UNIVERSITI PUTRA MALAYSIA

EPIDEMIOLOGY OF INFLUENZA A VIRUSES IN THE AVIAN AND SWINE POPULATIONS IN PENINSULAR MALAYSIA

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

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January 2008

Chairman : Latiffah Hassan, PhD

Faculty : Veterinary Medicine

Avian influenza (AI) that has emerged from animal reservoirs represents one of the greatest concerns to public health. To date, 16 hemagglutination subtypes and nine neuraminidase subtypes are found in many different combinations.

The general objective of this study is to describe the epidemiology of influenza A viruses in the animal population in Malaysia. The specific objectives of the study are to describe the pattern and geographical distribution occurrence of the various AI strains in the avian species in Peninsular Malaysia based on retrospective examination of records and data from disease surveillance conducted in previous years, to identify, describe and determine the risk factors of the influenza strains circulating in the pig populations, and detect and isolate the influenza virus in the pigs, and molecularly characterised the strains that were isolated.

A descriptive analysis was performed on the AI data based on the surveillance that was conducted by the Department of Veterinary Services (DVS) between 2000 to

2005. A three-page questionaire was developed and administered to the participating states. Only Kedah, Perak, Pulau Pinang, Johor, Negeri Sembilan and Pahang responded to the questionnaire. Data that were collected were compared to the total number of poultry farms and avian related establishments in each state. The sample size for each state was calculated using the formula given by Dahoo et al (2003) and using FreeCalc software. Using the assumptions of 0.5% prevalence and confidence level of 95%, neither highly pathogenic AI nor low pathogenic AI has been detected in samples.

A cross-sectional study was carried out to determine the seroprevalence of H1N1 and H3N2 swine influenza virus (SIV) and the risk factors of SIV in the pig population. Between May and August 2005, 41 randomly selected farms were visited where a total of 727 serum samples from 4 to 6-months-old pigs were collected. Each subtypes of H1N1 and H3N2 were detected at 17 farms (41.4%). Eight-nine animals (12.2%) and 88 animals (12.1%) were seropositive for H1N1 and H3N2 respectively. Using binary logistic regression, four common risk factors were identified for SI H1N1 and H3N2: Farm size, farms that import pigs or purchase pigs from elsewhere, farms where animals such as cats were observed and farms that were closely located to another pig farm.

The study proceeds with virus detection and isolation from randomly selected samples that were seropositive for H1N1 or H3N2. Allantoic fluids were collected from inoculated eggs and tested using hemagglutination test, One-Step Real time Polymerase Chain Reaction (RRT-PCR) and Conventional Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). All samples were tested negative using HA and RRT-PCR. The failure to isolate the H1N1 and H3N2 viruses was possibly due to pigs that were not in the acute phase of the disease during the period when samples were collected, thus they did not shed the virus.

The study found that a high percentage of pigs in Peninsular Malaysia were seropositive for H1N1 and H3N2. However, no isolates could be obtained to further characterise the virus to determine whether the virus strain was avian or human-related. This study revealed some of the deficiencies and issues with the existing disease surveillance that must be addressed to face the potential global influenza pandemic.

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

KAJIAN EPIDEMIOLOGI VIRUS INFLUENZA A DI DALAM POPULASI AYAM DAN BABI DI SEMENANJUNG MALAYSIA

SURIYA KUMARI RAMIAH

Januari 2008

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Selsema burung (AI) yang berasal dari perumah merupakan satu daripada ancaman besar terhadap kesihatan awam. Sehingga kini, 16 subtip hemagglutinin dan sembilan subtip neuramidase dalam banyak kombinasi berbeza telah dikenalpasti.

Projek ini adalah dicadangkan sebagai sebahagian daripada satu objektif jangka panjang untuk menghuraikan epidemiologi virus influenza A dalam populasi haiwan di Malaysia. Objektif spesifik bagi kajian ini bermatlamat ke arah menghuraikan penularan pelbagai virus AI di dalam populasi unggas melalui pemeriksaan data pengawasan yang sedia ada, menentukan prevalens virus yang berkaitan dengan AI di dalam populasi babi, mengenalpasti faktor risiko virus yang menular di dalam populasi babi, dan mengesan serta mengisolasi virus influenza dari babi, dan secara molekul mencirikan jenis virus influenza yang terpencil.

Satu analisis perihalan telah dijalankan ke atas data AI berdasarkan pengawasan yang dijalankan oleh Jabatan Perkhidmatan Haiwan (DVS) dari 2000 ke 2005. Soal selidik sebanyak tiga muka surat disediakan dan diberi kepada negeri di semenanjung Malaysia. Hanya Kedah, Perak, Pulau Pinang, Johor, Negeri Sembilan dan Pahang memberi respon untuk soal-selidik tersebut. Menurut doktor veterinar yang ditemubual di negeri tersebut, pengawasan aktif hanya bermula pada 2004 sementara pengawasan pasif bermula pada 2000. Data yang dikumpul dibandingkan dengan jumlah bilangan ladang ternakan ayam di dalam setiap negeri. Saiz sampel untuk setiap negeri dikira mengikut formula yang diberikan oleh Dahoo et al (2003) dan ‘FreeCalc software’. Dengan andaian 0.5% prevalens and 95% interval keyakinan, tiada kes AI telah dikenalpasti dari sample.

Satu kajian keratan-rentas telah dijalankan untuk menentukan seroprevalen H1N1 dan H3N2 virus influenza babi (SIV) dan faktor risiko SIV dalam populasi babi. Di antara Mei dan Ogos, 2005, 41 ladang babi yang dipilih secara rambang telah dilawati, di mana sejumlah 727 serum diambil dari babi berumur 4-6 bulan. Setiap subtip H1N1 dan H3N2 telah dikesan di 17 buah ladang (41.4%). Lapan puluh sembilan babi (12.2%) dan 88 babi (12.1%) adalah seropositif untuk H1N1 dan H3N2. Menggunakan regresi logistik binari, empat faktor risiko telah dikenalpasti yang berkeertian secara statistik untuk SI H1N1 dan H3N2: saiz ladang, ladang yang mengimport babi atau membeli babi di tempat lain, ladang di mana haiwan lain seperti kucing dilihat, ladang yang berdekatan dengan ladang babi yang lain.

Kajian diteruskan dengan pengesanan virus dan pengasingan daripada sampel seropositif H1N1 atau H3N2 yang dipilih secara rambang. Cecair allantois diambil

dari telur yang diinokulasi dan diuji menggunakan ujian penghemaglutinatan (HA), RRT-PCR satu langkah dan RT-PCR konvensional. Semua sampel diuji negatif menggunakan HA dan RRT-PCR. Kegagalan untuk mengasingkan virus H1N1 dan H3N2 mungkin disebabkan oleh pendedahan lampau. Babi tersebut bukan dalam tahap akut penyakit pada masa sampel diambil, oleh itu mereka tidak mengeluarkan virus.

Kajian mendapati peratusan yang tinggi dari babi di Semenanjung Malaysia adalah seropositif bagi H1N1 dan H3N2, dua subtip virus dibabitkan dalam wabak influenza manusia. Bagaimanapun, tiada isolat diperolehi untuk mencirikan virus bagi menentukan sama ia berhubungkait dengan influenza virus dari unggas atau manusia. Kajian ini mendedahkan beberapa kekurangan dan masalah dalam pengawasan penyakit yang mesti diatasi bagi menghadapi wabak influenza global.

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I certify that an Examination Committee has met on 30th January 2008 to conduct the final examination of Suriya Kumari Ramiah on her Master of Science thesis entitled "Epidemiology of Influenza A Viruses in the Avian and Swine Population in Peninsular Malaysia" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the student be awarded the degree of Master of Science.

Members of the Examination Committee were as follows:

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

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations, which have been duly acknowledged. I also declare that it has not been previously and concurrently submitted for any other degree at UPM or other institutions.

SURIYA KUMARI RAMIAH

Date:

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LIST OF ABBREVIATIONS

°C	Degree Celcius
α	alpha
µg	microgram
µl	microliter
µm	micrometer
g	gram
ml	mililiter
AGID	Agar Gel Immunodiffusion
AI	Avian Influenza
bp	base pair
CI	Confidence Interval
Ct	Cycle Threshold
D	estimated minimum number of diseased animal in the group
DNA	Deoxyribo nucleic acid
DVS	Department of Veterinary Services
ECE	Embryonated chicken egg
EDTA	Ethylenediaminetetraacetic Acid
EID	Embryo Infectious Dose
ELISA	Enzyme linked immunosorbent assay
HA	Hemagglutination
HI	Hemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
IgG	Immunoglobulin G

JPHPK	Jabatan Perkhidmatan Haiwan Perak
LPAI	Low Pathogenic Avian Influenza
$MgSO_4$	Magnesium sulphate
N	Population size
n	Sample size
NA	Neuraminidase
NAI	Notifiable avian influenza
NS	Negeri Sembilan
NP	Nucleoprotein
NPF	Nucleoprotein forward
NPR	Nucleoprotein reverses
OD	Optical Density
OIE	Office International Des Epizooties
OR	Odd ratio
PI	Post Inoculation
PP	Pulau Pinang
PBS	Phosphate Buffer Solution
PCR	Polymerase Chain Reaction
RAT	Rapid Action Team
RBC	Red Blood Cell
RNA	Riboxynucleic Acid
rpm	revolutions per minute
RRT-PCR	Real-time Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S/P	Sera-to-positive ratio

sd	Standard deviation
SI	Swine Influenza
SIV	Swine Influenza Virus
SPP	Standing pig population
SPSS	Statistical Package for the Social Sciences
SYBR	Syber
TBE	Tris-borate-EDTA
T_m	Melting temperature
UPM	Universiti Putra Malaysia
UV	Ultra violet
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

Influenza viruses are the cause of outbreaks of acute respiratory disease, known as influenza or ‘flu’ in humans and wide ranged of animals (Hatta and Kawaoka, 2002). Influenza viruses belong to the family of Orthomyxoviridae which are divided into three types (A, B, and C) (Branson, 1995). Members of the Orthomyxoviridae are single-stranded RNA viruses; influenza A and B virions consist of an eight-segmented RNA genome and influenza C virus genome has seven segments (Hsiung, Caroline and Marie, 1994). To date, 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9) of influenza A viruses have been identified (Fouchier, Munster, Wallensten, Bestebroer, Herfst, and Smith, 2005).

Influenza A virus can infect variety of host species, the ability that is of great importance in determining its infectivity towards man. Highly pathogenic avian influenza virus which causes, an acute generalised disease in which mortality may be as high as 100%, is restricted to subtypes H5 and H7, although not all viruses of these subtypes necessarily cause highly pathogenic avian influenza. All other AI virus strains are low pathogenic avian influenza viruses and cause a much milder, primarily respiratory disease with loss of egg production in layers.

The most important aspect of the virus in terms of the public health impact is the ability of the virus to cross the species barriers. Viral subtypes that occur in animals can infect humans under the right conditions. In fact influenza pandemics that occurred in the year 1918, 1957 and 1968 were as a result of the animal influenza viral subtype that have been adapted thus enabling it to infect humans.

The most successful influenza virus of the 20th century from the perspective of transmissibility among and pathogenicity to humans was the H1N1 virus that caused the Spanish flu pandemic of 1918, estimated to have killed up to 100 million persons (Taubenberger, Ried, Janczeqski, and Fanning, 2001). The virus subtype was linked to the swine influenza H1N1 avian reservoir (Hatta and Kawaoka, 2002). The next most successful viruses were those that caused the Asian flu pandemic in 1957 (H2N2), which killed 70,000 persons in the United States, and the Hong Kong flu pandemic in 1968 (H3N2), which killed 34,000 persons in the United States (Lipatov, Govorkova, Webby, Ozaki, Peiris, Guan, Poon, and Webster, 2004). Both H2N2 and H3N2 subtypes were caused by hybrid viruses, that are, reassortants that harboured a combination of avian and human viral genes (Hatta and Kawaoka, 2002).

The H5N1 subtype is the most recent influenza subtype known to infect humans. H5N1 initially caused only mild disease with symptoms, but after months of circulation in chickens, the virus mutated to a highly pathogenic form that could kill chickens within 48 h, with a mortality approaching 100% (Barrera and Reyes Terán 2005). Human infections during the southeast Asian H5N1 outbreaks were first reported in the early 2004 from Vietnam and Thailand, followed by the ongoing resurgences of human cases in Vietnam, Cambodia, and Indonesia from then onwards (Chotpitayasunondh, Ungchusak, Hanshaoworakul, Chunsuthiwat, Sawanpanyalert and Kijphati, 2005; Tran, Nguyen, Nguyen, Luong, Pham, and

Nguyen, 2004). At the time of this writing (July, 2007), the total number of confirmed human cases of influenza H5N1 in the 10 countries amounts to 318 (Azerbaijan: 8, Cambodia: 7, China: 25, Djibouti: 1, Egypt: 37, Indonesia: 102, Iraq: 3, Thailand: 25, Turkey: 12, Vietnam: 95), of which 192 were fatal (WHO, 2007).

Based on OIE report (2005), the first ever outbreak of H5N1 highly pathogenic avian influenza (HPAI) in Peninsular Malaysia was on 17th August 2004. The outbreak occurred in the northern part of the state of Kelantan, which borders Thailand, and involved village chickens. An outbreak of highly pathogenic avian influenza H5N1 was confirmed in Wilayah Persekutuan State in Peninsular Malaysia on 19th February 2006. Subsequently, 4 more outbreaks were confirmed in the States of Perak and Pulau Pinang dated 30th March 2006. Since then, a new H5N1 outbreak was found Kampung Paya Jaras Hilir near Sungai Buloh in Selangor on 2nd June 2007 (New Strait Times Online, 2007).

However, no human infection has been reported in Malaysia due to this subtype. Worldwide, active research and investigation are being conducted on influenza viruses epidemiology using various surveys and systematically designed studies. Currently, the virus occurrences in the animal populations are of much interest. In Malaysia, not much is known about the occurrence of the influenza viruses in the animal populations. There are no baseline data or knowledge on the circulating influenza viruses with respect to the species of animals.

To effectively prevent or control the disease, comprehensive information about the disease existence and the extent of its occurrence need to be investigated. The