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Research Article
Pathogenicity, Histological and Molecular Characterization of Fowl Adenovirus 8b Propagated in Eggs and Chicken Embryo Liver Cells in a Bioreactor

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Abstract

Background and Objective: Fowl adenovirus 8b vaccine development is critical for the control of Inclusion Body Hepatitis (IBH) in chickens and scale-up for large volume production could be herculean. This study was conducted to determine the pathogenicity, histological and molecular changes of a field isolate of FAdV 8b (UPM11142) passaged in chicken embryonated eggs (CEE), chicken embryo liver (CEL) cells and propagated in microcarrier adapted CEL cells in a bioreactor. **Materials and Methods:** The virus isolate was inoculated into 9 days old specific pathogen-free (SPF) CEE, incubated at 37°C and monitored daily for mortality. Virus from liver homogenates of the dead embryo was passaged 5x on freshly prepared CEL cells and 1x in Cytodex 1 microcarrier adapted CEL cells in a bioreactor and $TCID_{50}$ was determined. The H&E, immunoperoxidase and immunofluorescence assays were carried out. Amplification, sequence alignments and phylogenetic analysis of hexon and fibre genes were conducted. **Results:** There was 100% mortality of CEE. Infected CEL cells produced cytopathic effects characteristic of FAdV. The CEL cells attached and proliferated in the microcarrier beads and were used to propagate CELP5 isolate to produce CELP5B1 with improved titre. Eosinophilic intranuclear inclusion bodies, depletion of cells and FAdV in the nucleus within 48 hrs post-inoculation were observed. All isolates were 98-100% identical and clustered together with group E, serotype 8b FAdV in the same evolutionary clade. **Conclusion:** Propagation of FAdV isolate in CEL cells offers the promise of further research and vaccine development while successful bioreactor propagation will be helpful in vaccine scale-up, large volume production and as the model for other viruses.

Key words: Fowl adenovirus 8b, chicken embryo liver cells, microcarrier, bioreactor, pathogenicity, hexon gene, fibre gene

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Fowl adenovirus (FAdV) is an emerging causal pathogen for Inclusion Bodies Hepatitis (IBH) in chicken responsible for heavy losses in poultry industries worldwide[1-3]. The FAdV is made up of 5 genotypes (A-E) and 12 species (FAdV1- FAdV8a, FAdV8b-FAdV11)[4]. Serotype 1 which causes gizzard erosion in chickens[5], serotype 4 which causes hydropericardium syndrome[6] and serotypes 2, 7, 8a, 8b and 11 which cause IBH[7-9] are the strains currently recognized as pathogenic in chickens.

The FAdV serotype 8b which has been reported to cause IBH in chickens worldwide[10] was first reported among broiler chickens in Malaysia in 2005 with 10% mortality[1] and later reported in other outbreaks in 2011[11] and 2015[3] with 16 and 30% mortality, respectively. In these outbreaks, the infection followed a short course with mortality occurring without many clinical signs. So adequate and early diagnosis especially with methods adaptable in simple laboratories is necessary. The disease predominantly affects chicks of 3-7 weeks of age[12] and is transmitted vertically and horizontally[13,14] with the liver being its primary target organ[15]. The trend of IBH in Malaysia indicates increasing mortality across outbreaks which necessitates interest in FAdV 8b prevention and control.

Although adenovirus has been an instrument of research, mainly as a vector for other vaccines[16], its rise as an emerging pathogen in Malaysia and worldwide has necessitated research interests in prevention and control[17], especially vaccine development. Vaccination has proven to be the most effective tool in controlling and preventing viral infections, to help improve production and overall animal health[18]. Major drawbacks to the availability of vaccines over the years have been technical manufacturing scale-up obstacles[19]. Vaccines are required in large volumes and availability could be easier through cell culture-based production than through egg-based production[20]. But *in vitro* growth of FAdV in established cell lines seems difficult and hinders vaccine development. Efforts to grow FAdV on adult hamster kidneys, green monkey kidney cells[21] and Vero cells[17,22] occurred with limitations and chicken embryo fibroblast (CEF) is insensitive to FAdV[23]. Chicken hepatoma cells[15,24] and chicken embryo kidney cells[25] showed encouraging results but with the liver being the major predilection site for FAdV infection *in vivo*[15], CEL cells could show better adaptability for long passages.

Although several methods exist for the growth of cells in large volumes, the use of microcarriers as a platform to support anchorage-dependent cell proliferation in suspension culture is the most desirable[26] and polymer microcarriers like Cytodex, a microporous microcarrier which has a small pore size that allows cell attachment and growth only on their external surface and has been used to produce follicle stimulating hormone and virus vaccines are highly recommended[27,28]. Operating conditions similar to *in vivo* conditions are required for optimum cell growth and this can be provided by a suitable bioreactor[29], like continuous stirred tank bioreactors[30-32], which are used in 70% of all fermentation and bioprocess operations[33]. A bioreactor is a piece of complex bioprocess equipment designed to carry out fermentation in a closed system but can be adapted and optimized to provide an ideal environment for all mammalian cell growth, with each operation requiring a specific set of parameters adequate for animal cell growth, yield and virus propagation[34].

The objectives of this study were to determine the pathogenicity, histological and molecular changes of a field isolate of FAdV 8b (UPM11142) initially passaged in CEE and CEL cells in a culture flask before propagation in the microcarrier-adapted CEL cells in a bioreactor.

MATERIALS AND METHODS

Study area: This research was carried out at the Virology Laboratory, Faculty of Veterinary Medicine, Universiti Putra Malaysia, from 2017 to 2021.

Virus: The FAdV isolate named UPM11142 was used. It was isolated from an outbreak of IBH in broiler chickens in Malaysia in 2011 with 0.5-1% mortality daily in broilers aged from 7 days to age[35] and maintained in the laboratory. Each of the isolates was filtered through a 0.45 µm syringe filter (Corning, USA) before use.

Propagation of FAdV isolates in specific pathogen-free (SPF) chicken embryonated eggs (CEE): An aliquot of 100 µL of UPM11142 isolate was inoculated into five, 9 days old SPF CEE (Valo Biomedia, USA) each through the chorioallantoic membrane (CAM)[36] with three eggs as control. The eggs were incubated (Memmert, Germany) at 37°C and monitored daily for mortality. The liver and CAM of the dead embryo were harvested. The liver from dead embryos was mixed with

2 parts PBS and 1-part liver (v/v) and macerated in a sterile mortar and pestle. The macerated liver in PBS was emptied into 50 mL centrifuge tubes and centrifuged (Hettich Zentrifugen, Germany) at 1500 rpm for 5 min. The supernatants were extracted, filtered through a 0.45 µm syringe filter (Corning, USA), labelled appropriately and stored at -20°C.

Preparation of chicken embryo liver cells: Liver from 15 days old SPF CEE was used to prepare CEL cells by aseptic harvesting and macerating them in a sterile petri dish. The macerated liver was treated with sodium bicarbonate-free trypsin-EDTA (0.25% trypsin and 2.25 mM EDTA) (Corning, USA) and centrifuged (Hettich Zentrifugen, Germany) at 1500 rpm for 5 min. The supernatant was discarded and CEL cells were suspended in DMEM media supplemented with 10% fetal bovine serum (FBS) and seeded into tissue culture flasks (Corning, USA), incubated in a CO_2 incubator (ThermoForma, USA) at 37°C and 5% CO_2. Until 80% confluency.

Propagation of the FAdV isolate in CEL cells: Confluent 25 cm^2 tissue culture (TC) flasks with primary CEL cells were used. The DMEM medium was decanted and each TC flask was washed with 6 mL of 2% DMEM 2x and inoculated with 0.1 mL of the CEE FAdV supernatant. The CEE isolate was inoculated into flasks and incubated in a CO_2 incubator (ThermoForma, USA) at 37°C and 5% CO_2 for 1 hr after which the inoculated cells were maintained in fresh DMEM media supplemented with 2% FBS and re-incubated at 37°C and 5% CO_2. Three uninfected flasks were used as control. The TC flasks with viral culture and the control were observed daily for CPE until 70% CPEs were visualized[37]. The CPE image for each passage and the corresponding control were visualized using an inverse microscope (Nikon Eclipse TS100, Japan) and the image was captured with a NIS Elements imaging system (Nikon Digital sight DS-U2, Japan) powered by PowerLogic computer with 17'' monitor (Lenovo, China).

At 70% CPE, the culture was freeze-thawed at -20°C, 3 times and pipetted into 50 mL centrifuge tubes (SPL, Korea) and centrifuged (Hettich Zentrifugen, Germany) at 1500 rpm for 5 min. About 1 mL of the supernatant was aliquoted into a sterile 1.5 mL Eppendorf tube and stored at -20°C (Fisher and Paykel, New Zealand), while the rest supernatant was stored at -80°C until use (Panasonic Ultra-low Freezer, Japan). They were labelled appropriately as cell culture passage 1 (CELP1) of their corresponding isolate. The isolate was passaged five times (CELP5).

Propagation in bioreactor: The UPM11142CELP5 isolate was propagated in stirred tank bioreactor as described by Ugwu *et al.*[38]. All glassware used was siliconized before sterilization. The B Braun Biostat® B Fermentation Cell Culture Bioreactor (Type 8840334) (Sartorius, Goettingen, Germany) was set up and Cytodex 1 microcarriers were prepared following the manufacturer's recommendation. Prepared 3.1×10^7 CEL cells were seeded into 8.6×10^6 microcarrier concentration with a surface area of 17.6 m^2 and inoculated with CELP5 isolate with a titre of $10^{7.5}$ mL^{-2}. Adaptation of CEL cells to Cytodex 1 microcarriers and propagation in stirred tank bioreactor was carried out as previously described by Ugwu *et al.*[38].

Determination of the infective dose ($TCID_{50}$) of the passage isolates: The FAdV CEL cell passages 1 and 5 of the isolate and the bioreactor isolate were diluted serially 10x and each dilution was used to infect 8 wells in 96-well tissue culture plates with confluent CEL cells and incubated in a CO_2 incubator (Thermo Forma, USA) at 37°C and 5% CO_2 [52, 50]. Each well was monitored daily for cytopathic effects (CPE) and the virus titre ($TCID_{50}$) was calculated using the formula of Reeds and Muench[39].

Haematoxylin and eosin (HE) staining: The HE staining for wet culture cells was performed[40]. Sterile coverslips were aseptically placed inside each well of 6-well tissue culture plates and placed under UV light overnight. The CEL cells were prepared as previously described and seeded onto the sterile coverslips and incubated in a CO_2 incubator (Thermo Forma, USA) at 37°C and 5% CO_2. The plates were observed daily until 80% confluency was achieved. The medium was discarded and washed 2x with PBS. Each well was infected with 0.1 mL of respective inoculum and incubated at 37°C and 5% CO_2 for 1 hr for adsorption. The inocula were CELP5 and CELP5B1 isolates. One hundred and eighty µl of DMEM media supplemented with 2% FBS was added while the uninfected control was maintained with only media and all incubated at 37°C and 5% CO_2 and observed for CPE for 48 hrs post inoculation (pi). On 40% CPE, media was discarded and washed twice with PBS. The wells were fixed with 4% buffered formalin for 30 min at room temperature and washed 2x with PBS. The slips were stained with haematoxylin directly for 5 min and washed with tap water for 2 min. The slips were

dipped in acid alcohol 3x and soaked in tap water for 5 min. The slips were stained with eosin for 5 min and washed with tap water for 2 min. The slips were dipped in 100% ethanol 3x, 90% ethanol 3x, 70% ethanol 3x, xylene 1 min and xylene again for 1 min and quickly mounted on a clean glass slide with DPX. The mounted slides were air-dried and viewed under a simple light microscope and images were captured[36,41].

Immunoperoxidase assay: The CEL cells preparation, seeding on sterile cover slip and infection with FAdV was carried out as described in the HE protocols above. On CPE, the wells were fixed with 4% buffered formalin for 30 min at RT after which they were washed 2x with ice-cold PBS containing 0.5% Tween 20 (PBST) for 5 min and quenched with 3% hydrogen peroxide for 30 min at RT. The plates were washed 2x with PBST for 5 min each. Forty microlitres of monoclonal FAdV hexon protein primary antibodies (Santa Cruz, USA) 1:500 dilution was added into the cells, allowed to stand and incubated at 4°C in a humidity chamber overnight after which the wells were rinsed 2 with PBST for 5 mins. Forty microlitres of mice anti-chicken Ig(H+L)/HRP-conjugated secondary antibody (eBioscience, USA) 1:1000 dilution was added and incubated at RT for 1 hr in a dark room. The plates were rinsed with PBST and 100 µL/well of 3.3′ diaminobenzidine for 5 min, rinsed again with PBST and stained with haematoxylin for 15 min. The cover slip was rinsed in slow-running water, dried, mounted on clean glass slides with DPX and observed under a microscope for brownish or golden colouration within the cytoplasm of the cells[30].

Immunofluorescence assay: The CEL cells preparation, seeding on sterile cover slip and infection with FAdV was carried out as described in the HE protocols above. On CPE, the wells were fixed with 4% buffered formalin for 30 min at RT after which they were washed 2x with ice-cold PBS containing 0.5% Tween 20 for 5 min and plates incubated in 0.5% Triton x-100 in PBST for 15 min to permeabilize the cells followed by rinsing with PBST 3x for 5 min. Unspecific bindings were locked with a blocking buffer (5% BSA in PBST) for 1 hr at RT and rinsed 3x with PBST for 5 min. Forty microlitres of adenovirus hexon protein (3GO) mouse monoclonal IgG (Santa Cruz, USA) 1:500 dilution was added onto the slips, allowed to stand and incubated at 4°C in a humidity chamber overnight after which the wells are rinsed 2x with PBST for 5 min. Cells were stained with 40 µL of m-IgGk BP-CPL 488 CruzFlor 488 conjugated secondary antibody (Santa Cruz, USA) 1:1000 dilution and incubated at RT for 1 hr in a dark room. The plates were rinsed with PBST and 20 µL of diamidino-2-phenylindole dihydrochloride (DAPI) added for 10 mins at RT. The cover slip was rinsed, dried, mounted on clean glass slides with DPX and examined with fluorescence microscope[30].

DNA extraction and PCR amplification of hexon and fibre genes: The DNA from UPM11142P0, UPM11142P1, UPM11142P5 and UPM11141P5B1 were extracted using innuPREP Virus DNA Kit (analytikjena, Germany) and the concentration was measured at 70 dilution factor using Ultraviolet-visible Spectrophotometer (UV-1601, PC, Shimadzu, Japan). The HexA1/HexB1 primers[42] were used to amplify the hexon gene while fbrF/fbrR primers[38] were used for fibre gene amplification with SensoQuest Labcycler Gradient (Germany) in a 25 µL reaction using MyTaq Red Mix (Bioline, UK). The amplification conditions were 95°C for 2 min 1x, 95°C for 1 min 35x, 55°C for 1 min 35x, 72°C for 1 min 2 cycles 35x and 72°C for 1 min 1x for hexon gene and 95°C for 2 min 1x, 95°C for 1 min 35x, 50°C for 1 min 35x, 72°C 2 cycles for 1 min 30 sec 35x and 72°C for 2 min 1x for fibre genes. The PCR products were separated by electrophoresis in EPS-300X (C.B.S. Scientific, Taiwan) in 1% agarose gel (GeneDireX, USA) at 70 V and 400mA for 60 min, stained with Redsafe stain and visualised through UV transillumination (Syngene, Gene Genius Bio Image system)[43].

Sequencing, blast, alignment and phylogenetic analysis: The PCR products were purified using a MEGAquick-spin™ total fragment DNA purification kit (iNtRON Biotechnology, USA) and sequenced (First base laboratories, Malaysia). Consensus sequences were assembled using the contig assembly application in BioEdit software v 7.2.5[44] and amino acids were deduced by using ExPasy software (www.expasy.ch/tools/dna.html). Sequences were subjected to NCBI Blast and amino acid residues of both hexon and fibre genes were aligned using BioEdit software v7.2.5 against UPM04217 reference strain with the sequences and 21 published strains for hexon (Table 1) with NCBI Genbank link in Table S1 and 14 published strains for fibre retrieved from Genbank (Table 2) with NCBI Genbank link in Table S2, phylogenetic trees were constructed by Neighbor-Joining Method using MEGA X software v 10.0.5[45].

Table 1: Aviadenovirus reference strains obtained from Genbank were used in the construction of a phylogenetic tree for hexon gene analysis.

Isolate name	Serotype	Origin	Accession number	Uploaded By
Celo	1	Australia	EU979367.1	NCBI Upload links in Table S1
Wroclaw 2015	1	Poland	KR259656.1	
SR48	2	Australia	EU979368.1	
SR49	3	Australia	EU070369.1	
KR5	4	Australia	EU979370.1	
CH/CQBS/1601	4	China	MF055642	
Indian	4	India	AJ459805.1	
340	5	Australia	EU979371.1	
2024682		Pakistan	KX377620.1	
CR119	6	Australia	YP009505663.1	
YR36	7	Australia	EU979373.1	
NSW-2/100642/1112	8a	Australia	KT037695.1	
UPM08158	8b	Malaysia	AEL21619.1	
FJ-3/100843-F	8b	Australia	KT037696.1	
UPM04217	8b	Malaysia	KU517714.1	
05-50052-3180-3	E	Canada	EF685409.1	
AO2	9	Australia	EU979376.1	
C2B	10	Australia	EU979377.1	
WA-1/100966-2	11	Australia	KT037713.1	
Duck 1	DAdV	-	EF09307.1	
Turkey Adv	3	UK	AC_000016	

Table 2: Aviadenovirus reference strains obtained from GenBank were used for alignment and in the construction of a phylogenetic tree for fibre gene analysis

Isolate Name	Serotype	Origin	Accession Number	Uploaded By
PL-060-08	1	Poland	GU952109.1	NCBI Upload links in Table S2
Punjab1	1	India	DQ864435.1	
NARC-3317	4	Pakistan	KT733569.1	
SX17	4	China	MF595799.1	
340	5	Austria	FR872928.1	
CR119	6	USA	NC_038332.1	
YR36	7	Austria	KT862809.1	
UPM04217	8b	Malaysia	KU517714.1	
UPM1137E2	8b	Malaysia	KY305950.1	
06-25854-1	11	Canada	JQ034219.1	
WA-1/100966-2	11	Australia	KT037713.1	
Strain 13-19395	-	Germany	MK572863.1	
EDS-76	EDSV	-	Z86065.1	
D11-JW-010	DAdV 1	South Korea	JX227930	

Table S1: NCBI GenBank link for reference adenovirus strains used for phylogenetic analysis of hexon gene

Accession number	GenBank link
Phylogenetic tree	
KU647775.1	https://www.ncbi.nlm.nih.gov/nuccore/KU647775.1
AEL21619.1	https://www.ncbi.nlm.nih.gov/nuccore/AEL21619.1
KT037696.1	https://www.ncbi.nlm.nih.gov/nuccore/KT037696.1
KT037695.1	https://www.ncbi.nlm.nih.gov/nuccore/KT037695.1
EU979367.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979367.1
EU979368.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979368.1
EU070369.1	https://www.ncbi.nlm.nih.gov/nuccore/EU070369.1
EU979370.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979370.1
KT037713.1	https://www.ncbi.nlm.nih.gov/nuccore/KT037713.1
EU979371.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979371.1
KX377620.1	https://www.ncbi.nlm.nih.gov/nuccore/KX377620.1
YP_009505663.1	https://www.ncbi.nlm.nih.gov/nuccore/NC_038332.1
EU979373.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979373.1
EU979376.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979376.1
EU979377.1	https://www.ncbi.nlm.nih.gov/nuccore/EU979377.1
KR259656.1	https://www.ncbi.nlm.nih.gov/nuccore/KR259656.1
MF055642.1	https://www.ncbi.nlm.nih.gov/nuccore/MF055642.1
AJ459805.1	https://www.ncbi.nlm.nih.gov/nuccore/AJ459805.1
KU517714.1	https://www.ncbi.nlm.nih.gov/nuccore/KU517714.1
EF685409.1	https://www.ncbi.nlm.nih.gov/nuccore/EF685409.1
AC_000016	https://www.ncbi.nlm.nih.gov/nuccore/AC_000016

Table S2: NCBI GenBank link for reference adenovirus strains used for phylogenetic analysis of fibre gene

Accession number	GenBank link
KU517714.1	https://www.ncbi.nlm.nih.gov/nuccore/KU517714.1
KY305950.1	https://www.ncbi.nlm.nih.gov/nuccore/KY305950.1
GU952109.1	https://www.ncbi.nlm.nih.gov/nuccore/GU952109.1
KT862809.1	https://www.ncbi.nlm.nih.gov/nuccore/KT862809.1
NC_038332.1	https://www.ncbi.nlm.nih.gov/nuccore/NC_038332.1
KT733569.1	https://www.ncbi.nlm.nih.gov/nuccore/KT733569.1
MF595799.1	https://www.ncbi.nlm.nih.gov/nuccore/MF595799.1
DQ864435.1	https://www.ncbi.nlm.nih.gov/nuccore/DQ864435.1
JQ034219.1	https://www.ncbi.nlm.nih.gov/nuccore/JQ034219.1
KT037713.1	https://www.ncbi.nlm.nih.gov/nuccore/KT037713.1
FR872928.1	https://www.ncbi.nlm.nih.gov/nuccore/FR872928.1
MK572863.1	https://www.ncbi.nlm.nih.gov/nuccore/MK572863.1
Z86065.1	https://www.ncbi.nlm.nih.gov/nuccore/Z86065.1
JX227930	https://www.ncbi.nlm.nih.gov/nuccore/JX227930.1

RESULTS

Specific pathogen-free CEE inoculated with three FAdV isolates: There was 100% mortality at 6 days post inoculation (dpi) for SPF CEE (Table 3). Mortality started within 48 hrs with one egg. At 5 dpi, the percentage of egg mortality for UPM11142 was 80.0%. The liver of the uninfected control was normal (Fig. 1a) while the liver of dead SPF embryos infected with FAdV isolates was discoloured, friable, necrotic, enlarged and haemorrhagic with distorted edges (Fig. 1b-d).

CEL cells preparation and propagation of FAdV isolate: Trypsinized liver from SPF CEE yielded liver cells which after seeding in cell culture flasks produced a monolayer of CEL cells that became confluent in 24-48 hrs of incubation (Fig. 2a). The isolate was passaged 5 times on CEL cells and produced CPE which included rounding, shining, clumping, detachment of cells and detachment of entire monolayer within 1-6 days dpi (Fig. 2b-d). At passage 1, no CPE was observed in cells inoculated with UPM11142P0 up to 7 dpi but at passage 5, detachment of the entire monolayer occurred on 1 dpi (Table 4).

Adaptation of CEL cells on microcarriers and propagation of FAdV 8b in microcarrier-adapted CEL cells in a bioreactor: The CEL cells attached and adapted to the Cytodex 1 microcarrier within 3 hrs of incubation (Fig. 3a) and grew confluent on the microcarriers beads after 24 hrs incubation in the bioreactor (Fig. 3b). The FAdV UPM11142CELP5 isolate caused CPE and detachment of cells from microcarriers from 24 hrs post-inoculation (Fig. 3c). At 48 hrs, most of the cells had detached from the microcarrier beads (Fig. 3d) indicating the suitability of the Cytodex 1 and STB for the propagation of FAdV serotype 8b. The final virus volume was 600 mL with a titre of $10^{8.3}$ mL^{-1}.

Titration of the FAdV isolates ($TCID_{50}$ determination) and volume of the bioreactor isolate: The tissue culture infective dose of the isolates were $10^{5.5}$, $10^{7.5}$ and $10^{8.3}$ $TCID_{50}$/1.0 mL for UPM11142P1, UPM11142P5 and UPM11142P5B1, respectively. The final volume of the microcarrier culture was 600 mL.

HE staining: This was different from the uninfected monolayer which maintained normal conformity (Fig. 4a). Cell depletion and detachment of cells from monolayer and eosinophilic intranuclear inclusion bodies were observed in CEL cells at 48 hrs pi (Fig. 4b-d).

Indirect immunoperoxidase assay: The uninfected control showed normal conformity (Fig. 5a). There was brownish colouration indicative of positive FAdV infection and cell depletion and detachment from the monolayer at 48 hrs pi (Fig. 5b-d).

Indirect immunofluorescence assay: The confluent CEL cells of uninfected control stained blue (DAPI) (Fig. 6a) and positive FAdV stained green (Fig. 6b-d). Viral infection of cells leading to mild cell depletion after 24 hrs pi (Fig. 6b-c) and almost complete depletion of cells after 48 hrs (Fig. 6d) were observed. Internalization of the viral genome in the nucleus of CEL cells indicating viral replication was also observed (Fig. 6b and d).

PCR amplification of the hexon and fibre genes of FAdV isolates inoculated in CEE and CEL cells: The three FAdV isolates from CEE were positive for hexon and fibre genes after PCR and electrophoresis with bands corresponding to 900 and 940 base pairs, respectively (Fig. 7a-b).

Sequence analysis and phylogenetic tree construction of the hexon and fibre genes of FAdV 8b isolates: Changes were observed in the amino acid sequences of the hexon gene between the FAdV isolates and the reference strain (Fig. 8). At position 246, UPM11142CEEP0 had alanine, but the reference strains possessed arginine and other changes were H▸247N and H▸248Q substitution in test isolate. In the fibre gene (Fig. 9) there were two R▸144T and K149N substitutions between CEEP0 and CELP5/CELP5B1 isolates. There was also RLSRPK▸SCPALS at position 144-149 between CEEP0 and reference strain. These changes show that CEL cell passages have effects on the genome of FAdV serotype 8b. Based on the phylogenetic tree constructed with nucleotide and amino acid of hexon (Fig. 10a) and fibre (Fig. 10b) genes, all the CEE and CEL cells isolates in the study clustered together with FAdV serotype 8b reference isolates

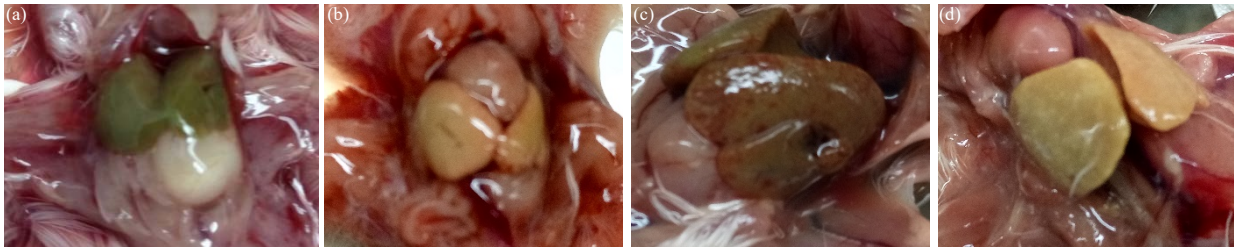

Fig. 1(a-d): SPF CEE infected with FAdV isolate showing haemorrhagic, friable, inflamed and necrotic liver and uninfected control, (a) Normal liver, uninfected control, (b) Discoloured liver of dead embryo inoculated with UPM11142 (5 dpi), (c) Inflamed and haemorrhagic liver and (d) Necrotic liver and enlarged heart (7 dpi)

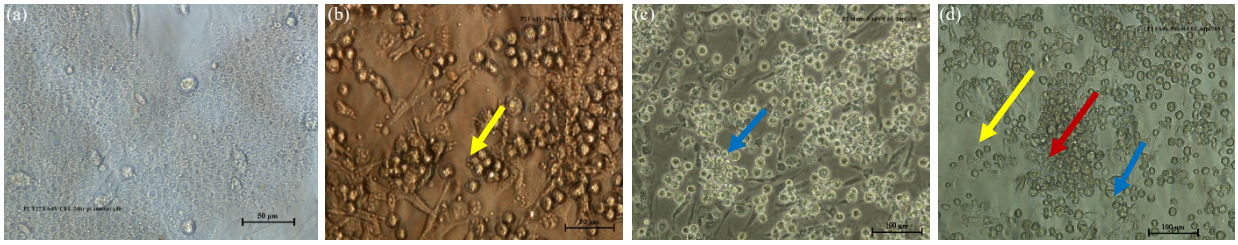

Fig. 2(a-d): Inverted microscopic image of chicken embryo liver cells infected with FAdV 8b, (a) Confluent monolayer CEL cells uninfected control (5 dpi x40), CEL cells infected with, (b) (UPM11142P1) x20 showing cell rounding and shining, (c-d) (UPM11142P5) x20 showing detachment of cells from monolayer, rounding (yellow arrow), clumping (red arrow) and detachment of cells (blue arrow)

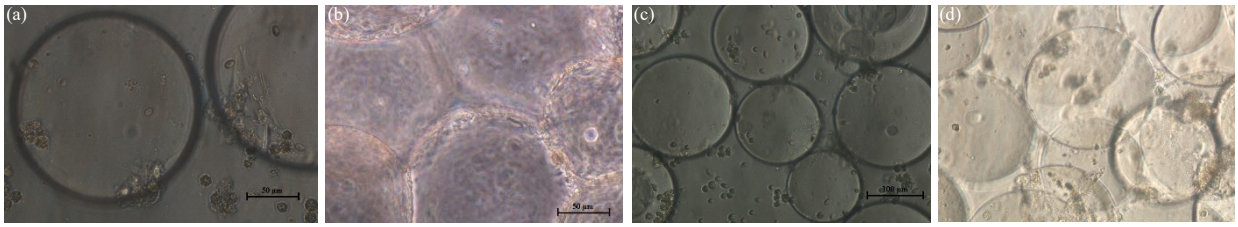

Fig. 3(a-d): Inverted microscopic image of Cytodex 1 microcarrier bead with attached CEL cells, (a) Attachment of CEL cells after 3 hrs (pi), (b) Attachment of CEL cells after 24 hrs, (c) Infected with UPM11142CELP5 with the detachment of cells from the microcarrier beads at 24 hrs pi and (d) Infected with UPM11142CELP5 with the detachment of cells from the microcarrier beads at 48 hrs pi

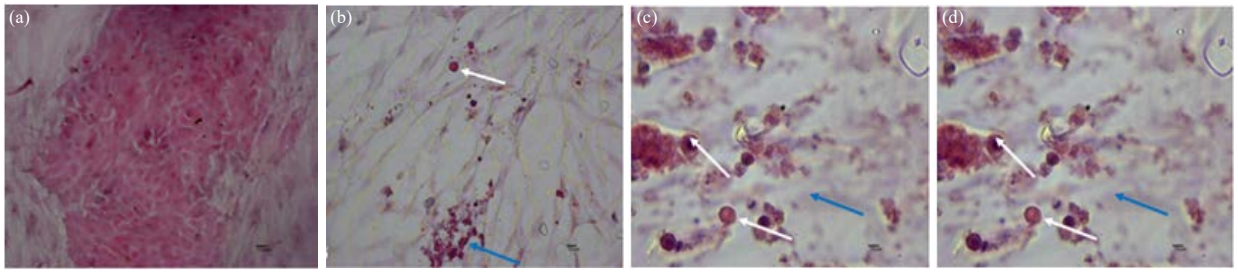

Fig. 4(a-d): HE staining of chicken embryo liver cells infected with FAdV serotype 8b isolates at 48 hrs pi, (a) Normal conformity of liver cells (uninfected control), (b) UPM11142CELP1, (c) UPM11142CELP5 and (d) UPM11142CELP5B1: Showing cell depletion (blue arrows) and eosinophilic intranuclear inclusion bodies in the hepatocytes (white arrows) (a-d, x40), HE

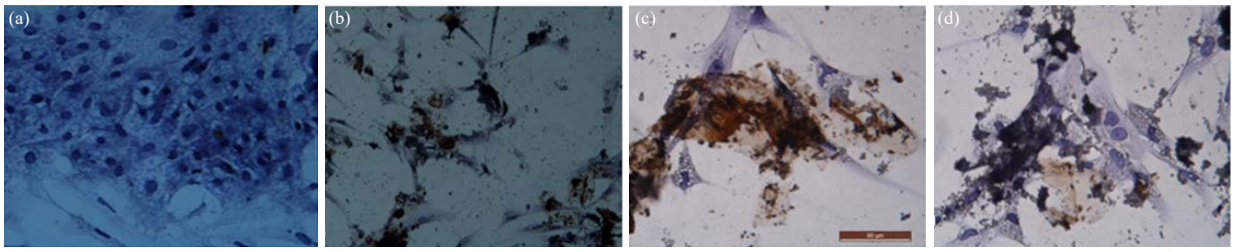

Fig. 5(a-d): Immunoperoxidase staining image of CEL cells infected with FAdV serotype 8b isolates, 48 hrs pi, (a) Normal conformity of liver cells (uninfected control), (b) UPM11142CELP1, (c) UPM11142CELP5 and (d) UPM11142CELP5B1, detachment of cells from monolayer

Cell necrosis, cell depletion and brownish colouration indicating positive FAdV infection (x40)

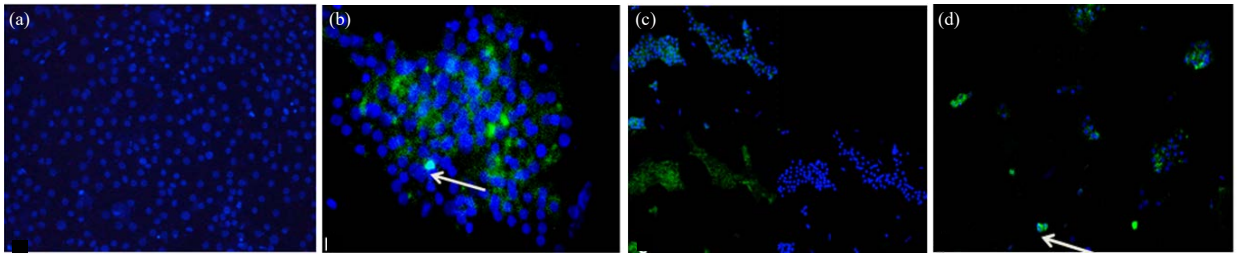

Fig. 6(a-d): Immunofluorescence microscopic image of CEL cells monolayer infected with FAdV serotype 8b isolates, (a) Uninfected control staining blue (DAPI) x20, (b) UPM11142CELP1: Staining blue and green indicating infection and depletion of cells after 24 hrs pi x40, (c) UPM11142CELP5: 3-dimensional image (DAPI, FITC and merged) indicating positive FAdV infection x20 and (d) UPM11142CELP5B1: Almost complete depletion of cells after 48 hrs pi x20

Evidence of internalization of the viral genome in the nucleus of CEL cells is indicated by arrows

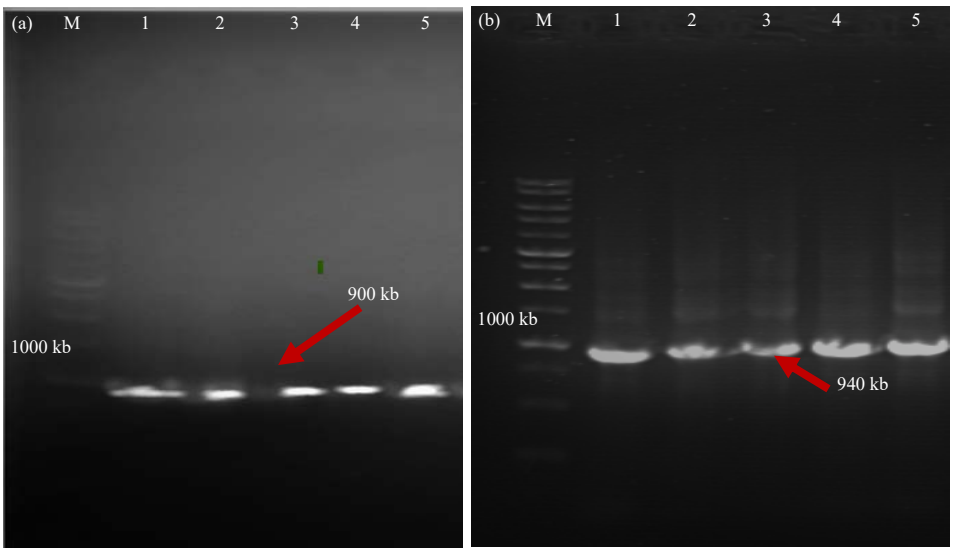

Fig. 7(a-b): Agarose gel electrophoresis image of bands from PCR amplification of hexon and fibre genes indicating FAdV positive samples, (a) Hexon gene profile using hexon-based primer pair HexA1/HexB and (b) Fibre gene profile using fbrF/fbrR

(a) Lane 1: FAdV hexon +ve control, Lane 2, 3, 4, 5 and UPM11142: P0, P1, P5 and P5B1, (b) Lane 1, FAdV fibre +ve control, Lane 2, 3, 4 and 5, UPM11142 (P0, P1, P5 and P5B1) and M: 1kb ladder (Promega, USA)

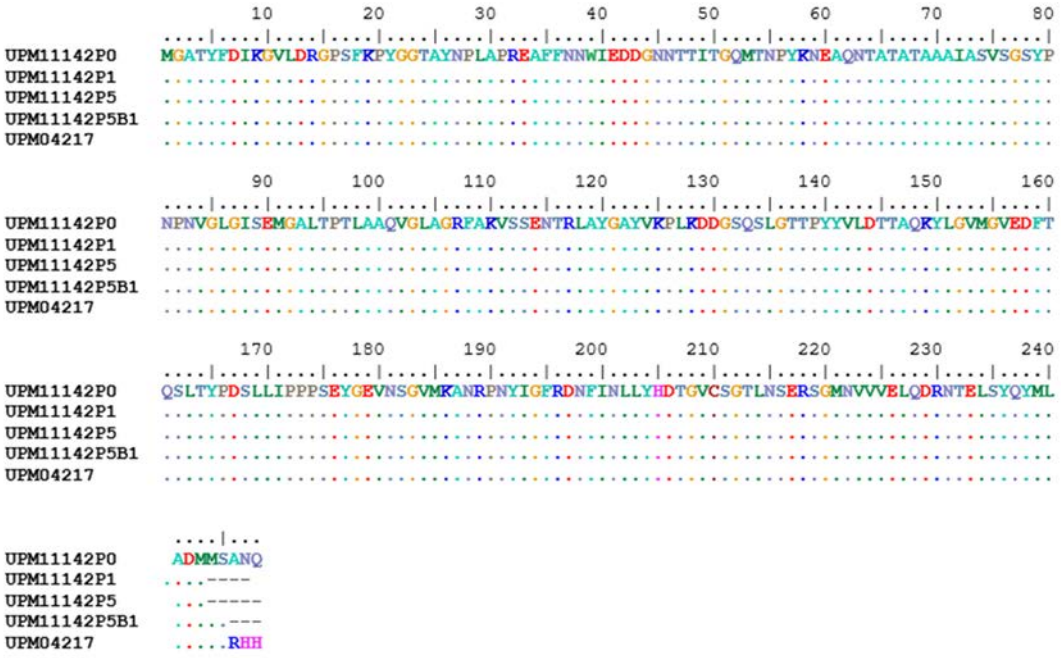

Fig. 8: Multi sequence alignment comparing the hexon gene amino acid residues of original, flask and bioreactor propagated isolates of UPM11142 with UPM04217 reference isolate from GenBank.

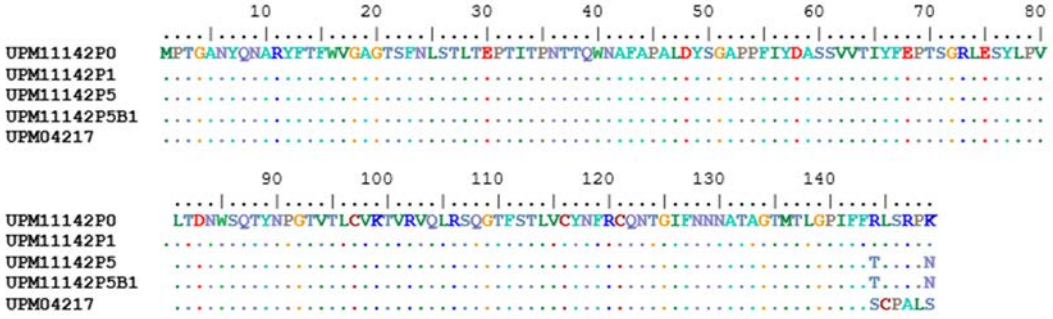

Fig. 9: Multi sequence alignment comparing the fibre gene amino acid residues of original, flask and bioreactor propagated isolates of UPM11142 with UPM04217 reference isolate from GenBank

Table 3: Cumulative mortality of SPF CEE following FAdV inoculation

	Time (day post inoculation)						
FAdV Isolate	1	2	3	4	5	6	Mortality (%)
UPM11142	0/5	1/5	1/5	2/5	4/5	5/5	100
Control	0/3	0/3	0/3	0/3	0/3	0/3	0

Table 4: Cumulative percentage of CPE of CEL cells following inoculation of UPM11142 FAdV isolate

	Time (day post inoculation)						
FAdV passages	1	2	3	4	5	6	7
1	No CPE	No CPE (0/3)	No CPE (0/3)	No CPE (0/3)	No CPE (0/3)	No CPE (0/3)	No CPE (0/3)
2	No CPE (0/3)	No CPE (0/3)	No CPE (0/3)	50% CPE (3/3)	70% CPE (3/3)	90% CPE (3/3)	
3	No CPE (0/3)	10% CPE (1/3)	30% CPE (3/3)	80% CPE (3/3)			
4	No CPE (0/3)	No CPE (0/3)	80% CPE (3/3)				
5	80% CPE (3/3)	-	-				

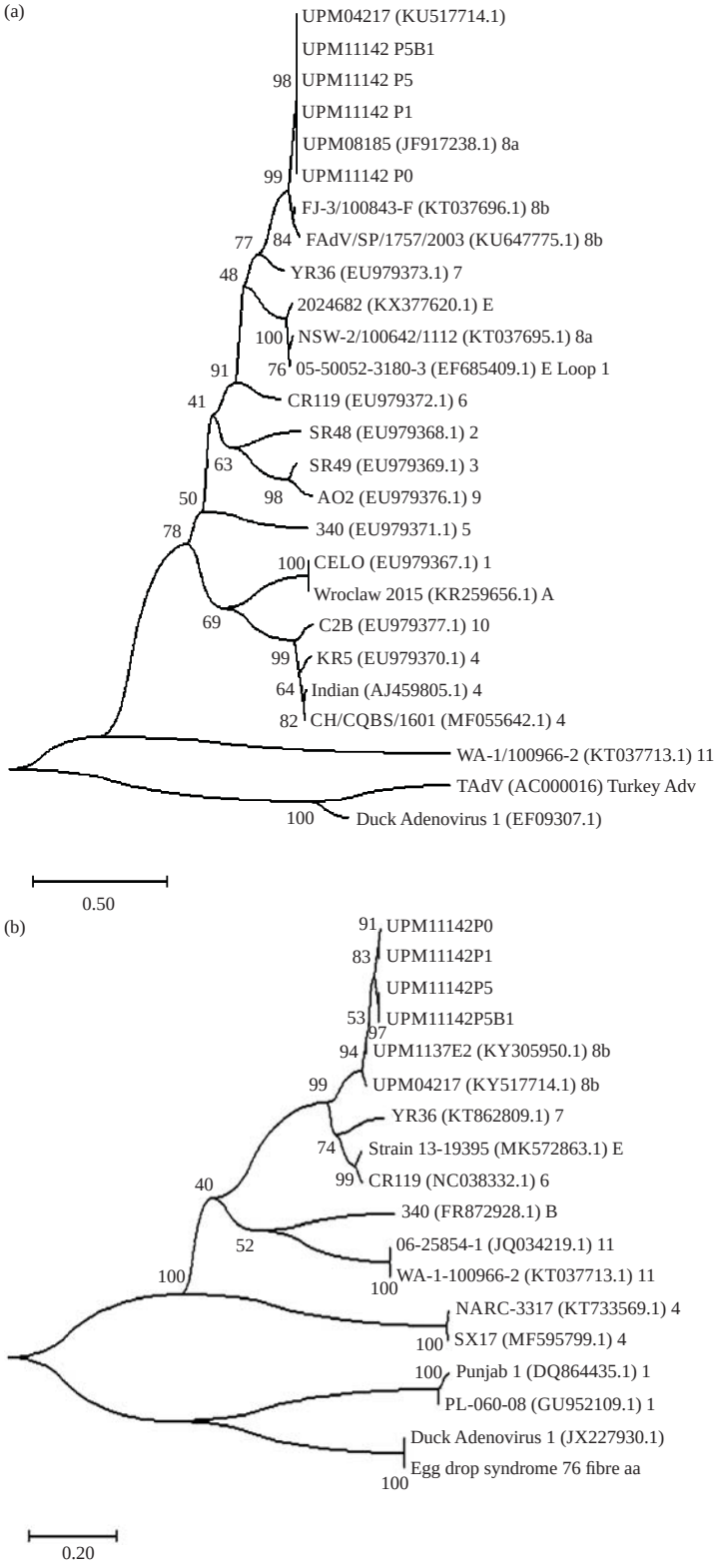

Fig. 10(a-b): Phylogenetic tree analysis of (a) Hexon and (b) Fibre gene based on the amino acids of the three test FAdV CEE, CEL cells isolates, bioreactor isolates and reference strains from GenBank

Root produced 2 branches with FAdV isolates on 1 evolutionary branch. The Group E FAdV all clustered together distinct from the other groups. All FAdV isolates in this study (labelled red) clustered together and were in the same evolutionary clade with another FAdV serotype 8b isolates retrieved from GenBank

retrieved from GenBank which confirms the study isolates as FAdV serotype 8b. All the hexon and fibre gene sequences in this study were deposited in the GenBank repository and were assigned accession numbers as follows: UPM1142CEEP0 (MT127099), UPM1142CELP1 (MT212040), UPM1142CELP5 (MT212043) and UPM11142CELP5B1 (MT561442) for hexon and UPM1142CEEP0 (MT479174), UPM1142CELP1 (MT525004), UPM1142CELP5 (MT525007) and UPM11142CELP5B1 (MT561446) for fibre genes.

DISCUSSION

One hundred percent mortality of SPF CEE inoculated with UPM11142, FAdV isolate in this study is evidence of the high level of pathogenicity of the isolate. Egg mortality is an indication of the pathogenicity of viruses[46]. The mortality rates of the eggs at days 4 pi and 5 pi exposed the pathogenicity of the isolate thereby projecting UPM11142 as very pathogenic. Pathological lesions observed from the dead SPF embryonated eggs which included friable, discoloured and inflamed liver with haemorrhages are typical FAdV lesions as reported by Morshed *et al*.[2] and Norina *et al*.[3].

Typical CPEs which included rounding, clumping, shining and detachment of cell and the entire monolayer were observed in the CEL cells inoculated with the isolate showing that FAdV has adapted very well in primary CEL cells and their suitability for the propagation of FAdV *in vitro* studies and future vaccine research. Similar CPE had been reported with FAdV serotype 4 inoculated onto CEL cells[37,47]. The complete CPE achieved by the isolate within 24 hrs at CELP5 indicates that FAdV at passage 5 in primary CEL cell culture has been fully revived and adapted to cell culture growth. Further passages should be possible which is a breakthrough for future FAdV serotype 8b research and the possible development of live attenuated vaccines. The uniformity in the pattern of CPE exhibited by the isolate indicated that FAdV serotype 8b elicits homologous characteristics on primary CEL cells. Such homologous characteristics when standardized could be a useful tool in the diagnosis and characterization of FAdV serotype 8b in future research. By causing 100% CPE, UPM11142 isolate could be reported as highly virulent. Cytopathic effects produced by virus in cells is regarded as a sign of pathogenicity since these are virus survival mechanisms leading to the expression of intrinsic factors responsible for viral replication, cell death and disease[48].

Chicken embryo liver cells adapted successfully to microcarrier culture which was shown by its attachment to the microcarrier beads within 3 hrs of incubation. The CEL cells, like most animal cells, are anchorage-dependent and most importantly need an attachment to a surface for optimum growth and function[49]. Growth in the beads after 24 hrs shows that Cytodex 1 microcarrier can be used for the proliferation of CEL cells and large volume propagation of FAdV 8b. This could improve cell yield and subsequent virus volume, particularly for large-volume production of FAdV 8b vaccines and could be adapted for other cells and viruses too. Growth of the cells in the beads and subsequent propagation of the virus in the microcarrier-adapted CEL cells is an indication that the stirred tank bioreactor provided a favourable *in vitro* environment required for cell proliferation. This was in line with the use of Cytodex™ 1 to propagate IBDV[30], Influenza virus[50], modified vaccinia ankara (MVA) virus[31] and *E. coli* to grow bacteriophages[51] in stirred tank bioreactor. Although several bioreactor systems have been developed and used, stirred tank bioreactor has been used in 70% of all bioprocess and fermentation operations[33]. But each operation still requires standardization and optimization specific for each cell type to enable optimum cell growth, cell yield and specific productivity[34]. An increase in titre was recorded with the bioreactor isolate in a volume of 600 mL which is an indication that this process is suitable for the large volume production of FAdV 8b virus. Although the volume is low compared to 6000 L produced with the propagation of the Influenza virus using Vero cells[50], it is encouraging, upon which higher volume can be obtained.

The eosinophilic intra-nuclear inclusion bodies (INIB) observed in CEL cells in this study are naturally characteristic of FAdV[52] and may have informed the name IBH. The presence of INIBs shows internalization of the virus in the cells which indicates infection and replication. Although FAdV could produce either basophilic or eosinophilic inclusion bodies or both, FAdV serotype 8b which is prevalent in Malaysia is mostly associated with eosinophilic INIBs[2,3]. The brownish colourations in the slides stained with hexon protein complementary secondary IgG conjugated with horseradish peroxidase indicate positive FAdV presence and infection. The depletion of cells in 48 hrs indicates the infectivity of the virus which could be a sign of its pathogenicity. Indirect immunoperoxidase can be used to detect the presence of viruses in tissue and monolayer and is suitable as a replacement serum neutralization and ELISA for serological diagnosis of viruses as confirmed by the

results of this study[53]. With the indirect immunofluorescence assay, the infectivity of FAdV could be confirmed as shown by the detection of FAdV in chicken kidney cells[54]. Infectivity being the capacity of viruses to invade host cells and utilize the cellular materials to replicate and cause infection[55] was established in this study by detecting green colour emissions. The pattern of infectivity of the FAdV isolates in CEL cells was also established. The depletion of CEL cells within 24-48 hrs is an indication of the high virulence of the FAdV strains. Since FAdV replicates in the nucleus[56], the localization of the virus in the nucleus observed in this study suggests that the virus replication could start 24-48 hrs after infection indicating high infectivity attribute of the viruses. This could explain the usually short course of the disease and high pathogenicity of FAdV infections resulting in the often-reported high mortality[25]. Several detection methods in this report establish simple and easily adaptable diagnostic protocols which can be used in any simple laboratory for monitoring IBH in chickens.

Hexon and fibre genes of the isolate were amplified with electrophoresis bands corresponding to about 900 and 940 bp, respectively. The primers used for the amplification of fibre and hexon genes were fbrF/fbrR[38] and HexA1/HexB1[42], respectively. The multi-sequence analysis of the hexon gene of the isolates shows that there was to change between the CEE, CEL cells and bioreactor isolates. In the fibre gene, there was R▸T and K▸N substitution between CEE and CELP5 isolates. This shows that the propagation of the isolates in CEL cells has effects on the isolate and could be a good indication of the possibility of attenuation in further passages. Interest in fibre gene profile has grown due to its role in the pathogenicity of FAdV[57]. Fibre plays a role in tissue tropism being responsible for attachment which could alter the infectivity of FAdV. Analysis of the fibre gene of FAdV had been carried out by previous researchers for identification, characterization and pathogenicity studies[12,35,58]. This substitution especially occurring at the Knobs Region could affect the interaction of the fibre knob with the CEL cell surface receptors which is necessary for endocytosis. Variations in the fibre gene were associated with virulence and difference in virus infectivity among canine adenovirus[59] and in CEE infected with FAdV[36]. Interestingly, the bioreactor isolate did not show any change with the flask isolate, which is good indicating that propagating FAdV 8b in stirred tank bioreactor using Cytodex 1 adapted CEL cells is suitable and does not exert much effect on the virus. This was similar to the report of Kluge *et al.*[30] where there was also no change between flask and stirred tank bioreactor isolates. This will be particularly very important in the propagation of attenuated isolates for large-volume vaccine production.

All the isolates were 98-100% identical to the UPM04217 isolate from GenBank and also phylogenetically clustered together with Group E, serotype 8b reference isolates, confirming the isolates as group E serotype 8b FAdV. The close relationship of the isolates with other Malaysian reference isolates could suggest that IBH in Malaysia are caused by circulating FAdV of the same ancestry. The evolutionary relationship of the test isolates with other reference strains phylogenetically could be helpful in future molecular epidemiologic studies of FAdV infections. Originally, FAdV had been adjudged a secondary pathogen, but it has established itself as a primary pathogen for IBH worldwide. This migration could probably be a result of the virus acquiring molecular virulence determinants across infections and becoming more pathogenic. Therefore, increased interest in the study of FAdV 8b is necessary to power the drive for vaccine development which is important in the control and prevention of IBH infections. And since the population of chickens keeps increasing, the need for large volume production of viral vaccines will always be a necessity.

CONCLUSION

Fowl adenovirus isolates from a field outbreak of IBH in Malaysia were isolated and pathogenicity confirmed in CEE and adapted to CEL cells culture for five passages. The CELP5 isolate was passaged once in Cytodex 1 adapted CEL cells in a stirred tank bioreactor. The CEL cells attached and grew on the microcarrier indicating adaptation to microcarrier culture in a stirred tank bioreactor. Virus titre was obtained while infectivity and characteristics of the CEL cells and bioreactor isolates were confirmed with immunoperoxidase, immunofluorescence assays, HE staining and PCR techniques which showed the suitability of stirred tank bioreactor for the propagation of FAdV 8b. The isolates were confirmed to be FAdV Group E serotype 8b, closely related to other Malaysian reference isolates from GenBank. There was a difference in the amino acid residues of fibre genes of CEE and CELP5 isolates which means they are affected by CEL cell passage indicating a potential for further research and vaccine development but no difference between CELP5 and CELP5B1 showing the suitability of the microcarrier culture for the propagation of FAdV 8b. It was also established that diagnosis of FAdV infection could be carried out using immunocytochemical analysis as well as molecular methods.

SIGNIFICANCE STATEMENT

Difficulty in the growth of FAdV in cells hampers FAdV research and confirmatory diagnosis of IBH could be herculean in simple laboratories. This article reports the successful adaptation of FAdV 8b in chicken embryo liver cells and the adaptation of these cells to Cytodex 1 microcarrier in a bioreactor. This will improve virus yield for large volume production of FAdV vaccines and can be adapted to other viruses. More research is needed on the improvement of cell yield and virus titre. This article also reports simple methods through HE, immunoperoxidase and immunofluorescence which offer confirmatory identification of FAdV 8b in combination and association with egg or cell culture-based isolation procedures utilizable in the simple laboratory.

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