UNIVERSITI PUTRA MALAYSIA

MOLECULAR CHARACTERIZATION OF AVIAN INFLUENZA VIRUS ISOLATE VRI1803/04 AND DEVELOPMENT OF REAL-TIME RT-PCR DETECTION METHOD

HAZALINA BINTI ZULKIFLI

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By

HAZALINA BINTI ZULKIFLI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science

December 2013

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

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December 2013

Chairman: **Prof. Datin Paduka Aini Ideris, PhD**
Faculty: **Veterinary Medicine**

Influenza virus can be divided into 3 subtypes; Influenza type A, B and C which belong to the Orthomyxoviridae family. Only influenza type A virus is responsible for infection in avian species. It is divided into low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI). Since 2003, outbreaks of HPAI strain H5N1 have occurred in poultry in Asia, Europe, and Africa. Human infections with this subtype have been reported and continued to occur. The ongoing transmission of avian influenza A virus strain H5N1 in Asia and Africa has raised global awareness of the threat of a new influenza pandemic which causes devastating economic lost in poultry industry. It has stimulated interests in the development of a rapid laboratory method for detection of influenza A viruses to reduce the time taken as compared to the conventional virus isolation methods.

An isolate of AIV designated as VRI1803/04 obtained from Veterinary Research Institute, Ipoh, Perak, Malaysia was used for identification and characterization using the conventional polymerase chain reaction (PCR). The complete sequence of the H and N genes of VRI1803/04 was determined. A total of 1701 and 1410 bp of H and N, respectively, were identified. The H gene has 89% homology to H3 gene from A/duck/Ukraine/1/63 (H3N8). Meanwhile, the N gene has 92% homology to N2 gene from A/Pekin duck/Singapore/F59/04/98 (H5N2). Thus, the VRI1803/04 can be subtyped as H3N2. The complete nucleotide sequence of H gene demonstrated that the VRI1803/04 virus isolate belongs to LPAI due to the absence of multiple basic amino acids in H cleavage sites.

Further study was carried out for AIV detection. This assay known as one step real-time RT-PCR universal assay was developed using cheaper dye which is SYBR Green 1. The time taken to complete the whole process was around 6 hours, starting from RNA extraction from the sample until result is obtained, whether the sample contains AI virus or not. The result can be monitored in

computer by looking at the existence of amplification curves. The melting temperature peak could be observed as soon as the test was completed. The melting temperature average of the positive amplification product is 83.4 ± 0.6^{o}C using the universal NP primer.

The detection limit of the assay was determined by performing serial dilution of total RNA and determination of HA titer. The detection limit for universal assay was 0.1 ng of total RNA and 0.1 HAU. In conclusion, the developed assay offers a rapid method for detection of AIV in a single tube reaction. This technique was used to detect presence of avian influenza virus in poultry samples during HPAI H5N1 outbreak in 2004.

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

PENCIRIAN MOLEKUL VIRUS AVIAN INFLUENZA ISOLAT VRI1803/04 DAN PEMBANGUNAN KAEDAH PENGESANAN BERDASARKAN TINDAK BALAS RANTAI POLIMERASE TRANSKRIPTASE BERBALIK MASA NYATA

Oleh

HAZALINA BINTI ZULKIFLI

Disember 2013

Pengerusi: **Prof. Datin Paduka Aini Ideris, PhD**
Fakulti: **Perubatan Veterinar**

Virus influenza boleh dibahagikan kepada 3 jenis; virus influenza jenis A,B dan C yang berasal dari famili Orthomyxoviridae. Hanya virus influenza jenis A yang bertanggungjawab menjangkiti spesis avian. Ianya dibahagikan kepada virus influenza avian berpatogenik tinggi (HPAI) dan virus influenza avian berpatogenik rendah (LPAI). Semenjak 2003, penularan virus influenza avian (AIV) berpatogenik tinggi strain H5N1 yang berlaku dalam poltri di Asia, Eropah dan Afrika serta jangkitan terhadap manusia dengan subjenis ini telah dilaporkan dan masih terus berleluasa. Penularan virus influenza avian yang terus berleluasa di kawasan Asia dan Afrika ini mewujudkan kesedaran di peringkat global mengenai ancaman yang akan timbul oleh pandemik influenza yang akan menyebabkan kerugian besar ekonomi dalam industri poltri. Kejadian tersebut juga menggalakkan kecenderungan untuk membangunkan kaedah pengesanan pantas virus influenza A di makmal bagi mengurangkan masa yang diambil jika dibandingkan dengan kaedah pemencilan virus.

Pada permulaan kajian ini, satu isolat yang dilabel sebagai VRI1803/04 telah dibekalkan dari Institut Penyelidikan Veterinar, Ipoh, Perak, Malaysia telah digunakan bagi tujuan pengenalpastian dan pencirian menggunakan kaedah tindakbalas rantai polimerase konvensional. Jujukan nukleotida yang lengkap bagi gen H dan N telah dikenalpasti. Sejumlah 1701 bp bagi gen H dan 1410 bp bagi gen N telah dikenalpasti. Gen H mempunyai homolog sebanyak 89% dengan gen H3 daripada virus A/ Itik / Ukraine / 1 / 63 (H3N8). Manakala gen N mempunyai homolog sebanyak 92% dengan gen N2 daripada virus A/Pekin duck/Singapore/F59/04/98 (H5N2). Oleh itu, virus VRI1803/04 ini disubjeniskan sebagai H3N2. Jujukan nukleotida lengkap bagi gen H membuktikan virus VRI1803/04 adalah daripada jenis virus influenza avian berpatogenik rendah (LPAI) kerana ketidakhadiran asas asid amino berganda di tapak ‘cleavage’ H.

Kajian seterusnya adalah untuk membangunkan satu kaedah pengesanan berdasarkan tindakbalas rantai polimerase transkriptase berbalik masa nyata (*real-time* RT-PCR), satu esei yang universal yang menggunakan SYBR Green 1 iaitu 'dye' yang murah, untuk mengesan virus influenza avian. Masa yang diambil bagi keseluruhan proses adalah sekitar 6 jam bermula daripada pengekstrakan RNA sampel sehingga memperoleh keputusan pengenalpastian samada sampel tersebut mengandungi virus influenza avian atau tidak. Keputusan tersebut boleh dipantau menggunakan komputer dengan melihat puncak pencairan. Purata suhu pencairan adalah 83.4 ± 0.6^{o}C menggunakan pencetus protein nukleokapsid (NP). Kesensitifan asai telah dikenalpasti dengan melakukan pencairan bersiri daripada jumlah RNA, dan pengenalpastian titer penggumpalan hemaglutinin (HA).

Had pengesanan bagi asai universal ini ialah 0.1 ng daripada jumlah RNA dan 0.1 unit HA. Sebagai kesimpulan, pembangunan esei ini menawarkan kaedah pantas dalam mengesan virus influenza avian. Teknik ini juga telah digunakan bagi mengesan kehadiran virus avian influenza pada sampel sampel poltri semasa penularan wabak HPAI H5N1 pada tahun 2004.

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I certify that a Thesis Examination Committee has met on 23 December 2013 to conduct the final examination of Hazalina Binti Zulkifli on her thesis entitled "Molecular Characterization of Avian Influenza Virus Isolate VRI1803/04 and Development of Real-Time RT-PCR Detection Method" 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.

Members of the Thesis Examination Committee were as follows:

Abdul Rani bin Bahaman, **PhD**
Professor Dato'
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Zeenathul Nazariah binti Allaudin, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Jalila binti Abu, PhD
Associate Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Internal Examiner)

Abd. Manaf Ali, **PhD**
Professor
Universiti Sultan Zainal Abidin
Malaysia
(External Examiner)

_____
NORITAH OMAR, PhD
Associate Professor and Deputy Dean
School of Graduate studies
Universiti Putra Malaysia

Date: 10 March 2014

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

Aini Ideris, PhD
Professor Datin Paduka
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Chairman)

Abdul Rahman Omar, PhD
Professor
Faculty of Veterinary Medicine
Universiti Putra Malaysia
(Member)

Sharifah Syed Hassan, PhD
Associate Professor,
Monash University (Malaysia Campus)
(Member)

_____

BUJANG BIN KIM HUAT, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

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Name and Matric No.: Hazalina Binti Zulkifli (GS13690)

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Signature: _____
Name of Chairman of Supervisory Committee: Prof. Datin Paduka Dr. Aini Ideris

Signature: _____
Name of Member of Supervisory Committee: Prof. Dr. Abdul Rahman Omar

Signature: _____
Name of Member of Supervisory Committee: Assoc. Prof. Dr. Sharifah Syed Hassan

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LIST OF ABBREVIATIONS

AF	Allantoic fluid
AGID	Agar gel immuno diffusion
AIV	Avian Influenza Virus
bp	Base pair
Ca	Calcium
cDNA	Complimentary deoxyribonucleic acid
cm	Centimeter
C_T	Cycle Threshold
^oC	Degree Celsius
DNA	Deoxyribonucleic acid
DVS	Department of Veterinary Services
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassays
ELISA	Enzyme-linked immunosorbent assay
Fig	Figure
FRET	Förster Resonance Energy Transfer
H	Hemagglutinin
HAU	Hemagglutination unit
HCl	Hydrochloric acid
HI	Hemagglutination Inhibition
HPAI	Highly pathogenic avian influenza
HPNAI	Highly pathogenic notifiable avian influenza
IVPI	Intravenous pathogenicity index
kb	Kilobase
kDa	Kilodalton
LPAI	Low pathogenic avian influenza
LPNAI	Low pathogenic notifiable avian influenza
M	Molar
Mg	Magnesium

min	Minute
ml	Milliliter
M1	Matrix
mM	Millimolar
µm	Micrometer
µg	Microgram
N	Neuraminidase
NaCl	Sodium chloride
ng	Nanogram
nm	Nanometre
NP	Nucleoprotein
NS1	Non Structural Protein 1
NS2	Non Structural Protein 2
PA	Polymerase A
PB1	Polymerase B1
PB2	Polymerase B2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RFLP	restriction fragment length polymorphism
RT- PCR	Reverse-transcriptase polymerase chain reaction
RNA	Ribonucleic acid
SPF	Specific-pathogen-free
SS	Single stranded
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
T_M	Melting temperature
Tris	2-amino-2-(hydroxymethyl)-1, 3 propandiol
UPM	Universiti Putra Malaysia
VRI	Veterinary Research Institute
WHO	World Health Organization
>	Greater than
~	Approximately

CHAPTER I

INTRODUCTION

Avian influenza virus (AIV) belongs to type A influenza virus (Alexander, 2007). Majority of AIV do not infect human. However, a few are zoonotic which can infect and cause disease in human. The most famous example is the avian influenza subtype H5N1 viruses which are still circulating in poultry, in parts of Asia and northeast Africa. The H5N1 virus is the utmost concern for human health from all influenza viruses that circulate in birds. The main reason is that the H5N1 virus has caused the largest number of human cases of very severe disease and the biggest number of deaths. It has beyond the species barrier to infect human. The first documented infection of avian influenza H5N1 in human was in Hong Kong in 1997 where there were 18 cases with six deaths. Six years later in 2003, another two cases of H5N1 infection in human with one death was reported. The H5N1 outbreak in Hong Kong was controlled by culling 1.5 million chickens. It has cost more than 245 million dollars in a month (Jalilian *et al,.* 2010).

Since their re-emergence in late 2003, avian influenza or bird flu has become one of the most publicized emerging infectious diseases. In several Southeast Asian countries, the outbreaks of H5N1 avian influenza viruses among poultry have reached their endemic levels. As of 31st December 2012, 610 human infected with H5N1 cases have been reported by the WHO in 15 countries worldwide, which include Cambodia, China, Laos, Indonesia, Vietnam, Thailand, Myanmar, Azerbaijan, Djibouti, Egypt, Bangladesh, Iraq, Nigeria, Pakistan and Turkey. As time goes on, the list of human infection and the number of countries will only get longer. The latest confirmation of death cases due to H5N1 avian influenza was announced by World Health Organization (WHO) on 10th August 2012. The case is a 37 year old male from Yogyakarta province. He developed fever on 24th July 2012, was hospitalized on 27th July 2012 and died on 30th July 2012. These continuing situations have ignited global fears of an imminent influenza pandemic (WHO, 2010).

A major concern of outbreak of H5N1 is the virus would exchange their gene and re-assort with human influenza virus, such as, H3N2 that is circulating in human population. The situation becomes worst when WHO announced the new pandemic of swine flu H1N1 in June 2009 (CDC, 2010). The concern of outbreak is that the virus H5N1 from avian will mix together with swine isolate H1N1 to spark off new hybrid influenza pandemic subtype, as we know that swine is the mixing vessel of avian and human flu (Gunson *et al.,* 2010).

The H5N1 avian influenza crisis has had wide ranging social and economic implications. The outbreaks across South East and East Asia, have resulted in large scale loss of birds, high costs of outbreak control to public and private

sectors, and indirect costs from lost markets and lost production value to producers, traders, the retail sector and sectors linked to livestock. As of mid-2006, it was widely estimated that at least 200 million domestic birds have either died or been culled as a results of H5N1 crisis. For poultry producing and exporting countries, great economic loss is due to export restriction (FAO, 2006).

The poultry industry is regarded as the most successful industry of the livestock sector in Malaysia. Perhaps, it has the highest output value per worker in the agriculture sector. Poultry meat and eggs are consumed chiefly as the main source of protein by Malaysians. According to the statistics data from Department of Veterinary Services (DVS), the local poultry production was 1,334,470 metric tonne in 2011 with the total consumption of 1,041,380 metric tonne. The production of chicken contributed about 80% of the total output of animal products. In 2004, export of live broilers decreased around 22% when only 34 million birds have been exported, compared to the previous year. Amount of broiler meat also experienced a decrease in 2004 where only 2,554 metric tonne had been exported compared to 5,195 metric tonne in 2003. The discovery of H5N1 virus in Kelantan in August 2004 provoked Singapore, Japan, The EU and Hong Kong which was Malaysia's major markets, to ban all Malaysian poultry and poultry products. Broilers were in excess supply in the local market when Singapore, the largest export market for live poultry and poultry products, imposed a temporary suspension on all Malaysian poultry. The local poultry industry was reported to be losing RM 10 million (US$2.6 million) daily (Loh *et al*., 2004).

Malaysia exported almost 51% of live ducks in 2002 for world consumption (FAO, 2004). Malaysia exported 14480 birds to the USA in 2003 compared with 57356 birds in 2002. The decreased amount was due to global HPAI outbreak in neighbouring countries. Based on data by DVS, Malaysia exported 5,163,444 ducks to Singapore in the year of 2008.

Data from DVS indicated that 503.19 million day-old chicks were produced in Peninsular Malaysia in 2010. Some 471.56 million broilers were marketed. Industry projections for 2008 have been for 548.8 million day-olds and 522.83 million broilers. This was after a forecast that the standing population of parent stock would raise in 2008 to 5.25 million birds, from 5.19 million in 2007 (Best, 2008).

There are several routine diagnostic methods for detection and subtyping of influenza A virus, including virus isolation, hemagglutination (HA), hemagglutination inhibition tests (HI) and agar gel immunodiffusion (AGID). Virus Isolation is the gold standard diagnostic test for influenza virus. This is a very sensitive test where the virus is isolated inside the embryonated chicken eggs or cell culture, but the result requires several days to be available. Since influenza viruses can cause severe illness, timely diagnosis is important for an

adequate intervention. The use of molecular technique such as RT-PCR to detect viruses can facilitate the rapid identification and genetic characterization of AIV. The goal for the development of molecular based test is to achieve a simpler, rapid and highly sensitive test which is prerequisite for the surveillance and control of influenza outbreaks. The use of RT-PCR followed by ethidium bromide staining gel electrophoresis of the PCR product is fairly laborious and therefore not really an option for routine analysis, due to many steps which would introduce more potential for contamination. The recent development of fluorogenic PCR-based format, termed as 'real-time PCR' promises a wide dimension in diagnostic approach. The introduction of real-time PCR has made it possible to accurately quantify starting amounts of nucleic acid during the PCR reaction without the need for post-PCR analyses (Bustin, 2000; Kearns *et al*., 2001; Komurian-Pradel, 2001).

Previously, before August 2004, only low pathogenic avian influenza (LPAI) viruses were isolated in Malaysia. These LPAI viruses were isolated from domestic ducks as a result of surveys for the presence of Newcastle disease virus (Aini and Ibrahim, 1986) and have been extended to include domestic, caged and feral birds of other species. As a result of that surveillance, two influenza isolates were obtained from passerines. One was from a magpie robin (*Copsychus saularis musicus*) which had died at a transit aviary in Selangor and the other from yellow vented bulbul (*Pycnonotus goiaver personatus*), which had been found sick on roadway and unable to fly. Those virus isolates were confirmed to be H4N3 subtype and designated as A/magpierobin/Malaysia/23/89 (H4N3) and A/bulbul/Malaysia/41/89 (H4N3).

Avian influenza viruses isolated in Malaysia need to be characterized in order to find preventive measures against infection of new type of virus that may be transmitted to local poultry and assess the likelihood of further dissemination. In this study, virus isolate VRI 1803/04 from duck was used for further molecular characterization. This study also described a development of a rapid and sensitive detection method that can be easily applied for routine diagnosis and surely would pave way for a more effective control of AIV.

The objectives of this study were:

1) To sequence the entire length of H and N genes of a low pathogenic AIV subtype VRI 1803/04

2) To determine the relationship of the virus with other influenza viruses based on phylogenetic analysis

3) To develop a one-step real-time RT-PCR assay based SYBR Green 1 for universal detection of all subtypes of AIV.

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