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Immunoprotection of FliBc chimeric fiber2 fusion proteins targeting dendritic cells against Fowl adenovirus serotype 4 infection

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ABSTRACT Hepatitis-hydropericardium syndrome (**HHS**) is a highly fatal disease in chickens caused by the highly pathogenic fowl adenovirus serotype 4 (**FAdV-4**), which has severe economic consequences. The fiber2 protein exhibits excellent potential as a candidate for a subunit vaccination against FAdV-4. Despite having a high safety profile, subunit vaccines have low immunogenicity due to their lack of infectivity, which leads to low levels of immune response. As a vaccine adjuvant, *Salmonella* flagellin possesses the potential to augment the immunological response to vaccinations. Additionally, a crucial strategy for enhancing vaccine efficacy is efficient presentation of immune antigens to dendritic cells (**DC**) for targeted vaccination. In this study, we designed FAdV-4-fiber2 protein, and a recombinant protein called FliBc-fiber2-SP which based on FAdV-4-fiber2 protein, was generated using the gene sequence FliBc, which retains only the conserved sequence at the amino and carboxyl termini of the flagellin B subunit, and a short peptide SPHLHTSSPWER (**SP**), which targets chicken bone marrow-derived DC. They were separately administered via intramuscular injection to 14-day-old specific pathogen-free (**SPF**) chickens, and their immunogenicity was compared. At 21 d postvaccination (**dpv**), it was found that the FliBc-fiber2-SP recombinant protein elicited significantly higher levels of IgG antibodies and conferred a vaccine protection rate of up to 100% compared to its counterpart fiber2 protein. These results suggest that the DC-targeted peptide fusion strategy for flagellin chimeric antigen construction can effectively enhance the immune protective efficacy of antigen proteins.

Key words: fowl adenovirus serotype 4, dendritic cells targeting peptide, flagellin, subunit vaccine, immunogenicity

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INTRODUCTION

Fowl adenovirus serotype 4 (**FAdV-4**) infection can cause hepatite-hydropericardium syndrome (**HHS**), which is an acute disease with a mortality rate of more than 80%, and broilers aged 4 to 5 wk are the most susceptible to HHS, causing serious economic losses to the poultry industry (Li et al., 2016; Pan et al., 2017; Wang et al., 2019). The first outbreak of HHS occurred in 1987 in the Ankara area near Karachi, Pakistan. Since then, it has been found in many parts of the world, such as Chile, Mexico, Peru, Iraq, South America, and Russia, and in recent years, and also been reported in India, Canada, Korea, and China (Dar et al., 2012; Ye et al., 2016; Schachner et al., 2018). A member of the *Adenoviridae* family, FAdVs are nonenveloped, icosahedral virions that contain linear dsDNA genomes (Davison et al., 2003; Benkő et al., 2022). The nuclear capsid is composed of 252 capsomers, including hexon bases and penton bases. Penton bases are located at each vertex of the capsome and serve as attachment points for fibers, from which fibers protrude (Russell, 2009; San, 2012). The fiber consists of 3 structural components: knob, shaft, and tail. The knob can bind to the Coxsackie adenovirus receptor (**CAR**), facilitating virus invasion into host cells (Sohaimi et al., 2021). Studies suggest that fiber2 plays a crucial role in virulence, making it the main antigenic protein for FAdVs immune protection (Zhang et al., 2018; Liu et al., 2020; Xie et al., 2021; Zhao et al., 2022). Researchers have expressed 3 structural proteins of FAdV-4, namely fiber1, fiber2, and hexon loop-1, using baculovirus system, which were subsequently evaluated for their immunogenicity in chickens, the results showed that the protective effect of subunit vaccine prepared with fiber2 was better than that of fiber1 and hexon loop-1, and the protection rate

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against the challenge of the powerful strain FAdV-4 was more than 95%, and no clinical symptoms were observed in vaccinated individuals (Schachner et al., 2014). Demonstrated the effective immunoprotection of fiber2 by expressing FAdV-4 structural protein in *Escherichia coli* prokaryotes (Wang et al., 2018). In addition, related studies have shown that the fiber2 protein provides complete protection against FAdV-4, with the recombinant protein eliciting faster and stronger immune responses than the inactivated oil-emulsion vaccine (Chen et al., 2018; Ruan et al., 2018).

The 3 fundamental components of vaccine research are the immunogen, adjuvant, and interaction between the vaccine and the host organism (Tacken et al., 2011 ; Caminschi et al., 2012). With advancements in frontier disciplines such as structural molecular biology and immunomics, recombinant subunit vaccine immunogens are gradually transition towards a new era of precise design. The key to further developing subunit vaccines lies in enhancing their immunogenicity (Slifka and Amanna, 2014). Dendritic cells (**DC**) possess a robust capacity for antigen presentation, it is frequently utilized as targets for antigens which making a major determinant of vaccination (Cohn et al., 2014; Macri et al., 2016; Matsuda et al., 2022). The recombinant *Lactococcus lactis* expressing FAdV-4-hexon fusion DC targeting peptide was constructed, which resulted in increased levels of serum IgG and secretory IgA (**sIgA**) in jejunal lavage fluid after oral immunization compared to the PBS and empty vector control groups, providing enhanced protection against FAdV infection (Jia et al., 2021). Short peptides (SP) capable of targeting DC can be screened and expressed through fusion with antigen proteins to prepare targeted vaccines, thereby achieving precise and effective delivery of antigens to DC, ultimately enhancing the immunogenicity of antigens (Chen et al., 2016; Shrestha et al., 2018; Xia et al., 2022). Our laboratory has previously conducted studies to screen targeted peptides (**SP**) that can specifically target DC derived from chicken bone marrow, and their ability to target DC was verified through in vitro experiments (Ma et al., 2019). The flagellin of *Salmonella* is a natural agonist for toll-like receptor 5 (**TLR5**), functioning as an antigen that binds with TLR5 to activate the proliferation and differentiation of various immune cells, thereby augmenting the body's innate immune response. As an immune adjuvant, flagellin can exert its activity regardless of the administration route, be it oral or injection. The fusion protein is constructed through gene fusion technology by combining the antigen gene sequence with the flagellin gene sequence while preserving their independent spatial structure and biological activity (Fitzgerald et al., 2020; Tran-Mai et al., 2022). Researchers engineered a flagellin enhanced green fluorescent protein (**EGFP**) fusion protein, and immunization of animals with flagellin-EGFP fusion protein produced specific anti-EGFP responses. Thus recombinant-flagellin fusion proteins can be used as adjuvants for the development of new vaccination strategies to induce and enhance immune responses against infectious diseases and cancers (Cuadros et al., 2004).

To investigate the immunogenicity of antigen proteins fused with targeted peptides under FliBc chimeric expression conditions, we constructed FliBc chimeric antigen-targeted DC recombinant subunit vaccine FliBc-fiber2-SP, explore the immune protective effect of the vaccine by SPF chicken challenge protection experiment. The development of a subunit vaccine targeting DC with FliBc chimeric antigen provides a new idea for the prevention and control of HHS and other poultry related diseases.

MATERIALS AND METHODS

Virus Propagation and Genomic DNA Extraction

Leghorn male hepatocellular (**LMH**) cells (ATCC, CRL-2117), were cultured in DMEM supplemented with 10% fetal bovine serum. The highly pathogenic FAdV-4 strain CH/SD15-21/2015 (GenBank accession No. KY364398) was isolated and preserved in the laboratory, and propagated by the LMH cells. Viral DNA was extracted from the supernatant of cultured cells using the TRAN Easy Pure Viral DNA/RNA extraction kit (TARN, Beijing, China).

Construction of Expression Recombinant Vector

Based on the sequence of FAdV-4-fiber2 and SP (SPHLHTSSPWER) that targeting chicken bone marrow DC, specific primers (Table 1) were designed to expand fiber2 and fiber2-SP. The gene sequence of flagellar protein B subunit from *typhimurium-Salmonella* was amplified by PCR using the cloned plasmid pMD-18Ts-FliBc, and N and C-terminal sequences of FliBc (FliBc-F and FliBc-R) were obtained (Figure 1). The recombinant gene fragment FliBc-fiber2-SP was generated through fusion PCR, wherein the fiber2-SP gene fused to the targeted peptide sequence was inserted between FliBc-F and FliBc-R using a flexible linker (Figure 2). Subsequently, the resulting recombinant gene fragments fiber2 and FliBc-fiber2-SP were individually ligated to pET-SUMO-His employing T4 ligase, leading to the construction of recombinant expression bacteria.

Expression and Purification of Recombinant Proteins

The recombinant bacteria were expanded and cultured in liquid Luria-Bertani (**LB**) medium (containing 100 μg/mL kanamycin) at a ratio of 1:100 until the OD600 value of the bacterial solution reached between 0.4 and 0.6. Subsequently, the final concentration of Isopropyl β-D-Thiogalactoside (**IPTG**) was adjusted to 1 mol/mL, and the recombinant bacteria were further

Table 1. Primer sequences used to amplify the genes of recombinant gene fiber2 and FliBc-fiber2-SP.

Designation	Primer sequences (5'to3')	Fragment
fiber2-F	F: *GAGCTC*GGATGCTCCGGGCCCCT	fiber2
fiber2-R	R: *GCGGCCGC*CGGGAGGGAGGCC	(1440 bp)
fiber2-SP-F	F: ATGCTCCGGGCCCCTAAA	fiber2-SP
fiber2-SP-R	R: **ACGTTCCCATGGACTACTAGTATGAAGATG TGGTGA**AGATCCGCCACCGCCCGGGAGGGAGGC	(1491 bp)
FliBc-F-F	F: *GAGCTC*ATGGCACAAGTA	FliBc-F
FliBc-F-R	R: TGAGGCCGGATCAACCGG	(540 bp)
FliBc-R-F	F: ACCGAAAACCCGCTGCAG	FliBc-R
FliBc-R-R	R: *GCGGCCGC*ACTCGAGACG	(288 bp)
A-F	GAGCTCATGGCACAAGTA	FliBc-fiber2-SP
B-F	TCCGGCCTCAATGCTCCGG	(2319 bp)
B-R	GGTTTTCGGTACGTTCCCATG	
C-R	GCGGCCGCACTCGAGACG	

The restriction site is *in italics*, the gene sequence of the SP short peptide is **bolded**, and the Linker gene sequence is underlined.

cultured for a duration of 8 h. The bacterial precipitate was collected for ultrasonic fragmentation, while the supernatant was obtained through centrifugation. Finally, purification of the target protein was performed using a denaturing-resistant dosage form His-tag protein purification kit (Beyotime, Beyotime Institute of Biotechnology, Jiangsu, China).

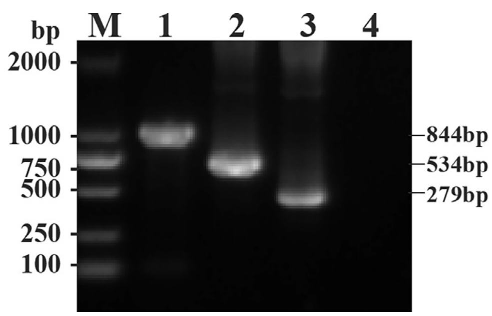

Figure 1. The FliBc-F and FliBc-R genes were amplified via PCR. The conserved sequences FliBc-F and FliBc-R at the C-and N-terminals of Salmonella flagellar protein FliBc were amplified via PCR, utilizing pMD18Ts-FliBc as the template. Lane 1 represent the pMD18Ts-FliBc, lane 2 represent the genes FliBc-F, lane 3 represent the genes FliBc-R, lane 4 represents a negative control.

Western Blot

The protein samples were separated by 10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (**PVDF**) (Millipore, Milford, MA). A 200-fold dilution of anti-FAdV-4-fiber2 serum was utilized as the primary antibody and incubated at 37°C for 1 h. Subsequently, a 5,000-fold dilution of horseradish peroxidase (**HRP**)-labeled goat anti-chicken IgG antibody served as the secondary antibody and was incubated at 37°C for 30 min. The samples were then washed 3 times with PBST (0.1% Tween-20). Finally, enhanced chemiluminescence (**ECL**) reagent (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) was employed to visualize the results.

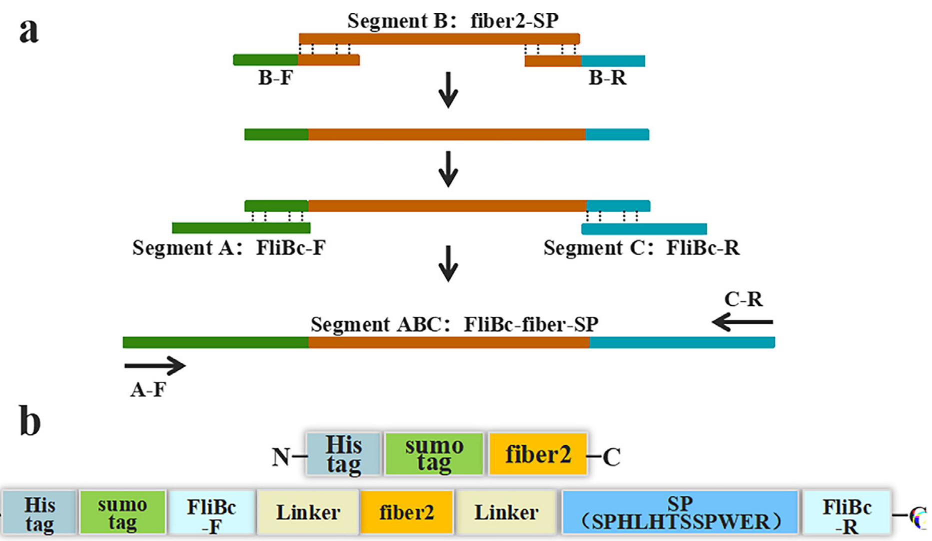

Figure 2. Fusion PCR amplification of the recombinant fragments. (A) The recombinant gene fragments containing the A and C parts were amplified using gene fragment B as a template, along with primers B-F and B-R. In the initial step of PCR, the recombinant gene fragment exhibited complementarity with gene fragments A and C through overlapping homologous sequences. Subsequently, in the second step of PCR amplification, the recombinant gene fragment ABC was obtained by incorporating primers A-F and C-R. (B) Design of recombinant vector construction in *Escherichia coli.*

Table 2. Animal experimental grouping.

Group	Vaccine	Challenge
Group I	fiber2 (50 μg)	$2 \times 10^{6.5}$ $TCID_{50}$ FAdV-4
Group II	FliBc-fiber2-SP (50 μg)	
Group III	PBS	
Group IV	PBS	PBS

Immunization and Challenge Experiment

40 of 14 d postbirth (**dpb**) SPF chickens (Experimental Animal Center, Harbin Veterinary Institute, Chinese Academy of Agricultural Sciences) were reared in hermetically poultry isolators with sufficient provisions of water and food, were randomly assigned to 4 groups with 10 chickens in each group. The experimental procedures employed in the animal experiments conducted in this study were all approved by the Laboratory Animal Welfare and Ethics Committee of Northeast Agricultural University and was conducted in compliance. The purified protein samples (0.5 μg/μL) were mixed with a white oil adjuvant and emulsified at a ratio of 1:2 to form a water-in-oil preparation vaccine. Group I and Group II chickens were injected with fiber2 and FliBc-fiber2-SP vaccines at a dose of 50 μg/chicken, respectively according to the Grouping outlined in Table 2. Group III and IV received an equivalent volume of sterile PBS. The status of chickens was observed following immunization, and blood samples were collected from the brachial vein at 7, 14, and 21 d postvaccination (**dpv**). At the 21 dpv 200 μL FAdV-4 virus ($2\times10^{6.5}$ $TCID_{50}$) was injected intramuscularly for challenge, and an equal volume of sterile PBS was injected in Group IV, the sampling procedure was performed with reference to Figure 3. At 5-d postchallenge (**5dpc**) 3 chickens in each group were randomly selected to humanely euthanized using isoflurane to evaluate the immunological protective effect of vaccines. All remaining chickens were humanely euthanized at the termination of the experiment on 42 d postbirth.

Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was employed to detect serum-specific IgG antibody levels. The recombinant proteins fiber2 and FliBc-fiber2-SP were utilized as antigens for coating 96-well microplate (100 ng per well). Meanwhile we coated 96-well microplates with virus as antigen (100 uL/well, $2\times10^{6.5}$ $TCID_{50}$) to determine serum specific IgG against FAdV-4 after immunization. The sera collected at different time points postimmunization was diluted 200-fold as the primary antibody, and a secondary antibody consisting of horseradish peroxidase (**HRP**)-labeled goat anti-chicken IgG was used at a dilution of 5,000-fold. Meanwhile, the chicken serum from the PBS group served as a negative control. The serum samples were collected from chickens prior to and 21 d postimmunization. The levels of Th1 (IL-2) and Th2 (IL-4) cytokines in the serum samples before and after immunization were determined using enzyme-linked immunosorbent assay kits (Meimian Industrial Co., Ltd., Jiangsu, China).

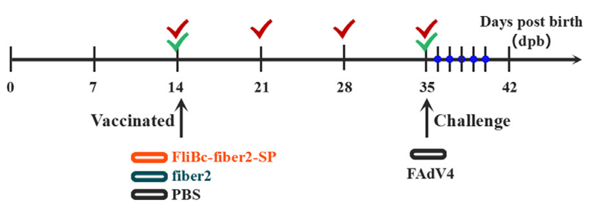

Figure 3. Procedure of intramuscular immunization and sampling. A 14 dpb SPF chickens were infected with FAdV-4 challenge at 21 d postvaccine (35 dpb). Red mark indicates serum samples were collected to monitor IgG antibodies, green mark indicates cytokine detection, blue mark indicates the time points of sampling feces.

Testing Viral Loads of Target Organs by Quantitative Polymerase Chain Reaction (qPCR)

A 0.1 g samples of each organ were weighed and placed in a 2 mL centrifuge tube, followed by the addition of 1 mL sterile PBS and magnetic beads. The mixture was thoroughly homogenized using a tissue homogenizer and then centrifuged for 10 min at 4℃. Viral DNA was extracted from the supernatant, and the viral load in each organ was quantified by qPCR using CFX96 real-time system (Bio-Rad, Hercules, CA) with iTag Universal SYBR Green Supermix (Bio-Rad, Hercules, CA). Table 3 lists the primers information used.

Testing Virus in Cloacal Swabs Samples

Cloacal swabs were collected from all chickens in each group 1 to 5 d after challenge and placed in a 2 mL centrifuge tube containing 800 μL sterile PBS. The supernatant was aspirated, viral DNA was extracted, and specific identification primers (Table 3) for FAdV-4 were designed to PCR the presence or presence of virus in cloacal swabs of chickens in each group, cloacal swabs samples with FAdV-4 were quantitatively analyzed by qPCR.

Clinical Signs and Histopathological Analysis

After the challenge, a 7-d observation period was established to monitor and record the clinical symptoms, morbidity, and mortality of chickens in each group. Additionally, the efficacy of the vaccine was calculated. The heart and liver specimens were collected from each

Table 3. Primers used for qPCR for viral loads of cloacal swabs and organs analyses.

Designation	Primer sequences (5'–3')	Fragment
fiber2-F250	F:AACTCAACCTAAAAGCGCAG	250 bp
fiber2-R250	R:GACAAAAGTAGCGATGGGTG	

group for pathological examination and recording of organ-specific changes. A suitable amount of organs were fixed in a 4% paraformaldehyde solution for over 24 h to prepare histopathological sections stained with HE dye.

Statistical Analysis

The data were analyzed using GraphPad Prism (version 7.0; GraphPad Software, San Diego, CA). The results are presented as mean ± SD of triplicate measurements. Tukey's multiple comparison test was employed to assess the differences between the control groups. Statistical significance was defined as $P < 0.05$, indicating a significant difference, and $P < 0.01$), indicating a highly significant difference.

RESULTS

Identification the Immunoreactivity of Recombinant Proteins

Recombinant bacteria's pSUMO-fiber2/BL21, pSUMO-FliBc-fiber2-SP/BL21, and pSUMO/BL21 were cultured overnight and subsequently lysed. Western blotting analysis revealed the presence of target bands of expected size in the supernatant of lysate from both groups of recombinant bacteria. However, these bands were absent in the supernatant of pSUMO/BL21 lysate, indicating efficient expression of the target protein in a soluble form. Furthermore, the recombinant protein exhibited specific binding to FAdV-4-positive serum with excellent reactivity (Figure 4).

IgG Antibody Titer Following Immunization

ELISA was employed to evaluate the level of anti-fusion protein IgG antibody induced by FliBc-fiber2-SP, fiber2 in the serum of both groups, as illustrated in Figure 5. Specific IgG antibody was detectable in the immunized groups (Group I, Group II) at 7 d postvaccination (**dpv**), and their levels increased rapidly during

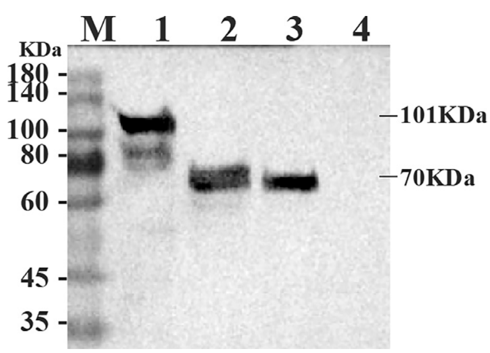

Figure 4. Expression of proteins by Western blot detection. Expression of interest proteins fiber2, FliBc-fiber2-SP by the recombinant *Escherichia coli* were detected with chicken anti-FAdV-4 positive serum. Lane 1 represent the strains pSUMO-FliBc-fiber2-SP/BL21, lane 2-3 represent the strains pSUMO-fiber2/BL21, lane 4 represent the strains pSUMO/BL21.

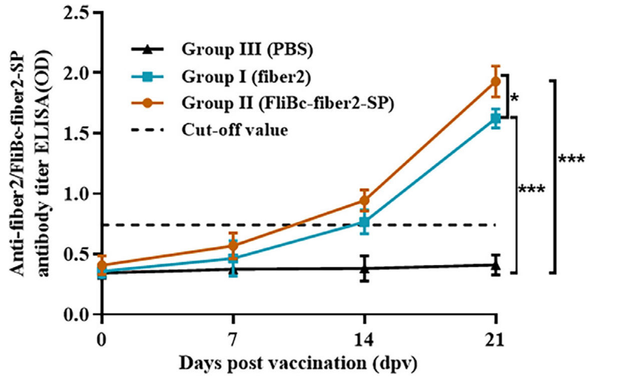

Figure 5. The levels of anti-fiber2/FliBc-fiber2-SP specific serum IgG. After immunization, sera (Three chickens were randomly selected from each group) were collected at weekly intervals for antibody IgG concentration by indirect ELISA for 3 wk (the value of 2 times the mean value of the negative serum assay results was used as the cut-off value) ($^{*}P < 0.05$, $^{***}P < 0.001$).

14 dpv to 21 dpv before reaching a peak at 21 dpv, and were significantly higher than PBS control group (Group III) ($^{***}P < 0.001$), the level of IgG induced by FliBc-fiber2-SP higher compared to that induced by fiber2 ($^{*}P < 0.05$). Using FAdV-4 as coating antigen, specific IgG antibody against FAdV-4 was detected. At 21dpv, the level of IgG antibody against FAdV-4 in Group III was significantly higher than that in Group I ($^{**}P < 0.01$) (Supplementary Figure 1). The levels of anti-FAdV-4 IgG were not significantly altered in the PBS control (Group III).

Serum Cytokine Levels Following Immunization

The levels of IL-4 and IL-2 in serum were quantified using ELISA kits prior to immunization and 21 d post-vaccination (21 dpv). As depicted in Figure 6, there was a significant increase in serum levels of IL-4 and IL-2 observed at 21 dpv in the immunization group (Group I, Group II), which were markedly higher than those detected in the PBS control (Group IV) ($^{**}P < 0.01$). Furthermore, the serum level of IL-2 in the FliBc-fiber2-SP immunization group exhibited a significant elevation compared to that seen in the fiber2 immunization group ($^{*}P < 0.05$).

Protection Against FAdV-4 Challenge Infection

The viral shedding of cloacal swabs and mortal situation of chickens in each group were recorded following FAdV-4 challenge to assess the protective effect of the vaccine (Table 4; Figure 7). The results of qPCR showed that the viral load of cloacal swabs in Group II was significantly lower than that in Group I ($^{*}P < 0.05$) (Supplementary Figure 3). In Group III (PBS +FAdV-4), at 2 d postchallenge (**dpc**) chickens appeared manifested as loss of appetite, listlessness, rough and disorderly feathers, and in severe cases,

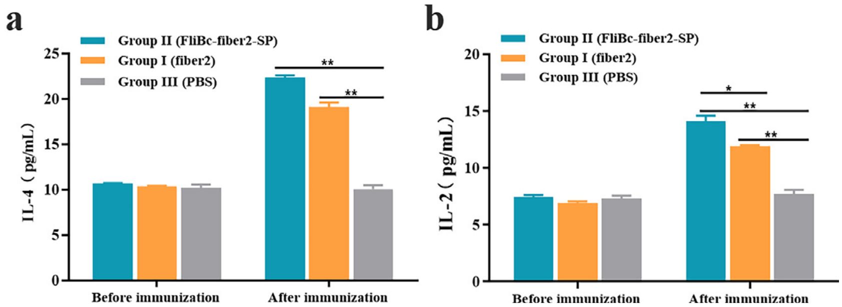

Figure 6. IL-4 and IL-2 mean values post-immunization. Three chickens were randomly selected from each group for serum collection, the concentrations of the respective cytokines in serum fell within the range determined by the standard curve generated from the standard substance. (A) Serum IL-4 levels before immunization and 21 d post vaccination (dpv). (B) Serum IL-2 levels before vaccination and 21 dpv ($*P < 0.05$, $**P < 0.001$).

Table 4. Virus shedding rate of chickens infected by FAdV-4.

	Virus shedding rate (%) Days post challenge (**dpc**)				
Group	1 dpc	2 dpc	3 dpc	4 dpc	5 dpc
Group I	0	30 (3/10)	40 (4/10)	50 (4/8)	50 (4/8)
Group II	0	10 (1/10)	30 (3/10)	30 (3/10)	40 (4/10)
Group III	0	50 (5/10)	80 (8/10)	100 (5/5)	100 (5/5)
Group IV	0	0	0	0	0

green watery feces appeared, mortality commences at 3 dpc, and all chickens succumbed to the infection at 5 dpc, virus shedding rate reach to 100%. Two chickens died in the Group I (fiber2+FAdV-4), resulting in a vaccine protection rate of 80% (8/10). No deaths were observed in Group II (FliBc-fiber2-SP+FAdV-4), indicating a vaccine protection rate of 100%.

Clinical Symptoms and Histological Lesions

After 5 d postchallenge (5 dpc), all chickens in the Group III (PBS+FAdV-4) postmortem exhibited characteristic pathological changes associated with pericardial HHS, included hepatomegaly with a swollen, yellowish, friable liver and the presence of a prominent yellow-green

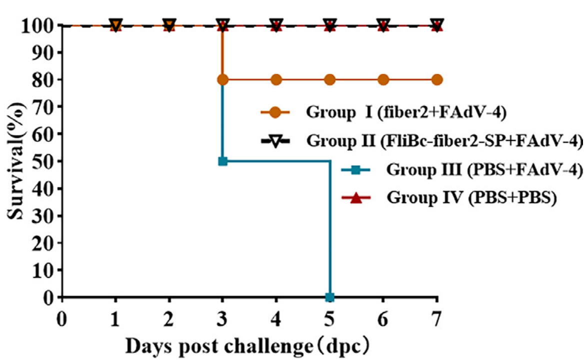

Figure 7. The chicken survival rate. The deaths of chickens in different groups at different time point postchallenge was recorded and the survival curve was drawn. In Group II (FliBc-fiber2-SP+FAdV-4) and Group IV (PBS+PBS) all the chickens survived (100%), whereas in Group I (fiber2+FAdV-4) 8 out of 10 (80%) survived. All the chickens of Group III (PBS+FAdV-4) died within 3 to 5 dpc.

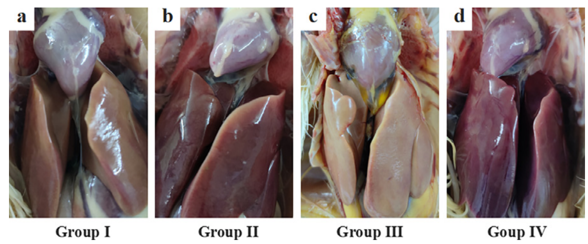

Figure 8. The postmortem lesions observed in the organs. Chickens that died after FAdV-4 challenge infection and those that survived were autopsied separately. Heart and liver of the chickens from Group I (fiber2+FAdV-4) and Group IV (PBS+PBS) did not show any gross lesion, whereas the control Group III (PBS+FAdV-4) presented with earthy yellow liver and yellow-green pericardium effusion in the heart. Liver of Group II (FliBc-fiber2-SP+FAdV-4) showed slight discoloration.

pericardial effusion in the heart (Figure 8C), none of the groups immunized with the FliBc-fiber2-SP vaccine displayed typical lesions indicative of HHS (Figure 8B). Histological examination revealed severe degeneration and necrosis of hepatocytes, numerous intranuclear inclusion bodies, congestion of certain capillaries with red blood cells, rupture of cardiac muscle fibers, and infiltration by a significant number of inflammatory cells (Figure 9C). In contrast, these histopathological findings were absent in both the immune group (Figures 9A and 9B) and PBS control group (Figure 9D).

Viral Loads of in Various Organs

RT-qPCR was used to calculate the viral copy numbers in tissues of each group chickens at 5 d postchallenge. The results showed that after infection, FAdV-4 was widely distributed in chicken tissues, liver of Group III (PBS+FAdV-4) with a viral load of $4.27\times10^{7.0}$ copies/g, followed by heart, spleen and lung. At the same time, a small amount of virus could be detected in immune-related organs such as thymus and bursa of Fabricius. The viral load of each organ in vaccine protected groups (Group I; Group II) were significantly lower than that in Group III (PBS+FAdV-4) ($***P < 0.001$) (Figure 10).

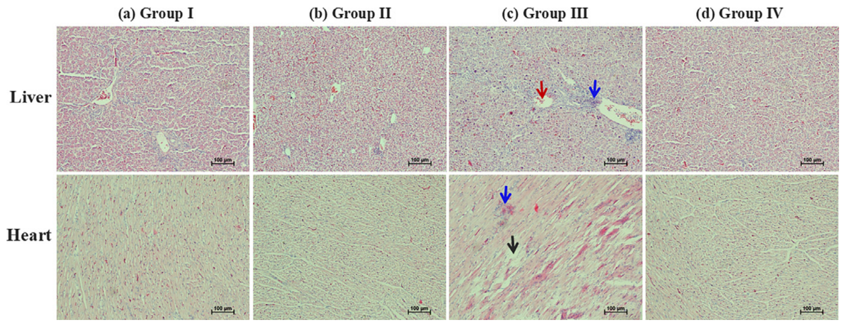

Figure 9. Histological lesions in heart and liver of chickens postchallenge. Histological lesions of Group I (fiber2+FAdV-4), Group II (FliBc-fiber2-SP+FAdV-4), Group III (PBS+FAdV-4), Group IV (PBS+PBS). Black arrow indicates red blood cells aggregated, black arrow indicates large number of inflammatory cells infiltrated, blue arrow indicates rupture of the myocardial fibers.

DISCUSSION

FAdV-4 is prevalent worldwide (Kiss et al., 2021) was highly lethal to poultry, causing sudden onset and death (Choi et al., 2012; Del-Valle et al., 2020; Rashid et al., 2020; Franzo et al., 2020). Currently, vaccination stands as the most cost-effective measure for preventing fowl adenovirus infection. As an important structural protein of FAdV, fiber has a better effect in inducing protective immunity and is considered as a candidate antigen protein for the preparation of subunit vaccines (Guan et al., 2018). The research on targeted vaccines has witnessed a remarkable expansion in recent years. The composition of subunit vaccines consists of purified proteins or inactivated pathogens derived from the pathogen, which upon immunization can elicit specific memory B and T cells, thereby conferring long-term protection to vaccinated individuals. DC possess a robust capacity for antigen capture and processing, making them pivotal players in vaccination strategies (Caminschi et al., 2012; Pugholm et al., 2015). Enhancing the effective delivery of antigens to DC represents a promising approach for augmenting vaccine immunogenicity.

Using a phage display library, researchers screened for 12 peptides (DC-pep) that specifically bind human DC, and used hepatitis C virus (**HCV**) nonstructural protein 3 (**NS3**) as the immunogen, and the "DC-pep" fusion was expressed at the C-terminus of NS3, and the fused protein enhanced DC activation and caused a significant increase in interferon IFN γ and tumor necrosis factor TNF-α secreted by $CD4^+$ and $CD8^+$ T cells in patients infected with HCV (Curiel et al., 2004). Therefore, in this study, primers were designed according to the sequence of SP gene that can target chicken bone marrow derived dendritic cells screened by a phage display library in our laboratory. The SP peptide was fused to the C-terminus of fiber2 by PCR and fusion sequence fiber2-SP was constructed. Studies have shown that Salmonella flagellin as an adjuvant can induce mixed Th1 and Th2 immune responses (St Paul et al., 2012; Gupta et al., 2013). Pertinent researches indicate that the truncated N-terminal and C-terminal functional regions are responsible for flagellin's immunoenhancement properties (Chauhan et al., 2005; Mizel et al., 2009). Previous research has utilized genetic engineering to embed key antigen genes from various pathogens among these regions, resulting in enhanced immune responses to the antigen proteins (Applequist et al., 2005; Asadi et al., 2013; Song et al., 2015; Tran-Mai et al., 2022). Studies have found that bacterial flagellin can activate myd88-dependent signal transduction pathway through TLR5 expressed on the cell surface to activate and mature TLR5-expressing cells, further activate and mature antigen presenting cells, enhance the ability to uptake antigen, and finally initiate adaptive immune response (Trachtenberg et al., 1988; Aizawa et al., 1990). Through the interaction with TLR5, flagellin can achieve the efficacy of immune adjuvants and significantly enhance the level of humoral and cellular immune responses to exogenous antigens (Muzio et al., 2000). Currently, there are 2 approaches to investigating the adjuvant effect of flagellin. One involves fusing the flagell the target antigen gene in an expression vector to generate a recombinant vaccine capable of expressing a fusion protein comprising both flagellin and the target antigen, another strategy is to express flagella protein and target antigen protein separately, and mix them for vaccine immunization (Okugawa et al., 2006).

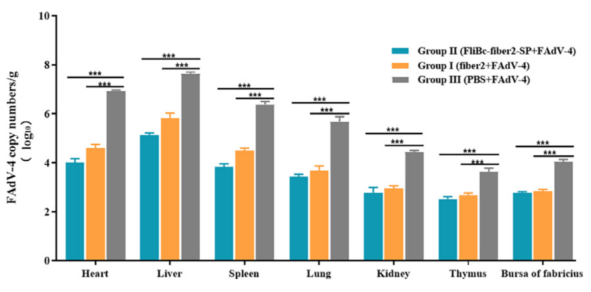

Figure 10. Viral copy numbers in chicken tissues post FAdV-4 challenge. The viral copy numbers in tissues from chickens (three chickens were randomly selected from Group I, Group II and Group III, respectively) at 5 dpc were collected to quantify by qPCR. All experiments were performed in triplicate, and each bar represents the mean value (***$P < 0.001$).

In order to be closer to the actual vaccine in production, the recombinant subunit vaccines fused with flagellin and antigen were prepared based on commercial Freund's adjuvant, based on the SP which can target DC and flagellin (FliBc) which has the potential of immune adjuvant screened in our laboratory, recombinant fusion protein FliBc-fiber2-SP was prepared based on the gene sequence of FAdV-4-fiber2. Western blot analysis showed that both histones could react specifically with anti-FAdV-4 positive serum, showing good reactivity. However, since FliBc-fiber2-SP is the fusion expression of FliBc-F, fiber2-SP, and FliBc-R gene fragments (Figure 2), we speculated that the FliBc-fiber2-SP protein was partially expressed without fusion, so a protein band of about 90KDa (FliBc-F-fiber2-SP) might appear, therefore in Figure 4, lane1, 2 bands were detected by the FAdV-4 positive serum. Proteins were purified according to the correct band size using His-tag protein purification kit (Beyotime, Beyotime Institute of Biotechnology, Jiangsu, China) for intramuscular immunization, the specific IgG level of FliBc-fiber2-SP immunized group was significantly higher than that of fiber2 immunized group at 21dpv. The specific IgG antibody against FAdV-4 in serum was measured by ELISA, and the results showed that FliBc-fiber2-SP (Group II) induced higher levels of specific IgG (Supplementary Figure 1). These results indicated that FliBc chimeric antigen targeted DC vaccine induced higher level of IgG antibody, and the antibody produced faster and lasted longer. This is consistent with the result that sIgA can be produced earlier in the trachea after induction of immunization with previously constructed fusion-expressing IBDV-VP2-SP recombinant bacteria (Ma et al., 2019). The levels of IL-2 and IL-4 in serum before and after immunization were determined using ELISA kits. IL-2 is primarily synthesized and secreted by antigen-stimulated T cells, promoting T cell differentiation and indirectly reflecting cellular immune response levels. As a Th2-related cytokine, IL-4 plays a crucial role in the body's humoral immunity (Kathania et al., 2013). The FliBc-fiber2-SP group exhibited significantly elevated serum IL-2 levels compared to the fiber2 group, indicating that targeted peptide (SP) facilitated antigen exposure to DC in tissues, thereby activating more B-cells to secrete IgG and activating T-cells to proliferate and differentiate, resulting in higher levels of specific immune response.

After challenge, cloacal swabs sampled from each chicken were tested for the presence of FAdV-4 DNA using PCR, the vaccine FliBc-fiber2-SP appeared to be more effective in diminishing virus shedding (Table 4, Supplementary Figure 2), and the results of qPCR indicated that the viral load of cloacal swabs in Group II was significantly lower than that in Group I ($*P < 0.05$), there were significant differences in viral titers of cloacal swabs between Group I and Group II (Supplementary Figure 3). Although the vaccine immunization played a crucial role in production of specific antibodies, but still part of some chickens still exhibited virus shedding, which could be attributed to fiber2 not inducing neutralizing antibodies, our previous study collected the positive serum after fiber2 immunization and measured its ability to neutralize virus, the results showed that the serum had no significant neutralizing activity compared with the control group. At the same time, studies has also shown that fiber2 can not induce the production of effective neutralizing antibodies (Schachner et al., 2014; Xie et al., 2022). The subsequent experimental designs investigated the correlation between immune dosage and protective efficacy. The liver was identified as the primary target organ of FAdV-4 infection (El-Shall et al., 2022), therefore, assessing the occurrence of liver and heart lesions in the vaccine group serves as a crucial indicator for evaluating efficacy of the vaccine. After FAdV-4 challenge, the viral load in the control group (Group III) subjected to challenge reached up to $4.27\times10^{7.0}$ copies/g, with prominent pathological changes observed in both the liver and heart during necropsy, included hepatomegaly and a significant accumulation of yellow effusion in the pericardium. Both groups of vaccines (Group I, Group II) exhibited significant reduction in liver lesions, and no pericardial effusion was observed during necropsy conducted 5 d after challenge. Vaccine immunization effectively mitigated viral load in target organs and ameliorated virus-induced damage to the heart and liver. At 3 d postchallenge, the challenge control group exhibited 100% mortality, while the subunit vaccine fiber2 conferred a protection rate of 80, 20% chickens could not be protected by the fiber2 subunit protein. In contrast, all chickens immunized with FliBc-fiber2-SP survived and achieved complete protection. Although previous studies that showed fiber2 could provide complete protection against FAdV-4 (Chen et al., 2018; Ruan et al., 2018), but study has also proved that the protection rate of the vaccine is related to the immunization dose (Wang et al., 2018), and the subsequent design experiments we can explore the relationship between immunization dose and vaccine protective effect.

CONCLUSIONS

From present study, it can be determined the recombinant subunit vaccine FliBc-fiber2-SP, which FliBc chimeric fiber2 protein targeting DC and is developed based on the FAdV-4-fiber2, higher levels of specific IgG was induced after immunization. The challenge protection experiments demonstrated that FliBc-fiber2-SP significantly reduced viral load in tissues and provided complete protection against FAdV-4 infection. These findings suggest that recombinant subunit vaccines prepared by antigen proteins fusing DC targeting peptide under flagellin chimeric conditions can greatly improve their utilization rate, and provide valuable concepts for developing new subunit vaccines targeting other chicken diseases, with broad potential application potential.

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Ethics Statement: The care and use of animals in this study adhered to all relevant international and national guidelines. Approval for animal experiments was obtained from the Committee on the Ethics of Animal Experiments at Northeast Agricultural University, Harbin, China (2016 NEFU-315, 13 April 2017).

Data Availability Statement: The original contributions presented in the study are included in the article. For any additional inquiries, please direct them to the corresponding authors.

DISCLOSURES

The authors declare no competing interests.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.103474.

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