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Molecular Characterisation of Partial Structural Genes of Fowl Adenovirus Serotype 8b UPMT1901 Field Strain Isolate Associated with the Inclusion Body Hepatitis in Malaysia's Commercial Broiler Chickens

Bahiyah Azli[1], Nur Farhana Salim[1], Abdul Rahman Omar[3], Mohd Hair-Bejo[3], Norfitriah Mohamed Sohaimi[4] and Nurulfiza Mat Isa[1,2]*

[1]Laboratory of Vaccine and Biomolecules, Institute of Bioscience, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[2]Department of Cell and Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Pura Malaysia, 43400 Serdang, Selangor, Malaysia
[3]Department of Veterinary Pathology and Microbiology, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia
[4]Department of Veterinary Medicine, Faculty of Veterinary Medicine, Universiti Putra Malaysia, 43400 Serdang, Selangor, Malaysia

ABSTRACT

Fowl adenovirus (FAdV) is reported to pose a severe risk to the poultry industry, affecting food and nutrient security nationally and globally. FAdV is identified as the primary pathogen for inclusion body hepatitis (IBH) disease in avians during outbreaks in farms. Numerous interventions have been employed to reduce the chicken's mortality rate in future outbreaks, such as local autogenous vaccine production that has yet to be successfully commercialised. Fibre and hexon protein are two out of the three major components of the adenoviral capsid, identified to contribute towards FAdV virulence. Hence, this study aims to determine the fibre and hexon gene molecular changes of a local isolate, FAdV UPMT1901, in Malaysia's recent IBH outbreak and identify the evolutionary relationship with known FAdV strains. Propagation of FAdV UPMT1901 was performed in specific pathogen-free embryonated chicken prior to genomic extraction. The genes were amplified, and the retrieved nucleotide sequences were aligned with the published FAdV sequences. The phylogenetic tree analysis showed that UPMT1901 partial fibre and hexon genes are 99% similar to other known FAdV serotype 8b (FAdV-8b) species, especially the published Malaysian FAdV-8b isolates. Interestingly, the amino

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E-mail addresses:
bahiyahazli@gmail.com (Bahiyah Azli)
nur.farhanasalim97@gmail.com (Nur Farhana Salim)
aro@upm.edu.my (Abdul Rahman Omar)
mdhair@upm.edu.my (Mohd Hair-Bejo)
fitriahsohaimi@upm.edu.my (Norfitriah Mohamed Sohaimi)
nurulfiza@upm.edu.my (Nurulfiza Mat Isa)
*Corresponding author

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acid residue analysis further supported consistent residues amongst Malaysian isolates at fibre positions T176A, Q195H, D213E, S243T, A258V, F335Y, and F353V and hexon position T422M. These findings elucidate the structural proteins' functional capacity and molecular diversity, specifically amongst Malaysian FAdV isolates and FAdV-8b, while contributing to global initiatives to establish biosecurity, such as a vaccine or antiviral production against future outbreaks.

Keywords: Adenovirus, FAdV, fibre, fowl adenovirus, hexon, inclusion body hepatitis, phylogenetic analysis, serotype

INTRODUCTION

Malaysia is among the top global consumers of poultry meats and eggs as 63 kg of poultry per capita consumption (PPC) volume was reported in the year 2019 (Kinsley, 2020), followed by a record of 66.9 kg (PPC) in the year 2021 (Department of Statistics Malaysia [DOSM], 2022b). Besides that, DOSM (2022a) has documented an annual increment of 5.6% in chicken production in 2021 compared to 2022, deeming the poultry industry significant and robust compared to other livestock such as red meat and pork. Constant outbreaks in farms are considered a major contributing factor that jeopardises the poultry industry and causes losses to the nation's revenue. For several years, pathogens such as bacteria, viruses, and parasites have been commonly isolated in commercialised farms hit with uncontrolled and sudden outbreaks. One of the viral pathogens being identified is fowl adenovirus (FAdV).

FAdV is a double-stranded DNA virus with a non-enveloped structure adenovirus. This avian-infecting pathogen is subgrouped into five species (A-E) and subtyped into 12 (CELO 1, 2, 3, 4, 5, 6, 7, 8a, 8b, 9, 10, 11) serotypes. The virus is reported to be a primary pathogen of a few common avian diseases called inclusion body hepatitis (IBH), hepatitis hydropericardium syndrome (HHS), and avian gizzard erosion (AGE) in chickens. However, interestingly, different diseases and clinical symptoms were reported with different FAdV serotypes, further indicating the serotypes' virulence capacity variability. The highly lethal HHS symptoms were commonly manifest among FAdV subgroup C–infected outbreaks (Zhang et al., 2018), while IBH was routinely seen in FAdV subgroups B, D, and E (Islam et al., 2023). In contrast, FAdV species A was generally reported as non-pathogenic (Gupta et al., 2018).

The major constituents of FAdV are fibre, hexon, and penton proteins. These three proteins are elucidated to play a massive role in the virulence of FAdV as the aetiological agent of IBH, HHS, and AGE. Additionally, the antigenic specificity among FAdV isolates was commonly determined and identified in the fibre and hexon gene regions. These major antigenic determinants located in fibre and hexon genes will be translated into the nucleus of host cells to manifest the infection's clinical signs, such as ruffled feathers, diarrhoea, huddling, and reduction in body weight.

The fibre protein is also speculated to aid the viral attachment and internalisation of the virus into host cells (Wang & Zhao, 2019). Meanwhile, the penton protein characteristics and functionality in infection are still understudied but mainly reported to enhance the interaction of cellular components and the virus-neutralising antibodies (Wang & Zhao, 2019).

Fibre protein is responsible for the antigenicity of the virus and poses type-specific epitopes. It is divided into three domains, positioned explicitly in the tail, shaft, and head domains, which specific domains carry specific features (Grgić et al., 2013). The three-dimensional structure of fibre protein is reported to be bound non-covalently and projected to the penton base, involved in viral entry during infection and compromised in the virulence variation among FAdV groups. The head (knob) domain is characterised as the receptor-binding domain (RBD) of FAdV, hence acting as the critical factor associated with adenoviral infection's virulence and tissue tropisms properties. While the shaft region constitutes the largest portion of fibre protein, its specific role towards FAdV virulence is not fully established. However, the "VYPF" residues at position 55-58 amino acid of fibre protein have been elucidated to be involved in the penton base adenoviral interaction (Grgić et al., 2014). The previous report has established that base substitution mutational changes in fibre gene sequence would result in different pathogenicity of strains (Pallister et al., 1996).

Among the three main structural proteins, hexon makes up the largest structure of the virus protein and is further divided into two major parts: variable and conserved loops. The conserved regions, divided into P1 and P2, located at the inner side of the capsid, were elucidated to provide stability for the hexon protein structure. Meanwhile, L1, L2, and L4 loops are found to be a part of the outer side of the capsid and observed to be non-conservative and vary between serotypes, exposing different surfaces to produce type-specific epitopes. Interestingly, compared to other loops, only the L4 loop is conserved and found internally in the capsid to stabilise the interface between P1 and P2 regions as hexon protein structures the global three-dimensional stability of the overall virus. Previously, the antigenic specificity region (epitope) was highlighted in the hypervariable region (HVR) of L1 and L2 of hexon, as it was reported to be the predominant target for serotype-specific neutralising antibodies during immune infection (Rux et al., 2003). Interestingly, 7 HVRs were identified within the region of three loops; four HVRs in L1, two in L2, and one in L4 (Crawford-Miksza & Schnurr, 1996). The type-specific antigenic determinants responsible for triggering an immune reaction in the host were elucidated in HVR between L1 and L2 (Niczyporuk, 2018). A previous study has also elucidated a cell-culture-adapted FAdV strain into a complete attenuation due to amino acid substitution in the variable region of the L1 loop (Majdi & Bejo, 2015).

The first FAdV in Malaysia was isolated in 2005 from an IBH outbreak and identified as the FAdV-8b strain (Hair-Bejo, 2005). To date, the mortality of IBH ranges from 10 to 30% per outbreak in Malaysian farms and continues to increase (Sohaimi, Omar, et al., 2018). To this date, FAdV pathogens isolates identified worldwide exhibit predominant nature to the reporting countries, such as FAdV-4 being the predominant serotypes in China and Japan, and FAdV-8b in Turkey, Indonesia, Malaysia, and Australia (Cizmecigil et al., 2020; Juliana et al., 2014; Majdi & Bejo, 2015; Steer et al., 2009; Wang & Zhao, 2019; Wei et al., 2019). However, suitable and efficient autogenous vaccines to combat all IBH-caused FAdV strains, especially against Malaysian local FAdV isolates, are unavailable in the market (Juliana et al., 2014). Despite being profiled with low mortality and morbidity rate, the presence of FAdV in the environment or the avian host system still poses horizontal or vertical transmission potential, respectively, which could eventually lead to continuity of problems within the avian industry (Sohaimi & Clifford, 2021). Hence, there is an urgent need for national-specific initiatives to propose a control and measure protocol for preventing future IBH outbreaks.

FAdV was established by a few researchers as an opportunistic pathogen as different strains have different pathogenicity and mortality rate against susceptible flocks (Jørgensen et al., 1995). Due to the previously discussed variations of FAdV activities, it is essential to fully understand essential gene sequences, amino acid residues, and their functional capacity. Hence, this study aims to identify the DNA sequence and amino acid changes of fibre and hexon of the newly isolated FAdV strain UPMT1901 isolate with other previous isolates, as well as establish the evolutionary relationship with other FAdV isolates reported from other countries globally.

MATERIALS AND METHODS

Isolation of the Field Strain

The liver of infected broiler chickens in the 2019 outbreak in Johor, Malaysia, were harvested and stored in Acson® International -40ºC freezer (Acson®, Malaysia) for future virus isolation in the lab. The virus isolated from the harvested liver is FAdV strain UPMT1901 isolate. The infected broiler chicken flocks were crouching and exhibiting IBH clinical symptoms such as ruffled feathers, low performance, and severe depression. These chickens were also pathologically reported to have friable, swollen, pale, and haemorrhagic livers gross lesions upon necropsy.

The collected liver tissue samples were homogenised thoroughly in phosphate buffer saline (PBS, 0.1 M, pH 7.4, Life Technologies™, USA) to produce 50% (w/v) suspension to isolate the virus. The homogenate liver suspension was centrifuged with a 32R centrifuge (Hettich Universal, Germany) at 350 x*g* for 5 min at 4ºC, post-three sets of freeze-thaw cycles. The retrieved supernatants were filtered using 0.45 and 0.22 µm polyethersulfone (PES) membrane syringe filters (Microlab Scientific Inc., China) prior to viral propagation and viral DNA extraction.

Viral Propagation in Specific Pathogen-free Chicken Embryonated Eggs

Specific pathogen-free (SPF) chicken embryonated eggs were purchased from Malaysian Vaccine Pharmaceutical® Sdn. Bhd. (Malaysia). The Committee of IACUC Universiti Putra Malaysia approved the animal experiment under the UPM/IACUC/AUP-R065/2020.

Eight-day-old SPF eggs were disinfected with 70% Systerm® ethyl alcohol solution (Classic Chemicals Sdn. Bhd., Malaysia) and proceeded with candling for viability, followed by marking the desired position for a new artificial route in the chorioallantoic membrane (CAM). The new artificial route marking was 90º from the original air sac position at the region, with fewer capillaries observed. Both original and newly marked routes were poked using a sterilised thumbtack, followed by making an artificial air sac via the suction of a sterilised rubber. Then, 0.1 ml virus supernatant from the virus isolation step was inoculated via the CAM route using a 1 cc/ml syringe (Terumo, China). The holes were sealed with thick fast-drying glue and positioned on the egg rack horizontally. An egg was inoculated with 0.1 ml of PBS during each passage, while one was non-inoculated to act as the negative control per batch. The inoculated eggs were then incubated in a sterile incubator with a 37ºC setting, and observations were made daily. The propagations were performed until a passage with 100% mortality was observed and tabulated. The total observation window frame is between 9 to 20 days, approximately until the control egg was observed with a physical sign of hatching. Dead eggs' CAM and liver organs were harvested within two days maximum to prevent significant loss of viral titre concentration.

The harvested CAM and liver organs proceeded with viral inoculum preparation using the method mentioned in the viral isolation step. The prepared 50% (w/v) suspension inoculum of CAM and liver for all passages were labelled and stored in Innova® U725 ultra-low temperature freezer -80ºC (Eppendorf, Germany) for future use such as deposition into institutes microbiology catalogue or subjected onto viral DNA extraction.

Extraction of Viral DNA

The total viral DNA extraction was performed on both viral supernatants retrieved from the field and subsequent passages from the viral propagation stage. InnuPREP® Viral DNA/RNA Extraction kit (Analytik Jena, Germany) was used according to the manufacturer guide with few optimisations. Two hundred (200) µl of freshly prepared viral inoculum (supernatant) were subjected to extraction. 200 µl of CBV/carrier mix mixture, 200 µl of viral inoculum and 20 µl of proteinase K were mixed vigorously and incubated for one hour in a shaking heat block Thermomixer® R Mixer (Eppendorf, Germany) with 400 rpm continuous shaking and 70°C setting. The remaining extraction and purification processes were followed per the manufacturer's guidelines. The final purified viral DNA was eluted into 50 µl and subjected to quantification via an

Eppendorf BioSpectrometer® (Eppendorf, Germany). Only extracted viral DNA with high concentration and good purity is further used in the amplification process.

Amplification via Polymerase Chain Reaction

The extracted viral DNA was amplified via the Polymerase Chain Reaction (PCR) method to obtain the partial gene sequence of fibre and hexon. The primers were designed with the available nucleotide sequence of FAdV-8b strain 764 (accession no.: KT862811) as the reference sequence. Only primer sets with suitable GC content, target region and optimum amplicon length were chosen. The primer sets were: FibreF/FibreR: 5'-AGCCACTACCGAGGGAATA-3'/5'-GCTCCAACCCAGAAGGTAAA-3' and HexonF/HexonR: 5'-CAGCTCGAACGCTACATCTT-3'/5'-ACGTAGTCCTCCTTGTCTCTAC-3'. The PCR protocol was performed using the MyTaq™ Mix kit (Meridian Bioscience, USA) on a PCR thermocycler C1000 Touch™ Thermal Cycler (Bio-RAD, USA). The PCR reaction mixture of 50 µl contained 1 µl of both 20 µmol of forward and reverse primers, 150–200 ng of DNA template, 25 µl of 2x MyTaq Mix and deionised distilled water. The thermal condition protocol was as follows: 95ºC for 1 min (initial denaturation), followed by 35 cycles of 95ºC for 15 s (denaturation), 55ºC for 15 s (annealing), 72ºC for 30 s (elongation), and final elongation at 72ºC for 1 min. Each gene was run with three technical replicates. The final product of the amplicons was then further subjected to gel electrophoresis for validation. A known FAdV-8b UPM11134CELP10 isolate was selected as the positive control of the concurrent PCR protocol.

The gel electrophoresis was done onto partial fibre and partial hexon gene amplicon products by running 1% gel agarose under 70 V, 400 A settings for 45 min. The 1% gel agarose was prepared using 1 g of HyAgarose™ LE agarose powder (Hydragene, China) in 100 ml 1x Tris-acetate-EDTA (TAE) (pH 8.0, 1st BASE™, Singapore). One microliter RedSafe nucleic acid staining solution (Chembio, UK) was mixed with the prepared gel agarose solution when the room temperature was reached, followed by gel casting. During electrophoresis, 1 µl 6x blue/orange loading dye (Promega®, USA) was mixed with 5 µl of the PCR products before loading into the well alongside 5 µl of 1 kb DNA ladder (1st BASE™, Singapore). Gel Doc™ Transillumination UV XR+ machine (Bio-RAD, USA) was used to visualise the conducted gel electrophoresis.

Sequencing and Molecular Analysis

Only amplicons samples with bright and linear bands from the gel visualisation process were pooled between technical replicates of each gene and further sent for a sequencing service (1st BASE™, Singapore). These amplicons were sequenced using the Sanger sequencing method. The retrieved raw sequences were

cleaned via Sequence Scanner Software v2.0 (Thermo Fisher Scientific, USA) to ensure that only high-quality sequences with nil noise were subjected to further molecular analysis. All clean nucleotide sequences were assembled and analysed using BioEdit v7.2.5 (Alzohairy, 2011), followed by a translation into amino acid residue sequences via ExPASY online tool (Gasteiger et al., 2003). Consensus nucleotide and amino acid sequences were functionally annotated using the Basic Local Alignment Search Tools (BLAST), a bioinformatics tool available from the National Centre for Biotechnology (NCBI) (Conesa et al., 2005).

Upon functional annotation, the high-quality sequences were aligned with homologous difference FAdV species available in the GenBank database of NCBI via MEGA-X v10.2.6 software (Kumar et al., 2018). All sequences were performed multiple sequence alignment (MSA) using the ClustalW algorithm (Thompson et al., 1994) for nucleotide and amino acid residues. Additionally, phylogenetic trees for fibre and hexon were generated to analyse the relationship between isolate and other known FAdVs (Tables 1 and 2), employing the maximum-likelihood (ML) analysis with 1,000 bootstrap replicates.

RESULTS

Mortality and Gross Lesion

The inoculation of UPMT1901 viral inoculum into viable SPF chicken embryonated eggs was observed within 12 days post-infection (dpi). In every passage, the inoculated eggs were candled in a 24-hr interval. Interestingly, inoculated eggs from Passage 0 (P0) and 1 (P1) were observed with 4/4 and 15/15 death of inoculated eggs, respectively. It suggests that UPMT1901 isolate virus propagated in P0 and P1 could reach 100% mortality within the expected observation window frame of a FAdV infection in chicken embryonated eggs, further implying UPMT1901 to be pathogenic. It is also worth noting that P0 achieved 100% mortality at 10 dpi, whereas P1 reached a full-mortality profile at 7 dpi (Table 3).

Subsequently, according to Table 3, the highest mortality rate throughout the infection window time frame for both passages was observed at 4 dpi. It suggests that 4-dpi of UPMT1901 isolate in SPF embryonated eggs have the highest virulence activity compared to another dpi. Meanwhile, the mock-inoculated eggs with PBS and non-inoculated eggs in both passages are still viable at 12 dpi, ensuring the propagation passages are in a controlled environment.

Upon harvesting, all embryos in the control group were normal without lesions throughout both passages, displaying a transparent and thin CAM (Figure 1). Meanwhile, gross lesions were mainly observed in all mortalities of FAdV-inoculated eggs, especially at the CAM and liver tissues. CAM of inoculated eggs was cloudy and thickening, while the embryos' liver organs were seen as swollen and friable, with pale yellow discolouration.

Table 1

Fibre gene sequences of fowl adenovirus from all species retrieved from NCBI for phylogenetic analysis

No.	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
1	Strain 8b 764	KT862811.1	ANJ02574.1	E	2016	Austria	Marek et al. (2016)
2	**UPMT1901	OQ162224	GenBank: WIF20012.1	E	2022	Malaysia	This study
3	UPM04217	KU517714.1	ANA50324.1	E	2017	Malaysia	Juliana et al. (2014)
4	UPM1137E15	KY911371.1	ATI09499.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
5	UPM1137E10	KY911370.1	ATI09498.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
6	UPM1137E5	KY911369.1	ATI09497.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
7	UPM1137CEL35	KY305957.1	AQZ26947.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
8	UPM1137CEL25	KY305955.1	AQZ26945.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
9	UPM1137CEL10	KY305952.1	AQZ26942.1	E	2017	Malaysia	Sohaimi, Bejo, et al. (2018)
10	Strain HG	GU734104.1	ADE58406.1	E	2011	Canada	Grgić et al. (2011)
11	SD1356	MG712775.1	AYC35477.1	E	2018	China	Qinghua et al. (2019)
12	ON NP2	KP231537.1	AKR76204.1	D	2016	Canada	Slaine et al. (2016)
13	HBQ12	KM096545.1	AIS19830.1	D	2015	China	Zhao et al. (2015)
14	MX95-S11	KU746335.1	AOS51019.1	D	2016	Mexico	Absalón et al. (2017)
15	CH/HNJZ/2015	KU558760.1	ANV21404.1	C	2016	China	Liu et al. (2016)
16	AG234-CORR	MK572849.1	QGQ62347.1	C	2019	Japan	Schachner et al. (2019)
17	HLJFAd15	KU991797.1	APA19531.1	C	2016	China	Pan et al. (2017)
18	Strain-340	NC_021221.1	YP_007985662.1	B	2020	Austria	Marek et al. (2013)
19	40440-M/2015	MG953201.1	QCC26484.1	B	2019	Hungary	Kaján et al. (2019)
20	Isolate-340	FR872928.1	CCB84856.1	B	2012	Austria	Marek et al. (2012)

Table 1 *(Continue)*

No	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
21	CELO-CORR	MK572875.1	QGQ63308.1	A	2019	Japan	Schachner et al. (2019)
22	Punjab 1	DQ864435.1	ABI20702.1	A	2016	India	Unpublished
23	Duck FJ12025	KF286430.1	-	-	2013	China	Fu et al. (2013)
24	Turkey A	AC_000016.1	-	-	2018	USA	Davison et al. (2003)

Note. - = Unrelated; ** = The sequence identified in this study

Table 2

Hexon gene sequences of fowl adenovirus from all species retrieved from NCBI for phylogenetic analysis

No.	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
1	Strain 8b 764	KT862811.1	ANJ02569.1	E	2016	Austria	Marek et al. (2016)
2	**UPMT1901	OQ162223	GenBank: WIF20011.1	E	2022	Malaysia	This study
3	SD1356	MG712775.1	AYC35472.1	E	2018	China	Qinghua et al. (2019)
4	SD15-24	KY426992.1	AUB29588.1	D	2017	China	Unpublished
5	ON NP2	KP231537.1	AKR76197.1	D	2016	Canada	Slaine et al. (2016)
6	JL/1407	KY012057.1	ATG30790.1	D	2017	China	Li et al. (2022)
7	ZZ	MN337322.1	QGS70289.1	C	2019	China	Unpublished
8	SDSX1	KY636400.1	AUO29782.1	C	2018	China	Unpublished
9	SD1601/FAdV-4	MH006602.1	AZJ17899.1	C	2018	China	Qinghua et al. (2019)
10	AG234-CORR	MK572849.1	QGQ62336.1	C	2019	Japan	Schachner et al. (2019)
11	CH/GDYF/201706	MK387062.1	QDY98296.1	C	2019	China	Unpublished

Table 2 *(Continue)*

No	Strain	Accession number		Group	Year	Country	Reference
		Nucleotide	Amino acid				
12	CH/HNJZ/2015	KU558760.1	ANV21395.1	C	2016	China	Unpublished
13	Strain-340	NC_021221	YP_007985654.1	B	2020	USA	Marek et al. (2013)
14	CELO-CORR	MK572875.1	QGQ63303.1	A	2019	Japan	Schachner et al. (2019)
15	CELO	AF339914.1	AAL13217.1	A	2001	Belgium	Meulemans et al. (2001)
16	Duck FJ12025	KF286430.1	-	-	2013	China	Fu et al. (2013)
17	Turkey A	AC_000016.1	-	-	2018	USA	Davison et al. (2003)

Note. - = Unrelated; ** = The sequence identified in this study

Table 3

Tabulated cumulative mortality frequency of specific pathogen-free (SPF) chickens embryonated eggs infected with FAdV strain UPMT1901 isolate in two subsequent passages

Passage	Cumulative mortality (Days post-inoculation, [dpi])													Total death	Mortality (%)
	0	1	2	3	4	5	6	7	8	9	10	11	12		
P0	$0^a/4^b$	0/4	1/4	2/4	2/4	2/4	3/4	3/4	3/4	3/4	4/4	-	-	4/4	100
P1	0/15	0/15	0/15	3/15	11/15	11/15	14/15	15/15	-	-	-	-	-	15/15	100

Note. The data are presented in a/b representation: a = The number of infected embryos that died in the passage; b = The total number of eggs inoculated in the passage

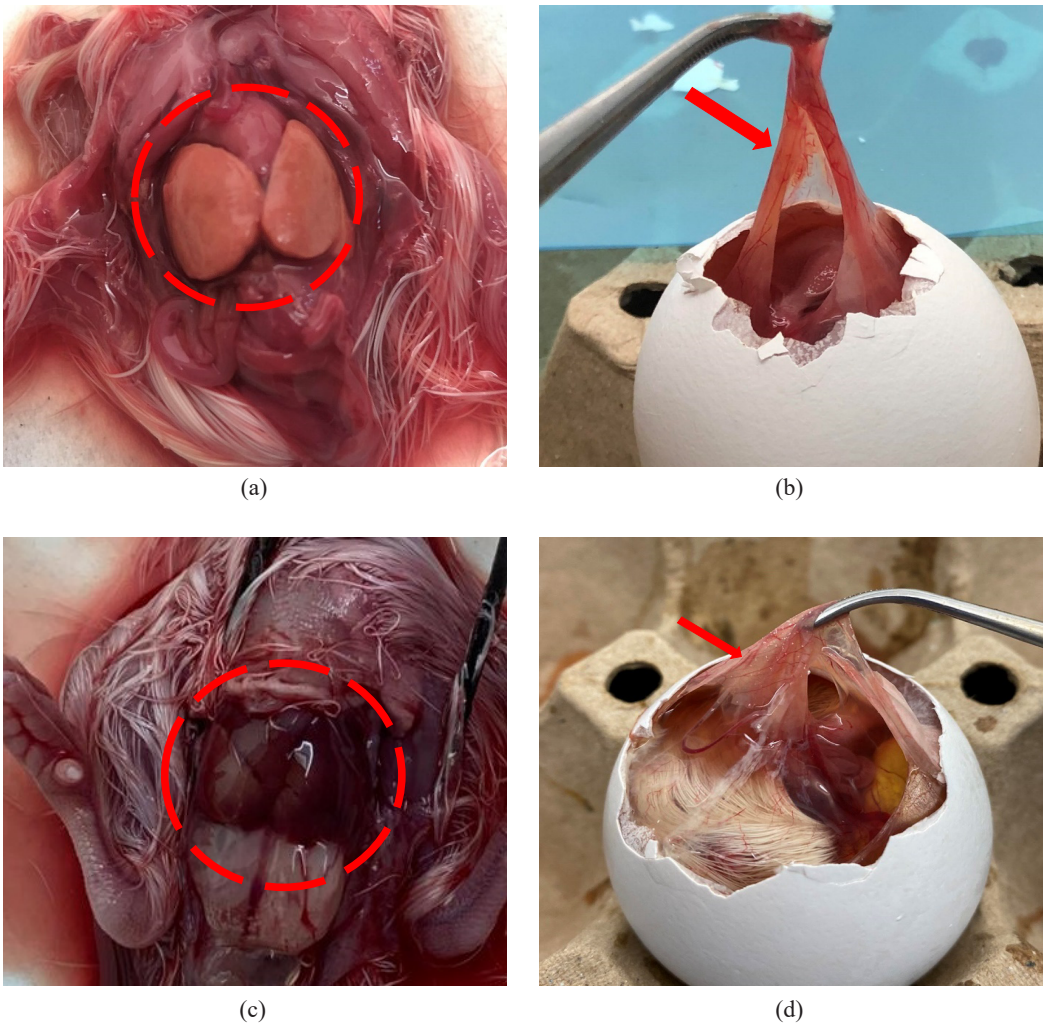


Figure 1. Gross lesion of the (a) liver and (b) chorioallantoic membrane (CAM) of FAdV strain UPMT1901-inoculated Specific pathogen-free (SPF) eggs, as well as the (c) liver and (d) CAM of phosphate buffer saline mock-inoculated SPF eggs

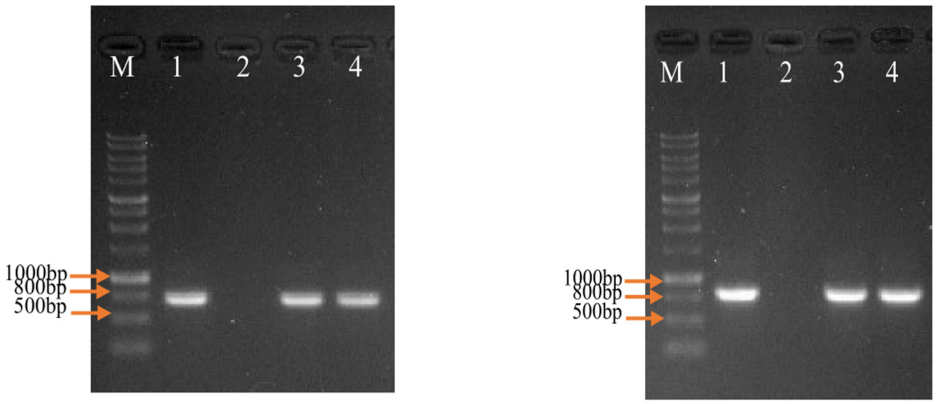
Note: FAdV = fowl adenovirus; PBS = phosphate buffer saline; SPF = specific pathogen-free

Partial Gene Prediction, Amino Acid Annotation, and Phylogenetic Analysis

DNA fragments of the expected amplicon lengths of 747 bp of partial fibre and 821 bp of hexon were successfully amplified, as observed in the agarose gel (Figure 2).

Next, only 723 bp of clean partial fibre and 789 bp of clean partial hexon nucleotide sequences proceeded to functional annotation via BLAST. According to BLAST hits of both partial genes, the nucleotide sequence revealed that the FAdV strain UPMT1901 isolate of this study has high nucleotide identity of other reported fibre genes [accession no.: KY911369.1 (99.86%), KY305954.1 (99.86%), KY305950.1

(99.86%), KY911370.1 (99.72%), KY305955.1 (99.72%), KY305952.1 (99.72%), KU517714.1 (99.72%), and MW885275.1 (99.72%)], and previous hexon gene sequences [accession no.: KU517714.1 (99.48%), KT2862809.1 (98.97%), KX258422.1 (98.71%), and MK572858.1 (98.58%)] of other Malaysian FAdV-8b isolates.



(a) (b)

Figure 2. Amplification of (a) partial fibre (747 bp) and (b) partial hexon gene (821 bp), using primer set Fibre F/R and Hexon F/R of fowl adenovirus serotype 8b (FAdV-8b) strain UPMT1901 isolate and other isolates

Note. M = 1 kbp DNA ladder; Lane 1 = FAdV-8b positive control FAdV UPMT11134CELP10F isolate (accession no.: MT525011.1); Lane 2 = No template control; Lanes 3 and 4 = FAdV-8b UPMT1901

Moreover, the phylogenetic tree analysis of both partial fibre (Figure 3) and partial hexon (Figure 4) nucleotide sequences also displayed that FAdV strain UPMT1901 isolate has a close relationship with other FAdV-8b Malaysian isolates, further supporting previous BLAST hits results. The constructed tree also reported UPMT1901 to share the same FAdV serotypes 8b subgroup ancestor into FAdV Group E.

Meanwhile, the multiple sequence alignment (MSA) performed via ClustalW of the partial fibre gene (Figure 5) presented similar and identical nucleotide sequence concurrently observed in FAdV-8b Malaysian isolates (KY911369.1, KY911370.1, KY305955.1, KY305952.1, and KU517714.1). However, a nucleotide base of UPMT1901 at 33,217 nt, which is sequenced as 'Y' (coded for any pyrimidine base), was identified, whereas the thymine (T) base was consistently reported at the same position in other Malaysian FAdV-8b isolates. Accordingly, X365F was exhibited in the UPMT1901 strain in the MSA analysis of the partial fibre amino acid residues analysis (Figure 6). Interestingly, 'X' was often discussed as a possibility of translating into more than one amino acid probability. Nevertheless, consistent amino acid substitution observed in other FAdV-8b Malaysian isolates are still present in UPMT1901, such as T176A, Q195H, D213E, S243T, A258V, F335Y, and F353V.

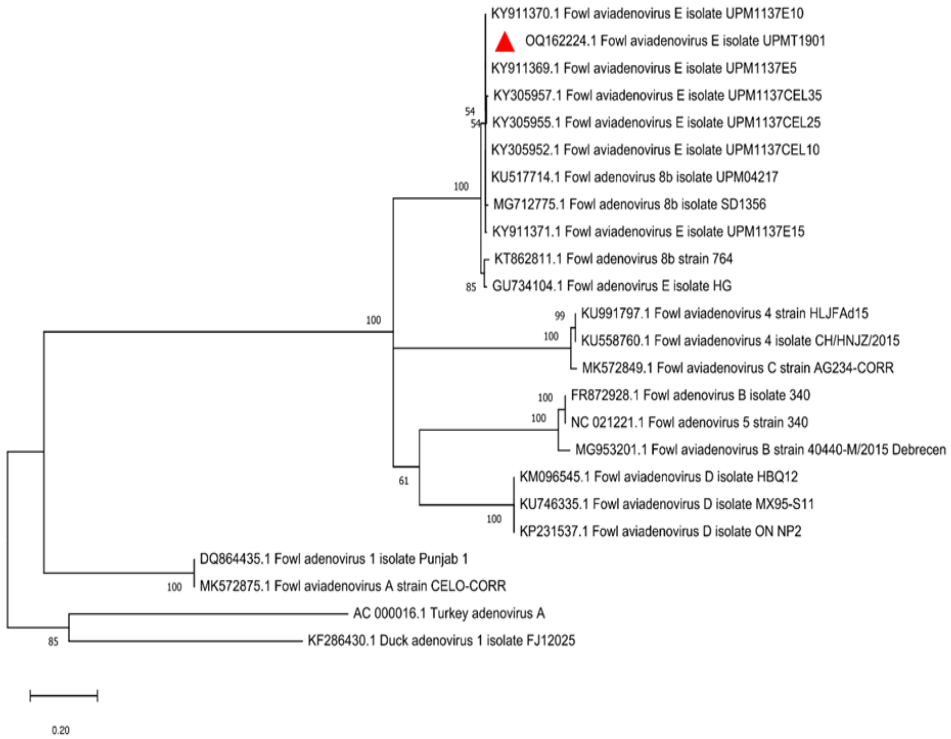


Figure 3. Phylogenetic analysis and sequence identity of partial fibre gene based on the 723 bp nucleotide in the shaft region, with selected Fowl adenovirus (FAdV) groups, performed via MEGA-X v10.2.6 software with maximum-likelihood algorithm applied with 1,000 bootstraps. The red triangle indicates FAdV strain UPMT1901 partial fibre gene

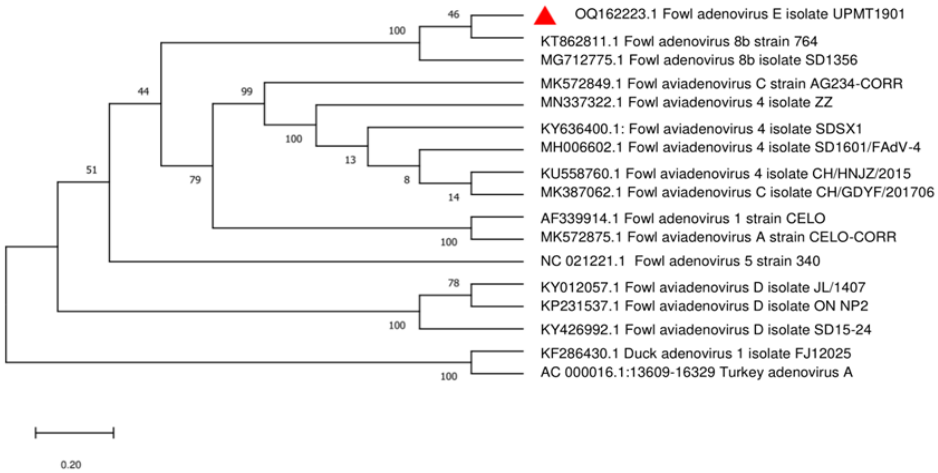


Figure 4. Phylogenetic analysis and sequence identity of partial hexon gene based on the 789 bp nucleotide in the L1 loop region, with selected Fowl adenovirus (FAdV) groups, performed via MEGA-X v10.2.6 software with maximum-likelihood algorithm applied with 1,000 bootstraps. The red triangle indicates FAdV strain UPMT1901 partial hexon gene

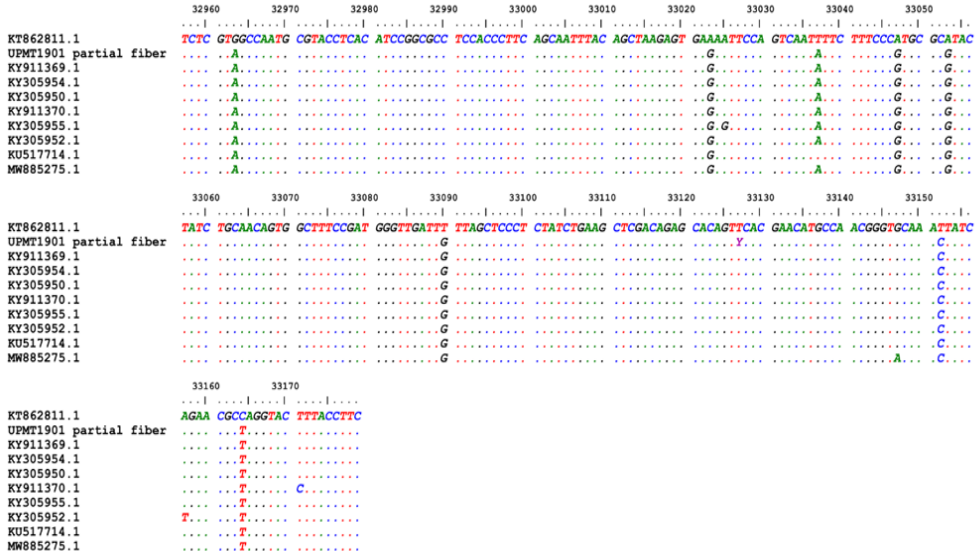


Figure 5. Multiple sequence alignment of partial fibre gene nucleotide sequence based on the 723 bp of nucleotide positioned within the shaft and head domain regions between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software. Only nucleotide positions 32,957-33,180 nt are shown

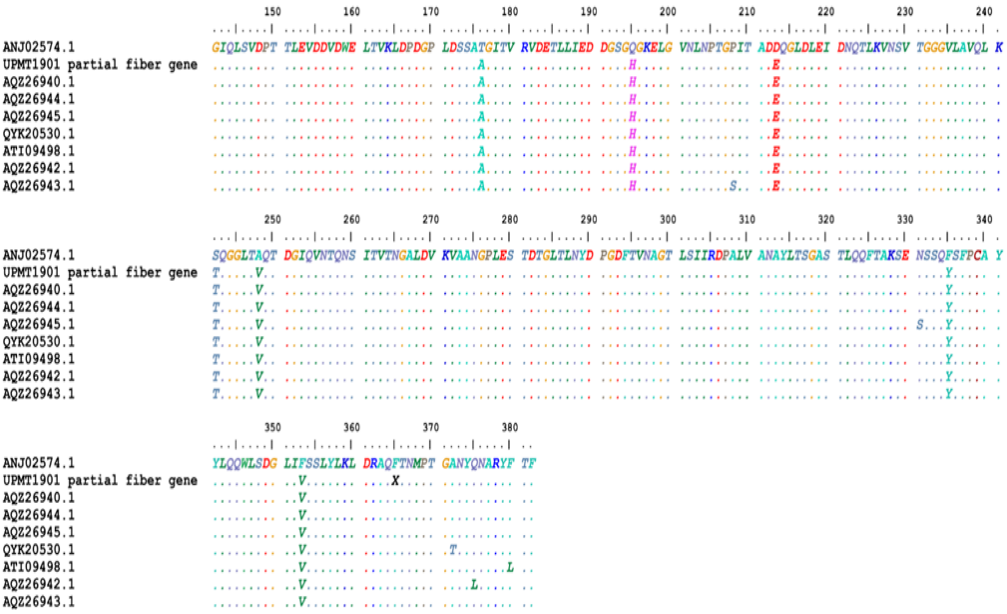


Figure 6. Multiple sequence alignment of partial fibre amino acid residues sequence based on the 241 amino acids positioned within the shaft and head domain region Open reading frame between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

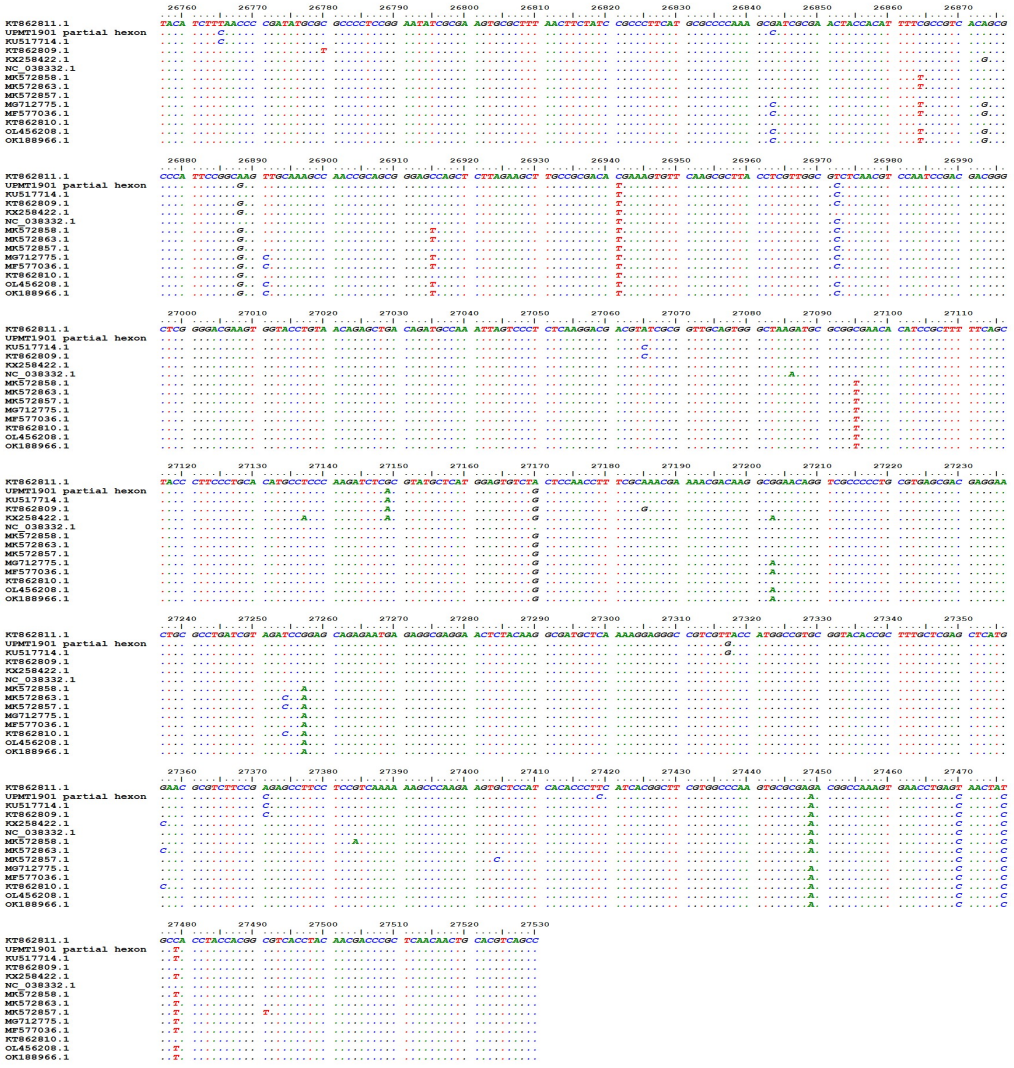


Figure 7. Multiple sequence alignment of partial hexon gene nucleotide sequence based on the 789 bp nucleotide positioned within the L1 loop region between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

On the contrary, the MSA analysis of both the partial hexon gene and protein of the UPMT1901 strain displayed high similarity with consistent nucleotide sequence (Figure 7) and amino acid residues (Figure 8) substitutions as observed in other Malaysian FAdV-8b isolates, specifically T422M.

DISCUSSION

The mortality pattern of FAdV-infected diseases such as HHS, IBH, and AGE significantly affects the poultry industries, nationally and globally. The loss of chicken as one of the many sources of proteins will lead to food and nutrition security, as well

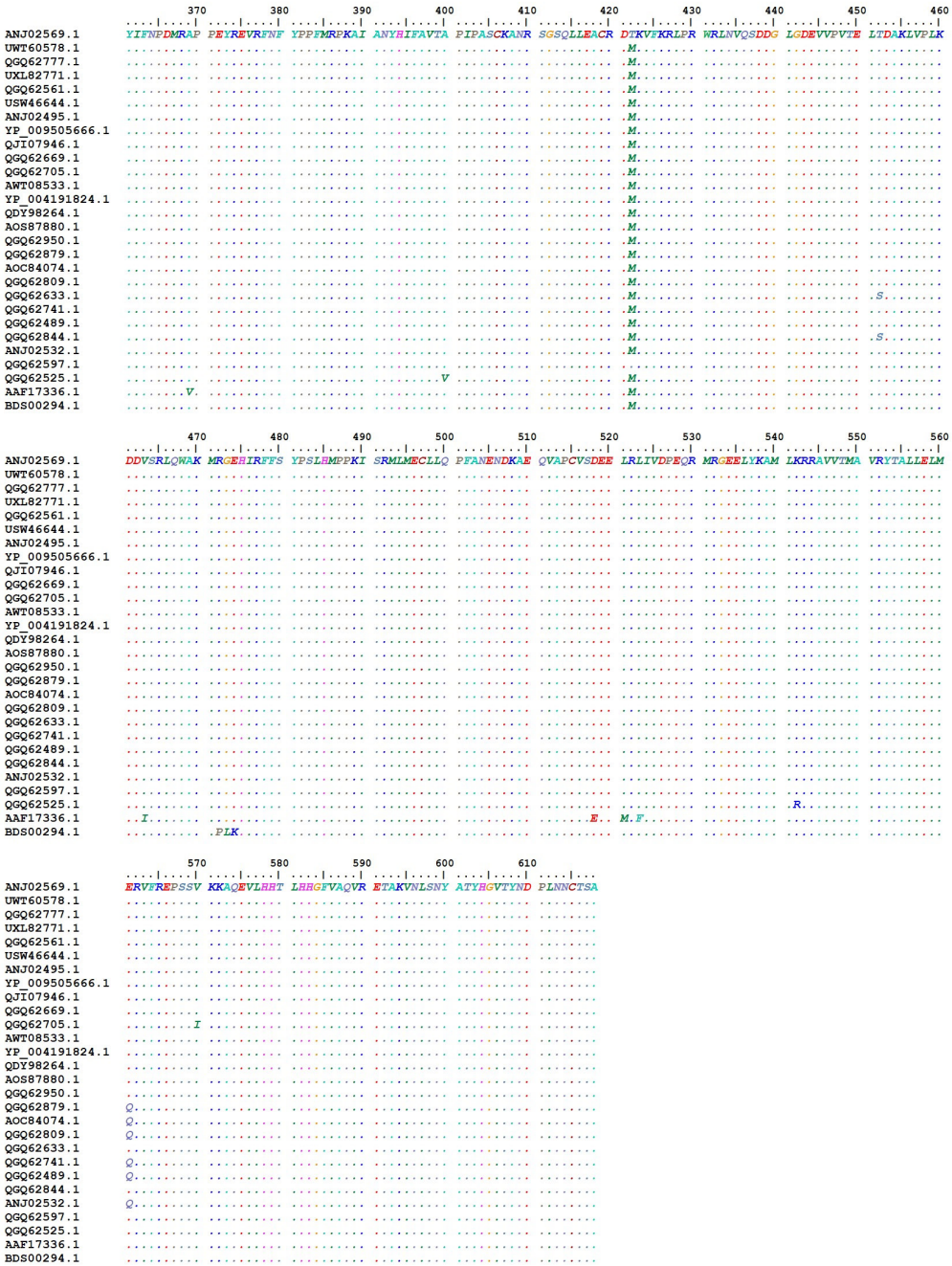


Figure 8. Multiple sequence alignment of partial hexon amino acid residues sequence based on the 263 amino acids positioned within the L1 loop region open reading frame between Fowl adenovirus (FAdV) strain UPMT1901 isolate and other FAdV isolates with BLAST hits similarity of >99%, performed using ClustalW algorithm in the BioEdit software

as food scarcity issues. Multiple records of FAdV-associated infection signs have been documented from every continent, with an increasing number of FAdV outbreaks reported for the past 15 years (Schachner et al., 2021). As tabulated in Table 3, UPMT1901 displayed 100% mortality occurrence in SPF chicken embryonated eggs in early passages as early in P0 and replicated in the subsequent passage of P1. Previous FAdV-8b UPM1137 Malaysian isolate propagation in a series of passages trial was reported to achieve 14.3% mortality (7-dpi; 1/7) in the first passage and only achieved 100% in the second passage (9-dpi; 5/5) (Sohaimi, Bejo, et al., 2018). The full-mortality occurrence observed in inoculated eggs between P0 and P1 suggests that UPMT1901 has a high virulence capacity.

Next, the designated Fibre F/R and Hexon F/R primers about FAdV-8b strain 764 isolate had successfully amplified partial regions of both fibre and hexon genes of UPMT1901, approximately 747 bp and 821 bp expected length of DNA fragments respective to each gene. However, only 723 bp of the sequenced partial fibre and 789 bp of sequenced partial hexon genes were produced after cleaning bases with high noise, further ensuring the significance and confidence in downline analyses performed in this study. As mentioned in the result, BLAST alignment outputs of both partial gene sequences also resulted in hits by FAdV serotypes 8b organisms (e-value = 0, percentage identity = 99%). This result indicates that UPMT1901 isolates retrieved from the farm outbreak with observed IBH signs are due to FAdV serotype 8b-infection, similar to previously reported IBH outbreaks cases in Malaysia (Ahmed et al., 2021; Majdi & Bejo, 2015; Mat Isa et al., 2019; Sabarudin et al., 2021; Sohaimi, Omar, et al., 2018). To date, most FAdV isolates identified in Malaysia are predominant by the FAdV-8b strain; it facilitates the veterinarians and researchers in prioritising potential target genomic composition to act as a template in autogenous vaccines or antiviral production as biosecurity to fight against local FAdV infection. Several numbers of countries with FAdV outbreaks were constantly reporting similar patterns of a predominant serotype as their nation's primary aetiological agent, such as FAdV-4 in China (Niu et al., 2022), FAdV-11 in Australia (Steer et al. 2011) and FAdV-2 in Japan (Nakamura et al., 2011), a study from the Caribbean has reported the identification of multiple serotypes within an outbreak in the same farm, with FAdV-8a, -8b, -9, and -11 isolates circulating among the low-performed chickens in the farm (Jordan et al., 2019). It suggests the probability of other FAdV serotypes emerging in Malaysian farms or currently residing in the environment is low and yet to cause an alarming outbreak.

Next, upon performing the MSA of the genes, both partial fibre and hexon exhibit similar nucleotide base substitutions with other previously published Malaysian FAdV isolates such as UPM04217 (Mat Isa et al., 2019) and UPM1137 (Sohaimi, 2018). The 723 bp of fibre and 789 bp of hexon genes phylogenetic analysis also

displayed that both partial gene sequences are closely related to known FAdV-8b isolates, especially the published Malaysian FAdV-8b isolates. This finding highlights that UPMT1901 shared common ancestors with FAdV species E serotype 8b. Although FAdV outbreak cases in Malaysia have yet to be reported with the identification of various serotypes, lack of biosecurity, such as vaccine intervention in the nation, could lead to a critical infection in the future due to uncontrolled low management husbandry ethics, such as movement of contaminated workers between poultry houses, sharing of contaminated water sources within farm perimeters and more (Butterworth & Weeks, 2010).

Next, the 789 bp of partial fibre sequence was observed to reside within the coding region of the shaft and head domain of the fibre protein. Albeit identified as FAdV-8b, there are still chances of having variety between isolates, which could lead to the rise of alterations in amino acid residues sequence upon translation as laid in central dogma protocol. As shown in Figure 6, base 'Y' is located at 33,217 nt (in reference to KT862811.1), whereas previously known Malaysian FAdV isolates were only reported with base T (thymine pyrimidine). The presence of the 'Y' base in the sequence suggested an equal chance of any pyrimidine bases, either C (cytosine) or T (thymine), to be recruited during viral replication. Hence, the expected amino acids translated from a codon TCC and TTC are serine (S) and phenylalanine (F). These amino acids differ in structures and chemical characteristics as S is a polar, uncharged amino acid (Group II), while F is identified as a non-polar, aromatic amino acid (Group I). A single base substitution mutation within genes of FAdV has the potential to alter the functional capacity of the protein performance, resulting in a variety of FAdV virulence as a primary pathogen of IBH, specifically during viral attachment and entry into the host cells (Pallister et al., 1996; Shah et al., 2017). Previous studies supported this notion as amino acid differences observed in the fibre knob domain region and hexon L1 loop domain result in a degree of virulence and tissue tropism variety for canine and human adenovirus (Zhang et al., 2018).

As two of the main major constituents of the structural capsid of adenovirus, alterations of the fibre and hexon genes would result in a better viral adaptation, depending on the various degree of pathogenicity of the FAdV isolates. Different FAdV serotypes are elucidated to have serotype-specific gene characteristics such as numbers of fibre proteins (long and short fibre proteins in FAdV group C) and epitope-region gene sequences, which suggested the difference in pathogenicity and FAdV-infections clinical symptoms observed in chickens. However, observing slight difference base substitution mutations between isolates of similar serotypes may also suggest an individualistic and opportunistic profile of each adenoviral isolate for viral adaptation. Interestingly, it was observed that changes in fibre genes among isolates of FAdV-8b lead to a different pathogenicity profile

(Ojkic & Nagy, 2000). It promotes the notion that investigating structural protein such as penton, fibre, and hexon, which play a huge role in viral survival, are essential to evaluate and determine the pathogenicity of groups, serotypes, and isolates as individuals. However, it is only possible to observe patterns between gene and amino acid residues sequence prior to concluding a definite indicator of FAdV isolates' pathogenicity by deciphering the overall virulence determinants of FAdV. The age of the chicken during inoculation, viral inoculum titre, route of administration, chicken susceptibility, chicken breed, and other factors contribute towards the chicken's mortality and the virus's pathogenicity.

Equally important, FAdV is also known to be resistant to disinfectants and high temperatures, increasing the threat posed by this pathogen when it is widespread globally (Hafez, 2011). Developing a vaccine against FAdV infection has not been a priority in research because of the absence of important diseases with severe mortality rates and morbidity profiles caused by an adenovirus (McFerran & Smyth, 2000). However, frequently reported outbreaks of HHS and IBH worldwide have sped up the development of an autogenous vaccine, either live attenuated, subunit, virus-like particles, or genetically-modified vaccine (Ahmed, 2020; De Luca et al., 2020; Gupta et al., 2018; Schachner et al., 2014; Schonewille et al., 2010; Sohaimi, 2018; Yin et al., 2021). In Malaysia, a commercialised vaccine developed from the country's local strain is yet to be produced to protect the flocks against FAdV infections (Juliana et al., 2014). However, the effort in establishing vaccines with reports of varying success are efforts worth acknowledging (Ahmed, 2020; Sohaimi, 2018). Indeed, any autogenous vaccine production as a potential biosecurity in controlling infectious diseases requires a thorough understanding and established molecular characteristics of its known isolates.

With the advance in technology in the research field, whole genome sequencing (WGS) could be performed on FAdV strain UPMT1901 isolate to evaluate further the isolate's evolutionary relationship with other known FAdV isolates molecular profiles, as well as establish the factors that lead to virulence of the virus.

CONCLUSION

The UPMT1901 isolate is a FAdV serotype 8b under the group E strain. This isolate was reported to have evolutionary relationships with other FAdV-8b, with precisely 99% identity with other known Malaysian FAdV isolates. The presence of a nucleotide base substitution in the partial fibre gene sequence of UPMT1901 indicates the possibility of having a variety of pathogenicity between and within the serotype, indicating a potential opportunistic nature as an individual isolate. The control and preventive measure protocols, such as vaccine production, are ongoing to curb avian FAdV diseases, especially in future farm outbreaks that would jeopardise the nations' food scarcity and economic growth.

The study of FAdV isolates will be a stepping point to elucidate the epidemiological factor of FAdV in Malaysia and provide essential information in building a detailed understanding at the molecular level to produce an effective vaccine against FAdV infections in the future.

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