MOLECULAR CHARACTERIZATION OF FOWL ADENOVIRUSES ISOLATED FROM CHICKENS IN MALAYSIA

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ABSTRACT. Recently, Fowl Adenovirus (FAdV) cases have been reported in many countries worldwide. FAdV is a contagious agent associated with inclusion body hepatitis (IBH) and hydropericardium syndrome (HPS) in chickens. It belongs to the *Aviadenovirus* genus of the family *Adenoviridae.* The virus is classified into five species (A to E) and further divided into 12 serotypes. Depending on the serotypes, they have diverse characteristics of virus that can either be pathogenic or nonpathogenic strain. From the viewpoint of epidemiological as well as vaccine development, it is very important to detect FAdV strains. Previous studies have been conducted on molecular research, but the continuity of this study in Malaysia has been limited. This study aims to identify the serotype classification of five Malaysian FAdV isolates obtained from field outbreaks during 2017-2019. In this study, polymerase chain reactions (PCR) were conducted based on Hexon gene. Results from the nucleotide sequence analysis discovered that the five isolates showed high similarity with FAdV-8b strains. High bootstrap values in phylogenetic analysis supported the clustering of the Malaysian FAdVs isolates into FAdVs species E. Consequently, the result of this study contributed important information on the epidemiology and culminated in the importance of control strategies against FAdV infection in Malaysia.

Keywords: fowl adenovirus, inclusion body hepatitis, FAdV-8b, chicken, Malaysia

INTRODUCTION

Of late, poultry farms are facing emerging problems of Fowl adenovirus (FAdV) which has been distributed worldwide. FAdV have been detected from sick birds as well as from birds without any clinical signs of infection (Jowita, 2015). Previous studies on this area have proven that the existence of low virulence of some strains and maternal antibodies to be the causes (McFerran *et al.,* 1972, Norina *et al.,* 2016). Some of FAdV strains can cause moderate to high mortality and lead to significant economic loss in poultry industries such as hepatitis hydropericardium syndrome (HPS), inclusion body hepatitis (IBH), gizzard erosions (GE), and respiratory tract disease (Wibowo *et al.,* 2019; Juliana *et al*.,2014).

IBH was first reported by Helmboldt and Frazier in 1963 in the United States and prevalent in other countries in the 1960s and 1970s (Hair-Bejo, 2005, Nakamura *et al.,* 2011*).* IBH outbreak in Malaysia was first reported by Hair-Bejo during 2005 in commercial broiler chickens and caused 10% mortality. Since that occurrence, the reported numbers of IBH cases have increased (Norfitriah *et al.,* 2019, Juliana *et al.,* 2018). Several serotypes such as FAdVs 2, 4, 5, 8b and 11 were associated with IBH outbreaks in many countries (Norfitriah *et al.,* 2019; Oliver-Ferrando *et al.,* 2016).

FAdVs are non-enveloped icosahedral viruses, with a linear double stranded DNA genome between 35-45 kb size and belongs to the genus *Aviadenovirus.* The viruses' cluster can be divided into five species (A-E) according to molecular

structure and 12 serotypes (FAdV 1–8a and 8b-11) as concluded using cross-neutralization test (Xia *et al.,* 2017; Domanska-Blicharz *et al.,* 2011). However, there are a number of methods that have been used routinely to diagnose FAdVs such as histological examination, isolation in cell culture, agar gel immunodiffusion (AGID), immunofluorescence techniques, enzyme-linked immunosorbent assay (ELISA) as well as electron microscopy (Manu *et al.,* 2013). Recently, genomic analysis was identified as a faster and more effective method for differentiating FAdV strains (Meulemans *et al.,* 2004).

In theory, the 937-amino acid (AA) hexon protein as a major surface-exposed capsid structure is the key mediator of antigenicity of adenoviruses that has wide array of epitopes which allow subgroup and type specificity (Niu *et al.,* 2018). That being the case, this study aims to determine serotypes of FAdV strain that existed in Malaysia during 2017-2019 and to compare them with other local and foreign isolates based on the hexon gene.

MATERIALS AND METHOD

A total of five suspected FAdV from different geographic regions in Malaysia were received by Veterinary Research Institute, Ipoh, Malaysia for disease investigation. The descriptions of the FAdV strains in this research are summarised in Table 1. The whole of the samples was propagated in specific-pathogen-free (SPF) embryonated chicken eggs via the chorioallantoic membrane (CAM) route (McFerran *et al.,* 2000). The CAM membrane was harvested as a source of virus stock and stored at -80^{O}C until further analysis.

Viral DNA Extraction

Viral DNA was extracted from the 200µl of CAM of each isolates using QIAamp Cador (Qiagen, USA) based on manufacturer's instructions. The DNA precipitate were dissolved in 50 µl nuclease free water and then stored at -80^{o}C.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Specific forward primer HexF1: 5'-GAYRGYHGGRTNBTGGAYATGGG -3' and HexR1: 5'-TACTTATCNACRGCYTGRTTCCA-3' were used (Masaji *et al*., 2009). Polymerase chain reaction (PCR) was conducted using GOTaq Green Master Mix PCR (Promega, USA). A thermal cycler (Bio-Rad, USA) was used to perform PCR, with a single cycle at 94^{o}C for 5 min. This was followed by 34 cycles of denaturation at 95^{o}C for 1 min, annealing at 55^{o}C for 1 min and elongation at 72^{o}C for 1 min. A final extension step was performed at 72^{o}C for 10 min (Masaji *et al*., 2009). The PCR products were then run through agarose gel electrophoresis (Promega, USA).

Table 1. Description of FAdV isolates included in this study

No	Strains	Geographic location	Breed
1	2542/2019/Malaysia	Melaka	Ross
2	6499/2019/ Malaysia	Selangor	Arbor Acres
3	3390/2018/ Malaysia	Pulau Pinang	Ross
4	9968/2017/ Malaysia	Perak	Cobb
5	4572/2017/ Malaysia	Perak	Ross

Nucleotide Sequencing

The PCR product was electrophoresed on 1.5% agarose gel (voltage 100V for 35 min). The hexon gene-specific products (800 bp) were purified from agarose gels using QIAquick PCR (Qiagen, USA) following manufacturer's instructions and sent to Apical Scientific Sdn. Bhd. for Sanger sequencing.

Sequence and Phylogenetic Analysis

Seqman Pro software (DNAStar Lasergene, USA) was used to edit the nucleotide sequences and aligned by using ClustalW multiple alignment in Bioedit Sequence Aligment Software Version 7.2.5 (Hall, 1999). Phylogenetic tree was produced using the neighbor-joining method in Molecular Evolutionary Genetic Analysis (MEGA) Version 6.06 with 1,000 bootstrap replicates (Tamura *et al*., 2013). Subsequent to that, twenty-three sequences were then downloaded from the Genbank database (http://www. ncbi.nlm. nih.gov/genbank/) for comparison as well as phylogenetic analysis (Table 2).

RESULTS AND DISCUSSION

In recent years, FAdV has been reported and the infection still continues in many countries (David, *et al*., 2018; Juliana *et al*., 2014). Virus isolation of FAdV is still practiced in many diagnostic laboratories worldwide (Gyozo *et al*., 2014). In this study, all organ samples collected were inoculated for FAdV in SPF embryonated chicken eggs. Based on the hexon gene, all isolates were detected positive for FAdV with the expected size of 800 bp (result not shown). Based on Nateghi *et al.* (2014), virus' isolation's sensitivity is found to be lower than molecular methods such as PCR.

Table 2. FAdV hexon genes used in the sequence and phylogenetic analysis.

No	Strain	Accession number	Group	Reference
1.	Celo	AF339914	A	Juliana *et al*.,2014
2.	PL/060/08	GU952110	A	Domanska-Blicharz *et al*.,2011
3.	TR22	AF508953	B	Juliana *et al*.,2014
4.	506	AF508950	C	Juliana *et al*.,2014
5.	J-2A	AF339917	C	Juliana *et al*.,2014
6.	C2B	AF339923	C	Juliana *et al*.,2014
7.	75	AF508949	D	Juliana *et al*.,2014
8.	SR49	AF508948	D	Juliana *et al*.,2014
9.	A2-A	AF339918	D	Juliana *et al*.,2014
10.	CH/GZXF/1511/2015/China	MF055635	D	Xia *et al*.,2017
11.	SA55-08/2008/South Africa	HQ117900	D	Unpublished
12.	380	AF339925	D	Juliana *et al*.,2014
13.	CR119	AF508954	E	Juliana *et al*.,2014
14.	YR36	AF508955	E	Juliana *et al*.,2014
15.	58	AF508957	E	Juliana *et al*.,2014
16.	TR59	AF508956	E	Juliana *et al*.,2014

No	Strain	Accession number	Group	Reference
17.	UPM04217	KU517714	E	Juliana *et al*.,2014
18.	UPM08158	JF917238	E	Juliana *et al*.,2014
19.	UPM08136	JF917239	E	Juliana *et al*.,2014
20.	764/Australia	AF508958	E	Juliana *et al*.,2014
21.	Stanford	DQ323986	E	Juliana *et al*.,2014
22.	vsn033pat17/2017/Indonesia	MK692960	E	Unpublished
23.	04-524856/2007/Canada	EF685515	E	Ojkic *et al*.,2008

The isolation of virus requires more passage than one for cytopathic effect (CPE) and time consuming, which takes about three weeks approximately. Nowadays, the PCR method has been known to be a sensitive, rapid, and specific test to discover various avian adenovirus infection's serotypes which could be used for surveillance work of avian adenovirus in many countries as routine diagnostic (Xie *et al.,* 1999).

The FAdV capsid is constructed from three major structural proteins called hexon, penton base, and fiber. As explained by previous studies, hexon is the subtype-specific antigenic determinant and major capsid protein against which antibodies are constructed (Liu *et al.,* 2016; Juliana *et al.,* 2014). In the past, a serological method like cross-neutralization test was used to detect 12 serotypes of FAdV which was a long process. Besides that, it is difficult for every laboratory to get a reliable set of antisera and reference strains to differentiate various serotypes of FAdV (Ojkic *et al*., 2008). Therefore, conventional serotyping tests of FAdV seem to be performed rarely nowadays (Gyozo *et al*., 2014). Recently, most of the molecular classification of FAdV was performed based on the hexon gene loop 1 region and the fiber gene (Liu *et al.,* 2016; Kajan *et al.,* 2013). Phylogenetic analysis and direct sequence of the variable loop region of the hexon gene was demonstrated as fast and appropriate alternative for FAdV typing (Gyozo *et al*., 2014). Phylogenetic trees in this study showed that 2542/2019/Malaysia, 6499/2019/Malaysia, 3390/2018/Malaysia, 9968/2017/Malaysia and 4572/2017/Malaysia isolates belong to FAdV-E, serotype 8b (Figure 1). Based on serotype FAdV-8b, two clusters were observed in the phylogenetic tree. The first cluster consists of Canadian and Indonesian strain, while the other cluster is among Malaysian isolate (UPM strains) and Australian strain. However, the phylogenetic tree showed that all isolates in this study were clustered together with Canadian and Indonesian strain. It indicates that those isolates have originated from other countries compared to the origin of the previous Malaysian strain. It is believed that the virus can be transmitted horizontally from flock to flock by the fecal-oral route as well as vertically in breeder chickens (Li *et al*., 2017). Broiler breeder farms that provide day old chicks might have been a source of infection (Wibowo *et al*., 2019). Wild birds have high positive rates of FAdV which might serve as a reservoir and spread the disease under natural conditions (Kumar *et al.,* 2010). Besides that, contaminated live poultry vaccines might be a factor to this situation. Live vaccines might fail to provide protection to the chickens. It is because the virus may revert to be pathogenic (Cecchinato *et al.,* 2014). However, the source of

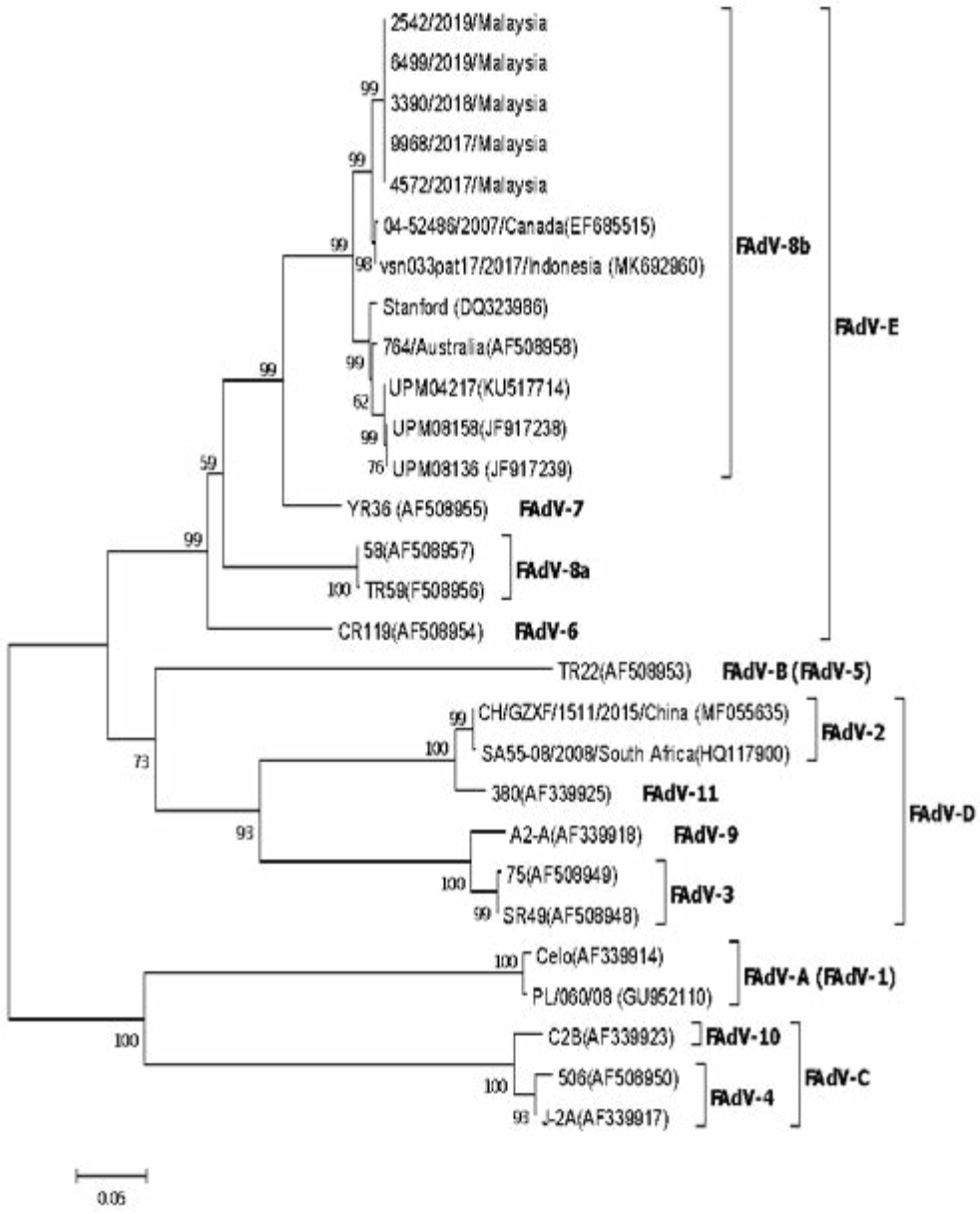

Figure 1. Phylogenetic tree of twenty-three reference strains from Genbank database and five isolates in this study were constructed based on 800bp long nucleotide sequence of hexon gene. The Accession numbers of the sequence from GenBank are shown in parenthesis. Tree was constructed using MEGA version 6.06 by the Neighbour-Joining statistical method with model number of differences and setting bootstrap 1,000 replicates.

infection in Malaysia is still unknown and needs further investigation.

The nucleotide similarity between all five Malaysian isolates was 99-100% to each other. Therefore, since they have high similarities, it is speculated that these isolates have the same origin of FAdV strain. Even though most of the isolates came from different states in Malaysia, these isolates have nucleotide similarity between 98.4% to 99.8% with 04-52486/2007/ Canadian strain and 98.6% to 99.8% with vsn033pat17/2017/Indonesian strain. Compared to previously reported Malaysian strains, all isolates in this study showed 95.1% to 96.4% nucleotide similarity (Table 3). Thus, this might be the reason for those isolates in this study (Table 1) to be clustered together with Canadian and Indonesian strains. The reason is unknown and needs further investigation. Furthermore, various strains in the same serotype might have pathogenicity differences. This is because the differences in pathogenicity of the different strains within the same serotype may be related to genetic differences between the strains or the differential susceptibility of the chicken (Pan *et al.,* 2017, Ye *et al.,* 2016). Therefore, more information of disease in various aspects such as epidemiology, molecular analysis and pathogenicity study are required.

This study showed that IBH outbreaks in several states in Malaysia were solely caused by FAdV group E and within serotype 8b. Many countries such as Japan, Canada, Australia and Brazil showed prevalence of IBH serotype 8b (Xia et al., 2017; Du *et al*., 2017). In Southeast Asia, Thailand has reported FAdV D, whereas Indonesia has both FAdV D and E (Wibowo *et al*., 2019). According to Chen *et al.* (2019) and David *et al.* (2018), the multiple FAdV serotypes will be a challenge to prevent and control the

Table 3. Nucleotide Similarity for group E,Serotype 8.

No	Strains	Nucleotide Similarity (%)											
		1	2	3	4	5	6	7	8	9	10	11	12
1	04-52486/2007/ Canada	100	99.8	98.9	98.9	98.9	98.9	98.4	96.1	96.0	96.0	96.4	96.3
2	vsn033pat17/2017/ Indonesia	99.8	100	99.0	99.0	99.0	99.0	98.6	96.2	96.1	96.1	96.5	96.3
3	4572/2017/Malaysia	98.9	99.0	100	100	100	100	99.3	95.5	95.4	96.4	95.8	95.7
4	9968/2017/Malaysia	98.9	99.0	100	100	100	100	99.3	95.5	95.4	95.4	95.8	95.7
5	3390/2018/Malaysia	98.9	99.0	100	100	100	100	99.3	95.5	95.4	95.4	95.8	95.7
6	6499/2019/Malaysia	98.9	99.0	100	100	100	100	99.3	95.5	95.4	95.4	95.8	95.7
7	2542/2019/Malaysia	98.4	98.6	99.3	99.3	99.3	99.3	100	95.3	95.1	95.1	95.5	95.5
8	UPM04217	96.1	96.2	95.5	95.5	95.5	95.5	95.3	100	99.8	99.8	98.4	98.3
9	UPM08158	96.0	96.1	95.4	95.4	95.4	95.4	95.1	99.8	100	100	98.2	98.2
10	UPM08136	96.0	96.1	95.4	954	95.4	95.4	95.1	99.8	100	100	98.2	98.2
11	764/Australia	96.4	96.5	95.8	95.8	95.8	95.8	95.5	98.4	98.2	98.2	100	98.9
12	Stanford	96.3	96.3	95.7	95.7	95.7	95.7	95.5	98.3	98.2	98.2	98.9	100

Nucleotide Similarity (%) for group E, Serotype 8

disease. Therefore, it has been suggested that maintaining good husbandry practices such as disinfection, maintaining strict biosecurity, and proper ventilation may significantly reduce the chances of infection (Balvinder *et al.,* 2014; Ameji *et al.,* 2012). In addition, vaccination is a common practice to prevent the disease and a suitable vaccine against the disease is important. In Malaysia, a vaccine against IBH is not available (Norfitriah *et al*., 2018). Development program of vaccine against specific serotype 8b for IBH is required. Besides that, using local isolates for the development of vaccines is more effective (Wubet *et al*., 2019). It is because local isolated viruses such as 8b are more specific and produce specific antibodies in chicken against specific serotypes of FAdV in Malaysia (Du *et al*., 2017). Therefore, the development of vaccines using local isolates might be an effective strategy to reduce outbreak and prevent the disease in Malaysia.

CONCLUSION

In conclusion, our results indicate the presence of FAdV E serotype 8b in all the isolates based on hexon gene and this serotype is circulating in Malaysia. This study provides meaningful information and better understanding about FAdV circulating poultry viruses in Malaysia

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