



# Recombinant fiber-1 protein of fowl adenovirus serotype 4 induces high levels of neutralizing antibodies in immunized chickens

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## Abstract

Virulent fowl adenovirus serotype 4 (FAdV-4) causes hydropericardium syndrome (HPS) with high mortality in chickens, leading to significant economic losses to the poultry industry. The development of an effective vaccine is essential for successful disease control. Here, we produced recombinant fiber-1 protein of FAdV-4, isolated from a Japanese HPS outbreak strain, JP/LVP-1/96, using a baculovirus expression system and evaluated its immunogenicity and protective efficacy. Recombinant fiber-1 protein induced high levels of neutralizing antibodies in immunized chickens, which were maintained for a minimum of 10 weeks. After being challenged with the virulent FAdV-4 strain JP/LVP-1/96, the immunized chickens did not exhibit clinical signs of infection or histopathological changes, there was a significant reduction in the viral load in various organs and total serum proteins, and albumin levels did not decline. These results suggest that the recombinant fiber-1 protein produced in this study can serve as a subunit vaccine to control HPS in chickens.

## Introduction

Hydropericardium syndrome (HPS), also known as hepatitis hydropericardium syndrome (HHS), is a disease affecting chickens that was first reported in Pakistan approximately 35 years ago [1]. HPS caused by virulent fowl adenovirus serotype 4 (FAdV-4) is characterized by the accumulation of amber-colored fluid in the pericardial sac and an enlarged discolored liver with foci of hemorrhage and/or necrosis [2]. HPS outbreaks in poultry have been reported mainly in Asian and Latin American countries, causing significant economic losses to the poultry industry [3–6]. In Japan, HPS outbreaks in chickens were reported on Shikoku Island from 1996 to 2001, and FAdV-4 was isolated from all of the cases [7]. Phylogenetic analysis based on the part of the hexon gene that includes the L1 region revealed that all of these

FAdV-4 isolates were identical and were distinct from the cluster including FAdV-4 strains from chickens with HPS in other countries [8, 9].

The FAdV-4 capsid consists of four major structural proteins: hexon, penton base, fiber-1, and fiber-2 [10]. Functional analysis of these proteins is essential for understanding the infectivity and virulence of FAdV-4. Fiber-1 directly mediates FAdV-4 adsorption to host cells and triggers FAdV-4 infection [11]. Wang et al. also reported that the adsorption of FAdV-4 through fiber-1 is essential for infection [12]. Fiber-2 is an important virulence factor of FAdV-4 [11], and fiber-2 and hexon play crucial roles in the pathogenicity of FAdV-4 strains [13]. Amino acid substitutions at positions 219 and 188 in the fiber-2 and hexon protein, respectively, are commonly found in virulent FAdV-4 strains, including Japanese HPS-associated isolates [9]. Importantly, these capsid proteins induce humoral and/or cellular immune responses in chickens and have been shown to be protective against HPS [14–17], suggesting their potential application as subunit vaccines.

Here, we report the production of recombinant FAdV-4 fiber-1 protein derived from a Japanese HPS isolate, using a baculovirus expression system, and we demonstrate its ability to induce high levels of neutralizing antibody titers in immunized chickens. The efficacy of this vaccine was verified by challenging immunized chickens with a pathogenic Japanese FAdV4 strain.

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## Materials and methods

### Virus

JP/LVP-1/96 (FAdV-4, GenBank accession number LC628937) was used in this study [9]. This strain was isolated from HPS-affected chickens in Japan [7, 8]. After plaque purification, the virus was grown in primary chicken kidney cell (CKC) cultures, and the culture supernatants were harvested, centrifuged, aliquoted, and stored at  $-80^{\circ}\text{C}$  for further experiments.

### Cloning, expression, and purification of recombinant FAdV-4 fiber-1 protein

The fiber-1 gene of JP/LVP-1/96 with a His-tag sequence at its C-terminus was amplified by polymerase chain reaction (PCR) and cloned into the baculovirus transfer vector pAcYM1 [18]. The recombinant transfer vector and the *LacZ* gene-recombinant *Autographa californica* nuclear polyhedrosis virus DNA, linearized with *Eco81I*, were co-transfected into *Spodoptera frugiperda*-derived Sf21AE cells. After plaque purification, the fiber-1-gene-containing recombinant baculovirus was isolated. To obtain the recombinant protein, Sf21AE cells were infected with recombinant baculovirus at a multiplicity of infection of 1.0, harvested after 3 days, and suspended in PBS containing protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). They were then sonicated using an LP-300N homogenizer (Titec, Tokyo, Japan), followed by centrifugation at 11,000 rpm for 20 min. The His-tagged recombinant protein was purified from the supernatant using Talon spin columns (Takarabio, Shiga, Japan) according to the manufacturer's protocol. Bound protein was eluted using 150 mM imidazole in 50 mM phosphate buffer (pH 7.0). The eluted fractions were collected and dialyzed against PBS for further analysis. To determine the purity of the recombinant proteins, samples were separated by SDS-PAGE under reducing conditions [19]. After electrophoresis, the gel was stained with Coomassie brilliant blue (CBB) G-250 or the proteins were transferred to a polyvinylidene difluoride membrane (Thermo Fisher Scientific, Waltham, USA). After blocking, the membrane was probed with anti-His antibody (MBL, Tokyo, Japan) and horseradish-peroxidase-conjugated goat anti-mouse immunoglobulin G (KPL Inc., Gaithersburg, MD, USA). Reactive bands were visualized using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ, USA). The concentration of the protein obtained was determined using a DC protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as the standard.

### Immunization of chickens and determination of serum neutralizing antibody titers

All experimental procedures and animal care were performed in compliance with the guidelines of the National Institute of Animal Health for the humane use of laboratory animals.

Specific-pathogen-free chicken (SPF) eggs were purchased from VALO Biomedica GmbH (Osterholz-Scharmbeck, Germany) and were incubated in an egg incubator. The hatched birds were transported and housed in a biosafety level 2 facility. Five 2-week-old chickens were immunized subcutaneously with 100  $\mu\text{g}$  of recombinant fiber-1 protein in 100  $\mu\text{L}$  of PBS with incomplete Freund's adjuvant and boosted with the same preparation 14 days after the first immunization. As negative controls, five chickens were injected with 100  $\mu\text{L}$  of PBS. Blood samples were collected from the jugular vein of the chickens until 10 weeks after the first immunization, and sera were recovered by centrifugation. The neutralization test was performed using micro-neutralization methods [20]. Briefly, sera were inactivated at  $56^{\circ}\text{C}$  for 30 min and serially diluted twofold in 96-well plates from a starting ratio of 1:20. Strain JP/LVP-1/96 at a concentration of 200 50% tissue culture infectious doses ( $\text{TCID}_{50}$ ) per 100  $\mu\text{L}$  was mixed with an equal volume of diluted serum, and the mixture was incubated at  $37^{\circ}\text{C}$  for 1 hour. Subsequently, CKC monolayers in 48-well plates were inoculated with the mixture and incubated at  $37^{\circ}\text{C}$  for 1 hour. After the addition of the cell culture medium, the plates were kept at  $37^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator and observed daily for 7 days for a cytopathic effect, and neutralization titers were calculated.

### Protective efficacy using recombinant fiber-1 protein against virulent FAdV-4 isolated in Japan

First, we inoculated four 6-week-old chickens with  $10^6$   $\text{TCID}_{50}$  of JP/LVP-1/96 in a volume of 0.1 mL, and none of the chickens died after the inoculation. Thereafter, we evaluated the efficacy of the recombinant fiber-1 vaccine by studying the histopathology and biochemistry of the vaccinated chickens.

The efficacy of the recombinant fiber-1 vaccine was evaluated as follows: Two-week-old SPF chickens ( $n = 30$ ) were randomly and equally divided into three groups. Group A was immunized with 100  $\mu\text{g}$  of recombinant fiber-1 protein in 100  $\mu\text{L}$  of PBS with incomplete Freund's adjuvant and boosted with the same preparation 14 days after the first immunization. As unvaccinated controls, chickens in groups B and C were injected with PBS. Chickens in groups A and B were challenged intramuscularly with  $10^6$   $\text{TCID}_{50}$  of JP/LVP-1/96 in 100  $\mu\text{L}$  of cell culture medium 28 days after the

first immunization. Chickens in group C remained unchallenged and served as a negative control. Four days after the virus challenge, all chickens were euthanized, their organs were collected, and their spleens were weighed.

### Histopathological analysis

Tissue samples of the liver, spleen, pancreas, and duodenum were fixed in 10% neutral-buffered formalin. Samples were embedded in paraffin, sectioned at 3  $\mu\text{m}$ , and stained with hematoxylin and eosin. Immunohistochemical analysis was performed to detect fowl adenoviral antigens. Enzyme digestion was performed for antigen retrieval by applying 0.1% actinase E solution (Kaken Pharmaceutical Corp., Tokyo, Japan) for 5 min at 35°C. A rabbit hyperimmune serum against FAdV-4 strain KR5 (diluted 1:12,000) was used as the primary antibody as described previously [21] and applied overnight at 4°C. A Histofine Simple Stain MAX PO (R) Kit (Nichirei Inc., Tokyo, Japan) was used as the secondary antibody. 3'-3-Diaminobenzidine tetrahydrochloride was used as the chromogen. All slides were counterstained with Mayer's hematoxylin. Archived samples of normal chickens and FAdV-4-infected chickens were included as controls for immunohistochemistry.

### Biochemical analysis

Prior to euthanasia, blood was collected from the jugular vein of the chickens. Serum was separated, and the total protein (TP) and albumin concentrations were determined using an automatic biochemical analyzer (HITACHI 7020; HITACHI, Tokyo Japan) and commercial assay kits for each of them (FUJIFILM Wako Pure Chemical, Tokyo, Japan).

### Distribution of the virus in organs determined using real-time PCR

Samples of the liver, spleen, kidney, and intestinal tract, including feces, were homogenized in Dulbecco's modified Eagle medium supplemented with 1% fetal calf serum. Viral DNA was purified from a 10% tissue homogenate using a QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany) as per the manufacturer's instructions. The copy numbers of viral genomes in various tissues were estimated using quantitative real-time PCR as described previously [22]. Real-time PCR was performed using TB Green<sup>TM</sup> Premix Ex Taq<sup>TM</sup> II (Tli RNaseH Plus) (Takara) with the primers 5'-ATGGCTCAGATGGCCAAGG-3' and 5'-AGCGCCTGGGTCAAACCGA-3'. The PCR conditions were as follows: 98°C for 2 min, followed by 40 cycles of PCR at 98°C for 10 s, 60°C for 10 s, and 68°C for 30 s, and finally, a dissociation stage was added to generate a melting curve (98°C for 15 s, 60°C for 1 min) to verify the

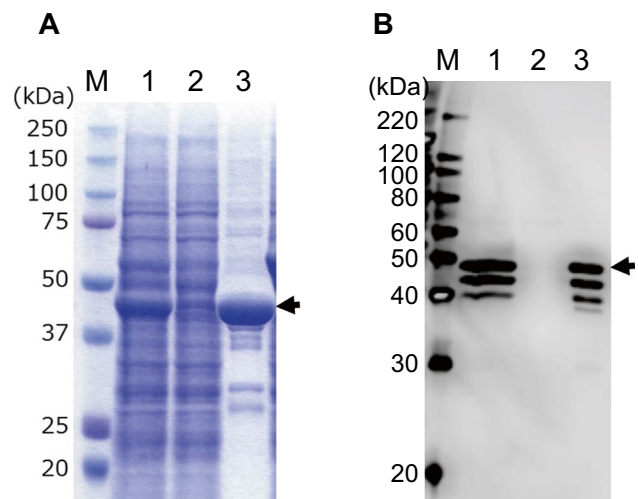
specificity of the PCR amplification. A 164-bp fragment with  $10^1$  to  $10^8$  copies was used to generate a standard curve.

**Statistical analysis** Data were analyzed using Student's *t*-test. A *p*-value < 0.05 was considered statistically significant.

## Results

### Expression of recombinant FAdV-4 fiber-1 protein

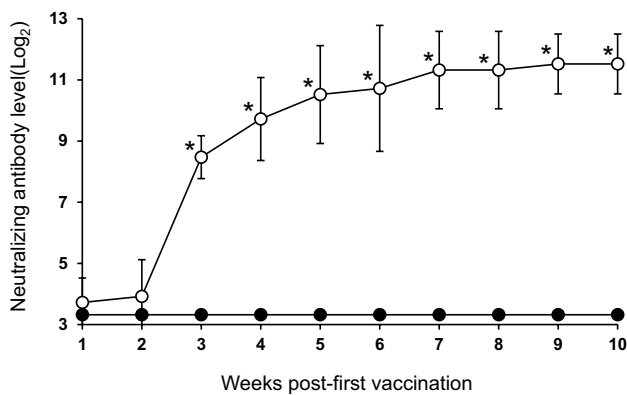
Recombinant fiber-1 with a His-tag (rFiber-1) was detected by SDS-PAGE in the sonicated supernatant of a recombinant-baculovirus-infected Sf21AE cell suspension. A band of approximately 45 kDa was observed, which was consistent with the expected molecular mass of the protein (Fig. 1A, lane 1). rFiber-1 was adsorbed to a  $\text{Co}^{2+}$  column, and purified rFiber-1 was eluted with 150 mM imidazole (Fig. 1A, lanes 2 and 3). rFiber-1 expression was confirmed by western blot analysis with anti-His monoclonal antibodies. A major band consistent with the expected molecular weight of rFiber-1 and minor bands with lower molecular weights were detected (Fig. 1B, lanes 1 and 3). Purified rFiber-1 was obtained at an approximate yield of  $1.8 \text{ mg}/1 \times 10^8$  cells.



**Fig. 1** Expression and purification of rFiber-1. rFiber-1 was purified on a  $\text{Co}^{2+}$  affinity column. Fractions were separated using SDS-PAGE and visualized using Coomassie brilliant blue staining (A) or anti-His antibody staining (B). M, molecular mass markers; lane 1, cell disruption supernatant of recombinant-baculovirus-infected insect cells; lane 2, flow-through fraction of  $\text{Co}^{2+}$  column; lane 3, fraction eluted with 150 mM imidazole. Molecular mass markers are shown on the left; the position of rFiber-1 is indicated by an arrow on the right.

## Persistence of neutralizing antibodies in immunized chickens

To evaluate the immunogenicity of rFiber-1, FAdV neutralizing antibodies were detected in the serum of rFiber-1-vaccinated chickens. Neutralizing antibody levels did not increase after the first rFiber-1 immunization; however, the levels increased when chickens received a booster immunization 2 weeks later. The levels of neutralizing antibodies increased statistically significantly one week after the second immunization, and statistically significant titers were maintained until 10 weeks after the second immunization (Fig. 2). Neutralizing antibodies were not detected in the control group at any time point. All chickens remained healthy and did not exhibit any clinical signs during vaccination (data not shown).



**Fig. 2** Time course for the development of neutralizing antibodies to JP/LVP-1/96 in immunized chickens. Immunized chickens (open circles) received 100 µg of purified rFiber-1 at 2-week intervals; control chicken (closed circles) received PBS. Neutralizing antibody levels are presented as the mean ± standard deviation (SD). Data were analyzed using Student's *t*-test. The *p*-value was always less than 0.05. Asterisks (\*) indicate that the values were significantly different from those of the control group (*p* < 0.05).

## Protective efficacy of rFiber-1 protein

Neutralizing antibodies were also induced in all group A chickens after the second immunization with rFiber-1 (the average titer was about log<sub>2</sub>7.8, data not shown). To evaluate the protective efficacy of rFiber-1, chickens in groups A and B were inoculated intramuscularly with the virulent strain JP/LVP-1/96 4 weeks after the first immunization. In group A (rFiber-1 vaccinated group) and group C (negative control group), none of the chickens exhibited any clinical signs throughout the experiment, whereas four of the 10 chickens in group B (challenge control group) exhibited clinical signs such as depression. The appearance of the liver and spleen in group A was comparable to that in group C. In contrast, all of the chickens in group B showed yellowish colored liver with necrotic foci (Supplementary Fig. S1A) and a swollen spleen compared with those in groups A and C (Supplementary Fig. S1B).

All birds in group B exhibited histological lesions in the liver (Supplementary Fig. S2B and Table 1). Many hepatocytes throughout the liver exhibited cytoplasmic vacuolar (fatty) degeneration. In addition, small foci of mixed inflammatory cells (lymphocytes, heterophils, and macrophages) were scattered throughout the liver of all birds in group B. Some hepatocytes intermittently exhibited adenoviral intranuclear inclusion bodies, single-cell necrosis, and mitosis. A few fibrotic thrombi were observed in sinusoids. Using immunohistochemistry, FAdV antigens were detected in the hepatocytes of all birds in group B (Supplementary Fig. S2B). In contrast, group A showed fewer lesions in the liver than group B. Most hepatocytes appeared normal with no viral inclusion body formation, although small inflammatory cell aggregates were sometimes observed in a few birds (Supplementary Fig. S2A, Table 1). The detection rate of viral antigens in group A was 10% in the liver. In the spleen and pancreas, the incidence of histological lesions in group B was slightly higher than that in group A. However, viral

**Table 1** Major histological findings and fowl adenovirus (FAdV) antigen distribution detected using immunohistochemistry

Organ	Histological findings	A	B	C
Liver	Vacuolar degeneration of hepatocytes	0/10*	8/10	0/10
	Inclusion bodies in hepatocytes	0/10	4/10	0/10
	Aggregates of heterophils and macrophages	2/10	10/10	0/10
	Increased number of lymphocytic aggregates**	4/10	10/10	0/10
	FAdV antigen	1/10	10/10	0/10
Spleen	Increased count of hemophagocytic macrophages**	0/10	4/10	0/10
	FAdV antigen	1/10	0/10	0/10
Pancreas	Increased amount of lymphoid aggregates**	0/10	2/10	0/10
	FAdV antigen	0/10	1/10	0/10
Duodenum	FAdV antigen	0/10	2/10	0/10

\*number positive/total chickens

\*\*in comparison with group C



antigen positivity in the spleen and pancreas was low in both groups (0–10%). In the duodenum, no histological lesions were observed in any groups, but viral antigens in mucosal epithelial cells were detected in group B (20%). Group C exhibited no histological lesions or viral antigens in any of the organs examined.

Figure 3 shows the serum levels of TP and albumin after virus challenge. TP (Fig. 3A) and albumin (Fig. 3B) levels were similar in groups A and C; however, the levels in group B were significantly lower than those in groups A and C.

Viral copy numbers were determined using real-time PCR 4 days postinfection (Fig. 4). The detection limit in this reaction was 10 copies/10  $\mu$ g of tissue. In the liver, the viral copy number in group A was significantly lower than that in group B, and the viral genome content was below the detection limit in 40% of the group A chickens. The viral genome content in group A was also below the limit of detection in the kidney, spleen, and intestinal tract, including feces. In contrast, the virus copy number in group B was significantly higher ( $p < 0.01$  for the liver, kidney, spleen, and intestinal tract, including feces) than that in the control group (group C). In group C, the viral genome content was below the detection limit in all organs (data not shown).

## Discussion

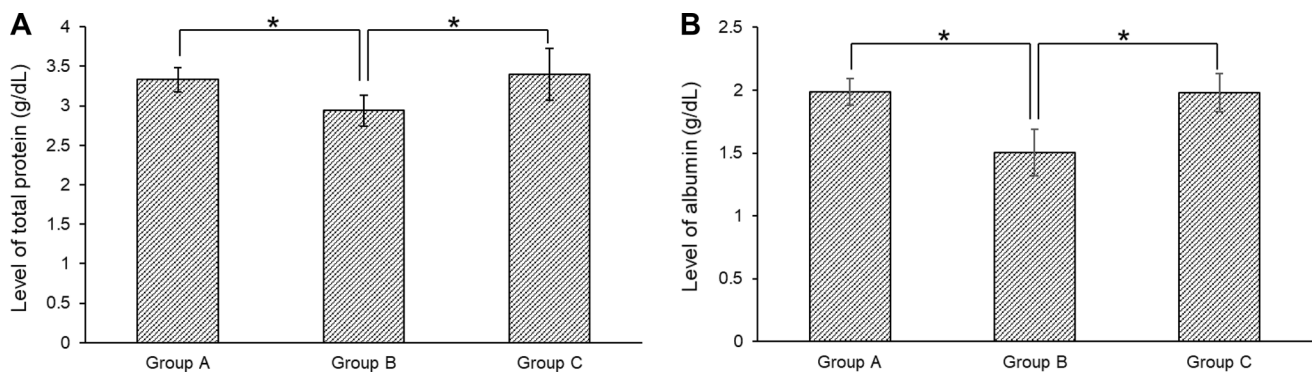
We successfully produced an immunogenic rFiber-1 protein from a Japanese FAdV-4 strain, using a baculovirus expression system, to develop a subunit vaccine for chickens. Chickens immunized with rFiber-1 were considerably protected against infection with a virulent Japanese FAdV-4 strain.

Subunit vaccines are a promising alternative for viral disease control, since they are safe, effective, and easy to produce. In particular, the baculovirus expression system allows a high level of expression of recombinant proteins; hence, it

has potential applications in the development of subunit vaccines for viral diseases [23–25]. Schachner et al. expressed recombinant FAdV-4 fiber proteins intracellularly using a baculovirus expression system [14]. In the present study, rFiber-1 was also expressed intracellularly, and it was purified after disrupting the recombinant baculovirus-infected cells using sonication. The minor bands detected by CBB staining and western blotting with molecular weights lower than that expected for rFiber-1 were presumably degradation products of rFiber-1.

A single immunization of 10-day-old chickens with fiber-2 – another fiber protein of FAdV-4 – was shown previously to induce humoral and cellular immunity that was protective against the virus, but it did not induce neutralizing antibodies [15]. Chickens immunized twice with a chimeric protein derived from fiber-2 of FAdV-4 and the fiber protein of FAdV serotype 11 at 1 and 7 days of age were also protected from challenge with FAdV of the homologous serotype and had high antibody titers, but no neutralizing activity was observed [26]. Moreover, immunization of 1-day-old chickens with recombinant fiber-1 or fiber-2 of FAdV-4 did not induce neutralizing antibodies, but a protective effect was nevertheless observed [14]. In the present study, immunization with rFiber-1 induced high levels of neutralizing antibodies in chickens. However, the first immunization of 2-week-old chickens did not induce neutralizing antibodies, and neutralizing antibodies were only observed after the second immunization in 4-week-old chickens. Therefore, the age of the chickens being immunized with fiber proteins of FAdV-4 might have an effect on the neutralizing antibody production.

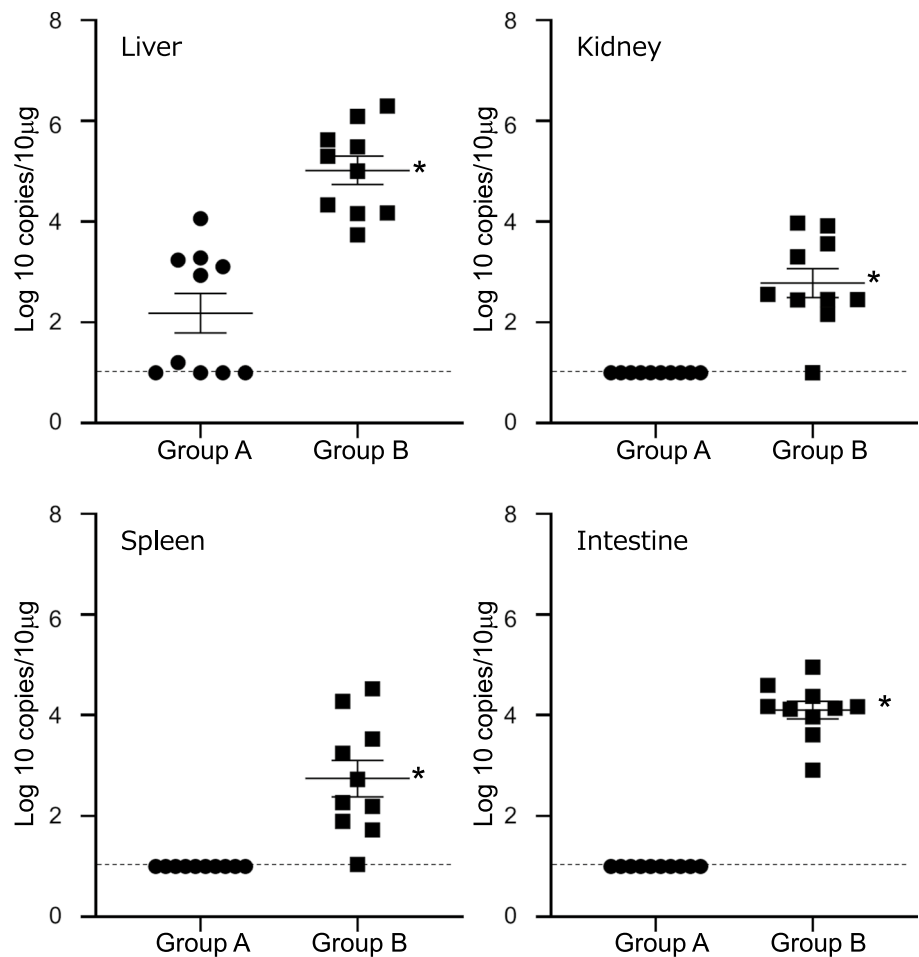
In the case of another fowl adenovirus, FAdV-E, immunization of adult chickens with fiber protein and virus-like particles of FAdV serotype 8b induced high neutralizing antibody titers [27]. De Luca et al. successfully induced serotype-homologous neutralizing antibodies in 1-day-old chickens immunized with a fiber protein derived from



**Fig. 3** Biochemical analysis after JP/LVP-1/96 infection. The levels of total protein (A) and albumin (B) in serum were measured at 4 days postinfection. Data are expressed as the mean  $\pm$  SD. Data were

analyzed using Student's *t*-test. Asterisks (\*) indicate viral copy numbers that were significantly different between the groups ( $p < 0.05$ ).

**Fig. 4** Viral DNA copy number in different tissues of immunized chickens at 4 days postinfection. Data are presented as the mean  $\pm$  SD. Data were analyzed using Student's *t*-test. Asterisks (\*) indicate significant differences between the groups ( $p < 0.05$ ). Dotted lines represent the detection limit of real-time PCR.



FAdV-serotype 8a [28]. Since immunity to a chimeric fiber protein with amino acids 1-441 derived from FAdV-8b also induced neutralizing antibodies [29], it is likely that FAdV-E has a neutralizing epitope that is highly immunogenic, even in young chickens.

Infection with highly pathogenic FAdV-4 is associated with a high mortality rate in chickens up to 6 weeks of age, and chickens show age-related resistance to FAdV [2]. Titers of neutralizing antibodies induced in parent chickens are maintained and protect chicks from FAdV as maternal antibodies [27]. In this study, high levels of neutralizing antibodies were induced in chickens after the second immunization with rFiber-1, and antibody titers were maintained over 10 weeks. Therefore, rFiber-1 may also be an effective immunogen for older chickens for the purpose of conferring maternal antibodies.

In the present study, we evaluated the protective efficacy of rFiber-1 using pathological, biochemical, and virological assays conducted four days after virus inoculation. In these experiments, there were no significant differences between the rFiber-1-vaccinated group and the negative control group. However, the challenge control group showed severe pathology due to virulent Japanese

FAdV-4 infection. The virus was not detected in any of the organs except the liver in the rFiber-1 immunization group, indicating that rFiber-1 immunization significantly suppressed the viral load in chickens. Since the virus was not detected in the intestinal tract, including feces, it can be assumed that the neutralizing antibodies detected in the serum suppressed virus shedding.

Biochemically, TP and albumin levels were significantly decreased in the challenge control group. Albumin is produced by the liver; it accounts for approximately half of the plasma TP and is associated with synthesis functions of the liver [30]. Thus, the TP and albumin levels might indicate hepatic injury. Liver damage caused by FAdV-4 infection leads to an increase in the levels of aspartate aminotransferase and lactate dehydrogenase and a decrease in the levels of glucose, TP, and albumin [31]. In this report, we show that immunization with rFiber-1 suppressed hepatocyte damage caused by FAdV-4 infection and maintained normal liver function. In chickens experimentally infected with FAdV, changes in biochemical test values correlate with disease progression, pathological lesions, and changes in viral load in the organs [32]. Therefore, biochemical indicators such as TP and albumin can serve as novel indicators of vaccine

efficacy for diseases such as that caused by FAdV infection, which manifests as liver lesions.

In conclusion, we successfully produced recombinant FAdV-4 fiber 1 protein, which induced the production of high levels of neutralizing antibodies in chickens, which were maintained for more than 10 weeks. Therefore, this recombinant protein can function as a novel subunit vaccine against FAdV-4 infection.

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1007/s00705-023-05709-6>.

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**Author contributions** SW designed and performed the study, analyzed the data, and wrote the manuscript. YY and AK performed pathological experiments and analysis. HI and TT performed the experiments. MM designed the study, analyzed the data, and wrote the manuscript. The manuscript was read and approved by all authors.

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**Data availability** The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Declarations

**Conflict of interest** The authors have no conflicts of interest to declare with regard to this work.

**Ethical approval** Animal experiments were carried out in accordance with the regulations and guidelines of the Animal Ethics Committee of the National Institute of Animal Health (approval number 19-057, 20-021, 21-012).

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