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BRIEF REPORT

Preparation and Serological Evaluation of an Inactivated Trivalent Oil Emulsion Vaccine for Avian Fowl Adenovirus (FAdV) Containing – 8a, 8b, and 11 Serotypes

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Abstract

Inclusion body hepatitis (IBH), caused by fowl adenovirus (FAdV) species D and E, results in significant economic losses for the poultry industry. This study aimed to evaluate the immunogenicity of an inactivated trivalent FAdV vaccine in SPF chickens, specifically assessing the effects of antigen payload, thimerosal addition, and booster immunization on antibody titers. Results demonstrated a clear dose-response, with higher antigen payloads eliciting a more prolonged immune response compared to the lowest dose. Thimerosal was confirmed to be a safe preservative, showing no negative impact on antibody development. Furthermore, a booster immunization significantly increased antibody levels, but this effect was observed only in the group receiving the high-payload vaccine, with peak titers at weeks 2 and 3 post-vaccination. These findings indicate that a high-dose trivalent inactivated FAdV vaccine, administered with a booster, is highly immunogenic and represents a promising strategy for the effective prevention and control of IBH.

Keywords IBH · FAdV · Vaccine · Immunization · Chicken

Introduction

FAdV are significant infectious agents and responsible for severe economic losses [1–3], and it can spread vertically [4] via the allantoic cavity, chick embryo chorioallantoic membrane, and yolk sac as well as horizontally through contact with contaminated utensils, feed, or water [5]. Vertical transmission is considered the main route method of transmission, and the virus replicates mainly in chickens' intestinal and respiratory systems [6]. The disease can be found year-round, with a peak prevalence during the rainy season and summer. Although majority of FAdV cause subclinical illness, some FAdV cause acute viral infections such as hepatitis-hydropericardium syndrome (HHS), inclusion body hepatitis (IBH), and gizzard erosions (GE) [6]. IBH has been reported globally, commonly influencing meat-producing chickens at 3 to 4-week-old and some layer chickens ranged between 25 and 27 weeks [7], resulting in mortality rate varies between 2% and 30%. Surveillance studies have documented that IBH is primarily caused by FdAV serotypes 2, 8a, 8b, and 11 [6], while HHS and gizzard erosion are mainly associated with FdAV serotypes 1 and 4. IBH is characterized by enlarged, friable, and swollen liver with a stellate hemorrhages [8]; bloated, pale, and mottled kidneys with a hemorrhagic renal cortex; and occasionally, hemorrhagic and an inflated spleen [9].

Fowl adenoviruses are non-enveloped, linear double-stranded DNA viruses with size 43–45 Kb. They belong to

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the Aviadeno virus genus in the Adenoviridae family [10]. In the middle of the last century FAdV were recognized and have been reported to infect poultry [11]. FAdVs are divided into five species (FAdV-A to FAdV-E) by their molecular structure and subdivided into 12 serotypes (FAdV-1 to 8a and 8b to 11) based on their serological relationships [12].

Fowl adenovirus (FAdV) infections are a significant concern in Egypt's poultry industry, with several serotypes identified in different regions. Several studies have showed the circulation of various serotypes in different poultry farms in Egypt, including FAdV species E/serotype 8a [10], FAdVs species D/serotype 2–11 [11, 12], and FAdV serotypes 1, 3 and 8b [13].

Fowl adenovirus (FAdV) vaccines are available in several forms, including inactivated, live attenuated, recombinant, and oral vaccines, each with specific advantages depending on the target poultry population and regional needs. Inactivated vaccines, which contain killed virus particles, are the most common and are typically administered via injection. These vaccines stimulate an immune response without causing disease and are highly effective in controlling FAdV infections [14, 15]. Furthermore, the use of cell culture is generally considered superior to chicken embryo-based methods for propagating Fowl Adenovirus (FAdV), particularly for vaccine production, due to several advantages including earlier and higher antibody responses, improved protection, and practical benefits in manufacturing [16–18]. Thus, the objectives of this study are to evaluate the immunogenicity of an inactivated trivalent FAdV vaccine following vaccination of commercial broiler chickens with different viral antigen payloads.

Materials and Methods

Molecular Detection of FAdV

Liver organs samples were collected from layers chickens that were suspected to be infected with Fowl adenovirus (FAdV) in Sharqia government in Egypt; the samples were stored in −80°c freezer for further processing. Liver samples were suspended and homogenized with phosphate buffered saline to a 10% suspension containing antibiotics at a concentration of 200U/ml penicillin and 0.2 mg/ml streptomycin. The homogenized suspension has been clarified by centrifuges at 2000 g at 4 °C for 10 min. The supernatant was filtered twice, the first was through 0.45 µm filter and the second with 0.2 µm filter then transferred to fresh sterile falcon for molecular detection of FAdV by PCR followed by virus propagation, identification and titration in cell culture.

Table 1 Sequences of the primers used for identification of FAdV in this study

Primer ID	Sequence (5'–3')	Reference
AdPol F	GCAGCGTGGTCTTGAAGATG	This study
AdPol R1	GCATGTGATTGCGACATCAAGTGC	This study
F0 hexone	GCT GCA GTA TTT TCA CAT CGC	This study
AdR5	ATGTCGGCGAGCATGTACTG	This study

The total DNA was extracted from the above supernatant using viral gene-spinTM viral DNA/RNA Extraction kit (Trans, China) according to the manufacturer's instructions. Conventional PCR was performed using in-house designed specific primers for the L1 region of the hexon gene of FAdVs. The specific oligonucleotide primers were used for the amplification of the L1 loop of the hexon gene of different adenovirus serotypes. The primers were synthesized by metabion (Munich, Germany). PCR amplification was accomplished using an EmeraldAmp Max PCR Master Mix (Takara, Japan) in a total volume of 25-µL consisting of 12.5 µL of EmeraldAmp Max PCR Master Mix, 1 µL of forward and reverse primers (working concentration 20 pmol), 5.5 µL of PCR-grade water, and 5 µL of extracted DNA. The reactions were run in Biometra T3000 thermal cycler as follows35 cycles of 95 °C for 15 s for denaturation, 60 °C for 20 s for annealing, and 72 °C for 60 s for extension. The predicted size of the PCR products was approximately 950 base pairs (bp). List of primers used in this study is presented in Table 1. Strong PCR.

Isolation and Propagation of FAdV

Preparation of Primary Chicken Embryo Liver Cell (CEL) Culture

Chicken embryo liver cell culture was prepared from 13 to 15 days old SPF chicken eggs according to standard protocol. The embryos were removed aseptically then the livers were collected by using sterile forceps and washed several times with sterile PBS (0.1 molar PH7.4), gently trypsinized with 0.25% trypsin solution for 7 min at 37 °C. The trypsinized cells were poured in sterile falcon. To get rid of the effect of residual trypsin, add small volume of the growth media, the growth medium is fresh Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotic (1% penicillin-streptomycin). The cells suspension was centrifuged at 1500 rpm at 4 °C for 10 min to obtain cell pellet. Withdraw The supernatant and resuspend the pellet with the growth media, cell concentration was approximately 5×10^6 cell/ml of the medium and cell suspension in 10 ml volume was seeded in 25 cm^2 tissue culture flasks and incubated at 37 °C incubator under 5% CO_2 with humidity 85%−90% until forming confluent monolayer sheet [19].

Propagation and Identification of FAdV

After checking the confluency of the flasks under the microscope (80%), the flasks are ready for inoculation, throw the old media gently and inoculate 0.5 ml of filtered liver tissue homogenate that was previously stored at −80°c freezer in the tissue culture flasks. After adsorption of the inoculum onto the cells at 37 °C for one hour, 10 ml of maintenance medium containing 2% FBS were added. The infected and control flaks were incubated at 37 °C under 5% CO_2 incubator, daily inspection of the flasks under the microscope for observation of cytopathic effect (CPE) for 5days. three cycles of freezing and thawing for the inoculated flasks, centrifuged at 2000 rpm for 10 min, then the supernatants were transported into sterile falcons for titration.

Identification of Propagated Fowl Adenoviral Isolates

The virus was identified by PCR as described above then all amplified products were subjected to DNA sequencing to confirm specific amplification of type-specific hexon genes.

Titration of FAdV

The FAdV infected CEL cells were titrated by the median tissue culture infective dose ($TCID_{50}$), according to the methods previously reported [20]. Briefly, a 10-fold serial dilution of the virus was prepared in culture medium from 10^{-2} to 10^{-10} of virus stock. Monolayer of CEL cells prepared in 96-well tissue culture plate (SPL life sciences, Korea) were infected by 100 ul of each dilution in 4 replicates and incubated at 37 °C incubator under 5% CO_2 with humidity 85%−90% for 5 days. The CPE was daily observed under an inverted microscope and confirmed by PCR.

Vaccine Preparation

Fowl adenovirus D, Fowl adenovirus 8a, and Fowl adenovirus 8b were propagated on primary chicken embryonic liver cells (CEL) and used for production of trivalent vaccine. For inactivation of FAdV serotypes, harvested virus from infected CEL cells was incubated with 0.2% formalin on stirrer for 48 h at room temperature. After conforming the completion of inactivation of the virus by the PCR through three successive passages in CEL, the formaldehyde inactivated antigen solutions were emulsified with mineral oil adjuvant at a ratio of 30:70 and sterile saline to make different doses of 10^7 $TCID_{50}$, $10^{6.8}$ $TCID_{50}$, and $10^{6.5}$ $TCID_{50}$ of each serotype. The final volume of the vaccine dose was 0.5 mL per bird.

Animal Experiment and Priming Immunization

Forty SPF chickens 10 weeks old have been divided into 4 groups (G1-G4), each group containing 10 chickens as illustrated in Fig. 1. The divided groups have been immunized with 0.5 ml of the trivalent vaccine per chick with different viral loads as shown in Fig. 1. briefly, the G1 was immunized with 10^7 $TCID_{50}$/dose and birds of G2 were vaccinated with the same concentration of the vaccine to which thiomersal at a concentration of 25 micrograms/dose has been added. However, the G3 and G4 groups have been immunized with low virus payload per dose, in concentration $10^{6.8}$ $TCID_{50}$/dose and $10^{6.5}$ $TCID_{50}$/dose, respectively, as shown in Fig. 1. the vaccinated groups were monitored and serum samples were collected separately from each group for 6 successive weeks post-vaccination to follow up the humoral immunity.

The Booster Immunization

This experiment has been designed to evaluate the effect of a single booster dose on the humoral immunity of the previously vaccinated groups after 6 weeks of vaccination. based on experiment 1, G1 and G3 were divided into 2 subgroups (A &B), each single group contained 5 chicks at 16 weeks old (6 weeks post-vaccination). As illustrated in Fig. 1, G1-A did not receive booster doses, while subgroup G1-B was immunized with a booster dose from the trivalent vaccine with a high virus payload (10^7 $TCID_{50}$). On the other hand, subgroup G3-A received a booster dose with a low virus payload ($10^{6.8}$ $TCID_{50}$), and subgroup G3-B got a high virus payload (10^7 $TCID_{50}$). All these subgroups have been incubated, and monitored, additionally serum samples have been collected for 3 successive weeks post boosting (until 9 weeks after the first dose) for monitoring the humoral immunity.

Monitoring of Humoral Immunity During the Experiments

Serum samples were collected from SPF chicken vaccinated with one or two doses of vaccine intramuscularly to confirm antibody response of inactivated traivalent FAdV vaccine. Levels of FAdV-specific antibodies have been examined by a commercial enzyme-linked immunosorbent assay (ELISA) kit (Biostone, USA) according to manufacturer's instruction.

Statistical Analysis

The means of antibody titers were statistically analyzed for significant differences at p-value$>$0.05, at different time points in each vaccinated group by Two-way ANOVA (with

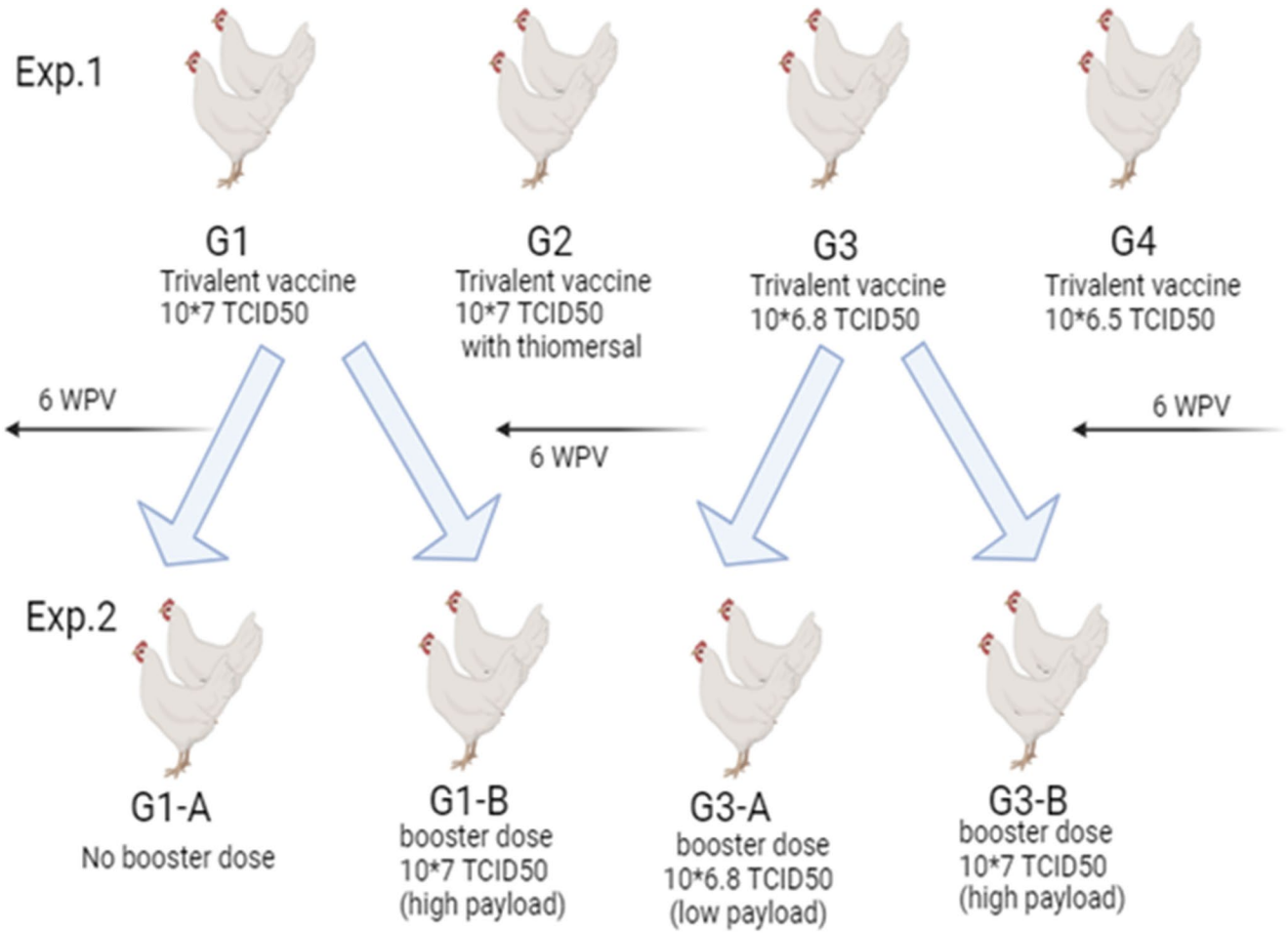

Fig. 1 The diagram of the Experimental design of the investigated work in this study

mixed model). The data were analyzed and visualized using GraphPad Prism version 9.

Results and Discussion

The inclusion body hepatitis (IBH) incidence has increased in recent years, worldwide [18]. IBH is an emerging disease caused by FAdV that causes significant economic losses in the global poultry sector. Most outbreaks are linked to FAd-2, FAd-11, FAd-8a, and FAd-8b [21]. In Egypt, it was reported the co-circulation of several serotypes including FAd-1, FAd-2, FAd-5, FAd-8a, FAd-8b, and FAd-11 [13]. In this study, we developed an inactivated FAdV trivalent oil-emulsion vaccine encompassing FAdV-11, FAdV-8a, and FAdV-8b serotypes and the immune responses were evaluated in SPF chickens to select a promising vaccine candidate to decrease and control the prevalence of disease syndromes related to FAdV in Egypt. Because the protective efficacy of the inactivated vaccine is based on induction of antigen-specific humoral immune responses, the level of antibody in vaccinated chickens might be a helpful criterion for efficacy evaluation of inactivated vaccine [20].

First, was isolated FAdV virus from liver organs obtained from chickens that were suspected of being infected with FAdV (Fig. 2) and confirmed by PCR, producing a single amplicon of 950 bp (Figure S1). Sequence analysis of the PCR-amplified products was in consistency with fowl adenovirus D (accession no. OR753244.1), Fowl adenovirus 8a (accession no. OR753245.1), and Fowl adenovirus 8b (accession no. OQ988005.1) that were used in our experiments. The propagated FAdV-8a/8b/-D exhibited 40%, 60%, and 85% cytopathic effect (CPE) at 24, 48 and 72 h post-infection in CELs, respectively (Figure S2). Compared to cell control, the observed CPE included rounding, clumping, and cell detachment from the cell monolayer. The $TCID_{50}$ was found to be 10^9/ml for FAdV-8a, 10^9/ml for FAdV-8b, 10^8/ml for FAdV-D, using the Reed-Muench method.

After successful inactivation of virus with formaldehyde in 0.2% final concentration [15], three different formulas with three different payloads to optimize vaccine dose were

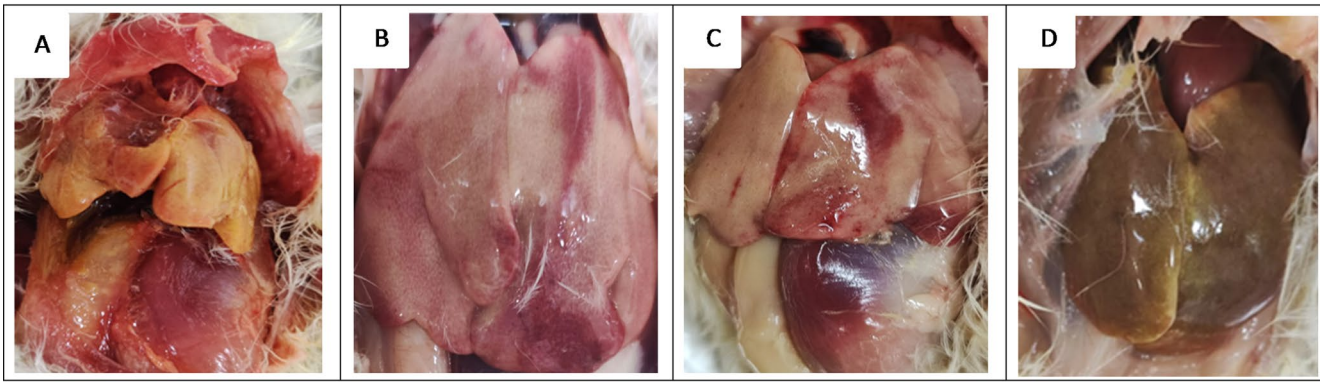

Fig. 2 Morphology of liver organs of chicken suspected to be infected with FAdV (**A-D**) that were used for the virus isolation

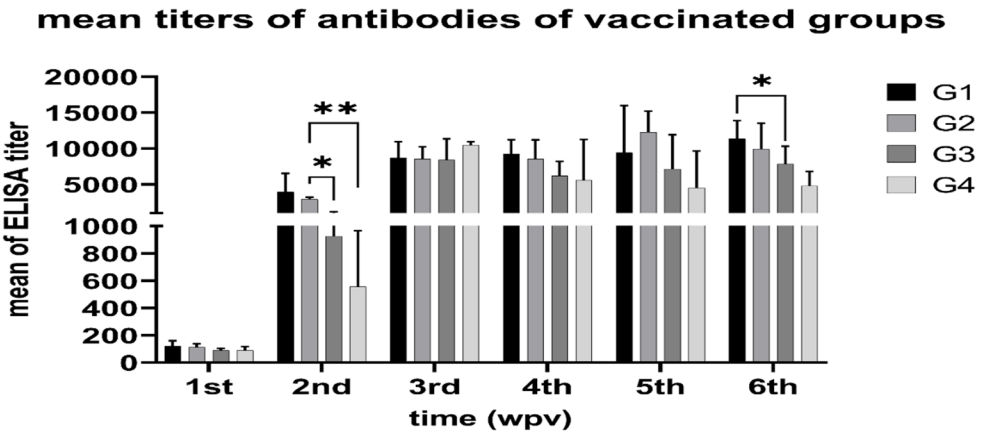

Fig. 3 The mean ELISA titers of antibodies against the trivalent FAdV vaccines in the 4 vaccinated groups through 6 weeks post-vaccination. Comparison test were performed using the GraphPad Prism 9.0 software; $p<0.01$and $p<0.001$ are expressed by * and **, respectively

prepared. The three doses achieved considerable ELISA titers; however remarkable difference was noticed for the least payload ($5 \times 10^{6.5}$ $TCID_{50}$). The mean titers of the 4 vaccinated groups showed that the G1 that was vaccinated with a high payload of the virus presented a prolonged immune response till the 6th week of vaccination without a sharp decline in the antibody's titer, as shown in Fig. 3. This finding support the results of some previous reports [20, 22–24] which showed the increasing antibody level at 1 st week and decreasing at 4th weeks post vaccination. Also, it was noticed that the G2 group, which was vaccinated with the same vaccine in addition to thiomersal as a preservative, showed no significant reduction in immune response in comparison with the G1 group that was vaccinated with the same doses without thiomersal. Steer-Cope et al. [25] demonstrated that the inactivated FAdV-8a serotype vaccine produced serum antibodies from vaccinated chickens that neutralized FAdV-8a with ELIZA titers ranging from 600 − 20,000 post immunization. The results obtained for inactivated FAdV trivalent vaccine under study are in consistence with those previous studies, suggesting that this vaccine could be helpful in controlling the FAdV infection [15].

Additionally, we explored the effect of booster dose following 6 weeks of the priming dose. According to experiment 1, we found a significant variation between G1 and G3 by the end of the incubation period at 6th week post vaccination. So, we choose those groups as a base for the experiment 2 which studied the effect of the booster dose with high and low virus pay load vaccine. As, shown in Fig. 4, G1-A group (non-boostered) and G3-A group (boostered with low virus payload) showed no increase in the antibody levels. In contrast, there were increase in the antibody titers in sera of G1-B and G3-B groups that were boostered with high virus payload (10^7 $TCID_{50}$).

In general, the antibodies titer increased considerably following the boosting dose, even for the group that has lower antibodies titer. This underpins the importance of serological monitoring following the first dose vaccine administration. In case of modest results following the prime dose, a different vaccine can be used for the booster where it can restore the high titers required for an efficient protection for the flocks. Furthermore, the control group that didn't receive a booster dose showed the need for receiving booster dose as antibodies titers can decline considerably. It is recommended to test the ability of vaccines to reduce or prevent the virus shedding in the environment and determining the duration of the protective efficacy prevented the organism shedding to the environment [26]. Cádiz et al. [27] showed

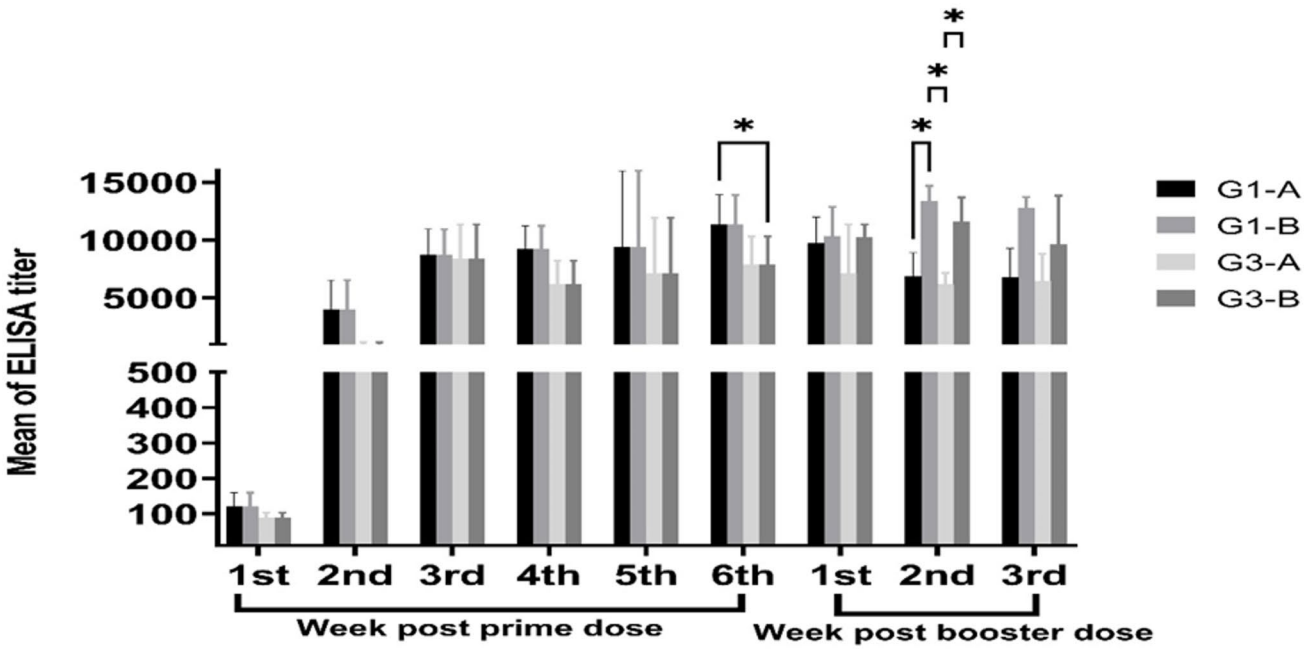

Fig. 4 The mean ELISA titers of antibodies against the trivalent FAdV vaccine booster dose in G1 and G3. Comparison test were performed using the GraphPad Prism 9.0 software; $p < 0.01$ is expressed by *

that the booster immunization significantly reduced shedding of virus, compared with non-booster inoculation.

So, a significant limitation of the present study is that no challenge trials against live virulent FAdV strains (homologous and heterologous) were performed to evaluate the protective efficacy of the vaccine in preventing virus shedding and/or spread among flocks. Another limitation is that the study could not evaluate the longer-term duration of immunity, such as protection lasting 3 to 6 months or beyond. This leaves an important unanswered question regarding the persistence of the immune response and the sustained efficacy of the vaccine over an extended period.

Thimerosal is considered as an important preservative agent to vaccine manufacturers [28], as it is one the most used preservative for chicken and veterinary vaccines. In 2008 the World Health Organization approved the addition of thimerosal in vaccines [29]. However, the effect of thiomersal on the immunogenicity is not understood. So, an additional group was added that explore the effect of the thiomersal on inactivated vaccine efficacy. Results showed low effect on the antibodies titers. Stone [30], showed that thimerosal at the recommended levels in commercial poultry vaccines does not significantly reduce vaccine efficacy.

An important note to consider, the ELISA results is influenced by the type of antigen coated within the kit. In this study, one ELISA kit didn't show any readings with our vaccine but gave good results with the monovalent inactivated FAdV vaccine control that contained FAdV-4 (serotype-C) as this kit coated with homologous antigen for FAdV-4 (data not shown). On the other hand, another ELISA kit showed positive results for both vaccines (vaccine under study and control vaccine). Therefore, the selection of the ELISA kit is important for vaccine manufacturers due to the limited serological relatedness between the different FAdV serotypes.

Besides the above immunological findings, The FAdV cell-culture based vaccine tested in this study showed some well-known advantages over embryo-adapted vaccines. Virus propagation on chicken embryo is limited for many reasons such as its dependance on a continuous supply chain of embryonated eggs, time-consuming, and labor-intensive, which make it is not suitable for the practical manufacturing [31]. Moreover, cell-based production technology is more flexible that allows manufacturers to react quickly to pandemic, and produce greater quantities of the vaccines with lower variations among vaccine batches in less time [32]. Additionally, cell-culture based production approach does not introduce greater or new adventitious agents when compared to egg-based vaccine production system [17]. Thereby, cell-culture derived inactivated FAdV vaccine has more useful benefits than the embryo-adapted vaccine.

This study gives preliminary recommendations regarding the optimal vaccine payload, the feasibility of using thiomersal preservative during formulation, and the importance of diagnostic kit selection. Still, additional work needs to be carried out to correlate the relation between antibodies ELISA titer and protection against challenge. Overall, FAdV is a continuing threat to the poultry industry and effective control measures are needed to be implemented.

Effective vaccination strategies should be considered as one of the main pillars for controlling the FAdV threat.

Conclusion

The inactivated FAdV trivalent FAdV-11, FAdV-8a, and FAdV-8b could be utilized as a vaccine to stimulate specific immunity against the FAdV-11, FAdV-8a, and FAdV-8b infection. Hence, humoral immunity induced by the inactivated FAdV trivalent vaccine containing the three serotypes could be a tool for IBH control in both breeders and their progenies.

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Author Contributions F.E.I. methodology, the conception and design, data curation, visualization, writing—original draft. M.N.F.S.: investigation, methodology, visualization, writing—review and editing. H.M.: investigation, methodology. M.I,: formal analysis, methodology, writing—review and editing. Y.E.S.: the conception and design, analysis and interpretation of the data, editing. S.A.H.A.: the conception and design, formal analysis, data curation. A.D.A.M.: the conception and design, formal analysis, data curation. A.S.: conception and design, formal analysis, data curation, writing—review and editing. All authors agree to be accountable for all aspects of the work.

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Declarations

Consent for Publication Not Applicable.

Conflict of interest No potential conflict of interest was reported by the authors.

Ethical Approval The study was approved by The Institutional Animal Care and Use Committee (ARC-IACUC), Agriculture Research Centre, Egypt on the date of 01.11.2024 (Ref No: ARC AHRI 183 24). Informed consent was obtained in accordance with the Ethics Committee and approval procedures.

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