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La Sota-vectored recombinant vaccine with chimeric hemagglutinin-neuraminidase for enhanced protection against highly pathogenic pigeon paramyxovirus type 1

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ABSTRACT

Pigeon Paramyxovirus Type 1 (PPMV-1), an antigenic and host variant of the Newcastle Disease Virus (NDV), can infect pigeons of all ages and cause severe economic losses in the poultry industry. The existing commercial vaccines are not capable of providing complete protection against the prevalent PPMV-1 strains. To address this issue, reverse genetic technology was employed to create a recombinant 167DM strain by incorporating the chimeric genotype VI hemagglutinin-neuraminidase (*HN*) with La Sota as the backbone. The optimal anti-PPMV-1 vaccine candidate was identified through a systematical comparison of biological characteristics and immune efficacy of the predominant PPMV-1 epidemic strain, the 167DM strain, and the La Sota strain. Results indicated that the 167DM strain exhibited the highest culture titers in allantoic fluid and the strongest heat resistance. The antibody titers in the 167DM vaccine group consistently surpassed those in other groups tested. Cross-hemagglutination inhibition (HI) tests revealed no detectable antigenic differences between the 167DM and the prevalent PPMV-1 strain. Furthermore, the 167DM strain conferred 100% protection by preventing PPMV-1 infection and completely inhibiting virus shedding. These findings provide valuable insights for the development of a novel vaccine targeting ND in pigeons, thus laying a foundation for further advancements in vaccine development within this avian population.

INTRODUCTION

Newcastle Disease (ND), caused by the Newcastle Disease Virus (NDV), is a highly contagious disease affecting most avian species and has resulted in severe economic losses in the global poultry industry (Mayo, 2002). NDV, also known as Avian Paramyxovirus Type 1 (APMV-1), is a member of the genus *Orthoavulavirus* in the family *Paramyxoviridae* (Dimitrov, et al., 2019). The negative-stranded and non-segmented genome of NDV is approximately 15.2 kb, adhering to the "rule of six" for genome length and encodes six major structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and large polymerase (L) (Millar, et al., 1988). Pigeon Paramyxovirus Type 1 (PPMV-1), a host-specific and antigenic variant of NDV, predominantly falls within genotype VI of class II (Liu, et al., 2003; Qiu, et al., 2017; Wei, et al., 2018). PPMV-1 was first identified in Middle Eastern pigeons in 1978 and triggered the third ND panzootic during the 1980s. Pigeons of all ages are susceptible to PPMV-1 with morbidity and mortality rates often exceeding 50% (Xie, et al., 2020). The typical clinical symptoms in pigeons infected with PPMV-1 include neurological signs, such as paralysis and torticollis (Śmietanka, et al., 2014; Ren, et al., 2017). The disease was first confirmed in China in 1985, where it has since posed a continual threat to pigeon breeding and wild bird populations (Aldous, et al., 2004; Tian, et al., 2020).

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Contemporary control measures for ND encompass vaccination programs and the culling of infected or potentially infected animals. However, there is a lack of commercial vaccines and specific antiviral therapies against PPMV-1 in China. Chicken genotype II vaccines, such as La Sota, have been widely utilized for vaccinating pigeon flocks to prevent ND (He, et al., 2020). Unfortunately, the commercial vaccine has not demonstrated complete efficacy, leading to outbreaks in numerous pigeon farms across China (Guo, et al., 2013; He, et al., 2020). The insufficient immune response is largely due to significant biological and genetic differences between the vaccine strains and the circulating NDV strains (Rui, et al., 2010; Dortmans, et al., 2014; Liu, et al., 2015a). Numerous studies have demonstrated that vaccines genotypically matched to the epidemic strains significantly enhance ND control by reducing viral shedding among affected birds (Miller, et al., 2007; Hu, et al., 2009; Dimitrov, et al., 2017). The immunogenicity of genotype VI NDV strains derived from pigeons differs from that of the chicken-origin vaccine strain La Sota (Qiu, et al., 2017; He, et al., 2020; Xie, et al., 2020). Immunization with inactivated strains of PPMV-1 has been shown to protect pigeons from lethal infection with the virulent PPMV-1 strains and reduce virus shedding (Amer, et al., 2013; Zhang, et al., 2022).

The two surface glycoproteins, the HN protein and the F protein, are key protective antigens of NDVs. Antibodies against the F protein protect chickens from lethal NDV challenge (Swett-Tapia, et al., 2016). Studies have demonstrated that the F protein plays a pivotal role in Newcastle disease virus (NDV) vaccine design, as its expression or incorporation across different genotypes significantly enhances protective immunity in animals (Śmietanka, et al., 2019; Sun, et al., 2020; Wei, et al., 2020). These antibodies exhibit broad-spectrum properties because the F proteins of different NDV strains exhibit highly conserved antigenicity (Abenes, et al., 1986), providing protection against both homologous and heterologous NDV strains (Ferreira, et al., 2021). The HN protein of NDV is crucial for eliciting immune protection against viral infections and is susceptible to immune response, making it an important target for vaccine development (Gong and Cui, 2011; Chang, et al., 2023). The HN protein comprises an intracellular region, a transmembrane region, and an extracellular region, which is further divided into a globular region and a stem-like region (Yuan, et al., 2011; Yuan, et al., 2012). The stalk region of the HN protein primarily serves to connect the globular region with the transmembrane region. The interaction between the homologous HN stalk region and the F protein is essential for cell fusion and syncytium formation (Stone-Hulslander and Morrison, 1997; Li, et al., 1998; Sergel, et al., 2000; Tsurudome, et al., 2015; Tsurudome, et al., 2018). The globular head region contains several critical functional domains, including the HN receptor recognition site, antigenic sites, and the hemagglutination (HA) active site (Yuan, et al., 2011). These domains collectively facilitate the virus's ability to recognize host cells, evade immune responses, and enter host cells, thus playing a crucial role in viral infectivity and transmission. Studies have demonstrated that replacing the globular region of La Sota HN can create effective vaccine markers while providing protection against the corresponding genotype (Peeters, et al., 2001; Liu, et al., 2017; Bu, et al., 2018). Research indicates that the HN protein of Newcastle disease virus (NDV) shows genotype-related variability in its immunogenicity (Connaris, et al., 2002). Differences in antigenic epitopes across genotypes can affect the immune responses elicited by vaccines that target these proteins, potentially altering the effectiveness of immunity (Zaitsev, et al., 2004; Doan, et al., 2022).

Previous studies have shown that the double mutation (DM) in the M protein (R247K and S263R) enhances its nuclear export capability, leading to increased proliferation of NDV (Peng, et al., 2022). Given the growing threat of PPMV-1 and the limitations of current vaccines, we used a reverse genetics platform to generate a recombinant virus with genotype VI antigen substitutions in the rLa Sota-DM backbone. This study aims to evaluate the biological characteristics and immune efficacy of this recombinant strain in comparison with the prevalent PPMV-1 TJ(WT) strain, its attenuated aTJ strain, and the La Sota strain. The recombinant virus demonstrated high proliferation efficiency, thermal stability, and improved immune protection against virulent PPMV-1strain, indicating its potential as a candidate vaccine.

MATERIALS AND METHODS

Ethics Statements

The protocols involving animals were approved by the Animal Welfare and Ethics Committee of the Institute of Animal Sciences (IAS), Chinese Academy of Agricultural Sciences (Approval number: ISA2023-120, CAAS, Beijing, China).

Virus and Plasmids

The PPMV-1 strain Pigeon/TJ/CH/020/2020, referred to as TJ(WT) and assigned the GenBank accession number PQ563353, was isolated from pigeons in China by our laboratory and identified as the dominant epidemic strain of subtype VI.2.1.1.2.2 (Zhang, et al., 2024a). The aTJ strain was rescued in our laboratory, where the F cleavage site of the TJ (WT) virus was replaced with that of the La Sota strain (Zhang, et al., 2024b). The PPMV-1 Strain pi/SH/CH/0167/2013 (ND167) was isolated and preserved in our laboratory with GenBank accession number KT163262.1 (Qiu, et al., 2017). The NDV strain La Sota/46 was purchased from the China Institute of Veterinary Drug Control (Beijing, China). pLaSota-DM, pCI-NP, pCI-P, and pCI-L were kept in our laboratory (Wang, et al., 2013).

Cells and Eggs

Baby hamster kidney fibroblast cells (BHK21) were purchased from the Cell Resource Center, Peking Union Medical College. BHK21 cells constitutively expressing T7 RNA polymerase (BSR T7/5 cells) were kindly provided by Zhigao Bu (Harbin Veterinary Institute, Harbin, China). Nine-day-old Specific Pathogen Free (SPF) embryonated chicken eggs and one-day-old SPF chickens were purchased from Beijing Boehringer Ingelheim Vital Biotechnology Company.

Construction of Infectious cDNA Clones of the Recombinant Virus

Using the full-length cDNA clone pLaSota-DM as the backbone, the C-terminal globular head domain of the HN protein was replaced with the corresponding region from the HN of NDV genotype VI. Initially, the pLaSota-DM plasmid served as the template. The primer pairs ZPB1-F/ZPB1-R and ZPB2-F/ZPB2-R were employed for PCR amplification, and the resulting amplified products were ligated using the pEASY-Uni Seamless Cloning and Assembly Kit (TransGen Biotech, Beijing, China) to construct the ZP-B plasmid. As illustrated in Fig. 1A, primers 6HN-F and 6HN-R were used to amplify the globular region of the HN from the ND167 strain, while primers ZPBHN-F and ZPBHN-R were utilized to amplify the corresponding fragment from ZP-B. The two amplified fragments were subsequently ligated using the same method to create the ZP-B-6HN plasmid. Following this, primers pLS-F/pLS-R and ZPB6HN-F/ZPB6HN-R were employed to amplify segments from plasmid pLaSota-DM and plasmid ZP-B-6HN, respectively. These two amplified fragments were then ligated to generate a full-length plasmid designated as pLSD-6HN. All primers mentioned above are presented in Supplementary Table 1.

Rescue and Propagation of Viruses

The full-length cDNA clone of pLSD-6HN, along with supporting plasmids expressing the NP, P, and L proteins, were co-transfected into BSR T7/5 cells using Lipofectamine 2000 (ThermoFisher, USA), following previously established protocols (Römer-Oberdörfer, et al.,

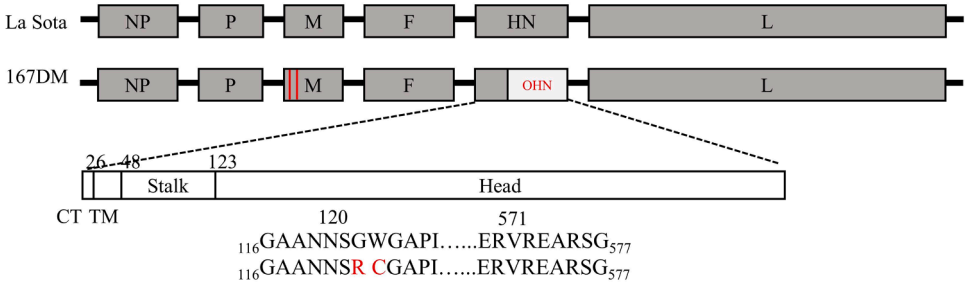

Figure 1. Schematic diagram of gene replacement at the head of hemagglutinin-neuraminidase (HN). Using the La Sota reverse genetics platform, the antigenic region of NDV genotype VI was substituted in the HN globular region, spanning amino acids 122 to 571 of the HN protein. The length was adjusted accordingly, while retaining the final 6 amino acids from the La Sota strain. A red vertical line indicates the R247K and S263R double point mutations on the M protein.

1999; Wang, et al., 2013; Peng, et al., 2022). At 4 h post-transfection, the medium was switched to Dulbecco's Modified Eagle Medium (DMEM, Life Technologies, UK) supplemented with 2% fetal bovine serum (FBS, Gibco) and 1 μg/mL Tosyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma, USA). Cells and supernatant were harvested, and a 100 μL mixture was inoculated into 9-day-old SPF chicken embryos. The presence of recombinant virus was confirmed through HA test as outlined in the World Organization for Animal Health (WOAH) reference manual (WOAH, 2023).

Virus Titration and Pathogenicity Assessment

The characteristics of viruses were measured using a standard HA assay and a 50% egg infective dose (EID_{50}) assay in 9-day-old SPF chicken embryos (WOAH, 2023). A total of 40 allantoic fluid samples from chicken embryos at the fifth passage were selected for HA titer measurement. Viral titers were also determined using a 50% tissue infectious dose ($TCID_{50}$) assay in BHK21 cells. Briefly, the virus was serially diluted tenfold in DMEM. A total of 100 μL of each dilution was added to a 96-well plate containing with a monolayer of BHK21 cells, with each dilution replicated six times. After incubation for 1 h, the supernatant was removed and replaced with 100 μL of fresh DMEM containing 2% FBS and 1 μg/mL TPCK-trypsin. Cells were incubated for 48 h and then fixed with 4% paraformaldehyde for 30 min. A primary antibody, Mouse anti-NP monoclonal antibody (1:1000 dilution), was added into 50 μL per well and incubated at 37°C for 2 h. Subsequently, a fluorescent secondary antibody, Alexa Fluor 488 nm goat anti-Mouse IgG (diluted 1:500), was added into 50 μL per well and incubated at 37°C for 40 min in the dark. NDV infection was determined by fluorescence observation using a fluorescence microscope (Nikon, Tokyo, Japan). The EID_{50} and $TCID_{50}$ values were calculated using the Reed-Muench method (Lei, et al., 2021). Pathogenicity of the viruses was assessed through the mean death time (MDT) and intracerebral pathogenicity index (ICPI) tests (WOAH, 2023).

Thermostability Tests

The NDV strains were incubated in a 56°C water bath and then immediately transferred to ice to halt the heating process at various time intervals. Subsequently, the HA titer and $TCID_{50}$ on BHK21 cells were measured.

Viral Growth Kinetics

To measure the virus growth curve, monolayer BHK21 cells were initially infected with the virus at a multiplicity of infection (MOI) of one and incubated at 37°C for 1 h, with regular shaking of the cell plate every 15 min. After 1 h incubation, the virus was removed and BHK21 cells were washed with phosphate-buffered saline (PBS, Gibco, USA). The medium was replaced with fresh DMEM supplemented with 2% FBS and 1 μg/mL TPCK-trypsin. The supernatant was collected at 12, 24, 36, 48, and 60 h post-infection. The $TCID_{50}$ of the supernatants was quantified using the endpoint method of Reed and Muench (Lei, et al., 2021).

Vaccines Preparation

All kinds of viruses were propagated in 9-day-old SPF chicken embryos. The virus in allantoic fluid was completely inactivated by 0.1% formaldehyde at 37°C in a rotary shaker (200 rpm) in the dark for 24 h. The loss of infectivity was confirmed by two serial blind passages in embryonated chicken eggs. The inactivated virus was then mixed with 70% water-in-oil adjuvant (ISA 763; Seppic, Puteaux, France) to produce the inactivated vaccine.

Animal Immunization and Viral Challenge

To evaluate the efficacy of recombinant strains, a total of fifty one-month-old domesticated homing pigeons were purchased from a pigeon farm (Beijing, China). None of the pigeons had any history of disease or vaccination against NDV, and all were confirmed serologically negative for NDV antibodies using the hemagglutination inhibition (HI) assay. The pigeons were randomly divided into five groups (n = 10/group) and immunized intramuscularly with either TJ(WT), aTJ,167DM, La Sota, or PBS (control) at a dose of 0.5 mL/pigeon. Twenty days post-vaccination (dpv), all pigeons were challenged intramuscularly with 10^6 EID_{50} of virulent NDV genotype VI strain TJ (WT). The pigeons were monitored daily for clinical signs and mortality for up to 14 days post-challenge (dpc). Clinical symptoms were assessed daily based on the following scoring criteria: 0 points for normal state, 1 point for depression or neck shrinkage, 2 points for drooping wings, torticollis or ataxia, 3 points for general paralysis or collapse, and 4 points for death (Paldurai, et al., 2014; Chen, et al., 2021). Blood samples were collected at 7, 14, and 21 dpv for antibody titer assays. Cloacal swabs taken at 5 and 10 dpc were analyzed using the chicken embryo isolation method to detect viral shedding. Protection rates were calculated based on the absence of both clinical symptoms (score 0) and viral shedding after the challenge. At the end of the observation period, all remaining pigeons were humanely euthanized using CO_2 asphyxiation.

HI Assay

Serum samples were collected from pigeons at weekly intervals for three weeks post-immunization. HI titers against NDV were determined according to the standard procedure (WOAH, 2023). Briefly, the HA units of genotype II NDV isolate La Sota or genotype VI NDV isolate TJ (WT) were measured by the HA assay, where one unit is defined as the reciprocal of the highest dilution of purified antigen that causes complete hemagglutination. The antigen was diluted to 4 HA units (HAU) in 25 μL. Pigeon serum samples were pre-treated with 20% volume of chicken red blood cells (RBCs) at 4°C for 1 h to remove nonspecific agglutination. The RBCs were then pelleted by centrifugation at 800 rpm for 5 min, and the adsorbed sera were collected. Processed serum

samples were serially diluted (1:2) in V-bottomed microwell plastic plates. Four HAU of HA antigen were added, followed by incubation at room temperature for 1 h. Finally, 25 μL of 1% (vol/vol) RBCs was added to each well and allowed to settle for 40 min at room temperature. NDV-positive controls, antigen controls, and PBS controls were included in each plate. HI titers were recorded as the highest dilution that completely inhibited four HAU of antigen. The HI titer was considered positive when it was equal to or greater than 4 $\log_2$. Additionally, the geometric mean titer (GMT) was calculated for each group to provide a robust assessment of the immune response, as this method is less affected by outliers (Ren, et al., 2020).

Analysis of Antigenic Variation

To assess the serological differences among the La Sota, TJ(WT), and 167DM strains, sera collected on 21 dpv were analyzed through cross-HI tests. The antigen correlation coefficient (R-value) between each pair of strains was calculated with the formula: $R = \sqrt{r1 \times r2}$, r means heterologous HI titer to homologous HI titer. The following interpretation criteria were applied: an R-value between 0.67 and 1.5 indicates no significant antigenic difference between the two viruses; an R-value between 0.50 and 0.67 indicates a minor difference; and an R-value less than 0.50 indicates a major difference between the two virus strains (Archetti and Horsfall, 1950; Li, et al., 2010).

Neutralization Assay

All serum samples collected at 21 dpv were used for neutralization assays. Serum samples were heat-inactivated at 56 °C for 30 min and serially diluted twofold. A total of 200 $TCID_{50}$ of TJ(WT) strain was mixed with an equal volume of serially diluted serum samples and incubated at 37 °C for 1 h. BHK21 cells, cultured in 96-well plates, were infected with the mixture and incubated for three days. Each dilution was tested in quadruplicate. Blank controls and no-serum control samples were prepared by inoculating cells with an equal volume of DMEM or virus. The cells were monitored for virus-specific cytopathogenic effects at 37°C daily. The neutralization titer was defined as the highest serum dilution inhibiting virus replication, and the median protective dose (PD_{50}) was calculated using the Reed-Muench method.

Virus Shedding

Cloacal swabs were collected from each pigeon in groups at 5 and 10 dpc for the detection of virus shedding. Samples were placed in 1 mL of PBS containing penicillin (10,000 units/mL), streptomycin (10 mg/mL), and gentamycin (250 μg/mL) and incubated at 37°C for 2 h. a total of 200 μL of the supernatant was incubated in 9-day-old chicken embryos. After 4 days, virus presence was confirmed by HA assay.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism version 9.5 (GraphPad Software Inc., USA). Data are presented as mean ± SD or mean ± SEM, as appropriate. Two-way analysis of variance (ANOVA) was used for comparing HI titers and cross-HI titers between groups. One-way ANOVA was conducted to analyze neutralizing antibody (NAb) titers. Clinical scores for each group were expressed as mean ± SEM, and survival curves were plotted to assess protection levels. Statistical significance was set at $p < 0.05$.

RESULTS

Generation of Recombinant Viruses

To develop a genotype-matching vaccine against PPMV-1 epidemic strains, the reverse genetic platform of La Sota was utilized to substitute its HN globular region with the corresponding region of the genotype VI NDV strain (Fig. 1a). The first passage of the rescued virus, designated as 167DM, exhibited an HA titer of 8 $\log_2$ in the allantoic fluid of chicken embryos, confirming the successful recovery of the virus.

Virus Titration and Pathogenicity Analysis

To assess the replication efficiency attenuated virus strains, HA titers were measured from 40 allantoic fluid samples collected from 9-day-old SPF chicken embryos at the fifth passage.The results, depicted in Fig. 2a, revealed that the average HA titer of 167DM was the highest at 14.08 ± 1.07 $\log_2$, followed by the aTJ strain at 10.78 ± 1.27 $\log_2$, and the classic vaccine strain La Sota at 10.45 ± 0.55 $\log_2$. The HA titer of 167DM was significantly the highest compared to both the aTJ strain ($p < 0.0001$) and La Sota strain ($p < 0.0001$), indicating that the 167DM strain proliferated most efficiently in chicken embryos compared to other attenuated strains. These findings suggested that 167DM may serve as a more efficient and cost-effective vaccine candidate due to its superior replication capacity.

$TCID_{50}$, EID_{50}, MDT, and ICPI of the rescued virus were measured and the results are presented in Table 1. Based on the virulence criterion, it was determined that 167DM was lentogenic.

Growth Characteristics and Thermostability

To compare the growth characteristics of the rescued virus and the parental strain, growth curves for the viruses were constructed using BHK21 cells. The TJ(WT), aTJ, 167DM and La Sota strains were inoculated into BHK21 cells at MOI of one, and the $TCID_{50}$ at different time points was measured. The results are presented in Fig. 2b. The rescued virus replicates normally in BHK21 cells, similar to the parent strain. The aTJ and 167DM strains showed similar growth curves, reaching their maximum titer 48 h after infection, although both were lower than the TJ(WT) strain. La Sota displayed the least proliferation at each time point.

The impact of heating at 56 °C on the activity of the viruses was investigated. The results indicated that the La Sota vaccine strain exhibited lowest heat resistance compared to other strains. The HA activity was completely diminished after heated at 56 °C for 5 min, and its $TCID_{50}$ loss rate was 100% after 30 min of heating. In contrast, the aTJ and TJ(WT) strains lost their activity after 60 min, indicating their better heat resistance. The 167DM strain, however, remained active after 60 min of heating, showing a 25% decrease in HA titer and a 64.67% decrease in $TCID_{50}$ assay (Fig. 2). These results suggest that the 167DM strain possesses the highest heat resistance among the strains tested.

Serological Responses Post-Immunization

Blood samples were collected on 7, 14, and 21 dpv. The aTJ antigen was used to detect HI antibodies. As showed in Fig. 3a, the 167DM group exhibited the highest average antibody level on 7 dpv, with an average of 2.0 $\log_2$, followed by the La Sota group (1.5 $\log_2$), suggesting that the genotype II inactivated vaccine could stimulate antibody production in pigeons earlier than the genotype VI inactivated vaccines. Over time, the antibody levels of the TJ(WT), aTJ, and La Sota vaccine groups gradually increased. The 167DM group consistently showed highest antibody levels, peaking at (6.8 ± 0.39) $\log_2$ (sample size, n = 10; degrees of freedom, df = 9) on 14 dpv. If the HI titer is positive at a level of 4 $\log_2$ or higher, the antibody positivity rates before the challenge were 100% in the 167DM group, 80% in the TJ (WT) group, 70% in the aTJ group, and 20% in the La Sota group.

GMT was calculated for each group using aTJ as the four-unit antigen. The GMT of the HI antibody titer on the 14 dpv and 21 dpv in the 167DM group was found to be greater than 4 $\log_2$ (16), displaying an inverted "V" shape with the peak observed on the 14 dpv (Fig. 3b). In

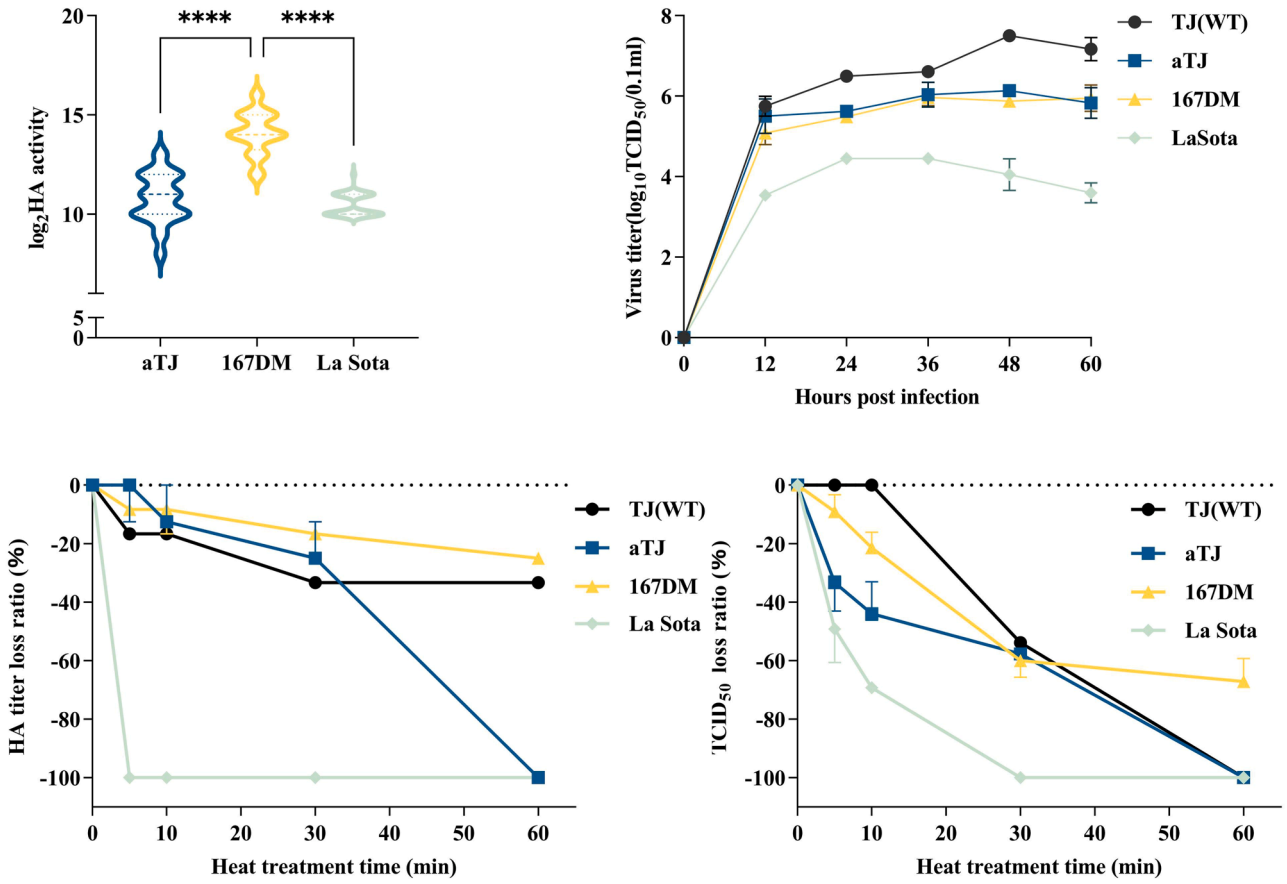

Figure 2. Biological properties of recombinant strains. (a) The hemagglutination (HA) titers of NDVs. The HA titers of 40 different viruses in their fifth-generation allantoic fluids were individually determined using 1% chicken red blood cells. GraphPad 9.5 software was utilized to calculate the titers of each strain, and a One-way ANOVA analysis was conducted to compare the HA titers. Statistical significance was denoted by p-values: $p < 0.0001$ (****). (b) One-step growth curve of NDVs. Different viruses were used to infect BHK21 cells at a multiplicity of infection (MOI) of one. The culture supernatant was collected every 12 h, and the 50% tissue infectious dose ($TCID_{50}$) at each time point was measured. Three independent repeated experiments were conducted. Statistical data was collected were represented as lg ($TCID_{50}$ /0.1mL) on the ordinate. The virus titers at different times were shown as mean ± SD, with the infection time on the abscissa to create the one-step growth curve of the virus. The thermo-stability of NDVs was assessed by subjecting viruses to heat treatment at 56°C at specified time intervals. Virus samples were then analyzed for their HA activity (c) and infectivity (d) using the HA assay and $TCID_{50}$ assay, respectively. The reduction in HA activity and infectivity of the viruses were calculated as a percentage relative to the untreated viruses, which were considered to have 100% activity.

Table 1
Virulence analysis results

Strains	lgTCID$_{50}$[1](0.1mL)	lgEID$_{50}$[2](0.1mL)	MDT[3](h)	ICPI[4]
TJ(WT)	-8.17	-8.38	62	1.19
aTJ	-8.63	-9.00	146	0.20
167DM	-8.64	-9.50	110	0.40

[1] lgTCID$_{50}$, logarithm base 10 of 50% tissue infectious dose ($TCID_{50}$).
[2] lgEID$_{50}$, logarithm base 10 of 50% egg culture infective dose (EID_{50}).
[3] MDT, mean death time.
[4] ICPI, intracerebral pathogenicity index.

comparison, the TJ(WT) group had a GMT higher than 4 log_2, while those of other groups did not exceed 4 log_2 until 21 dpv. The GMT relationship between the three groups was as follows: 167DM > TJ(WT) > aTJ > La Sota.

We also compared the NAb titers of the vaccinated pigeon before the TJ(WT) challenge. The results, depicted in Fig. 3c, show that the average numbers of neutralizing antibodies in 167DM, aTJ, TJ(WT), and La Sota were $10^{2.28}$, $10^{1.79}$, $10^{1.67}$, and $10^{1.73}$, respectively. Notably, the titers in the 167DM group were significantly higher than those in the La Sota group ($p = 0.05$) and TJ(WT) group ($p = 0.02$).

Antigenic Variation Assessment

At 21 dpv, HI titers were measured using sera from pigeons, with aTJ, 167DM, and La Sota as antigens. Cross-HI tests were performed to evaluate antigenic variation among the strains. The antigen correlation coefficient (R-value) between aTJ and La Sota, as well as between 167DM and La Sota, was calculated as 0.18, indicating a significant antigenic difference. In contrast, the R-value between aTJ and 167DM was 0.90, suggesting no significant antigenic difference between these two strains (Fig. 3d). According to the predefined criteria, an R-value above 0.67 implies no significant antigenic difference, while a value below 0.50 suggests a major antigenic difference between strains (Archetti and Horsfall, 1950; Li, et al., 2010).

Efficacy of Inactivated Vaccines Against TJ(WT) Challenge

Each group was challenged with the TJ(WT) strain on 21 dpv. Clinical manifestations of the pigeons in each group were observed and recorded daily for a period of 14 days. Fig. 4a and Fig. 4b show the clinical scores and survival curves of the pigeons in each group. Pigeons in the PBS group began to exhibit symptoms on 4 dpc, with clinical scores gradually increasing. Symptoms included depression, ruffled feathers, lethargy, and yellow-green or white loose feces. Three pigeons developed severe symptoms within two days and succumbed to the

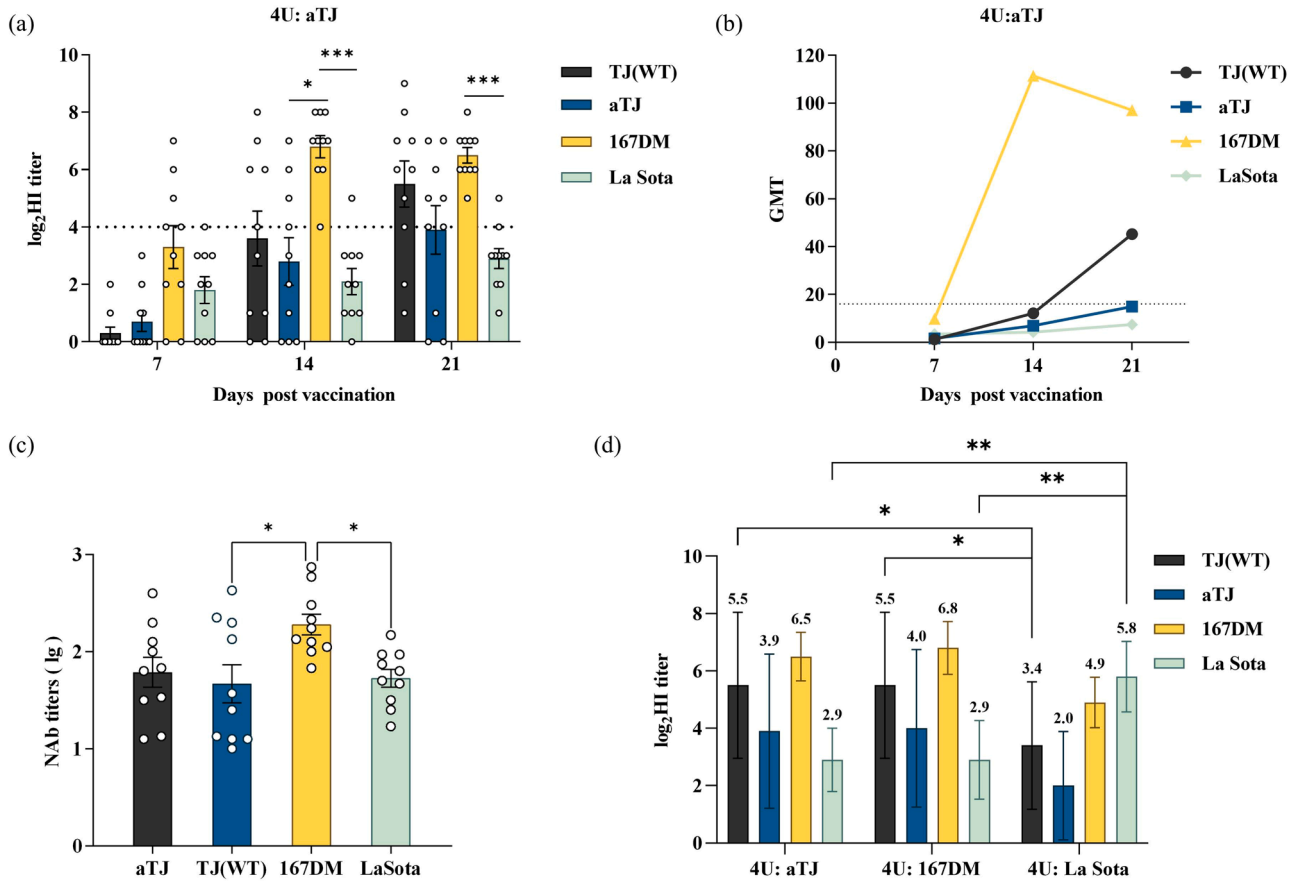

Figure 3. Serological responses to NDV strains post-vaccination. Statistical significance is indicated as follows: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$. (a) Hemagglutination inhibition (HI) titers of vaccinated pigeons. HI titers of pigeons vaccinated with TJ(WT), aTJ, 167DM, and La Sota strains at 7, 14, and 21 days post-vaccination (dpv). aTJ was used as the antigen. HI titers are expressed as $\log_2$ values, with 4 $\log_2$ considered positive (dotted line). two-way ANOVA was used for statistical comparisons. (b) Geometric mean titers (GMT) of HI antibodies over time. GMT of HI antibodies at 7, 14, and 21 dpv for each group. aTJ was used as the antigen. 167DM showed the highest GMT, peaking at 14 dpv. (c) Neutralizing antibody (NAb) titers at 21 dpv. NAb titers at 21 dpv for each vaccine group, expressed as $\log_{10}$ (lg) values. One-way ANOVA was conducted to determine statistical significance. (d) Cross-HI titers using different strains as the four-unit (4U) antigen. Titers are expressed as $\log_2$ values, with statistical comparisons among strains shown using two-way ANOVA.

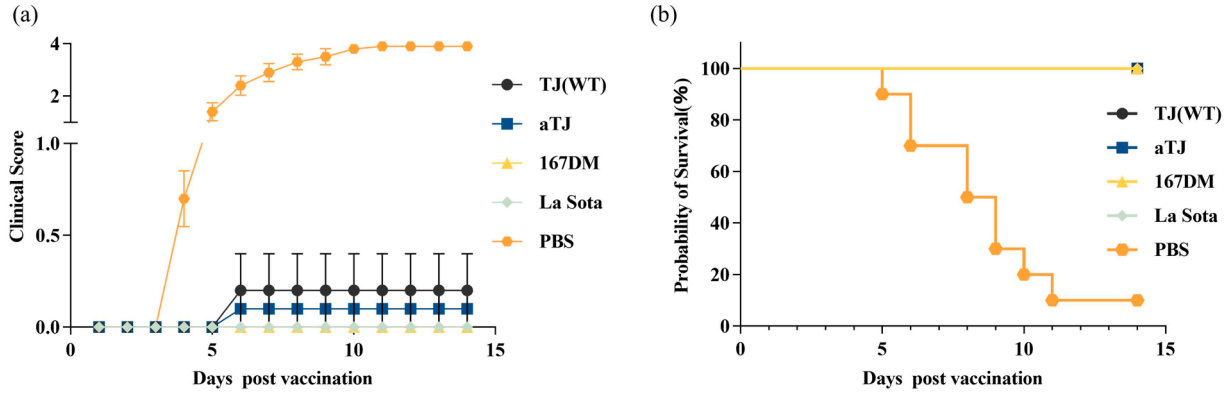

Figure 4. (a) Clinical scores for each group of inactivated vaccines, presented as mean ± SEM of 10 pigeons per group. (b) Survival curves for each group of inactivated vaccines.

infection, resulting in a 100% incidence rate by 6 dpc, with clinical scores peaking on 11 dpc and a mortality rate of 90%. Conversely, in the TJ(wt) and aTJ groups, only one pigeon in each group exhibited mild clinical signs, such as ruffled feathers, lethargy, and drooping wings since 6 dpc, with no further progression of symptoms and a mortality rate of 0%. Throughout the observation period, all pigeons in the 167DM and La Sota groups remained asymptomatic, with morbidity and mortality rates of 0%.

Virus Shedding and Protection Rate after TJ(WT) Challenge

To better evaluate the protective efficacy against TJ(WT) infection, virus shedding was assessed in all four groups on 5 and 10 dpc. As shown in Table 2, The virus-shedding rate in the PBS group remained at 100%

Table 2
Frequency of isolation of challenge virus in different vaccine groups

Groups	Number	Virus shedding[1]		Protection rate (%)[3]
		5 dpc[2]	10 dpc[2]	
TJ(WT)	10	2/10	4/10	60
aTJ	10	5/10	2/10	50
167DM	10	0/10	0/10	100
La Sota	10	4/10	2/10	60
PBS	10	10/10	2/2	0

[1] The number "n/m" indicates the number of positives detected and the total number of related samples.
[2] "dpc" represents days post-challenge.
[3] The vaccinated pigeons chickens that showed no clinical signs, no mortality, and no virus shedding after challenge were considered to be protected.

on both 5 dpc and 10 dpc. In the TJ (wt) group, the cloacal virus shedding rate was 20% on 5 dpc, increasing to 40% by 10 dpc. The aTJ group had a cloacal virus-shedding rate of 50% on 5 dpc, which decreased to 20% on 10 dpc. In the La Sota group, the rate was 40% on 5 dpc and dropped to 20% on 10 dpc, indicating partial efficacy in reducing virus shedding. The 167DM group demonstrated the highest protection, with no virus shedding on either 5 or 10 dpc. The protection rates, defined as the absence of both clinical symptoms and virus shedding, were 100% in the 167DM group, followed by 60% in both the TJ(WT) and La Sota groups, and 50% in the aTJ group. The protective efficacy of vaccines was further analyzed based on HI titers (Supplementary Table 2). Pigeons with HI titers ≥ 6 $\log_2$ demonstrated no virus shedding or morbidity, achieving 100% protection. Those with titers ≤ 1 $\log_2$ showed no protection (0%), while intermediate titers exhibited partial protection, with varying levels of virus shedding and morbidity.

DISCUSSION

PPMV-1 was first introduced to China in 1985, and has since become the most significant pathogen affecting the development of pigeon breeding in the region (Tian, et al., 2020; Xie, et al., 2020). Currently, inactivated and attenuated vaccines based on the La Sota strain are widely used to prevent PPMV-1 infection. However, sporadic cases of ND still occur, indicating that existing vaccines may not provide optimal protection. PPMV-1 is an antigenic variant genotype of NDV that has evolved into a distinct branch, classified as genotype VI. In China, the predominant strains in recent years have been categorized into subtypes VI.2.1.1.2.1 and VI.2.1.1.2.2, with VI.2.1.1.2.2 gradually becoming the dominant strain in outbreaks (Tian, et al., 2020; Zhan, et al., 2022). Through phylogenetic analysis, the TJ(WT) strain was identified as the predominant PPMV-1 strain circulating in China since 2010 (Zhang, et al., 2024a). It has been shown to exhibit severe pathogenicity in pigeons, causing clinical symptoms and complete viral shedding in 100% of infected pigeons, making it an ideal candidate for evaluating vaccine efficacy. Moreover, previous research used this strain to assess vaccine protection, further validating its suitability as a challenge virus in vaccine trials (Zhang, et al., 2024b). PPMV-1 exhibits significant genetic divergence from the La Sota vaccine strain, which belongs to genotype II, resulting in notable antigenic differences. Much research demonstrates that vaccines with a close antigenic relationship to currently circulating strains may offer superior protection compared to commercial vaccines (Hu, et al., 2009; Liu, et al., 2015b; Yang, et al., 2017). PPMV-1 typically exhibits lower HA titers, around 7 $\log_2$, compared to the higher titers of classical NDV strains like La Sota, reflecting reduced immunogenicity(Stone, 1989; King, 1996; Shan, et al., 2021). La Sota is known for its high titers and excellent safety profile (Su, et al., 2018). A recombinant virus that integrates PPMV-1's antigenicity with La Sota's high titers may represent a more effective vaccination strategy.

The F and HN proteins are the primary protective antigens of NDVs. Research has demonstrated that F protein, highly conserved across NDV strains, exhibits broad-spectrum activity, providing protection against both homologous and heterologous strains (Ferreira, et al., 2021). Due to this conservation, the F protein is commonly used as a primary antigen in various viral vector-based vaccines, including those utilizing herpesvirus of turkeys (HVT) (Hu, et al., 2022; Rajab, et al., 2024), poxvirus (Hu, et al., 2022), adenovirus (Jogi, et al., 2024), and subunit vaccines. The HN protein plays a crucial role in determining serological characteristics and antigenic variation (Peeters, et al., 2001; Gong and Cui, 2011; Chang, et al., 2023). The antigenic similarity of HN protein between the vaccine strain and the prevalent NDV strains is important in reducing the shedding of virulent virus (Liu, et al., 2017; Soliman, et al., 2024). NDV proteins, particularly the F, HN, and M proteins, are generally thought to co-evolve within each genotype to ensure efficient viral replication. This co-evolution is vital for maintaining compatibility among viral proteins, which supports proper viral assembly and function (Bose, et al., 2015; Kai, et al., 2015; Firouzamandi, et al., 2023). Incompatibility between proteins from different genotypes can impair viral replication, as evidenced by recombinant viruses with heterologous proteins exhibiting reduced replication capacity (Kai, et al., 2015; Izquierdo-Lara, et al., 2019). The interaction between the HN stalk region and the F cytoplasmic tail is particularly critical for viral particle assembly and release (Bose, et al., 2015; Kubota, et al., 2020). To avoid potential issues of protein incompatibility and preserve functional interactions, we focused exclusively on modifying the globular region of the HN protein in this study. The globular region contains most of the antigenic sites, making it an ideal target for antigenic enhancement while maintaining the crucial protein interactions required for viral replication. In this study, the globular region of the HN from the La Sota strain was replaced with that of the circulating PPMV-1 strain (ND167), aiming to improve antigenic compatibility without compromising protein function. PPMV-1 belongs to the NDV genotype VI and has an HN length of 571 amino acids (aa), slightly shorter than the 577-aa length of the La Sota strain. Extending the carboxyl terminus of the HN from 571aa to 577 or 616 aa has been shown to decrease viral pathogenicity in 1-day-old and 3-week-old chickens (Kim, et al., 2013). This study focused on replacing only the *HN* genes of equal length, ensuring that the total length of the *HN* gene remained constant. Pathogenicity test revealed that the recombinant strain 167DM was a lentogenic strain, with MDT and ICPI values of 110 h and 0.40, respectively.

The biological properties of 167DM showed that the average HA titer of chicken embryo allantoic fluid was (14.08 ± 1.07) $\log_2$, indicating the highest virus yield among all tested strains. The use of 167DM as a vaccine may significantly reduce production costs. This discovery is in agreement with previous studies, which reported that the DM strain with mutations R247K and S263R in the M protein of the La Sota strain exhibits increased viral proliferation efficiency and higher titer (Peng, et al., 2022). Additionally, 167DM demonstrated the highest heat resistance compared to aTJ and La Sota strains, maintaining HA titer and infectivity even after exposed to 56°C for 60 min, thus lowering vaccine transportation expenses. It is speculated that the ND167 strain exhibits similar heat resistance to TJ(WT) strain and aTJ strain. The heat resistance of NDV has been linked to the HN gene (Ruan, et al., 2020; Shang, et al., 2022), which was replaced in the context of this study.

Furthermore, the immune protection experiment indicated that the HI titer of the 167DM strain was the highest among all groups. Pigeons immunized with the 167DM strain exhibited a rapid antibody response, peaking at 14 dpv with an average titer of (6.8 ± 0.39) $\log_2$ ($n = 10$; $df = 9$). This enhanced response may be attributed to the specific virus content of 167DM during vaccine preparation. All pigeons in the 167DM group exhibited antibody levels ≥ 4 $\log_2$, resulting in a 100% antibody positivity rate. Following the experimental challenge, these pigeons were completely protected from clinical symptoms and virus shedding, reinforcing the relationship between high HI titers and effective immunity. Previous studies have consistently demonstrated that a higher HI antibody titer is correlated with a stronger protective effect (Yang, et al., 2017; Ferreira, et al., 2021). Specifically, when using the aTJ

strain as the four-unit antigen, an HI titer of ≥ 6 $\log_2$ was identified as the protective threshold, as pigeons with this level of antibodies demonstrated complete protection against clinical symptoms and virus shedding. In contrast, HI titers ≤ 1 $\log_2$ were associated with no measurable protection. These findings underscored the critical role of achieving sufficient HI titers to inhibit viral attachment and provide protection. HI titers reflect only one aspect of the humoral immune response, whereas NAb titers provide a more comprehensive measure of protective immunity by encompassing broader mechanisms of viral neutralization (Corti, et al., 2011; Wohlbold and Krammer, 2014; Chumbe, et al., 2017). This distinction underscores the need for future studies to evaluate how HI and neutralizing antibodies collectively contribute to vaccine-induced protection. The antigen correlation coefficient determined through the cross-HI test revealed that the 167DM strain exhibited no antigenic variation in comparison to the aTJ strain, but displayed a notable antigenic difference when compared to La Sota. This finding further illustrates that NDV strains can be serologically distinguished from one another by modifying the globular region of the HN protein (Peeters, et al., 2001). In addition, the result is consistent with previous studies showing that the similarity of the HN protein to the challenge strain seems to be important for improving clinical protection and decrease virus shedding (Liu, et al., 2017). However, the immune response elicited by the inactivated PPMV-1 strains was less effective compared to La Sota. This discrepancy could be attributed to variations in individual pigeons and the viral content in the vaccine. Analysis of HI titers and NAb titers revealed that although the average antibody levels (HI and neutralizing) did not differ significantly, there were fluctuations in PPMV-1 antibody titers among pigeons. Some pigeons exhibited lower antibody levels, leading to inadequate vaccine-induced immunity.

In conclusion, we developed a recombinant virus incorporating the globular region of the HN of genotype VI NDV into the rLa Sota-DM backbone. The recombinant virus, 167DM, exhibited increased viral production and stimulated a more rapid and robust antibody response in pigeons. Furthermore, 167DM remained infectivity even after exposure to 56°C for 60 min, demonstrating enhanced heat resistance, which supports its potential as a promising vaccine candidate that simplifies storage and transportation requirements. Importantly, no antigenic distinction was observed between the 167DM strain and the PPMV-1 strain. Pigeons immunized with the 167DM inactivated vaccine displayed 100% protection against virulent PPMV-1 challenge, effectively preventing clinical symptoms and virus shedding. These findings provide valuable insights for the development and application of PPMV-1 vaccines.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Shan Zhang reports statistical analysis was provided by Beijing Xinhexiang Technology Co., LLC, Beijing 100085, China. Baojing Liu reports a relationship with Beijing Xinhexiang Technology Co., LLC, Beijing 100085, China that includes: non-financial support. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2025.104874.

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