EPIDEMIOLOGICAL INVESTIGATION OF EQUINE INFLUENZA VIRUS INFECTION IN PENINSULAR MALAYSIA

Abdul Rahman D. Abdul Hadi

MASTER OF VETERINARY SCIENCE
UNIVERSITI PUTRA MALAYSIA

2009
EPIDEMIOLOGICAL INVESTIGATION OF EQUINE INFLUENZA VIRUS INFECTION IN PENINSULAR MALAYSIA

By

Abdul Rahman D. Abdul Hadi

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

April 2009
DEDICATED

To my late father “may Allah bless him with his supreme benevolence”.

To my caring, lovely mother and father who have shown me the way to the right path.

To my wife, brother, sisters, and all those who passed away in struggle for sovereignty of my fatherland.

To my patient and bleeding country, may Allah grant you peace.
Abstract of thesis presented to the Senate of Universiti Putra Malaysia In fulfillment of the requirement for the Degree of Master of Veterinary Science

EPIDEMIOLOGICAL INVESTIGATION OF EQUINE INFLUENZA VIRUS INFECTION IN PENINSULAR MALAYSIA

By

Abdul Rahman D. Abdul Hadi

April 2009

Chairman: Assoc. Prof. Dr. Bashir Ahmad Fateh Mohamed, PhD

Faculty: Veterinary Medicine

A cross-sectional study was conducted in Peninsular Malaysia with the objectives of determining the serological prevalence, molecular evidence and risk factors of equine influenza virus (EIV) distribution among different geographical regions. A total of 435 serum samples and 172 nasopharyngeal swabs were collected during July 2007- July 2008. Our study showed that the prevalence of antibodies against EIV was recorded in 215 of the 435 sera (49.4%). The prevalence of circulating antibodies against equine influenza virus in relation to states were recorded as; Selangor 127 out of 170 sera (74.7%) from vaccinated horses, and 9 out of 12 sera (75%) from unvaccinated horses, in Kelantan 37 out
of 165 sera (22.4%) are unvaccinated horses, in Melaka 11 out of 25 sera (44%) are unvaccinated horses, in Negeri Sembilan 2 out of 23 sera (8.7%) are unvaccinated horses, in Johor 16 out of 17 sera (94.1%) are vaccinated horses, in Kedah 11 out of 14 sera (78.6%) are unvaccinated horses and in Pahang 2 out of 9 sera (22.2%) are vaccinated horses. The viral nucleic acid was detected in 77 of the 172 nasopharyngeal swabs (44.7%). The prevalence of positive nasopharyngeal swabs among vaccination status, were also recorded as, 44 out of 102 (43.1%) from vaccinated horses and 33 out of 70 (47.1%) nasopharyngeal swabs from non-vaccinated horses.

The association between several putative risk factors from vaccinated and unvaccinated groups on the seroconversion of equine influenza virus using binary logistic regression was recorded as; Age factor showed to be not significant factor in vaccinated groups against possible EIV infection as compared to unvaccinated groups which is recorded as 5.5 times chances to seroconversion. Thoroughbred groups showed a significant risk above unity whereas the pony groups breed showed a significant low risks. All others group of horses had non-significant, low risks. Sex did not contribute significantly to the epidemiology of the infection since there were no significant risk differences between sexes.
This moderate antibody level detected from horses might indicate exposure of these animals to the virus or evidence of recent infection. The horses that were detected positive for equine influenza might be shedding the virus among naïve population, and likely represent an important role in the epidemiology of respiratory disease outbreaks. In conclusion, the data presented in this study revealed that the EIV circulates among vaccinated and non-vaccinated horses in Malaysia and the incidence rate of EIV is relatively high. The periodic movement of sub-clinically infected horses at the international level provides the potential for interaction with susceptible populations and may serve as a crucial factor in transmission of infections among horse population. Absence of mandatory of vaccination program against EIV in Malaysia most probably contributed to the spread of the disease between provinces. Therefore, it is advisable to update equine influenza vaccine regularly.
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah sarjana Veterinar Sains

KAJIAN EPIDEMIOLOGI JANGKITAN VIRUS INFLUENZA EKUIN DI SEMENANJUNG MALAYSIA

Oleh

Abdul Rahman D. Abdul Hadi

April 2009

Pengerusi: Prof. Madya Dr. Bashir Ahmad Fateh Mohamed, PhD

Fakulti: Perubatan Veterinar

Kajian rentasan telah dijalankan bagi menentukan prevalen serologi, bukti molekular dan faktor risiko penyebaran virus influenza ekuin di antara wilayah geografi yang berlainan di Semenanjung Malaysia. Sebanyak 435 sampel serum dan 172 sampel calit naso far nks telah dikutip sepanjang Julai 2007 hingga Julai 2008. Kajian ini telah menunjukkan bahawa prevalen antibodi terhadap EIV telah direkodkan pada 215 sampel daripada 435 sampel keseluruhan (49.4%). Prevalen ataran antibodi melawan virus ekuin influenza mengikut negeri telah direkodkan seperti; di Selangor daripada 170 sera sebanyak 127 sera (74.7%) adalah kuda yang telah divaksinkan, dan 9 daripada 12 sera (75%) adalah daripada kuda yang belum divaksinkan, di Kelantan sebanyak 37 daripada 165
sera (22.4%) adalah kuda yang belum divaksinkan, di Melaka 11 daripada 25 sera (44%) adalah kuda yang belum divaksinkan, di Negeri Sembilan sebanyak 2 daripada 23 sera (8.7%), di Johor sebanyak 16 daripada 17 sera (94.1%) adalah kuda yang belum divaksinkan, di Kedah sebanyak 11 daripada 14 sera (78.6%) adalah kuda yang belum divaksinkan, dan di Pahang 2 daripada 9 sera (22.2%) adalah yang belum divaksinkan. Virus asid nukleik telah dikesan dalam 77 sampel daripada 172 sampel calit nasofarinks (44.7%). Prevalen sampel calit nasofarinks yang positif bagi status pemvaksinan, juga telah direkodkan iaitu sebanyak 44 daripada 102 (43.1%) sampel kuda yang telah disuntik vaksin dan 33 daripada 70 (47.1%) sampel calit nasofarinks daripada kuda yang tidak disuntik vaksin.

Hubung kaitan di antara beberapa faktor risiko anggapan dari kelompok yang telah disuntik vaksin dan tidak disuntik vaksin pada penukaran sero kuda dari virus influenza menggunakan binari logistik regresi mencatat sebagai, usia merupakan faktor yang tidak signifikan dalam kelompok yang disuntik vaksin terhadap berkemungkinan jangkitan EIV jika dibandingkan dengan kelompok yang tidak disuntik vaksin yang mana telah direkodkan sebanyak 5.5 kali lebih baik. Baka kuda Throughbred menunjukkan risiko yang signifikan melebihi kesatuan sedangkan baka kuda padi menunjukkan risiko signifikan yang
rendah. Semua baka kuda yang lain telah menunjukkan faktor tidak signifikan yang rendah. Jantina tidak menyumbang secara signifikan kepada jangkitan epidemiologi kerana tiadanya risiko perbezaan yang signifikan di antara jantina.

Paras antibodi yang sederhana yang telah dikesan pada kuda tersebut telah menunjukkan bahawa haiwan tersebut telah terdedah kepada virus atau jangkitan terkini. Kuda yang telah dikenal pasti positif terhadap influenza ekuin berkemungkinan boleh menyebar virus tersebut di kalangan populasi yang lemah, dan berkemungkinan memainkan peranan yang penting dalam epidemiologi wabak penyakit pernafasan. Kesimpulannya, data yang ditunjukkan dalam kajian ini menyatakan bahawa virus influenza ekuin berlegar di kalangan kuda yang telah disuntik vaksin dan yang tidak disuntik vaksin di Malaysia. Dan kadar insidens EIV secara relatifnya adalah tinggi. Kekurangan pemantauan terhadap program vaksinasi terhadap EIV di Malaysia mungkin turut menyumbang terhadap penyebaran penyakit. Oleh itu, adalah disyorkan vaksin EIV dikemas kini dengan lebih kerap.
ACKNOWLEDGEMENTS

Life is a challenge to most people and success is measured in many ways. I have always believed that you should challenge yourself everyday and strive to achieve success, or at least satisfaction, through attacking each challenge with a balance of knowledge and ability while maintaining sanity. The tools needed to attack each challenge have been gained through the help, advice, and leadership of many people. This belief was imposed upon me, not in words but by actions, first by my parents and secondly by counselors and instructors.

First, I would like to thank Almighty Allah by the number of my heart beats, this dissertation would not have been completed without the support and his spiritual guidance and for blessing me with all those wonderful people that I met.

I would like to express my gratitude to my supervisor Assoc. Prof. Dr. Bashir Ahmad Fateh Mohamed, who has been very helpful in my research. I thank him for his kindness and willingness to help. The opportunities that Assoc. Prof. Dr. Bashir Ahmad and Assoc. Prof. Dr. Abdul Rahman Omar gave me in joining the Biologic Laboratory at Universiti Putra Malaysia allow me to pursue my interest
of influenza research. While conducting the studies, I really appreciated the great comments and suggestions from Assoc. Prof. Dr. Abdul Rahman Omar who always provided support and guidance. I would like to express my gratitude to Dr. Goh Yong Meng for his guidance in statistical analysis. There were a lot of support and encouragement from the faculty friends in the Universiti Putra Malaysia, especially, Dr. Majed Ahmad. Also, I would like to acknowledge my colleagues in the Biologics Laboratory. Special thanks go to the staff of Biologics Lab, and everybody who has helped me in this study, especially, Dr. Tan Sheau Wei, for her assistance, and the great comments on my work, Dr. Mustapha Abubakar, for his scientific editing, and guidance and I will always remember the great time we had together and for his company late at night and on the weekends and my thanks also goes to my dear friends, Siti, Drs. Tan, Kartini, Lim Jin Nee, Lim, Hid, Mohamad Ghrici, Ayalew, and Zizu, for their invaluable friendship and love.

Finally, my deep thanks go to my family and close friends, who were now in a very bad and terrible situation in Mosul-Iraq for your encouragement and emotional support. To my parents and my lovely sisters, thank you for your endless love and support, this dissertation is dedicated to them.
I certify that an Examination Committee has met on 2 April 2009 to conduct the final examination of Abdul Rahman D Abdul Hadi on his thesis entitled “Epidemiological Investigation of Equine Influenza Virus Infection 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 Master of Veterinary Science.

Members of examination committee are as follows:

**Hassan Hj. Mohd Daud, PhD**
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Chairman)

**Siti Suri Arshad, PhD**
Associate Professor,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Internal Examiner)

**Jalila Abu, PhD**
Lecturer
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Internal Examiner)

**Hussni Omar Mohammed, PhD**
Professor,
College of Veterinary Medicine
Cornell University
United States
(External Examiner)

\[signature\]

**BUJANG B. KIM HUAT, PhD**
Professor and Deputy Dean
School of Graduate Studies
Universiti Putra Malaysia

Date: 21 May 2009
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:

**Bashir Ahmad Fateh Mohamed, PhD**
Associate Professor,
Department of Veterinary Clinical Studies,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Chairman)

**Abdul Rahman Omar, PhD**
Associate Professor,
Department of Veterinary Pathology and Microbiology,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Member)

**Rasedee @ Mat Bin Abdullah, PhD**
Professor,
Department of Veterinary Pathology and Microbiology,
Faculty of Veterinary Medicine,
Universiti Putra Malaysia,
(Member)

**HASANAH Mohd. GHAZALI, PhD**
Professor and Dean,
School of Graduate Studies,
Universiti Putra Malaysia

Date: 8 June 2009

xii
DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

__________________________
Abdul Rahman D. Abdul Hadi

Date: 20 – 4 - 2009
TABLE OF CONTENTS

DEDICATION ii
ABSTRACT iii
ABSTRAK vi
ACKNOWLEDGEMENTS ix
APPROVAL xi
DECLARATION xiii
LIST OF TABLES xviii
LIST OF FIGURES xx
LIST OF ABBREVIATIONS xxii

CHAPTERS

1 INTRODUCTION 1
  1.1 GENERAL BACKGROUND 1

2 LITERATURE REVIEW 7
  2.1 EQUINE INFLUENZA 7
  2.2 General Structure of Equine Influenza Virus 9
  2.3 General Properties and Replication Cycle of Influenza Virus 12
    2.3.1 Overview of Influenza Replication 12
    2.3.2 Adsorption of the Virus 12
    2.3.3 Entry of the virus 13
    2.3.4 Un-coating of the virus 13
    2.3.5 Synthesis of viral RNA and viral proteins 14
  2.4 Epidemiology of Equine Influenza 15
  2.5 Influenza Zoonosis and Interspecies Transmission 19
  2.6 Pathogenesis of Equine Influenza 21

xiv
2.7 Clinical Signs of equine Influenza 23
2.8 Immunity of Equine Influenza 25
2.9 Vaccination against Equine Influenza Virus Infection 27
   2.9.1 Vaccination Strategies 27
2.10 Vaccination Program 31
   2.10.1 Primary Vaccination of Adult Horses 31
   2.10.2 Vaccinations of Foals 32
   2.10.3 Vaccinations of Pregnant Mares 33
   2.10.4 Routine Re-Vaccination 33
   2.10.5 Vaccination In The Face of an Outbreak 34
2.11 Diagnosis and Control of Equine Influenza Virus Infection 35
2.12 Prevention and Control of Equine Influenza Virus Infections 41

3 MATERIALS AND METHODS 43
3.1 SAMPLING TECHNIQUES 43
   3.1.1 Study Backgrounds 43
   3.1.2 Study Design 44
   3.1.3 Questionnaires 45
   3.1.4 Estimation of Required Sample Size 45
   3.1.5 Sampling Areas 46
   3.1.6 Serum Samples Collections 50
   3.1.7 Collections of Nasopharyngeal Swabs 50
3.2.1 Indirect IgG-ELISA Test procedure 51
   3.2.2 Processing of Nasopharyngeal Swabs 53
   3.2.3 Extraction Viral RNA 53
   3.2.4 Determination of RNA Concentration and Purity 54
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Number of serum samples and nasopharyngeal (N) swabs from vaccinated and unvaccinated groups</td>
<td>47</td>
</tr>
<tr>
<td>2</td>
<td>Number of serum samples from different breed in relation to their vaccination status</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>Distribution of samples from different states in relation to the horse breeds</td>
<td>48</td>
</tr>
<tr>
<td>4</td>
<td>Distribution of nasopharyngeal swabs from different states in relation to horse breeds</td>
<td>48</td>
</tr>
<tr>
<td>5</td>
<td>Distribution of samples from different states in relation to sexes</td>
<td>49</td>
</tr>
<tr>
<td>6</td>
<td>Seroconversion of EIV among states and breeds as detected by ELISA</td>
<td>60</td>
</tr>
<tr>
<td>7</td>
<td>Seroconversion of EIV among sexes and ages as detected by ELISA</td>
<td>61</td>
</tr>
<tr>
<td>8</td>
<td>Seroconversion of EIV from different states amongst breed as detected by ELISA test</td>
<td>62</td>
</tr>
<tr>
<td>9</td>
<td>Molecular detections of EIV nucleic acid among states and breeds as detected by RT-PCR</td>
<td>65</td>
</tr>
<tr>
<td>10</td>
<td>Molecular detections of EIV nucleic acid among ages and sex as detected by RT-PCR</td>
<td>66</td>
</tr>
<tr>
<td>11</td>
<td>Molecular detections of EIV antigen amongst different breed from different states as detected by RT-PCR</td>
<td>67</td>
</tr>
<tr>
<td>12</td>
<td>Molecular detections of EIV antigen from sexes amongst different states as detected by RT-PCR</td>
<td>68</td>
</tr>
</tbody>
</table>
Association between several candidates’ risk factors from vaccinated groups on the prevalence of EIV using binary logistic regression

Association between several candidates’ risk factors from unvaccinated groups on the prevalence of equine influenza virus using binary logistic regression
## LISTS OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Amplification products of equine influenza virus with 244 bp M-gene detected from unvaccinated horses in Kelantan</td>
<td>69</td>
</tr>
<tr>
<td>2</td>
<td>Amplification products of equine influenza virus with 244 bp M-gene detected from vaccinated horses in Selangor</td>
<td>70</td>
</tr>
<tr>
<td>3</td>
<td>Amplification products of equine influenza virus with 244 bp M-gene detected from vaccinated horses in Selangor</td>
<td>71</td>
</tr>
<tr>
<td>4</td>
<td>Amplification products of equine influenza virus with 244 bp M-gene detected from unvaccinated horses in Kelantan</td>
<td>72</td>
</tr>
</tbody>
</table>
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µM</td>
<td>micromolar</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CI</td>
<td>confidence intervals</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>EI</td>
<td>equine influenza</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>EIV</td>
<td>equine influenza virus</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>HA</td>
<td>haemagglutinin activity</td>
</tr>
<tr>
<td>HN</td>
<td>haemagglutinin neuraminidase</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IM</td>
<td>intramuscular</td>
</tr>
<tr>
<td>IN</td>
<td>intranasal</td>
</tr>
<tr>
<td>M</td>
<td>matrix protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MEM</td>
<td>minimal essential medium</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby Canine kidney</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>N</td>
<td>nasopharyngeal</td>
</tr>
<tr>
<td>NA</td>
<td>neuraminidase</td>
</tr>
<tr>
<td>NP</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>NS</td>
<td>nonstructural</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>OR</td>
<td>Odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>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>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SRH</td>
<td>single radial hemolysis</td>
</tr>
<tr>
<td>Taq</td>
<td><em>Thermus aquaticus</em></td>
</tr>
<tr>
<td>TB</td>
<td>Thoroughbred</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>---------------------------------------</td>
</tr>
<tr>
<td>UK</td>
<td>United Kingdom</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
<tr>
<td>VTM</td>
<td>virus transport medium</td>
</tr>
<tr>
<td>w/v</td>
<td>weight/volume</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 GENERAL BACKGROUND

Influenza is Italian for "influence" (Crosby, 1993), Latin: influential. It used to be thought that the disease was caused by a bad influence from the heavens. In the Middle Ages influenza was also called "Knock-me-down-fever". Ancient descriptions have suggested the possibility of influenza as a cause of respiratory disease, which was the case where Hippocrates described an outbreak of flu-like illness in 412 BC (Sovinova et al., 1958).

Equine influenza virus is a species–type A influenza virus from the Orthomyxoviridae family, and is comprise of eight segments of RNA. These RNA segments are coated by nucleoprotein (NP), which along with a complex of polymerase enzymes, is responsible for transcription and replication of the virus within the nucleus of the host cell. The segments are surrounded by matrix protein (MP) and the entire structure is enclosed within a lipid bilayer called the virion envelope, two major surface glycoprotein's, hemagglutinin and