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Comparative Sequence Analysis, Pathogenicity and Immunogenicity of Attenuated Fowl Adenovirus Isolates as Experimental Vaccines against Inclusion Body Hepatitis of Commercial Broiler Chickens

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A R T I C L E I N F O

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A B S T R A C T

Introduction: Fowl adenoviruses (FAdV) cause inclusion body hepatitis (IBH) in chickens and new vaccination strategies against IBH are needed as an effective control measure in the poultry industry. **Methods:** The attenuated FAdV isolates from from chicken embryonated (UPM1137E20) and cell culture (UPM1137CEL35) were evaluated and compared based on sequence analysis of hexon and fiber genes. Their pathogenicities and immunogenicities were then determined in the commercial broiler chickens. Groups of chicken were inoculated with 0.5ml chicken-embryonated-derived attenuated FAdV isolates via oral and IP routes and sera, trachea, liver and gizzard samples were collected at days 3, 7, 14 and 21post-inoculation. **Results:** Molecular analysis revealed both isolates had 99.1% and 97.3% homologies in the L1 loop region of hexon gene and knob region of fiber gene, respectively. Molecular changes in UPM1137E20 were prominent in the knob of fiber gens with 3 amino acid changes, while for UPM1137CEL35, notable in the L1 loop region with 3 amino acid changes. It was demonstrated that both attenuated isolates are non-pathogenic and safe in commercial broiler chickens. Neither gross nor histopathological lesions were recorded in all tested groups. Both isolates induced high antibody response significantly via intraperitoneal route when compared to the control. **Conclusion:** UPM1137E20 isolate had a high potential to be further evaluated as a live attenuated vaccine against viral poultry diseases such as IBH.

Citation:

INTRODUCTION

Fowl adenoviruses (FAdV) are non-enveloped DNA viruses that have been classified into 5 molecular species (A-E) and 12 serotypes. Based on the International Committee on Taxonomy of Viruses (ICTV) nomenclature, FAdV are comprised of groups A (FAdV-1), B (FAdV-5), C (FAdV-4 and -10), D (FAdV-2,-3,-9 and -11), and E (FAdV -6,-7,-8a and -8b)[1]. FAdV mostly infect broiler chickens at age of 3 to 6 weeks and are readily transmitted both horizontally by the fecal-oral route and vertically by embryonated eggs [2, 3]. The virus agent has been reported as a hardy virus, ubiquitous, and highly resistant to most disinfectants [4]. FAdV are a major threat among the avian viruses and have been identified as a causative agent of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS) and gizzard erosion, in the poultry industry [5, 6]. These diseases cause significant economic losses to commercial poultry farms, globally [4, 7]. In Malaysia, FAdV serotype 8b is a primary agent of IBH and gizzard erosion outbreaks in the local commercial poultry industry [8]. To date, the number of clinical cases of IBH have been increased over a year in Malaysia due to unavailable vaccines against the primary FAdV pathogen [9, 10]. Thus, there is a need to develop a suitable local vaccine against the disease.

FAdV are routinely diagnosed in chicken embryonated eggs or cell cultures, typically primary chicken embryo liver (CEL) cells, chicken embryo kidney (CEK) cells, or chicken kidney (CK) cells due to the high sensitivity of the medium [11-13]. Adaptation and attenuation of high pathogenic FAdV in cell culture or chicken embryonated eggs are necessary for alteration of FAdV genes to diminish virus virulence [8, 10]. Consecutive passages of FAdV in alternative host induces virus attenuation with molecular changes in hexon and fiber genes [8, 14].

Molecular analysis followed by animal testing have been used extensively as a tool for detection of virus infectivity between the passaged isolates, specifically based on the L1 loop

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region in hexon gene and knob of the fiber gene [8, 10]. Recently, genetic diversity of FAdV along with the emergence of new strains have enhanced the challenge of vaccine efficacy against the pertaining diseases in the chicken farms [4, 15]. Thus, several studies for the vaccine development have been attempted for control and prevention against various serotypes and strains of FAdV worldwide [16-18].

To date, the assessment of attenuated FAdV isolates from different alternative hosts has remained scanty since most of the works have involved only a single attenuated strain [14, 19]. To clarify the best vaccine candidate, knowledge on the molecular characteristics, the virus infectivity, and the immune response in the chicken are crucial for excellent protection against such diseases in commercial poultry farms. The objective of this study was to evaluate and compare the attenuated isolates from chicken embryonated (UPM1137E20) and cell culture (UPM1137CEL35) on sequence analysis of hexon and fiber genes and to determine their pathogenicity and immunogenicity in the commercial broiler chickens.

MATERIALS AND METHODS

Ethical Statement

Ethical approval for treatment of the chickens was granted under the approval of the Institutional Animal Care and Use Committee (IACUC), Universiti Putra Malaysia with AUP No. R072/2015.

FAdV Isolate

FAdV isolate, UPM1137 was originated from an outbreak of IBH and gizzard erosion in 25-to-27-week-old commercial layer chickens in Malaysia. Samples of the liver were cultivated into chicken embryonated eggs (CEE) for 2 passages and the homogenate liver was named UPM1137E2. This isolate was subsequently passaged into CEE 20 times and the virus suspension at the 20th passage was named UPM1137E20 [8]. In addition, UPM1137E2 isolate was propagated into primary chicken embryo liver (CEL) cells for 35 passages and named as UPM1137CEL35, as described in the previous works [10]. The UPM1137CEL35 isolate was confirmed as attenuated in SPF chickens, following propagation into cell culture [10]. The virus titers of the UPM1137E20 and UPM1137CEL35 isolates were $10^{8.7}$ 50% tissue culture infective dose ($TCID_{50}$)/ml and $10^{6.7}$ TCID50/ml, respectively, based on Reed and Muench protocol [20].

Sequence Analyses

Nucleotide and amino acids sequences for UPM1137E2, UPM1137CEL35, and UPM1137E20 were retrieved from NCBI GenBank database for comparative analysis for hexon and fiber genes. For hexon gene, accession number were as follows: KF866370 (UPM1137E2), KY305949 (UPM1137CEL35) and KY911368 (UPM1137E20). The sequences for the fiber genes sequences were KY305950 (E2), KY305957 (CEL35), and KY911372 (E20). All the sequences belonged to Fowl adenovirus group E under serotype 8b [8, 10]. ClustalW program in Bioedit Version 7.0.9 software was used for detection of nucleotide and amino acids differences between the attenuated isolates. Sequence identity matrix was calculated by Bioedit software to determine the percentage of nucleotide homologies between the isolates.

Experimental Design of Pathogenicity and Immunogenicity

Hundreds of 4-day-old commercial broiler chickens (Cobb500) were divided into five major groups, namely, A1, A2, B1, B2, and C. Each group of chickens was separated into individual cages in three different rooms in Animal Research Facility (ARF), Faculty of Veterinary Medicine, UPM Serdang. Each group was divided further into two groups (i.e., the sacrificed and the mortality groups). Twenty chickens were assigned in each group with 16 chickens labeled as sacrifice group and 4 chickens in the mortality group. Whereas 24 chickens were assigned in group C with 20 chickens in the sacrifice group and 4 chickens in the mortality groups. All chickens in the mortality group were used to determine the percentage of mortality throughout the trial. At a day old of age, all chickens in group A1 and A2 were inoculated with 0.5ml chicken-embryonated-derived attenuated FAdV isolate, UPM1137E20 with titer $10^{8.7}TCID_{50}$/ml via oral and intraperitoneal (IP) route, respectively. Another 40 chickens in groups B1 and B2 were inoculated with a cell culture derived from attenuated FAdV isolate UPM1137CEL35 with titer $10^{6.7}TCID_{50}$/ml via oral and IP routes, respectively. All chickens in Group C remained uninoculated and used as a control group. All chickens were observed daily until 3 weeks post-inoculation (pi) for any abnormality throughout the trial. Water and feed were given ad-libitum until the end of the experimental period. Prior to inoculation at day 0pi, 4 chickens in group C were sacrificed and followed by sampling at day 3, 7, 14, and 21pi in all groups. Prior to sacrifice by cervical dislocation, the body weight and blood samples of the chickens were recorded.

Clinical Signs and Mortality Observation

Throughout the trial, chickens in all groups were monitored daily for mortality and clinical signs of FAdV infection such as diarrhea, depression, inappetence, and recumbence until 21 days pi [21].

Gross and Histopathological Examinations

Upon necropsy, any gross changes were recorded throughout the trial. Sample of trachea, liver, and gizzard were collected and fixed into 10% buffered formalin for histological examination. All samples were stained with haematoxylin and eosin [22] and examined under the light microscope.

Detection of FAdV Antibody Titer

Serum samples were collected on day 0 for the control group (Group C) and day 3, 7, 14 and 21pi for all other groups. Detection of FAdV antibody response was done by using a commercial ELISA kit (BioChek, Ltd., UK) according to the manufacturer`s instruction.

Statistical Analysis

Mean antibody titers from all the groups were analyzed by IBM SPSS Statistics version 22. Following the significant outcome with value of $P < 0.05$, a multiple group comparison test was performed using Dunnett`s test to compare means between inoculated and control groups. For within-group means, Tukey HSD (Honest Significant Difference) test was applied [23]. An independent t-test was conducted to compare means between two groups [24].

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RESULTS

1) Sequence Analysis Between Attenuated and Original Isolates

Nucleotide sequences

Comparison of L1 loop region in nucleotide sequences between the attenuated and the original isolates (UPM1137E2), revealed 99.4% and 99.3% homology recorded in E20 (UPM1137E20) and CEL35 (UPM1137CEL35), respectively (Table 1). Based on the entire 1166 nucleotide bases, both isolates exhibited 99.6% homologies compared to the original isolate. The homology between the attenuated isolates based on the L1 loop region was 99.1%, compared to 99.4% homology on the entire length of partial hexon gene.

Based on the nucleotide sequence of knob region in fiber gene, comparison with original isolate (UPM1137E2) indicated 97.3% homology in E20 (UPM113E20) and CEL35 (UPM1137CEL35). The homology based on the entire 1094 nucleotide bases revealed 99.4% homologies between the passaged isolates and the original isolates. Analysis between the passaged isolates showed 99.2% homology based on the entire length of partial fiber genes; however, only 97.3% homology based on the knob region.

Table 1: Sequence homology matrix (%) between the original isolate (UPM1137E2) and the attenuated FAdV isolates (UPM1137E20 and UPM1137CEL35).

	FAdV Passaged Isolate		Comparative analysis between UPM1137E20 and UPM1137CEL35
	UPM1137E20[a]	UPM1137CEL35[b]	
Hexon gene			
Nucleotide (%)	99.6	99.6	99.4
Nucleotide at L1 loop (%)	99.4	99.3	99.1
Amino acid (%)	99.2	99.2	98.4
Amino acid at the L1 loop (%)	98.9	98.4	97.4
Fiber gene			
Nucleotide (%)	99.4	99.4	99.2
Nucleotide at knob region (%)	97.3	97.3	97.3
Amino acid (%)	99.1	98.9	98.6
Amino acid at knob region (%)	93.8	95.9	93.8

[a]UPM1137E20: Passaged 20 isolate in chicken embryonated derived
[b]UPM1137CEL35: Passaged 35 isolate in cell culture-derived

Deduced Amino Acid Sequences

Considering the deduced amino acid sequences, the homology between UPM1137E20 and UPM1137E2 were 99.2% and 98.9% based on the entire partial hexon gene length and L1 loop regions, respectively (Table 1). Analysis of UPM1137CEL35 isolate revealed 99.2% homology based on the entire partial hexon gene; however, only 98.4% homology in the region of the L1 loop compared to UPM1137E2 isolate. Comparison of amino acid sequence between the passaged isolates exhibits 98.4% and 97.4% homologiess, in the entire 388 deduced amino acid and in the L1 loop region, respectively.

For fiber gene analyses, the sequence homologies of UPM1137E20 and UPM1137CEL35 isolates were 99.1% and 98.9%, respectively, on the entire partial 364 deduced amino acids compared to that UPM1137E2 isolate. Adding to that, high amino acid variability was recorded in the knob region with only 93.8% and 95.9% homologies in UPM1137E20 and UPM1137CEL35 isolates, respectively, compared to the UPM1137E20 isolate. Our data of the passaged isolates in the current study showed 98.6% homology based on the entire partial fiber genes and 93.8% homology in the knob region.

Molecular Changes between the Attenuated FAdV isolates

Based on the nucleotide sequence analyses, several molecular changes were detected in hexon and fiber genes for the attenuated isolates (UPM1137E20 and UPM1137CEL35) compared to that original isolate (UPM1137E2). The L1 loop region was determined at position 6 to 601 nucleotide (nt) bases. Analysis of the nt sequences revealed 1nt substitution was identified in both isolates at position T90C. For UPM1137E20, additional 3 nt changes were detected at A147G, C199T, and A1134T, where 2 out 3 nt bases were located within the L1 loop region. Compared to the isolate attenuated in the cell culture (UPM1137CEL35), the substitution of other 3nt bases occurred within the L1 loop region at positions A133T, C400T, and T556A.

Molecular analyses of the nt sequences in the fiber gene involved regions of the tail, the shaft, and the knob at positions 1-66, 67-907, and 946-1094, respectively. There are 6nt base substitutions at position 879(A-G), 918(T-C), 952(C-G), 964(A-T), 1062(A-C), and 1078(G-C) where 4 out 6 bases located within the knob region for UPM1137E20 isolate. On

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the other hand, 6nt bases substitution at position 556(T-C), 821(T-A), 1042(A-C), 1050(A-C), 1062(A-C), and 1078(G-C) for UPM1137CEL35 isolate were detected. It is shown that those changes were detected in both the shaft and the knob regions with 2nt and 4nt bases, respectively.

Based on the amino acid sequence analyses, the variable loop, L1, in hexon gene was determined at positions 2 to 199 in the amino acid sequence. In the UPM1137E20 isolate, 2 out of 3 amino acid substitutions were noticed in the L1 loop region at positions T49A and A66V, whereas the other located at M378L was excluded from the L1 loop region. On the other hand, all 3 amino acid substitutions in UPM1137CEL35 were located in the L1 loop regions at D44V, S133F, and V185E.

Analysis of the deduced amino acids in fiber gene sequences involved the tail, the shaft, and the knob regions at positions 1-22, 23-303, 316-364, respectively. Amino acid substitution at position A360P in the knob region was detected in both attenuated isolates. Two amino acid changes in UPM1137E20 at position P318A and N322Y were in the knob region. Compared to the UPM1137CEL35 isolate, substitution of amino acid at position T348P in the knob region and other 2 amino acid changes in the shaft area were identified at positions L189P and F274Y.

2) Clinical Signs and Mortality

Neither clinical signs nor mortality were recorded in all the studied groups throughout the trial until day 21 pi.

3) Gross and Histological Lesions

Liver and gizzard samples were normal in all the groups without any significant changes until day 21 pi. Grossly, the livers appeared in normal size, brown color with a firm texture and a smooth and glistening surface after inoculation with the both passaged isolates and the control groups. The gizzard of the chickens showed a normal and intact koilin layer without gross changes in all the groups. Microscopically, the liver sample appeared as normal architecture and hepatocytes lining by sinusoid without histological changes in all the groups.

4) FAdV Antibody Response in Commercial Broiler Chickens

The antibody titers in the control group were 7795 ± 1414, 4776 ± 1170, 8536 ± 1542, and 2445 ± 1472 at days 0, 3, 7, and 14 days pi, respectively, with an exception at day 21pi due to no detectable antibody. For group A1, inoculated with UPM1137E20 via the oral route, the antibody titers were 3946 ± 1704, 3742 ± 1946, 3278 ± 1997, and 1700 ± 1332 at day 3, 7, 14, and 21pi, respectively. The antibody tires for group A2 (IP route) were 5581 ± 2641, 2168 ± 441, 5746 ± 2034, and 1833 ± 792, at day 3, 7, 14, and 21 pi, respectively (Fig. 1A). Both groups had an identical pattern of antibody titer throughout the trial. Thus, there were no significant differences ($P > 0.05$) in mean antibody response between the route of inoculation and day post-inoculation.

On the other hand, the antibody titers in group B1 inoculated with UPM1137CEL35 via the oral route were 4943 ± 2049, 2243 ± 433, 2595 ± 1326, and 190 ± 136, at day 3, 7, 14, and 21 pi, respectively. For group B2 (IP), the antibody titers were 4188 ± 1310, 3634 ± 2315, 2866 ± 1281, and 2348 ± 1800 at 3, 7, 14, and 21 pi, respectively. Both groups had identical patterns of antibody titer throughout the trial regardless of differences in route of inoculation. There are no significant differences in mean antibody response between day post-inoculation and route of inoculation.

Mean antibody responses between-groups were not significantly different from days 3 to 14 pi throughout the trial. However, at day 21pi, there was a significant difference of mean antibody response between groups via the IP route. The antibody response was significantly higher in group A2 (1833 ± 792) and B2 (2348 ± 1800) when compared to group C (not detected; Fig. 1B. Statistical analysis revealed there is no significant different in mean antibody response between the type of inoculum for either chicken embryonated or cell culture origin isolates.

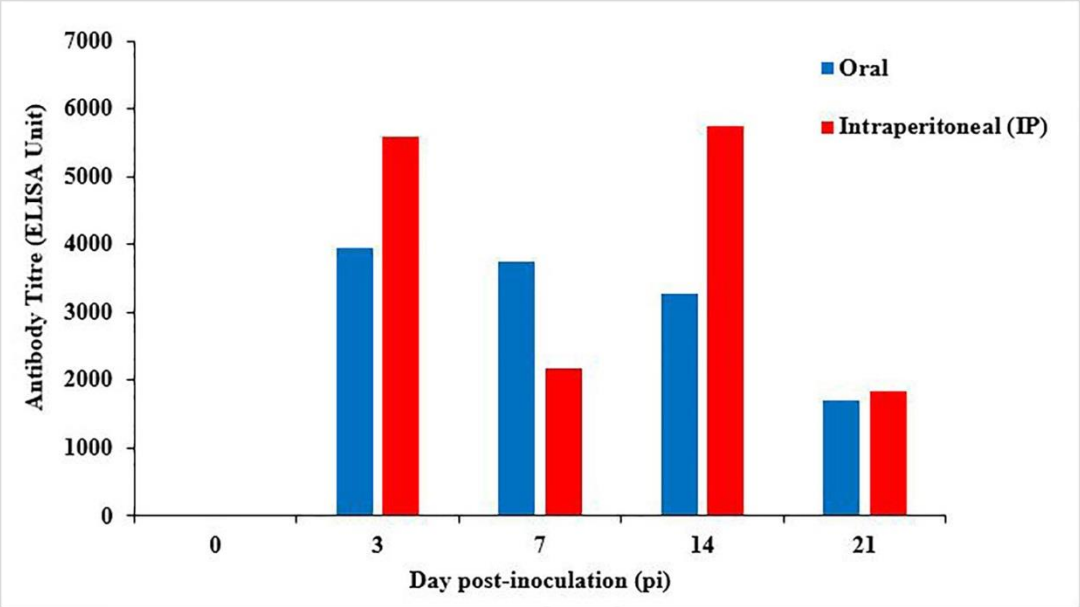

Fig. 1A. FAdV antibody titer in group A inoculated with chicken-embryonated attenuated isolate, UPM1137E20 at days 3, 7, 14 and 21 days post-inoculation (pi) via oral and IP routes. Both route of inoculation had an identical pattern of antibody titer throughout the trial. Thus, there are no significant differences ($P > 0.05$) in mean antibody response between the route of inoculation and day post-inoculation.

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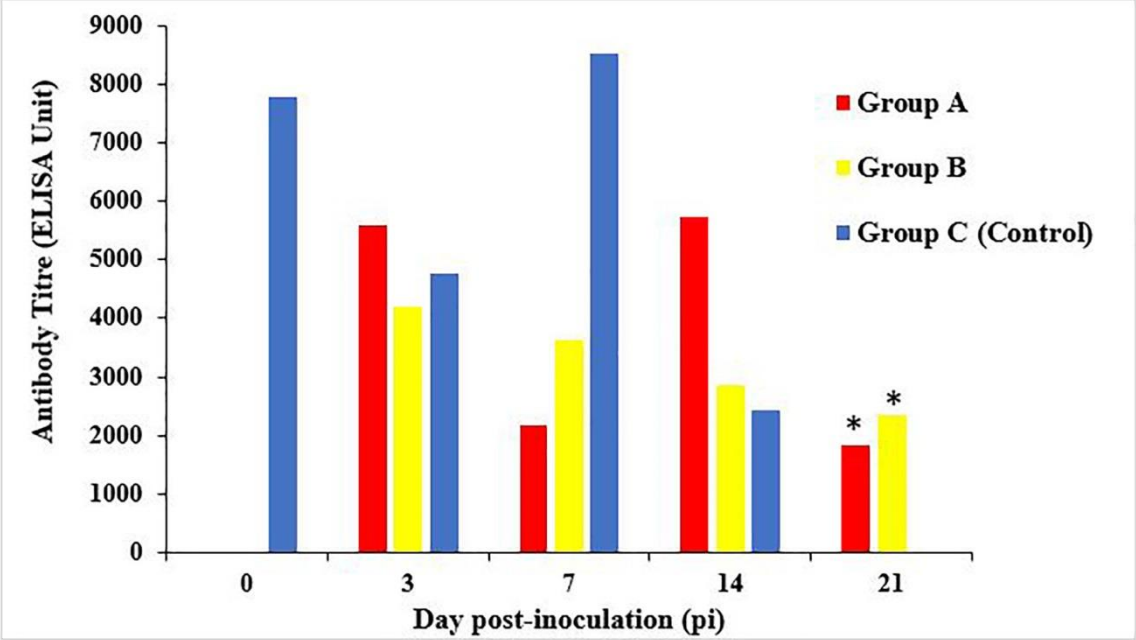

Fig. 1B. FAdV antibody titer between groups A, B, and C following 21 days post-inoculation (pi) via IP route. Group A inoculated with chicken-embryonated attenuated isolate, UPM1137E20. Group B inoculated with cell-culture derived attenuated isolate, UPM1137CEL35. Chickens in group C remained uninoculated throughout the trial. The antibody titer of FAdV was significantly high in Groups A and Group B compared to that in the control group at day 21pi (*$P < 0.05$).

DISCUSSION

Molecular analysis of nt sequences between attenuated FAdV isolates revealed 99.1% homology in the L1 loop region of the hexon gene and 97.3% homology in the knob region of the fiber gene. Similarly among the isolates, for the deduced amino acids, they had 97.4% and 93.8% homologies in the L1 loop and knob regions, respectively. This showed high amino acid variability in the fiber genes due to the protruding structure of the protein compared to hexon protein which constitutes large surfaces of the viral capsid [25].

Comparative sequence analysis between the attenuated FAdV isolates from chicken embryonated eggs, UPM1137E20, cell culture and UPM1137CEL35, based on nucleotide and deduced amino acids exhibited several molecular changes in both hexon and fiber genes at varied positions. Interestingly, both isolates induced similar nucleotide changes at T90C in L1 loop regions of hexon gene and amino acid substitution at A360P in the knob region of the fiber gene regardless of the types of media used for the virus attenuation. It seems that molecular changes in the current finding could be used as an indicator of adaptation of the genes in both alternative media [8].

Numerous molecular changes were detected in an attenuated isolate from chicken-embryonated derived, UPM1137E20, with 2 amino acid substitutions in the variable L1 loops of hexon gene and 3 amino acid changes in the knob of the fiber gene. On the other hand, for cell culture-derived attenuated isolate (UPM1137CEL35), amino acid changes were prominent in the L1 loop regions with 3 amino acid substitutions and 2 amino acid changes in the knob regions. This indicated that the molecular changes in UPM1137E20 are notable in the knob region of the fiber gene; however, for UPM1137CEL35, the changes were pronounced at the L1 loop region of the hexon gene. Furthermore, the isolate from cell culture required more passages to induce molecular changes, up to 35 times, compared to the chicken embryonated-derived isolate with 20 passages only. The current finding is compatible with a previous work, conducted by Mansoor et al. [14] which required 16 passages for attenuation of FAdV isolate in the chicken embryos. The differences in the media used for the virus attenuation perhaps can influence the passage number to induce the viral mutation by the major capsid proteins [8, 10].

It was demonstrated that both attenuated FAdV isolates were non-pathogenic in commercial broiler chickens without disease manifestations throughout the trial. Neither gross nor histopathological lesions were observed in all the groups. Trachea, liver, and gizzard tissues were normal without any significant changes in the inoculated chickens from day 3 to 21post-immunization similar to the control or the uninoculated group. The isolates were immunogenic and induced FAdV antibody response in the inoculated chickens.

Moreover, molecular changes existed in hexon and fiber genes that caused a significant impact on the virus infectivity in broiler chickens and were unable to induce disease due to low virulence after continuous passages in the cell culture and chicken embryos [10, 21]. Although earlier studies speculated that only the fiber gene is important for the virus virulence, it has been proved that those changes in the knob of the fiber gene along with a variable L1 loop in hexon gene are highly related to the attenuation of the gene based on animal trial findings [8]. Thus, both isolates were safe regardless of different inoculums used in commercial broiler chickens. The current finding was in agreement with previous studies in which passaged isolates induced low pathogenicity without mortality in chickens [14, 26]. As compared to the field isolate, high mortality and severe depression had been observed in the infected chickens as shown in various studies [27-29].

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The assessment of the antibody response between the attenuated FAdV isolates revealed that UPM1137E20 isolate induced high antibody response via oral and IP routes at day 21 pi without interference by the maternally-derived antibodies. Live attenuated vaccines are typically applied by drinking or spray methods in commercial farms, thus, the UPM1137E20 isolate has high potentials to be used as a vaccine candidate in the future [14, 19]. However, based on the level of antibody titer for the cell culture-derived isolate, (UPM1137CEL35), it induced a high antibody response compared to that chicken embryonated derived isolate via IP route at day 21 pi. This finding was in agreement with Steilz et al. [30] since the parenteral route induced a satisfactory level of antibody response than other routes. Thus, the UPM1137CEL35 isolate had high potential to be used for hatchery vaccination in day-old chicks against FAdV diseases via the parenteral route and perhaps confers full protection at an early age of chickens [10, 31]. Further efficacy study is necessary to evaluate the effectiveness of this attenuated isolate against a challenge by FAdV strains.

In addition, it showed that chicken inoculated with an attenuated isolate from chicken embryonated-derived UPM1137E20, had a high antibody response at day 3 to 21 pi via both routes. As compared to the group inoculated with UPM1137CEL35, the isolate induced high antibody response via IP than the oral route, typically at day 21 pi. Increased antibody titers is perhaps due to direct absorption of antigen to the systemic circulation after circumventing from the neutralization process from the maternally derived antibody [21, 32]. However, the antibody titers were not significantly different between the attenuated FAdV isolates throughout the trial. The declined pattern of antibody titer at day 21pi via an oral route from UPM1137CEL35 isolate is perhaps as a result of neutralization of the viral antigens by the mucosal immunity lining the GIT tract, particularly at gut-associated lymphoid tissue (GALT) and thus, the antigens were unable to replicate in the target organ for induction of an antibody response [33].

The high antibody titer in group A1 (1700 ± 1332) was observed after being inoculated with UPM1137E20 via oral route at day 21pi compared to group B1 (190 ± 136). It seems that differences in antibody titer are perhaps highly associated with virus doses given to chicken at day-old of age. The UPM1137E20 isolate had a high virus titer with 108.7TCID50/ml due to the origin of inoculum from homogenate liver embryos which was more concentrated than attenuated cell culture isolate (UPM1137CEL35) with 106.7TCID50/ml titer. High virus concentration was sufficient enough to cause escaping from the mucosal immunity and further replication in the target organ. This current finding was compatible with the previous study as described by Ojkić and Nagy [34]. For future recommendations, challenges and protection studies are essential to evaluate the effectiveness of both attenuated isolates against the disease associated with FAdV infection.

In conclusion, attenuated FAdV isolates, UPM1137E20 and UPM1137CEL35 exhibited several molecular changes in the hexon and fiber genes which caused a significant impact on the virus infectivity in commercial broiler chickens. Both isolates induced significant antibody response via the parenteral route; thus, the attenuated FAdV isolates exhibited high potentials to be used for future hatchery vaccination against the disease. Moreover, it was concluded that UPM1137E20 isolate had a high potential to be used for a future live attenuated vaccine against the disease.

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CONFLICT OF INTEREST

The authors declare they have no conflict of interests.

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