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Molecular Characterization of Fowl Adenoviruses Isolated from Inclusion Body Hepatitis Outbreaks in Commercial Broiler Chickens in Malaysia

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ABSTRACT

Fowl adenoviruses (FAdVs), belonging to the *Aviadenovirus* genus of the family *Adenoviridae*, have been classified into five species (A to E) and further divided into 12 serotypes. The objective of this study was to identify the serotype classification of five Malaysian FAdV isolates obtained from field outbreaks of IBH in commercial broiler chickens. Hexon-based polymerase chain reactions (PCR), combined with restriction enzyme analysis (REA), were applied. Viral DNA reacted positively with H1/H2 and H3/H4 primer pairs which hybridised to highly conserved regions of the hexon genes. The restriction enzyme profiles of the H1/H2 fragment digested with *HaeII* and the H3/H4 fragment digested with *Hpa*II revealed that all five isolates shared identical patterns and are characterised as being FAdV-8b, species E. Meanwhile, sequence analysis of the L1 loop region of the hexon gene revealed 98.1% identity with FAdV-8b strain 764. High bootstrap values in phylogenetic analysis supported the clustering of the Malaysian FAdV isolates into FAdV species E. The present study has provided a very useful reference for further studies of FAdVs in Malaysia. Vaccination strategies should be developed against FAdVs infection in commercial broiler chickens to prevent IBH outbreaks in the country.

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INTRODUCTION

Fowl adenoviruses (FAdVs) are common infectious agents in poultry farms and are distributed worldwide. The *Aviadenovirus's* members are classified into five species (A to E) based upon their restriction enzyme fragment patterns, phylogenetic relationships, pathogenicity, cross-neutralization and recombinant potential (Zsák & Kisary, 1984; Virus Taxonomy, 2011). In particular, FAdVs consist of 12 serotypes with varying pathogenicities and can be isolated from both healthy and sick birds (McFerran *et al*., 1972). This is due to the presence of maternal antibodies and low virulence of some strains. Economically, these icosahedral viruses are mainly responsible for naturally acquired outbreaks of inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome, respiratory tract disease and gizzard erosions that have high economic impacts (Alvarado *et al*., 200; Grime *et al*., 1977; Nakamura *et al*., 1999; Mase *et al*., 2009; Ono *et al*., 2004).

There are several methods that have been routinely employed for the diagnosis of FAdVs including isolation in cell culture, immunodiffusion test, immunofluorescence techniques, enzyme-linked immunosorbent assay (ELISA) and electron microscopy (Balamurugan & Kataria, 2004; Cowen *et al*., 1978). However, despite their time-consuming and expensive nature, these techniques are only of minor relevance due to the widespread occurrence of antibodies to avian adenoviruses. In recent years, several methods based on the polymerase chain reaction (PCR), combined with restriction enzyme analysis (REA), have been developed and proven to be not only rapid but more sensitive and specific for the detection of group 1 avian adenovirus (Ganesh *et al*., 2002; Hess, 2000; Jiang *et al*., 1999; Raue & Hess,1998; Singh *et al*., 2002).

Furthermore, variable regions that exist in the loop regions of the hexon protein, specifically the L1 and L2 loops (Crawford-Miksza & Schnurr, 1996) had been manipulated to generate a more precise evolutionary profile compared to the restriction profiles of the whole genome. These regions of the hexon gene consisting of seven hypervariable regions in mastadenovirus have been shown through neutralization test to have type-specific domains (Toogood *et al*., 1992). Previous studies used L1 loop sequences for the generation of a phylogenetic tree of FAdVs owing to this loops greater variability when compared to the L2 loop in FAdV (Meulemans *et al*., 2004). The L1 loop was also found to be the longest and most complex loop in mastadenovirus.

In an endeavour to identify and characterise FAdV serotypes associated with IBH outbreaks in Malaysia, hexon-based PCR and REA, as described by Raue and Hess (1998), was employed to test its usefulness for the detection of our isolates,

followed by the analysis of the nucleotide sequences and the phylogenetic clustering of the isolates based on the L1 loop region.

MATERIALS AND METHODS

Viruses

The FAdVs isolates (designated as UPM04217, UPM08158, UPM08136, UPM11142 and UPM11134) used in this study were obtained from the collection maintained by the Faculty of Veterinary Medicine, Universiti Putra Malaysia. They were isolated previously from IBH field outbreaks in commercial broiler chickens from different states in Malaysia in 2004, 2008 and 2011. Samples in the form of CAM (UPM04217, UPM08158) and liver (UPM08136, UPM11142 and UPM11134) tissues were frozen and thawed three times before being macerated with a sterile mortar and pestled to prepare a 1 in 2 (w/v) suspension in sterile phosphate buffer saline (PBS; pH 7.4, 0.1 M). The suspension was centrifuged at 3000 rpm for 30 minutes for clarification. The collected supernatant was filtered through a 0.45-µm filter and treated with a commercial antibiotic and antimycotic preparation (GIBCO Laboratories, New York, USA) at a 1 in 10 (v/v) dilution and incubated at 4°C for 1 hour prior to inoculation. All the isolates were then inoculated into 10-day-old SPF embryonated chicken eggs via the chorioallantoic membrane (CAM) route following a standard procedure (Dagmar & Becht, 1975). The CAM and embryonic liver from dead embryos were harvested under sterile conditions.

Viral DNA Extraction

The salting-out DNA extraction method was used with some modifications (Mirmomeni *et al*., 2010). DNA was extracted from 500 µl homogenates of CAM (UPM04217 and UPM08158) and liver (UPM08136, UPM11142 and UPM11134). Aliquots of 50-70 µl of 10% sodium dodecyl sulfate (SDS) and 1 µl of proteinase K were added into each tube, which were then vortexed. The tubes were placed into a warm water bath (65°C) for 30 minutes and shaken every minute. After keeping the cells in a freezer (-20°C) for 5 minutes, 5M ammonium acetate was added into each tube, and the mixture was then vortexed. The cell debris was pelleted and 850 µl of supernatant was transferred into a new Eppendorf tube. Ice-cold isopropanol (700 µl) was added into each tube and it was inverted 30-40 times. The DNA was pelleted and purified by two ethanol washes. The pellets were dried and resuspended in ddH_20 and the resultant DNA extract was qualitatively checked and quantified at 260/280 nm using a spectrophotometer (Beckman, USA).

PCR and Restriction Enzyme Analysis

DNA amplification was carried out using H1/H2 and H3/H4 primer sets (Raue & Hess, 1998). The PCR products were then separated by using electrophoresis in 1% agarose gel (Promega, USA) at 75 V for 50 minutes, stained with ethidium bromide and visualised through UV transillumination. PCR fragments amplified by the H1/H2 and H3/H4 primer sets were cleaved by *Hae*II and *Hpa*II restriction enzyme

respectively according to the manufacturer's recommendations (Fermentas, Life Sciences). The *Hae*II cleavage products were separated by electrophoresis in 1% agarose, 80 V for 45 minutes while the *Hpa*II cleavage products were separated in 3% agarose gel at 88 V for 75 minutes followed by ethidium bromide staining and visualisation by UV transillumination. The profiles created by restriction enzyme digestion were compared with those of the FAdV 1-12 reference strains (Raue & Hess, 1998).

Nucleotide Sequencing and Phylogenetic Analysis of the Hexon Genes

The PCR products for H1/H2 of each isolate containing the loop 1 and loop 2 regions were cloned into the pCR®2.1-TOPO® vector using the TOPO TA Cloning kit (Invitrogen, USA). Then, plasmid from the positive colonies were extracted and purified using GeneAll® Exprep™ Plasmid Quick (Generabiosystems, Australia) and subjected to DNA sequencing at least three times for each isolate on an automatic sequencer (ABI PRISM 377 DNA) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin Elmer) to get a precise consensus sequence. The sequences were assembled, edited, and analyzed using the BioEdit version 7.0.9 package and translated using ExPASy Proteomic server available online.

Twenty-nine available FAdVs hexon sequences representing each serotype were retrieved online from GenBank including 11 sequences published by Meulemans (2004) (Table 1). The nucleotide and deduced amino acid sequences were aligned and compared using BioEdit™ version 7.0.9, GeneDoc version 2.7.000 and Clustal-X™ version 2.0.12. For the generation of a phylogenetic tree, a segment of 198 amino acids was selected to correspond to residues 101 to 298 of the reference strain HG (Steer *et al.*, 2011). MEGA software version 5 was used to compute the distance matrix using the Jones-Taylor-Thorton (JTT) model and subsequently used to generate a phylogenetic tree using the neighbour-joining (NJ) method with 1000 bootstrap replicates.

GenBank Accession Numbers

The sequences were submitted to GenBank and the accession numbers assigned were: UPM04217 [GenBank: JF917237], UPM08158 [GenBank: JF917238] and UPM08316 [GenBank: JF917239].

RESULTS AND DISCUSSION

The main structural component of the capsid of fowl adenovirus is the hexon, which plays important roles in establishing immune responses (Russel, 2009). The hexon determines the type, group and subgroup antigenic determinants of fowl adenovirus (Norrby, 1969) and has been proven to be very useful in serotype identification. Therefore, several classification methods have been developed recently based on the hexon region for FAdV typing (Marek *et al*., 2010; Meulemans *et al*., 2001; Steer *et al*., 2011).

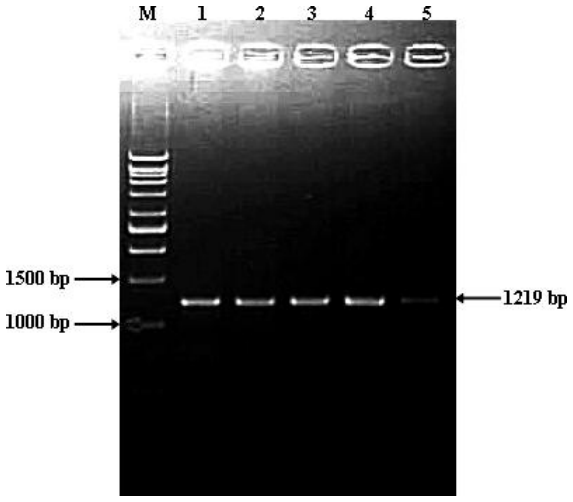
TABLE 1
FAdV hexon genes used in the sequence and phylogenetic analysis

No	Strain	Accession number	Reference
1	CELO	AF339914	Meulemans *et al*., 2001
2	340	AF508952	Meulemans *et al*., 2004
3	IBH-2A	AF339916	Meulemans *et al*., 2001
4	TR22	AF508953	Meulemans *et al*., 2004
5	506	AF508950	Meulemans *et al*., 2004
6	J-2A	AF339917	Meulemans *et al*., 2001
7	KR5	AF508951	Meulemans *et al*., 2004
8	C2B	AF339923	Meulemans *et al*., 2001
9	685	AF508947	Meulemans *et al*., 2004
10	SR48	AF508946	Meulemans *et al*., 2004
11	75	AF508949	Meulemans *et al*., 2004
12	75-1A-1	AF339921	Meulemans *et al*., 2001
13	SR49	AF508948	Meulemans *et al*., 2004
14	A2-A	AF339918	Meulemans *et al*., 2001
15	380	AF339925	Meulemans *et al*., 2001
16	CR119	AF508954	Meulemans *et al*., 2004
17	X11	AF339920	Meulemans *et al*., 2001
18	X11A	AF339924	Meulemans *et al*., 2001
19	YR36	AF508955	Meulemans *et al*., 2004
20	58	AF508957	Meulemans *et al*., 2004
21	TR59	AF508956	Meulemans *et al*., 2004
22	T8-A	AF339919	Meulemans *et al*., 2001
23	764	AF508958	Meulemans *et al*., 2004
24	B-3A	AF339922	Meulemans *et al*., 2001
25	430-06	GU120266	Steer *et al*., 2011
26	607-06	GU120267	Steer *et al*., 2011
27	Australian FAdV Vaccine	GU120268	Steer *et al*., 2011
28	Stanford	DQ323986	Alvorado *et al*., 2007
29	HG	GU734104	Grgić *et al*., 2011

The present study describes the molecular characterisation of five Malaysian isolates of FAdVs that were associated with field outbreaks of IBH. Among the five isolates, only UPM04217 was studied in detail. Meanwhile, UPM04217 had been previously isolated (Hair-Bejo, 2005) from a commercial broiler farm in Perak and identified as FAdV by electron microscopy (Alemnesh *et al*., 2012) and other molecular techniques (Jason *et al*., 2008). This isolate caused 100% mortality in SPF embryonated chicken eggs but showed low pathogenicity to 9-day-old SPF chicks since no clinical signs, mortality and gross lesions were found. This finding led to the present study

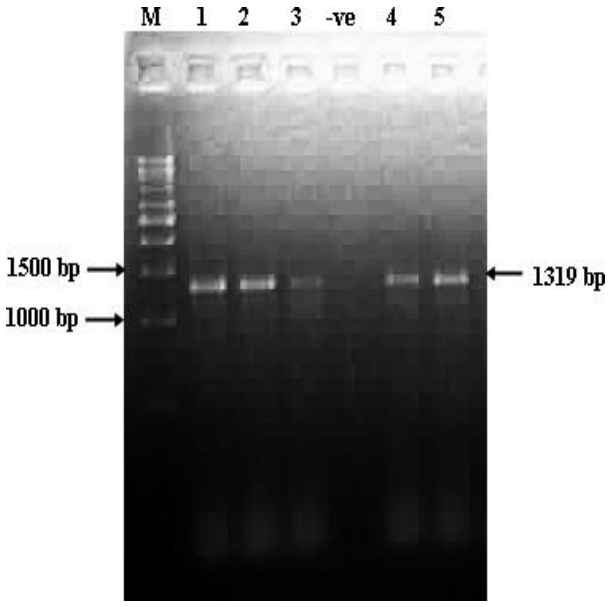
for the characterisation of the Malaysian isolates as no such report on them has been published to date.

The presence of FAdVs was detected by virus isolation. All infected embryos inoculated with UPM04217, UPM08158, UPM08136, UPM11134 and UPM11142 isolates showed obvious gross lesions of swelling, paleness, haemorrhages and multi-focal necrosis on the liver (data not shown). The presence of adenovirus was demonstrated by PCR using the H1/H2 and H3/H4 primer sets to amplify conserved regions of the hexon genes (Fig.1 and Fig.2), where fragments of expected sizes with the estimated lengths of 1219 bp and 1319 bp, respectively, were obtained. When subjected to RE analysis, all the isolates produced identical cleavage patterns. The *Hae*II restriction profiles of the H1/H2 PCR products of UPM04217, UPM08158, UPM08136, UPM11142 and UPM11134 isolates yielded three segments between 200 and 1200 bp (Fig.3A and Fig.3B), whilst the cleavage of the H3/H4-amplified segments generated five fragments between 100 to 400 bp (Fig.4). Interestingly, the digestion patterns of *Hae*II are similar to the RE profiles for FAdV strain 764 (Raue and Hess, 1998) that had been used as a reference strain of FAdV-9, on the basis of molecular weight in agarose gels. However, in accordance with the current ICTV nomenclature system (Benko *et al*., 2000), FAdV strain 764 which had formerly been classified as European serotype 9 was renamed as FAdV-8b (Pizzuto *et al*., 2010; Steer *et al*., 2009). The serotype identification was further strengthened by the generation of five fragments when the *Hpa*II restriction enzyme digested the H3/H4 PCR products of these isolates. These



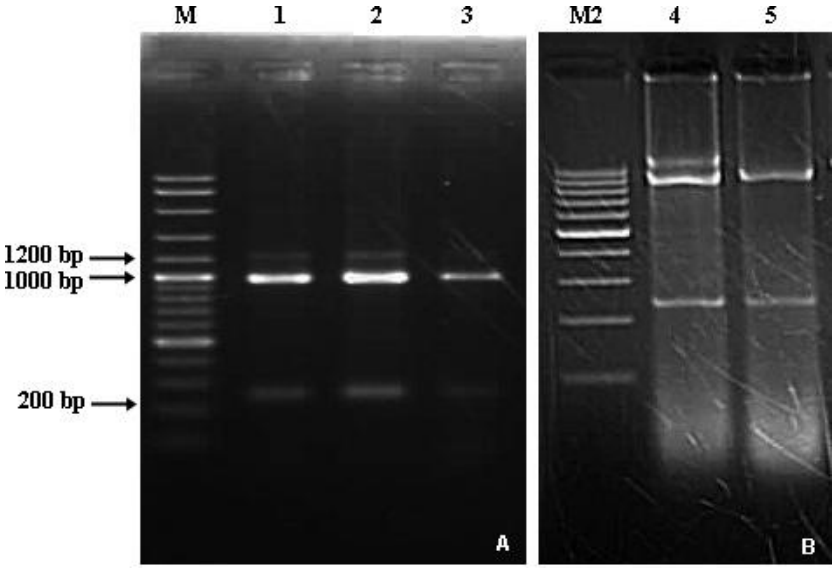
Lane 1, UPMPH04217; 2, UPM08158; 3, UPM08136; 4, UPM11142; 5, UPM11134 and M, molecular weight DNA marker (1kb DNA ladder, Promega).

Fig.1: Demonstration of the presence of FAdV using the hexon-based primer pair H1/H2



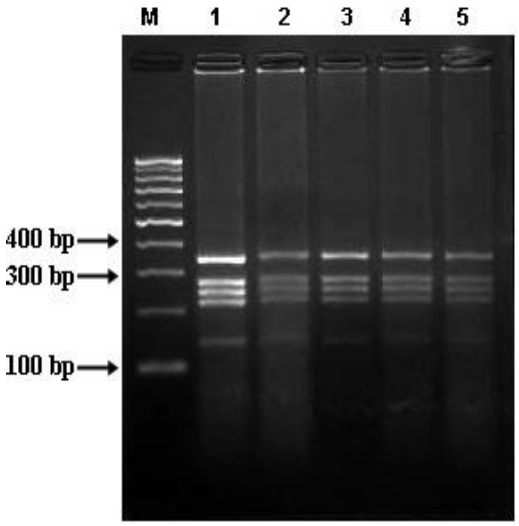
Lane 1, UPMPH04217; 2, UPM08158; 3, UPM08136; 4, UPM11142; 5, UPM11134; M, molecular weight DNA marker (1kb DNA ladder, Promega) and –ve, negative control.

Fig.2: Amplification of the hexon gene using the H3/H4 primer set



A) Lane 1, UPM04217; 2, UPM08158; 3 UPM08136; M, DNA size marker (VC 100bp Plus DNA ladder, Vivantis). B) Lane 1, Positive control of UPM04217; Lane 2, UPM11142; Lane 3, UPM11134; M, DNA size marker (VC 100 bp DNA ladder, Vivantis).

Fig.3: Ethidium bromide-stained agarose gel showing *Hae*II restriction enzyme patterns of the FAdV local isolates



Lane 1, UPM04217; 2, UPM08158; 3, UPM08136; 4, UPM11142 and 5, UPM11134 and M, DNA size marker (VC 100 bp DNA ladder, Vivantis).

Fig.4: Ethidium bromide-stained agarose gel showing *Hpa*II restriction enzyme patterns of the FAdV local isolates

profiles were identical to those of DNA group E fowl adenoviruses (Raue & Hess, 1998; Singh *et al*., 2002) which comprised FAdV-6, -7, -8a and -8b (Pizzuto *et al*., 2010; Steer *et al*., 2009).

Strong evidence for this classification was demonstrated when comparing the amino acid sequences of the L1 loop of the hexon gene of the Malaysian isolates with several reference strains representing each of the 12 serotypes. The sequence analysis of the deduced amino acids of 388 residues of the hexon protein between Malaysian isolates revealed 100% identity among them. The pairwise comparisons and the phylogenetic analysis of the 198 amino acids corresponding to residues 101 to 298 of the reference strain HG (Benko *et al*., 2000) confirmed the classification of UPM04217, UPM08158, UPM08136, UPM11134 and UPM11142 as being FAdV species E. The number of nucleotide differences between Malaysian and selected FAdV strains is shown in Table 2. Low nucleotide differences between Malaysian and several FAdV-8 species E (strains X11-A, T8-A, 764, Stanford, HG, 430-06, 607-06 and Australian FAdV vaccine) ranging from 13 to 23 nucleotides were also observed. In contrast, FAdV-A to -D showed up to 257 nucleotide differences. The highest identity of 98.1% was revealed between Malaysian strains and FAdV strain 764 followed by strains HG, 430-06, 607-06, Australian FAdV Vaccine and T8-A which showed 97.6% identity. An overall identity percentage of only 68% sequence identity was shown between the Malaysian isolates and FAdV species D, followed by FAdV species B and A with the identity percentage

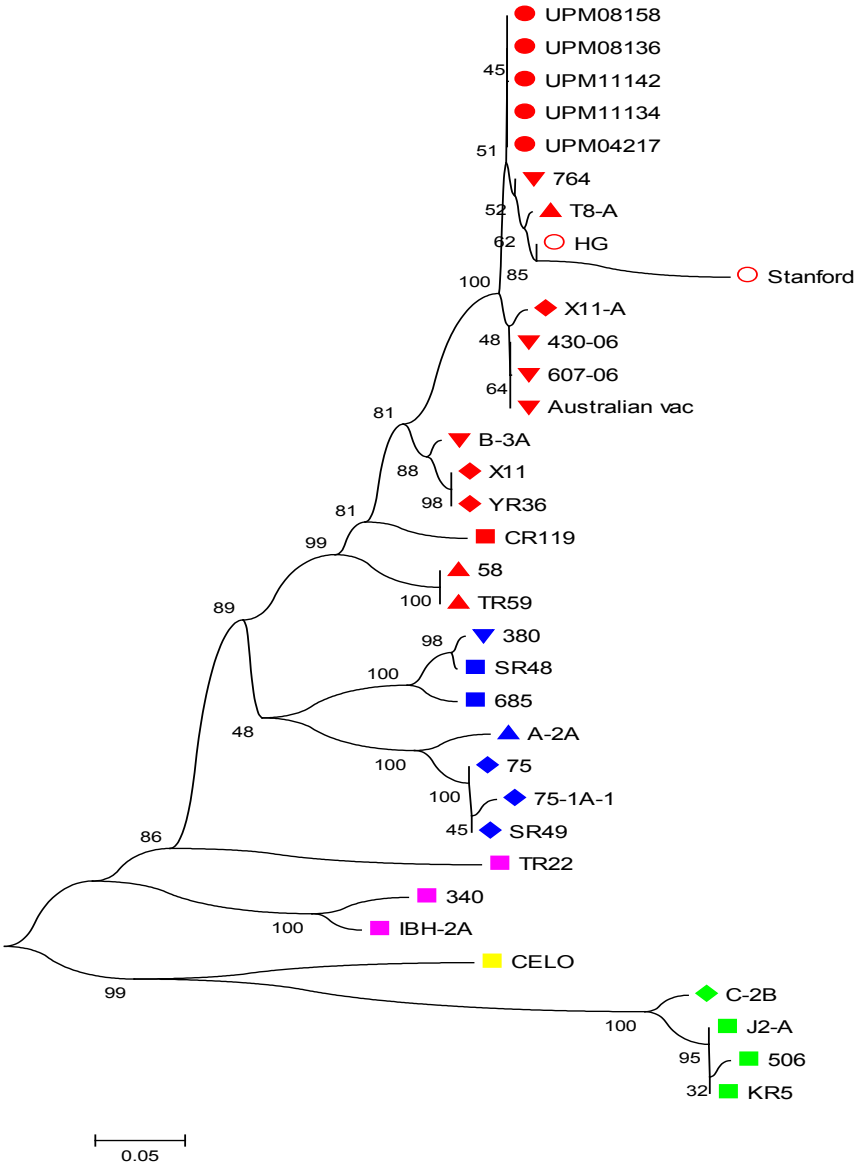
TABLE 2
Comparisons between the L1 sequences of Malaysian isolates with other published sequences

Strain	Species	Serotype	N. diff	Sequence identities (%)
CELO	A	1	237	60.4
340	B	5	213	64.6
IBH-2A	B	5	210	65.1
TR22	B	5	206	65.4
506	C	4	257	57.0
J2-A	C	4	254	57.5
KR5	C	4	254	57.5
C-2B	C	10	255	57.4
685	D	2	187	68.6
SR48	D	2	189	68.2
75	D	3	192	67.7
75-1A-1	D	3	194	67.4
SR49	D	3	191	67.9
A-2A	D	9	193	67.6
380	D	11	191	67.9
CR119	E	6	117	80.3
X11	E	7	77	87.0
X11-A	E	7	23	96.1
YR36	E	7	75	87.4
58	E	8a	106	82.2
TR59	E	8a	106	82.2
T8-A	E	8a	13	97.8
764	E	8b	11	98.1
B-3A	E	8b	75	87.4
Stanford	E	8[a]	14	97.6
HG	E	8[a]	13	97.8
430-06	E	8b	13	97.8
607-06	E	8b	13	97.8
Australian FAdV Vaccine	E	8b	13	97.8

[a] Unspecific group of FAdV-8 published data.

of 65% and 60%, respectively. FAdV species C showed the lowest evolutionary relationship with the Malaysian isolates, with only 57% identity.

The results of the distance-based method analysis on the 34 aligned amino acid sequences of the L1 loop hexon sequences are summarised in Fig.5. Five major groups are clearly shown in the phylogenetic tree. Each group represents species of fowl adenovirus from A to E of the current ICTV classification. The Malaysian isolates were most likely to have evolved together with several FAdV-8 such as strains 764, HG,



The datasets were bootstrapped 1000 times before being analysed using the Neighbour-Joining method. Species of FAdV are represented by colours; FAdV-A (yellow), FAdV-B (pink), FAdV-C (green), FAdV-D (blue) and FAdV-E (red). FAdV serotypes are represented by symbols as follows; FAdV-1 (yellow, square), FAdV-2 (blue, square), FAdV-3 (blue, diamond), FAdV-4 (green, square), FAdV-5 (pink, square), FAdV-6 (red, square), FAdV-7 (red, diamond), FAdV-8a (red, triangle), FAdV-8b (red, inverted triangle), FAdV-8 of unknown group (red, empty circle), Malaysian isolates (red, circle), FAdV-9 (blue, triangle), FAdV-10 (green, diamond) and FAdV-11 (blue, inverted triangle).

Fig.5: Phylogenetic analysis of amino acid sequences of 34 FAdV strains

Stanford and T8-A and seemed to be derived from a common ancestor. This was validated by the good matches to these viruses when analysed using pairwise alignments. A close relationship was also found between Malaysian and Australian field isolates (430-06 and 607-06) and an Australian FAdV vaccine strain that were identified as FAdV-8b (Steer *et al*., 2011).

In addition, a positive correlation was also found between FAdV-6 and -7 and our isolates but they were subgrouped into different subclusters by using the distance clustering method. The distance between these serotypes was increased equivalent to every mismatch occurring in the pairwise calculation. This topology was supported by the new ICTV classification of fowl adenovirus with five independent clusters representing FAdV-A, -B, -C, -D and –E. Contrary to expectation, strain TR22 was diverged from the phylogenetic clustering with FAdV-5. This evidence for the existence of six clusters has also been reported in some previous studies based on amino acid sequences of the loop regions (see Marek *et al*., 2010; Meulemans *et al*., 2001) and thought to result from cross-reactions among the serotypes.

In other Asian countries like India, Pakistan, Japan and Korea, most FAdVs infections were reported to be associated with hepatitis-hydropericardium syndrome and the main causative agent was FAdV-4 species C (Asthana *et al*., 2011; Kim *et al*., 2008; Mase *et al*., 2009; Mansoor *et al*., 2009; Park *et al*., 2011). Recently, IBH cases reported in Japan are associated with FAdV-2 (Nakamura *et al*., 2011). An increased number of IBH outbreaks associated with FAdVs infection were also reported in Korea (Lim *et al*., 2011). The researchers have identified four serotypes of FAdVs (FAdV-4, -5, -8b and -11) responsible for the IBH outbreaks and these are considered as dominant serotypes in Korea with FAdV-4 isolated in most cases. In Malaysia, outbreaks of IBH have been reported from several states, yet the serotype of the FAdV responsible is not known. Interestingly, the IBH cases in Malaysia caused solely by FAdV-8b, species E and are similar to those strains originating from Northern Ireland (strain 764), U.S.A (strain Stanford), Canada (strain HG) and Australia (strains 430-06, 607-06 and Australian FAdV Vaccine) (Alvarado *et al*., 2007; Calnek & Cowen, 1975; Grgić *et al*., 2011; McFerran *et al*., 1972) that have minimal number of nucleotides different. In previous studies, FAdV species E was also reported to be isolated in majority of the outbreaks in Ireland, England, Australia and New Zealand with FAdV-8 exhibiting the highest virulence and being the dominant serotype in Ontario, Canada, although the presence of other serotypes was also encountered (El-Attrache & Villegas, 2001; Erny *et al*., 1991; Grgić *et al*., 2011; McCracken *et al*., 1976; Steer *et al*., 2011; Toro *et al*., 1999; Ojkic *et al*., 2007). However, the relationship between geographical areas and genotype of FAdV remains poorly understood and we are unable to conclusively establish the origin.

An accurate identification of the serotypes involved in IBH outbreaks is very useful for epidemiological tracing and controlling the disease. Thus, vaccination

strategies against FAdV-8b should be developed for the prevention of IBH outbreaks in Malaysia. However, additional numbers of isolates are preferable for the establishment of the role of this virus in relation to IBH outbreaks.

CONCLUSION

This study describes the molecular characterisation of fowl adenoviruses isolated from several states of Malaysia through a combination of hexon-based PCR, REA and phylogenetic analysis. The outbreaks of IBH investigated in this study were solely caused by FAdV-8b and seemed to be the dominant serotype in Malaysia. Therefore, there is a need for the development of vaccines against FAdV-8b to control FAdV infections in the poultry industry.

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