Original Article

Safety and Immunogenicity of Inactivated Fowl Adenovirus Serotype 8b Isolate Following Different Inactivation Time Intervals in Broiler Chickens

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ABSTRACT

In recent years, significant attention has been directed towards the development of inactivated fowl adenovirus (FAdV) vaccines within the poultry industry to combat outbreaks of inclusion body hepatitis (IBH). This study aimed to assess the safety and immunogenicity of an inactivated FAdV vaccine following different inactivation time intervals in commercial broiler chickens. The FAdV isolate UPM1137 was treated with binary ethyleneimine (BEI) at intervals of 20, 24, 28, 32, and 36 hours. All treated isolates underwent safety testing in specific pathogen-free (SPF) chicken embryonated eggs (CEE), followed by safety and immunogenicity trials in SPF chickens. The findings demonstrated that isolates treated at 20, 24, and 28 hours induced over 80% mortality in SPF CEE, while no mortality was observed in isolates treated at 32 and 36 hours. In SPF chickens, there were no clinical signs or gross and histological lesions recorded throughout the trial for those inoculated with isolates treated at 32 and 36 hours, indicating that these treatments rendered the virus completely inactivated and safe for use in commercial broiler chickens. Based on ELISA results, chickens vaccinated with the inactivated FAdV at 32 hours (group B) exhibited a higher antibody response compared to those vaccinated with the inactivated FAdV at 36 hours (group A) at days 14 and 28 post-inoculation (pi). At day 28 pi, the mean antibody titers for the booster groups in both group A and group B were significantly higher ($p < 0.05$) compared to the control group. These results indicate that both FAdV vaccines, using inactivated inocula at 32 and 36 hours, are safe and immunogenic in broiler chickens, particularly by day 28 pi following booster vaccination. Thus, it was concluded that the optimal duration for FAdV inactivation is 32 hours, making it highly suitable for future vaccine formulations.

Keywords: Aviadenovirus, Vaccines, Safety, Chickens, Antibody Response.

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1. Introduction

Fowl adenoviruses (FAdVs) are double-stranded DNA viruses with a non-enveloped capsid, belonging to the family Adenoviridae and the genus Aviadenovirus (1). FAdVs cause several clinical diseases in chickens, resulting in significant economic impacts due to mortality and reduced productivity (2, 3). Inclusion body hepatitis (IBH) has been reported worldwide, primarily affecting meat-producing chickens at 2 to 3 weeks of age, as well as some layer chickens between 25 and 27 weeks of age (4). Epidemiological studies have confirmed that IBH is commonly caused by serotypes 2, 8a, 8b, and 11 (5), while hepatitis-hydropericardium syndrome (HHS) and gizzard erosion are primarily associated with serotypes 1 and 4. In Malaysia, recent reports indicate occurrences of IBH and gizzard erosion in broiler and layer chickens due to FAdV serotype 8b infections (6). Currently, virus inactivation is primarily achieved through chemical treatments, such as formalin or binary ethyleneimine (BEI), for a specified duration depending on the virus type or structure, with the aim of developing inactivated vaccines against the disease (7, 8). The application of these vaccines in other countries has been effective in controlling virus spread at both vertical and horizontal levels (5, 9). Several studies have focused extensively on vaccine development against serotype 4 due to the lack of commercially available vaccines. For example, an inactivated vaccine in China provided full protection against the novel FAdV serotype 4 (10). Recent work has shown that bivalent live and inactivated vaccines can protect chicks against IBH through breeder vaccination (11). Additionally, an inactivated vaccine targeting serotype 2 was developed in Thailand, offering 90 to 100% protection in progeny stocks via vaccination of broiler breeders (12). Earlier studies indicated that formalin and BEI are commonly employed chemicals for virus inactivation in poultry vaccine production (13). In vaccinated chickens, membrane alterations in formalin-treated FAdV particles resulted in poor immunogenicity compared to those inactivated using BEI (14). Malaysia is one of the top global consumers of poultry meat, with a per capita consumption of 50 kg in 2022 (15). However, IBH caused by FAdV serotype 8b has emerged as a significant threat to the poultry industry in recent years, leading to substantial economic losses due to high mortality rates and decreased production in commercial farms. Therefore, effective biosecurity measures and vaccination strategies are essential for sustaining food security in the country. The number of clinical cases of IBH has continued to rise in recent years due to the absence of locally available vaccines against high-pathogenic FAdV serotype 8b in commercial chickens. The development of suitable inactivated vaccines requires a thorough understanding of optimal virus inactivation, which remains insufficiently documented for FAdVs across various serotypes and strains. Thus, the objectives of this study are to determine the safety and immunogenicity of inactivated fowl adenovirus (FAdV) following different inactivation time intervals in broiler chickens.

2. Materials and Methods

2.1. Inactivated FAdV Inoculum

The FAdV isolate, designated UPM1137CEL5, was obtained from the fifth passage in primary chicken embryo liver (CEL) cells, yielding a virus titer of 10^{11.5} TCID₅₀/ml (6). The virus suspension was stored at -20°C for future use.

2.2. Virus Inactivation by Chemical Treatment The virus suspension was inactivated using a chemical treatment with a 0.002 M concentration of binary ethyleneimine (BEI) at five different time intervals: 20 hours (h), 24 h, 28 h, 32 h, and 36 h. The BEI solution at a concentration of 0.1 mol/L was prepared from 2-bromoethylamine hydrobromide (Sigma) in a 0.175 mol/L NaOH solution under cyclization at 37°C for one hour (16). BEI was then added to the virus suspension at a rate of 2% to achieve a final concentration of 0.002 mol/L and was subsequently incubated at 37°C for 20 h, 24 h, 28 h, 32 h, and 36 h. After incubation, the residual BEI was neutralized with 1 mol/L sodium thiosulfate at a volume of 10% of the BEI used, at room temperature for 1 hour, and then filtered through a 0.2 µm filter before being stored at 4°C.

2.3. Virus Infectivity Assay in SPF Chicken Embryonated Eggs (CEE)

Each treated isolate (0.1 mL) was inoculated into 9-day-old SPF CEE via the chorioallantoic membrane (CAM) in triplicate and incubated for 12 days post-inoculation (pi). Embryonic mortality was recorded throughout the trial. Five eggs remained uninoculated and served as the control group (12).

2.4. Preparation of Inactivated FAdV Formulated with Adjuvant

Two different inoculums treated for the optimum period were selected based on safety test findings in SPF CEE and primary CEL cells. For vaccine preparation, the inactivated FAdV inoculums were formulated with Montanide ISA 71 VG (SEPPIC, Inc.) adjuvant at a ratio of 70:30 (adjuvant) (v/v). Each virus suspension from specific periods was mixed with the adjuvant and stored in separate tubes prior to testing for safety in commercial broiler chickens (9).

2.5. Experimental Design for Safety and Immunogenicity in Commercial Broiler Chickens

The study was conducted under IACUC approval number: UPM/IACUC/AUP-U012/2022. Forty-five (45) day-old commercial broiler chicks (Cobb500) were reared in a wire-floored isolated house with ad libitum access to food and water. The chicks were divided into three major groups: A, B, and C. Fifteen (15) chicks were assigned to each of the individual groups A and B, while seventeen (17) chicks were assigned to group C. Groups A and B were further divided into booster (A1 and B1) and non-booster groups (A2 and B2). All chicks in groups A1 and A2 were inoculated with 0.2 mL of vaccine prepared from the inactivated FAdV inoculum at period A, while groups

B1 and B2 received 0.2 mL of vaccine prepared from the inactivated FAdV inoculum at period B. Inoculation was conducted via the subcutaneous (SQ) route at a dose of 10^{11.5} TCID₅₀/mL on day 0. All chicks in group C remained uninoculated and served as the control group. At day 14 pi, all chicks in groups A2 and B2 received a booster dose of 0.2 mL of vaccine via the SQ route. All chicks were monitored daily for clinical signs at least twice daily. At day 0 pi, five (5) chicks from group C (control) were sacrificed through cervical dislocation. At day 24 pi, five chicks from groups A1, B1, and C were similarly sacrificed. At day 28 pi, all chicks from all groups were slaughtered through cervical dislocation. Body weight and serum samples were collected from each chicken prior to slaughter (6).

2.6. Gross and Histopathological Lesions

Upon necropsy, gross lesions in the chickens were observed and recorded during sampling. Both the liver and trachea were collected for histological examination, fixed immediately in 10% buffered formalin, and subsequently processed and stained with hematoxylin and eosin (HE) (2).

2.7. FAdV Antibody Detection

Serum samples from both the inoculated and control groups were collected and analyzed for FAdV antibody titers using a commercial enzyme-linked immunosorbent assay (ELISA) as described by BioChek, London, UK.

2.8. Statistical Analysis

Mean body weights and FAdV antibody titers were analyzed using one-way analysis of variance (ANOVA) with IBM SPSS Statistics version 22. The significance level was set at an alpha value of $p<0.05$. For significant outcomes, multiple group comparison tests were conducted using Tukey's HSD (Honest Significant Difference) test. An independent t-test was performed to compare means between two groups (17).

3. Results

3.1. Virus Infectivity Assay in SPF Chicken Embryonated Eggs (CEE)

At the 20 h, 24 h, and 28 h intervals, FAdV demonstrated infectivity, resulting in embryo mortality across all three replicates (Table 1). As shown in Figure 1, embryonic mortality rates ranged from 25% to 100% throughout the trial for FAdV treated at 20 h, 24 h, and 28 h. The BEI-treated FAdV inoculum at 20 h caused mortality rates of 50% to 75% in CEE, followed by 25% to 50% at 24h. Mortality rates of 25% to 100% were recorded in CEE inoculated with the 28 h treatment. No mortality was observed in CEE following inoculation with the 32 h and 36 h treatments. The FAdV isolate was completely inactivated at both the 32 h and 36 h intervals, showing no infectivity in embryos and exhibiting characteristics similar to control eggs throughout the trial.

3.2. Safety Test in Commercial Broiler Chickens

FAdV inocula treated at the 32-hour (h) and 36 h periods were found to be safe in commercial broiler chickens, with no clinical signs associated with FAdV infection observed.

3.2.1. Body Weight of Chickens

The initial body weight of the day-old chicks was 0.074 kg ± 0.003. On day 14 post-inoculation (pi), the body weights of the chickens in the Control Group, Group A (FAdV treated at 36 h), and Group B (FAdV treated at 32 h) were 0.753 kg ± 0.017, 0.700 kg ± 0.027, and 0.678 kg ± 0.022, respectively (Figure 2). After 28 days pi, the body weights of the chickens in the control, A1 (FAdV treated at 36 h, non-booster group), A2 (FAdV treated at 36 h, booster group), B1 (FAdV treated at 32 h, non-booster group), and B2 (FAdV treated at 32 h, booster group) were 1.888 kg ± 0.101, 1.646 kg ± 0.165, 1.893 kg ± 0.061, 1.813 kg ± 0.060, and 2.103 kg ± 0.027, respectively. There were no significant differences ($p > 0.05$) in body weight among all groups at both day 14 pi and day 28 pi. Additionally, there were no significant differences ($p > 0.05$) in body weight between the non-booster (Groups A1 and B1) and booster (Groups A2 and B2) groups.

3.2.2. Gross and Histopathological Findings

Upon necropsy, the livers of day-old chicks were observed to be yellowish, while the trachea appeared normal relative to the chicks' size. At days 14 and 28 pi, all livers exhibited a dark and glistening appearance across all groups, while the trachea remained normal. No gross lesions were observed in the liver and trachea samples from any group at both days 14 and 28 pi. Histopathological examinations revealed no significant findings in either the liver or trachea for all groups at days 0, 14, and 28 pi. The livers retained their normal architecture, with no intranuclear inclusion bodies present in the hepatocytes. Similarly, there was no evidence of inflammatory cells in the tracheal tissue.

3.2.3. FAdV Antibody Response

The initial FAdV antibody titer of day-old chicks was 4469±1175 (Figure 3). For the control groups, the FAdV antibody titers on days 14 and 28 were 112 ± 106 and 147 ± 60, respectively. On day 14 pi, the antibody titers for the chickens in Groups A and B were 78 ± 35 and 490 ± 360, respectively, which were not significantly different from each other ($p > 0.05$). On day 28 pi, the antibody titers for the control group and non-booster inoculated groups A1 and B1 were 1 ± 0, 321 ± 189, and 690 ± 484, respectively; the FAdV antibody titers of Groups A1 and B1 were significantly higher ($p < 0.05$) compared to the control group. Likewise, the antibody titers for the booster groups A2 (602 ± 367) and B2 (874 ± 317) were significantly elevated ($p < 0.05$) compared to the control group. However, there was no significant difference ($p > 0.05$) in antibody titers between the booster and non-booster groups, regardless of the period of inactivation time.

4. Discussion

The present findings demonstrate that complete inactivation of the FAdV isolate, representing other non-enveloped DNA viruses (18), requires suitable chemical treatment, specifically with binary ethyleneimine (BEI).

Table 1: Effect of 0.002M binary ethyleneimine (BEI) on the infectivity of FAdV in SPF chicken embryonated eggs (CEE)

BEI	Replication	Inactivation time (h)	Number of dead embryo/Inoculated egg
0.002M	1	20	3/4
		24	1/4
		28	2/4
		32	0/4
		36	0/4
	2	20	2/4
		24	1/4
		28	4/4
		32	0/4
		36	0/4
	3	20	2/4
		24	2/4
		28	1/4
		32	0/4
		36	0/4

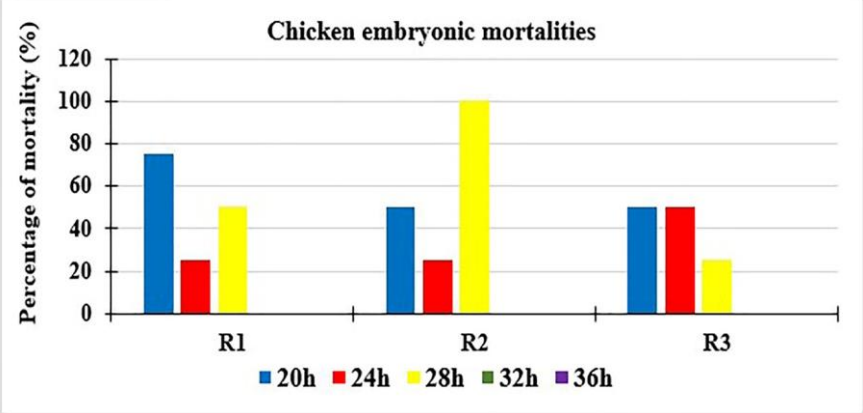

Figure 1: Percentage mortality (%) of SPF chicken embryonated eggs (CEE) following inoculation with treated FAdV inoculum with binary ethyleneimine (BEI) at 20 hours (h), 24h, 28h, 32h and 36h.

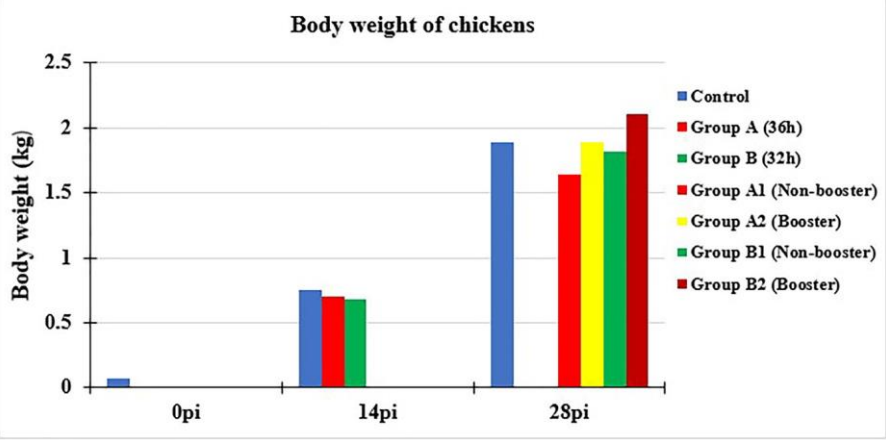

Figure 2: Mean body weight of chickens at days 0, 14 and 28 post-inoculation (pi) between FAdV inoculums treated with binary ethyleneimine (BEI) at 32 hours (h) (group B), 36h (Group A) and control groups.

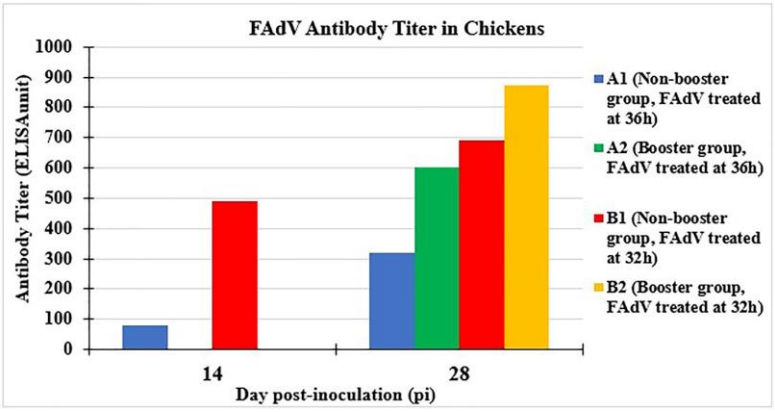

Figure 3: Mean FAdV antibody titer of chickens at days 14 and 28 post-inoculation (pi) between booster and non-booster groups following inoculation with FAdV inoculums treated with binary ethyleneimine (BEI) at 32 hours (h) (group B) and 36h (Group A).

The periods of BEI treatment at a concentration of 0.002 M in the FAdV inoculum at 20 h, 24 h, and 28 h showed evidence of FAdV infectivity, resulting in embryonic mortality rates ranging from 25% to 100%, indicating incomplete virus inactivation during these intervals. Conversely, FAdV was completely inactivated at 32 h and 36 h, with no infectivity observed in embryos throughout the trial. Compared to previous studies, which indicated that a longer inactivation period of 72 h was necessary to achieve optimal inactivation of FAdV serotype 2 isolates using a higher concentration of 0.01 M BEI (10), it appears that different serotypes of FAdV necessitate varying concentrations of BEI to achieve optimal inactivation, potentially due to differences in the capsid structure of the virus prior to genome passage. In the case of enveloped viruses, such as the avian influenza H5N1 isolate, the application of a high concentration of BEI at 0.01 M induces rapid virus inactivation within 6 h, while a lower concentration of 0.001 M requires 24 h for complete inactivation (19). The selection of a chemical for virus inactivation during vaccine development is crucial for preserving the virus structure and allowing for the induction of virus-neutralizing antibody responses (20). Alkylating agents such as β-propiolactone (BPL), BEI, and radiation agents such as gamma irradiation and ultraviolet light primarily act on the viral genome and are likely to preserve viral-neutralizing epitopes. In contrast, cross-linkers such as formaldehyde, glutaraldehyde, and 2,2'-dithiodipyridine, or denaturing procedures involving pH and temperature, act on viral proteins and can modify viral-neutralizing epitopes, potentially leading to protein degradation (7). In this study, BEI was used for the development of inactivated FAdV vaccines due to its stability and low reactivity with proteins, thus preserving the antigenic components of the virus (10, 21). BEI inactivates non-enveloped viruses by targeting the viral RNA/DNA genome. Ethylenimine, the active chemical reagent in the aziridine group of BEI, specifically interacts with the nucleic acids on viral RNA/DNA, while exhibiting minimal reactivity with other proteins present in the virus (22). This specificity renders non-enveloped viruses more challenging to inactivate, necessitating longer exposure times, as demonstrated in this study. In contrast, BEI is a photoactive hydrophobic alkylating compound that covalently binds to the hydrophobic domains of viral proteins present in the envelope lipid bilayer for enveloped viruses (7). This process renders the enveloped virus non-infectious by disrupting its fusion proteins on the lipid bilayer, preventing binding to receptor molecules and thereby inhibiting its ability to infect (23). Thus, enveloped viruses are generally easier and faster to inactivate, as evidenced by previous studies involving Newcastle disease virus isolates, which were inactivated within 18 h using 0.001 M BEI (24). The safety of the inactivated FAdV treated with BEI at 32 h and 36 h, formulated with Montanide ISA 71 VG adjuvant, was further assessed in commercial broiler chickens as a vaccine candidate. No clinical signs or gross or histological lesions were observed in either group inoculated with inactivated FAdV at 32 h and 36 h. Similarly, the control groups exhibited no significant findings throughout the trial. The body weight of the chickens increased significantly in all groups up to day 28 pi. Based on the broiler performance objectives and guidelines from Cobb Vantress (25), female and male Cobb 500 should weigh 1.4 kg and 1.6 kg, respectively, by 28 days of age, as recorded in the present study. Therefore, these results indicate that inoculation with the inactivated FAdV inoculum did not adversely affect the growth performance of the broiler chickens. Based on the findings regarding FAdV antibody titers, chickens in the control group (C) exhibited the highest FAdV antibody titer (9605 ± 3555) on day 0 post-inoculation (pi) due to the presence of maternal-derived antibodies. However, this titer dropped significantly by day 14 pi (112 ± 106) and further to day 28 pi (147 ± 60). On day 14 pi, chickens administered the inactivated FAdV at 32 hours in Group B produced a higher antibody response (490 ± 360) compared to those receiving the inactivated FAdV at 36 hours in Group A (78 ± 35) and the control group. Similarly, on day 28 pi, chickens in the booster Group B, following inoculation with the inactivated FAdV at 32 hours, exhibited the highest antibody response compared to the other groups. Although there were no significant differences between the

booster and non-booster groups, this suggests that the booster dose is crucial for inducing antibody titers that confer full protection against infectious bursal disease (IBH) in commercial farms. Additionally, administration of the treated FAdV inoculum during both periods in chickens receiving the booster dose did not adversely affect their performance throughout the trial. Supporting this, previous studies on vaccinated chickens reported no obvious clinical signs or pathological damage in either single or double immunization groups, indicating the safety and adequate protection provided by the vaccine. In conclusion, a 32-hour inactivation period for the FAdV serotype 8b isolate UPM1137 using binary ethyleneimine (BEI) at a concentration of 0.002 M is optimal. The inactivated FAdV at 32 hours, formulated with Montanide ISA 71 VG adjuvant, is safe and immunogenic in commercial broiler chickens when administered via subcutaneous injection. Thus, this inactivated FAdV shows significant potential as a vaccine candidate against IBH in local poultry farms.

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Authors' Contribution
NMS performed conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, validation, visualization and writing for review and editing. AQMA involves in investigation, methodology, software and writing for original draft. MHB and NYAR performed conceptualization, supervision, validation and visualization. All authors read and approved the final manuscript.

Ethics
The authors declare that all the ethical considerations were observed in animal experiments and the preparation of the manuscript.

Conflict of Interest
No conflict of interest is declared

Data Availability
The data that support the findings of this study are available on request from the corresponding author.

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