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A novel attenuated and marker *M. bovis*-BoAHV-1 combined vaccine provides broad protection against diverse bovine alphaherpesvirus type 1 genotypes

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ABSTRACT

Mycoplasma bovis (*M. bovis*) and bovine alphaherpesvirus 1 (BoAHV-1) are two major infections that seriously impair cattle's reproductive and respiratory systems. We created an *M. bovis*-BoAHV-1 combined vaccine and assessed its safety and effectiveness in our earlier work. This study evaluated a novel combined vaccine comprising attenuated *M. bovis* and marker BoAHV-1 antigens for its immunogenicity and efficacy in protecting cattle against diverse BoAHV-1 genotypes. The cattle were divided into four groups: *M. bovis*-BoAHV-1 combined vaccine, inactivated vaccine, non-immune challenge, and blank control groups. RT-PCR was used to assess viral shedding after the groups challenged with either the BoAHV-1 1.1a or 1.2b strains. The results demonstrated that the combined vaccine group prompted a high degree of production of cytokines, B cells, and T cell-associated signaling pathways and was able to elicit considerably greater antibody titers ($p < 0.01$). Under experimental challenge conditions, the combined vaccine reduced BoAHV-1 viral shedding by 70% in vaccinated cattle compared to controls, shorter duration of virus shedding was also observed in the vaccinated groups compared to the non-immune challenge group for different BoAHV-1 strain challenges, about 6–8 d earlier clearance of virus shedding compared to the non-immune challenge group. Notably, the combined vaccine provided cross-protection against multiple BoAHV-1 genotypes, demonstrating its potential as a broad-spectrum solution for controlling bovine respiratory disease. These findings providing important insights into effective disease control strategies for cattle herds.

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Introduction

Bovine alphaherpesvirus type 1 (BoAHV-1) is a major viral pathogen belonging to the genus *Varicellavirus* in the *Herpesviridae* family and Alphaherpesvirinae subfamily. It is a significant cause of economic losses in the cattle industry worldwide [1]. BoAHV-1 can lead to a variety of clinical manifestations, including infectious bovine rhinotracheitis, conjunctivitis, abortion, and systemic infections [2]. The virus can also establish lifelong latent infections in cattle, posing an ongoing threat of reactivation and transmission.

BoAHV-1 is classified into three subtypes, 1.1a, 1.2a, and 1.2b [3,4]. In China, subtypes 1.1a and 1.2b are predominant circulating strains. These different BoAHV-1 genotypes are associated with distinct clinical manifestations in infected cattle [5]. Subtype 1.1 is primarily linked to respiratory symptoms, whereas subtype 1.2 can cause a broader range of clinical signs, including respiratory infections, abortion, laryngitis,

and vulvovaginitis [6,7]. Interestingly, BoAHV-1 infected cattle in China often exhibit only mild clinical signs, typically without severe respiratory distress, and the virus is predominantly found in calves aged 0–10 months [8]. Acute high-level viral shedding is the first phase of BoAHV-1's biphasic life cycle, which is followed by the development of a latent infection that lasts a lifetime [9].

Notably, BoAHV-1 infection can result in the suppression of the immune system in cattle, consequently rendering them more vulnerable to secondary viral or bacterial infections [10]. This can contribute to the development of Bovine Respiratory Disease (BRD), which is a widespread and economically significant condition in the livestock industry, and it has been shown to be a major cause of morbidity and financial loss in cattle production [11].

The genetic diversity of BoAHV-1 strains and the complex interplay between viral infections and

secondary respiratory diseases highlight the need for effective and broadly protective vaccination strategies to safeguard cattle health and productivity.

Existing BoAHV-1 vaccines, while effective in reducing disease severity and achieve cross-species protection effects [12,13], there is a lack of support for whether a single inoculation with one marker vaccine can provide cross-protection against the genetic diversity of circulating BoAHV-1 genotypes. This limitation is particularly problematic in regions with high genotype variability, where vaccine escape mutants contribute to ongoing outbreaks. Additionally, conventional vaccines may exhibit variable efficacy in eliciting robust and durable immune responses, emphasizing the need for more versatile solutions [14].

Previous attempts to develop combined vaccines targeting multiple pathogens, such as BoAHV-1 and *M. bovis*, have shown promise in reducing co-infection rates and disease severity [15]. However, these efforts have been limited by the challenges in maintaining antigen stability and immunogenicity when combining attenuated and marker vaccine components [16].

In our previous study, we developed an attenuated marker-based combined vaccine targeting both *M. bovis* and BoAHV-1 [17]. Vaccination of cattle with this combined vaccine resulted in no clinical signs, demonstrating the safety and tolerability of the formulation. Importantly, the combined vaccine was effective in protecting cattle against challenge with both *M. bovis* and BoAHV-1, confirming its potent immunogenicity and protective efficacy. However, these studies have not proved whether the use of the combined vaccine can provide effective protection against different genotypes of BoAHV-1 wild-type strains. And there are few researches evaluating the cross-protective effects of BoAHV-1 relevant marker vaccines.

To meet this demand, this study builds upon these findings by exploring whether an attenuated and marker *M. bovis*-BoAHV-1 combined vaccine inoculation in cattle could provide strong cross-protection against diverse BoAHV-1 genotypes, aiming to enhance immune responses. Specifically, we challenged vaccinated cattle with different BoAHV-1 subtypes that are prevalent in China and compared the vaccine's performance against a commercially available inactivated BoAHV-1 vaccine. This comprehensive assessment will provide valuable insights into the breadth of protection conferred by our novel combination vaccine candidate against diverse BoAHV-1 genotypes circulating in the field.

Materials and methods

Cells and viruses

The study involved the isolation of two subtypes of the wild-type strains of BoAHV-1 from respiratory clinical samples. These two strains were designated as BoAHV-1 HB06 and BoAHV-1 SZ01, corresponding to subtypes 1.1a and 1.2b, respectively, and the virus strains of the two subtypes HB06 and SZ01 had known titers of $10^{7.7}$ TCID₅₀/mL and $10^{7.4}$ TCID₅₀/mL, respectively. The BoAHV-1 HB06 strain (GenBank accession number: AJ004801.1) was maintained alongside the BoAHV-1 SZ01 strain and BoAHV-1 gG-/tk- strain at the National Key Laboratory of Agricultural Microbiology. For experimental purposes, Madin-Darby bovine kidney (MDBK) cells were utilized, which were obtained from the China Institute of Veterinary Drug Control.

Culture of BoAHV-1

BoAHV-1 strains HB06, SZ01, and gG-/tk- were cultured as previously described [18]. Briefly, the viruses were cultivated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with the addition of 10% fetal bovine serum (Inner Mongolia Opcel Biotechnology Co., Ltd., Hohhot, China) in MDBK cells at a temperature of 37°C within a 5% CO₂ incubator.

Visual diagram summarizing the study workflow

A schematic workflow summarizing the experimental design, from vaccine preparation to post-challenge assessments, is presented in Figure 1. This diagram provides an overview of key steps, including vaccine formulation, animal grouping, immunization schedules, challenge infection, and subsequent immune and clinical evaluations.

Animal experiments

A total of 21 Holstein calves, aged between two to four months, were sourced from a pasture and exhibited serological responses to infections caused by *M. bovis*, BoAHV-1, *Pasteurella*, and *Mannheimia hemolyticus*. These calves were randomly assigned to four distinct groups, and the groups were as follows: a combined vaccine group ($n = 6$), an inactivated vaccine group ($n = 6$), a non-immune challenge group ($n = 6$), and a blank control group ($n = 3$). To prevent cross-infection, all calves were maintained in isolated housing. Sample sizes for each group were determined based on a power analysis to detect a minimum 30%

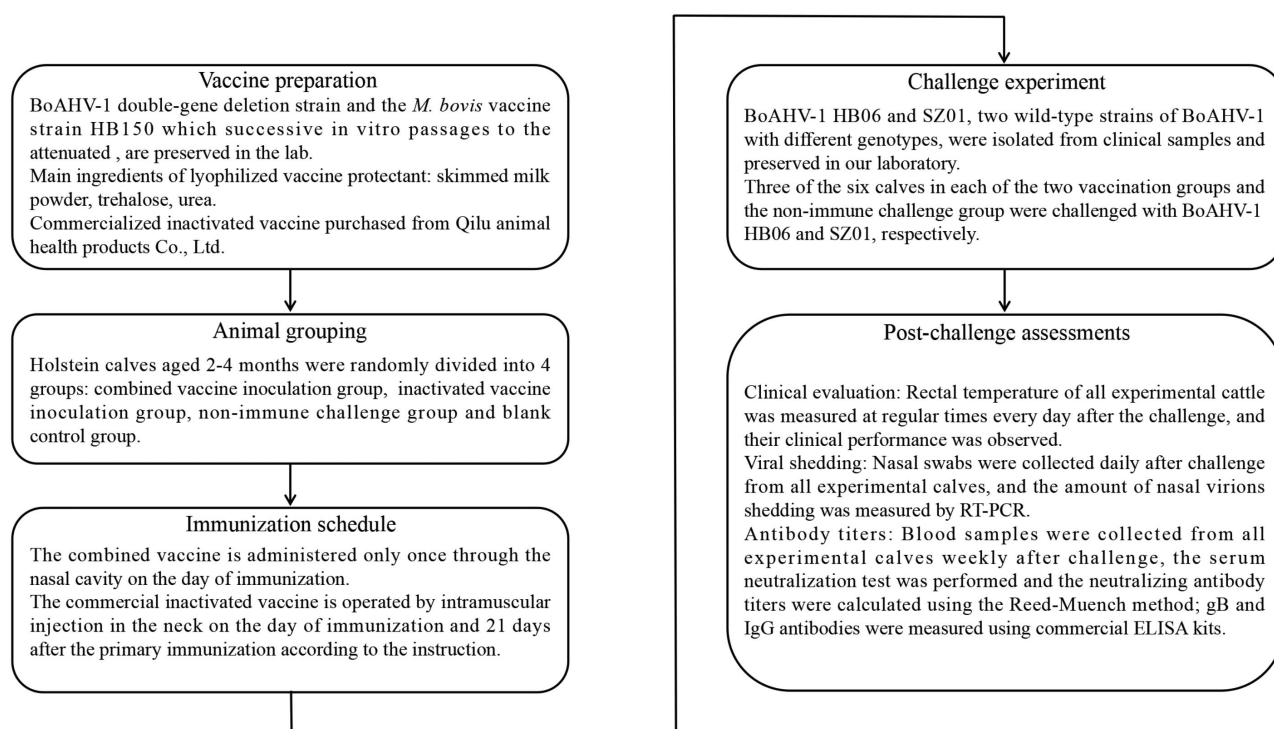


Figure 1. The visual diagram of the experimental design and workflow summary of this study.

reduction in viral shedding between vaccinated and control groups at a significance level of 0.05 and a power of 95%. Each group consisted of six or three animals, which allowed for statistical comparisons while minimizing animal use in accordance with ethical guidelines. The specific operations were as described below, and the animal immunization and challenge information was shown in Table 1.

For the experimental procedure, the combined vaccine was initially prepared by dissolving in a 2 mL of saline solution. In the subsequent step, each calf within the combined vaccine group was administered 1 mL of the vaccine solution into each nasal cavity, facilitated by the use of a 2 mL syringe. For the non-immune challenge and control groups, an equivalent volume of PBS was administered through the intranasal

route, utilizing the identical procedural steps as those employed for the vaccine group.

In accordance with the prescribed guidelines, each calf in the inactivated vaccine group received an intramuscular injection of 2 mL of the commercial inactivated vaccine containing $2.0 \times 10^{7.5}$ TCID₅₀ BoAHV-1 virion into the neck. A booster vaccination was subsequently administered 21 d following the initial immunization with the same dose.

Intranasal challenge was performed on the combined vaccine group, the inactivated vaccine group, and the non-immune challenge group with BoAHV-1 1.1a and BoAHV-1 1.2b at 28 d post-vaccination. Both two challenge strains were administered at a dose of 4.0×10^7 TCID₅₀.

Following immunization and challenge, nasal swabs were collected daily in the morning after

Table 1. Animal immunization and challenge information.

Group	No	Vaccination content and dose	Vaccination route	Challenge strain and dose
1	3	Combined vaccine; 1.0×10^6 TCID ₅₀ BoAHV-1 gG ⁻ /tk ⁻	Intranasal	BoAHV-1 HB06 strain; 4.0×10^7 TCID ₅₀
	3	Combined vaccine; 1.0×10^6 TCID ₅₀ BoAHV-1 gG ⁻ /tk ⁻	Intranasal	BoAHV-1 SZ01 strain; 4.0×10^7 TCID ₅₀
2	3	Commercial inactivated vaccine; $2.0 \times 10^{7.5}$ TCID ₅₀ BoAHV-1 C1 strain	Intramuscular	BoAHV-1 HB06 strain; 4.0×10^7 TCID ₅₀
	3	Commercial inactivated vaccine; $2.0 \times 10^{7.5}$ TCID ₅₀ BoAHV-1 C1 strain	Intramuscular	BoAHV-1 SZ01 strain; 4.0×10^7 TCID ₅₀
3	3	PBS	Intranasal	BoAHV-1 HB06 strain; 4.0×10^7 TCID ₅₀
	3	PBS	Intranasal	BoAHV-1 SZ01 strain; 4.0×10^7 TCID ₅₀
4	3	PBS	Intranasal	\

feeding over a 28-d period. Blood samples were collected on a weekly basis throughout the experiment. Additionally, blood samples were taken on days 0, 1, and 3 post-immunization for further analyses. All samples were stored at a temperature of -80°C . The animals were euthanized upon completion of the experiment.

Clinical evaluation and sample collection

Throughout the experimental period, clinical signs, such as rectal temperature and respiratory status, were continuously monitored. Rectal temperature was measured by inserting a thermometer approximately 10 cm into the rectum until a stable reading was obtained. The average temperature value was subsequently recorded.

Following immunization and subsequent challenge, nasal swabs were obtained on a daily basis for a period of 28 d. Each sample was thoroughly vortexed in tubes containing 1 mL of PBS. Then, the samples were filtered through a $0.45\text{-}\mu\text{m}$ filter. The processed samples were then stored at -20°C for subsequent RT-PCR.

Blood samples were collected for the purpose of conducting antibody detection and whole blood transcriptome analysis.

Virus and bacteria shedding

To quantify the shedding of BoAHV-1 1.1a and BoAHV-1 1.2b after the challenge, DNA was extracted from nasal swabs and used for RT-PCR targeting the envelope glycoprotein B (gB) gene of BoAHV-1. The primer and probe sequences of gB gene can be found in our previous studies [19]. The RT-PCR program was as follows: 95°C for 30 s, 95°C for 10 s, 60°C for 20 s, and 40 cycles of 95°C for 15 s, 60°C for 20 s, and 95°C for 15 s.

Transcriptome preparation and analysis

Whole blood samples (4 mL) were collected in EDTA-containing anticoagulant tubes and stored at -80°C . cDNA libraries were sequenced on an Illumina sequencing platform by Metware Biotechnology Co., Ltd. (Wuhan, China).

The samples were analyzed for differential gene expression using DESeq2, followed by the Benjamini-Hochberg method to test this hypothesis. The adjusted p -value was set at less than 0.05, and a fold change greater than or equal to 1.

Neutralization assay

Serum samples were subjected to a 30-min incubation at 56°C to inactivate and then serially diluted in a 96-well cell culture plate. Diluted serum samples were incubated with 100 TCID₅₀ of either BoAHV-1 1.1a or BoAHV-1 1.2b virus at 37°C in a 5% CO₂ incubator for 1 h. Following this step, the serum-virus mixture was transferred to a 96-well cell culture plate containing MDBK cells and cultured in a 5% incubator at 37°C . The mixture was left to culture for a period of 3 d.

The neutralizing antibody titers, which represent the highest serum dilutions capable of inhibiting BoAHV-1 infection, were calculated using the Reed–Muench method.

Detection of ELISA antibodies

Commercial ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China) were utilized for the detection of alterations in serum antibody levels against BoAHV-1 gB and IgG antibodies, as well as fluctuations in serum cytokines, including IFN- γ and TNF- α .

Ethics statement

The experimental procedures on animals were formally ratified by the Animal Experiment Ethics Committee of Huazhong Agricultural University, and the conduct of these experiments was undertaken in strict compliance with the Guidelines for the Care and Use of Laboratory Animals of Wuhan, Hubei, China (Huazhong Agricultural University Ethics Approval Number: HZAUCA-2024-0036).

Statistical analysis

Statistical analyses were conducted using ANOVA for comparing antibody titers and viral shedding between groups, with Tukey's post-hoc tests applied for multiple comparisons. Normality and homoscedasticity assumptions were verified using the Shapiro-Wilk and Levene's tests, respectively. These tests were selected to ensure accurate and reliable interpretation of differences across experimental groups. The following p -value thresholds were used to determine the statistical significance: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****). Error bars indicate standard error of the mean.

Confirmation of the ARRIVE guidelines

The manuscript is provided with a ARRIVE checklist, all authors have carefully examined and checked to confirm that the ARRIVE guidelines are followed in this study.

Results

Clinical signs

It was observed that all calves in the immunized groups exhibited normal rectal temperature and mental status, with no evidence of respiratory signs throughout the entire observation period; Only some of the cattle vaccinated with the inactivated vaccine experienced a slight rectal temperature elevation for two consecutive days after the initial immunization, and a transient increase after booster immunization (Figure 2a).

In the unimmunized groups, rectal temperatures were recorded as being higher than 39.5°C on days 3–7 and days 4–7 after challenge with BoAHV-1 1.1a and BoAHV-1 1.2b, respectively (Figure 2b–c). Two cattle in the BoAHV-1 1.1a and BoAHV-1 1.2b challenge groups also presented with conjunctivitis accompanied by increased ocular discharge and typical nasal mucosal bleeding, respectively.

Vaccination elicits strong antibody response

The presence of BoAHV-1 neutralizing antibodies was measured by a serum neutralization assay. On day 7 after vaccination, the combined vaccine group was able to detect the production of neutralizing antibodies (1:9.3), and there was a significant difference between it and the inactivated vaccine group ($p < 0.05$). The

neutralizing antibody titer of the combined vaccine group increased slowly and remained high throughout the immunization period. In contrast, the production of neutralizing antibodies in the inactivated vaccine group was relatively slow until 21 d after the initial immunization, when booster immunization was administered. After the booster, the neutralizing antibody titer increased rapidly until 28 d after vaccination and was significantly higher than that in the combined vaccine group ($p < 0.05$) (Figure 3a).

The gB antibody levels increased slowly in all vaccinated cattle within 14 d after immunization until 21 d after immunization, when the gB antibody levels increased sharply in both immunized groups, and it is noteworthy that all calves in the combined vaccine group turned positive at 28 d after immunization. In addition, there was a significant difference in the gB antibody levels between the combined vaccine and control groups during the entire immunization period ($p < 0.05$) (Figure 3b).

Following the administration of vaccination, elevated levels of IgG antibodies were recorded, and statistically significant differences were found between the two immunized groups and the control group during the entire immunization period ($p < 0.01$). Moreover, IgG antibody levels in the combined vaccine group were consistently higher than those in the inactivated vaccine group, peaking at 286.1 µg/mL 21 d post-

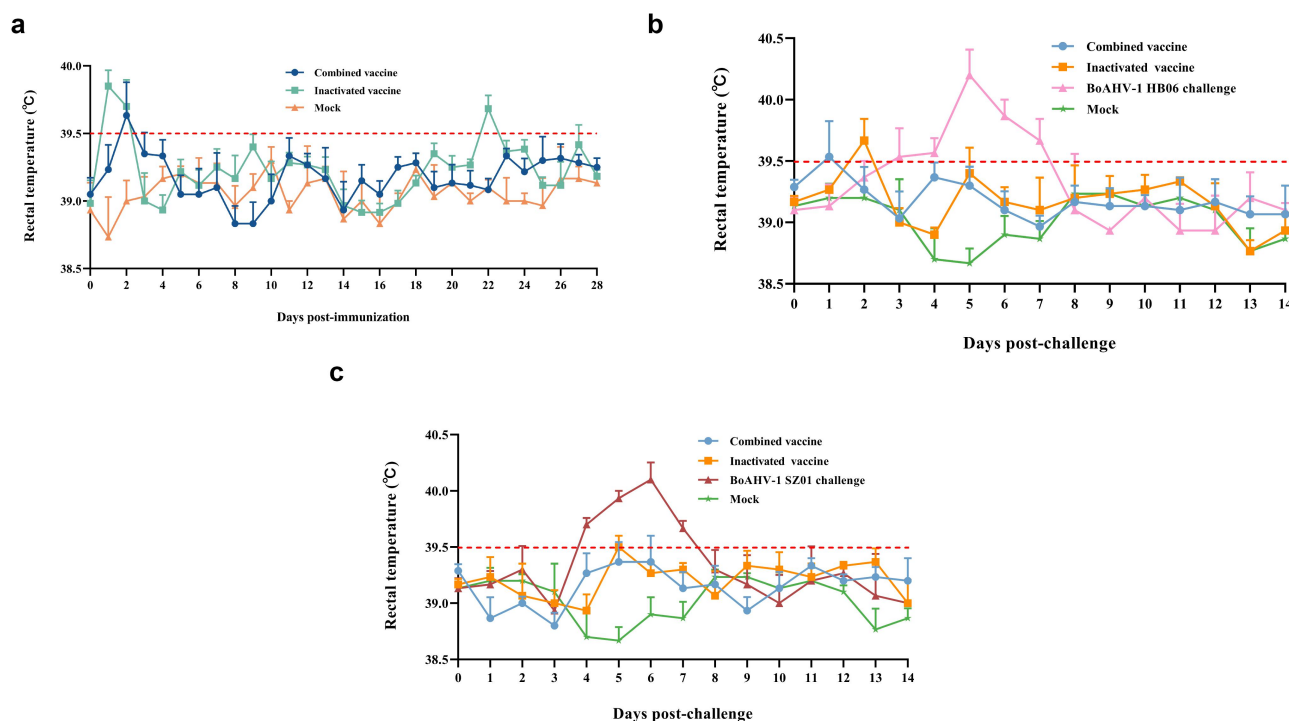


Figure 2. Changes in rectal temperature after (a) vaccination and (b) BoAHV-1 1.1a challenge and (c) BoAHV-1 1.2b challenge, respectively. The red line denotes the upper threshold of normal rectal temperature.

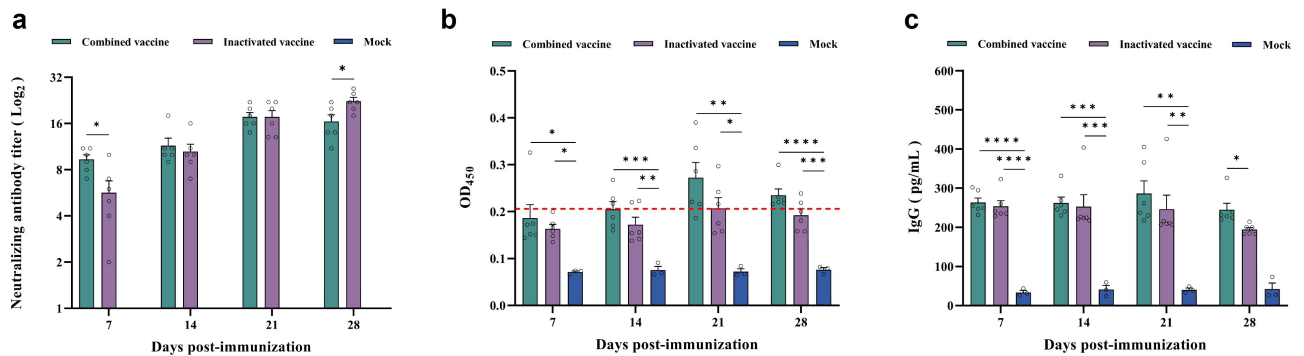


Figure 3. Humoral immune responses elicited after immunization with *M. bovis*-BoAHV-1 combined vaccine and inactivated vaccine. Serum samples were collected on a weekly basis for the purpose of detecting (a) BoAHV-1 neutralizing antibody titers and (b) BoAHV-1 gB antibody and (c) IgG levels. The red line denotes the negative-positive threshold for gB antibodies.

immunization, and a considerable discrepancy was identified between the two vaccine groups 28 d after immunization ($p < 0.05$) (Figure 3c).

Antibody response after challenge

Antibody response after BoAHV-1 1.1a challenge

The combined vaccine and inactivated vaccine groups were able to generate strong humoral immune response facing the challenge with BoAHV-1 1.1a. Both immunized groups induced extremely high levels of neutralizing antibodies at 7 d post challenge, which were significantly higher than the unimmunized challenge group ($p < 0.01$). At 14 d post challenge, the neutralizing antibody titer in the inactivated vaccine group reached the highest value during the entire observation period (1:52.7) (Figure 4a).

When the immunized groups were challenged with BoAHV-1 1.1a, the gB antibody levels of the combined vaccine and inactivated vaccine groups increased sharply, and the gB antibodies of all the vaccinated cattle turned positive. Specifically, the gB antibody level of the

combined vaccine group peaked at 0.402 14 d after challenge. Furthermore, the gB antibody levels of both vaccinated groups were significantly higher than those of the unimmunized challenge group at 21–28 d after challenge ($p < 0.05$) (Figure 4b).

IgG is pivotal in the secondary immune response. Following the challenge with BoAHV-1 1.1a, a significant elevation in IgG antibody levels was observed in both immunized groups. Notably, IgG antibody levels in the combined vaccine group were consistently and significantly higher than those in the unimmunized challenge group throughout the challenge period ($p < 0.05$) (Figure 4c).

Antibody response after BoAHV-1 1.2b challenge

When the vaccinated groups were challenged with BoAHV-1 1.2b, the neutralizing antibody titers of the combined and inactivated vaccine groups increased rapidly and remained at a considerably high level over 7–14 d, which was statistically different from that of the non-immune challenge group ($p < 0.01$) (Figure 5a).

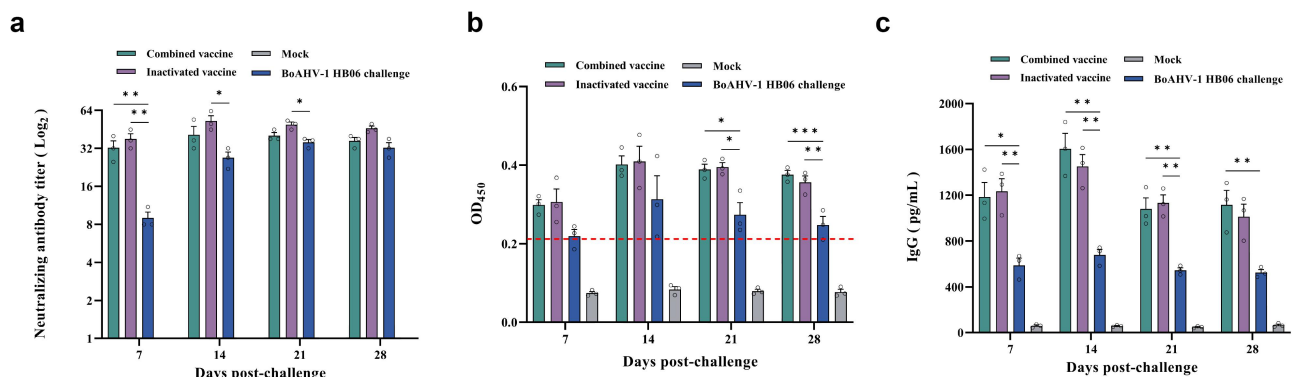


Figure 4. Humoral immune responses induced by *M. bovis*-BoAHV-1 combined vaccine and inactivated vaccine after challenge with BoAHV-1 1.1a. (a) BoAHV-1 neutralizing antibody titers and (b) BoAHV-1 gB antibody were determined. Also, (c) IgG levels were monitored. The red line denotes the negative-positive threshold for gB antibodies.

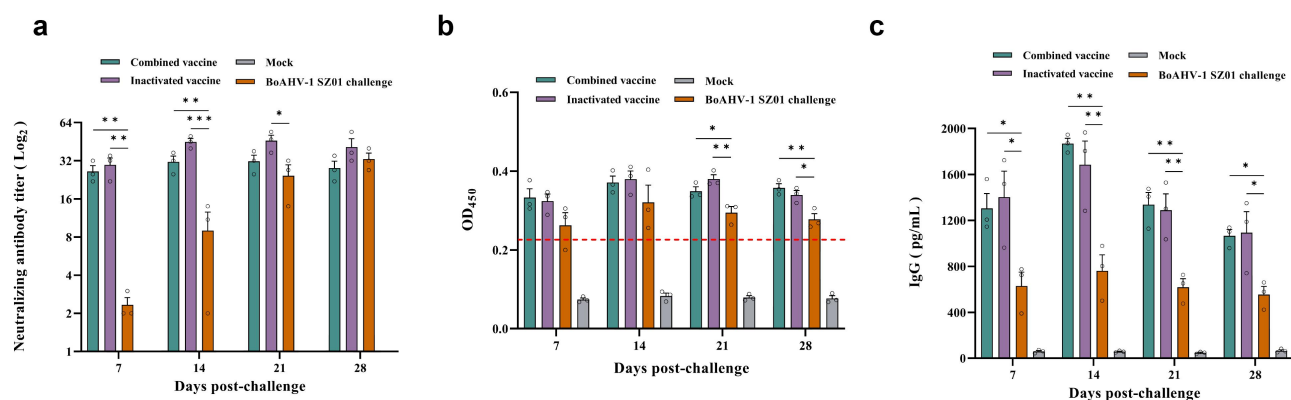


Figure 5. Humoral immune responses induced by *M. bovis*-BoAHV-1 combined vaccine and inactivated vaccine after challenge with BoAHV-1 1.2b. (a) BoAHV-1 neutralizing antibody titers and (b) BoAHV-1 gB antibody were determined. Also, (c) IgG levels were monitored. The red line denotes the negative-positive threshold for gB antibodies.

After facing the challenge with BoAHV-1 1.2b, the gB antibody levels of both immunized groups surpassed the negative-positive threshold and remained positive throughout the observation period. On day 7, the gB antibody level in the combined vaccine group could reach 0.333. Moreover, gB antibody levels were much higher in the combined vaccine and inactivated vaccine groups than in the unimmunized challenge groups at 21–28 d after challenge, and there was a statistical difference between them ($p < 0.05$) (Figure 5b).

After challenge with BoAHV-1 1.2b, an increase in IgG antibody levels was noted in both immunized groups, particularly in the combined vaccine group, whose IgG levels peaked on day 14 to 1869 µg/mL. During the whole observation period, IgG antibody levels in both inoculated groups were statistically different from those in the non-immune challenge group ($p < 0.05$) (Figure 5c).

Detection of virus shedding after challenge

Virus shedding after BoAHV-1 1.1a challenge

The quantity of BoAHV-1 1.1a and BoAHV-1 1.2b shedding post-challenge was measured using RT-PCR. When the experimental groups were challenged with BoAHV-1 1.1a, all experimental groups except the blank control group could detect high titers of BoAHV-1 1.1a shedding on the first day after the challenge, and a marked increase in the shedding of virus was observed in the non-immune challenge group in comparison to the two vaccinated groups ($p < 0.001$). Until day 9, both immunized groups continued to detect high titers of BoAHV-1 1.1a shedding. The shedding amount in the inactivated vaccine group was higher than that in the combined vaccine group, peaked at $10^{6.5}$ /mL on the ninth day, but there was no statistical difference between the two groups. The amount of

shedding in the two vaccinated groups decreased by day 11 and disappeared by day 15. Conversely, the non-immune challenge group exhibited elevated levels of shedding after challenge, with a peak concentration of $10^{7.67}$ /mL recorded on day 7, and the magnitude of shedding on day 13 was found to be significantly higher compared to the two immunized groups ($p < 0.0001$) (Figure 6a).

Virus shedding after BoAHV-1 1.2b challenge

When cattle were challenged with BoAHV-1 1.2b, virus shedding was detectable in the combined vaccine, inactivated vaccine, and unimmunized challenge groups on the first day, and shedding was significantly higher in the unimmunized challenge group than in the two immunized groups ($p < 0.05$). High titers of shedding could be detected in both vaccinated groups until day 9 and peaked on days 9 and 7, respectively. However, shedding declined by day 11 and disappeared by day 19. The non-immune challenge group maintained a high level of shedding from day 5–9, peaking at $10^{7.77}$ /mL on day 7, and a statistically significant disparity was detected between the two immunized groups on day 17 ($p < 0.001$) (Figure 6b).

Compared to existing commercial vaccines, the combined *M. bovis*-BoAHV-1 vaccine demonstrates superior cross-protection, as evidenced by significant reduction in viral shedding across multiple BoAHV-1 genotypes.

Post-vaccine transcriptome analysis demonstrates robust immune responses

We analyzed whole-blood gene expression profiles in the combined vaccine and inactivated vaccine groups. Samples available for the analysis were

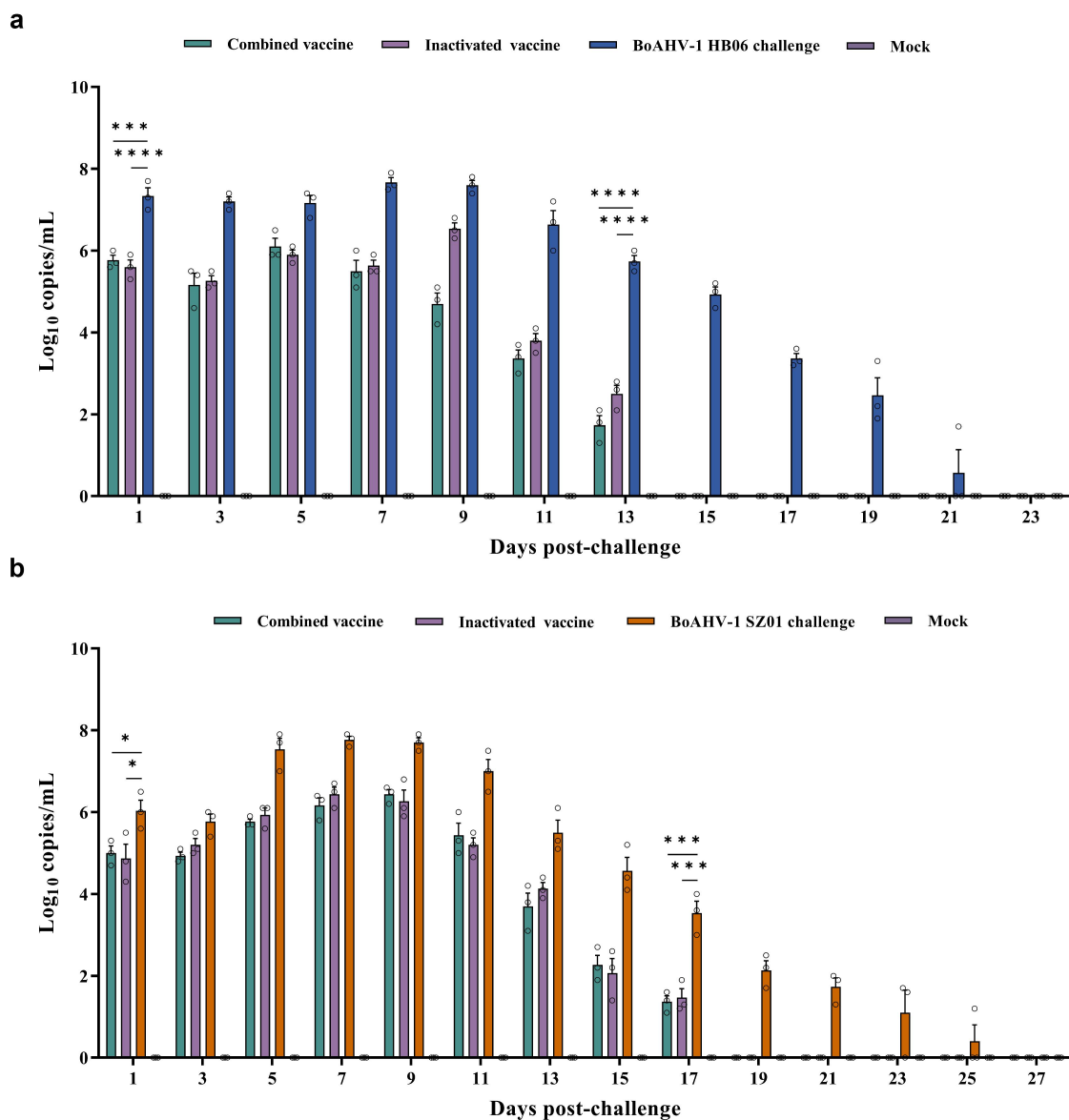


Figure 6. Detection of (a) BoAHV-1 1.1a and (b) BoAHV-1 1.2b shedding after challenge. Nasal swabs of calves immunized with combined vaccine and inactivated vaccine were collected on a daily basis.

obtained on day 0 ($n=6$; baseline), day 1 ($n=6$), day 3 ($n=6$), day 7 ($n=6$), and day 14 ($n=6$). We applied established modular tools to screen some of the differentially expressed genes [20–22]. Compared to inactivated vaccine-immunized cattle, the combined vaccine-immunized cattle overexpressed most cytokine-related genes within 14 d after immunization. Notably, the combined vaccine-vaccinated cattle expressed significantly higher levels of nearly half of the cytokine-related genes than those of the inactivated vaccine-vaccinated cattle on day 1, including IL-1B, IL-6, IFNAR, and IFNGR. Until day 14, the expression levels of IL-1B, IFNGR, and TRAP1 still remained statistically

different between the two vaccine groups (Figure 7a).

In addition, we found overexpression of the B-cell module on day 1 in cattle vaccinated with the combined vaccine compared to those vaccinated with the inactivated vaccine. Interestingly, the expression of B-cell module-related genes remained significantly higher in the combined vaccine group than in the inactivated vaccine group until day 7 ($p<0.05$), suggesting that the combined vaccine could induce a faster antibody response during the early stage of vaccination.

Moreover, transcripts overexpressed within 14 d after vaccination also included monocyte-related genes, and their expression during this period was

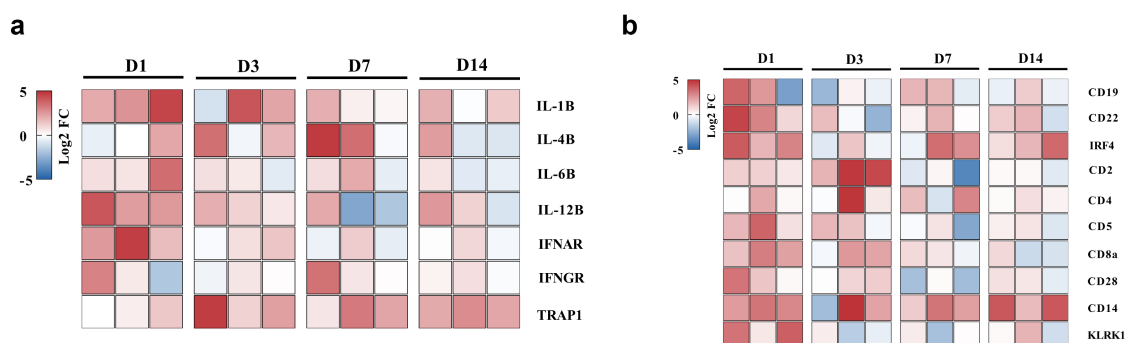


Figure 7. Transcriptomic analysis reveals elevated expression of (a) cytokines, (b) B cells, T cells, NK cells and monocyte factor-associated modules. Heat maps of transcriptome expression on days 1, 3, 7, and 14 after vaccination were compared between the two vaccines, using day 0 as the baseline and the inactivated vaccine as the reference. Each module represents one cattle. The color legend represents the Log2-fold change. Bubble plots show the fold change and statistical significance of the target genes (two-sided Student's t test).

significantly different between the two vaccine groups ($p < 0.05$).

Conversely, the T-cell-associated modules in the combined vaccine group decreased slightly from their previous overexpression and a few shifted to under-expression on day 7–14 compared to the inactivated vaccine group. However, in the first 3 d after immunization, the combined vaccine group still induced a robust T-cell immune response compared to the inactivated vaccine group ($p < 0.05$) (Figure 7b).

Combined vaccine induce cellular immunity

Based on the whole blood transcriptomics results, we quantified the differentially expressed cytokines, with IFN- γ and TNF- α exhibiting the highest levels of expression. The level of IFN- γ increased rapidly in the combined vaccine group and was significantly higher than that in the inactivated vaccine group on the first and third day after vaccination ($p < 0.01$). In addition,

a statistically significant disparity between these two immunized groups was observed 14 d post-immunization ($p < 0.0001$) (Figure 8a).

The level of TNF- α increased slightly after inoculation with the combined vaccine, but the level of TNF- α produced by the group received the combined vaccine exhibited a consistent and significant increase compared to those inoculated with the inactivated vaccine throughout the observation period ($p < 0.01$) (Figure 8b).

Current vaccines often exhibit limited efficacy against diverse genotypes, whereas this novel approach elicited robust humoral and cellular immune responses, including significantly higher antibody titers ($p < 0.05$) and enhanced IFN- γ production.

Discussion

BoAHV-1 is a successful herpesvirus that adapts to its bovine host and often causes only mild clinical symptoms, but establishes lifelong latent infections with

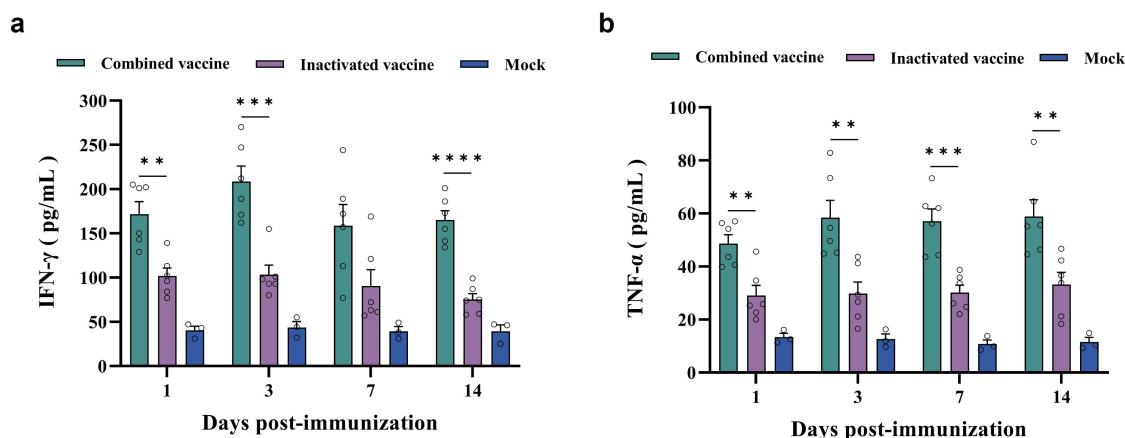


Figure 8. Cellular immune response following immunization of cattle with *M. bovis*-BoAHV-1 combined vaccine and inactivated vaccine, respectively. (a) and (b) represent the level of IFN- γ and TNF- α , respectively.

intermittent viral shedding. Currently, the accepted taxonomic classification of BoAHV-1 divides the virus into two main types, type 1 and type 2, based primarily on restriction enzyme analysis (RAE) profiles obtained during viral purification [23] or BoAHV-1.1 and BoAHV-1.2 by other methods [24]. Although BoAHV-1.2b is commonly associated with diseases of the reproductive system, it is frequently detected in calves with respiratory problems. Typical BoAHV-1 infection manifests as inflammation of the respiratory mucosa, including the nasal and tracheal regions, accompanied by hemorrhage of the nasal mucosa and conjunctivitis. In our study, when unimmunized cattle were challenged with the BoAHV-1 1.1a and 1.2b subtypes, most exhibited these characteristic clinical symptoms, underscoring the ability of these viruses to induce the expected pathological presentation in the absence of prior immunity.

Importantly, while BoAHV-1 1.1a and 1.2b are the predominant subtypes circulating in China, there is currently no published evidence demonstrating whether existing BoAHV-1 vaccines are capable of providing cross-protective immunity against these two specific viral variants. This knowledge gap highlights the need for further investigation of the cross-protective efficacy of available BoAHV-1 vaccines, particularly in regions where these subtypes are known to be endemic.

In our preceding study, we developed a novel attenuated and marker *M. bovis*-BoAHV-1 combined vaccine for the first time, and a preliminary evaluation of its safety and immunogenicity was also undertaken. In this study, we further challenged vaccinated cattle with the two predominant subtypes of BoAHV-1 in China and compared its protective efficacy with that of an inactivated vaccine already on the market.

The results demonstrated that both the combined vaccine and inactivated vaccine were effective in reducing virus shedding after challenge compared with the unimmunized challenge group. In the BoAHV-1 1.1a challenge, all vaccinated groups showed significantly lower virus shedding on the first day post-challenge than the unimmunized group. Additionally, the vaccinated groups continued to shed high titers of the virus until day 9, but the amount of shedding was significantly lower than that in the non-immunized group, especially on day 13. A similar pattern was observed in the BoAHV-1 1.2b challenge, in which the vaccinated groups had significantly lower virus shedding levels than the unimmunized group throughout the experimental period. This could be attributed to the additional immune priming provided by the subunit component of the combined vaccine, which might

have enhanced the overall protective immune response. The findings presented here align closely with the study's objectives, providing strong evidence of the vaccine's ability to reduce viral shedding and alleviate clinical symptoms. This highlights the vaccine's potential to address key challenges in veterinary vaccinology, particularly the need for cross-protection against diverse BoAHV-1 genotypes.

The differences in viral shedding between the vaccinated and unimmunized groups were likely mediated by both humoral and cell-mediated immunity. The induction of robust antibody-mediated and cellular immune responses by the vaccines likely contributed to the reduced virus shedding observed in vaccinated animals compared to unvaccinated controls.

The neutralizing antibody titers increased more rapidly in the combined vaccine group than in the inactivated vaccine group, reaching significantly higher levels by day 7 post immunization. This rapid induction of neutralizing antibodies is likely attributable to the stimulation of Th2-type immunity by the combined vaccine [25], as evidenced by the overexpression of the Th2 cytokine IL-4. Importantly, the average neutralizing antibody titer in the combined vaccine group (1:9.3) exceeded the protective threshold of 1:8 [26], underscoring the potential of the vaccine to provide effective humoral immunity against BoAHV-1 infection.

In line with the production of a high titer of neutralizing antibodies, we found that the combined vaccine was able to induce higher production of gB- and IgG-specific antibodies compared to the inactivated vaccine. The importance of IgG-mediated humoral immunity, particularly in the context of bovine udder defense, is well-established [27]. The superior IgG response elicited by the combined vaccine suggested its ability to confer robust systemic humoral protection against BoAHV-1. When challenged with different BoAHV-1 subtypes, both vaccinated groups were able to mount high titers of neutralizing and gB-specific antibodies. However, the inactivated vaccine group exhibited stronger humoral immune response. This observation is consistent with the known mode of action of inactivated vaccines, which typically induce more pronounced humoral immunity and short-term memory than attenuated vaccines [28].

Furthermore, according to the transcriptome analysis, we found that a number of genes related to various immune response pathways, including cytokines, NK cells, monocytes, B cells, and T cells, were expressed at higher levels after immunization with the combined vaccine than with the inactivated vaccine. The most pronounced increases were observed for genes

associated with IL-1 β , IL-6, type I IFN, IFN- γ , and TNF- α , as well as genes linked to NK cell, B cell, and T cell activation. The findings indicate that the combined vaccine has the capacity to stimulate a vigorous cellular immune response, thereby engaging both the innate and adaptive arms of the immune system [29,30]. The enhanced expression of cytokines, NK cells, B cells, and T cell-related genes indicates that the combined vaccine formulation was more effective in stimulating a comprehensive cellular immune response than inactivated vaccine alone [28]. The increased activation of pathways involved in innate immunity (e.g. type I IFN, IL-1 β , IL-6, and TNF- α) and adaptive immunity (e.g. IFN- γ , NK cells, monocytes, B cells, and T cells activation) likely contributed to the improved protection observed with the combined vaccine in previous virus shedding experiments [31–39].

The rapid clearance of the virus in the vaccinated groups, with shedding becoming undetectable by days 15–19, also highlights the ability of vaccines to stimulate a robust and durable immune response. This is an important consideration for the practical application of these vaccines, as it suggests that they could provide effective protection against BoAHV-1 infection and transmission within herds.

Taken together, our findings indicate that the *M. bovis*-BoAHV-1 combined vaccine elicited a rapid and comprehensive immune response compared with the inactivated vaccine, engaging both cellular and humoral arms of the immune system. The ability of the combined vaccine to induce elevated levels of neutralizing antibodies, gB-specific antibodies, and IgG, as well as the upregulation of key cytokine and immune cell-related genes, suggests its potential to provide effective protection against BoAHV-1 infection. These findings have significant implications for the development of next-generation vaccines in veterinary medicine. In addition, compared with our previously developed single-component BoAHV-1 double gene-deleted vaccine, the two antigenic components of the combined vaccine did not show antagonistic effects [17], and the combined vaccine showed good immune efficacy against BoAHV-1 1.1a subtype compared with the single-component BoAHV-1 double gene-deleted vaccine [19], besides, it effectively protects vaccinated individuals from the challenge of BoAHV-1 1.2b subtype at the same time. Although some studies have shown that existing commercial marker vaccines can also effectively protect animals, including dairy cows and water buffalo, from BoAHV-1 wild-type strains, increase antibody levels in vaccinated individuals, and reduce the duration and amount of viral shedding after

challenge [40,41], all these marker vaccines have a common limitation, that is, they can only resist challenge from a single genotype of BoAHV-1 wild-type strain. The cross-protective efficacy of a vaccine against BoAHV-1 challenges of different genotypes has never been addressed in previous studies. By providing broad-spectrum protection against diverse BoAHV-1 genotypes, our combined vaccine offers a cost-effective solution for addressing genotype-specific outbreaks, potentially reducing economic losses in the cattle industry. Furthermore, the success of this approach could inform policy decisions aimed at promoting the adoption of combination vaccines, particularly in regions with high genetic variability of BoAHV-1.

While this study demonstrates robust protection afforded by the combined *M. bovis*-BoAHV-1 vaccine under controlled experimental conditions, mainly in terms of in reducing viral shedding, alleviating clinical symptoms, and providing cross-protection against diverse BoAHV-1 genotypes. But there are limitations that warrant further investigation, the short duration of post-vaccination observation primarily assesses acute immune responses, leaving questions about the long-term durability of immunity unanswered. Future research should explore its long-term protective effects, and extending observation periods will provide insights into the durability of immunity. Additionally, the vaccine's field applicability and efficacy in diverse environmental and management conditions remain to be evaluated in large-scale trials, and large-scale field trials are essential to assess the efficacy of the vaccine. Furthermore, the incorporation of additional antigens could enhance protection against other co-infecting pathogens, offering a more comprehensive solution to bovine respiratory diseases. Future studies should focus on addressing these gaps to validate the vaccine's broader utility in real-world scenarios.

In conclusion, the results of this study demonstrated that both the combined vaccine and inactivated BoAHV-1 vaccine were effective in significantly reducing and clearing virus shedding after challenge with either BoAHV-1.1a or 1.2b strains in comparison with the non-immune challenge group. It is worthy of note that the combined vaccine group demonstrated slightly superior performance in terms of the limitation of virus shedding from various BoAHV-1 subtypes, in comparison to the inactivated vaccine group. This indicated that the combined vaccine can provide cross-protection against diverse BoAHV-1 subtypes, which is crucial for implementing effective disease control strategies in cattle herds. Further investigation of the immunological mechanisms and long-term protective effects will help

fully elucidate the advantages of these vaccine approaches. By demonstrating these effects in a controlled setting, this study establishes a foundation for developing next-generation combination vaccines that are both cost-effective and broadly protective. Also, our results underscore the potential of this combined vaccine to serve as a cost-effective and scalable solution for improving cattle health. Its application in regions with high genetic variability of BoAHV-1 genotypes could significantly reduce economic losses and inform policy decisions aimed at advancing veterinary vaccination strategies.

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No potential conflict of interest was reported by the author(s).

Data availability statement

The data supporting the findings of this study are available within the article and are openly available in “science data bank” at <https://doi.org/10.57760/sciencedb.11553>.

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