Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergikafkas.org
E-ISSN: 1309-2251

Kafkas Univ Vet Fak Derg
31 (3): 293-321, 2025
DOI: 10.9775/kvfd.2025.33751

Review Article

Comprehensive Review of Fowl and Duck Adenovirus Vaccines Development: Innovations, Challenges, and Future Directions

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Abstract

Fowl adenovirus (FAdV) and Duck adenovirus are key poultry pathogens, causing inclusion body hepatitis, hydropericardium syndrome, Egg Drop Syndrome (EDS), and sudden mortality in broilers, layers, and ducks. These pathogens contribute significantly to economic losses in the global poultry industry. Consequently, measures such as vaccine development to control and prevent these agents have been extensively researched, with recent advancements showing promise. This review discusses recent advancements in vaccines for avian adenovirus species, challenges faced in studies, and future directions for developing effective vaccines against these viruses. Our study highlights that research has focused on 2nd (subunit) and 3rd (recombinant viral vector) generation vaccines, which combine multiple immunogenic proteins for single-shot protection against various avian diseases. Studies show that capsid proteins, particularly fiber, provide the highest protection rates, with reduced viral shedding and clinical signs in poultry. Significant discrepancies exist among studies evaluating vaccines for poultry due to variations in bird type, age, challenge strains, vaccine strains, dosage, administration frequency, small sample sizes, and unexamined immune responses or pathogenic mechanisms. These challenges hinder optimal vaccine identification, as many fail to protect chickens fully. Future studies should focus on real-life testing, FAdV infection mechanisms, and passive immunity transfer to progeny post-immunization.

Keywords: Duck adenovirus (DAdV); Fowl adenovirus (FAdV); immunity; poultry; vaccine

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How to cite this article?

Afshar MF, Mohamed Sohaimi N, Bejo MH, Abd Rahaman NY, Mazlan M, Mat Isa N: Comprehensive review of fowl and duck adenovirus vaccines development: Innovations, challenges, and future directions. *Kafkas Univ Vet Fak Derg*, 31 (3): 293-321, 2025.
DOI: 10.9775/kvfd.2025.33751

Article ID: KVFD-2025-33751
Received: 28.01.2025
Accepted: 04.06.2025
Published Online: 13.06.2025

Introduction

The Fowl Adenovirus (FAdV) belongs to the genus Aviadenovirus of the Adenoviridae family. FAdV is a non-enveloped DNA virus with icosahedral symmetry. Fowl adenovirus (FAdV) is a non-enveloped, double-stranded DNA virus composed of three major structural proteins: hexon, penton, and fiber [1]. Among 12 serotypes of fowl adenovirus (FAdV), FAdV-1, FAdV-4, and FAdV-10 both carry two fiber genes (i.e., fiber-1 and fiber-2), whereas other serotypes have only one [2].

High amino acid variability in the fiber protein, particularly in the head domain or knob region, leads to binding with different receptors [3]. The knob region of the fiber protein contains a significant portion of the antigenic site across all serotypes and includes a type-specific epitope for antibody neutralization [1,4].

Fowl adenoviruses (FAdVs) are classified into five species (A-E) and 12 serotypes. Serotypes 2, 11, 8a, and 8b are linked to inclusion body hepatitis, while serotype 4 is primarily associated with Hydropericardium syndrome. Species classifications follow ICTV nomenclature based on serotype groupings [5,6].

Hydropericardium syndrome (HPS), also known as hepatitis hydropericardium syndrome (HHS), affects chickens and was first reported in Pakistan 35 years ago [7]. Caused by virulent FAdV-4, HPS leads to amber-colored

fluid accumulation in the pericardial sac and an enlarged liver with hemorrhages or necrosis [8]. HPS outbreaks in Asia and Latin America cause major economic losses, including up to 80% mortality, reduced productivity, and the need for antibiotics due to adenovirus-induced immunosuppression [6,9-13].

Over the past two decades, the number of IBH outbreaks has risen across various geographic regions, highlighting the global spread of the disease. IBH affects broilers up to five weeks of age in the field, though sporadic cases have also been observed in layers and broiler breeders. Mortality during IBH outbreaks typically peaks within 3-4 days, reaching up to 10% and, in some instances, as high as 30% [6,14].

Vaccination is the most effective method for control and prevention of the disease, either by horizontal or vertical transmission in poultry farms [1,6,15]. Fowl adenovirus (FAdV) significantly impacts poultry due to mortality and treatment costs. Despite vaccines, poor cross-protection, incomplete efficacy, and emerging strains demand improved solutions. Technological advancements, including recombinant and vector-based platforms, offer innovation opportunities. A global approach is essential to address disparities in vaccine development, distribution, and accessibility, guiding future strategies.

First-Generation Vaccines (Conventional Vaccines)

1. Live Attenuated Vaccines

Fowl Adenovirus Species A

Adenoviral gizzard erosion (AGE), caused by FAdV-1, has been linked to significant economic losses in broiler flocks due to growth retardation and reduced slaughter weight [15]. AGE has also been reported in broilers infected with FAdV-8a and -8b [16,17]. Recent outbreaks in pullets and layers, especially in cages or alternative systems, have led to increased mortality and decreased egg production or weight [18-21]. These outbreaks in layer-type chickens are attributed to FAdV-1 infections, confirmed by virus detection and experimental reproduction in SPF birds [18,20,21].

Recently, the development of efficacious protection against the disease due to live vaccination with an apathogenic FAdV-1 was demonstrated in broilers [22]. However, the recent increase of reported AGE cases in layers and documented economic losses in natural outbreaks indicate the need for an efficacious protection strategy in older birds. Therefore, a study by Grafl et al.[18] produced a live-attenuated vaccine against AGE.

The study demonstrated that a live vaccine prevents symptoms and gross pathological changes in the gizzards. Additionally, no negative impacts on the development of the reproductive tract were observed in pullets and layers at 20 weeks of age. In vaccinated groups with single dose and double dose, homologous antibodies were detectable starting one week post-vaccination, with peak titers averaging 10.4±2.1 $\log_2$ at 0 days post-challenge (DPC) and 9.7±2.5 $\log_2$ at 7 DPC, indicating robust immune responses *(Table 1)* [18].

Fowl Adenovirus Species C

After identifying Fiber-2 as a critical factor in FAdV-4 pathogenicity, several studies explored the development of Fiber-2 recombinant vaccines [60,80,81]. Concerns over FAdV-4 vaccine efficacy and antibody detection led to development a live-attenuated vaccine. Serial passages of the virulent FAdV-4 KNU14016 strain in LMH cells reduced pathogenicity, evidenced by delayed and absent cytopathic effects after the 20th and 100th passages. Genetic analysis revealed a C-base insertion at 39,197 bp and a 26-bp sequence shortening in LMH80, supporting vaccine attenuation and improved delivery methods. Chickens injected with LMH10 had 89% mortality within five days and high viral shedding. LMH80 showed delayed mortality (starting at day 9) and minimal shedding. Different administration routes for LMH80 provided protection, with oral and intramuscular groups showing no mortality *(Table 1)* [40].

Recent research suggests that local immune responses triggered by vaccination play a crucial role rather than neutralizing antibodies [44]. However, the vaccine-induced cellular immune response and the involvement of various immune genes have not been fully explored. Thus, another study aimed to enhance understanding of the immune response elicited by live-attenuated viruses and the role of attenuated vaccines in immune defense during avian viral infections [69].

Most immunized animals were observed to survive and remain active, whereas LMH10 infection proved fatal in the absence of LMH80 pre-treatment. The immunization with LMH80 was shown to influence viral clearance, as the virus was detected less frequently among immunized animals, which corresponded with their higher survival rate *(Table 1)*. Moreover, the residual virus levels in the immunized group were determined to be lower than those in the control group.

LMH80 immunization boosted CD44+ expressing CD8+ T cells regardless of FAdV-4 infection. Helper T cells expanded significantly only after both immunization and viral challenge. Total T (CD3+) and γδ-T cell populations remained unchanged. Monocytes expressing MHCII and secreting cytokines increased upon FAdV-4 exposure, despite no pre-immunization monocyte expansion.

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
1	India	144-day-old Cobb broiler chicks	HHS (hepatitis-hydropericardium disease), IBH (Inclusion body hepatitis)	FAdV4 and FAdV11	Not mentioned	Subcutaneous (SC); 10^7 $TCID_{50}$ of FAdV serotype 4 and 11	Against FAdV serotype 4 and 11 was 100%	[5]
2	Austria	20-week-old specific-pathogen-free (SPF) layer-type chickens.	AGE (Adenoviral gizzard erosion)	FAdV1,	Live- attenuated	Oral; 0.5 mL of an apathogenic FAdV-1 (CELO strain)	Not mentioned 100% as no chicken died or revealed clinical signs.	[18]
3	Japan	2 weeks old SPF chickens	Hydropericardium syndrome (HPS)	FAdV-4	Recombinant subunit	SC; 100 µg of recombinant fiber-1 protein in 100 µL of PBS with incomplete Freund's adjuvant and boosted with the same preparation 14 days after the first immunization	Not mentioned, but all chickens showed no signs and gross anatomical changes (100%).	[12]
4	Malaysia	chicken embryo liver (CEL) cells adapted	-	Serotype 8b	-	-	-	[23]
5	Malaysia	SPF chickens	IBH	FAdV8b	FAdV8b attenuated vaccine	SC and oral; $10^{6.7}$ $TCID_{50}$/mL for SC and 0.1 mL of $10^{6.7}$ $TCID_{50}$/mL for oral immunization	100% of vaccinated chickens survived and did not develop any clinical symptoms.	[1]
6	Malaysia	day-old commercial broiler chicks, Cobb500	IBH	8b	Inactivated vaccine using binary ethyleneimine (BEI)	SC; vaccine formulated with Montanide ISA 71 VG at vaccine dose $10^{11.5}$ $TCID_{50}$/mL	100% of vaccinated chickens did not die or show signs and symptoms.	[24]
7	China	2-week-old specific pathogen-free (SPF) chickens	HHS	4	Recombinant vector vaccine (live attenuated)	Intramuscular inoculated with $2.5x10^4$ $TCID_{50}$ of the indicated virus in 200 mL of culture medium	100%	[25]
8	China	32- week-old SPF chickens	HHS, IBH	Recombinant	Recombinant vector vaccine from rR188I mutant strain	Intramuscular (IM); 10^5 PFU (plaque forming unit) of the indicated viruses and monitored daily for 1 week	100%	[26]
9	Iran	Chicken embryonated eggs	Egg drop syndrome (EDS)	*Duck Atadenovirus A*	-	-	-	[27]
10	China	Leghorn male Hepatocellular cells	IBH	FAdV- 11	-	-	-	[28]
11	Pakistan	Commercial broiler chickens	HPS (hydropericardium syndrome)	FAdV serotype 4	Subunit vaccine	SC; fiber-2 protein (25 µg/mL dissolved in PBS and emulsified in Freund's complete adjuvant (FCA))	80%	[29]
12	Austria	14-day old SPF chickens	IBH	FAdV serotype 8	Recombinant vaccine	IM; 50 µg of crecFib-8b/8a with 40% (wt/vol) antigen-oil-based adjuvant phase GERBU adjuvant	100%	[30]
13	Austria	Day-old SPF chickens	IBH	FAdV-7 and 8b	Recombinant subunit vaccine	IM; For the Pb-7 study, 50 µg of Pb-7 was combined in a 1:1 ratio with GERBU adjuvant P. In the Pb-8b study, a booster vaccination was administered at 25 days of age, consisting of 100 µg of Pb-8b mixed with Freund's incomplete adjuvant	64.3%	[31]
14	China	Babcock SPF chickens aged 2 weeks	HHS, IBH	FAdV4 and FAdV8a)	Recombinant chimeric vector virus vaccine	IM; $1x10^6$ $TCID_{50}$ of the recombinant virus FAdV4-F/8a-rF2	100% against both FAdV-4 and -8a	[32]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
15	China	7 day-old chickens seronegative for FAdv-4	HHS	FAdV-4	rFiber2 subunit vaccine (which was mixed with an oil adjuvant) and Fiber2 DNA vaccine candidates	IM ($10^{6.75}$ $TCID_{50}$/0.1 mL)	rFiber2 subunit vaccine: 100% and Fiber2 DNA vaccine: 60%	[33]
16	India	7-day-old SFP chickens	IBH-HHS	Serotype 2/11	Recombinant fiber protein of FAdV-2/11	For dose-effect study: Subcutaneous; (10 µg/bird (group I), 25 µg/bird (group II), 50 µg/bird (group III), 75 µg/bird (group IV)) of recombinant fiber protein with Freund's complete adjuvant (FCA) (200 µL + 200 µL) For adjuvant effect study: 25 µg IM of (montanide (antigent to adjuvant ratio 3:7), resiquimod (1:2), and saponin (1:1)) and SC for FCA (1:1)	Dose effect study: 95% protection for all groups; adjuvant effect study: 100% protection for all groups	[34]
17	China	2-weeks old SPF chickens	HHS, swollen and hemorrhagic liver and kidney in ducks	FAdV-4 and DAdV-3	Recombinant viral vector vaccine rFAdV-4-Fiber-2/DAdV-3-RFP	The route of administration was not mentioned; $2x10^6$ $TCID_{50}$/200 µL	100%	[35]
18	Malaysia	1-day-old commercial broiler chickens	IBH	FAdV-8b	Inactivated vaccine	0.5 mL inactivated FAdV isolate of UPM08136P5B1 ($10^{7.5}$ $TCID_{50}$/mL)	100%	[36]
19	China	Two-week-old SPF chicken	HHS	FAdV-4	Recombinant vector vaccine live rHN20, rDL3-EGFP, or rHN20-EGFP, and rHN20-vvIBDV-VP2 (EGFP: Enhanced Green Fluorescent Protein) and Recombinant rHN20-vvIBDV-VP2	IM; 200 µL	100 %	[37]
20	Malaysia	Day-old broiler chickens	IBH	FAdV-8b	Attenuated	SC; 0.5 mL of UPM08136CEL20B1 $10^{6.5}$ $TCID_{50}$	100%	[38]
21	China	one-day-old SPF chickens	HHS	FAdV-4	Recombinant vector live-attenuated vaccine virus FA4-EGFP expressing EGFP-Fiber-2 fusion protein	IM 10^6 $TCID_{50}$ of the indicated virus in 200 µL of 1% culture medium	100%	[39]
22	South Korea	3-days old SPF chickens	HHS	FAdV-4	Live attenuated FAdV-4 after 80 passages (LMH80)	IM; 10^5 $TCID_{50}$	100%	[40]
23	China	6-day old specific pathogen-free (SPF) chicken eggs were	HHS	FAdV-4	Recombinant vector vaccine	yolk sac route; purified virus of $2x10^8$ vp (virus particle) in 100 µL PBS from two recombinant viruses: FAdV4-GFP, FAdV4-GX4C, and FAdV4-CX19A	0%, as all the embryos died at different time points.	[41]
24	China	6-day-old SPF chicken eggs	HHS	FadV-4	Recombinant vector vaccine XHE-CX19A, or XGAM1-CX19A	Egg yolk; $1x10^8$ Vp in 100 µL PBS	100% only for the XGAM1-CX19A virus group	[42]
25	China	21-day-old SPF chickens were	HHS and Avian influenza (AI) signs and symptoms (Sudden death, respiratory distress, swelling, discoloration, diarrhea, decreased egg production, lethargy, loss of appetite, nervous signs)	FadV-4	Triple vaccine (FAdV-4 fiber 2 as a recombinant subunit vaccine) and inactivated vaccines against NDV and AI	Subcutaneous injection with 4 µg of fiber-2 protein, $10^{8.5}EID_{50}$ of H9N2 AI SZ virus and$10^{8.2}$ EID_{50} of ND N7a virus	100%	[43]
26	Austria	One-day-old SPF chicks	IBH, HHS	FAdV-4 and FAdV-11	Chimeric recombinant subunit vaccine	0.5 mL intramuscular injection of the vaccine containing 50 µg of crecFib-4/11 homogenized in a 40% (wt/vol) antigen oil-based adjuvant	100%	[44]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
27	Austria	One-day-old SPF chicks	IBH, HHS	FAdV-4 and FAdV-11	Chimeric recombinant subunit vaccine named crecFib-4/11	IM; 50 µg of crecFib-4/11 homogenized in an oil-based adjuvant	100%	[45]
28	China	7-day-old SPF chickens	IBH, HHS	FadH-4,8b and 11	Recombinant vector trivalent vaccine Oil-Adjuvant Inactivated rFAdV-4- Fiber/8b + 11	IM; a dose of rFAdV-4-fber/8b + 11 in the oil- emulsion vaccine was 10^6 $TCID_{50}$ in 200 µL per bird	100%	[46]
29	China	SPF chickens	HHS	FAdV-4	Probiotic recombinant subunit bacteria vaccine	PO; *L. lactis* NZ9000/pTX8048, $1.0x10^{10}$CFU; *E. faecalis* MDXEF-1/ pTX8048, $5.0x10^9$ CFU; *L. lactis* NZ9000/pTX8048-Fiber2-CWA, $1.0x10^{10}$ CFU; *L. lactis* NZ9000/ pTX8048-DCpep-Fiber2-CWA, $1.0x10^{10}$CFU; *E. faecalis* MDXEF-1/ pTX8048-Fiber2-CWA, $5.0x10^9$ CFU; *E. faecalis* MDXEF-1/pTX8048-DCpep-Fiber2-CWA, $5.0x1.0x10^9$ CFU	Survival rates of 60%, 80%, 90%, and 100% in groups *L. lactis*/pTX8048-Fiber2-CWA, *L. lactis*/pTX8048-DCpep-Fiber2-CWA, *E. faecalis*/ pTX8048-Fiber2-CWA, and *E. faecalis*/ pTX8048-DCpep-Fiber2-CWA, respectively	[47]
30	Saudi Arabia	Not- applicable	IBH, HHS, AGE	All serotypes	Multi-epitope	Not- applicable	Not mentioned *in-vivo* and *in-vitro*	[48]
31	Pakistan	One-day-old broiler chickens	HHS	FAdV-4	Virus-like-particle (VLP) subunit vaccine	SC; 100 µg of recombinant HBc-fused hexon epitopes at the neck region	Up to 90% using HBc-hexon (Asp348-Phe369)	[49]
32	China	21-day-old SPF chickens	IBH	FAdV-8a	Inactivated oil emulsion vaccine has	Group A received 0.3 mL of the vaccine with a concentration of $10^{6.5}$ $TCID_{50}$ per 0.1 mL through IM injection. Groups B and C were administered 0.3 mL of the vaccine with concentrations of $10^{5.5}$ $TCID_{50}$ per 0.1 mL and $10^{4.5}$ $TCID_{50}$ per 0.1 mL, respectively	100%	[50]
33	China	2-weeks old SPF chickens	HHS	FAdV-4	Recombinant subunit vaccine	A total of 46 chickens were randomly assigned to six groups: four immunization groups (A-D), a negative control group (E), and a challenge control group (F). Groups A, B, and D received IM injections of rFH protein at doses of 2.5 µg, 5 µg, and 7.5 µg per bird, respectively. Group C was given an initial dose of 5 µg rFH protein per bird, followed by a booster of 5 µg per bird 14 days later. Groups E and F served as controls. Three weeks post-immunization, Groups A-D and F were exposed to the FAdV-4 HB1505 strain, with each bird receiving 0.2 mL (106.0 $TCID_{50}$) of the virus intramuscularly and 0.1 mL orally	100%	[51]
34	Pakistan	14-day-old broiler chickens	HHS	FAdV-4	Inactivated	IM; LD_{50} infectivity titer $1x10^{5.6}$, LD_{50} of $1x10^{4.6}$, and LD_{50} of $1x10^{3.6}$	Not mentioned	[52]
35	China	SPF chickens and Layer hens	EDS	DAdV-1	Subunit fiber vaccine	HA (haemagglutination) titer of 11 log_2 through IM route	90-100%	[53]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
36	China	Chicken	HHS	FAdV-4	Recombinant yeast expressing Fiber-2 of FAdV-4	The yeast culture mixture was prepared with feed and administered to chickens via oral immunization, followed by two booster doses at five-day intervals. Each immunization included an oral dose of $1x10^9$ CFU of ST1814G/Fiber-2 per chicken	100%	[54]
37	China	20-day-age Muscovy ducks	yellowish livers with hemorrhagic spots, kidney enlargement, and bleeding	DadV-3	Recombinant DadV-3 expressing VP1 protein (rDAdV3-VP1-188)	The rDAdV3-VP1-188 and DAdV3 were inactivated with 2% formaldehyde and mixed with Freund's adjuvant in equal volumes. The resulting mixture, along with PBS and DAdV3 at a concentration of $10^{3.00}$ $TCID_{50}$, was injected subcutaneously in the neck of the subjects every two weeks. This approach was used to assess the vaccine's immunogenicity and efficacy	100%	[55]
38	China	2-week-old specific-pathogen-free (SPF) chickens	HHS	FAdV-4	Subunit vaccine (fusion protein containing His-knob)	IM in the pectoral muscle. 150 µg of the purified His-knob-containing fusion protein mixed with a polymeric adjuvant	100% with purified His-knob	[2]
39	China	2-week-old White Leghorn SPF chicken	HHS	FAdV-4	Recombinant subunit fiber-2 vaccine	IM; 2.5 µg, 5 µg, 10 µg, 30 µg of protein per 0.3 mLof vaccine	100 % when at least 10 µg of subunit vaccine (F2-knob protein) is used	[56]
40	China	seven-day-old SPF chickens	HHS	FAdV-4	Subunit Fiber2 and penton vaccine with oil adjuvant	SC; purified recombinant proteins were emulsified in a ratio of 1:2 (v:v) with Marcol™ 52 white oil. 300 µL of 40 µg, 20 µg, 10 µg, and 5 µg of protein at one site on the nape	100%	[57]
41	China	2-week-old SPF chickens	HHS	FAdV-4	Recombinant Subunit vaccine from Fiber-2	Five groups of chickens received IM injections: Group A received 0.3 mL of vaccine with 2.0 µg of WZ-Fiber-2 protein, Group B with 2.0 µg of ON1-Fiber-2 protein, and Group C with 0.3 mL of inactivated WZ strain virus ($\geq 1x10^{6.0}$ $TCID_{50}$). Group D was given 0.3 mL of PBS-emulsified vaccine as a negative control, while Group E remained unvaccinated as a blank control	100 %	[58]
42	China	SPF) chickens aged 2 weeks	HHS	FAdV-4, H9N2 influenza virus	Recombinant vector bivalent FAdV4-HA(H9) vaccine	Chickens were injected with $1x10^5$ $TCID_{50}$ of attenuated virus in 200 µLof culture medium intramuscularly	75% against FAdV-4	[59]
43	South Korea	10 weeks-old SPF chickens	HHS	FAdV-4	Recombinant subunit yeast vaccine (inactivated yeast)	IM; The chickens were allocated into five groups, each consisting of five birds, including three groups for vaccine candidates: IBH-Fiber1-His (Fiber-1), IBH-Fiber2-His (Fiber-2), and IBH-hexonL1-His (Hexon-L1), along with a negative control group and a positive control group. The *S. cerevisiae* cells, containing their respective recombinant proteins, were inactivated by heating at 60°C for 1 h. A volume of 0.5 mL, containing 10^6 cells, was combined with incomplete Freund's adjuvant (Sigma-Aldrich, MO, USA) in a 1:1 ratio and administered into the thigh muscle	100% protection using fiber-1 and fiber-2	[60]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
44	Austria	(SPF) broiler chicks	IBH	FAdV-8a and 8b	Recombinant subunit vaccine	On the first day of life, chickens in groups I, II, and III were vaccinated intramuscularly with 50 µg of recombinant Fib-8a protein, combined in a 1:1 ratio with GERBU Adjuvant P (GERBU Biotechnik GmbH, Heidelberg, Germany). Chickens in groups IV and V (challenge controls) received a mixture of phosphate-buffered saline (PBS) and adjuvant, while group VI (negative control) received PBS alone	100% when challenged with homologous serotype	[61]
45	China	2-week-old specific SPF chickens	HHS	FAdV-4	Recombinant live-attenuated vaccine	IM; $2x10^5$ TCID50 in 200 µL of 1% culture medium	100%	[62]
46	China	SPF chicken embryonated eggs	HHS	FAdV-4	Recombinant virus	Yold sack route; purified FAdV4- GFP or FAdV4XF2- GFP of $2x10^8$ vp in 100 µL PBS	40% of embryos survived when infected with FAdV4XF2- GFP	[63]
47	China	SPF White Leghorn chickens	HHS	FAdV-4	Recombinant bacterial vector vaccine expressing hexone protein on their cell wall.	Oral; *L. lactis* NZ9000/pTX8048, $1x10^{10}$ CFU; *E. faecalis* MDXEF-1/pTX8048, $5x10^9$CFU; *L. lactis* NZ9000/pTX8048-SP-ΔHexon-CWA, $1x10^{10}$CFU; *L. lactis* NZ9000/pTX8048-SP-DC-ΔHexon-CWA, $1x10^{10}$ CFU; *E. faecalis* MDXEF-1/pTX8048-SP-ΔHexon-CWA, $5x10^9$CFU; *E. faecalis* MDXEF-1/pTX8048-SP-DC-ΔHexon-CWA, $5x10^9$CFU	90% for MDXEF-1/DC- 1Hexon-CWA	[64]
48	China	LMH cells	lethargy, drooping neck, closed eyes, huddling, decreased appetite, and respiratory distress	DAdV-3	Monoclonal antibody against fiber-2 of DAdV-3	Serial dilutions of mAb (1:50, 1:100, 1:200, 1:400, 1:800, 1:1600, and 1: 3200) were mixed with 200 TCID50 of DAdV-3, incubated for 1 h at 37°C, and then inoculated into fresh LMH cells in a 96-well plate	CPE was barely observed in infected LMH cells treated with mAb 3E7 The mAb effectively neutralized DAdV-3 at 1:50-1:400 dilutions, while mAb 3C2 (control antibody) showed no neutralizing activity	[65]
49	India	Day-old SPF chickens	IBH-HHS	FAdV-2/11	Recombinant subunit vaccine	SC; Group-1 (penton base): 100 µg; Group-2 (fibre): 100 µg; Group-3 (penton + fibre): 50 µg + 50 µg	Up to 80% protection from fiber-immunized group	[66]
50	China	7-d-old SPF chickens	HHS	FAdV-4	Subunit vaccine	IM; Subunit vaccines (Fiber1/2 knob subunit vaccine and fiber-2 subunit vaccine) were prepared by emulsifying recombinant proteins with Freund's complete adjuvant (1:1) at a protein concentration of 100 mg/mL	100% for vaccinated groups	[67]
51	China	one-day-old SPF chickens	HHS	FAdV-4	Recombinant live attenuated vaccine candidate	IM; $2.5x10^4$ $TCID_{50}$ of indicated virus in 200 µL 1% culture medium	100%	[68]
52	Korea	One-day-old SPF chickens were	HHS	FAdV-4	Live-attenuated vaccine	Chickens were injected intramuscularly with 0.1 mL of 80-times passaged FAdV-4 (LMH80)	Over 80%	[69]

Table 1. *Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)*

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
53	China	2-week-old SPF White Leghorn chickens	HHS and Newcastle disease signs and symptoms (gasping, coughing, sneezing; nervous signs such as tremors, twisted necks, paralysis, and inability to stand; digestive issues like greenish diarrhea; a drop in egg production)	LaSota strain of NDV and FAdV-4	Attenuated recombinant virus	Groups one and two of chickens were immunized intramuscularly (IM) with 100 µL (10^7 EID_{50}) of live rLaSota-fiber2 and 100 µL (10^7 EID_{50}) of inactivated rLaSota-fiber2, respectively	100%	[70]
54	China	SPF Chickens	HHS, and infectious bursal disease signs and symptoms (Ruffled feathers, depression and reluctance to move dehydration diarrhea (watery, white, or greenish) vent picking and swelling around the vent)	FAdV-4 and very virulent IBDV vvIBDV HLJ0504 strain (vvIBDV)	Recombinant- viral vector inactivated bivalent FAdV-4/IBDV vaccine	IM; 0.3 mL of vaccine (10^7 PFU) inactivated with 0.1% formaldehyde at 2 weeks of age	100% protection against IBVD and FAdV-4	[71]
55	China	2-week-old specific-pathogen-free (SPF) chickens were	HHS	FAdV-4	Recombinant inactivated vaccine	IM or SC; the virus was inactivated with 0.1% formaldehyde and mixed with oil adjuvant at a ratio 1:2; chickens were immunized with 0.3 mL (10^7 PFU) of inactivated rHN20 vaccine at 2 weeks of age	100%	[72]
56	China	day-old chickens	HHS	FaDV-4	Recombinant vaccine	100 µg of the purified MLFRPs-FAdV4:F1-P-H-F2, FAdV4:F1-P-F2-H, FAdV4:F1-H-F2-P, FAdV4:F1-F2-P-H, and FAdV4:F1-F2-H-P-were emulsified with an equal volume of Freund's complete adjuvant (FCA) and utilized as immunogens for intramuscular injection	100% for FAdV4:F1-P-F2-H with a single shot	[73]
57	China	day-old chickens	HHS and IBH	FAdV-4 and -8b	Inactivated recombinant vaccine	IM; The inactivated FA4-F8b vaccine was prepared by mixing with oil adjuvant (1:3), yielding a final dose of 10^6 $TCID_{50}$ in 0.4 mL per chicken	100% against serotypes 4 and 8b	[74]
58	Malaysia	day-old broiler chickens	IBH	FAdV-8b	Attenuated UPM11142CEL20B1 strain	Chicks in groups B, C, and D were subcutaneously inoculated on day 0 with 0.5 ml of attenuated FAdV isolate UPM11142CEL20B1 ($10^{5.8}$ $TCID_{50}$/mL), while group A remained uninoculated. On day 14 post-inoculation (pi), group C received an attenuated booster, and group D received an inactivated booster ($10^{8.3}$ $TCID_{50}$/ml) via the same route	100%	[75]

Table 1. Fowl adenovirus and duck adenovirus vaccines development in worldwide (continued)

No.	Country	The Type of Animal Studied	Syndrome	Serotype of FAdV	Type of Vaccine	Route of Administration and Dose	Protection Rate	Ref.
59	China	3-week-old SPF chickens	HHS and IBH	FAdV-4 and 8b	Inactivated recombinant rFAdV-4-fiber/8b	Fifty 3-week-old SPF chickens were divided into five groups: vaccinated with the FAdV-4 challenge, vaccinated with the FAdV-8b challenge, unvaccinated with the FAdV-4 challenge, unvaccinated, and a sham control group. The vaccinated groups received 200 µL of the inactivated rFAdV-4-fiber/8b ($2x10^5$ $TCID_{50}$) vaccine intramuscularly	100% protection against both serotypes 4 and 8b	[76]
60	China	SPF white Leghorn chickens	HHS	FAdV-4	Live recombinant viral vector rHN20 vaccine	Different routes: Two groups of chickens served as non-inoculated and challenged controls with no immunization. Three groups were immunized with 10^6 PFU of rHN20 in 200 µL DMEM/F12 via different routes (intranasal, subcutaneous, and intramuscular) at 2 weeks of age. Additionally, two groups were intramuscularly immunized with varying doses of rHN20 (10^5 or 10^4 PFU per bird) in 200 µL DMEM/F12 at 2 weeks of age	100%	[77]
61	Pakistan	Non-vaccinated broiler chickens	HHS	FAdV-4	Subunit	Seven-day-old birds were subcutaneously injected with the specified amount of freshly purified or stored protein (100 µg of the protein per dose), which was adjuvanted with Montanide™ ISA71 VG at a 1:1 (w/v) ratio of adjuvant to protein	100%	[78]
62	China	14-day postbirth (dpb) SPF chickens	HHS	FAdV-4	Recombinant subunit vaccine	The purified protein samples (0.5 mg/mL) were emulsified with a white oil adjuvant at 1:2 to create a water-in-oil vaccine preparation. Chickens in Group I and Group II were administered 50 µg of fiber2 and FliBc-fiber2-SP vaccines per chicken, respectively. Groups III and IV were injected with an equivalent volume of sterile PBS	100% protection from FliBc-fiber2-SP+FAdV-4	[79]

FAdV-4 infection caused a threefold reduction in splenic B cells, prevented by LMH80 pre-treatment. LMH80 immunization regulated T cells, macrophages, and B cells, enhanced memory T cell subsets and CD44+, CD4+, CD8+ cells, and maintained MHCII+ macrophages. Activated CD4+ and CD8+ T cell infiltration increased in the liver [69].

Fowl Adenovirus Species E

Vaccine development faces manufacturing scale-up challenges [82]. Cell culture-based production offers a more efficient alternative to traditional embryonated egg culture, enhancing vaccine availability to meet the demands of the expanding production animal population [23].

Anchorage-dependent cells require attachment surfaces for proliferation [83]. Tissue culture flasks improved contamination control but limited large-volume vaccine production. Microcarriers, particularly spherical bead-type ones like Cytodex™ 1, enable industrial-scale mammalian cell proliferation, supporting ADC growth in stirred tank bioreactors for vaccine production [84]. It is a multipurpose microcarrier that can be used to grow various cells. While culturing the influenza virus vaccine in a stirred tank bioreactor on Vero cells, Cytodex™ 1 was used and optimized to the production capacity of 6000 L [85].

For virus propagation, cells are usually needed, and they are necessary in higher volumes for vaccine production. This is because cell concentration is of significant importance in determining viral titer [86], and viral titer has control on the efficacy of vaccines, which makes the need for a high volume of cells for volume production of vaccine an ongoing requirement. Therefore, a study aimed to propagate fowl adenovirus serotype 8b (FAdV-8b) in chicken embryo liver (CEL) cells adapted to Cytodex™ 1 microcarriers using a bioreactor *(Table 1)*.

FAdV isolate (UPM08136) was successfully propagated in chicken embryo liver (CEL) cells using Cytodex™ 1 microcarriers in a stirred tank bioreactor (STB), enabling large-scale virus production for vaccine development. No molecular changes were observed in the hexon and fiber proteins. This method was used to create an attenuated vaccine against FAdV-8b [23].

In another study by the same author [38], an inactivated and attenuated vaccine was inoculated in chickens to see the difference between each and the booster dose effect *(Table 1)*.

No clinical symptoms or histopathological changes were observed in unchallenged chickens. Inoculated groups (B, C, D) had higher liver weights at 14 and 21 dpi and lower liver-to-BW ratios at 35 dpi, indicating protection. Group B had the highest antibody titers at 42 dpi (>2000 ELISA units), while Group D exceeded 4000 units at 35 dpi.

Inoculated chickens showed significantly higher CD3+, CD4+, and CD8+ T lymphocytes than controls, with elevated CD3+ cells in the liver, spleen, and thymus at multiple intervals. CD4+ and CD8+ cells increased significantly, especially in the thymus at 42 dpi. Inoculation reduced FAdV viral load and shedding while confirming attenuated isolate stability in a stirred tank bioreactor, enhancing virus production and cost-efficiency. The study provided novel insights into chickens' cell-mediated immune response to FAdV8b vaccines, showing booster doses significantly accelerate higher antibody levels by 35 dpi [38].

A study using subcutaneous FAdV vaccination showed no clinical signs or histological changes in vaccinated chickens, unlike controls with liver discoloration and splenomegaly. Vaccinated groups had higher body weights and FAdV antibody titers at 35 and 42 dpi. CD3+ and CD4+ T lymphocytes increased in vaccinated chickens, with reduced viral genome copies in the liver and cloaca, indicating lower viral shedding and better clearance [75].

2. Inactivated Vaccines

Fowl Adenovirus Species C

With the rise of viral resistance to numerous antiviral drugs, controlling viral issues becomes increasingly challenging and can lead to significant economic losses [87]. Few commercial HPS vaccines use traditional liver homogenates, potentially causing allergic reactions due to non-specific proteins. A study explored developing a cell-free vaccine to address these concerns [52].

The study showed that a 20-dose HPS-infected liver vaccine induced a higher serum anti-HPS ELISA antibody titer (1110.4) at 40 days than 25 doses (1071.9) or 30 doses. The infectivity titer of $1x10^{5.6}$/mL BLD50 produced stronger antibody responses (1052.5±18.04) than $1x10^{4.6}$/mL (772.6±133.1) and $1x10^{3.6}$/mL (588.00±61.97). The liver homogenate vaccine achieved higher antibody titers (2009.3) than primary hepatocyte culture vaccines. Oil-based tissue culture vaccines (1148.45) outperformed gel-based ones (1137.2). Findings support liver homogenate and Montanide-adjuvanted cell culture vaccines for inducing robust anti-HPS responses [52].

Fowl Adenovirus Species E

Inactivated vaccines are easier to administer and distribute globally without specialized storage, benefiting regions with limited medical resources or infrastructure [50]. Findings of the literature reveal that several inactivated vaccines have been developed. For instance, one study used Fowl adenovirus 8b (UPM08136) isolated from an IBH outbreak in Malaysia.

The study used Montanide adjuvant with an inactivated virus and compared booster and non-booster groups. No clinical signs or lesions were observed in vaccinated groups, while challenged controls showed pale livers and symptoms. Antibody titers in the non-booster group reached nearly 4000 ELISA units by 35 DPI. CD4+ T-lymphocytes in the spleen and CD8+ T-lymphocytes in the liver were significantly higher ($P<0.05$) in vaccinated groups. Viral copy numbers in the liver were markedly lower in booster and non-booster groups, demonstrating the vaccine's effectiveness in reducing viral replication and shedding [36].

Selecting the right chemical for virus inactivation is essential in vaccine development, preserving the virus's structural integrity and entry-associated domains to effectively trigger virus-neutralizing antibody responses [88]. Alkylating agents like β-propiolactone (BPL) and binary ethyleneimine (BEI), as well as gamma and ultraviolet radiation, target the viral genome while preserving neutralizing epitopes. Cross-linking agents like formaldehyde and glutaraldehyde, or denaturing methods involving pH and temperature changes, modify viral proteins, risking epitope degradation and reduced immunogenicity [89].

Among these agents, BEI stands out for its mechanism of inactivating non-enveloped viruses by targeting their RNA/DNA genome. The active component of BEI, ethylenimine, reacts explicitly with nucleic acids, leaving other viral proteins unaltered [90]. This selective interaction makes BEI a promising agent for preserving viral-neutralizing epitopes, which are critical for vaccine efficacy. Building on this understanding, a study was conducted to evaluate the inactivation of FAdV-8b using BEI. The methodology and findings of this study contribute valuable insights into the development of vaccines targeting FAdV-8b while ensuring the preservation of its immunogenic properties.

The vaccine inactivated with BEI for 32 hours was safe and immunogenic in broiler chickens. At 28 DPI, antibody titers were 1±0 for the control group, 321±189 for Group A1, and 690±484 for Group B1, with Groups A1 and B1 showing significantly higher titers ($P<0.05$). Booster groups A2 (602±367) and B2 (874±317) also had significantly higher titers ($P<0.05$) than the control. No significant difference ($P>0.05$) in antibody titers was found between booster and non-booster groups, regardless of inactivation time [24].

Commercial FAdV vaccines exist for FAdV-4 and FAdV-8b but not for FAdV-8a. An inactivated FAdV-8a vaccine using the CY21 strain (15 LMH cell passages) was tested. Group A ($10^{6.5}$ $TCID_{50}$/0.1 mL) had antibody titers exceeding 6000 ($P<0.0001$), significantly higher than the control. Group B ($10^{5.5}$ $TCID_{50}$/0.1 mL) showed titers below 2000 ($P<0.05$). Group C ($10^{4.5}$ $TCID_{50}$/0.1 mL) displayed clinical symptoms, liver lesions, and viral DNA in organs. Viral shedding was fully inhibited in Group A, partially (40%) in Group B, and not prevented in Group C. Group A demonstrated complete protection with no viral DNA in organs *(Table 1)* [50].

Second Generation Vaccines

1. Subunit Vaccine

Fowl Adenovirus Species C

Subunit vaccines are highly immunogenic and eliminate the risk of incomplete inactivation associated with whole-virus vaccines, making them a promising option for controlling HHS. Several studies have shown that subunit vaccines can effectively protect against virulent FAdV-4 challenges [91,33]. However, most current research on subunit vaccines has concentrated on the Fiber-2 protein of FAdV-4, with the protective potential of vaccines targeting the knob domains of Fiber-1 and Fiber-2 yet to be explored. Therefore, subunit vaccines from the Fiber-1/2 knob and Fiber-2 proteins of FAdV-4 was produced [67].

AST and ALT levels were significantly higher ($P<0.05$) in unvaccinated challenged chickens at 3, 5, and 7 dpc compared to controls and vaccinated groups. Necropsies revealed severe HHS lesions in unvaccinated challenged chickens, while vaccinated groups showed healthy organs. The Fiber-1/2 knob vaccine offered superior protection against FAdV-4, with no lesions, compared to minor lesions in the Fiber-2 vaccine group.

qPCR revealed significantly lower viral DNA copy numbers in the heart, liver, spleen, lungs, and kidneys of vaccinated groups compared to unvaccinated controls. The Fiber-1/2 knob vaccine group showed significantly reduced viral loads at 3 and 5 dpc ($P<0.05$) and earlier antibody detection (7 dpv) compared to 14 dpv in the Fiber-2 group. Neutralizing antibody titers in the Fiber-1/2 group increased to 4.1, 5.6, and 6.5 at 14, 21, and 28 dpv, respectively, compared to 3.3, 4.1, and 4.3 in Fiber-2. Body weight loss was also minimized with Fiber-1/2 vaccination [67].

Similarly, another study [73] explored the development of subunit vaccines by combining multiple capsid protein-derived epitopes into multilinked fusion recombinant proteins (MLFRPs), which were recombinantly expressed in *E. coli*. Unlike previous research that primarily assessed the immunogenicity of individual capsid proteins by expressing their complete amino acid sequences [33,51], this approach focused on leveraging the combined immunogenic potential of multiple epitopes within a single recombinant construct.

The study designed multiantigen epitope tandem proteins (MAETPs) from four FAdV-4 capsid proteins (hexon,

penton, fiber1, and fiber2), selecting efficient antigenic epitopes using bioinformatics tools. The epitopes were linked with GGGGS linkers, and DNA sequences encoding MAETPs were chemically synthesized and assembled into multilinked fusion recombinant proteins (MLFRPs) using T4 ligases. The resulting constructs were cloned into pET-28a vectors and expressed in *E. coli*. Five MLFRPs (FAdV4:F1-P-F2-H, FAdV4:F1-F2-P-H, FAdV4:F1-F2-H-P, FAdV4:F1-P-H-F2, FAdV4:F1-H-F2-P) were produced and evaluated for their potential as protective antigens through chicken immunization.

Microneutralization assays showed that MLFRP-immunized chicken sera effectively neutralized FAdV-4, with FAdV4:F1-P-F2-H (over 1200) and FAdV4:F1-F2-P-H (nearly 1200) eliciting the highest neutralizing antibody titers. FAdV4:F1-P-F2-H provided full protection with a single immunization, while other proteins required two doses. After one immunization, protection rates were 83.33% for FAdV4:F1-F2-P-H and the inactivated vaccine, 66.67% for FAdV4:F1-F2-H-P, and under 50% for FAdV4:F1-P-H-F2 and FAdV4:F1-H-F2-P. Hepatic lobules in the FAdV4:F1-P-F2-H group appeared intact, while other groups displayed vacuolated or blurred cells. Further studies could investigate alternative adjuvants to enhance immune responses [73].

Bioinformatics, especially immunoinformatics, aids in designing multi-epitope subunit vaccines efficiently. A study by Mugunthan et al.[48] used these techniques to create a cost-effective FAdV vaccine, activating B and T cells with sustained activity over 50 days, promoting long-term immune memory.

2. Recombinant Subunit Vaccines

Fowl Adenovirus Species A and Duck Adenovirus Serotype 1 Vaccine

Duck Atadenovirus A (DAdV-1), responsible for egg-drop syndrome '76 (EDS '76) in laying hens, belongs to the Atadenovirus genus within the Adenoviridae family, with a linear double-stranded DNA genome of 30-35 kb [92]. First identified in 1976, EDS '76 has one serotype [93]. DAdV-1 typically infects waterfowl, such as ducks and geese [94]. In laying hens, it reduces egg production and quality. Vaccination is a control measure [95,96]. DAdV-1's capsid protein contains neutralizing epitopes, aiding vaccine development [97,98]. The fiber protein, which includes the N-terminal tail, shaft, and C-terminal knob domain, is crucial for inducing virus-neutralizing antibodies and serves as a target for subunit vaccines [99-102].

Although the production of subunit vaccine from the fiber was successful and induced with the fiber protein also induced lymphocyte proliferation response, cytokine secretion, and reduced viral load in SPF chickens [103], there is no data regarding its efficacy in layer hens. Thus, a study [53] was conducted to fill this gap.

The study [53], the first to evaluate the subunit fiber vaccine of DAdV-1 in layer hens, demonstrated superior efficacy over the inactivated vaccine. HI titers at 21 and 28 dpi reached 11.1±1.0 $\log_2$ and 12.5±1.4 $\log_2$, surpassing the inactivated vaccine. Egg production rates remained at 90-100% for vaccinated hens, while unvaccinated hens dropped to 12%. A critical HI titer of at least 7 $\log_2$ was essential to prevent production losses, protecting against the virus's effects for up to 180 days post-challenge [43].

Fowl Adenovirus Species C Vaccines

Evaluation of the fiber gene of FAdV has become necessary because, through the interaction of the fiber knob with host cells, the fiber gene is responsible for tissue tropism, which is also very important in the virulence of FAdV [23]. As one of the capsid proteins of FAdV-4, Fiber2 has been identified as an efficient protective immunogen for subunit vaccine candidates [16]. In one study [12], immunization with rFiber-1 was evaluated.

The study showed that neutralizing antibody levels increased significantly one week after the second immunization and remained significant for up to 10 weeks. All chickens stayed healthy, with viral genome content in tissues below detection limits. The rFiber-1 protein provided protective efficacy, with an average antibody titer of $\log_2$ 7.8 *(Table 1)* [11].

Similarly, A subunit vaccine candidate was developed using the recombinant Fiber2 protein (the rFiber2 subunit vaccine) expressed in bacteria from the hypervirulent FAdV-4 GZ-QL strain, isolated in Guizhou province. Additionally, a DNA vaccine candidate, the Fiber2 DNA vaccine, was created using the recombinant plasmid pVAX1-Fiber2 [33].

Both vaccine candidates induced significant Fiber2-specific antibody levels (rFiber2 subunit 50μg: 24 pg/mL; 100 μg: nearly 26 pg/mL; 150μg: 22 pg/mL; Fiber2 DNA vaccine: 20-22 pg/mL). The rFiber2 subunit vaccine showed superior efficacy (80-100%) compared to the Fiber2 DNA vaccine (50-60%) and commercial inactivated vaccine (80%). Higher dosages, especially 100 μg, produced significantly higher antibody titers. Both vaccines elicited robust cellular and humoral immune responses without significant histopathological changes [33].

Building on the development of the fiber-2 recombinant subunit vaccine, a triple vaccine was formulated by combining the fiber-2 protein antigen with inactivated H9N2 AI and NDV antigens, offering a multivalent approach to protect against multiple avian pathogens [43].

No significant difference in ELISA antibody titers against FAdV-4 was observed between the triple vaccine group (19 log_2) and the monovalent vaccine group (over 19 log_2) containing 4 mg of fiber-2 protein (P>0.05), both showing higher levels than unvaccinated controls (P<0.0001). Fiber-2 did not interfere with other antigens. Immunization with fiber-2 protein induced stronger IFN-γ secretion and FAdV-4-specific cellular immunity (P<0.05). The triple vaccine provided complete protection, with no viral shedding or histopathological changes observed [43].

Expanding on recombinant fiber-based proteins in vaccine development, a chimeric fiber vaccine, crecFib-4/11, was engineered to combine epitopes from FAdV-4 and FAdV-11, highlighting another innovative approach to enhance immunogenicity.

Vaccination with crecFib-4/11 led to elevated systemic antibody levels against the vaccine antigen (over 3 OD at 27 days post-challenge), as measured by ELISA, though neutralizing antibodies against FAdV-4 were not produced. Nonetheless, vaccinated birds challenged with virulent FAdV-4 exhibited significantly reduced clinical symptoms and pathological lesions.

Vaccinated birds showed increased B lymphocytes in the liver throughout the post-challenge period, correlating with hepatic lymphoid infiltration. The vaccine primed a rapid rise of these cells in the blood, followed by their presence in the liver. Monocyte/macrophage levels increased in the blood, liver, and spleen of vaccinated+challenged and control groups, while thymus levels decreased in challenge controls. CD4+ T lymphocyte levels remained stable, except for a late decrease in the bursa fabricius in challenge controls, which vaccination prevented. Cytotoxic CD8α+ T cells were maintained across groups, with a vaccine-induced rise in target organs. Vaccination reduced viral loads in the liver, spleen, and bursa fabricius [44].

In line with the development of chimeric fiber vaccines, another study [51] explored a different strategy by focusing on a subunit vaccine using the combination of fiber-2 protein from FAdV-4 HB1505 and hexon, aiming to evaluate its immunogenic potential and efficacy through various dosage levels.

The study assessed rFH protein vaccine doses: 2.5 μg (75% survival, 6/8), 5 μg (100%, 8/8), double 5 μg (100%, 8/8), and 7.5 μg (100%, 8/8), compared to 0% (0/7) in challenge controls. Vaccinated groups maintained body weight and showed significantly higher OD ELISA titers (~2 for 2.5 μg; nearly 3 for higher doses), indicating strong humoral responses. Viral loads in vaccinated livers were significantly reduced. Truncated fiber two proteins (Gly275 to Pro479) expressed in *E. coli* improved solubility, while adding a hexon epitope (Met21 to Val55) with a G3S linker enhanced immunogenicity [51].

Expanding on the investigation of mixed subunit vaccines comprising hexon and fiber proteins to identify the optimal protective dose, the subsequent study concentrated on assessing fiber and penton proteins, individually and in combination, to optimize further dosing for adequate protection [57].

Complete protection (10/10) was achieved 21 dpi with fiber-2 doses of 20 μg/bird and 200 μg/bird, while penton base protein provided complete protection only at 200 μg/bird. At seven dpi, vaccinated chickens had significantly elevated OD450 values (P<0.001): 1.006 (Fiber-20 μg), 1.458 (Fiber-200 μg), 2.059 (Penton-20 μg), and 2.576 (Penton-200 μg). Viral loads in tissues were reduced compared to the challenge control group, though cloacal swab loads remained higher. Fiber-2 provided full protection at 10 μg/bird, with 5 μg offering 90% protection. Penton base required 200 μg for complete protection, with lower doses showing 70%-60% protection. No adverse effects on body weight gain or inflammation were observed [57].

The prokaryotic expression system was used for its high yield, low cost, and ease of management in subunit vaccine production. To address inclusion body formation during fiber-2 expression, the culture temperature was lowered to 16°C, and Rosetta (DE3) cells were used, yielding 1.5 mg/mL of soluble protein. Fiber-2 and penton base proteins were successfully expressed with strong immunogenicity. The use of Marcol™ 52 white oil as an adjuvant enabled cost-effective, scalable subunit vaccines for FAdV-4 [57].

Studies have shown that the trimeric knob domain of the EDS virus from fowl adenovirus group III, when used as a subunit vaccine, can induce hemagglutination inhibition titers and serum-neutralizing activity comparable to those of the full-length fiber protein [98,103,104]. However, the immune efficacy of the knob protein in FAdV-4 has not yet been reported. Subunit vaccines are effective for controlling FAdVs due to their safety, ease of mass antigen production, and low cost. Therefore another study focused on producing a subunit vaccine from the knob region of the FAdV-4 and a minimum dose for complete protection [46]. The study evaluated F2-knob subunit vaccine doses (2.5 μg, 5 μg, 10 μg, and 30 μg) combined with ISA 71 VG adjuvant. Antibody levels exceeded the OD 0.125 cut-off by day 14, with Groups C (10 μg) and D (30 μg) surpassing 0.4 OD and exceeding 0.8 by day 21. Groups C and D had significantly higher antibody titers than Group E (inactivated whole virus) at 14 days post-immunization (P<0.0001).

All chickens immunized with 5 μg, 10 μg, or 30 μg of F2-knob protein and the inactivated whole virus vaccine were fully protected against FAdV-4, showing no clinical symptoms. FAdV-4 virus shedding remained negative in

Groups C, D, and E. Symptoms like lethargy and green feces appeared in Groups F (challenge control) and A (2.5 μg) but not in Groups B (5 μg), C (10 μg), D (30 μg), and E. Group E had significantly higher neutralizing antibody titers than Groups A ($P<0.05$), B ($P<0.001$), C ($P<0.001$), and D ($P<0.01$) after the challenge [46].

Genome sequence alignment of all FAdV-4 strains revealed that pathogenic and non-pathogenic strains are classified into two genotypes. Recent Chinese isolates exhibit a natural 1966 bp deletion and other genomic differences compared to the classical non-pathogenic strain ON1. However, the biological properties of Fiber-2 proteins in FAdV-4 strains with varying virulence remain unexplored. In a study, the Fiber-2 proteins of the highly virulent WZ strain and the non-virulent ON1 strain of FAdV-4 were successfully expressed and purified [58].

Chickens immunized with 2 μg of WZ-Fiber-2 protein or the inactivated vaccine achieved complete protection (10/10) with no morbidity, mortality, or histopathological changes. FAdV-4 DNA shedding remained at background levels, contrasting with the ON1-Fiber-2 and PBS groups, where viral shedding persisted until death. Viral DNA levels in the WZ-Fiber-2 group were significantly lower than in the ON1-Fiber-2 group, comparable to the inactivated vaccine group. Blood titers in the WZ-Fiber-2 group rose from 0.2 OD at two weeks post-priming to over 0.4 OD at three weeks, significantly surpassing the ON1-Fiber-2 and PBS groups ($P<0.0001$) [58].

In recent years, lactic acid bacteria (LAB) have been extensively used as delivery systems for key pathogen antigens, including the circumsporozoite protein of *Plasmodium falciparum* [105], the spike protein of SARS-CoV-2 [106], and the heavy-chain antigen of *Clostridium botulinum* serotype A neurotoxin [107]. Research has demonstrated that FAdV-4 structural proteins, such as Hexon, Penton, Fiber 1, and Fiber 2, can be expressed in *Escherichia coli* and other systems to develop subunit vaccines [70,91,108]. However, the effectiveness of live recombinant LAB in delivering FAdV-4 structural proteins to protect against homologous challenges has not yet been evaluated. Building on previous research, it was hypothesized that oral immunization, capable of inducing strong mucosal and humoral immune responses, could serve as an effective strategy to prevent HPS caused by FAdV.

Chickens immunized with recombinant strains expressing 1Hexon-CWA or DC-1Hexon-CWA exhibited significantly higher Hexon-specific IgG (nearly 1.5 to over 1.5 OD, 14 days post-immunization) and sIgA levels (over 1 to over 1.5 OD) compared to control groups ($P<0.01$). DC-1Hexon-CWA strains, incorporating the DCpep, induced higher antibody levels ($P<0.01$). Elevated mRNA levels of ChIL-2, ChIFN-γ, ChIL-4, and ChIL-10 were observed, with MDXEF-1/DC-1Hexon-CWA achieving the highest cytokine expression ($P<0.01$). Peripheral blood lymphocytes (PBLs) showed enhanced proliferation in the MDXEF-1/DC-1Hexon-CWA group ($P<0.01$). *E. faecalis* strains provided superior protection, with the highest survival rates and delayed mortality after FAdV challenge. Mild HPS symptoms occurred [64].

Intramuscular injections of inactivated or attenuated vaccines have not successfully triggered intestinal mucosal immunity. Recently, subunit vaccines, which share similarities with inactivated vaccines, have been shown to possess stable and safe properties [109,110]. In theory, vaccination methods that can stimulate effective immune responses in the intestinal mucosa offer a promising approach to preventing pathogens transmitted orally [110]. There remain significant risks of active infection when using live, attenuated, and even inactivated vaccines. Additionally, commercially available vaccines that effectively stimulate intestinal mucosal immunity are lacking. Therefore, exploring new types of vaccines is essential. Henceforth, a recombinant bacteria for the vaccine (probiotics surface-delivering Fiber2 protein) was created.

Two weeks after primary, secondary, and third immunizations, IgG and sIgA levels steadily increased in groups immunized with Fiber2-expressing probiotics (IgG: nearly 1 to over 1.5 OD; sIgA: over 1 to over 1.5 OD) and remained higher than controls. DCpep-fused groups (*L. lactis*/pTX8048-DCpep-Fiber2-CWA and *E. faecalis*/pTX8048-DCpep-Fiber2-CWA) showed significantly higher antibody levels ($P<0.01$). Elevated mRNA levels of IL-2, IFN-γ, IL-4, IL-10, IL-6, and IL-17 were observed in all groups except *L. lactis*/pTX8048-Fiber2-CWA. Peripheral blood lymphocytes (PBLs) exhibited significant responses to rFiber2 protein. Viral loads and clinical symptoms were significantly reduced in vaccinated groups. Elevated serum IgG and sIgA confirmed the vaccine's success in inducing humoral and mucosal immunity, contributing to immune protection in all vaccinated chickens [47].

Building on the development of recombinant bacteria for probiotic surface delivery of the Fiber-2 protein, the focus has also been shifted to dendritic cells (DCs) as key antigen-presenting targets and the exploration of fusion proteins, such as flagellin-antigen constructs, to enhance immune responses and advance vaccination strategies. Dendritic cells (DCs), known for their strong antigen-presenting ability, are crucial targets for vaccines, enabling precise and effective antigen delivery to enhance immunogenicity [111-113]. Short peptides targeting DCs can be fused with antigens for vaccine preparation [114]. *Salmonella* flagellin, a Toll-like receptor five agonist, stimulates immune responses and is an adjuvant via oral

or injection routes. Fusion proteins combining antigens with flagellin retain activity, facilitating new vaccination strategies against infections and cancers [115,116].

Recombinant FAdV-4-fiber2 and FliBc-fiber2-SP proteins were produced by fusion PCR, inserted into the pET-SUMO-His vector, and expressed in *E. coli*. Purified via His-tag kits, proteins were verified by SDS-PAGE and Western blot using anti-FAdV-4-fiber2 serum.

ELISA results showed anti-fusion protein IgG antibodies in Groups I (fiber2) and II (FliBc-fiber2-SP) at 7 dpv, significantly increasing by 21 dpv ($P<0.001$). FliBc-fiber2-SP induced higher IgG levels (nearly OD2) than fiber2 (over 1.5 OD) ($P<0.05$). Serum IL-4 and IL-2 levels were significantly elevated in immunized groups ($P<0.01$), with FliBc-fiber2-SP showing higher IL-2 levels ($P < 0.05$). Post-challenge, Group II had lower viral loads ($P<0.05$) and a 100% survival rate, while Group I had 80% protection. Group III exhibited severe pathological changes. RT-qPCR confirmed significantly reduced viral loads in vaccinated groups ($P<0.001$), confirming the superior efficacy of FliBc-fiber2-SP [117].

In addition, recent efforts to control FAdV-4 have focused on developing subunit vaccines using viral capsid proteins like hexon, fiber, and penton, as well as non-structural proteins such as the 100k protein. *Saccharomyces cerevisiae*, the first fully sequenced eukaryote, is a cost-effective eukaryotic expression system widely used for producing pharmaceutical products, including subunit vaccines. *S. cerevisiae* and other yeast species trigger immune responses by promoting the maturation of dendritic cells, facilitating the presentation of yeast-expressed antigens through MHC class II and MHC class I proteins [118]. Consequently, *S. cerevisiae* has been utilized as a vaccine carrier in various studies.

Recombinant yeast expressing Hexon-L1, Fiber-1, and Fiber-2 proteins effectively stimulated immune responses against FAdV-4 in chickens. Antibodies were undetectable at zero and one wpi but appeared at two wpi, with Fiber-1 showing the highest titer (2.4 $\log_2$), followed by Fiber-2 (1.8 $\log_2$) and Hexon-L1. By three wpi, Fiber-1 (4 $\log_2$) and Fiber-2 (3.4 $\log_2$) titers were significantly higher than Hexon-L1 (3 $\log_2$) ($P<0.05$). Protection was observed in Fiber-1 and Fiber-2 groups, with no gross or histopathological lesions post-challenge. The Fiber-2 group exhibited superior serum neutralization, highlighting the potential of yeast-expressed Fiber-1 and Fiber-2 proteins as protective FAdV-4 vaccine candidates [60].

Fowl Adenovirus Species D Vaccine

FAdV-2/11 has become the most commonly isolated type from cases of Inclusion Body Hepatitis (IBH) and Hepatitis-Hydropericardium Syndrome (HHS) in chickens, leading to significant economic losses globally. FAdV-2 and FAdV-11 are closely related both serologically and molecularly. As a result, isolates from either serotype are collectively referred to as FAdV-2/11 [79,119].

E. coli-produced recombinant proteins fiber-1, fiber-2, hexon loop-1, and penton base were evaluated for FAdV-4 protection, with fiber-2 showing superior efficacy. However, no comparative studies exist for FAdV-2/11, highlighting the need to assess these proteins individually and in combination for developing suitable recombinant subunit vaccines.

The study validated the successful expression and characterization of recombinant penton base and fiber proteins, which are critical for advancing vaccine development [66]. This study addresses a research gap by comparing the immune potency of penton base, fiber, and their combinations for fowl adenoviruses beyond FAdV-4. The fiber group demonstrated the highest survivability rate at 80%, compared to 68% mortality in the challenged control group, 44% in the penton base group, and 34% in the penton + fiber group. Antibody titers peaked at seven days post-immunization (1960.09±87.06) and increased sharply post-challenge (28 dpc: 6536.23±227.06). Viral shedding in feces ceased by day 8 in the fiber group, earlier than other groups, underscoring the fiber protein's superior protective efficacy [66].

Fowl Adenovirus Species E Vaccines

Despite uncertainties surrounding the immune mechanisms behind fiber-induced protection and the variability of fiber types across species, a recombinant fiber from F AdV-8a was tested for protective efficacy against homologous (-8a) and heterologous (-8b) IBH strains [61]. This study is the first to extend the immune response profile beyond the challenge time, comparing pre-stimulated and naïve responses. Cellular immune subpopulations, including CD4+ T lymphocytes, were stimulated by FAdV fiber subunits, with an increase in CD8α+ T cells after a booster immunization with FAdV-8b fiber [120].

The recombinant fiber vaccine provided significant protection against FAdV-8a but limited cross-protection for FAdV-8b. Vaccinated birds showed normal liver-to-body weight ratios and reduced viral loads. Neutralizing antibodies were detected in 73.5% for FAdV-8a/TR59 (4.4 $\log_2$±3.1), 38.8% for FAdV-8a/11-16629 (1.7 $\log_2$±2.3), and minimally for FAdV-8b/764. Cellular immunity revealed elevated B cells and TCR+ T cells [61].

In another study, information on multiple linear epitopes predicted in the Fowl Aviadenovirus E (FAdV-E) fiber head (knob) was utilized to develop chimeric fibers by exchanging sequences between two serotypes, each

containing the proposed epitopes [30]. Two consecutive segments of amino acid positions 1 to 441 and 442 to 525/523 in the fibers of FAdV-8a and -8b, types of Fowl Aviadenovirus E that cause inclusion body hepatitis, were swapped reciprocally to result in novel chimeras, crecFib-8a/8b and crecFib-8b/8a.

The bivalent crecFib-8b/8a vaccine protected against FAdV serotypes 8a and 8b, with viral loads undetectable in vaccinated groups. Antibody titers exceeded 3 OD by 2 wpv, peaking at nearly 3.5 OD at four wpv. VV8b/8a achieved higher peak titers (3.14±0.73 OD) compared to VV8a/8b (0.51±0.65 OD) [30].

Cross-protection is essential for IBH, which involves different serotypes from two FAdV species, but it holds less significance for HHS, which is linked to a single serotype. The penton base protein gene is relatively conserved within serotypes of the same FAdV species, indicating its potential for broad protection against IBH. Thus, another study marks the first evaluation of the recombinant penton base protein as a subunit antigen for IBH, aiming to determine its effectiveness as a standalone vaccine [31]. Recombinant subunit vaccines for FAdV were produced by cloning and expressing penton base proteins Pb-7 and Pb-8b from FAdV-7 and FAdV-8b strains, respectively. Full genome sequencing confirmed strain identities. Each recombinant antigen was tested separately *in vivo* to assess immunogenicity and antigenic differences between serotypes.

The Pb-7 study reported mortality rates of 35.7% in vaccinated and 40% in control groups, with clinical signs by 5 dpc. In the Pb-8b study, only one control bird showed mild signs. Hepatic lesions indicative of IBH were common post-infection. Pre-challenge antibody levels were low in both studies, with OD means at 20 dpv of 0.11±0.17 (Pb-7) and 0.09±0.14 (Pb-8b). A Pb-8b booster increased antibody titers to 1.23±0.70 OD at 6 dpb, compared to 0.08±0.04 in controls. Despite boosted titers, no pre-challenge sera showed *in vitro* neutralizing activity against FAdV-8b [31].

3. Virus-Like Particle (VLP) Vaccines

Fowl Adenovirus Species C

Hexon, a key capsid protein of adenoviruses, is highly immunogenic [121]. It contains conserved pedestal regions (P1 and P2), shared across adenovirus types, and seven hypervariable regions (HVR1-7) that vary among adenoviruses and are found in three loops (L1, L2, and L4) [122,123]. Due to its immunogenic properties, hexon has been used as an antigen in vaccine development against adenoviral infections. However, vaccines that are both readily producible and capable of using hexon to provide complete protection against adenoviral infections are still unavailable [100,124]. Recently, virus-like particles (VLPs) based on the hepatitis B virus core protein (HBc) have gained significant attention as vaccine carriers. HBc can be efficiently produced as VLPs across various expression systems, can hold large foreign antigens at its central immunodominant region (MIR), and can stimulate a humoral response when in a properly folded, particulate form [125]. Hence, a study focused on the production of VLP vaccine [49].

The HBc-hexon (Asp348-Phe369) construct provided 90% protection against pathogenic FAdV-4, outperforming HBc-hexon (Ser19-Pro82) and HBc-hexon (Gly932-Phe956) constructs, which offered 70% and 40% protection, respectively. The control groups vaccinated with a commercial inactivated vaccine or PBS had 50% survival rates. Histopathological analysis showed no inclusion bodies in the HBc-hexon (Asp348-Phe369) group, though mild vacuolar degeneration was observed. HBc-hexon (Ser19-Pro82) and HBc-hexon (Gly932-Phe956) groups displayed moderate degeneration and necrosis with cellular infiltration. The superior protection from HBc-hexon (Asp348-Phe369) likely stems from the immune response elicited by this conserved epitope displayed at HBc's major immunodominant region. Serum antibody titers exceeded 0.4 OD for HBc-hexon (Asp348-Phe369) and HBc-hexon (Ser19-Pro82).

Moreover, the sequence and structure conservation of the epitope region among adenovirus hexon proteins suggests potential broader applications of this vaccine strategy against infections caused by other adenovirus strains [49].

4. Recombinant Virus Vaccines

Fowl Adenovirus Species C Vaccines

Recent studies found that Fiber-1, not Fiber-2, directly triggered the viral infection of FAdV-4 via its shaft and knob domains [2,126]. However, the molecular basis of Fiber-2 in the pathogenesis of the highly pathogenic FAdV-4 needs to be further elucidated. A study found that fiber-2 interacts with karyopherin alpha 3/4 (KPNA3/4) through its N-terminal 1-40 amino acids, with KPNA3/4 shown to promote the replication of FAdV-4. The study used CRISPR-Cas technology to omit the fiber-2.

Co-IP and western blot analyses identified the N-terminal 1-40 amino acids of Fiber-2 as crucial for KPNA3/4 interaction. FAV4_Del replicated significantly slower than wild-type FAdV-4, with viral titers 100 times lower (10^5 $TCID_{50}$/mL vs. $6x10^7$ $TCID_{50}$/mL) at 96 hpi. Immunofluorescence and western blot analyses confirmed these results. FAdV-4-infected chickens exhibited symptoms at 2 dpi, with mortality rates of 10%, 90%, and 100% at 3, 4, and 5 dpi, respectively, along with severe necropsy findings. Conversely, FAV4_Del-infected

chickens showed no symptoms or organ damage. Viral titers from cloacal swabs in FAdV-4-infected chickens ranged from 10^3-10^4 $TCID_{50}$/ml, but no virus was detected in FAV4_Del-infected chickens from 2 to 8 dpi. Liver viral titers in FAdV-4-infected chickens reached 10^5–10^7 $TCID_{50}$/mL, whereas FAV4_Del-infected chickens had titers below 10^3 $TCID_{50}$/mL, with similar findings in kidney and spleen tissues. Chickens previously infected with FAV4_Del displayed complete protection upon challenge, with no symptoms or virus detection, unlike the control group, where high viral titers (10^3-10^4 $TCID_{50}$/mL) were found in cloacal swabs and liver, spleen, and kidney tissues at 2 to 4 dpc. These results highlight FAV4_Del's potential as an attenuated vaccine candidate [127].

Ensuring the safety of vaccine production and administration is a critical priority in preventing unintended exposure to virulent pathogens. To reduce the potential biosafety risks of the virulent strain-inactivated vaccine during production or clinical immunization, the development of inactivated vaccines comprising non-pathogenic strains should be pursued. Therefore, an inactivated recombinant vaccine was produced [72].

Neutralizing antibodies exceeding 4 log_2 at seven dpv and 8 log_2 at 14 dpv were detected in IM and SC vaccinated chickens. By two dpi, 40% of challenge control chickens died, while immunized groups remained symptom-free throughout.

Anatomical and histopathological analysis revealed no abnormalities in the immunized and healthy control groups. In contrast, the control group showed yellow-brown pericardial effusion, liver enlargement, and extensive necrosis. High FAdV-4 DNA levels were detected in their liver, kidney, and spleen, while viral loads were negligible in the immunized and healthy controls. Given FAdV-4's ability to infect various avian hosts, the vaccine could be applied to commercial chickens, ducks, and wild birds, reducing environmental biosafety risks [68,72,128-131].

Third Generation Vaccines

1. Recombinant Vector Vaccines

Fowl Adenovirus Species C Vaccines

Hepatitis-hydropericardium syndrome (HHS) caused by the highly pathogenic fowl adenovirus serotype 4 (FAdV-4) has resulted in substantial economic losses to the poultry industry globally [48]. The fiber-2 gene, a significant virulence determinant, is also a vital vaccine target against FAdV-4. Therefore, the CRISPR/Cas9-based homology-dependent recombinant technique was used to replace the fiber-2 gene with EGFP (enhanced green fluorescent protein) and generate a novel recombinant virus, designated FAdV4-EGFP-rF2. Although FAdV4-EGFP- rF2 showed low replication ability compared to the wild-type FAdV-4 in LMH cells, FAdV4-EGFP-rF2 could effectively replicate in LMH-F2 cells with the expression of Fiber-2 [25]. FAdV4-EGFP-rF2 was highly attenuated in chickens and protected FAdV-4. Without fiber-2, it induced neutralizing antibodies comparable to those with fiber-2. Fiber-1 triggers infection, while fiber-2 determines virulence and serves as a protective immunogen. FAdV-1, FAdV-4, and FAdV-10 uniquely possess fiber-1 and fiber-2, highlighting fiber-2's significance in pathogenesis and vaccine development [2,25,126].

Although the hexon and fiber-2 genes are associated with the pathogenic CH/HNJZ/2015 strain's virulence [132], the roles of these two genes in other virulent strains and their exact locations remain unknown. A single amino acid at position 188 of the hexon protein was further identified as the determinant for FAdV-4 pathogenicity. Virulence based on a single amino acid often appears in RNA viruses but is rarely reported in DNA viruses. Amino acid 367 of the Tembusu virus E protein plays a critical role in pathogenesis [133], and amino acid 431 of the H1N1 swine influenza virus (SIV) PB2 protein determines its virulence in mice [134]. Recently, there was a report that the single amino acid R188 of the hexon protein is responsible for novel FAdV-4 pathogenicity. Thus, the hexon gene, but not fiber-2, was identified as the critical virulence gene for FAdV-4 [26].

All chickens survived and showed no symptoms when inoculated with the rR188I mutant strain, and their serum neutralized the non-pathogenic E188I mutant strain. FAdV-4 hexon sequences from natural non-pathogenic strains (ON1, KR5, B1-7) showed a conserved isoleucine at position 188, whereas pathogenic strains had arginine. The R188I mutant may activate the innate immune or complement systems to neutralize non-pathogenic FAdV-4 [26].

The infection of FAdV-8a alone cannot cause severe disease [135,136]; however, the outcome may become complicated when co-infected with other pathogens or other serotypes of fowl adenoviruses [135,137], which raises concerns for the prevention of FAdV-8a. Previously, De Luca et al.[61] and Schachner et al.[30] demonstrated that either wild-type fiber or chimeric fiber derived from FAdV-8a could protect against homologous challenge. So far, only subunit chimeric Fiber proteins (crecFib-4/11 and crecFib-8a/8b) and inactivated chimeric FAdV-4 with FAdV-8b Fiber were developed [30,44,74]. However, the chimeric FAdV-4 with a fiber of FAdV-8a has not yet been developed. Thus, in a study, A new recombinant virus, FAdV4-F/8a-rF2, which expresses the Fiber protein of FAdV-8a, was created using the CRISPR-Cas9 and Cre-LoxP systems, with FA4-EGFP serving as the template virus.

FAdV4-F/8a-rF2 showed vaccine potential, with chickens displaying no symptoms, lesions, or virus shedding. Histopathology matched negative controls, indicating attenuation. High neutralizing antibody titers were observed for FAdV-4 (938.7) and FAdV-8a at 21 days post-infection. This first bivalent vaccine candidate against FAdV-4 and FAdV-8a in China offers promising protection [32].

Additionally, while various inactivated and subunit vaccines have been developed for FAdV-4 or FAdV-8 individually [61,120,138], no inactivated or recombinant genetically engineered vaccine targeting both FAdV-4 and FAdV-8 simultaneously has been documented.

The recombinant FA4-F8b expressed FAdV-8b fiber and FAdV-4 Fiber-1/Fiber-2. The inactivated vaccine induced neutralizing antibodies against FAdV-4 and FAdV-8b, with NT averages at 7, 14, 21, and 28 dpv reaching 0, 1.3, 3.5, 7.8 (FAdV-8b) and 0, 1.8, 3.8, 9.8 (FAdV-4). No antibodies formed for FAdV-8a. All vaccinated chickens survived; challenge controls died at 3 dpc with HHS lesions. Histopathology showed no symptoms in vaccinated groups. Elevated viral titers were detected in the liver, spleen, kidney, and cloacal swabs of challenge control groups (3, 4, 5, and 7) at 1-9 dpc, while Groups 2 and 6 showed minimal to no viral presence. FA4-F8b demonstrates robust protection, effectively preventing infection and HHS symptoms [74].

Due to FAdV-4's emergence and high pathogenicity, vaccine development is limited. Live FAdV-4 vector bivalent or multiple vaccines could reduce production costs and workload. Therefore, deleting 10 left-end and 13 right-end ORFs identified non-essential regions for replication using an EGFP-indicator virus, marking the first systematic identification. This provides insertion sites for exogenous genes and valuable information for gene function studies, supporting the development of live FAdV-4 vector bivalent or multiple vaccines to reduce production costs and workload.

The FAdV-4 vector expressed vvIBDV VP2 protein, and rHN20-vvIBDV-VP2 conferred complete protection against FAdV-4 and vvIBDV, with nearly $\log_2 8$ antibody titers at 21 dpv. Seven combinations (rDL1-EGFP to rDR3-EGFP) enable multivalent vaccine development, demonstrating successful exogenous gene delivery and protection against HHS and related diseases [37].

Although several inactivated or subunit vaccines have been developed against FAdV-4, live-attenuated vaccines for FAdV-4 are rarely reported. Hence, a recombinant virus FA4-EGFP, expressing the EGFP-Fiber-2 fusion protein, was generated by the CRISPR/Cas9 technique.

Necropsy and histopathology showed no lesions in FA4-EGFP-inoculated chickens, unlike severe hepatic damage in wild-type FAdV-4 infections. Viral loads were significantly lower, with no spleen detection and shedding ceasing by three dpi. Neutralizing titers at 14 dpi for 10^6, 10^5, and 10^4 $TCID_{50}$ were 2.8, 3.0, and 2.3, rising to 7.5, 5.2, and 3.5 at 21 dpi, outperforming the inactivated vaccine (0.8, 3.9) [127].

Adenoviral genes are genus-common or genus-specific. Genus-common genes, conserved in the genome's central region, are crucial for structural proteins, replication, and encapsidation. Genus-specific genes at genome ends encode non-structural proteins for virus-host interactions. While HAdV-C genus-specific genes are well-studied, FAdV genes remain unexplored. Understanding these functions supports attenuated vaccine development, offering high efficacy, easy inoculation, and low costs [81,91,139]. A reverse genetics system was developed to modify the FAdV-4 genome using cell-free restriction digestion and Gibson assembly. Three recombinant viruses were created: FAdV4-GFP (replacing ORF1, ORF1b, and ORF2 with GFP), FAdV4-GX4C (replacing ORF4 with mCherry), and FAdV4-CX19A (deleting ORF19A). Inoculation of chicken embryos with FAdV4-GX4C resulted in 100% mortality between days 5-10, while FAdV4-GFP caused embryo deaths starting on day 8. FAdV4-CX19A had delayed mortality on day 11, suggesting that ORF19A is linked to virulence but is not essential for replication. Enhanced replication was observed in FAdV4-CX19A, though not statistically significant [41].

Understanding viral gene roles is crucial for vaccine development and adenoviral vector optimization. Identifying essential genes aids vector construction, while deleting nonessential genes increases cloning capacity. Essential gene deletion creates replication-defective vectors, and trans-expressed essential genes enable virus-packaging cell lines. Genus-specific essential genes in FAdV remain unidentified. An adenoviral plasmid carrying deletions spanning all 22 genus-specific ORFs of FAdV-4 was constructed to investigate this. Four out of 14 embryos died in the XHE-CX19A group, while all embryos survived in the XGAM1-CX19A group, highlighting differences in virulence among mutants. Using reverse genetics, 21 FAdV-4 mutants with deletions across the genome's ends were constructed. No genus-specific gene was essential for replication in LMH cells or primary chicken hepatocytes, providing a foundation for FAdV-4 vector development. Growth differences in mutants between LMH cells and chicken embryos indicate potential for attenuated FAdV-4 vaccine construction [42].

Identifying and manipulating viral essential and non-essential genes supports adenoviral vector construction and recombinant vaccine development, exemplified by a trivalent vaccine targeting serotypes 4, 8, and 11.

A recombinant FAdV-4 virus containing fiber genes of FAdV-8b and FAdV-11 was constructed using an amp-ccdB cassette and a p15A-cm-HNJZ-fber/8b plasmid [46]. The chimeric virus rFAdV-4-fber/8b + 11, inactivated with formaldehyde and formulated into an oil-emulsion vaccine, induced detectable antibodies in SPF chickens by one week post-immunization. Anti-FAdV-4 Fiber-2 and FAdV-11 antibody levels reached nearly 1 OD (450 nm), and anti-FAdV-8b levels approached 2 OD by the second week. All vaccinated chickens survived FAdV-4, FAdV-8b, and FAdV-11 challenges without clinical signs.

Vaccinated chickens showed no gross or histopathological lesions, unlike the challenge control groups. Viral DNA copy numbers in the liver, heart, spleen, kidney, lung, cecal tonsil, pancreas, bursa fabricius, proventriculus, and duodenum were significantly lower in vaccinated chickens. FAdV-4, FAdV-8b, and FAdV-11 excretion ceased by 3, 4, and 5 days post-challenge, respectively.

A novel recombinant virus, rFAdV-4-fber/8b + 11, was developed in another study to address the common clinical co-infections with different FAdV serotypes. This recombinant virus co-expresses the Fibers of FAdV-8b and FAdV-11 by inserting the FAdV-11 fiber gene into the 1966-bp deletion region of the rFAdV-4-fber/8b genome, providing a potential trivalent vaccine to prevent and control HHS and IBH [46].

To address challenges in co-infections and enhance antiviral strategies, novel recombinant FAdV-4 viruses have been developed, employing advanced genetic editing techniques to efficiently express foreign genes.

Traditional reverse genetics for recombinant fowl adenovirus is inefficient. Using FA4-EGFP as a template, the fiber-2 gene was edited via CRISPR/Cas9 and Cre-Loxp technologies, creating an efficient double-fluorescence system. This method successfully developed recombinant virus FAdV4-HA(H9), expressing the HA gene of H9N2 influenza. A rapid and efficient method for generating fiber-2-edited attenuated recombinant FAdV-4 was demonstrated for the first time in this study [59] utilizing CRISPR-Cas9 and Cre-LoxP systems. The recombinant virus FAdV4-HA(H9) showed vaccine potential against FAdV-4 and H9N2 AIV. Chickens challenged with XZ491 exhibited significantly lower oropharyngeal viral titers at 5 dpc when inoculated with FAdV4-HA(H9) compared to controls, as confirmed by qRT-PCR. The recombinant virus demonstrated attenuation and protective efficacy against H9N2 AIV [59].

The Fiber protein of FAdV is crucial for infection and pathogenicity. Unlike most FAdV serotypes, FAdV-4 has two Fiber proteins: Fiber-1 and Fiber-2 [140]. Fiber-2 is closely linked to virulence, with fiber-2-edited recombinant viruses showing significant attenuation in SPF chickens [132]. Fiber-1, however, directly facilitates viral infection via its knob and shaft domains interacting with the CAR homology receptor [126]. Building on these advancements, further efforts have focused on targeting additional structural proteins to develop novel recombinant FAdV-4 viruses with enhanced functionality.

In a study by Mu et al.[62], another structural protein, Fiber-1 of FAdV-4, was targeted to rescue a novel recombinant virus, FAdV4-RFP_F1, which expresses a fusion protein of RFP and Fiber-1. To explore the potential for editing Fiber-1 and to create a live-attenuated FAdV-4 vaccine or vector, researchers used CRISPR/Cas9 to modify the region between the tail and shaft at the 87th amino acid, generating the recombinant virus FAdV4-RFP_F1 expressing a fusion protein of Fiber-1 and Red Fluorescent Protein (RFP). FAdV4-RFP_F1 was successfully generated.

In vivo evaluation showed FAdV4-RFP_F1 caused no clinical symptoms or mortality in SPF chickens, unlike WT FAdV-4, which caused 100% mortality by four dpi and severe hepatitis-hydropericardium syndrome. WT FAdV-4 reached 10^8 $TCID_{50}$/mL in organs, while FAdV4-RFP_F1 showed no detectable virus by $TCID_{50}$. PCR confirmed early tissue presence, indicating high attenuation and reduced pathogenicity.

Chickens infected with FAdV4-RFP_F1 produced high neutralizing antibody levels (mean titer ~27.4) by 21 dpi, unlike controls. Upon WT FAdV-4 challenge, 82% of control chickens died with severe lesions and high viral titers, whereas FAdV-4-RFP_F1-infected chickens showed no symptoms, mortality, or detectable viral titers, confirming effective protection. The N-terminal domain of fiber-1 was identified as a potential insertion site for foreign gene expression [62].

FAdV-C is another species of fowl adenovirus that contains two fiber genes. Recently, a novel FAdV-C4 strain has been identified as the cause of hepatitis-hydropericardium syndrome (HHS) outbreaks in chickens in China, leading to substantial economic losses in the poultry industry [141, 142]. Despite its impact, the virology of FAdV-4 remains poorly understood. Another study aimed to investigate the distinct roles of FAdV-4 fibers in viral infection using reverse genetics techniques.

Recombinant FAdV-4 viruses expressing Fiber-1 and Fiber-2 were developed. Fiber-1 was essential for rescuing FAdV-4, as no GFP+ foci formed in pKFAV4XF1-GFP-transfected LMH cells, whereas Fiber-2 was dispensable, enabling replication in pKFAV4XF2-GFP cells. Cytopathic effects and GFP signals confirmed virus growth, and sequencing validated the Fiber-2 mutation. Fiber-1 knob protein (F1H6) inhibited FAdV-4 infection in LMH cells by up to 75% at 1-4 µg/mL, while Fiber-2 knob (F2H6)

had no effect, confirming Fiber-1 as the primary binding ligand. In chicken embryos, FAdV4-GFP caused 100% lethality by day 12, while 40% of FAdV4XF2-GFP-infected embryos survived up to 14 dpi. FAdV4-GFP exhibited 2-3 orders of magnitude higher viral levels in the liver than FAdV4XF2-GFP [63].

Vaccination with live attenuated and inactivated vaccines continues to be the most practical approach to controlling Newcastle Disease (ND). The naturally avirulent NDV strain LaSota has been widely used as a live vaccine globally for over 60 years, demonstrating excellent safety and stability [143]. Advances in reverse genetics have enabled the development of the LaSota strain and other NDV strains as vectors for expressing foreign antigens, offering applications in vaccine development and gene therapy [144,145]. The NDV LaSota strain was used as a vector to generate a recombinant NDV virus expressing the full-length fiber-2 gene from a novel FAdV-4 genotype isolated in China [146]. Since unpublished data indicated that live rLaSota-fiber2, delivered via drinking water or ocular administration, did not protect against hypervirulent FAdV-4 challenge, despite offering full protection against NDV, the efficacy of rLaSota-fiber2 as a bivalent vaccine candidate against FAdV-4 and NDV was assessed through intramuscular administration in another study [70].

The NDV LaSota strain expressing the fiber-2 gene of hypervirulent FAdV-4 was developed as an attenuated recombinant vaccine. Single-dose vaccination of 2-week-old SPF White Leghorn chicks with live or inactivated rLaSota-fiber2 induced strong antibody responses against NDV (over 6 $\log_2$) and FAdV-4 (over 1.5 OD). The live vaccine generated higher and earlier titers (over 0.5 OD for FAdV-4 and over 6 $\log_2$ for NDV) compared to the inactivated vaccine (less than 0.5 OD for FAdV-4 and over 2 $\log_2$ for NDV).

Complete protection was observed with the live vaccine, while the inactivated formulation conferred 70% protection against FAdV-4. NDV shedding ceased by day 3 post-challenge in the live vaccine group and by day 6 in the inactivated group. Both groups showed reduced FAdV-4 shedding compared to non-vaccinated birds, which continued shedding until death. The live vaccine induced higher HI antibody titers and better overall protection [70].

Given the need for vaccines offering dual protection against FAdV-4 and NDV, other viral pathogens have also been recombined with FAdV to confer dual immunogenicity. Co-infections of novel FAdV-4 and vvIBDV have been observed in farms due to the overlapping susceptible ages of chickens, resulting in more severe diseases and posing challenges to the poultry industry [146]. Developing a vaccine providing simultaneous protection against both viruses is considered essential. In previous research, an artificial non-pathogenic FAdV-4 strain expressing vvIBDV VP2 was constructed [26]. In a recent study, the immunogenicity of this recombinant virus as an inactivated vaccine was evaluated.

An inactivated vaccine was developed using the recombinant FAdV-4 rHN20-vvIBDV-VP2 strain, created by inserting the vvIBDV VP2 gene into a non-pathogenic FAdV-4 backbone. The inactivated bivalent vaccine, containing 10^7 PFU/mL of virus and stored at −80°C, induced 100% neutralizing antibody positivity against FAdV-4 and vvIBDV three weeks post-immunization. All vaccinated chickens survived FAdV-4 and IBDV challenges without clinical signs. Histopathology and viral load analysis at four dpi showed no liver or bursal lesions in immunized groups, unlike non-immunized chickens, which exhibited severe hepatic damage and lymphocyte depletion.

The vaccine effectively inhibited FAdV-4 and vvIBDV replication, prevented pathological damage, and reduced environmental shedding. High viral loads were detected only in non-immunized chickens. Strong neutralizing antibody responses were observed, with titers exceeding 8 $\log_2$ for FAdV-4 and nearly 8 $\log_2$ for IBDV. Derived from a non-pathogenic strain, the vaccine minimized risks associated with incomplete inactivation or contamination. It was suitable as a standalone bivalent vaccine and a replacement for monovalent and VP2 subunit vaccines. Identified FAdV-4 genome regions offer potential for future vaccine development [26].

Although several inactivated or subunit vaccines have been developed against FAdV-4 and DAdV-3, such as the recombinant viral vector FAdV-4 and inactivated IBDV vaccine [26], there remains an urgent need to develop a novel bivalent vaccine candidate targeting both FAdV-4 and DAdV-3.

The Fiber-2 protein of DAdV-3 can induce neutralizing antibodies and be used as an efficient protective immunogen to offer complete protection against DAdV-3 infection [65,147]. Based on the previous study, fiber-2-edited or fiber-2-deleted FAdV-4 is a highly attenuated and protective vaccine candidate [25,39]. However, a bivalent vaccine against both FAdV-4 and DAdV-3 is not available. Previous studies revealed that Fiber-1 of FAdV-4 directly triggered the viral infection via its shaft and knob domains, and Fiber-2 of FAdV-4 was identified as a significant virulent determiner [2]. More recently, it was found that Fiber-2 of FAdV-4 was not necessary for viral replication and induction of neutralizing antibody, and fiber-2-edited or fiber-2-deleted FAdV-4 was a highly attenuated and protective vaccine candidate [25,39], highlighting that fiber-2 can be as an editable or inserting site for generating live-attenuated recombinant FAdV-4 vaccines against both

FAdV-4 and other pathogens. Therefore, a recombinant FAdV-4 expressing Fiber-2 protein of DAdV-3 using CRISPR/Cas9 and Cre-LoxP systems were generated [35].

The recombinant virus rFAdV-4-Fiber-2/DAdV-3 replicated efficiently in LMH cells, reaching a peak titer of $10^{8.5}$ TCID50/mL. In SPF chickens, it induced high antibody levels and neutralizing titers against FAdV-4 and DAdV-3 without clinical symptoms. This study is the first to generate rFAdV-4-Fiber-2/DAdV-3 using CRISPR/Cas9 and Cre-LoxP. However, its efficacy in ducks and potential to induce cellular immunity were not tested [35].

In addition to recombinant vector vaccines from other viral families, vaccines derived from the same genus, but different serotypes have also been introduced.

Isolation of multiple FAdV serotypes from the same diseased bird is common, highlighting the lack of cross-protection between different serotypes [45,148]. Mixed infections involving HHS and IBH have also been observed in field cases [149]. However, there is currently no commercially available vaccine that targets both FAdV-4 and FAdV-8b infections. FAdVs from different species exhibit significant structural and genomic differences. Previous research has shown that the virulence of FAdV-4 is not dependent on fiber-1, although fiber-1 plays a direct role in mediating infection by pathogenic FAdV-4 [2, 150]. In a recent study, a novel hypothesis was proposed for the first time: replacing the fiber-1 of FAdV-4 with the fiber of FAdV-8b. To test this, a chimeric FAdV-4 virus containing the fiber of FAdV-8b, named rFAdV-4-fiber/8b, was successfully constructed.

Chickens vaccinated with the inactivated rFAdV-4-fiber/8b vaccine developed antibodies against FAdV-4 fiber-2 and FAdV-8b fiber, reaching over 1.5 OD and nearly 1.5 OD by the third week, respectively. All vaccinated chickens survived, while the control group showed 50% mortality 102 h post-infection. Necropsy confirmed protection, with vaccinated chickens displaying healthy organs, unlike unvaccinated ones challenged with FAdV-4 or FAdV-8b, which exhibited severe lesions, including liver necrosis and hemorrhages.

qRT-PCR revealed significantly lower FAdV-4 and FAdV-8b loads in vaccinated chickens, while unvaccinated ones showed high viral copies (10^4-10^{12}). Vaccinated and control groups exhibited no lesions, unlike unvaccinated chickens with severe organ damage, confirming the robust protective efficacy of the inactivated rFAdV-4-fiber/8b vaccine.

PCR analysis revealed that viral shedding was minimal in vaccinated chickens. In the FAdV-8b challenge group, only 2 of 10 vaccinated chickens shed the virus on day 1 but shedding ceased entirely by day 2. Vaccinated chickens in the FAdV-4 challenge group showed no viral shedding throughout the experiment. A single dose provided full protection against both serotypes [76].

Recent studies on FAdV-4 vaccines have mainly focused on inactivated, subunit, and genetically engineered vaccines, while live FAdV-4 vaccines have not been extensively studied [70,138]. Live vaccines are typically based on low-pathogenic or non-pathogenic strains. It has been reported that three naturally non-pathogenic FAdV-4 strains have been isolated: ON1 (Canada), KR5 (Japan), and B1-7 (India). However, the protective effectiveness of these strains against FAdV-4 remains uncertain [77].

The FAdV-4 virulent strain HLJFAd15 (GenBank No. KU991797) from Heilongjiang, China, caused 100% mortality in SPF chickens. Sequencing revealed a 1966-base pair deletion at the genome's right end, identifying it as a novel FAdV-4 genotype [151]. The hexon of the HLJFAd15 strain was replaced with that of the non-pathogenic ON1 strain, creating the recombinant FAdV-4 strain rHN20, which maintained similar viral titers to the wild-type virus but lacked pathogenicity. Chickens immunized with 10^6 PFU of rHN20 showed strong neutralizing activity against FAdV-4 at 7 and 14 days post-vaccination. The intramuscular group exhibited the highest activity (over $6\log_2$) compared to intranasal ($6\log_2$) and subcutaneous (over $4\log_2$) groups. At 7 days, only the 10^6 and 10^5 PFU groups showed neutralizing activity. Post-challenge with 2000 PFU of FAdV-4, vaccinated chickens showed no clinical symptoms and achieved 100% protection, unlike the control group, which experienced mortality within 4 days.

High FAdV-4 copy numbers were detected only in the viscera of dead chickens from the unimmunized challenge control group. Immunized groups, regardless of route or dose, showed background levels similar to non-inoculated controls. The rHN20-based live vaccine provided effective protection, eliminating the need for antigen purification and adjuvant addition. Severe hydropericardium and liver lesions were only seen in challenge controls. Histopathological analysis confirmed healthy liver structures in immunized groups, demonstrating the vaccine's efficacy against HHS [77].

Fowl Adenovirus Species D Vaccines

FAdV-11-associated IBH is increasingly reported worldwide [152,153]. However, its pathogenesis remains poorly understood due to limited genome sequences and technical challenges in manipulating its large genome. Only 14 complete FAdV-11 genome sequences exist, with just one being non-pathogenic. A recent study [28] introduced the first reverse genetics platform for FAdV-11, offering an efficient tool to study its virulence genes and develop multivalent recombinant vaccines.

The FAdV-11 reverse genetics platform enables identifying virulence-associated genes and developing multivalent recombinant vaccines. ORF11 was found non-essential for *in vitro* replication, making it a suitable site for foreign gene insertion, facilitating future vaccine development [28].

2. Duck Adenovirus Vaccines

Duck adenovirus includes DAdV-1 and DAdV-2. In 2014, strain CH-GD-12-2014 was isolated in Guangdong Province, potentially representing DAdV-3 due to low genetic similarity with DAdV-2. Infected ducks exhibited yellowish livers with hemorrhagic spots, kidney enlargement, and bleeding [154]. To address the absence of an effective DAdV-3 vaccine, the VP1 protein of DHAV-1 was recombined into the DAdV-3 genome, creating a recombinant virus for dual prevention against DHAV-1 and DAdV-3 [55].

The antibody response to DAdV-3 in rDAdV3-VP1-188 and DAdV3 groups peaked at 4 weeks post-vaccination (over 3 OD values) and declined by 7 weeks. No antibodies were detected in the negative control group. Ducks in DAdV3 and rDAdV3-VP1-188 groups showed no significant histopathological damage [55].

3. DNA Vaccines

Fowl Adenovirus Species C Vaccine

A subunit vaccine using recombinant Fiber2 protein from the hypervirulent FAdV-4 GZ-QL strain and a Fiber2 DNA vaccine were developed. Both induced significant Fiber2-specific antibody levels (rFiber2 subunit: 50 µg at 24 pg/mL, 100 µg at nearly 26 pg/mL, 150 µg at 22 pg/mL; Fiber2 DNA vaccine: 20-22 pg/mL). The rFiber2 subunit vaccine achieved higher efficacy (80-100%) compared to the Fiber2 DNA vaccine (50-60%) and a commercial inactivated vaccine (80%). No significant histopathological changes were observed [33].

Challenges to FAdV Vaccine Development

Live-attenuated fowl adenovirus (FAdV) vaccines face limitations in efficacy, safety, and production. Long-term effects on layers remain unassessed, with concerns about potential viral shedding through cloaca and gizzard routes, raising questions about their safety and effectiveness in pullets [18]. Small sample sizes in studies limit generalizability, highlighting the need for further exploration of alternative vaccination routes and dosages to optimize immunization protocols [75].

The lack of vaccine production from propagated virus strains, as noted by Ugwu et al., raises concerns about genetic stability, including point substitutions in key viral genes. The absence of details on cell requirements and bioreactor conditions complicates large-scale vaccine production. The lack of vaccine production from propagated virus strains, as noted by Ugwu et al.[23], raises concerns about genetic stability, including point substitutions in key viral genes. The absence of details on cell requirements and bioreactor conditions complicates large-scale vaccine production. Some studies reported reduced pathogenicity in vaccinated chickens, but liver and tissue abnormalities persisted, along with viral shedding and suboptimal antibody responses, especially following intramuscular administration [40].

Undefined vaccine formulations and unspecified commercial vaccine types in studies hinder the comparison and assessment of vaccine effectiveness [5]. Further research is needed to enhance vaccine development by improving immunogenicity, understanding cellular immune responses, and refining production methods.

Live-attenuated and inactivated FAdV vaccines face limitations, including insufficient cellular immunogenicity research and the need to explore alternative vaccination routes, adjuvants, and doses. For example, Wu et al.[50] suggest further research on cross-protection against different FAdV serotypes, while Ugwu et al.[36] emphasize the need for alternative adjuvants beyond Montanide. Similarly, Mohamed Sohaimi et al.[24] note that the potential of adjuvants in enhancing cellular and humoral immunity has yet to be fully explored, particularly for inactivated FAdV-8b vaccines.

Concerns about the limited duration of vaccine efficacy studies, such as those monitoring efficacy for only four weeks [24] and the failure to assess the impact of booster doses on immune responses [36], also remain prominent. While some studies show improvement in viral shedding and body weight with boosters, more comprehensive investigations are needed to determine their long-term impact.

The lack of research on various adjuvants and formulations is another key limitation. Mehmood et al. [52] emphasize the need to compare adjuvants and determine optimal vaccine doses based on age and regimen. They highlight the importance of exploring alternative cell sources for vaccine production, assessing cross-protection against multiple serotypes, and developing more effective inactivation methods [72].

Many studies stress the need for field trials to confirm vaccine efficacy, highlighting the importance of optimizing formulations, dosages, and including cellular immunity, varied adjuvants, and diverse regimens for effective FAdV vaccine development [36,52].

Developing recombinant subunit vaccines for FAdV faces challenges, notably the insufficient evaluation of cellular immune responses, including T-cell activation

and cytokine production, limiting insights into vaccine-induced immunity [12,21,53,60,66,67]. Additionally, several studies did not assess the potential impact of different adjuvants, highlighting the need to explore alternative adjuvant formulations to enhance vaccine efficacy [30,49,67].

Several studies observed adverse clinical signs such as dullness, ruffled feathers, liver changes, and gastrointestinal issues post-vaccination, suggesting potential safety concerns that should be addressed in future research [34,51,57,60,66]. Mortality rates, although low, were reported in some groups, raising concerns about vaccine-associated risks [30,34]. Moreover, viral shedding and histopathological lesions were still detected in vaccinated birds, indicating that vaccine formulations and delivery methods might require refinement to eliminate these issues [47,73].

Vaccine efficacy concerns exist between chicken breeds, such as layer SPF and broilers, due to immune response variations impacting effectiveness [44]. Many studies focused on short-term effects, lacking data on long-term immunity or humoral response durability [46,49]. Some vaccines require multiple doses for optimal protection, posing challenges for large-scale vaccination programs [47,73].

Alternative vaccination routes, such as oral, spray, or intramuscular, require further exploration alongside optimal dosing strategies and dose comparisons [12,43]. Cross-protection against different FAdV serotypes remains underexplored, necessitating further research for broader vaccine coverage [61,117]. Identifying specific mutations in virulent FAdV-4 strain domains linked to unique immunogenic properties also warrants investigation [58].

Finally, the small sample sizes used in some studies limit the generalizability of the findings and call for larger-scale trials and extended monitoring to understand vaccine efficacy and safety under real-world conditions [30,57].

The limitations of recombinant vector vaccines against various FAdV serotypes, as discussed in multiple studies, highlight several common challenges and areas for further investigation.

1. **Inactivation and Antigenic Alterations:** The use of 2% formaldehyde for inactivating recombinant viruses, such as FAdV-1 and DAdV-3, may alter viral antigens and reduce immune response efficiency, raising concerns about the impact of inactivation methods on vaccine integrity and protective efficacy [55].
2. **Unexplored Immune Responses:** Many studies did not assess humoral or cellular immunity, which is crucial for a comprehensive understanding of vaccine efficacy [42]. The lack of investigation into cellular immune responses, especially in live vaccine studies, is a common limitation across various serotypes, including FAdV-4 and DAdV-3 [37,39]. Additionally, the long-term protection rate in chickens was not consistently evaluated [76], and more studies are needed to measure the effectiveness of these vaccines in poultry.
3. **Lack of *In Vivo* Testing and Protection in Chickens:** Many recombinant vector vaccines have not been tested in chickens [63], with some studies lacking data on protection rates or immune responses [28,41,42]. The ability of FAdV-4 Fiber-1 to induce neutralizing antibodies requires further investigation, along with the roles of Fiber-1 and Fiber-2 in protection against FAdV-1 [25]. The absence of *in vivo* data emphasizes the need for additional research to confirm vaccine and antibody efficacy in chickens [65].
4. **Cost and Production Challenges:** The preparation of recombinant vaccines, mainly inactivated ones, is costly [55], which raises concerns about the scalability and cost-effectiveness of these vaccines for widespread use, suggesting that production methods need optimization to reduce costs and improve feasibility.
5. **Optimization of Administration Route and Dose:** variations in vaccination doses and routes, such as oral or subcutaneous delivery, have not been sufficiently tested in many studies, limiting the potential for improving vaccine administration and effectiveness [25,46,62,68].
6. **Viral Replication and Protection Levels:** While some vaccines have demonstrated replication *in vitro,* their performance *in vivo* has been inconsistent. For example, FAdV-4 vaccines have shown slow replication in cell cultures, which may hinder their protective capabilities *in vivo* [39]. The effectiveness of vaccines in inducing robust immune responses and achieving full protection against FAdV infections in chickens remains a key area for further exploration since some studies reported incomplete protection [33,59,64].
7. **Co-infection and Cross-Protective Efficacy:** The impact of co-infections, such as FAdV-4 combined with IBDV or other poultry pathogens like NDV, has not been thoroughly evaluated, despite the potential for co-infection to alter vaccine efficacy [35,37,70]. Furthermore, vaccines developed for one serotype may not offer cross-protection against others, as shown in studies of FAdV-8a and FAdV-4 vaccines [74].
8. **Safety Concerns:** Some recombinant vaccines have raised safety concerns, including minor liver inflammation and potential viral replication in tissues [25,77]. Additionally, the risk of reversion to virulence in recombinant live attenuated vaccines [32] necessitates further studies to assess long-term safety and stability.

9. **Lack of Molecular Investigation Towards Pathogenicity and Mechanism of Neutralization:** For instance, the molecular basis of pathogenicity and the mechanism of virus neutralization FAdV-4 and 8b remain poorly understood, highlighting the need for further investigation [26,76].
10. **Geographical and Environmental Considerations:** Most studies often focused on specific strains or geographical regions, limiting the generalizability of findings. Broader investigations are necessary to account for diverse environmental factors and interactions with other pathogens that influence vaccine efficacy.
11. **Maternal Antibodies and Field Conditions:** The impact of maternal antibodies and field conditions on vaccine performance has not been thoroughly addressed. These factors can significantly influence the immune response and effectiveness of vaccines in commercial poultry operations.

Recombinant vector vaccines for FAdV show promise but have challenges, such as preserving antigen integrity, testing adjuvants and vaccination methods, and evaluating immune responses. *In vivo* testing is crucial for safety and efficacy. Future research should focus on improving production, cross-protection, and addressing safety concerns to enhance vaccine effectiveness for poultry.

Future Directions Towards Fowl Adenovirus Vaccine Development

Future FAdV vaccine development focuses on targeting multiple serotypes, viral components, and innovative methods. Genomic studies on FAdV-9 suggest non-essential genes like ORF1 and ORF19 could be used for foreign gene expression, enhancing FAdV-based vaccines or gene therapy tools [155,156]. Fiber gene variations in FAdV-8b indicate potential for fiber-based vaccines [42], while FAdV-4's unique use of shorter fiber and CAR receptor provides opportunities for targeted vaccine development [126].

Multi-epitope vaccine strategies, incorporating T and B cell-activating peptides, offer cost-effective, faster production than traditional vaccines [48,157,158]. For FAdV-4, studies on fiber-1 and penton show they are vital for replication and immune response, with complex roles in pathogenicity, suggesting future vaccine potential [80,81,150]. Host-virus interaction studies highlight Hsp70 and DnaJC7 as modulators of FAdV-4 replication [159]. miRNA-based strategies, like gga-miR-181a-5p, show promise in antiviral responses and vaccine development [160].

A study showed FAdV-4 Fiber-1, particularly its shaft and knob domains, conferred superinfection resistance against FAdV-8b in LMH cells, unlike FAdV-8b's hexon, penton, or Fiber proteins. Knocking out the CAR receptor suppressed FAdV-8b replication, but CAR is not its primary receptor. These findings suggest targets for controlling FAdV-4 and FAdV-8b infections [74]. In duck adenovirus (DAdV), Fiber-2 of DAdV-3 shows potential as a subunit vaccine, with epitope 108LALGDGLE115 identified [65].

Conclusion and Recommendation

Future vaccination strategies should account for genotype, bird age, and microbiota diversity. Large-scale application of developed vaccines is essential to assess their effectiveness or need for optimization. The transfer of passive immunity to progeny warrants further investigation. Integrated research between vaccinologists and immunologists is crucial to better understand cell-mediated immune responses and improve vaccine efficacy [161].

Highlight Keypoints

- FAdV and duck adenovirus are major pathogens in poultry and necessitates an effective vaccine strategy against the disease outbreak in poultry farms.
- Recent advances in the development of various vaccines against numerous avian adenovirus species, such as FAdV species A-E and Duck adenovirus 1 and 3 were discovered, in addition to the challenges that the conducted studies faced and the future aspects that must be focused on towards the production of effective vaccines.
- Multiple studies show that capsid proteins, especially fiber, provide the highest protection rates and the least viral shedding and clinical signs in poultry.
- Significant discrepancies exist among studies evaluating vaccines for poultry due to variations in bird type, age, challenge strains, vaccine strains, dosage, administration frequency, small sample sizes, and unexamined immune responses or pathogenic mechanisms.
- Future studies should prioritize testing vaccine candidates under real-life conditions, exploring FAdV infection mechanisms, and assessing passive immunity transfer to progeny post-immunization.

Declarations

Availability of Data and Materials: Not applicable

Competing Interests: The authors declared that there is no conflict of interest.

Declaration of Generative Artificial Intelligence (AI): The article and table were not written by AI and AI-assisted technologies.

Author`s Contributions: NMS made contributions to conception and design of the study. MFA writing original draft. NMS, MM and NMI revised the manuscript critically and together with MHB prepared the final draft of manuscript. All authors read and approved the final manuscript.

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