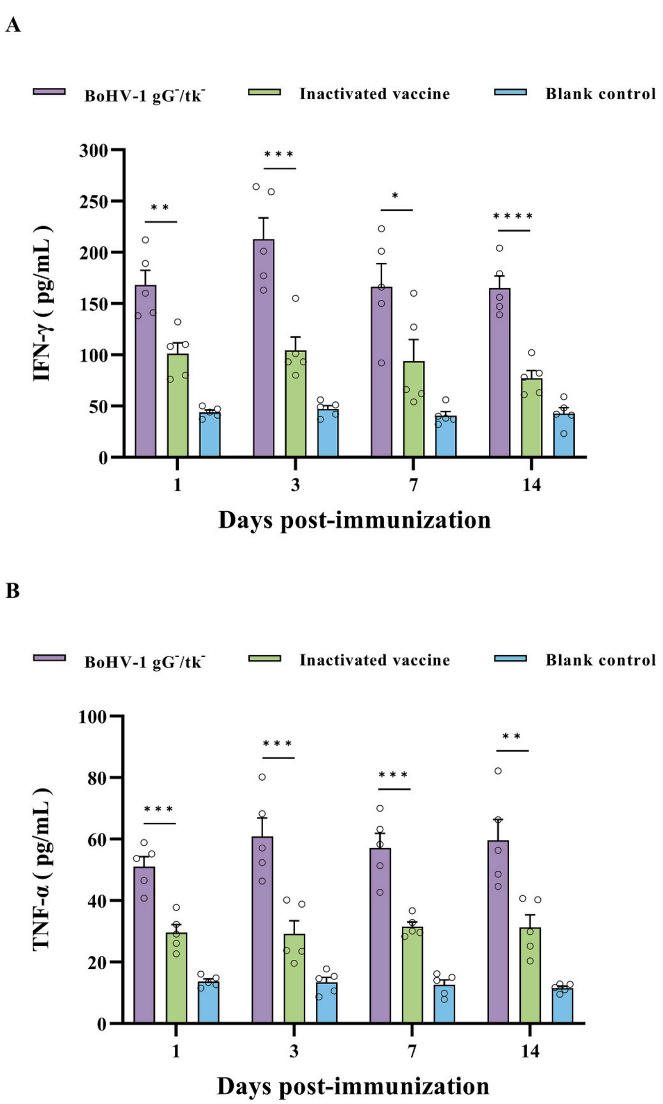

Fig. 2. Cytokine changes following immunization of cattle with BoHV-1 gG−/tk- gene-deleted vaccine and inactivated vaccine, respectively. **(A)** and **(B)** represent the level of IFN-γ and TNF-α, respectively.

3.3.2. BoHV-1 serum-specific gB antibody

In the gene-deleted vaccine group, gB antibody levels increased gradually, reaching a detectable level by day 7 post-immunization. In contrast, the inactivated vaccine groups achieved a gB antibody level of 0.626 at the same time. Subsequently, the gene-deleted vaccine group exhibited a robust increase in gB antibody levels, with all cattle testing positive by 14 days post-vaccination. Notably, at 28 days post-immunization, the gB antibody levels were 0.827 and 0.862 for the gene-deleted and inactivated vaccine groups, respectively (Fig. 3B).

Following the challenge, gB antibody levels in both vaccinated groups remained extremely high. Importantly, the antibody levels induced by the two immunized groups were significantly greater than those in the non-immune challenge group on day 7 ($p < 0.0001$) (Fig. 3B). These findings highlight the strong humoral immune response elicited by both vaccine types in the face of the viral challenge.

3.3.3. Detection of virus shedding after challenge

BoHV-1 HB06 shedding was assessed using RT-PCR. All experimental groups, except the blank control group, detected high titers of viral shedding on the first day post challenge. Throughout the observation period, the shedding titers in the two vaccinated challenge groups remained nearly identical, with levels decreasing by day 6 and completely vanishing by day 9. In contrast, the non-immune challenge group maintained significantly higher levels of viral shedding, peaking at $10^{5.28}$/mL on day 5. Notably, shedding levels in this group were significantly greater than those in the two immunized groups from days 4 to 8 ($p < 0.01$) (Fig. 4). These results indicate that vaccination effectively reduces viral shedding following challenge with BoHV-1 HB06.

3.4. Evaluation of the pathological changes post-challenge

Lung damage was significantly less severe in the immunized groups, primarily localized to the lung lobules such as apical and cardiac lobes of the lungs, characterized by mild bruising and fleshy lesions. In contrast, the non-immune challenge group exhibited more extensive damage, with lesions spread throughout the lungs. This group displayed notable hemorrhages in the lung lobes, along with interlobular adhesions and granulomatous changes (Fig. 5).

Following the BoHV-1 HB06 challenge, both immunized groups displayed no significant pathological changes. The alveolar structure remained relatively intact, and there was no inflammatory exudate present in the alveolar lumen. Only the inactivated vaccine group exhibited a small amount of inflammatory cell exudation within the alveolar cavity. And the hyperplasia of the alveolar walls was more pronounced than in the gene-deleted vaccine group. In contrast, the non-immune challenge calves exhibited the most severe lesions, characterized by a loss of normal lung tissue structure. This group showed marked interstitial hyperplasia and significant inflammatory infiltrates, underscoring the protective effects of vaccination against severe lung pathology (Fig. 6).

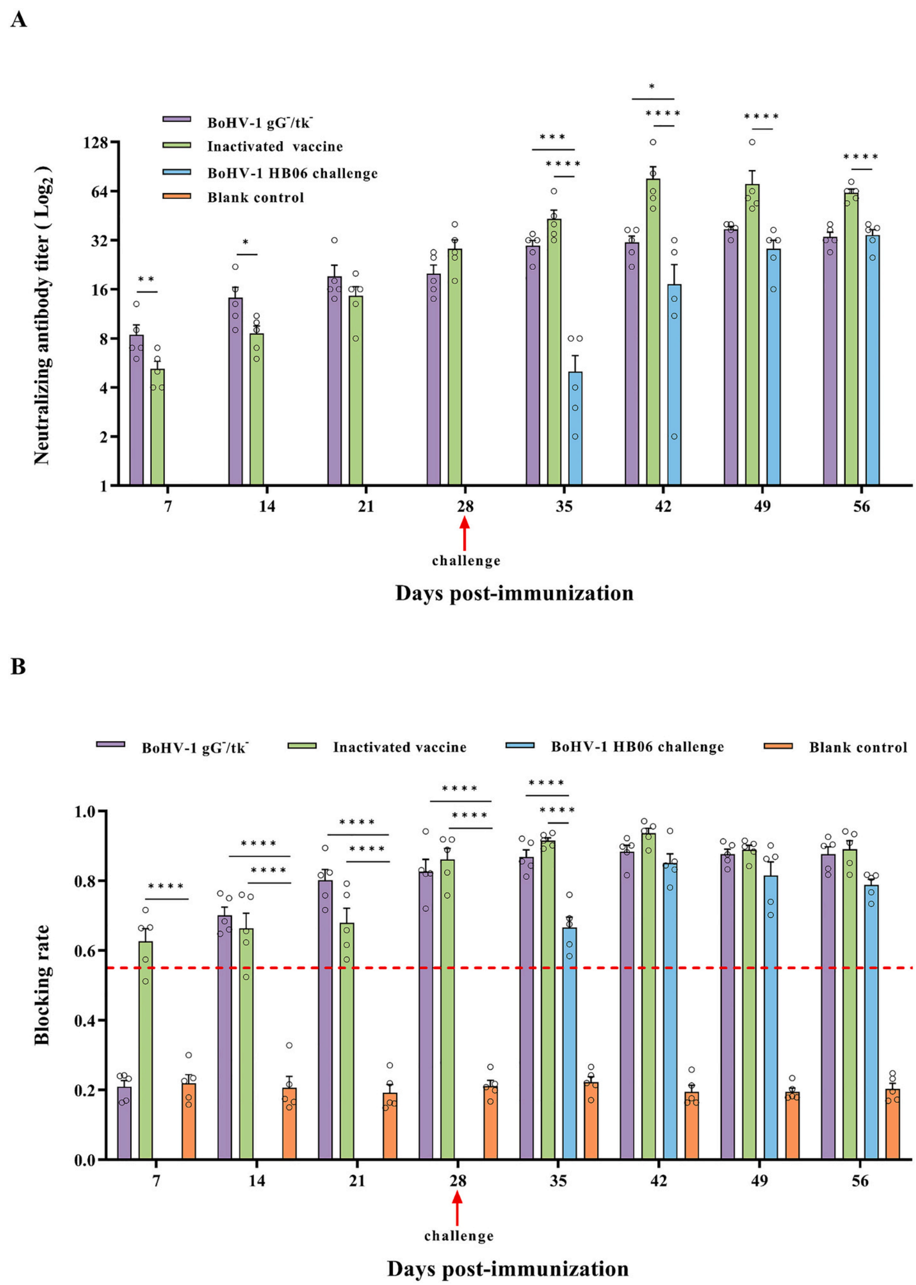

Fig. 3. Humoral immune responses induced by BoHV-1 gG−/tk- gene-deleted vaccine and inactivated vaccine after immunization and challenge with BoHV-1 HB06. **(A)** BoHV-1 neutralizing antibody titers and **(B)** BoHV-1 gB antibody were determined. The red line indicates the negative-positive threshold for gB antibodies. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Calculation of protective rate of the BoHV-1 gG−/tk- gene-deleted vaccine after challenge

As detailed in the Materials and Methods section, we calculated the protective rate following the BoHV-1 HB06 challenge. As shown in Table 2, the clinical symptom score of the gene-deleted vaccine group was lower than those of the inactivated vaccine group after challenge, but there was no statistic difference between the two groups. However, the lung gross lesion score was significantly lower than that of the inactivated vaccine group. Calculations based on the formula given above, it can be concluded that the gene-deleted vaccine group achieved the higher protection rate of 90.22 %, with a protection rate of 77.89 % in inactivated immunization group. This indicates that the gene-deleted vaccine had the same efficacy as the inactivated vaccine in terms of phenotypic analysis after challenge, but the gene-deleted vaccine group had milder lung gross lesions and a higher protection rate compared to the commercial inactivated vaccine group, offering superior protection when challenged with BoHV-1.

4. Discussion

BoHV-1 is a potent herpesvirus that poses significant health risks to cattle, causing severe respiratory diseases across all breeds. This virus primarily infects the respiratory mucosa, leading to symptoms such as nasal inflammation, hemorrhaging, and conjunctivitis. Our findings align with this, as unimmunized cattle exhibited typical symptoms when exposed to the wild-type BoHV-1 strain, highlighting the serious threat it poses to herd health and productivity. Infected cattle often enter an incubation period during which the viral genome can be detected in the trigeminal ganglion, with potential latency in other distant ganglia

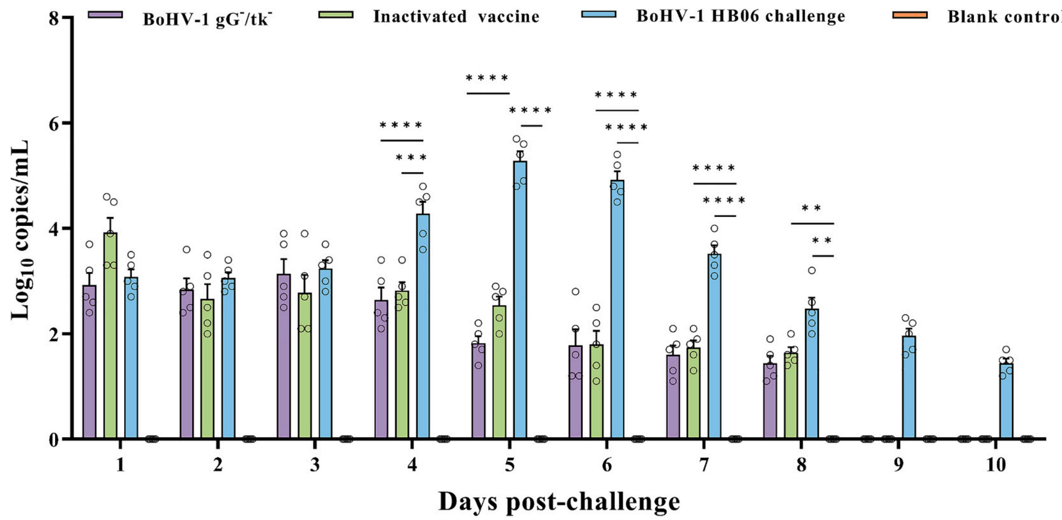

Fig. 4. Detection of BoHV-1 HB06 shedding after challenge. Nasal swabs of calves immunized with gene-deleted vaccine and inactivated vaccine were collected every day.

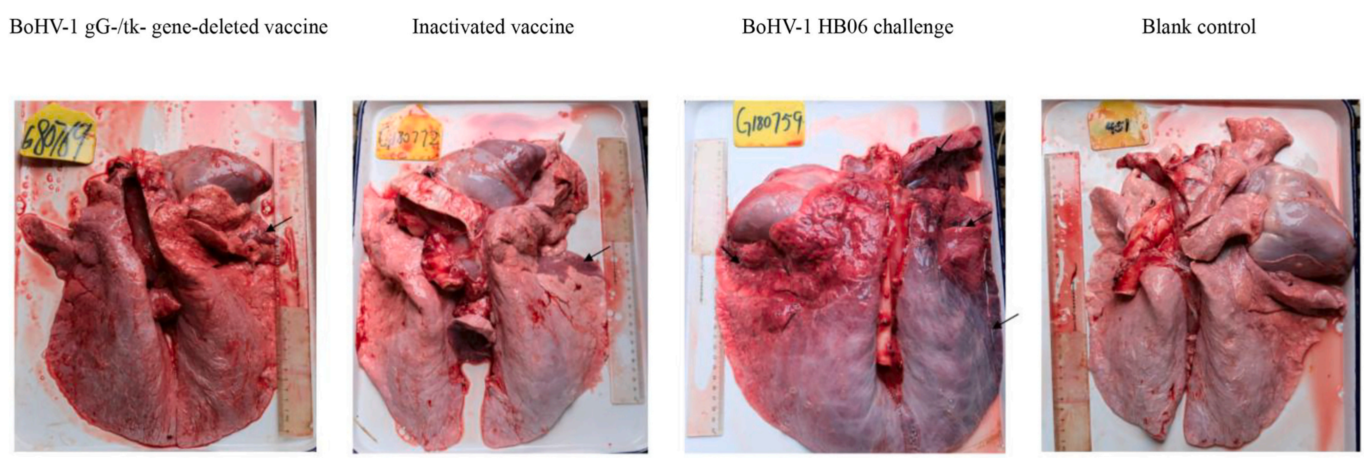

Fig. 5. Gross lesions of lung tissue after BoHV-1 HB06 challenge. Black arrows indicate the typical areas of lung lesions.

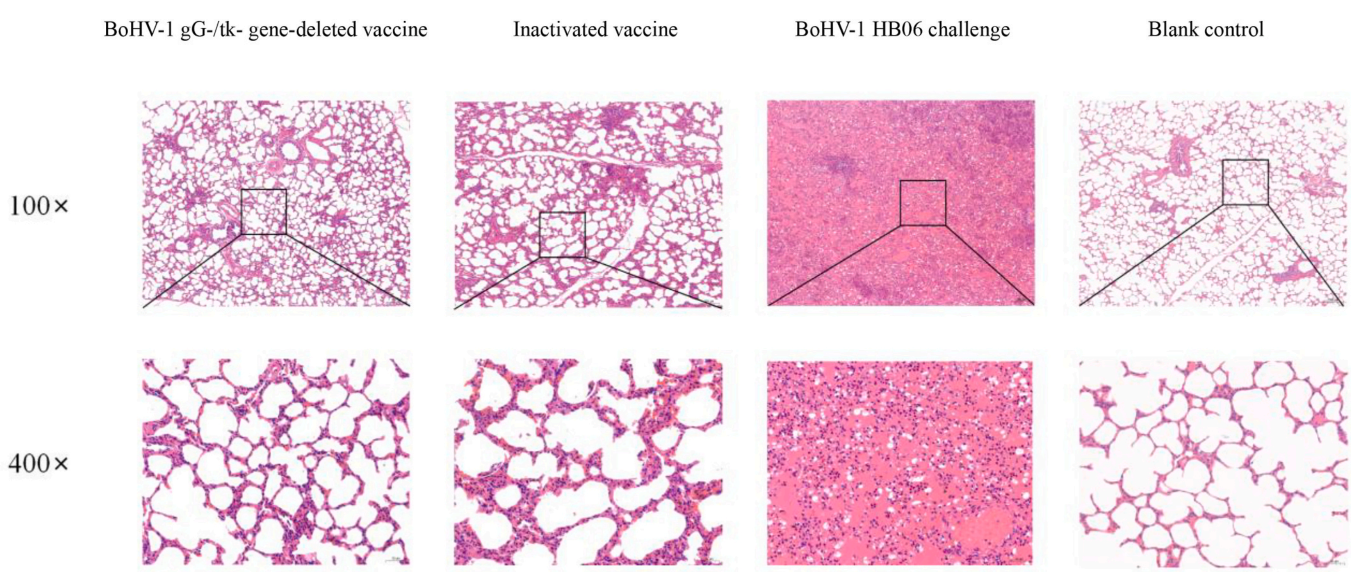

Fig. 6. Histopathological images of lung tissues after BoHV-1 HB06 challenge tained by H&E. The scale sizes are 200 μm (top) and 50 μm (bottom), respectively. The figure below is an enlargement of part of the area of the upper figure.

Table 2
Protective rate after BoHV-1 HB06 challenge.

Group	Average score		Protective rate (%)
	Clinical score	Lung lesion score	
BoHV-1 gG−/tk- gene-deleted vaccine	0.16 ± 0.049 [ns]	3.333 ± 0.678 **	90.22 %
Commercial inactivated vaccine	0.166 ± 0.077	6.0 ± 1.073	77.89 %
BoHV-1 HB06 challenge	1.714 ± 0.195	21.333 ± 1.152	\
Blank control	0.04 ± 0.026	1.333 ± 0.399	\

[ns] and ** indicate the statistical analysis of clinical symptoms and gross pathological lesions in the lungs between gene-deleted vaccine group and inactivated vaccine group.

[16,17]. Factors such as transportation, harsh weather, and changes in feeding can trigger reactivation of the virus, leading to further shedding and transmission [18]. Additionally, BoHV-1 causes extensive immunosuppression by infecting immune cells, which hampers the body's ability to respond effectively to infections [19]. This creates an environment conducive to secondary infections by other respiratory pathogens, ultimately increasing the risk of bovine respiratory disease.

The results from the gene-deleted vaccine group were particularly promising, showing rapid virus clearance with undetectable shedding by day 9. Throughout the experimental period, vaccinated groups demonstrated significantly lower virus shedding levels compared to unimmunized cattle. This is crucial for practical applications, as it suggests that vaccination can effectively reduce viral transmission, thereby protecting herds. The reduced shedding duration and quantity of BoHV-1 in vaccinated calves may help slow the virus's recirculation in pastures, thus reducing transmission risk [20]. The differences in shedding levels between vaccinated and unvaccinated groups likely result from the robust immune responses prompted by vaccination, leading to quicker and lower levels of viral shedding.

In this study, we evaluated the effectiveness of the BoHV-1 gG−/tk-gene-deleted vaccine by challenging immunized cattle with a wild-type strain and comparing its protective efficacy to that of currently available inactivated vaccines. We observed higher expressions of IFN-γ and TNF-α in the gene-deleted vaccine group compared to the inactivated vaccine group. The cell-mediated immune (CMI) response plays a crucial role in protecting against secondary infections [21]. IFN-γ, as the primary type II interferon in cattle, is a key marker for evaluating CMI responses and is essential for stimulating immunoglobulin production and activating cytotoxic T lymphocytes [22,23]. Its levels correlate positively with reduced clinical symptoms in infected animals [24]. TNF-α, a proinflammatory factor, also plays a significant role in activating immune responses and enhancing leukocyte recruitment to inflamed areas [25]. Our findings indicate that the gene-deleted vaccine effectively triggers a robust cytokine response, leading to greater protection against subsequent infections.

Additionally, we found that the gene-deleted vaccine induced a high production of neutralizing antibodies and serum-specific gB antibodies, both after immunization and following challenge. Notably, neutralizing antibody titers rose more quickly in the gene-deleted vaccine group, surpassing those in the inactivated group by day 7 post-immunization, reaching a titer of 1:8.4, which is sufficient to prevent BoHV-1 [26]. However, the inactivated vaccine group exhibited stronger overall humoral immune responses, which is consistent with the typical performance of inactivated vaccines that elicit more pronounced humoral immunity [13].

Additionally, an important advantage of the gene-deleted vaccine is its ability to facilitate the Differentiating Infected from Vaccinated Animals (DIVA) strategy. This capability is crucial for effective disease management and surveillance, allowing for better control of BoHV-1 spread in herds. Furthermore, our evaluation showed that the gene-deleted vaccine achieved the highest efficacy scores, reinforcing its potential as a superior vaccination option.

5. Conclusion

In conclusion, both the gene-deleted vaccine and the inactivated vaccine were effective in reducing and clearing virus shedding after exposure to the BoHV-1 wild-type strain compared to the non-immune challenge group. The gene-deleted vaccine effectively increased the cytokine concentration of the vaccinated individuals and generated a comparable antibody response to the inactivated vaccine, suggesting that it promotes a balanced immune response and provides long-term memory [27]. This balanced immune response is vital for effective infection control [28,29]. Moreover, histopathological evaluations revealed that immunized cattle had healthier lungs and more intact lung tissue than their unvaccinated counterparts. Overall, the BoHV-1 gG−/tk- gene-deleted vaccine offers more comprehensive protection than inactivated vaccines, providing valuable insights into controlling BoHV-1 infections and transmission. Nevertheless, further large-scale randomized trials are necessary to confirm its efficacy and support its eventual commercialization.

Ethics approval and consent to participate

The animal experiment was approved by the Animal Experiment Ethics Committee of Huazhong Agricultural University and conducted in strict accordance with the Guidelines for the Care and Use of Laboratory Animals of Wuhan, Hubei, China (Huazhong Agricultural University Ethics Approval Number: HZAUCA-2018-0018).

Consent for publication

All authors have read and approved the final work and approved the manuscript for publication.

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CRediT authorship contribution statement

Sen Zhang: Conceptualization. **Guoxing Liu:** Conceptualization. **Chen Wang:** Conceptualization. **Aizhen Guo:** Writing – review & editing. **Yingyu Chen:** Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data supporting the findings of this study are available within the article. Further inquiries can be directed to the corresponding authors.

Acknowledgement

The authors would like to thank the owner of the farm for allowing us to have access to the animals.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2025.126704.

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