UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A LIVE ATTENUATED VACCINE AGAINST FOWL ADENOVIRUS BY MUTATING *FIBER* GENE USING CRISPR –CAS9 TECHNOLOGY

AHMED SALISU

FBSB 2021 21

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By

AHMED SALISU

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

October 2020

DEDICATION

This thesis is dedicated to the memory of my beloved late further, Alhaji Ahmed Musah who passed away on Tuesday 7th September 2009. May your gentle soul continue to rest in *Aljannatul Firdaus*, ameen. And my mother, Hajiya Umma Babandi. May Allah continue to spare your life in good health and enable you to reap sufficiently the fruit of your labour, ameen.

Abstract of thesis presented to the senate of Universiti Putra Malaysia in Fulfillment for the Requirements for the Degree of Doctor of Philosophy

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Chairperson : Associate Professor. Nurulfiza binti Mat Isa, PhD
Faculty : Biotechnology and Biomolecular Science

Inclusion body hepatitis (IBH) is a commonly distributed poultry disease that has a huge economic impact on the poultry industry worldwide. Clinical finding is associated with bird age between 3 to 6 weeks old and is economically important due to significantly high mortality rates in young broiler birds that may reach up to 50%. Epidemiological studies demonstrated that all 12 serotypes of Fowl adenovirus are associated with the IBH where serotype-8b is a major concern due to highly pathogenic in chickens. The first IBH outbreaks due to FAdV serotype-8b was reported in Malaysia in 2005, since then the disease has been reported in several states of Malaysia involving major poultry producing areas. Vaccination against FAdV is not a common practice in Malaysia and a suitable vaccine against the disease is unavailable. Therefore, the need for the development of a safe and effective vaccine against IBH disease that can improve the health of poultry is highly important. The main objective of this study was to develop a live attenuated vaccine against FAdV by mutating its *fiber* gene using CRISPR-Cas9 technology. The Malaysian FAdV isolate (UPMT27) was propagated in specific pathogen free (SPF) chicken embryonated eggs and chicken embryonated liver (CEL) cells. Classical IBH lesions such as thickening and cloudy of the chorioallantoic membrane (CAM) were observed. The embryos show pale, petechial haemorrhages, multifocal area necrosis, swollen and yellowish liver, and formation of hydropericardium after eight days post inoculation (*dpi*). Cytopathic effect (CPE) of rounding, clumping, and detachment of the cells in CEL were observed at 72 *hpi*. Virus identification was successfully amplified by polymerase chain reaction (PCR) using FAdV specific primers of both *fiber* and *hexon* genes with an expected length of 882 bp and 2900 bp respectively. Nucleotide sequences of the *fiber* and the *hexon* genes was performed and phylogenetic tree was constructed. Phylogenetic analysis confirmed that the UPMT27 belongs to FAdV group E of serotype-8b. The result shows the UPMT27 had a high nucleotide identity (99%) with the previous Malaysian isolates (UPM1137E5; UPM1137E10 and UPM04217; UPM1137CEL10 for *fiber* and *hexon* respectively). Both *fiber* and *hexon* sequences were submitted to Genbank and the accession number

was given as MT233531 and MT233532 respectively. The *fiber* gene of UPMT27 was successfully mutated and amino acid substitution was observed in the shaft region at position 179 (Tyrosine-Aspartate). The mutated virus (*cf*UPMT27) was subsequently rescued in the SPF chicken embryonated eggs. Pathogenicity study of the *cf*UPMT27 in SPF chicken embryonated eggs showed a predominant delayed pattern of mortality. Interestingly, the attenuated study showed that the mutated region of c*f*UPMT27 was genetically stable even after ten (10) consecutive passages. Pathogenicity study of the *cf*UPMT27 in CEL cell shows reduced in CPE activity. Morphological features of the transfected cells indicate that the *cf*UPMT27 was unable to localize at 48 *hpi*, while, it replicate and cause low CPE at 72 hours post inoculation (*hpi*). Meanwhile, the UPMT27 start to localize at 48 hrs and cause higher CPE at 72 *hpi*. Apoptosis assay demonstrated that the apoptotic rates in CEL cells infected with UPMT27 were significantly higher than those infected with *cf*UPMT27 at $p<0.01$. Interestingly, histopathological examination indicates that the *cf*UPMT27 caused less pathological damage to the liver tissue with the few concentration of the necrotizing lesion in the tubular cell. Meanwhile, the UPMT27 caused basophilic intranuclear inclusion surrounded with unaffected fibroblast in CELs. The viral copy number (VCN) ($> \log10^9$) observed was significantly higher at 72 *hpi* in the UPMT27 infected cells compared with the *cf*UPMT27 with a significant reduction (3.6×10^{1} and 4.2×10^{2} copies) at 24 and 48 hrs post-infection respectively. Pathogenicity and immunogenicity study of the *cf*UPMT27 was conducted in SPF chickens. Clinical appearance of the *cf*UPMT27 infected chickens is normal with no clinical signs observed throughout the trial. The chickens body and liver weight was significantly increased in all the three groups subcutaneous route (A), oral route (B), and control (C) from day 0, 7, and 21 *dpi* ($p< 0.05$) with highest mean number of body and liver weight in the chickens inoculated subcutaneously. No antibody was detected in group C throughout the trial. Surprisingly, antibodies against FAdV was detected at 7 *dpi* with the highest titre recorded at 21 *dpi* regardless of the route of inoculations; subcutaneous (A) and oral (B) with 1568 ± 890.4 and 1265 ± 318.9 antibody titer respectively. Pathogenicity and immunogenicity evaluation demonstrated that the *cf*UPMT27 was safe and exhibited a good immune response in SPF chickens. Therefore, it was concluded that the CRISPR- based mutated FAdV (*cf*UPMT27) might be suggested as a potential vaccine in preventing the spread of FAdV serotype-8b in the poultry industry.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN VAKSIN TERATENUAT HIDUP TERHADAP VIRUS ADENO UNGGAS MELALUI PENGUBAHSUAIAN GEN *FIBER* MENGGUNAKAN TEKNOLOGI CRISPR-CAS9

Oleh

AHMED SALISU

Oktober 2020

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Hepatitis jasad rangkuman (IBH) merupakan satu penyakit poltri biasanya tersebar yang mempunyai kesan ekonomi ke atas industri poltri di seluruh dunia. Dapatan klinikal adalah berkaitan dengan anak ayam berusia antara 3 hingga 6 minggu dan ia adalah penting dari segi ekonomi disebabkan kadar kematian tinggi yang ketara pada ayam pedaging muda yang mencapai sehingga 50%. Kajian epidemiologikal memperlihatkan bahawa semua 12 serotip virus adeno unggas berkaitan dengan IBH di mana serotip 8b merupakan kebimbangan utama disebabkan patogenik yang tinggi dalam kalangan ayam. Wabak IBH pertama akibat FAdV serotip 8b telah dilaporkan di Malaysia pada tahun 2005, sejak itu penyakit tersebut telah dilaporkan di beberapa buah negeri di Malaysia yang melibatkan kawasan pengeluaran poltri major. Vaksinasi terhadap FAdV bukan merupakan amalan yang biasa di Malaysia dan vaksin yang sesuai terhadap penyakit tersebut masih belum didapati. Oleh sebab itu, keperluan untuk pembangunan vaksin yang selamat dan berkesan terhadap penyakit IBH yang dapat meningkatkan kesihatan poltri adalah paling penting. Objektif utama kajian ini adalah untuk membangunkan vaksin teratenuat hidup terhadap FAdV melalui pengubahsuaian gen *fiber* menggunakan teknologi CRISPR-Cas9. Isolat FAdV Malaysia (UPMT27) telah dibiakkan dalam telur berembrio bebas patogen spesifik (SPF) dan sel hati berembrio ayam (CEL). Tanda IBH klasikal, seperti penebalan dan pertukaran warna keruh pada membran korioallantoik (CAM) telah dikesan. Embrio menunjukkan hati pudar, pendarahan petesial, nekrosis kawasan multifokal, hati membengkak dan kekuningan, dan pembentukan hidroperikardium selepas lapan hari pascainokulasi (*dpi*). Kesan sitopatik (CPE) seperti pembundaran, pengumpalan dan pelepasan sel dalam sel CEL primer telah dikesan pada 72 *hpi*. Pengenalpastian virus telah berjaya dilakukan melalui reaksi rantai polimerase (PCR) menggunakan primer spesifik FAdV bagi kedua-dua gen *fiber* dan *hexon*, masing-masing dengan jangkaan kepanjangan 882 bp dan 2900 bp. Jujukan nukleotid bagi gen *fiber* dan *hexon* telah dijalankan dan rajah filogenetik telah dikonstruk. Analisis filogenetik mengesahkan bahawa UPMT27 tergolong dalam kumpulan E FAdV bagi serotip 8b. Dapatan menunjukkan bahawa UPMT27 mempunyai

identiti nukleotid yang tinggi (99%) dengan isolat Malaysia terdahulu (UPM1137E5; UPM1137E10 dan UPM04217; UPM1137CEL10, masing-masing bagi *fiber* dan *hexon*). Kedua-dua jujukan *fiber* dan *hexon* telah dihantar ke Genbank dan diberikan kod akses, masing-masing sebagai MT233531 dan MT233532. Gen *fiber* UPMT27 telah berjaya diubah suai dan penggantian asid amino telah dikesan dalam bahagian shaf pada posisi 179 (Tirosin-Aspartat). Virus yang diubah suai (*cf*UPMT27) kemudiannya telah diselamatkan dalam telur berembrio ayam SPF. Kajian kepatogenan *cf*UPMT27 dalam telur berembrio ayam SPF menunjukkan pola kelewatan mortaliti telur SPF yang predominan. Menariknya, kajian teratenuat tersebut menunjukkan bahawa bahagian ubah suai bagi *cf*UPMT27 secara genetik adalah stabil walaupun selepas sepuluh (10) laluan berturutan. Kajian patogenisiti *cf*UPMT27 dalam sel CEL menunjukkan penurunan dalam aktiviti CPE. Ciri morfologikal bagi sel yang ditransfeksi menunjukkan bahawa *cf*UPMT27 tidak berupaya untuk dilokalisasikan pada 48 *hpi*, manakala, ia replikat dan menyebabkan kesan CPE yang rendah pada 72 jam pascainokulasi (*hpi*). Walau bagaimanapun, UPMT27 mula dapat dilokalisasikan pada 48 jam dan menyebabkan CPE yang lebih tinggi pada 72 *hpi*. Esei apoptosis pula memperlihatkan bahawa kadar apoptotik dalam sel CEL yang dijangkiti UPMT27 secara signifikan adalah lebih tinggi daripada yang dijangkiti dengan *cf*UPMT27 pada $p<0.01$. Menariknya, pemeriksaan histopatologikal menunjukkan bahawa *cf*UPMT27 menyebabkan kerosakan patologi yang kurang pada tisu hati dengan kepekatan lesi menekrosis yang rendah dalam sel tubular. Sementara itu, UPMT27 menyebabkan inklusi intrauklear basohilik yang dikelilingi dengan fibroblast tak terkesan dalam CEL. Bilangan salinan viral (VCN) ($> \log10^9$) dikesan adalah secara signifikan lebih tinggi pada 72 hpi dalam sel dijangkiti UPMT27 berbanding dengan *cf*UPMT27 dengan penurunan yang signifikan (3.6×10^1 dan 4.2×10^2 salinan), masing-masing pada 24 dan 48 jam pascainfeksi. Kajian kepatogenan dan imunogenisiti *cf*UPMT27 telah dilaksanakan pada ayam SPF. Penampilan klinikal ayam terinfeksi *cf*UPMT27 adalah normal tanpa tanda klinikal yang kelihatan di sepanjang percubaan. Berat badan dan hati ayam meningkat dengan signifikan dalam semua ketiga-tiga kumpulan, laluan subkutaneous (A), laluan oral (B) dan kawalan (C) dari hari 0, 7, dan 21 *dpi* ($p<0.05$) dengan min bilangan berat badan dan hati tertinggi pada ayam yang diinokulasi secara subkutaneous. Tiada antibodi dikesan dalam kumpulan C di sepanjang percubaan. Anehnya, antibodi terhadap FAdV telah dikesan pada 7 *dpi* dengan titer tertinggi direkodkan pada 21 *dpi*, tanpa mengambil kira laluan inokulasi; laluan subkutaneous (A) dan oral (B) dengan titer antibodi masing-masing, 1568 ± 890.4 dan 1265 ± 318.9. Kepatogenan dan penilaian keimunogenan memperlihatkan bahawa virus diubah suai adalah bukan patogenik dalam ayam SPF dengan tiada tanda klinikal yang berkaitan dengan FAdV, dan menunjukkan titer antibodi yang tinggi. Oleh sebab itu, kajian menyimpulkan bahawa FAdV diubah suai berasaskan CRISPR (*cf*UPMT27) dapat disarankan sebagai vaksin berpotensi dalam mengelakkan penyebaran FAdV serotip-8b dalam industri poultri.

ACKNOWLEDGEMENTS

Bismillahi rahman raheem. I give praises and thanks to Allah, the Lord of the world for strengthening and protecting me towards realizing my dream for the pursuit of a PhD in Genetic engineering and Molecular Biology. Allah the most exalted, your names will forever be praised.

Special gratitude goes to the chairperson of my Supervisory Committee, Associate. Professor. Dr. Nurulfiza Mat Isa for your scholarly guidance, support, constructive criticism and most essentially your timely response to all matters that needed attention. This goal would not have been realized without your commitment and passion towards shaping and improving the scholarship of my thesis I am greatly indebted to you Ma.
In particular, I would like to express special appreciation and thanks to my amiable and highly cherished co-supervisor Professor. Dr. Abdul Rahman bin Omar Indeed, I can unequivocally affirm that I have been greatly touched academically through your lively practical discussion and troubleshooting and most importantly through your open door policy to all students. I verily remember those countless numbers of scholarly and beneficial engagement I have had with you in your office during the course of my studies. Professor. Dr. Mohd Hair Bejo was also my co-supervisor who is immensely contributed to scholarship of this work. At every stage of my thesis and during my personal interaction with you on my research, your invaluable contribution and advice have been very unique sources of academic orientation to me. I cannot appreciate you sufficiently for your consistent academic guidance throughout the period of my studies. Also worthy of mentioning is Professor. Dr. Aini Ideris. I have benefited significantly from your observation and suggestion Dr. Mariatulqabtiah Abdul Razak was also my co-supervisor who has immensely contributed to the scholarship of this work. At every stage of my thesis and during my personal interaction with you on my research, your invaluable contribution and advise. My gratitude also goes to my parents. Alhaji Ahmed Musah of blessed memory. May Allah blessed you with eternal rest in *Aljannatul Firdaus*, amin. And my mother Umma Babandi for your love and prayers throughout the period of my studies.

I must appreciate the love, support and encouragement from my dear wife, Zainab Jamel. Your patient and encouragement throughout my period of studies have been highly inspiring and motivating. I am glad and proud to be blessed by a woman of virtues. And my children, Ummulkhairi (Khairat), Ummussalama (Nabiha) and Abdullahi (Safwan). I appreciate you all for your patience and prayers. My brother's sisters and colleagues postgraduate student. Nasharuddeen and Najwa who assisted in my Malay version of my abstract and a proofread by my Malay teacher Siti Amhar and the setting of my laboratory bench. Khalida thanks for the bioinformatics knowledge and all staff of laboratory of vaccine and Immunotherapeutics IBS. My profound appreciation also goes to Jigawa state polytechnic Dutse for the nomination and Tertiary Education Trust Fund, my sponsor (TETfund) for its full provision of my living expenses and overall cost of the programme

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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aa	Amino acid
AdV	Adenovirus
AGE	Adenovirus gizzard erosion
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine Serum Albumin
CEE	Chicken embryonated egg
CEF	Chicken Embryonic Fibroblast
CEK	Chicken Embryonic Kidney
CEL	Chicken embryo liver
CMI	Cell-mediated immunity
CPE	Cytopathic effect
CO_2	Carbon dioxide
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
dpi	Day post-inoculation
DAdV	Duck adenovirus
dsDNA	Double-stranded deoxyribonucleic acid
EDSV	Egg-drop syndrome virus
ELISA	Enzyme-linked immunosorbent assay
FaAdV- A	Falcon adenoviruses A
FAdV	Fowl adenovirus

FBS	Fetal Bovine Serum
GCD	Genomic cleavage detection assay
GoAdV-A	Goose adenovirus A
gRNA	guide ribonucleic acid
HAdV	Human adenovirus
HHS	Hydropericardium syndrome
hpi	Hours post inoculation
hpt	Hours post transfection
IACUC	Institutional Animal Care and Use Committee
IBH	Inclusion body hepatitis
ICTV	International Committee on Taxonomy of Viruses
Ig	Immunoglobulin
IM	Intramuscular
INIB	Intranuclear inclusion bodies
IP	Intraperitoneal
ITR	Inverted terminal repeats
kDa	kiloDalton
kB	kilobyte
kb	kilobase
LAMP	Loop Mediated Isothermal Amplification
L1	Loop 1
LIVEs	Laboratory of Vaccine and Immunotherapeutics
MEGA	Molecular Evolutionary Genetics Analysis
MHC	Major histocompatibility complex

MLP	Major Late Promoter
mRNA	messenger RNA
$NaHCO_3$	Sodium Hydrogen Carbonate
NCBI	National Centre for Biotechnology Information
NDV	Newcastle Disease Virus
nt	Nucleotide
OIE	World Organization of Animal Health
ORF	Open reading frame
OD	Optical Density
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween
qPCR	Quantitative polymerase chain reaction
QT35	Quail fibroblast cell line
RE	Restriction Enzyme
RFLP	Restriction Fragment Length Polymorphism
RGD	Arginine-Glycine-Aspartic Acid
RNA	Ribonuclease
RNP	Ribonuclease protein
RNase	Ribonuclease
rpm	revolution per minute
SEM	Standard error mean
SPF	Specific-pathogen free
SPSS	Statistical Package for the Social Science

sc	Subcutaneous
TAdV-1	Turkey adenovirus
TALEN	Transcription Activator-Like Effector Nucleases
$TCID_{50}$	50% tissue culture infectious dose
TCR	T cell receptor
Tc	Cytotoxic T cells
Th	T helper cell
TP	Terminal proteins
TR	Tandem Repeat
µg	Microgram
µl	Microlitre
µm	Micrometre
UV	Ultraviolet
UPM	Universiti Putra Malaysia
V	Volt
VCN	Virus copy number
VN	Virus neutralization
wt	Wild type
w/v	Weight/volume
ZFN	Zinc-Finger Nucleases

CHAPTER 1

INTRODUCTION

Poultry trades has emerged as one of the integral and fastest-growing public sectors in the developed and developing countries (Shah *et al*., 2017). Regrettably, this sector encounters a major threat of emerging and re-emerging diseases that are caused by parasitic, bacterial, and virus. Among those disease etiological agent are aviadenovirus. Aviadenovirus (AdV) is one of the etiological agent of viral disease occurring specifically in the areas of the world were chickens especially broilers are raised under heavy poultry farm conditions (Shah *et al*., 2017). The disease has been reported in many countries ascribed with diverse group of pathogens causing a variety of disease in poultry production (Hess, 2000; De la Torre *et al*., 2018). Aviadenovirus referred to as fowl adenovirus (FAdV). The diseases cause by FAdV exclusively infect avian hosts (Marek *et al*., 2016). They are responsible for a wide range of clinical presentation of diseases which include inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HHS), adenoviral gizzard erosions (AGE) and enteric conditions in chickens (McFerran and Adair 2003; Adair and Fitzgerald, 2008; De la Torre *et al*., 2018). Those diseases can be transmitted both vertically through embryonated chicken eggs and horizontally via faecal-oral route, direct content and fomites. They affect layers and broiler chickens production in an infected farm. The disease agents is routinely diagnosed by virus isolation in embryonated eggs or chicken embryonated liver cell followed by PCR (Raue and Hess, 1998), across the global regions with different categorized group and serotypes (Niczyporuk *et al*., 2017).

FAdV are non-enveloped viruses, with a linear, double-stranded (ds) DNA genome, belonging to the genus *Aviadenovirus*, that are clustered into 5 (A-E) molecular subgroups with 12 serotypes base on its complete genomic sequence and sera cross-neutralization (Hess, 2000; Harrach *et al*., 2011; Niczyporuk, 2016; Xia *et al*., 2017). Earlier detailed molecular studies reported that, the major antigenic determinant was located on the *fiber* and *hexon* genes, the virus encoded those proteins during viral replication, which penetrate into the nucleus of host cells (Zeng and Jun, 2019). However, the antigenic fragment comprise high amount of amino acids changeability between genotype and serotype for FAdV taxonomy (Hess *et al*., 1998; Meulemans *et al*., 2001). Based on the previous literatures it has been reported that, there is scanty of information on the role of those proteins. Hence, this information are necessary in an attempt to understanding there role in the virus lifecycle, but there is speculation that the *fiber* protein is directly responsible for viral attachment and internalization of the virus into the host cell (Zeng and Jun, 2019). Whereas *hexon* protein play a major role in virus neutralization activity (Rux and Burnett, 2003) and consequently *penton* protein play a vital role in interaction with cellular components, and neutralizing antibodies (Zeng and Jun, 2019). As reported earlier its play a major role in virus infectivity and normally use for analysis to differentiate between pathogen and non-pathogenic strains.

Previous finding revealed that the preponderance of the FAdV in infected chickens, show subclinical symptoms, whereas the acute infections can cause inclusion body hepatitis (IBH), hepatitis-hydropericardium syndrome (HPS), gizzard erosion and ulceration

(GEU) (Okuda *et al*., 2004; Niczyporuk, 2016). Infection associated with FAdV, especially IBH and HPS, result in substantial economic damage in many countries globally due to sudden and high mortality in young chickens (McFerran and Smyth, 2000; Balamurugan and Kataria, 2004; Hafez, 2011). The IBH cases are associated with some FAdV serotypes (FAdV 2, 3, 8a, 8b and 11) (McFerran and Smyth, 2000; Ojkić *et al*., 2008), while most HPS outbreaks are associated with FAdV type 4 (Toro *et al*., 1999; Dahiya *et al*., 2002; McFerran and Adair, 2003; Balamurugan and Kataria, 2004; Mase *et al*., 2009). However, different serotypes were isolated both in broilers and layer chickens (Choi *et al*., 2012; Kajan *et al*., 2013).

In Malaysia, cases of IBH was first isolated in 2005 from commercially broilers chickens with high mortality and poor broiler and neonatal chicks (Adair *et al*., 2008). A similar observation regarding the concurrence of IBH was also noted by Gaweł *et al.* (2016) in Poland. The infection is characterized by liver necrosis, haemorrhage and large basophilic intranuclear inclusion bodies in the hepatocytes (Steer *et al*., 2011; Dar *et al*., 2012). Mortality related to the disease ranges by a sudden onset of mortality (2–30%) and high morbidity (60–70%).

Historically, IBH is one of the major viral diseases causing significant economic loss to the poultry sector. The disease were concurrently reported and isolated in 1963 in USA, from domestic chicks and then rapidly spread to other parts of the world including Canada and Mexico (Helmboldt and Frazier, 1963). It is an acute disease due to infection by FAdV (Dar *et al*., 2012).

Multiple evidence from epidemiological studies confirmed that the disease occurs mainly in chickens 3 to 7 weeks of age, but it has been reported in chicken's less than 1 week old (Pilkington *et al*., 1997). Majority of the FAdVs commonly infect liver cells of the broilers chickens. Currently, infection with FAdV serotype-8b is the primary cause of IBH in Malaysia, with high mortality rate in SPF chickens and severe lesion of disease associated with FAdV which comprise, swollen livers with focal to extensive necrosis, pale, friable and large basophilic inclusion bodies in hepatocytes in the infected chickens (Reece *et al*. 1986).

Various strategies have been used by researchers for prevention and control of IBH since the initial outbreaks. Among the control measures, biosecurity practices is the essential and primary steps for preventing infection against IBH infection which gives due regard to strict cleaning and disinfection procedures (Kataria *et al*., 2013). Vaccination against FAdV could be considered as the only effective control measures of preventing IBH outbreak globally (Kim *et al*., 2014). However, different vaccine technology that involve application of live attenuated FAdV vaccine is more effective than inactivated vaccine due to high protection rate in vaccinated chickens with long lasting immunity (Kuar *et al*., 1997; Mansoor *et al*., 2011). IBH disease was initially, controlled by an inactivated liver homogenate vaccine that was formulated after the first outbreak, and many researchers reported that the vaccine had several issues regarding vaccine administration doses, secondary bacterial infection, unpredictable immune response (Mahmood *et al*., 2014). This limitation of the inactivated vaccine has pave way for the development of live attenuated FAdV isolate for future production of live attenuated vaccine.

Development of a live attenuated vaccine against FAdV. Involved the adapting of FAdV isolate in chicken embryonated eggs and subjected to four blind passages (Mansoor *et al*., 2011). The chicken-embryo-adapted virus was further passage to 12 times for its complete attenuation (Shah *et al*., 2017.

Isolation of the FAdV is an advantageous procedure for the subsequent identification and typing of the FAdV. The fowl adenovirus can be grown in primary cell cultures prepared from chicken kidney [CEK] (Khaweja *et al*., 1988), chicken embryonic liver (CEL) cells (Naeem *et al*., 1995). And chicken embryo fibroblast [CEF] (Soumyalekshmi *et al*., 2014). FAdV can be isolated from liver tissues of the birds infected with IBH- HPS. It has been reported that CEL and CEK derived from embryonated eggs is a sensitive medium for the isolation and propagation of FAVs. However, continuous cell line are also suitable media for FAdV propagation and attenuation. Previous finding, indicate that QT35 is stabilized cell line suitable for FAdV propagation and able to produced CPE. The CPE observed were in form of rounding, clumping and detachment of cells, which indicate the viral kinetics (Soumyalekshmi *et al*., 2014), other continuous cell line Hepatoma cell line (CH-SAH) is also suitable for propagation of FAdV.

In Malaysia, cases of IBH among commercial chickens rise exponentially and the vaccine against IBH is not yet available to prevent the outbreaks (Juliana *et al*., 2014). Subsequently, number of clinical cases of IBH and gizzard erosion were reported across Malaysian poultry farms in alarming rate. Though, several precautionary measures have been applied to prevent IBH as secondary disease in broiler chickens associated with immunosuppression (Popowich *et al*., 2018) live attenuated vaccines against FAdV are in the preliminary stages of development (Schonewille *et al*., 2010; Mansoor *et al*., 2011; Steer *et al*., 2011; Gupta *et al*., 2017a). Primarily, the use of inactivated liver homogenate vaccine formulated after the first outbreak was the practice used to control the virus outbreak and many research reported that the vaccine was not successful on experimental trial due to horizontal transmission (Shah *et al*., 2017).

Development of a safe oral live attenuated vaccine against FAdV through continues passages in chicken embryonated eggs or cell culture. However, this techniques is laborious, time consuming and lack of study regarding the influence of attenuation process at high consecutive passage toward molecular changes in the structural proteins. Though, it has been reported that amino acids changes in the major structural protein especially fiber and hexon may reduce the virulence nature of the virus (Schonewille *et al*., 2010; Mansoor *et al*., 2011).

Despite the use of autogenous vaccines, periodic outbreaks of IBH occur in the immunized birds (Khan *et al*., 2005; Ojkic *et al*., 2008a) incurring considerable economic damages to the poultry farms (Senties *et al*., 2010; Dar *et al*., 2012). Henceforth, the development of safe and effective IBH vaccine is highly anticipated. Among the numerous types of vaccines are virus-like particle (subunit) based vaccines a potentially best alternatives to autogenous vaccines due to its easy in production at industrial scale, effectiveness and above all its safety and simplicity in administration. The composition of subunit vaccine comprise a recombinant of small immunogenic fragments which can

stimulates a protective immune response in combination with an adjuvant (Moyle *et al*., 2013).

Subunit vaccine is a type of vaccine that was developed from a recombinant small immunogenic component of a microorganism which elicits a protective immune response in combination with an adjuvant. However, the virus like particles (VLP) is a type of subunit vaccine, which mimics the natural virus conformation but lacks complete genome (Noad and Roy *et al*., 2003; Kushnir *et al*., 2010). Conversely, the FAdV capsid protein, mainly comprises of 3 exposed structural proteins which comprise, *fiber, hexon* and *penton* have been proven as potential subunit vaccine (Pitcovski *et al*., 2005; Schachner *et al*., 2014; Dar *et al*., 2015; Gupta *et al*., 2017a). Nevertheless, among these structural proteins, fiber protein is the most essential immunogenic fragment for a VLPs subunit vaccine due to its ability to elicit neutralizing antibody responses (Fingerut *et al*., 2003; Pitcovski *et al*., 2005) and provide protection against clinical disease of FAdV in birds upon contact with the viral load (Schachner *et al*., 2014).

However, the *fiber* gene is categorized by the formation of projection and bound noncovalently to the *penton* base which involved in virus entry into the host cell and implicated in the variation of virulence of FAdVs. The *fiber* protein is divide into 3 domains which involved tail, shaft, and head or knob comprising some specific features (Grgić *et al*., 2011). The shaft domain is located between the tail and head of the *fiber* protein. The unique special, feature in the shaft domain of adenovirus is the detection of 22 pseudo-repeats'' of 15 amino acid residues, most with a Proline (P) or Glycine (G) in a common position and with common substituting of hydrophobic and hydrophilic amino acids (aa), and play an important role in the b-strand formation. However, most of the serotype -8 contain 17 pseudo-repeats in the fiber shaft region with each repeat containing 14–21 amino acids. The shaft domain constitutes the largest portion of the *fiber* protein its specific role toward virulence was not established but, the presence of corresponding amino acid motif ''VYPF'' at position (55-56 amino acid), involved in the *penton* base interaction, (Grgic ´ *et al*., 2014). However, the detection of amino acid variation, especially in the shaft region. The presence of the ''TLWT'' motif marks the beginning of the *fiber* head regions of the AdV which is not detected in some FAdV isolates and might be associated with virulence. Equally, the presence of 1 amino acid conserved poly G stretch starting at amino acid 64 detected at the carboxyl end of the tail region that connects between the tail and shaft domains. (Grgic ´ *et al*., 2014). Moreover, the variability of the amino acids in the shaft domain might play important role in pathogenicity. However, the rationale behind the used of CRISPR- cas9 technology in mutating the shaft region of the FAdV *fiber* gene might significantly reduce the *penton-fiber* interaction which is associated with the FAdV virulence. Though, the use of CRISPR-cas9 technology in vaccine development and therapeutics is at infant stage of development (Bi *et al*., 2014).

CRISPR/Cas9 system is a simple and powerful tool for genome editing in various organisms including livestock, which provides a robust and multiplexable editing tools by enabling researchers to precisely manipulate specific genomic elements, and facilitating the elucidation of target gene function in biology and diseases (Xiquan *et al*., 2017). CRISPR in the molecular field can be programmed to target specific genetic codes and to edit the DNA sequences at target location. Researchers can now permanently edit

the genes either in cells or organisms easily. The main components of CRISPR include a guide RNA (gRNA) and Cas enzyme. A gRNA is a ~20 nucleotide spacer that includes the specific targeted sequence to be mutated which will then steer the Cas9 enzyme to the said target (Edgar and Qimron, 2010). Initially, CRISPR was bound to be used for gene-knockout purposes before researchers start modifying the enzyme so that it can selectively activate or repress target genes, purify specific regions of DNA, and even image DNA in live cells using fluorescence microscopy (Makarova *et al*., 2011). This cutting-edge technology saves a handful of time in laboratory as most works could be done online. These technologies are now being used to develop vaccines to protect agriculturally important animals against important diseases.

Therefore, the main objective of this study was to develop a live attenuated vaccine against FAdV by modifying its virulence associated gene using CRISPR Cas9 technology. To determine the effective modification of the fiber gene and understanding the pathogenicity and immunogenicity of the mutated virus, in vivo and invitro study is imperative. Though the modify isolate will remain similar with UPMT27, as the technique is designed to target few nucleotide substitutions randomly at the shaft fragment of the *fiber* gene, the viral replication will vary with the original isolate, the pathogenicity will subsequently differ when compared with UPMT27 in both CELs and SPF chickens. The immunogenic status of the mutated virus will be determined by the chicken experimental trial in one day old SPF chickens to determine whether the inoculated chickens are safe from IBH disease and able to elicit an antibody response.

The hypothesis of this study was the shaft region of the *fiber* gene of UPMT27 can be successfully mutated using CRISPR Cas9 technology and the mutated virus might be genetically stable. The amino acid identified will be consistent throughout the passages. The mutated virus is non-pathogenic and immunogenic in the SPF chickens and able to protect against FAdV infection. The mutated FAdV can be considered as a master seed for future vaccine development against FAdV. This is to our knowledge will be the first vaccine development against FAdV base on gene editing technology.

Therefore the objective of this study were:

1. To characterize the *hexon* and *fiber* genes of fowl adenovirus (FAdV) isolate UPMT27 using phylogenetic analysis.
2. To mutate and validate the shaft region of the fiber protein using CRISPR-Cas9 technology
3. To characterize the pathogenicity of the mutated virus in chicken embryo liver cells and SPF chicken embryonated eggs
4. To determine the pathogenicity and immunogenicity of the mutated virus in specific pathogen free chickens

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LIST OF PUBLICATIONS

Salisu, A., A.R, Mariatulqabtiah., Hair-Bejo, M., Omar, A. R., Aini. I and Nurulfiza, I. (2021). Molecular Markers and Phylogenetic Analysis of UPMT27, a Field Isolate of the Malaysian Fowl Adenovirus Associated with Inclusion Body Hepatitis. Published *Journal of Science and Technology Pertanika*. 29 (1): 547 - 563

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Salisu, A., A.R, Mariatulqabtiah., Hair-Bejo, M., Omar, A. R., Aini. I and Nurulfiza, I. (2020). Pathogenicity, Immunogenicity and Efficacy of Modified Live Attenuated Fowl adenovirus Candidate Vaccine *cf*UPMT27 *Clinical vaccine and immune* to be submitted.

Conferences attended

Salisu, A., Mariatulqabtiah, A.R., Hair-Bejo, M., Omar, A. R and Aini. I and Nurulfiza, I [Oral Presenter] Molecular Detection and Phylogenetic Analysis of UPM T27 Field Isolate of Malaysia Fowl adenovirus Associated with Inclusion Body Hepatitis. *Proceedings of 2nd International Symposium on Bioinformatics (InSyB2018). [International] 20-21, December 2018, Perdana University, Serdang, Malaysia. p 17.*

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