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Immunogenicity and protective efficacy of recombinant fiber-2 protein in protecting SPF chickens against fowl adenovirus 4

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ABSTRACT

Since a novel hyper-virulent fowl adenovirus serotype 4 (FAdV-4) infection occurred in 2015, the novel FAdV-4 has been widely spreading across China, causing significant economic losses to the poultry industry. As the urgency of the issue calls for effective and efficient solutions, the present study investigated the possibility of the fiber-2 protein of the FAdV-4 to serve as a vaccine candidate against the novel FAdV-4. In the research, fiber-2 proteins were expressed in *Escherichia coli*, and then purified. To evaluate the immunogenicity of the recombinant fiber-2 protein, we investigated both the humoral and cellular immune responses in chickens immunized with fiber-2. The humoral immunity was assessed by detecting IgY antibodies and virus-neutralizing antibodies in chicken serum at 7, 14, 21 days post-immunization (dpi). We examined cellular immune responses by detecting CD3+CD4+ and CD3+CD8+ changes in chickens' peripheral blood through using flow cytometry at 7, 14, 21 dpi. The cytokine production in the serum of the immunized chickens was detected by ELISA at 7, 14, 21 dpi to further explore the impact of the recombinant protein on the regulation of cytokines. The protective efficacy was determined by the survival rate of the immunized chickens challenged with the novel FAdV-4. The results show that the level of IgY antibodies of the chickens immunized with fiber-2 protein was significantly higher than that of the chickens immunized with an inactivated vaccine against FAdV-4. Moreover, 7 days after immunization, the CD4+ T-cell proliferative response of the chickens immunized with fiber-2 was significantly higher than that of the chickens immunized with the inactivated vaccine. Challenge experiment showed that the fiber-2 protein could provide full protection and the inactivated vaccine could provide 90 percent protection against the FAdV-4. These results suggest that the recombinant fiber-2 protein can be an ideal candidate for subunit vaccines against the disease.

1. Introduction

Fowl adenovirus serotype 4 (FAdV-4) is a non-enveloped double-stranded DNA virus, belonging to the genus *Aviadenovirus* in the family of *Adenoviridae*. It is the causative agent of hydropericardium syndrome (HPS), hepatitis-hydropericardium syndrome (HHS) and inclusion body hepatitis (IBH), causing significant economic losses to poultry industry [1]. Characterized by hepatitis, nephritis, and the accumulation of clear or straw-colored fluid in the pericardial sac of the infected animals, the disease caused by FAdV-4 affects broilers at the age of 3–6 weeks with a high mortality rate ranging from 10% to 100% [2,3]. After the first report of FAdV-4 in Pakistan in 1987, the virus has been spreading to vast regions of Asia, Central and South America, as well as some European countries [4]. Currently, various vaccine formulations are widely used in some countries to prevent and control the disease. Oral live attenuated vaccines [5] and parenteral inactivated whole cell culture vaccines [6] are available for the prevention against the conventional strains of the virus. However, the incomplete attenuation or inactivation of adenoviruses contained in the vaccines and the oncogenic potential of these vaccines have limited their use as routine vaccines [7]. Since 2015, severe outbreaks of the disease caused by a novel hyper-virulent FAdV-4 with a high mortality rate of 30–90% have occurred in different areas in China. As no commercial vaccine is available against the novel strain of FAdV-4, the development of an efficacious, safe, and economic vac-

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cine candidate is highly desired for the disease prevention and control.

The viral particle of FAdV-4 consists of three major structural adenovirus proteins including hexon, fiber and penton base, and they are identified as potential subunit vaccine immunogens [3,8–12]. With a major surface-exposed capsid structure, the fiber protein of FAdV-4 binds noncovalently to the penton base protein from its N-terminal side and contains two fibers, including a long fiber (fiber-1) and a short one (fiber-2). The fiber protein is responsible for viral virulence [13] and plays an important role in the attachment of the viral capsids to the host cells through interacting with cellular receptors [14,15]. In terms of antigenicity, the fiber-2 (the short fiber) is regarded as a protective immunogen because of its ability to induce neutralizing antibodies [3,12] and provide effective protection from the adenovirus infection [3].

Here, we successfully expressed the fiber-2 protein of the novel hypervirulent FAdV-4 in *E. coli* and used it as a subunit vaccine in chickens. In the research, the immunogenicity and protective efficacy of the recombinant fiber-2 protein were assessed to determine whether fiber-2 protein can serve as a viable and effective vaccine against FAdV-4. The results confirmed our assumption that the recombinant fiber-2 protein is a possible candidate for subunit vaccine against the disease.

2. Material and methods

2.1. Virus and cell line

The novel hypervirulent FAdV-4 was isolated from a clinical case of HPS and IBH in a broiler chicken farm in Guangdong Province, China. The serotype of the virus was defined by phylogenetic analysis based on the hypervariable Loop L1 hexon protein sequence according to ICTV system [16]. Leghorn male hepatoma (LMH) cell line (ATCC #CRL-2117) was used to cultivate FAdVs [17].

2.2. Cloning, expression and purification of recombinant fiber-2 proteins

To create a recombinant FAdV-4 *fiber-2* construct, the *fiber-2* gene was amplified from FAdV-4 genome DNA through PCR by using the following primers pair: Forward: 5′-GAGCTCATGCTCCGGGCCCCTAAAAGA-3′; Reverse: 5′-GCTCGAGTTACGGGAGGGAGGCCGCTGGACA-3′. The 5′ ends of the forward and reverse primers contained *Sac I* and *Psp XI* restriction sites (underlined), respectively. The PCR product was cloned into the pSYNO-1 vector (Convenience Biology Corporation in Changzhou, China. product number: CV-001) encoding maltose-binding protein (MBP) which is widely used as a highly effective solubilizing agent. The resulting plasmid was named pSYNO-1-*fiber-2*. All the primers were synthesized by Sangon Company (Shanghai, China), and pSYNO-1-*fiber-2* was confirmed with sequencing analysis by Huada Company (Beijing, China). To express the fiber-2 protein, pSYNO-1-*fiber-2* was transformed into *E. coli* BL21 (DE3) cells, and a single colony was grown in LB medium with kanamycin (50 μg/mL) at 37 °C overnight with 220 rpm shaking. The culture was diluted 1:20 in 100 mL fresh LB broth containing 100 μg/mL kanamycin and incubated at 37 °C at 220 rpm until its OD_{600} raised to 0.5–0.6. After isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM, the medium was incubated at 16 °C overnight. *E. coli* cells which contain wild type pSYNO-1 plasmid were processed likewise to serve as a negative control.

The cells were harvested by centrifugation at 4000*g* for 10 min at 4 °C and resuspended in 16 mL binding buffer (20 mM Tris, 500 mM NaCl, pH 8.0). Sonication was performed to lyse the cells on ice, and cell lysates were centrifuged at 13,000*g* at 4 °C for 20 min. The supernatant was passed through 0.22 μm filter before being loaded on a nickel-nitrilotriacetate (Ni-NTA) agarose column that had been preequilibrated with binding buffer. Then the supernatant was loaded onto the column by gravity flow and washed with washing buffer (20 mM Tris, 500 mM NaCl, 50 mM imidazole, pH 8.0). The bound proteins were then eluted with elution buffer (20 mM Tris, 500 mM NaCl, 200 mM imidazole, pH 8.0). Afterwards, the elution fractions containing target proteins were pooled together, with tobaccoetch virus (TEV) enzyme added to remove the N-terminal fusion tags (MBP), before they were dialyzed in binding buffer at 4 °C overnight, and applied to the Ni-NTA agarose column that had been preequilibrated with binding buffer. After flowing through the Ni-NTA agarose column, the target proteins were then concentrated using Amicon Ultra-15 centrifugal filter unit (Millipore, cut-off: 50 kDa) at 3500*g* at 4 °C, and the buffer was exchanged to PBS. All steps of protein harvesting and purification were performed at 4 °C. The content or concentration of the recombinant fiber-2 protein was analyzed and determined using SDS-PAGE gel electrophoresis the results of which were detected by GeneSnap and GeneTools from SynGene software with BSA (bovine serum albumin) as standard.

2.3. SDS-PAGE and western blot

The recombinant fiber-2 proteins were boiled for 10 min at 100 °C in the loading buffer with 1% β-mercaptoethanol and separated on 10% SDS polyacrylamide gels, and then electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After they were blocked with 4% BSA in TBST (Tris-buffered saline + 0.1% Tween-20) for 1 h, the PVDF membranes were then incubated overnight at 4 °C with primary chicken polyclonal serum (1:3000; China Institute of Veterinary Drugs Control), and subsequently probed with secondary HRP-conjugated rabbit anti-chicken IgY (1:6000; Bioss, China) for 1 h at RT. After washing the membranes three times, the signals of the proteins were visualized using the commercial ECL kit (Pierce) according to the manufacturer's instructions.

2.4. Animals, immunization, and viral challenge

Collected from the SPF Experimental Animal Center (Guangdong Wens Dahuanong Biotechnology Co., Ltd., Guangdong, China), sixty 10-day-old SPF chickens were housed in individual isolators under positive pressure and randomly divided into three groups (n = 20 chickens/group). The virulent FAdV-4 was propagated on leghorn male hepatoma (LMH) cells and used for vaccine production and challenge strain. For inactivation of the virus, formaldehyde (0.2% in final product) was added to the culture medium harvested from FAdV-4 infected LMH cells [18]. The purified fiber-2 protein, inactivated virus and PBS were emulsified with Montanide™ ISA 71 VG at a 3:7 ratio (w:w; antigens:adjuvant) as recommended by the manufacturer (Seppic, Paris, France) respectively and then used in intramuscular (i.m.) injection. Chickens in group I were immunized i.m. with 10 μg fiber-2 protein per chicken, while those in group II were immunized i.m. with the final dose 10^6 $TCID_{50}$ of the inactivated vaccine per bird. Group III were injected with PBS and set up as an infection control group.

21 days after the immunization, animals in groups I to III were intramuscularly challenged with the virulent FAdV-4 (2×10^7 $TCID_{50}$/chicken), and observed daily for clinical signs over a two-week period. Any chicken that died or had to be euthanized during the observation period was immediately necropsied. All the remaining chickens at the end of the observation period were euthanized. Blood was collected at 7, 14, 21 dpi. The test and all the

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