Propagation and Molecular Characterization of Fowl Adenovirus Serotype 8b Isolates in Chicken Embryo Liver Cells Adapted on Cytodex™ 1 Microcarrier Using Stirred Tank Bioreactor

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Keywords: PCR, bioreactor, fowl adenovirus 8b, Cytodex 1™ microcarrier, chicken liver cell

Abstract:

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Abstract: Large volume production of vaccine virus is essential for prevention and control of viral diseases. The objectives of this study were to propagate Fowl adenovirus (FAdV) isolate (UPM08136) in chicken embryo liver (CEL) cells adapted to Cytodex™ 1 microcarriers using stirred tank bioreactor (STB) and molecularly characterize the virus. CEL cells were prepared and seeded onto prepared Cytodex™ 1 microcarriers and incubated first in stationary phase for 3 h and in STB at 37 °C, 5% CO_2, and 20 rpm for 24 h. The CEL cells were infected with FAdV isolate (UPM08136) passage 5 (UPM08136CELP5) or passage 20 (UPM08136CELP20) and monitored until cell detachment. Immunofluorescence, $TCID_{50}$, sequencing, alignment of hexon and fiber genes, and phylogenetic analysis were carried out. CEL cells were adapted well to Cytodex™ 1 microcarriers and successfully propagated the FAdV isolates in STB with virus titer of $10^{7.5}$ (UPM08136CELP5B1) and $10^{6.5}$ (UPM08136CELP20B1) $TCID_{50}/_{mL}$. These isolates clustered with the reference FAdV serotype 8b in the same evolutionary clade. The molecular characteristics remained unchanged, except for a point substitution at position 4 of the hexon gene of UPM08136CELP20B1, suggesting that propagation of the FAdV isolate in STB is stable and suitable for large volume production and could be a breakthrough in the scale-up process.

Keywords: chicken liver cell; Cytodex 1™ microcarrier; fowl adenovirus 8b; bioreactor; PCR

1. Introduction

Fowl adenovirus (FAdV) is associated with inclusion body hepatitis (IBH) in chickens which is responsible for heavy losses in poultry industries worldwide [1–3]. Vaccination has proven to be the most effective tool in the control and prevention of viral diseases in animals and humans [4,5]. Despite the successes of vaccines against a wide range of animal diseases, developing a vaccine is a very challenging process, and one of the major drawbacks to the availability of vaccines over the years has been technical manufacturing scale-up obstacles [6]. Availability of vaccines in large volumes to meet the ever-growing production animal population could be easier through cell culture-based

production than the traditional embryonated egg culture [7]. Cell culture also offers advantages like reduced contamination, product purity, ability to use wild type viruses efficiently, reduced immunogenic changes, large volume production, and quick response during pandemic such as influenza outbreaks or the recent COVID-19 pandemic [8,9]. Cell culture involves harvesting cells from the organs of animals, insects, or from plants, growing them in vitro [10] and then maintaining them in medium for use to express enzymes, antibodies, therapeutics, and to grow viruses for vaccine development [11,12]. Anchorage-dependent cells like liver cells require surfaces to attach for stabilization to enable proliferation. The early protocol for cell culture made use of glass slides, petri dishes, and other glass-based surfaces for attachment of cells for growth, but due to difficulty encountered in primary cells not being able to attach to glass surfaces, George Gey in 1956 used rat tail collagen to coat glass surfaces which pioneered the development of plastic flasks, dishes, and 96-well plates, which were all commercially available by 1960 [13]. Tissue culture flasks have offered a lot of advantages and are still widely used in cell biology and vaccine development to date [13]. Although the use of tissue culture flasks provided a solution to the issue of cell culture contaminations and improved cell proliferation, its limitation with regards to large volume production of vaccines has been inability to provide enough space [14]. Several platforms for large volume proliferation of cells has been experimented with at different stages, but one of the breakthroughs for the industrial application of mammalian cells has been the invention of microcarriers for vaccine production to support growth of adherent or anchorage-dependent cells (ADC) [15]. A variety of microcarriers are available for vaccine development, but spherical bead type microcarriers such as Cytodex™ 1 are suitable especially for stirred tank bioreactors [16]. It is a multipurpose microcarrier which can be used to grow a variety of cells. While culturing influenza virus vaccine in stirred tank bioreactor on Vero cells, Cytodex™ 1 was used and optimized to the production capacity of 6000 L [16].

For a successful suspension culture of adherent cells with microcarriers as attachment surface, in vivo conditions which promote growth and differentiation of target cell types should be replicated as closely as possible in vitro. A wide variety of bioreactors has been developed by researchers to provide an in vitro environment that recapitulates the in vivo environment as accurately as possible [17]. These include continuous stirred tank bioreactors, which are used in 70% of all fermentation and bioprocess operations [18]. Bioreactors are complex and expensive devices and are not optimized to provide an ideal environment for all mammalian cell growth due to high local fluid shear and bubble aeration. Each bioprocess operation requires optimization with respect to a specific set of parameters, such as cell growth, cell yield, and specific productivity, because there is no universal approach to optimize conditions for all animal cell culture systems [19]. A stirred tank bioreactor was used to propagate Infectious bursal disease (IBD) virus in BGM70 cell line [20], Modified vaccinia Ankara (MVA) virus in avian AGE1.CR.pIX cell line [21], and bacteriophages in *E. coli* [22]. It was expected that primary chicken embryo liver (CEL) cells could be adapted to Cytodex™ 1 microcarrier culture and used to propagate FAdV isolate in a stirred tank bioreactor. The objectives of this study were therefore to adapt primary CEL cells to Cytodex™ 1 Microcarrier and propagate FAdV isolates in the microcarrier adapted CEL cells in a bioreactor and determine the molecular characteristics of the virus.

2. Materials and Methods

2.1. Ethics Statement

The design of this experiment including the embryonated chicken egg utilization protocol was done following the guidelines and ethics of the UPM institutional animal care and use committee (IACUC) and was approved with ref number; UPM/IACUC/AUP-R086/2018.

2.2. Virus Isolates

Fowl adenovirus serotype 8b isolate, which was passaged in CEL cells for 5× (UPM08136CELP5) with Genebank accession number MT561443 and passaged in CEL cells 20× (UPM08136CELP20) with Genebank accession number MT561445, was used in this study. Each of the isolates was freeze-thawed three times and centrifuged at 1500 rpm for 5 min and the supernatant filtered through 0.45 µm syringe filter. Filtered isolates were used as inocula for this study.

2.3. Siliconization of Glassware

All glassware used in this study was first siliconized by applying dichlorodimethylsilane (Sigma-Aldrich, Shanghai, China) with ≥99.5% concentration on all surfaces that came in contact with microcarrier beads [23]. After siliconization, the glasswares were then sterilized by autoclaving.

2.4. Setting up the BIOSTAT® B Bioreactor

A B Braun Biostat® B Fermentation Cell Culture Bioreactor (Type 8840334) (Sartorius, Goettingen, Germany), which is a continuous stirred tank bioreactor (STB), was used in this study. The BIOSTAT® B bioreactor vessel (chamber) was disconnected from the control unit, washed thoroughly, and dried. The inner chamber was then siliconized and dried. The bioreactor was then assembled according to manufacturer's recommendation and reconnected to the control unit. The pH and temperature regulators were then calibrated. After calibration, the unit was disconnected from the control unit and all the openings in the chamber and all the flow tubes were closed with aluminium foil. The prepared chamber was then sterilized by autoclaving at 121 °C 103.42 kpa for 15 min. After sterilization, the chamber was then reconnected to the control unit ready for use.

2.5. Preparation of Cytodex™ 1 Microcarrier Beads

Cytodex™ 1 was prepared according to manufacturer's recommendation to obtain a concentration of 3 g/L. Three grams of Cytodex™ 1 was measured into a siliconized conical flask and suspended in 150 mL of $CaCl_2$ and $MgCl_2$ free PBS (Nacalai tesque, Kyoto, Japan) and hydrated for 3 h at room temperature after which PBS is decanted. The microcarriers were washed in fresh 150 mL of PBS for 30 min and PBS decanted. The washing process was repeated, after which fresh 200 mL of PBS was added and the microcarriers in PBS were sterilized by autoclaving. After sterilization, the PBS was decanted and 150 mL of DMEM media was added to dislodge retained PBS. The media was decanted before cells were seeded onto the microcarrier beads. The surface provided by the microcarrier is shown in Table 1.

Table 1. Microcarrier concentration, virus titer, and CEL cells volume used for the bioreactor propagation.

No.	Isolate	Microcarrier Concentration (/mL)	Surface Area	Virus Innoculum Titre (/mL)	Inoculation Volume (mL)	CEL Cells Inoculated (/mL)
1	UPM08136 P5	8.6×10^6	17.6 m^2	$10^{7.67}$	10	3.1×10^7
2	UPM08136 P20	8.6×10^6	17.6 m^2	$10^{5.5}$	10	3.1×10^7

2.6. Preparation of Chicken Embryo Liver Cells

The chicken embryo liver (CEL) cells were prepared using 15-day-old specific pathogen-free (SPF) chicken embryonated eggs (CEE) [24]. The liver from the embryo was aseptically harvested and macerated in sterile petri dish with sterile forceps. Macerated liver was transferred into sterile conical flask and cells dissociated with sodium bicarbonate-free Trypsin-EDTA (0.25% Trypsin and 2.25 mM EDTA) (Corning, Manassas, VA, USA) in a two-step dissociation. Liver from 2 SPF CEE was dissociated initially with 12 mL of Trypsin-EDTA and pipetted into centrifuge 50 mL tube, and dissociation was repeated subsequently with 8 mL Trypsin-EDTA. Dissociated liver cells were centrifuged (Hettich Zentrifugen, Tuttlingen, Germany) at 1500 rpm for 5 min, after which Trypsin-EDTA was discarded and the CEL cells were suspended in fresh DMEM media supplemented with 10% FBS and 2% (*v*/*v*) antibiotics (penicillin–streptomycin) [25]. The CEL cells were seeded onto already prepared Cytodex™ 1 microcarriers.

2.7. Adaptation of CEL Cells to Cytodex™ 1 Microcarrier Culture

Freshly prepared CEL cells were seeded onto the prepared Cytodex™ 1 microcarriers and incubated in CO_2 incubator (ThermoForma, Champaign, IL, USA) at 37 °C and 5% CO_2 for 1 h. The culture was made up to 1/3 of the final volume and incubated for additional 2 h. The sample was drawn into tissue culture flask and observed under the inverted light microscope (Leica Microsystems, Wetzlar, Germany) for cell attachment and confluency and images for each passage were captured using an inverse microscope (Nikon Eclipse TS100, Tokyo, Japan) with the aid of NIS Elements imaging system (Nikon Digital sight DS-U2, Tokyo, Japan) powered by PowerLogic computer with 17" monitor (PowerLogic, Brownsville, TX, USA). The culture was then transferred into the bioreactor [20]. The cell volume is shown in Table 1.

2.8. Propagation of FAdV in Cytodex™ 1 Microcarrier Adapted CEL Cells in STB

The microcarriers with CEL cells attached were transferred into the bioreactor chamber using peristaltic pump drive (Longer Pump, Baoding, China). The bioreactor was preset at 37 °C, pH 7.4, dissolved oxygen (DO) 40% air saturation [20]. The rotor spinning was set at 10 rpm and increased to 20 rpm after 3 h and the culture made up to final volume with DMEM media supplemented with 10% FBS and 2% penicillin–streptomycin [26]. After 24 h of incubation in STB, cells were inoculated with 10 mL of FAdV inoculum (Table 1) and the rotor was increased to 30 rpm and observed closely for pH changes. Sterile NaOH and HCl were used for pH controls. Samples were drawn at 24 and 48 h post-inoculation and observed for cytopathic effects (CPE). At 48 h post-inoculation, the culture was harvested. Harvesting of virus culture was achieved by increasing the stirring to 500 rpm for 2 min to enable complete detachment of cells after which the microcarrier beads were allowed to sediment. Harvested cells with virus was removed from bioreactor by reverse flow using the peristaltic pump drive (Longer Pump, Baoding, China) into a siliconized, sterile bottle while the sedimented microcarrier beads were collected separately, washed and kept for reuse. The culture was freeze-thawed 3 times at −20 °C after which aliquots were centrifuged in 50 mL tubes at 1500 rpm for 5 min. The supernatant was extracted, filtered through 0.45 µm syringe filter and stored at −80 °C. The titer of each FAdV inoculum is shown in Table 1.

2.9. Determination of the Infective Dose ($TCID_{50}$) of the Passage Isolates

Ten-fold serial dilution of each isolate was carried out [27]. One 96-well tissue culture plate for each isolate to be tested was placed under UV light overnight and 0.2 mL of CEL cells, prepared as earlier described (2.5), was seeded into each well and incubated in CO_2 incubator (ThermoForma, Champaign, IL, USA) at 37 °C and 5% CO_2. The plates were observed daily until the cell became 100% confluent. At confluency, the plates were washed twice with PBS and infected with 0.1 mL of serially diluted isolate. Each dilution was used for 8 wells in a column (Columns 1–10). Columns 11 and

12 were infected with sterile distilled water as control. Infected plates were incubated for 1 h and 0.2 mL of DMEM media supplemented with 2% FBS was added to each well, re-incubated in CO_2 incubator (ThermoForma, Champaign, IL, USA) at 37 °C and 5% CO_2 and observed for CPE for 7 days. The number of wells in each column with more than 50% CPE was recorded and $TCID_{50}$ calculated as described by Reeds and Muench [28].

2.10. Indirect Immunofluorescence Assay

CEL cells were prepared as previously described and seeded onto sterile cover slips inside 6-well tissue culture plates and incubated until confluency. The medium was discarded and washed 2× with PBS after which each well was infected with 0.2 mL of respective bioreactor passage isolates (UPM08136CELP5B1 or UPM08136CELP20B1) and incubated at 37 °C and 5% CO_2 for 1 h for adsorption. Then, 1.8 mL of DMEM maintenance media 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 h. On CPE, the wells were fixed and inactivated with 4% formalin for 30 min at room temperature, after which they were washed 2× 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 3× for 5 min. Unspecific bindings were blocked with blocking buffer (5% BSA in PBST) for 1 h at room temperature and rinsed 3× with PBST for 5 min. Forty microlitres of monoclonal FAdV hexon protein primary antibody (Santa Cruz, Dallas, TX, USA) was added into the cells, allowed to stand, and incubated at 4 °C in humidity chamber overnight after which the wells are rinsed 2× with PBST for 5 min. Cells were suspended in 40 µL of mice anti-chicken IgY-FC-FITC-conjugated secondary antibody (Santa Cruz, Dallas, TX, USA) 1:1000 dilution was added and incubated at room temperature (RT) for 1 h in a dark room. The plates were rinsed with PBST and 20 µL of diamidino-2-phenylindole dihydrochloride (DAPI) added for 10 min at room temperature. The cover slip was rinsed, dried, mounted on clean glass slides with dibutylphthalate polystyrene Xylene (DPX), and observed with fluorescence microscope [20].

2.11. DNA Extraction and PCR Amplification of Hexon and Fiber Genes

DNA from UPM08136CELP5B1 and UPM08136CELP20B1 was extracted using an innuPREP Virus DNA Kit (analytikjena, Jena, Germany) and the concentration measured at 70 dilution factor, using ultraviolet–visible spectrophotometer (UV-1601, PC, Shimadzu Europa, Duisburg, Germany). HexA1/HexB1 primers [29] were used to amplify hexon gene while fbrF/fbrR primers Fbr F-5′ ACCGATTACGGCCGACGAAC -3′ and Fbr R-5′ -GAGCGTTGGCTGTGCTTAGG -3′ designed from KU517714.1 reference strain for this study were used for fiber gene amplification with sensoquest labcycler gradient (Biomedizinische Elektronik, Goettingen, Germany) in a 25 µL reaction using MyTaq Red Mix (Bioline, London, UK). The amplification conditions were 95 °C for 2 min 1×, 95 °C for 1 min 35×, 55 °C for 1 min 35×, 72 °C for 2 min 35× and 72 °C 1 min 1× for hexon gene and 95 °C for 2 min 1×, 95 °C for 1 min 35×, 50 °C for 1 min 35×, 72 °C for 3 min 35×, and 72 °C for 2 min 1× for fiber genes. The PCR products were separated by electrophoresis using EPS-300 X (C.B.S. Scientific, San Diego, CA, USA) in 1% agarose gel (GeneDireX, Taoyuan, Taiwan) at 70 V and 400 mA for 50 min, stained with redsafe stain (iNtRON Biotechnology, Burlington, MA, USA) and visualised through UV transillumination (Syngene, Cambridge, UK).

2.12. Sequencing, Blast, Alignment, and Phylogentic Analysis

The PCR products were purified using MEGAquick-spin™ Total fragment DNA purification kit (iNtRON Biotechnology, Burlington, MA, USA) and sequenced (Apical Scientific, Serdang, Malaysia). Consensus sequences were assembled using contig assembly application and multiple sequence alignment to determine changes in the genes were carried out using BioEdit software v 7.2.5 [30,31] and sequences were subjected to NCBI Blast. Amino acids were deduced by using ExPasy software (www.expasy.ch/tools/dna.html) [32]. With the sequences and 37 published strains for hexon (Table 2) with NCBI Genebank links to the sequences in Table S1 and 27 published strains for fiber (Table 3) with NCBI Genebank links to the sequences in Table S2, phylogenetic trees were constructed by neighbor-joining method [33] using MEGA X software v 10.0.5 [32].

Table 2. The aviadenovirus reference strains obtained from Genebank used in the construction of phylogenetic tree for hexon gene analysis.

No	Isolate Name	Serotype	Origin	Accession Number	Uploaded By
1	UPM04217	8b	Malaysia	ANA50319.1	Juliana et al., 2016
2	UPM08158	8b	Malaysia	AEL21619.1	Juliana et al., 2011
3	UPM1137CEL20	8b	Malaysia	AQZ26936.1	Norfitriah et al., 2017
4	UPM08158	8b	Malaysia	AEL21619.1	Juliana et al., 2011
5	Celo	1	Australia	EU979367.1	Steer et al., 2009
6	SR48	2	Australia	EU979368.1	Steer et al., 2009
7	SR49	3	Australia	EU070369.1	Steer et al., 2009
8	KR5	4	Australia	EU979370.1	Steer et al., 2009
9	340	5	Australia	YP 007985654.1	Marek et al., 2013
10	CR119	6	Australia	YP 009505663.1	Steer et al., 2009
11	YR36	7	Australia	ACL68141.1	Steer et al., 2009
12	AO2	9	Australia	EU979376.1	Steer et al., 2009
13	C2B	10	Australia	EU979377.1	Steer et al., 2009
14	Wroclaw 2015	1	Poland	KR259656.1	Wieliczko et al., 2015
15	USP-EC-01	6	Ecuador	ASU91620.1	De la Torre et al., 2017
16	TR59	8b	Australia	ACL68142.1	Steer et al., 2008
17	40440-M/2015	5	Hungary	QCC26479.1	Kajan et al., 2018
18	LYP	5	China	QJP03679.1	Chen, 2019
19	CH/CQBS/1601	4	China	MF055642	Xia et al., 2017
20	CH/CBQS/1512	8b	China	AUD09312.1	Xia et al., 2017
21	05-51425	8b	Canada	ABS81057.1	Ojkic et al., 2007
22	Indian	4	India	AJ459805	Barua et al., 2002
23	10-3678	8b	Poland	CUT98160	Schachner et al., 2016
24	HG	8b	Canada	YP 004191821.1	Grgic et al., 2011
25	14-259	8b	France	QGQ62947.1	Schachner et al., 2019
26	HUNGG	8b	Hungary	QGQ62522.1	Schachner et al., 2019
27	HLJ/151129	8b	China	AOS87877.1	Wang et al., 2016
28	FAdV-HNQX-101017-B	8b	China	ANG57906.1	Li et al., 2016
29	TR/8VKE/R/B-8	8b	Turkey	QGP73412.1	Sahindokuyucu et al., 2019
30	04-53357-105	8B	Canada	ABS81134.1	Ojkic et al., 2007
31	SA38D-08	8b	South Africa	ADV90772.1	Joubert et al., 2010
32	SA38C-08	8b	South Africa	ADV90771.1	Joubert et al., 2010
33	USP-BR-420.12	8b	Brazil	AQX17387.1	De la Torre et al., 2016
34	764	8b	United Kingdom	ANJ02566.1	Marek et al., 2016
35	764	8b	Canada	AER40292.1	Dar et al., 2012
36	ID-HCI-037	8b	Peru	AWF93664.1	Caballero-Garcia et al., 2018
37	Turkey Adv	3	United Kingdom	AC_000016	Davison et al., 2003

Table 3. The aviadenovirus reference strains obtained from Genebank used in the construction of phylogenetic tree for fibre gene analysis.

No	Isolate Name	Serotype	Origin	Accession Number	Uploaded By
1	UPM04217	8b	Malaysia	KU517714.1	Juliana et al., 2015
2	UPM1137E2	8b	Malaysia	KY305950.1	Norfitriah et al., 2017
3	PL-060-08	1	Poland	GU952109.1	Domanska-Blicharz et al., 2011
4	YR36	7	Austria	KT862809.1	Marek et al., 2015
5	CR119	6	USA	NC_038332.1	Schachner et al., 2018
6	NARC-3317	4	Pakistan	KT733569.1	Jabeen et al., 2015
7	SX17	4	China	MF595799.1	Feng et al., 2017
8	Punjab1	1	India	DQ864435.1	Bhan et al., 2006
9	06-25854-1	11	Canada	JQ034219.1	Grgic et al., 2014
10	WA-1/100966-2	11	Australia	KT037713.1	Steer et al., 2015
11	340	5	Austria	FR872928.1	Marek et al., 2015
12	HLJ151129	8b	China	AOS87884.1	Wang et al., 2016
13	764	8b	United Kingdom	ANJ02574.1	Marek et al., 2015
14	06-41265-07	8b	Canada	AFD32283.1	Grgic et al., 2014
15	QD2016	8b	China	AWT08538.1	Hu et al.,2017
16	NZ-1/101151-1	8b	New Zealand	ANQ43486.1	Steer et al., 2015
17	Vac-2005	8b	Australia	ANQ43481.1	Steer et al., 2015
18	VIC-2/430-6	8b	Australia	ANQ43482.1	Steer et al., 2015
19	NSW-3/100931	8b	Australia	ANQ43483.1	Steer et al., 2015
20	FJ-1/100842-C	8b	Fiji	ANQ43487.1	Steer et al., 2015
21	VIC-8/100719	8b	Australia	ANQ43484.1	Steer et al., 2015
22	NSW-5/100931	8b	Australia	ANQ43485.1	Steer et al., 2015
23	Strain 13-19395	-	Germany	MK572863.1	Schachner et al., 2019
24	LYG	5	China	QJP03687.1	Chen, 2019
25	340	5	Northern Ireland	YP 007985662.1	Marek et al.,2013
26	127	DAdV 1	Russia	Z86065.1	Akopian et al., 1997
27	D11-JW-010	DAdV 1	South Korea	JX227930	Cha et al., 2012

3. Results

3.1. Adaptation of CEL Cells on Cytodex™ 1 Microcarriers

The CEL cells attached and adapted to the Cytodex™ 1 microcarriers within 3 h of incubation (Figure 1a,b) and grew confluent on the microcarriers beads after 24 h incubation in the bioreactor (Figure 1c,d).

3.2. Propagation of FAdV in CEL Cells Adapted on Cytodex™ 1 Microcarrier

The FAdV UPM08136CELP5 (Figure 2a) and UPM08136CELP20 (Figure 2c) isolates caused CPE and detachment of cells from microcarriers from 24 h post-inoculation. At 48 h, most of the cells had detached from the microcarrier beads (Figure 2b,d) indicating suitability of the Cytodex™ 1 and STB for propagation of FAdV serotype 8b.

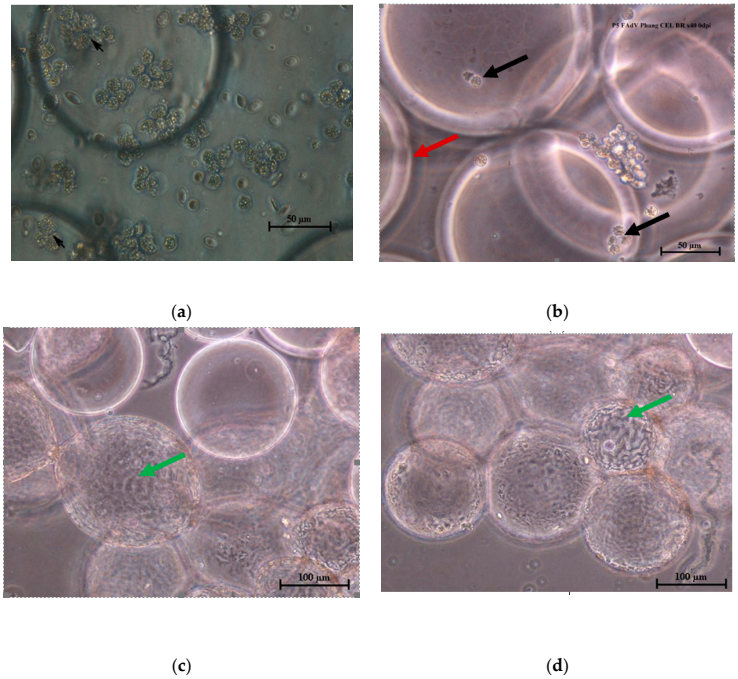


Figure 1. Inverted microscopic image of chicken embryo liver (CEL) cells attachment on the surface of Cytodex™ 1 microcarrier indicating adaptation of the cells to microcarrier culture. (**a**,**b**) CEL cells attached to Cytodex™ 1 microcarrier at 3 h of incubation. (**c**,**d**) CEL cells confluent on Cytodex™ 1 microcarrier beads at 24 h of incubation in bioreactor. Black arrow shows cells attached to Cytodex™ 1 microcarrier, red arrow shows normal Cytodex™ 1 microcarrier beads without any cells attached, and green arrow shows confluent Cytodex™ 1 microcarrier beads.

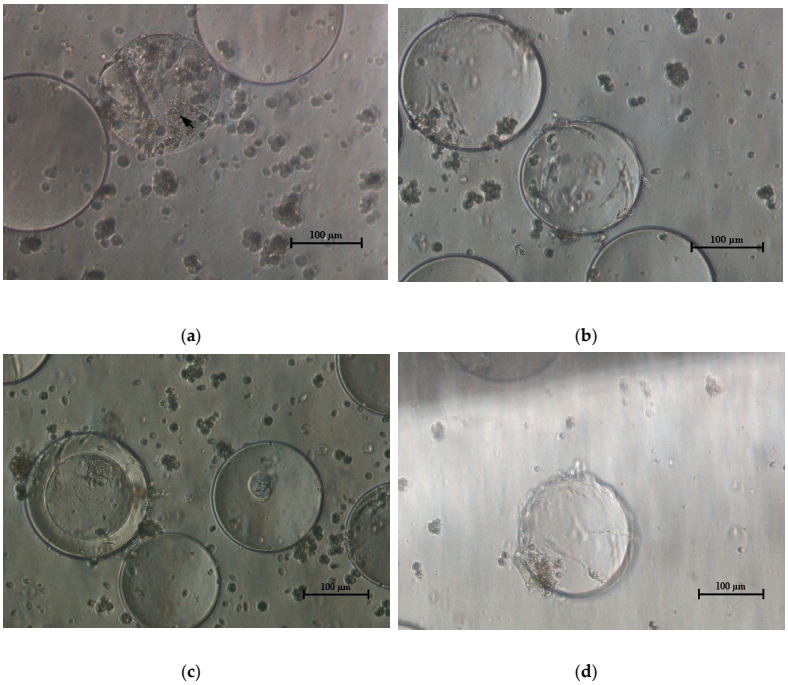


Figure 2. Inverted microscopic image of Cytodex™ 1 microcarrier beads with attached CEL cells infected with FAdV showing detachment of cells from the microcarrier beads. (**a**) Infected with UPM08136CELP5 at 24 h post-inoculation (pi). (**b**) Infected with UPM08136CELP5 48 h pi. (**c**) Infected with UPM08136CELP20 at 24 h pi. (**d**) Infected with UPM08136CELP20 at 48 h pi.

3.3. Infective Dose ($TCID_{50}$) of UPM08136P5B1 and UPM08136P20B1 Isolates

The UPM08136CELP5B1 showed higher virus titer of $1 \times 10^{7.5}$ $TCID_{50}/_{mL}$ in a final volume of 500 mL when compared to UPM08136CELP20B1 with virus titer of $1 \times 10^{6.5}$ $TCID_{50}/_{mL}$ in a final volume of 600 mL. These could be due to final culture volume or infectivity of the isolate (Table 4).

Table 4. Final volume and virus titer ($TCID_{50}$) of FAdV propagated in CEL cells adapted on Cytodex™ 1 Microcarriers in a Stirred Tank Bioreactor.

No	FAdV Isolate	Initial Titre (/mL)	Final Titre (/mL)	Final Volume (/mL)
1	UPM08136CELP5B1	$10^{5.5}$	$10^{7.5}$	500
2	UPM08136CELP20B1	$10^{7.67}$	$10^{6.5}$	600

3.4. Indirect Immunofluorescence Assay of FAdV Isolates Propagated in CEL Cells Adapted Cytodex™ 1 Microcarrier in a Stirred Tank Bioreactor

There were green particles indicating presence of FAdV antigens (Figure 3a,b). In contrast, in the control, there were only blue particles, denoting a negative result (Figure 3c). The isolates also showed affinity for the CEL cells and caused cell depletion after 48 h post-infection (Figure 3a,b), while the uninfected control shows intact monolayer (Figure 3c).

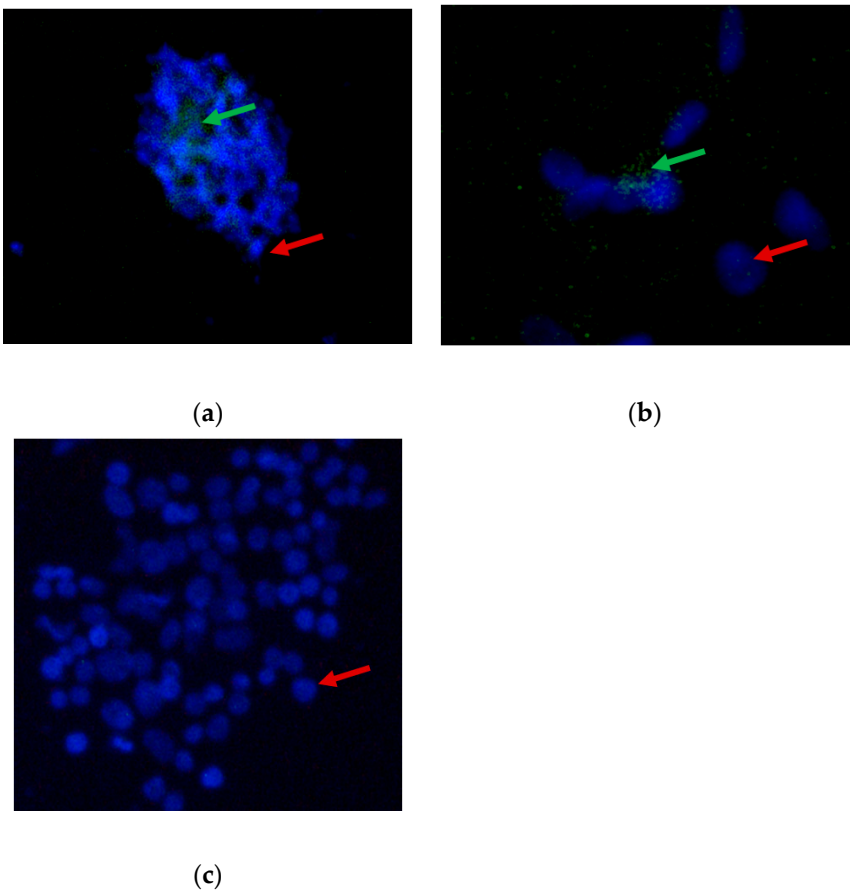
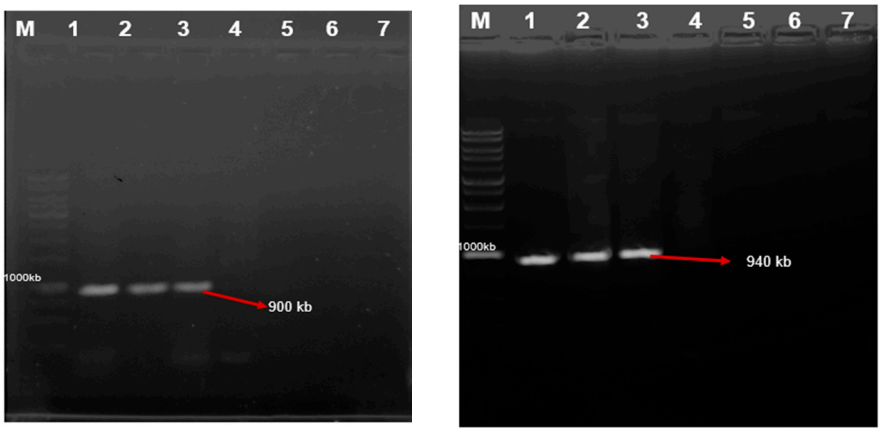


Figure 3. Indirect immunofluorescence images of CEL cells adapted Cytodex™ 1 microcarrier infected with FAdV isolates and propagated in a stirred tank bioreactor after 48 h incubation. (**a**) UPM08136CELP5B1 ×40; (**b**) UPM08136CELP20B1 ×40; (**c**) uninfected Control ×40. Red arrow indicates CEL cell nucleus; green arrow indicates FAdV in the cytoplasm of CEL cells.

3.5. Polymerase Chain Reaction Amplification of Hexon and Fiber Genes of the Propagated FAdV Isolates in CEL Cells Adapted on Cytodex™ 1 Microcarrier

The UPM08136CELP5B1 and UPM08136CELP20B1 yielded 900 bp (Figure 4a) and 940 bp (Figure 4b), which corresponds to the hexon and fiber genes of FAdV, respectively.



(**a**) (**b**)

Figure 4. Transillumination image of PCR fragments showing agarose gel electrophoresis bands of (**a**) hexon and (**b**) fiber genes. Lane 1, FAdV Hexon positive control; Lane 2, UPM08136CELP5B1; lane 3, UPM08136PCELP20B1; Lane 4 negative control; M, molecular weight DNA marker (1kb DNA ladder, Promega). Red labels show the band sizes.

3.6. Multisequence Alignment and Phylogenetic Analysis of UPM08136CELP5B1 and UPM08136CELP20B1 with Reference Strains

The translation of the nucleotides of hexon gene yielded 244–245 amino acid residues while the translation of the nucleotides of fiber gene yielded 148–149 amino acid residues. The nucleotide sequences and their corresponding amino acids were uploaded to Genebank and were assigned accession numbers, UPM08136CELP5B1 (MT561443 and MT561447) and UPM08136CELP20B1 (MT561445 and MT561449) for the sequences of hexon gene and fiber genes, respectively. There was no change in the hexon gene of UPM08136CELP5B1 compared to UPM08136CELP5. There was a G►^{4}T substitution (Figure 5) in the hexon gene of UPM08136CELP20B1 compared to UPM08136CELP20, which was the only change found in their hexon gene. This translated to an amino acid change from cysteine to glycine at position 2 (Figure 6) in the red box, which alone may not have effect on the stability of the virus. There was no change observed in the nucleotide sequences (Figure 7) and amino acid residues (Figure 8) of fiber gene of the bioreactor isolates compared to the flask isolates, suggesting suitability in FAdV serotype 8b propagation.

The hexon and fiber genes of UPM08136CELP5B1 and UPM08136CELP20B1 were 99–100% identical to UPM04217 and other FAdV serotype 8b isolates in Genebank, which confirms their non-variability with the flask isolates. The phylogenetic tree results based on the amino acid residues of hexon gene showed that all the test isolates clustered together in the same evolutionary clade with other Universiti Putra Malaysia (UPM) strains, the Australian vaccine strain, and other FAdV serotype 8b strains. Isolates in the FAdV genus branched out from the root away from the Duck and Turkey adenovirus strains (Figure 9a). The fiber amino acid phylogenetic tree results also showed that all the test isolates clustered together in the same evolutionary clade with other UPM strains and other FAdV serotype 8b strains. All the isolates in the FAdV genus branched out from the root away from the Duck adenovirus 1 strains (Figure 9b).

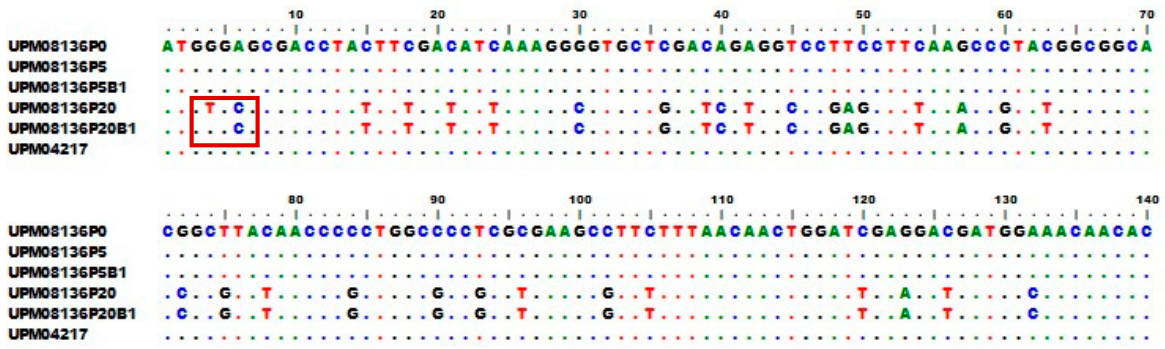


Figure 5. Multisequence alignment comparing the hexon gene nucleotide sequences of original, flask, and bioreactor propagated isolates of UPM08136 and UPM04217 reference isolate from Genebank. There was a point substitution from T to G at position 4 between bioreactor UPM08136CELP20B1 and flask UPM08136CELP20 (red box). There was no change between UPM08136CELP5B1 and UPM08136CELP5.

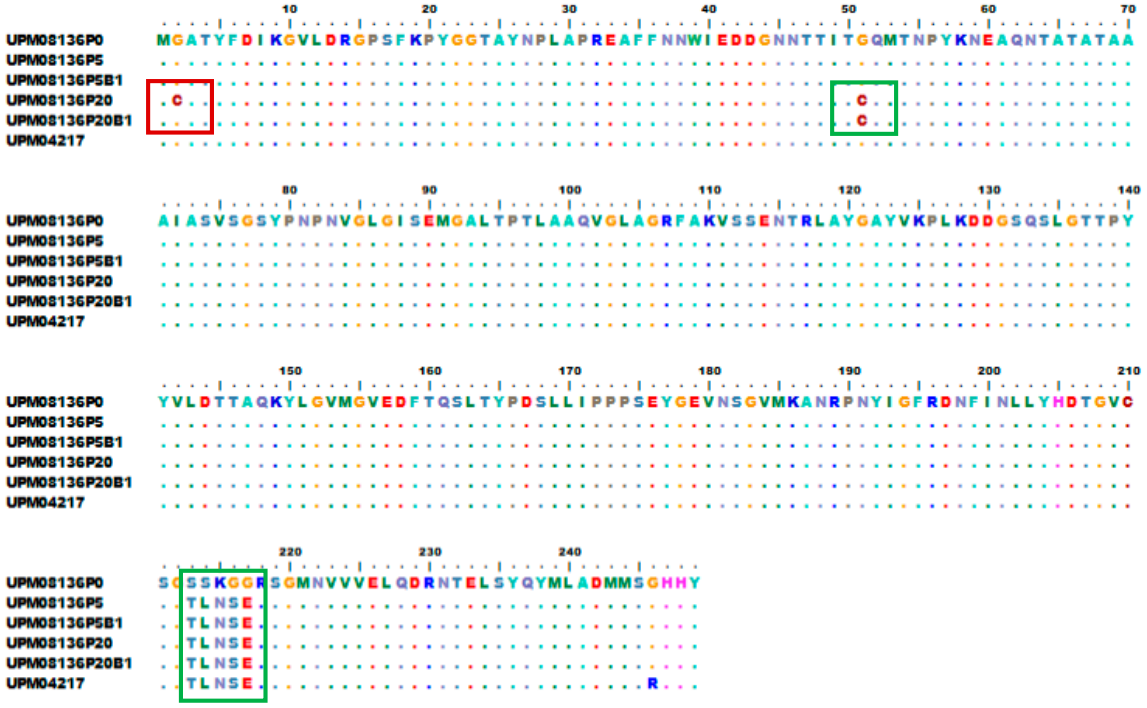


Figure 6. Multisequence alignment comparing the amino acid residues of hexon gene of the original, flask and bioreactor propagated isolates of UPM08136 and UPM04217 reference isolate from Genebank. There was a C to G substitution at position 2 (Red box) which is the only change observed between the bioreactor and flask isolates tested. Other changes (green box) remained the same between the flask and bioreactor isolates.

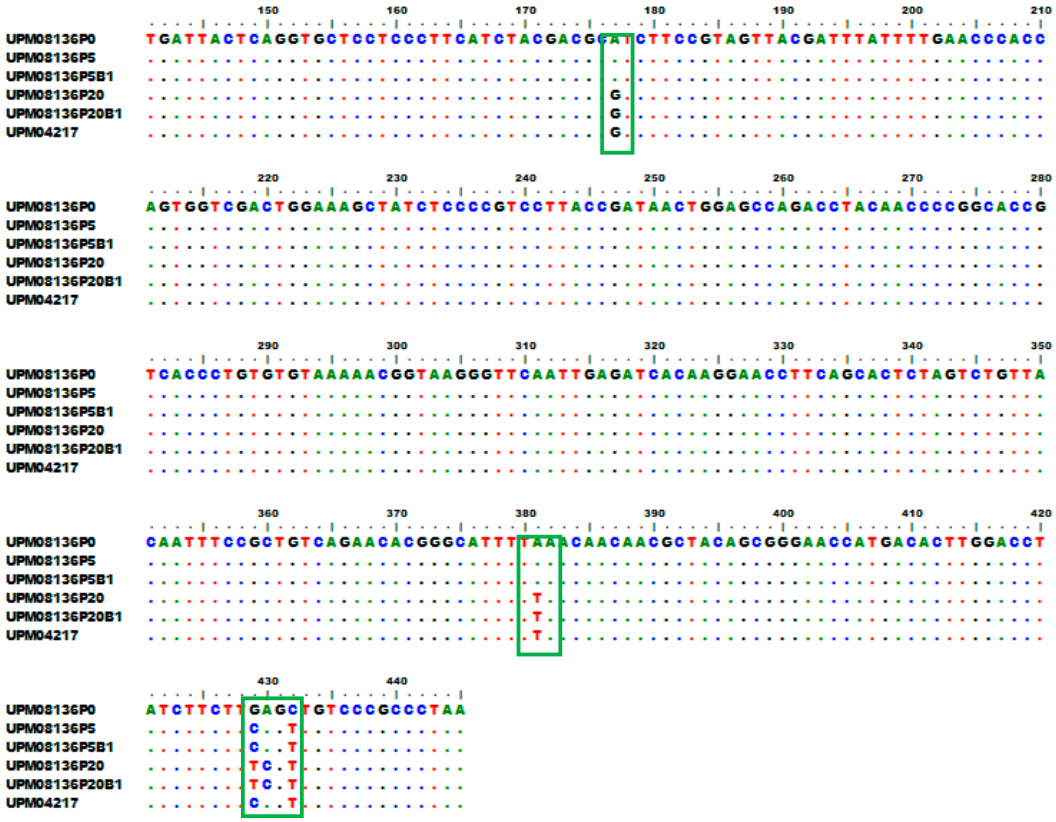


Figure 7. Multisequence alignment comparing the nucleotide sequences of fiber gene of the original, flask and bioreactor propagated isolates of UPM08136 and UPM04217 reference isolate from Genebank. There were no changes between the flask and bioreactor isolates observed in the fiber gene of the test FAdV strains. The changes in the CEL passages (green boxes) remained the same.

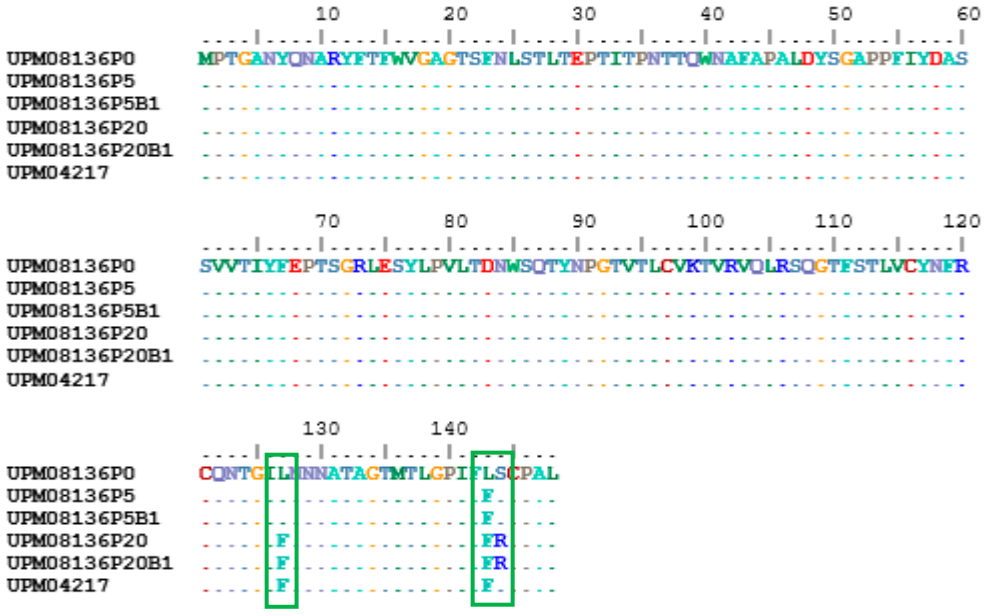
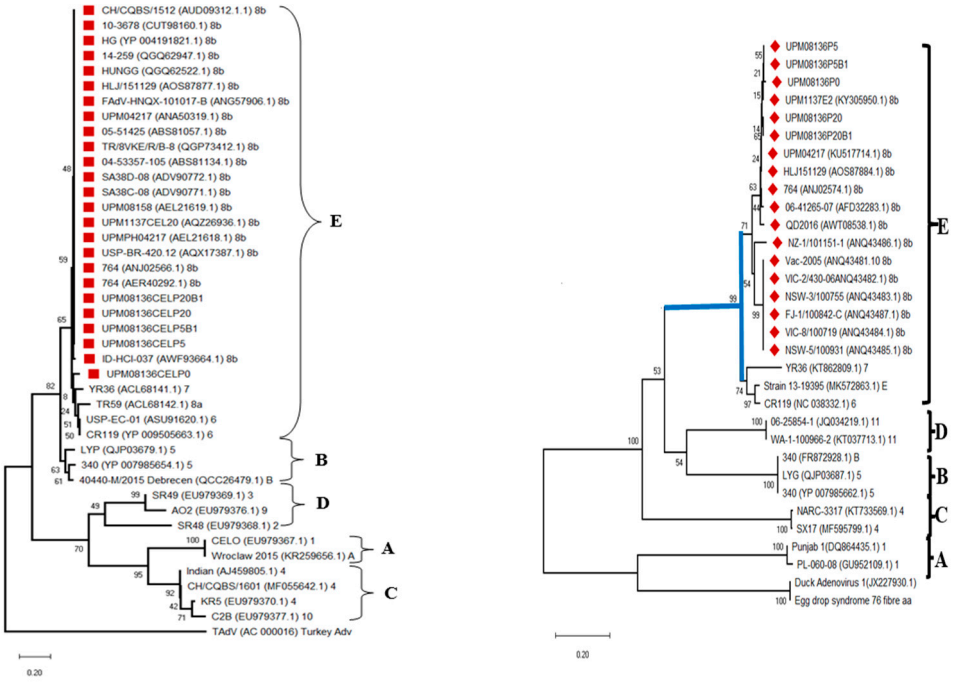


Figure 8. Multisequence alignment comparing the fiber gene amino acid residues of original, flask and bioreactor propagated isolates of UPM08136 and UPM04217 reference isolate from Genebank. There were no changes between the flask and bioreactor isolates observed in the fiber gene of the test FAdV strains. The changes in the CEL passages (green boxes) remained the same.



(**a**) (**b**)

Figure 9. Phylogenetic tree based on amino acid residues of (**a**) hexon and (**b**) fiber genes of the bioreactor propagated FAdV isolates and reference strains from GeneBank. The analysis involved 42 and 32 amino acid residues of hexon and fiber genes, respectively. The evolutionary history was inferred using the neighbor-joining method. Evolutionary analyses were conducted in MEGA X. Each serotype is indicated by name while the FAdV serotype 8b is labeled in red. FAdV isolates branched off in the same clade while all study isolates clustered together with other serotype 8b isolates.

4. Discussion

The CEL cells attached to the surface of the microcarrier beads within 3 h of incubation which indication adaptation of the cells to microcarrier culture. Most animal cells are anchorage dependent and can usually survive and are most productive when attached to a surface. Even suspension-type cell, like hybridomas, tends to give increased product yields when attached [34]. Attachment of cells to a surface is a critical need and one of the most essential points in the optimal functioning of all anchorage-dependent cells [35,36]. Attachment to the microcarrier is indicative of CEL cells adaptation to microcarrier culture. It proves that Cytodex™ 1 microcarrier is suitable for growth of CEL cells. Cytodex™ 1 was engineered to meet all properties essential for a successful microcarrier and has been used to culture more than sixty different cell lines successfully [37]. It is also cost effective as it can be reused [20]. The cells grew confluent in the cells within 24 h which shows that proliferation of cells in the microcarrier was successful. For virus propagation, cells are usually needed, and it is more needed in higher volumes for vaccine production. This is because cell concentration is of significant importance in determining viral titer [38], and viral titer has control on the efficacy of vaccines, which makes the need for a high volume of cells for volume production of vaccine an ongoing requirement.

The infected CEL cells exhibited cytopathic effects synonymous with FAdV on CEL cells which included rounding and detachment of cells [24,39] from the microcarrier beads. CPE is a sign of pathogenicity of virus on cells [40] and confirms infection of the cells. This means that CEL cells grown in the Cytodex™ 1 microcarrier are suitable for propagation of FAdV in a continuous stirred tank bioreactor system. This corroborates the reports of Lawal et al. [20] and Blumi [16] who used Cytodex™ 1 to propagate Infectious bursal disease virus (IBDV) and Influenza virus, and Tapia et al. [21] who used stirred tank bioreactor to propagate modified vaccinia ankara (MVA) virus in avian AGE1.CR.pIX

cell line. Mancuso et al. [22] also reported the successful use of *E. coli* to grow bacteriophages in stirred tank bioreactor. Stirred tank bioreactors are used in 70% of all fermentation and bioprocess operations and have been found to be suitable for propagation of virus vaccine candidates for large volume production [18]. The experimental volume was low especially when 6000 L was reported in influenza virus propagation [16], but it is a huge improvement when compared to flask propagation. UPM08136CELP20B1 had a lower titer than the flask propagated isolate which was similar to the report of Lawal et al. [20]. This could be attributed to the isolate having a lowered infectivity than the earlier passages. The titer values obtained in the study were $10^{6.5}/^{mL}$ and $10^{7.5}/^{mL}$ $TCID_{50}$ which were similar to the report of Chen et al. [41] who obtained a titer of 7.6 log10 $TCID_{50/mL}$ of influenza vaccine virus grown in Vero cells and $10^{6.4}$ $TCID_{50}/_{mL}$ reported by Lawal et al. [20] in vvIBDV passaged once in BGM-70 cell line before bioreactor passage in Cytodex™ 1 adapted BGM-70 cell line. The result is also comparable to 10^6 $TCID_{50}$ reported by Trabelsi et al. [42] in Measles vaccine virus grown in mammalian cells in microcarrier.

The amplification of the hexon gene of UPM08136CELP5B1 and UPM08136CELP20B1 yielded a partial hexon DNA 738–750 bp in size, which corresponds to the report of Meulemans et al. [29] who initially published HexA1/HexB1 primers. The primer had been used by several researchers for identification and studies of FAdV [43–48]. The amplification of the fiber gene using the novel fbrF/fbrR primers yielded partial fiber gene, mainly the knob region. Evaluation of fiber gene of FAdV has become necessary because through the interaction of the fiber knob with host cells, the fiber gene is responsible for tissue tropism which is also very important in the virulence of FAdV [49]. In fact, it had been reported that fiber alone could determine virulence of FAdV 8 [50]. The primer pair used in this study was designed in our laboratory and first published in this report. Analysis of fiber gene of FAdV had been carried out by previous researchers for identification, characterization and pathogenicity studies [25,51–53].

The NCBI blastn and blastp for nucleotide and protein sequences respectively of hexon and fiber genes showed 99–100% homology to FAdV serotype 8b in Genebank. This was synonymous with other FAdV sequences of Malaysian origin which suggests that the organisms in our study belong to the same serotype with those reported earlier in the country. Since the development of genomic analysis technology, researchers rely on NCBI Genebank blast as one of the repositories for virus taxonomical classification, species identification; and nucleotide and amino acid sequences identity search [54–56]. The major primary sequence databases are the International Nucleotide Sequence Database Collaboration (INSDC) databases, which includes GenBank (NCBI), European Nucleotide Archive (ENA), and the DNA Data Bank of Japan (DDBJ) [57], and Uniprot [58] which constitutes the main repositories of nucleotide and protein sequence data. In line with this, the sequences of hexon and fiber gene of isolates from this study were deposited in Genebank.

There were no changes observed in the hexon and fibre genes of UPM08136CELP5B1. There was also no change in the fiber gene of UPM08136CELP20B1. This means that the propagation of this FAdV isolate in CEL cells adapted in Cytodex™ 1 microcarrier in stirred tank bioreactor is suitable and had no adverse effect on the virus. This could be a model for adoption in the propagation of FAdV, especially serotype 8b when large volume of virus is needed. The bioreactor passage did not affect the virus so much to disturb its normal conformity. This is similar to the report of Lawal et al. [20] who found that vvIBD virus propagated in stirred tank bioreactor did not show molecular change in their VP2 protein. Other researchers did not report any change in the characteristics of the bioreactor propagated viruses which suggests that use of bioreactor for propagation of vaccine virus is suitable for large volume production of vaccines [41,59,60]. However, there was a point substitution in the hexon gene of UPM08136CELP20B1 which led to change in the amino acid residues at position 2 from cysteine to glycine. This alone would not affect the stability of the virus. However, following this change, it has become pertinent that all viruses propagated in a stirred tank bioreactor for a live-attenuated vaccine should be monitored for molecular changes in their antigenic proteins to forestall reversion to virulence. All the bioreactor propagated FAdV clustered together with serotype 8b viruses of Malaysian

origin and those from other sources. This shows that they are FAdV serotype 8b. The closeness of the flask and bioreactor isolates in the phylogram of hexon and fibre genes shows their similarity which confirms the suitability of propagating FAdV serotype 8b in CEL cells adapted Cytodex™ 1 microcarrier using stirred tank bioreactor. As the data on substrate requirement, metabolism, and specific CEL cells needs and bioreactor conditions for growth of primary CEL cells were not readily available, further studies should explore to establish the optimal growth conditions for CEL cells in stirred tank bioreactor and other conditions most appropriate for the propagation of FAdV in CEL cells adapted in microcarrier for maximum yield, to standardize the scaling-up and industrialization of FAdV vaccine production. In this way scale up for production of FAdV vaccine up to 5000–10,000 L could be achieved as this study could only achieve 600 mL volume. Due to paucity of literature on this area, it is possible that adaptation of primary CEL cells in Cytodex™ 1 microcarrier and using it in stirred tank bioreactor to propagate FAdV serotype 8b is a novel finding. To our knowledge, there are no reports on propagation of FAdV serotype 8b in primary CEL cells using the bioreactor technology. This finding could therefore be a breakthrough in the scale up process of FAdV vaccines and could be useful for researchers interested in scaling up their developed FAdV serotype 8b vaccines. It will offer opportunity for large scale production and commercialization of the FAdV vaccine which will aid the prevention and control of IBH and other FAdV infections worldwide.

5. Conclusions

Chicken embryo liver cells were adapted well to Cytodex™ 1 microcarriers and used to propagate Fowl adenovirus serotype 8b isolates in a stirred tank bioreactor. These bioreactor isolates were similar to their corresponding flask isolates in their molecular characteristics, which show that bioreactor propagation of Cytodex™ 1 adapted chicken embryo liver cells was well tolerated. This could be a breakthrough in FAdV serotype 8b vaccine scale up process.

Supplementary Materials: The following are available online at http://www.mdpi.com/2227-9717/8/9/1065/s1, Table S1: NCBI Genebank link for reference adenovirus strains used for phylogenetic analysis of hexon gene, Table S2: NCBI Genebank link for reference adenovirus strains used for phylogenetic analysis of fibre gene.

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