CONSTRUCTION AND CHARACTERIZATION OF AVIAN PAPILLOMAVIRUS L1 VIRUS-LIKE PARTICLES

By

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Chair : Mariatulqabtiah binti Abdul Razak, PhD
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Papillomaviruses (PVs) are a large group of pathogens that foster the proliferations of cells into urogenital or skin tumours in a wide spectrum of vertebrate species. Host-switching and broad host range were becoming more common in PVs than expected. Information on avian papillomavirus (AvPV) especially on their molecular and structure are still lacking due to insufficient study on the papillomavirus viral infection and detection among avian species compared to human papillomaviruses (HPV). This study focuses on molecular analysis of the L1 gene which is the most conserved region in the viral genome of AvPV. Potential discovery of novel papillomavirus in avian species will enable development of a specific model research that can be referred to as a probe to screen for papillomavirus infection in avian as well as expanding the current papillomavirus taxonomy or genome databank. In addition, little is known about its protein ability in self-assembling into capsid protein as observed in L1 protein of HPV. The main objective of this study is to assess the self-assembling properties of recombinant avian papillomavirus L1 capsid protein in the *E. coli* expression system. Fresh faecal samples and cloacal swabs from Rockhopper penguins (*Eudyptes chrysocome*) that showed papillomatosis symptoms were sampled. Then, the DNA was isolated and sent for sequencing. The sequencing result was then analysed by multiple sequence alignment (MSA). The L1 sequence of the Rockhopper penguin *Eudyptes chrysocome* papillomavirus (EcPV) was then submitted to GenBank with accession number MW715602. In this study, the phylogenetic analysis will provide more understanding of the evolutionary pattern of the L1 gene from Rockhopper penguin (*Eudyptes chrysocome*)'s papillomavirus. The L1 gene was further chemically synthesized as the template of interest. The synthesized gene then was amplified by polymerase chain reaction (PCR) before being cloned into pTrcHis2-TOPO expression vector. The recombinant plasmid was then transformed into *E. coli* TOP10 competent cells. Positive transformants were verified by colony PCR, restriction endonuclease digestion using *NcoI* enzyme and sequencing. Expression of the L1 capsid proteins was induced by isopropyl β-d-

1thiogalactopyranoside (IPTG) and analysed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting using mouse monoclonal antibodies targeting the His-tag. The proteins have been purified using sucrose density ultracentrifugation, immobilized metal affinity chromatography (IMAC) and size-exclusion chromatography by fast protein liquid chromatography (SEC-FPLC) before evaluating the assembling ability of the recombinant capsid proteins into virus-like particles (VLPs) by transmission electron microscopy (TEM). The L1 conserved region within another AvPV sequence has been validated by multiple sequence alignment (MSA). Results also showed that the amplified L1 EcPV capsid gene with the size of 1578 bp was successfully cloned and transformed. The L1 protein was expressed in the *E. coli* expression system producing approximately 37 kDa products. The purified recombinant L1 EcPV proteins were able to self-assemble into VLPs with size ranging from 30 – 50 nm. Different purification techniques gave different yields, whereby IMAC purification showed the most distinct self-assembled particles under TEM. Analysis and characterization of this L1 capsid protein is crucial for possible development of virus-like particle-based vaccines and immunological studies for AvPV.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMBINAAN DAN PENCIRIAN L1 DARIPADA VIRUS PAPILLOMA UNGGAS SEBAGAI PARTIKEL SEPERTI VIRUS

Oleh

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Papillomavirus (PV) terdiri daripada sekumpulan besar patogen yang menyebabkan pertumbuhan abnormal sel urogenital dan sel kulit menjadi tumor pada kumpulan besar spesis vertebrata. Penukaran hos dan rangkaian hos yang luas oleh PV adalah lebih kerap daripada yang disangkakan. Maklumat mengenai virus papilloma unggas (AvPV) terutamanya berkaitan molekul dan strukturnya masih tidak banyak kerana kurangnya kajian mengenai jangkitan dan pengesanan virus papilloma ini pada spesies unggas berbanding manusia (HPV). Kajian ini memfokuskan pada analisis molekul gen L1 AvPV di mana ianya merupakan bahagian paling terpelihara dalam genom virus ini. Potensi penemuan virus papilloma baru dalam spesies unggas akan memungkinkan pengembangan model penelitian khusus yang dapat digunakan dalam kajian penyelidikan untuk menyaring jangkitan virus papilloma pada unggas di samping pengembangan taksonomi atau bank data genom virus papilloma itu sendiri. Selain itu, tidak banyak yang diketahui mengenai kemampuan protein L1 daripada AvPV dalam pembentukan sendiri menjadi protein kapsid seperti yang diperhatikan dalam protein L1 HPV. Objektif utama kajian ini adalah untuk menjana pembentukan sendiri partikel seperti virus (VLPs) daripada protein kapsid L1 virus papilloma unggas rekombinan dalam sistem ekspresi *E. coli.* Sampel segar najis dan calitan kloaka daripada penguin Rockhopper (*Eudyptes chrysocome)* yang menunjukkan simptom papillomatasis telah diambil. DNA yang didapati daripada sampel tersebut dihantar untuk mendapatkan jujukan. Hasil jujukan yang berkemungkinan menemukan kawasan konservasi gen L1 baru itu kemudiannya dianalisa dengan penjajaran sekuen berganda (MSA). Jujukan gen L1 virus papilloma penguin Rockhopper *Eudyptes chrysocome* (EcPV) yang telah dikenal pasti kemudiannya dimasukkan ke dalam data GenBank dengan nombor aksesi MW715602. Dalam kajian ini, analisis pokok filogenetik akan memberikan lebih banyak pemahaman tentang corak evolusi gen L1 daripada virus papilloma penguin Rockhopper (*Eudyptes chrysocome*.
Gen L1 itu kemudiannya disintesis secara kimia untuk digunakan sebagai templat gen pilihan. Gen yang telah disintesis itu diperbanyakkan melalui tindak

balas rantai polimerase (PCR) dan diklon ke dalam vektor ekspresi pTrcHis2TOPO. Plasmid rekombinan itu kemudiannya ditransformasikan ke dalam sel kompeten *E. coli* TOP10. Transformasi yang berjaya telah disahkan dengan PCR koloni, pencernaan enzim sekatan menggunakan enzim *NcoI* dan penjujukan. Pengekspresan protein kapsid L1 diinduksi dengan IPTG dan dianalisis dengan SDS-PAGE dan *Western blotting* menggunakan antibodi monoklonal tikus yang mensasarkan *His-tag.* Protein telah ditulenkan dengan menggunakan pengultraemparan ketumpatan kecerunan sukrosa, afiniti logam tidak bergerak (IMAC) dan kromatografi pengecualian saiz oleh kromatografi cecair protein cepat (SEC-FPLC) sebelum menilai keupayaan pembentukan kapsid oleh virus rekombinan itu kepada VLP melalui mikroskop elektron penghantaran (TEM). MSA telah mengesahkan kawasan konservasi gen L1 bersama AvPV yang lain. Hasil mendapati gen kapsid L1 EcPV yang bersaiz 1578 bp telah berjaya diklon dan ditransformasikan. Protein L1 yang terekspesi dalam sistem ekspresi *E. coli* menghasilkan kira-kira 37 kDa produk. Protein L1 EcPV rekombinan yang telah ditulenkankan berjaya membentuk sendiri menjadi VLPs yang mempunyai saiz di antara 30 – 50 nm. Teknik ketulenan yang berbeza memberikan hasil yang berbeza di mana purifikasi oleh IMAC menunjukkan partikel yang paling jelas di bawah TEM. Analisis dan pencirian protein kapsid L1 ini adalah penting untuk perkembangan penghasilan vaksin berasaskan partikel seperti-virus dan kajian imunologi untuk AvPV.

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I certify that a Thesis Examination Committee has met on 11th October 2021 to conduct the final examination of Nur 'Atikah binti Abdul Latif on her thesis entitled "Construction and Characterization of Avian Papillomavirus L1 Virus-like Particles" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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LIST OF ABBREVIATIONS

%	Percentage
°C	degree celsius
AvPV	Avian Papillomavirus
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BLAST	Basic Local Alignment Tools
bp	base pair
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
E1	Early Protein 1
E2	Early Protein 2
EcPV	*Eudyptes chrysocome* avian papillomavirus
EDTA	Ethylenediaminetetraacetic acid
et al.	et alii
FPLC	Fast Protein Liquid Chromatography
g	gram
h	hour
HCl	Hydrochloric acid
HPV	Human Papillomavirus
Hz	Hertz
ICTV	International Committee of Taxonomy of Virus
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl ß-D-1-thiogalactopyranoside
kb	kilo base pair

kDa	kilodalton
L1	Late Protein 1
L2	Late Protein 2
Lac O	Lac Operon
LB	Luria Bertani
M	Molar
mg	milligram
min	minutes
MSA	Multiple Sequence Alignment
MWCO	Molecular Weight Cut-Off
NBT	Nitro blue tetrazolium
NCBI	National Institute for Biotechnology Information
ng	nanogram
nm	nanometre
OD	optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl Fluoride
PV	Papillomavirus
rpm	revolution per minute
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SOC	Super optimal growth with catabolite repression
TAE	Tris-Acetic-EDTA
Taq	Thermus aquaticus thermostable DNA
TBS	Tris-buffered saline

TEM	Transmission electron microscope
U	Unit
VLP	Virus-like particle
µL	microlitre
µM	micro-Molar

CHAPTER 1

INTRODUCTION

There may be a preamble at the beginning of a chapter. The purpose may be to introduce the themes of the main headings. Papillomavirus (PV) comes from the *papillomaviridae* family and have been infecting mammalian hosts' skin and mucosal epithelium since the 1930s. Up until now, more than 320 PV types have been discovered and there are not more than twenty non-mammalians PVs reported coming from birds, turtles, snakes, and fish. Avian papillomavirus (AvPV) has been associated with proliferation growths on the unfeather skin of some birds at various locations (Rector et al, 2013). This means that papilloma in birds can be external as well as internal. The majority of PV infections are asymptomatic. However, due to the ability of PVs to influence cell growth, some PV types can cause self-resolving hyperplastic lesions (warts) while others have been associated with the development of cancer (Munday et al., 2021). The genomic structure of PV is having a resemblance across different species. However, only the four core open reading frames (ORFs) consisting of early and late genes E1, E2, L1 and L2 are present in all PV genomes with variation of other early genes. The late genes, L1 and L2, expressed proteins that are responsible for the formation of PV capsid. L1 proteins have the ability to self-assemble into virus-like particles (VLPs) that are morphologically identical with the native virion as L1 protein contains all the crucial information to form the icosahedral capsid proteins with 72 capsomeres. This discovery leads to the development of VLP-based vaccines that offer protection against infection of the PV (Buck et al, 2013). In addition, L1 ORF has been used for the identification of new PV types over the past 15 years as it is the most conserved region within the genome (IARC, 2007).

With only rare cases of cross-species transmission that indicates a long virus-host association and subsequent tendency of viruses to co-diverge with their host, PV were originally thought to be species-specific (Shah et al, 2010). However, new PVs findings from host-switching and broad host range were more common than expected (Bravo and Felez-Sanchez, 2015). Although birds are the most diverse existing lineage of tetrapod vertebrates with more than 10,000 living species (Prum et al, 2015) with a global distribution known to harbour microbes and act as reservoir hosts for many pathogenic bacteria and viruses (Canuti el at, 2019), knowledge on AvPV is still very limited considering the vast viral diversity known for other vertebrates. To date, only eight different viral types of PV have been described in seven bird species with many more to be discovered.

Isolation of PVs in avian species will lead to the discovery of more novel PV as well as the expansion of PVs taxonomy as there is still limited data recorded for non-mammalian PV especially avian PV compared to human papillomavirus (HPV). The taxonomy classification is based on the identity of pairwise nucleotide sequences across the L1 ORF (Van Doorslaer et al., 2018).

Phylogenetic analysis will provide more information about the evolutionary relationship of avian papillomavirus. Furthermore, potential discovery of novel PV in avian species will enable development of a specific model research that can be referred to as a probe to screen for PV infection in avian. Animal models have contributed significantly to the understanding of papillomavirus pathogenesis, tissue tropism, and disease (Spurgeon and Lambert, 2020).

Furthermore, the AvPV L1 capsid protein self-assembling properties into VLPs can lead to the identification of protein components required for VLPs viral assembly in virus research. VLPs are made of viral structural proteins that assemble spontaneously when expressed in recombinant systems that form multimeric protein complexes which resemble the organisation and conformation of the native viruses without the viral genome (Roldao et al, 2017). These VLPs are not only safe, easy to produce, can be loaded with a wide range of different cargoes, but can also be modified for presentation of epitope or specific delivery. In addition to studying the intrinsic properties of viruses such as their structure and assembly, VLPs can be used to evaluate the interaction between viruses and their environment (i.e their intracellular trafficking, binding to cells and uptake by cells). These are useful for wild-type viruses that are not easy to be cultured in cells (Teunissen et al, 2013). With the recent development in molecular biology and virology, VLPs can also be used for biomedical applications such as vaccination, gene delivery and diagnostics.

In this study, a putative L1 sequence of rockhopper penguin, *Eudyptes chrysocome* avian papillomavirus (EcPV) was determined by bioinformatics analysis and the sequence was synthesized as the template of interest. The L1 gene was amplified and cloned into pTrcHis2-TOPO expression vector before being transformed into *E. coli* TOP10 competent cells. The expression of the proteins was induced by IPTG and further purified with different purification techniques such as sucrose gradient ultracentrifugation, immobilized metal affinity chromatography (IMAC) and size exclusion chromatography by fast protein liquid chromatography (SEC-FPLC). The self-assembling ability of the recombinant L1 capsid proteins into VLPs was then analyzed using transmission electron microscopy (TEM).

The general objective of this project is to evaluate the self-assembling ability of AvPV L1 capsid protein as a potential VLP.
Specific objectives:

1. To analyse a potential novel avian papillomavirus L1 gene.
2. To produce reconstruct of avian papillomavirus L1 capsid proteins from the *E. coli* expression system.
3. To determine the size and morphology of the assembled L1 virus-like particles.

The hypotheses of this study were: 1) the potential novel avian papillomavirus L1 gene of about 1.5 kbp was able to be identified and cloned into TOPO vector; 2) the L1 capsid protein could be expressed and purified from the *E. coli* expression system, with expected size of around 55 kDa; and 3) the recombinant L1 capsid proteins would be able to self-assemble into icosahedral virus-like particles with a known size, ranging from 20 nm to 60 nm.

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