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Recombinant adenovirus expressing pdhβ-pdhD fusion protein produces robust immune responses and partial protection against Mycoplasma Synoviae challenge in chickens

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ABSTRACT

Mycoplasma synoviae (MS) stands as a pivotal pathogen, responsible for triggering arthritis and airsacculitis in both chickens and turkeys. Given the pressing need for safe and efficacious vaccine candidates, we engineered recombinant adenoviruses expressing a fusion antigen. This antigen consisted of the pyruvate dehydrogenase E1 subunit beta (pdhβ) and dihydrolipoyl dehydrogenase (pdhD) of MS. We then systematically evaluated the immune effect and protective efficacy of these recombinant adenoviruses against MS challenge in a chicken model. Our results demonstrated the successful construction of recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD. The pdhβ, pdhD, and pdhβ-pdhD proteins were efficiently expressed in cells infected with the respective recombinant adenoviruses. Animal experiments further revealed that vaccination with recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD elicited significant specific humoral and cellular immune responses ($P < 0.05$). Notably, rAd-pdhβ-pdhD exhibited superior immunogenicity compared to rAd-pdhβ and rAd-pdhD. Moreover, all three recombinant adenovirus vaccine candidates conferred partial protection to chickens against MS challenge. They effectively alleviated MS-induced footpad and joint swelling, as well as inflammation. Among them, rAd-pdhβ-pdhD demonstrated a better protective effect. In conclusion, vaccination with recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD can evoke immune responses and provide partial protection against MS in chickens. In particular, rAd-pdhβ-pdhD holds greater potential as a vaccine candidate against MS.

Introduction

Mycoplasma synovium (MS), a prominent avian pathogen, elicits potent inflammatory responses in chickens while evading immune surveillance, frequently resulting in chronic infection and immunosuppression (Omotainse et al., 2022). Chickens aged 3–6 weeks were more susceptible to MS, and the affected chickens frequently exhibited symptoms such as depression, rough feathers, and swelling and deformity of the joints and footpads, thereby leading to lameness, recumbency, and even the development of air sacculitis (Zhang et al., 2022). A research team sampling over 9, 000 broiler farms across 16 provinces in China revealed that MS infection was widely prevalent in chickens of diverse ages and breeds, with an antibody-positive rate as high as 63.2 % and a positive rate of 16.29 % in breeding eggs (Sun et al., 2017). Mixed infections of multiple viruses, *Escherichia coli*, and *Mycoplasma gallisepticum* have been reported to increase the pathogenicity of MS, causing more severe clinical manifestations and mortality rate (Croville et al., 2018; Gowthaman et al., 2017), which brings great economic losses to the poultry industry.

Although antimicrobial therapy alleviates clinical symptoms in MS-infected chickens, it fails to eradicate the pathogen and accelerates antimicrobial resistance development, thereby invalidating prolonged antibiotic regimens (de Jong et al., 2021). MS culture *in vitro* requires the addition of nicotinamide adenine dinucleotide (NAD) and animal serum, and the substantial expense associated with MS culture has limited the scale-up of inactivated MS vaccine production (Zhu et al., 2019). At present, the Australian MS attenuated vaccine strain MS-H is a widely used commercial attenuated vaccine (Omotainse et al., 2023).

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However, it has been reported that this vaccine colonizes the respiratory tract of chickens after immunization, thus destroying the mucosa and leading to a more severe inflammatory response in MS-infected flocks, which can easily cause secondary infections (Noormohammadi et al., 2003). Consequently, there is an imperative need to develop novel vaccines for MS.

Genetically engineered vaccines have become a focus in the research and development of new vaccines for the prevention and control of animal diseases due to its high safety and easy mass production. Adenoviral expression vectors have been demonstrated to exhibit superiority over other vectors due to their capacity to produce high viral titers, mediate high levels of protein expression, and induce robust humoral, cellular, and mucosal immune responses (Matsuda et al., 2021; Zhang et al., 2023). Leveraging the replication-incompetent nature of adenoviral vectors, adenovirus-vectored vaccines integrate the safety advantages of inactivated vaccines with the efficacy of live vaccines. In the present study, the pyruvate dehydrogenase E1 subunit beta (*pdhβ*) gene and the dihydrolipoyl dehydrogenase (*pdhD*) gene were fused and cloned into the adenoviral shuttle vector pDC316-mCMV-EGFP. The recombinant shuttle plasmid and pBHGlox(delta)E1, 3 Cre were then homologously recombined in human embryo kidney (HEK) 293A cells to construct a recombinant adenoviral vector vaccine candidate for MS. The immune efficacy of this vaccine candidate was then evaluated in chickens. This study will establish the foundation for the development of novel vaccines against MS infections.

Materials and methods

Mycoplasma, cells, and plasmids

The MS inactivated vaccine based on the YBF-MS1 strain was produced and supplied by Qingdao Yebio Bioengineering Co., Ltd. (Shandong, China; product batch number: 150132337). MS strain MS-ZJ was isolated and stored in our laboratory. The Ad5Max adenovirus vector system derived from human adenoviral serotype 5 (HAdV-5) and the pDC316-mCMV-EGFP vector were obtained from Miaoling Biotechnology Co., Ltd. (Wuhan, China). HEK293A cells were purchased from Procell Life Science & Technology Co., Ltd. (Wuhan, China) and were maintained in DMEM with 10 % heat-inactivated fetal bovine serum (FBS; Gibco^{TM}, USA). *E. coli* DH5*α* competent cells were obtained from Beijing Tsingke Biotech Co., Ltd. (Beijing, China) and were grown in Lysogeny Broth (LB) or on agar media.

Animals and ethics statement

Fifty 18-day-old White Leghorn chickens were purchased from Guizhou Foster Biotechnology Co., Ltd. (Guizhou, China) and were seronegative for MS specific antibodies prior to immunization. The animal study was approved by the Ethics Committee of Guizhou University (EAE-GZU-2021-E028). All standard procedures concerning animal care and management were performed, and all efforts were made to alleviate animal distress during the experiment.

Construction of recombinant shuttle plasmid

The sequence encoding the *pdhβ* and *pdhD* in the MS genome sequence (accession number:LS991953.1) were retrieved from NCBI GenBank database. A linker sequence $(GGGGS)_2$ was designed to link the *pdhβ* gene to the *pdhD* gene. The Kozak sequence was introduced before the initiation codon, and then the restriction enzyme sites of *Not*I and *Eco*RV were introduced at the 5′ and 3′ termini of the sequence, respectively. The primer pairs were designed and synthesized by Sangon Biotech (Shanghai, China). The primer sequences are shown in Supplementary Table 1. The *pdhβ, pdhD, pdhβ*-linker (*pdhβ*-l), linker-*pdhD* (l-*pdhD*), and *pdhβ-pdhD* genes were PCR-amplified and cloned sequentially into the adenoviral shuttle vector pDC316-mCMV-EGFP, and positive clones were confirmed by restriction enzyme digestion and sequencing. The correct recombinant transfer plasmids were respectively designated as "pDC-*pdhβ*, pDC-*pdhD*, and pDC-*pdhβ-pdhD*".

Generation and characterization of recombinant adenovirus

HEK293A cells were maintained in DMEM with 10 % heat-inactivated FBS (Gibco^{TM}, USA), inoculated onto a 6-cm dish placed in a 37°C incubator with 5 % CO_2 until a 60–70 % confluency, and then transfected with 0.65 μg recombinant shuttle plasmid and 1.35 μg of pBHGlox(Δ)E1,3 Cre plasmid using 2 μL Neofect™ DNA transfection reagent (NeoFect Biotech, beijing, China). The pDC316-mCMV-EGFP empty vector served as the negative control. After incubation for 6–10 h, the fresh growth medium (containing DMEM and 10 % heat-inactivated FBS) was added, and the cell culture continued at 37°C and 5 % CO_2, with daily observation for the expression of EGFP and cytopathic effects (CPEs). At 10-14 days post-transfection, when more than 90 % of cells showed CPEs, the cell cultures were harvested. The cell cultures were then subjected to three freeze-thaw cycles and a centrifugation procedure to yield the subsequent passage of the recombinant adenovirus. The recombinant adenoviruses rAd-pdhβ, rAd-pdhD, rAd-pdhβ-pdhD, and rAd-EGFP were obtained.

After three rounds of amplification, the P3 recombinant adenoviruses were purified using Vivapure AdenoPACK^{TM} (Sartorius Stedim Biotech GmbH, Germany), and the viral titers were determined by a fluorescent focus assay (FFA) as previously described (Liu et al., 2019). Furthermore, HEK293A cells were respectively infected with rAd-pdhβ, or rAd-pdhD, or rAd-pdhβ-pdhD, and then cells were collected at 72 h post-infection for the detection of pdhβ, pdhD, and pdhβ-pdhD by RT-PCR, total cellular RNA was extracted using the TRIzol method. The PrimeScript™ RT kit (RR036Q, TAKARA) was utilized for reverse transcription to synthesize cDNA. The PCR primer sequences are presented in Supplementary Table 1. The amplification program was configured as follows: pre-denaturation at 95°C for 3 min; 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, and extension at 72°C for 70 s; and a final extension at 72°C for 5 min. The amplified products were separated by 1 % agarose gel electrophoresis and were analyzed using a gel imaging system. The procedure of western blotting was executed as previously described (Hashida and Coffey, 2022). The antibodies utilized in this study included MS positive serum as primary antibodies and goat anti-chicken IgG H&L (HRP) antibody (Bioss Biotechnology, Beijing, China) as secondary antibodies.

Chicken immunization and challenge experiment

Fifty 18-day-old healthy White Leghorn chickens were randomly allocated into seven groups ($n = 10$ per group). The chickens in PBS group were received phosphate-buffered saline (PBS) at the dose of 0.2 mL/bird through the leg muscles. The chickens in inactivated vaccine group were immunized with MS inactivated vaccine (0.5 mL/bird) by subcutaneous route in the cervical region. The chickens in the remaining groups were administered with rAd-pdhβ, rAd-pdhD, or rAd-pdhβ-pdhD recombinant adenoviruses (10^8 $TCID_{50}$/bird) through the leg muscles, respectively. All chickens were booster immunization at 14 days post-vaccination (dpv). Serum samples from all groups were collected at 7, 14, 21, 28, 35, and 42 dpv for antibodies and cytokines detection. At 28 dpv, to evaluate the efficacy of recombinant adenovirus, all chickens of each group were challenged with 2×10^7 color-changing units (CCU)/mL of MS-ZJ strain culture via footpad injection.

ELISA

This study employed two ELISA methods: (1) a pdhβ/pdhD-specific antibody detection assay based on 6 × His-tagged fusion proteins, which was used to evaluate antibody responses in chickens immunized with rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD vaccines via indirect ELISA;

and (2) commercial ELISA kits for cytokine detection. In brief, a concentration of 0.1 μg/mL purified pdhβ or pdhD protein in 0.05 M $NaHCO_3$ coating buffer was used to coat ELISA plates (Corning, USA) and then incubated with serum samples (1:150 dilution) at 37°C for 1 h. After the plate was washed with PBST 3 times, a Goat Anti-Chicken IgG $H + L$ (HRP) secondary antibody (1:6000 dilution) was added and incubated at 37°C for 0.5 h. Then, the plate was washed with PBST 3 times, followed by the addition of the 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate and incubation at 37°C for 15 min. Finally, 50 μL of stop solution was terminated, and the absorbance was determined at 450 nm. In addition, the concentrations of interleukin-2 (IL-2, JYM0026Ch), interferon-γ (IFN-γ, JYM00007Ch), interleukin-4 (IL-4, JYM0034Ch), and interleukin-6 (IL-6, JYM0028Ch) in the serum of chicken were quantified by chicken cytokine ELISA kit (Wuhan ColorfulGene Biological Technology Co., Ltd., China) according to the manufacturer's instructions.

Examination of clinical signs and pathological changes

The mental state, swollen joints and foot pads of the chickens were observed daily. All chickens were euthanized and necropsied at 14 days post challenge, and the footpads tissues were subjected to Clinical symptoms examination. The histopathological changes in footpad tissues were then examined. Briefly, footpads from each experimental group were fixed in 4 % paraformaldehyde for 36 h, followed by rinsing under running water for 12 h. Next, a dehydration process was carried out using a series of ethanol solutions: 75 %−95 % ethanol and absolute ethanol, with each step lasting 25 min. Subsequently, samples underwent a clearing process using a mixture of ethanol and benzene (an equal-volume mixture of absolute ethanol and xylene) and then pure xylene. After being immersed in molten paraffin at 60°C, the specimens were embedded and allowed to cool. The embedded wax blocks were trimmed and sectioned into 5-μm-thick slices. These slices were flattened in a 39°C water bath and then transferred onto slides. The slides with sections were baked at 60°C for 6-8 h. After dewaxing with xylene and rehydrating with ethanol, the sections were stained successively with hematoxylin and eosin (H&E), air-dried, and mounted with neutral balsam. Finally, histopathological changes in footpad tissues were observed microscopically.

Statistical analysis

Statistical analyses were conducted using IBM SPSS Statistics 27.0 (IBM Corp Armonk, NY, USA). Statistical significance among different experimental groups was assessed using one-way ANOVA with Tukey's multiple-comparison test. The datasets were visualized in GraphPad Prism 7.0 (San Diego, CA, USA).

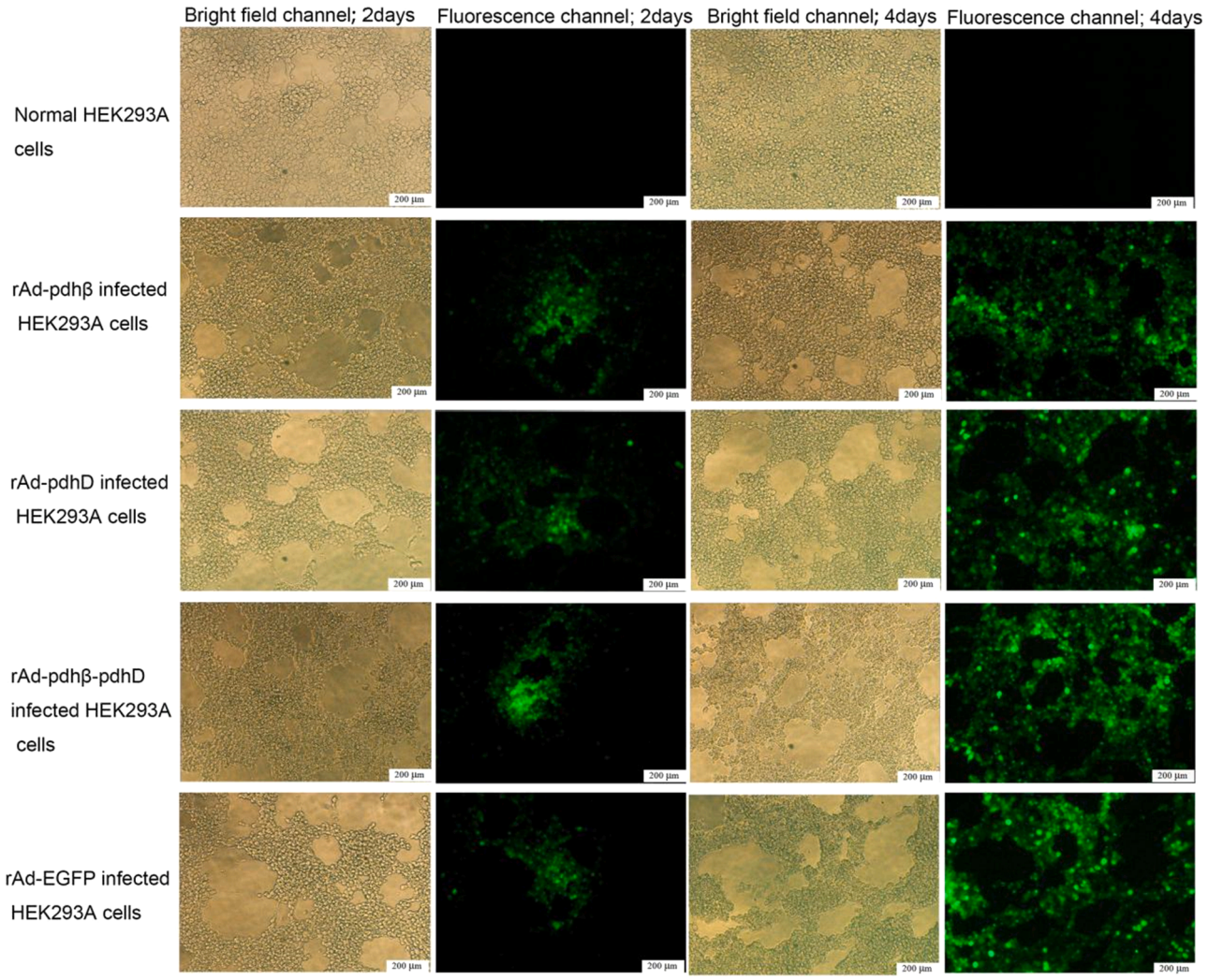

Fig. 1. Replication of recombinant adenoviruses in HEK293A cells. HEK293A cells were infected with rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD, and viral propagation was observed by fluorescence microscopy at 2 and 4 days post-infection. The virus-uninfected HEK293A cells served as control, Scale ba: 200 μm.

Results

Construction of Recombinant Shuttle Plasmid

The *pdhβ, pdhD, pdhβ* with linker (*pdhβ*-l), *pdhD* with linker (l-*pdhD*), and *pdhβ-pdhD* genes were amplified and sequenced. The lengths of the sequences were 993 base pairs (bp), 975 bp, 1023 bp, 1005 bp, and 1998 bp, respectively (Supplementary Figure 1 A-E). The *pdhβ, pdhD*, and *pdhβ-pdhD* genes were sequentially cloned into the adenovirus shuttle vector pDC316-mCMV-EGFP. The resulting recombinant transfer plasmids pDC-*pdhβ*, pDC-*pdhD*, and pDC-*pdhβ-pdhD* were verified through restriction enzyme digestion analysis (Supplementary Figure 2 A-C) and DNA sequencing.

Generation and characterization of recombinant virus

HEK 293A cells were individually infected with recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD. As depicted in Fig. 1, at 2 days post-infection, CPEs, including cell swelling, rounding, and detachment, were observed under fluorescence microscopy. Concurrently, the green fluorescence of EGFP expression in the infected cells exhibited a comet-like diffusion pattern. At 4 days post-infection, pan-cellular green fluorescence was observed exclusively in infected cells. In contrast, no green fluorescence was observed in uninfected cells. These results indicate that the packaged recombinant adenoviruses are capable of proliferating in HEK 293A cells. Results of the dilution assay indicated that the titers of recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD reached $10^{8.58}$ $TCID_{50}$/100 μL, $10^{8.5}$ $TCID_{50}$/100 μL, and $10^{9.11}$ $TCID_{50}$/100 μL, respectively.

The *in vitro* expression levels of the recombinant viruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD were evaluated via RT-PCR. DNA bands of approximately 993 bp, 975 bp, and 1998 bp were identified in 1 % agarose gel electrophoresis (Fig. 2A-C). Western blot analysis was further employed to assess the expression of pdhβ, pdhD, and pdhβ-pdhD proteins. The results revealed 36 kDa, 34.5 kDa, and 70.8 kDa protein bands, consistent with the predicted size of the pdhβ, pdhD, and pdhβ-pdhD protein, in HEK293A cells infected with rAd-pdhβ, rAd-pdhD, rAd-pdhβ-pdhD, respectively (Fig. 2d-F). No specific protein bands were observed in uninfected HEK 293A cells.

Three vaccine candidates elicited high levels of specific antibodies in the chicken sera

To assess the humoral immune response induced by the recombinant adenoviruses rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD, the secretion levels of pdhβ- and pdhD-specific antibodies in the sera of immunized chickens were determined by indirect ELISA. When compared with the PBS control group, all three vaccine candidates, along with the inactivated vaccine, induced a significant elevation in pdhβ/pdhD-specific antibody levels. The antibody levels peaked at 28 days post-vaccination (dpv). Notably, the serum specific antibody levels in the rAd-pdhβ-pdhD recombinant vaccine group and the inactivated vaccine group were significantly higher than those in the rAd-pdhβ and rAd-pdhD groups (Fig. 3). These results suggest that all three vaccine candidates are capable of eliciting robust humoral immune responses. Among them, the rAd-pdhβ-pdhD recombinant vaccine demonstrated a more potent immune effect.

Three vaccine candidates elicited robust Th1- and Th2-type responses in the immunized chickens

To explore the cellular immune responses induced by the vaccines, the secretion levels of Th1-type cytokines (IFN-γ and IL-2) and Th2-type cytokines (IL-4 and IL-6) in the sera of immunized chickens were quantified using ELISA. Compared to the PBS control group, all three vaccine candidates, along with the commercial inactivated vaccine,

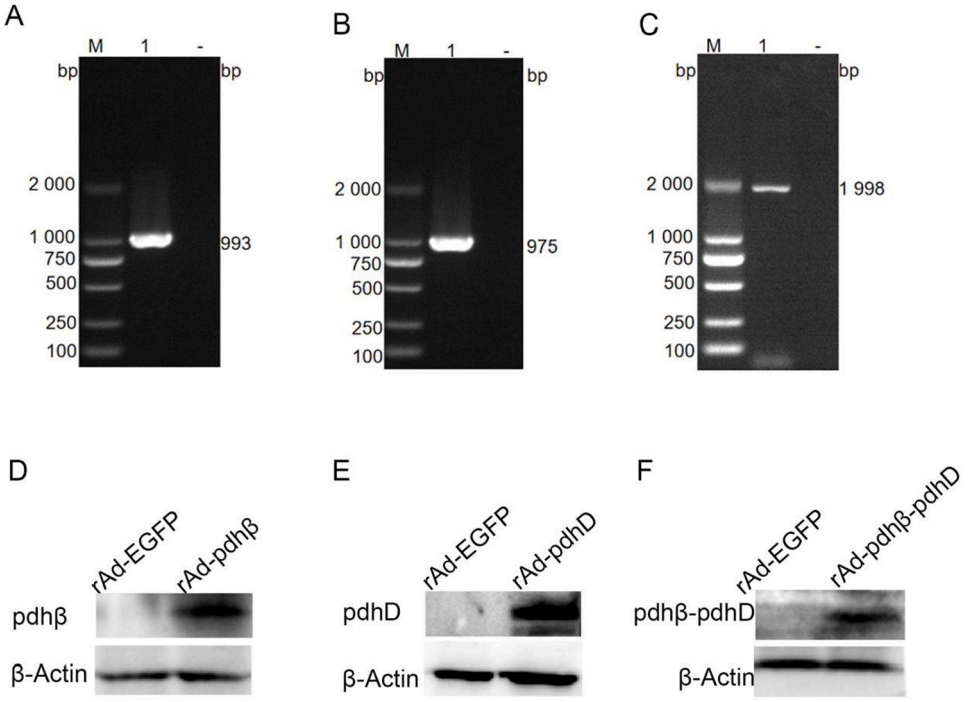

Fig. 2. Characterization of the recombinant adenoviruses.
HEK293A cells were respectively infected with rAd-pdhβ, or rAd-pdhD, or rAd-pdhβ-pdhD, and then cells were collected at 72 h post-infection for the detection of pdhβ and pdhD by RT-PCR (A-C) and Western blot (D-F), respectively. (A)M: DNA marker; Lane 1: rAd-pdhβ infected cells; Lane -: HEK293A cells); (B)M: DNA marker; Lane 1: rAd-pdhD infected cells; Lane -: HEK293A cells); (C)M: DNA marker; Lane 1: rAd-pdhβ-pdhD infected cells; Lane -: HEK293A cells. The expressions of pdhβ(D), pdhD (E), and pdhβ-pdhD (F) protein were conducted by Western blot, and the HEK-293 cells infected with adenovirus control (empty plasmid) were used as control. In this experiment, β-actin was selected as the internal reference.

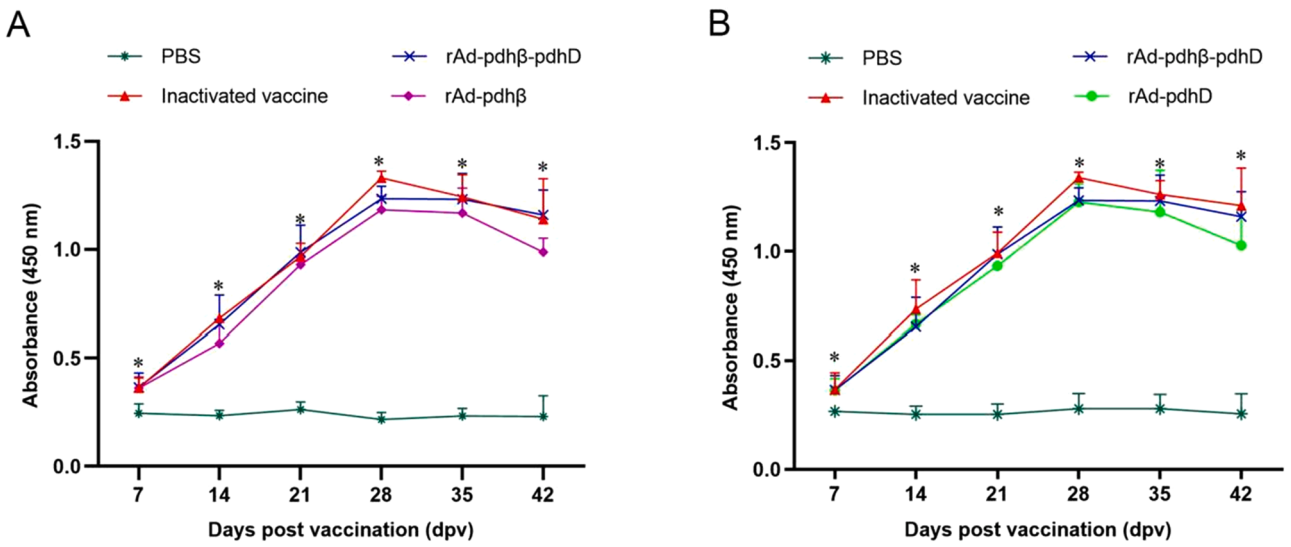

Fig. 3. Antibody responses following vaccination in chicken serum. Chickens were vaccinated at 0 dpv, and serum samples were collected at 7, 14, 21, 28, 35, and 42 dpv. (A) Anti-pdhβ antibody responses against MS from serum samples from vaccinated chickens determined using an indirect ELISA. (B) Anti-pdhD responses against MS in serum samples from vaccinated chickens as determined by an indirect ELISA. Statistical significance among different experimental groups was determined using one-way ANOVA with Tukey's multiple-comparison test. The asterisk denote a significant difference, with the *P* value less than 0.05.

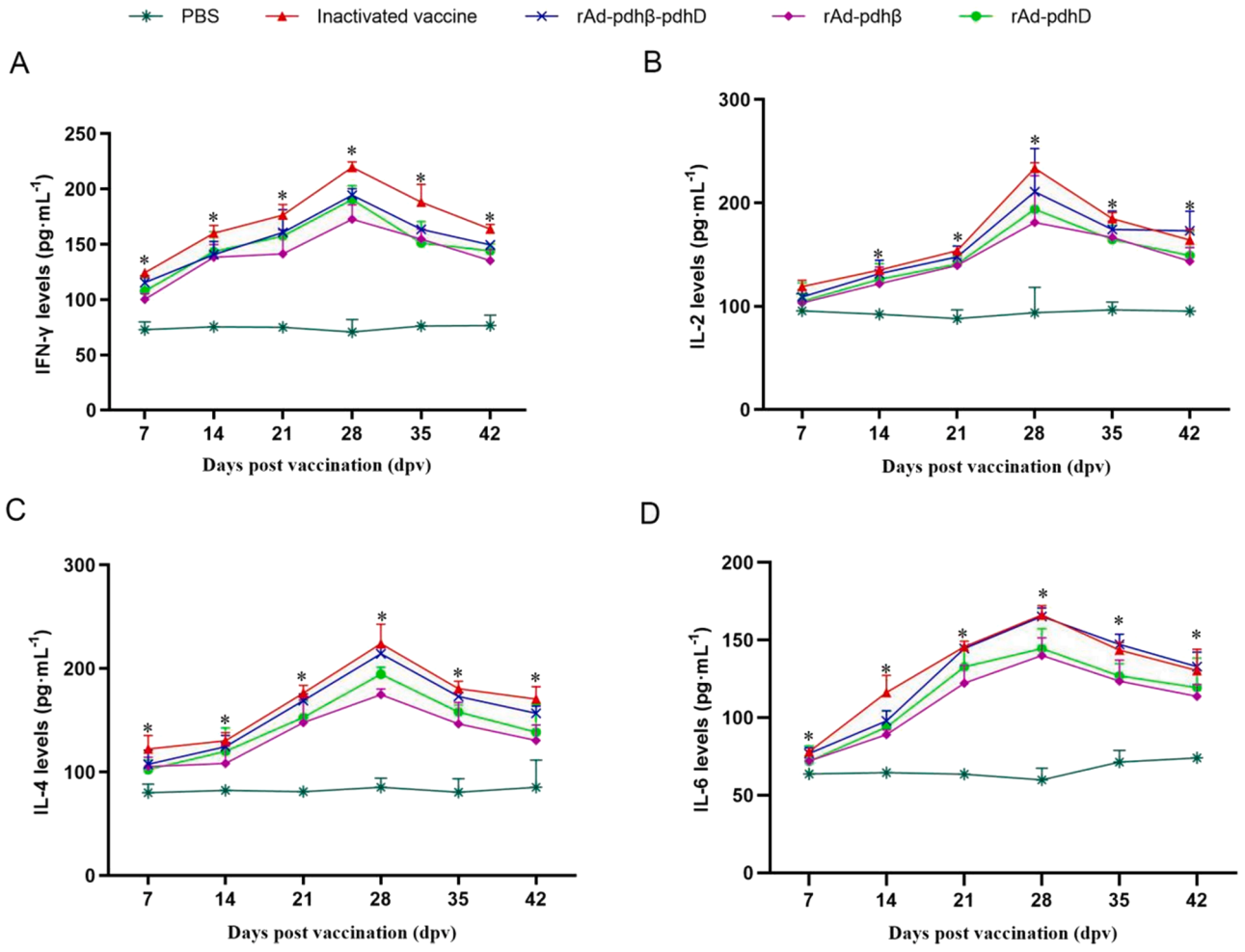

Fig. 4. Vaccination enhances the levels of IFN-γ (A), IL-2 (B) IL-4 (C), and IL-6 (D) **in chicken serum.** The serum samples from the PBS group, inactivated vaccine group, and recombinant adenovirus vaccine candidate group were analyzed for IFN-γ (A), IL-2 (B) IL-4 (C), and IL-6 (D) using ELISA. Statistical significance among different experimental groups was determined using one-way ANOVA with Tukey's multiple-comparison test. The asterisk indicates a significant difference, with the *P* value less than 0.05.

elicited a significant increase in the levels of IFN-γ, IL-2, IL-4, and IL-6. The cytokine levels peaked at 28 dpv. Notably, the cytokine levels in the rAd-pdhβ-pdhD group and the inactivated vaccine group were significantly higher than those in the rAd-pdhβ and rAd-pdhD groups ($P < 0.05$). In contrast, there was no significant difference in serum cytokine levels between the rAd-pdhβ and rAd-pdhD groups (Fig. 4). These results suggest that all three vaccine candidates were capable of eliciting robust cellular immune responses. Among them, the rAd-pdhβ-pdhD recombinant adenovirus vaccine demonstrated a better immune effect.

Protective efficacy of the vaccine candidates against MS challenge in chickens

To evaluate the protective efficacy of the recombinant adenoviruses, all chickens in each group were challenged via footpad inoculation with 2×10^7 CCU/mL of the pure culture of the MS-ZJ strain. After the challenge, no abnormalities were observed in the mental state of chickens across all groups. However, all chickens in the PBS control group exhibited obvious swelling of the footpads and joints. In contrast, only a few chickens in the MS inactivated vaccine group and the recombinant adenovirus groups had slight footpad swelling (Fig. 5). As depicted in Fig. 6, histological examination of footpad sections revealed that normal footpad sections of chickens were devoid of inflammatory cells, with intact fibrous tissues and acini. In the PBS control group, the fibrous tissues infiltrated by a large number of inflammatory cells, accompanied by vascular proliferation and disrupted acinar integrity. The rAd-pdhβ group and rAd-pdhD group showed a small amount of inflammatory cell infiltration and vascular proliferation, with slightly damaged acini, suggesting alleviation of inflammatory responses. Notably, the MS inactivated vaccine group and the rAd-pdhβ-pdhD group exhibited minimal inflammatory cell infiltration and acinar destruction. These results demonstrate that all three vaccine candidates to some extent mitigate the MS-induced pathological damage to footpad tissues.

Discussions

Current commercially available MS vaccines are constrained by critical shortcomings: live attenuated vaccines may pose inadvertent infection risks through colonization in the respiratory tract of seropositive chickens (Jones et al., 2006), whereas inactivated vaccines face challenges of high production costs and limited immunogenicity (Zhang et al., 2021). As a key avian pathogen listed by the World Organization for Animal Health (WOAH) (Yadav et al., 2022), MS presents difficulties in cultivation, necessitating the development of novel vaccines. However, research on such vaccines remains scarce. Existing studies primarily focus on subunit vaccines sporadically reported in the past four years, including single-protein subunit vaccines (Han et al., 2024), multi-epitope subunit vaccines (Zhang et al., 2023), and multi-component subunit vaccines (Sun et al., 2024). These vaccines still suffer from inadequate protective efficacy (incomplete prevention of infection), high production costs (particularly for multi-antigen combination vaccines), and complex manufacturing processes. Against this backdrop, recombinant live-vector vaccine technology has emerged as a promising new direction due to its significantly stronger immunogenicity, which closely mimics the natural infection process. It is usually produced through cell culture technology, ensuring a relatively stable production process. Moreover, the adenovirus vector boasts a large

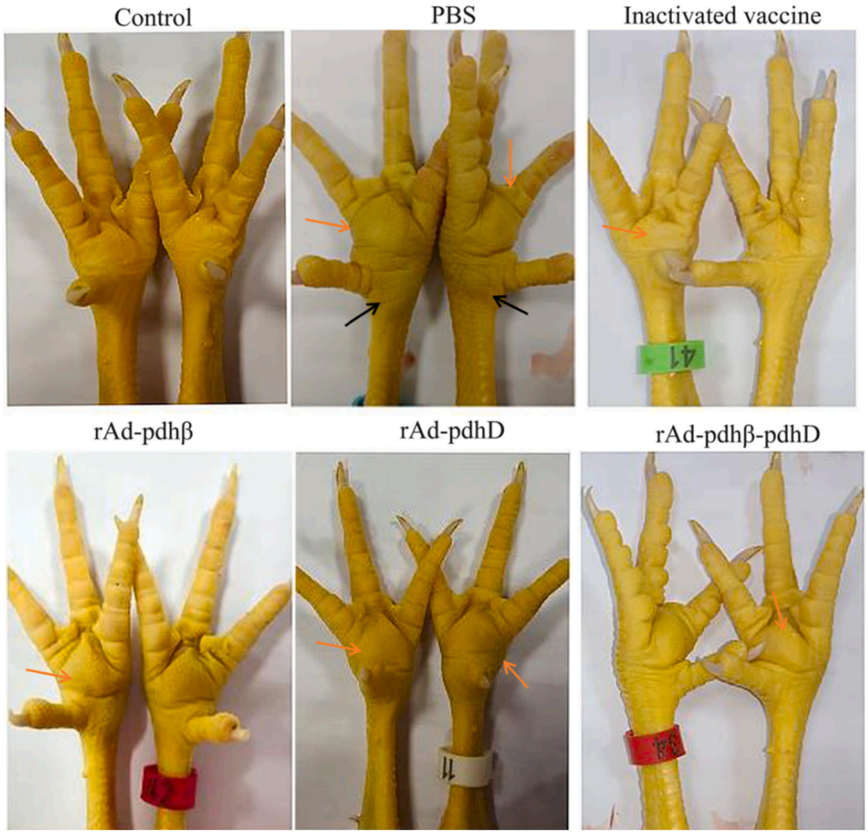

Fig. 5. Clinical symptoms of chicken footpads.
To evaluate the protective efficacy of the recombinant adenovirus vaccine, all chickens in each group were challenged by intramuscular injection into the footpad with a pure culture of the MS-ZJ strain at a concentration of 2×10^7 CCU/mL. After the challenge, there were no abnormalities in the mental state of the chickens in each group. However, all the chickens in the PBS control group showed obvious swelling of the footpads (indicated by red arrows) and joints (indicated by black arrows). In contrast, only a few chickens in the MS inactivated vaccine group and the recombinant adenovirus vaccine group showed mild swelling of the footpads.

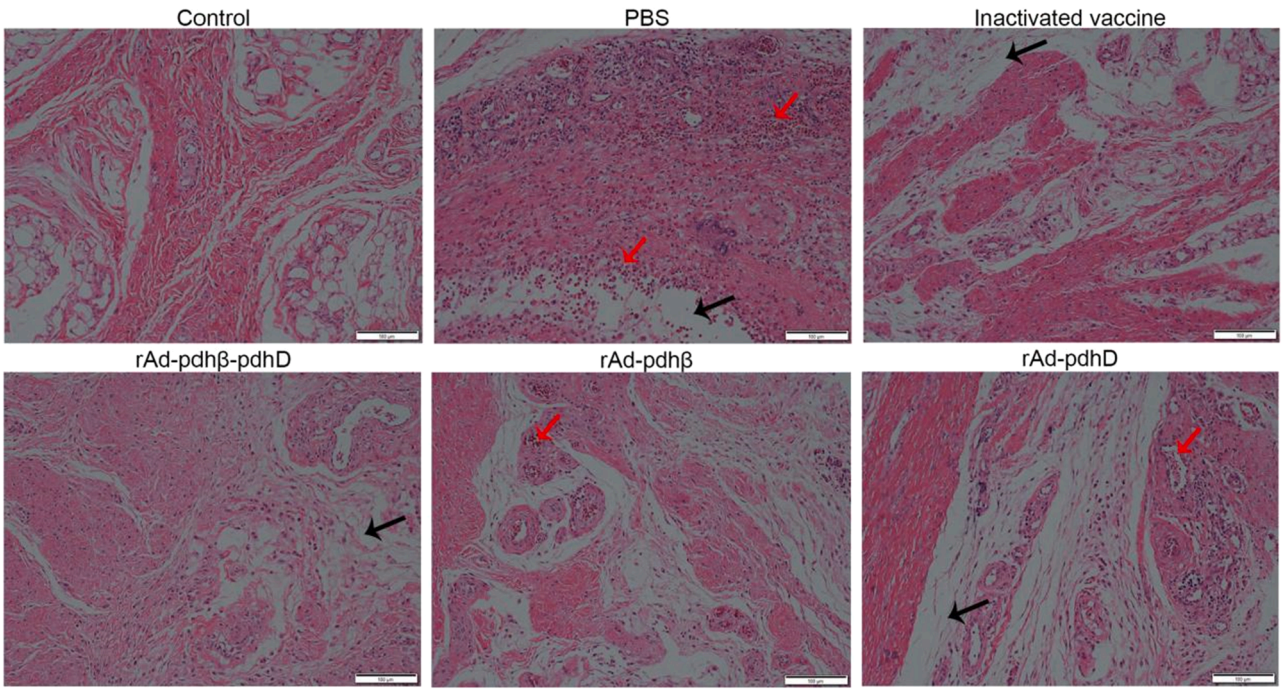

Fig. 6. The foot pads of chickens in the recombinant adenovirus vaccine candidate group, inactivated vaccine group, PBS group, and healthy control group were fixed in 4 % neutral buffered formalin for histological examination. The main pathological changes in the foot pad after artificial infection included inflammatory cell infiltration within fibrous connective tissue (red arrow) and disruption of structural integrity (black arrow). Scale bar: 100 μm.

capacity, allowing for the simultaneous insertion of multiple antigen genes to construct multivalent vaccines. Extensive studies have demonstrated the strong potential of recombinant adenoviral vector vaccines in human and animal vaccine development. (Lu et al., 2022; Miao et al., 2023; Sreenivasa et al., 2017; Zhu et al., 2020) Research (Fernandez-Sainz et al., 2017; Li et al., 2023) has demonstrated the efficacy of recombinant adenovirus vaccines in inducing protective immune responses, highlighting the technology's substantial potential as a vaccine delivery platform. Consequently, the development of a novel MS vaccine based on recombinant adenoviral vectors holds substantial research value and practical significance.

Previous studies have identified that MS immunogenic proteins pdhβ and pdhD (Berčič et al., 2008; Rebollo et al., 2012) are capable of inducing specific antibodies that effectively block MS adhesion to DF-1 cells (Qi et al., 2022; Bao, 2014). However, no studies have evaluated the advantages of pdhβ and pdhD as candidate antigens for recombinant live-vector vaccine. This study assess the efficacy of pdhβ/pdhD as candidate antigens for MS vaccines. Similar to Rojas' study in which sheep were immunized with recombinant adenoviruses expressing ovine OX40L or CD70, leading to improved adaptive immune responses against the model antigen OVA(Rojas et al., 2022), The recombinant adenovirus candidate vaccine for multiple sclerosis (MS) constructed in this study can simultaneously activate humoral and cellular immune responses. Specifically, after chickens were vaccinated with the recombinant adenovirus candidate vaccine, IL-4 and IL-6 secreted by Th2 cells, as well as interleukin-2 and interferon-γ secreted by Th1 cells, showed coordinated upregulation. Research has proven that interleukin-2 maintains immune memory by promoting the proliferation and differentiation of T cells, while interferon - γ can effectively activate effector cells such as natural killer cells, cytotoxic T lymphocytes, and macrophages, collectively enhancing cellular immunity (Foulds et al., 2006). In addition, interleukin-4 promotes the differentiation of B cells into plasma cells to enhance humoral immunity, and interleukin - 6 enhances antibody affinity maturation and regulates the Th1/Th2 balance, which plays an important role in humoral immunity (Mitchell et al., 2017). This suggests that the recombinant adenovirus candidate vaccine constructed in this study exhibits excellent immune responses in immunized chickens. Notably, the bivalent recombinant adenovirus rAd-pdhβ-pdhD elicited significantly stronger Th1 and Th2 immune responses than monovalent recombinant adenoviruses, likely attributable to its broader spectrum of antigenic epitopes. This observation correlates well with findings from Cao et al.'s Chikungunya virus vaccine study, where the E2-6K-E1 fusion protein-expressing adenovirus rAd-CHIKV-E2-6K-E1 demonstrated 1.45-fold and 1.38-fold higher IFN-γ and IL-4 levels respectively compared to controls, outperforming single-antigen vaccines. Collectively, these results demonstrate that multivalent antigen fusion strategies can significantly enhance the immunogenicity of recombinant adenovirus vaccines by presenting more comprehensive antigenic profiles.(Cao et al., 2022), further validating the candidate vaccine's advantage in eliciting balanced and potent immune responses.

MS infection in chickens is characterized by chronic disease pathology (Kamathewatta et al., 2024). Challenge experiments revealed that although no abnormal mental status was observed in any group, all PBS control chickens developed marked footpad and joint swelling, whereas only a few vaccinated chickens showed mild clinical signs. Histopathological evaluation revealed that MS infection primarily induced focal inflammatory cell infiltration, vascular hyperplasia, and follicular destruction in the footpad tissues. PBS-vaccinated chickens demonstrated significantly exacerbated lesions compared to vaccinated cohorts. Notably, while inactivated vaccines and recombinant adenoviral vaccines alleviated pathological severity, they conferred only incomplete protection against tissue damage. This observation aligns with recent studies (Han et al., 2024; Zhang et al., 2023). Han et al. evaluated six single-protein subunit vaccines targeting MS, and Zhang et al. developed a multi-epitope subunit vaccine incorporating five proteins. Both studies showed that while the vaccines could not completely prevent infection, they significantly mitigated clinical symptoms (Han et al., 2024; Zhang et al., 2023). A recent study reported a better novel tetravalent subunit vaccine comprising Mycoplasma synoviae Surface Protein B (MSPB), Phosphopyruvate Hydratase (Ppht), Class II Fructose-1,6-bisphosphate Aldolase (Cfba), and Elongation

Factor G (EF-G) proteins of MS, demonstrating 90–100 % protective efficacy with sustained immunity for 180 days post-vaccination in a chicken challenge model (Sun et al., 2024). Moreover, MSPB exhibits considerable variability among circulating MS strains, with amino acid sequence similarity in the NCBI database ranging only from 73.9 % to 100 %, potentially compromising the vaccine's effectiveness against evolving MS variants. pdhβ and pdhD proteins of MS, two conserved immunogenic proteins used in our study, displayed their potential in recombinant adenoviral vector vaccines. The pdhβ-pdhD dual-gene recombinant adenovirus vaccine constructed in this study demonstrated immune effects comparable to those reported by Sun and Zhang, suggesting that multi-antigen epitope synergy may be a key strategy for improving MS vaccine efficacy.

Studies have suggested that the immunogenicity of adenoviral vector vaccines may be influenced by the intensity of immune responses (Appaiahgari and Vrati, 2015; Lokhandwala et al., 2017). Zhang et al. found that the recombinant adenovirus rAd-E0-E2 vaccine against classical swine fever virus in piglets exhibited dose-dependent protective efficacy, with a minimum effective immunization dose of 3.16×10^7 Infectious Unit (IFU) (Zhang et al., 2022). In this study, the partial protection observed with recombinant adenoviral vaccines rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD may be attributed to suboptimal immunization doses or variations in immune response intensity due to antigen-specific differences. Optimization of immunization doses and combinations of different immunogens are critically necessary for immunization protocols in further studies. A critical limitation of this study is the absence of multidimensional protective efficacy assessments for the recombinant adenovirus-vectored vaccines, particularly quantification of bacterial loads in blood and tracheal swabs through qPCR and culture-based methods. Future studies should include pathogen load detection and other comprehensive methods to refine the system for evaluating vaccine protective efficacy.

Conclusion

In summary, the results of this study demonstrate the successful generation of three recombinant adenovirus vaccine candidates targeting the pdhβ and pdhD proteins of MS. Subsequent animal immunization and challenge experiments revealed that the rAd-pdhβ, rAd-pdhD, and rAd-pdhβ-pdhD vaccine candidates effectively elicited robust humoral and cellular immune responses. Among these candidates, rAd-pdhβ-pdhD conferred a superior immunoprotection against MS challenge in chickens. These findings provid scientific insights for the development of innovative MS vaccines.

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Declaration of competing interest

The authors declare that they have no conflicts of interest regarding the design or conduct of this study.

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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.2025.105185.

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