CLONING AND EXPRESSION OF INFLUENZA A VIRUS PROTEINS AND ITS USE IN SEROLOGICAL ASSAY

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A thesis submitted
in fulfilment of the requirements for the degree of Master of Science

INSTITUTE OF HEALTH AND COMMUNITY MEDICINE
UNIVERSITI MALAYSIA SARAWAK
2009
Dedications

To my mum and dad, this is as much my success as it is yours. Thank you.
Acknowledgments

First of all, I would like to dedicate my appreciation to Professor Mary Jane Cardosa for giving me the opportunity to do my Master’s degree under the Institute of Health & Community Medicine, Unimas and also thanks for her encouragement and support throughout my studies. I would also like to express my sincere gratitude to:

Tio Phaik Hooi, my supervisor, for introducing me to AIV, for all the precious idea and suggestion throughout this study and writing thesis. Thank you for all your assistance and also valuable advice, personally or professionally, it’ll be my guidance in future. I believed that the success of my work was due to the good supervision I was given. Without you I would not have made it! Thank you.

Dr. Andrea, Rosnani, Anita and all the staff in State Veterinary Diagnostic Laboratory (SVDL), Sarawak for kindly providing me with all the samples and important data. I am greatly in debt to your group.

The lecturers of IHCM, Dr. David, Dr. Magdine and Yuwana, thank you for all your assistance, guidance and support toward my studies.

The Ministry of Science, Technology and Innovation (MOSTI) for financially supporting this project. Without them, this project would not have been completed.

And last but not least, to all the lab techs and all my friends in the lab, thanks for all the help and support. Friendship, help and support appreciated.

To my parents and brothers, thanks for everything.
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<th>Description</th>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>bp</td>
<td>basepair</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathogenic effect</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>DNP</td>
<td>duck negative pooled</td>
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<tr>
<td>dNTPs</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetate</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>FBS</td>
<td>foetal bovine serum</td>
</tr>
<tr>
<td>HPAI</td>
<td>high pathogenic avian influenza</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HA</td>
<td>hemagglutinin</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl thiogalactosidase</td>
</tr>
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<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>luria bertani</td>
</tr>
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<td>LPAI</td>
<td>low pathogenic avian influenza</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>M1</td>
<td>matrix protein 1</td>
</tr>
<tr>
<td>M2</td>
<td>matrix protein 2</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine Kidney</td>
</tr>
<tr>
<td>M-MLV</td>
<td>Moloney Murine Leukemia Virus</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>nt</td>
<td>nucleotide</td>
</tr>
<tr>
<td>OPD</td>
<td>(\sigma)-phenylenediamine</td>
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<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-tetramethylethylenediamine</td>
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Abstract

Avian influenza poses a threat to many species including human, as proven by the current situation in Southeast Asia region. It appears that avian influenza of influenza A virus can spread to many species causing severe disease in these new species. The aim of this study is to clone and expressed the major influenza A virus proteins namely matrix 1 (M1), matrix 2 (M2), hemagglutinin 1 (HA1), hemagglutinin 2 (HA2) and nucleoprotein (NP) and also to assess its use in serological assay. Although all genes studied were successfully cloned, only the M1 construct protein resulted in good protein expression. The matrix 1 protein is a conserved protein. The viral gene encoding matrix protein 1 (M1) of influenza A virus is a multifunctional protein that plays essential structural and function roles in virus life cycle. M1 forms the major structural component of the virion, lying beneath the lipid envelope containing the viral hemagglutinin (HA) and neuraminidase (NA) glycoproteins and the M2 ion channel and it is presumed to play an important factor in initiating progeny virus assembly. The standard method required for determination of antibody level against influenza virus is hemagglutination inhibition (HAI). This method is not proficient because of the various subtypes and strains of influenza virus which consist of 16 hemagglutinin and 9 neuraminidase. Alternative methods are required for rapid screening especially in surveillance and large scale examinations. The matrix 1 (M1) gene of infectious avian influenza virus (AIV) strain isolated from infected duck was expressed using the pET SUMO system and was purified as recombinant matrix 1 (M1) protein. SUMO fusion protein was also expressed and used as controlled in ELISA assay. Expressed proteins were purified by affinity
chromatography column under denaturing conditions and tested for antigenicity by western blot and probed with commercially available antibody. The viability of using the M1 recombinant protein in M1-Sumo ELISA assay was evaluated. In this study, it shows matrix 1 (M1) recombinant protein had a potential usefulness in serological assay by showing the seroprevalance in ducks based on their reactivity level against avian influenza M1 protein. This can be useful for screening and monitoring in ducks poultry industry.
'Avian influenza' merupakan satu ancaman kepada kebanyakan spesis termasuk manusia, ini terbukti berdasarkan keadaan semasa yang melanda Asia Tenggara. Ini menunjukan bahawa virus influenza ini mampu tersebar kepada kebanyakan spesis lain dan menyebabkan penyakit teruk kepada spesies tersebut. Objektif kajian ini adalah untuk mengklon dan mengekspresikan protein matriks 1 (M1) influenza A virus dan mengaplikasikan kegunaanya sebagai salah satu kaedah alternatif asai serologikal. Gen virus yang mengkodkan protein matriks 1 (M1) membentuk lapisan yang mengelilingi nukleokapsid virion di bawah envelop virion dan ia merupakan faktor penting dalam memulakan penyusunan virus progeni. Kaedah standad yang digunakan untuk menentukan tahap antibodi terhadap virus influenza adalah 'hemagglutination inhibition' (HI). Kaedah ini kurang sesuai untuk kebanyak subtaip dan strain virus influenza, oleh itu kaedah alternative diperlukan untuk penyaringan yang cepat terutamanya dalam pemantauan dan pemeriksaan berskala besar. Gen protein matriks 1 (M1) yang diasingkan daripada itik yang dijangkiti 'avian influenza' telah diekspresikan menggunakan sistem pET SUMO dan ditulenkan menjadi protein rekombinasi matriks 1 (M1). Protin SUMO gabungan juga diekspresikan dan dijadikan sebagai kawalan dalam asai ELISA. Protein yang telah diexpres telah ditulenkan melalui kaedah penulisan kolumdi afiniti dibawah keadaan denaturasi dan keantigenikannya dikaji melalui kaedah "Western blot" dengan menggunakan antibody yang boleh didapati secara komersial. Kemungkinan untuk menggunakan protein rekombinan M1 dalam asai ELISA M1-Sumo telah diuji. Dalam ujikaji ini, menunjukan, protein rekombinan matriks 1 (M1) boleh
dijadikan alternatif serologikal asai yang cepat dan sesuai untuk pemantauan antibody influenza A, terutamanya dalam industry perladangan itik.
Chapter 1: Literature Review

1.1 Influenza History

Influenza is a highly contagious acute respiratory disease of avian origin that has caused epidemics and pandemics in humans for centuries, resulting in natural disasters (Taubenberger, 1998). Influenza was responsible for the most devastating plague in human history – the “Spanish” flu. During the pandemic of 1918 to 1919, more than 20,000,000 people died (Hentges, 1955). Its relationship with other milder viruses isolated from birds and with mammalian influenza A viruses (first isolated in the 1930s) was demonstrated (Metreveli, 2006). An antigenically distinct virus, isolated by Francis (1940), was classified as type B strain (B/Lee/40) to differentiate it from the 1930s isolate and finally, influenza C, the third major type of influenza virus was first isolated in 1947 by Taylor (1949) (Collier et. al., 1998).

Two influenza pandemics have swept the world since the “Spanish” flu of 1918 which is the “Asian” flu pandemic in 1957 and the “Hong Kong” flu pandemic of 1968. It was suggested that influenza in humans between 1889-1898 was caused by influenza A (H2N2) viruses based on testing of sera from older adults for influenza virus antibodies (seroarchaeology), whereas epidemics and pandemics in 1899-1917 and in 1918-1957 were the result of H3N8 and H1N1 viruses, respectively (Mulder et. al., 1960). Current pandemic strains share the hemagglutinin (HA) and neuraminidase
(NA) subtypes of these earlier strains; however, they are probably not direct descendants, as they show close relation to avian viruses (Levison et. al., 2000).

1.2 Epidemiology of Influenza A Virus Disease

Animals played a major role in the past influenza epidemics, for example in the eighteenth and nineteenth centuries, outbreaks of respiratory disease among horses were recorded concurrently with outbreaks in human. In present times, pigs have been accorded a prominent role in the generation of major influenza outbreaks (Ryan et al., 2003).

In severe influenza A epidemics, number of deaths reported in a given area of a country often exceed the number expected for that period and this significant increase, referred to as excess mortality, is another indicator of severe, widespread illness (Ryan et al., 2003). Pandemics outbreaks are probably a more recent development, paralleling the increase in the growth of mass transportation system and world population (Collier et. al., 1998).

1.3 Malaysian Scenario

The first Highly Pathogenic Avian Flu (HPAI) outbreak in Malaysia occurred on 17 August 2004 coinciding with outbreaks of avian influenza in the region. 8 more outbreaks followed, which ended with the last case in September 2004. All outbreaks were confined to the state of Kelantan and identified as H5N1 subtyped. 101 deaths recorded among poultry involving free-range village chickens, ducks and quail caged
on raised floors. The second national outbreak was then recorded in a village near Kuala Lumpur after it appeared in free range chickens. The outbreak triggered the slaughter of thousands of birds. Since then, five other outbreaks of H5N1 have occurred among poultry in Perak and Pulau Pinang and later in June 2006, Malaysia was declared free from H5N1 (http://www.unicef.org/malaysialmedia_5401.html).

Currently Malaysia has not reported any cases of avian influenza among human in Malaysia. A close collaboration has been made between the Ministry of Health and the Department of Veterinary to handle the avian influenza situation in the country. Surveillance of ‘influenza like illness’ and ‘atypical pneumonia’ is been currently held by the Ministry of Health in hospitals and health centers (MyHealth Portal, 2009). They were no increase in number of cases to date, as all health departments were advised to report of any unusual number of deaths in poultry in their localities. Activities at the poultry and bird farm are also closely monitored by the Department of Veterinary.

1.4 Introduction to Influenza A Virus Disease

Influenza A is divided into several types based on serological typing of the major outer envelope proteins of the virus, hemagglutinin (HA) and neuraminidase (NA). Influenza viruses that infect birds are called avian influenza viruses. Only influenza A viruses infect birds, and all known subtypes of influenza A viruses can infect birds. However, there are substantial genetic differences between the subtypes that typically infect both people and birds (CDC, 2005).
1.4.1 Avian Influenza A Virus

Avian influenza (AI) is a serious infectious zoonotic disease caused by avian influenza virus. Avian influenza, which is caused by influenza A viruses, can affect a variety of domestic and wild bird species. Infection can range from asymptomatic to severe, depending on the virulence of the virus and the susceptibility of the avian host (Prescott et al., 1993). Avian influenza virus is usually diagnosed either by serology or isolation and characterization of the virus. Infections in birds can lead to a wide variety of clinical signs that can vary according to the host, strain of virus, the host immune status, presence of any secondary exacerbating organism and environmental conditions. The virus has been recovered from domestic and wild avian species throughout the world and had affected international trade in poultry product (WHO, 2007). Many countries around the world have been reported to encounter with avian influenza cases since 2003. But most cases were reported in the Asian region especially in countries like China, Hong Kong and Indonesia (Figure 1.1) (http://www.who.int/mediacentre/factsheets/avian_influenza/en/).

Avian influenza is defined by the World Organization for Animal Health (OIE) as "an infection of poultry caused by any influenza A virus of the H5 or H7 or by any avian influenza virus with an intravenous pathogenicity index (IVPI) greater than 1.2 or at least 75% mortality (http://www.oie.int/eng/info/en_sam.htm). Various types of influenza A have been isolated from birds, but not all avian strains are pathogenic. The pathogenicity varies depending on birds species infected.
There are two forms of avian influenza;

i.) Highly pathogenic avian influenza (HPAI) - characterized by acute systemic disease with rapid onset high mortality.

ii.) Low pathogenic avian influenza (LPAI) - characterized by high morbidity and low mortality. From the clinical perspective, the low pathogenic conditions can be misdiagnosed for other respiratory diseases or syndromes, even with rolling vaccine reaction.

Within subtypes of avian influenza A viruses there also are different strains. Avian influenza A H5 and H7 viruses can be distinguished as “low pathogenic” and “high pathogenic” forms based on the genetic features of the virus and the severity of the illness they cause in poultry. Influenza H9 virus has been identified only in a “low pathogenicity” form. Each of these three avian influenza A viruses (H5, H7, and H9) theoretically can be partnered with any one of nine neuraminidase surface proteins; thus, there are potentially nine different forms of each subtype (e.g., H5N1, H5N2, H5N3, H5N9). At least three H9 infections in humans have been confirmed (http://www.cdc.gov/flu/avian/gen-info/pdf/avian_influenza.pdf).