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Recombinant FAdV-4 fiber-2 protein protects chickens against hepatitis–hydropericardium syndrome (HHS)

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ABSTRACT

Virulent fowl adenovirus (FAdV) serotype 4 strains are the etiological agents of hepatitis–hydropericardium syndrome (HHS), a highly infectious disease in chickens with severe economic impact.

In the present study, three different FAdV-4 derived capsid proteins, fiber-1, fiber-2, and hexon loop-1, were expressed in a baculovirus system and tested for their capacity to induce protection in chickens. Purified recombinant proteins were administered to day-old specific pathogen-free (SPF) chickens allocated in three separate groups and challenged with virulent FAdV-4 at 21 days of life. Two additional groups served as controls, a challenge control group with mock-vaccinated but infected birds and a negative control group with PBS injection substituting both vaccination and challenge.

The fiber-2 vaccinated group displayed high resistance against the adverse effects of the challenge with only one dead bird out of 28, as compared to the challenge control group where the infection caused 78% mortality. A moderate protective effect resulting in 38% mortality was observed for fiber-1, whereas the hexon loop-1 vaccinated group was not effectively protected as manifested by 73% mortality.

While a fiber-2 specific ELISA showed a gradual antibody increase after immunization of birds with the homologous protein, a commercial ELISA did not detect vaccination-induced antibodies in any of the groups but displayed a difference in challenge virus-directed response in protected and non-protected birds. Although immunoblotting confirmed the presence of specific antibodies in all vaccinated groups, the anti-protein sera did not exhibit neutralizing activity.

Fecal excretion of challenge virus DNA was detected with a real-time PCR in the majority of tested birds until termination of the study independent of protection, indicating the prevention of clinical symptoms, but not infection, by vaccination.

In conclusion, recombinant fiber-2 was identified as a protective immunogen and is proposed as an attractive candidate for a subunit vaccine to prevent hepatitis–hydropericardium syndrome in chickens.

1. Introduction

Hepatitis–hydropericardium syndrome (HHS) is an infectious disease of chickens, characterized by high mortality and severe economic losses, mainly in broiler flocks. After first reports of the disease in 1987 from Pakistan [1], outbreaks have been documented mainly in Asia, and Central and South America. Initial assumptions pointed toward the involvement of an unknown agent in addition to an adenovirus [2] which was later revised by reproducing the disease in specific pathogen-free birds following oral infection with virulent *Fowl adenovirus C* (FAdV-C) serotype 4 strains [3].

Fowl adenoviruses are members of the family *Adenoviridae* and genus *Aviadenovirus*. Five species (FAdV-A to FAdV-E) and 12 serotypes (FAdV-1 to 8a and 8b to 11), identified by cross-neutralization test, have so far been recognized [4,5].

Adenoviruses are non-enveloped particles with a double-stranded DNA genome and a diameter of 70–90 nm.

The major structural adenovirus proteins are hexon and penton, constituting an icosahedral capsid of 252 subunits (capsomers), with hexons forming the facets and pentons capping the vertices of the icosahedron. The penton base anchors the antenna-like fiber protein, whose distal head domain, termed knob, harbors

Abbreviations: CEL, chicken-embryo liver; CPE, cytopathic effect; ELISA, enzyme-linked immunosorbent assay; FAdV, fowl adenovirus; Fib-1, fiber-1; Fib-2, fiber-2; h, hour(s); Hex L1, hexon loop-1; HHS, hepatitis–hydropericardium syndrome; min, minute(s); MOI, multiplicity of infection; OD, optical density; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SN(T), serum neutralization (test); SPF, specific pathogen-free; $TCID_{50}$, 50% tissue culture infective dose.

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the receptor-binding site and is thus essential for initiating virus attachment to the host cell [6,7].

The FAdV capsid is characterized by a morphological peculiarity of two fiber proteins associated with each penton base [8], whereas mammalian adenoviruses feature only one fiber per vertex. Although the existence of dual fibers is common to all FAdVs, two fibers distinct in sequence and length, each encoded by a separate gene, are a specific feature of FAdV-1 (FAdV-A) [9,10]. On basis of the novel finding of two separate fiber-encoding genes in an FAdV-4 isolate [11], analysis of further sequence data generated recently from strains representing FAdV-B to -E provides evidence that this reflects, among all FAdV species with equally long fiber proteins, a feature exclusively attributed to members of FAdV-C [12].

Characterization of the knob as receptor-binding domain has established the fiber molecule as a critical factor associated with infection properties of adenoviruses, such as alterations in tissue tropism [7] and virulence [13]. However, many questions are still open in regard to the individual functionality of the dual fibers present in FAdVs, particularly in the context of interaction with host cell receptors.

As major surface-exposed capsid structures, fiber and hexon are key mediators of antigenicity in adenoviruses and carriers of a panoply of epitopes of subgroup- and type-specificity [14]. It has also been shown that hexon- and fiber-specific antibodies account for most of the neutralizing activity in mammalian humoral response against adenovirus [15–17]. Recently, *in vitro* trials demonstrated different degrees of neutralizing capacity of antibodies raised against recombinant hexon and fiber proteins of the egg-drop syndrome virus (DAdV-1) [18].

Owing to their antigenic properties, adenovirus capsid structures have been proposed as potential candidates for the design of epitope-based vaccines [19]. In the present study, fiber-1, fiber-2 and the loop-1 region of hexon of an FAdV-4 reference strain, were recombinantly expressed in a baculovirus system. In a vaccination trial, the efficacy of these capsid components to induce protective immunity in chickens was assessed by challenging birds with virulent FAdV. For the first time, FAdV capsid proteins were assessed as subunit vaccine candidates for the control of HHS by individual testing of each of the two FAdV fibers in an *in vivo* experiment.

2. Materials and methods

2.1. Virus propagation and DNA extraction

Virus strains KR5 and AG234 [3,20,21] were used as template for cloning and as challenge strain, respectively, and were propagated on primary chicken-embryo liver (CEL) cells according to a protocol by Schat and Sellers [22]. Viral titer was determined by endpoint titration [23]. DNA extraction from cell culture supernatant was carried out with the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany).

2.2. Cloning and protein expression

The entire encoding regions for fiber-1 (Fib-1) and fiber-2 (Fib-2) and the hexon loop-1 (Hex L1) were amplified from FAdV-4 reference strain KR5. Primers were designed on the basis of the complete genomic KR5 sequence (GenBank accession number HE608152) and contained the 5′-terminal restriction sites *BamHI*/*StuI* (Fib-1), *StuI*/*XbaI* (Fib-2) and *NcoI*/*XhoI* (Hex L1) for cloning the amplicons into the pFastBac transfer vector (Invitrogen, Vienna, Austria) (Table 1). Following transfection of *Spodoptera frugiperda* Sf9 cells (Invitrogen, Vienna, Austria) with recombinant baculovirus DNA isolated from transformed *Escherichia coli* DH10Bac (Invitrogen, Vienna, Austria), the proteins of interest were expressed as His-tag fusion proteins according to the manufacturer's protocol.

2.3. Expression and purification of recombinant proteins

Recombinant proteins were harvested from baculovirus infected Sf9 suspension cultures. Briefly, the cells were pelleted and disrupted by resuspension in lysis buffer (20 mM sodium phosphate, 0.5 M NaCl, 20–40 mM imidazole, 0.2 mg/ml lysozyme, 20 μg/ml DNAse, 1 mM $MgCl_2$, 1 mM PMSF and proteinase inhibitors) and sonication. The clarified cell lysate was subjected to purification on affinity chromatography columns (His GraviTrap, GE Healthcare, Freiburg, Germany). For detailed protocol see S1. Prior to *in vivo* administration, the recombinant proteins were transferred into sterile PBS (Gibco/Invitrogen, Vienna, Austria) using Slide-A-Lyzer 7K Dialysis Cassettes (Thermo Scientific, Vienna, Austria). The concentration of the proteins of interest was determined by Bradford assay (Thermo Scientific, Vienna, Austria).

2.4. Animal experiment

A total of 112 specific pathogen-free (SPF) chickens (VALO, Lohmann Tierzucht GmbH, Cuxhaven, Germany) were individually identified with subcutaneous tags (Swiftack, Heartland Animal Health Inc., Fair Play, USA) and divided into five groups, housed separately in isolator units (Montair Andersen bv, HM 1500, Sevenum, Netherlands). At first day of life, each animal was administered a 500 μl intramuscular injection, containing 50 μg recombinant protein, with group I ($n = 26$) receiving Fib-1, group II ($n = 28$) receiving Fib-2 and group III ($n = 26$) receiving Hex L1, mixed 1:1 with GERBU Adjuvant LQ no. 3000 (GERBU Biotechnik GmbH, Heidelberg, Germany).

Birds of group IV ($n = 23$) were injected purified non-infected insect cell material mixed 1:1 with adjuvant to serve as a challenge control. Birds of group V ($n = 9$) were treated as a negative control and were injected sterile PBS.

At day 21 of life, animals of groups I to IV were intramuscularly challenged with 200 μl of 10^7 50% tissue culture infective dose ($TCID_{50}$)/ml of the virulent FAdV-4 virus AG234. Birds of group V were administered the same amount of sterile PBS.

Upon challenge, the birds were monitored daily for clinical signs. Necropsy was performed on all animals that died or had to be euthanized in the course of the study. Samples taken at regular intervals included blood (collected on days 7, 11, 14, 21, 28, 35 and 42) and cloacal swabs (collected on days 21, 28 and 35) or tissue from the large intestine (taken on day 42).

All remaining birds were killed at the termination of the experiment on day 42 of life.

The trial and all of the included procedures on experimental birds were discussed and approved by the institutional ethics committee and licensed by the Austrian government (license number BMWF-68.205/0196-II/3b/2012).

2.5. Antibody response

2.5.1. Commercial FAdV enzyme-linked immunosorbent assay (ELISA)

Commercially available FAdV Group 1 Antibody Test Kit was obtained from BioChek (Reeuwijk, Holland) to determine antibody levels in sera of each group before (day 21) and after challenge (days 28, 35 and 42).