UNIVERSITI PUTRA MALAYSIA

POLYMERASE CHAIN REACTION BASED-ASSAYS FOR RAPID DETECTION AND SUBTYPING OF TYPE A INFLUENZA VIRUSES

BROOMAND CHAHARAEIN

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By

BROOMAND CHAHARAEIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

To my parents, wife, children, and siblings.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Doctor of Philosophy

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Chairman: **Professor Aini Ideris, PhD**

Faculty: **Veterinary Medicine**

Highly pathogenic avian influenza (HPAI) virus causes high morbidity, mortality and still is a big threat in poultry industry. The recently raised awareness of the threat of a new influenza pandemic has stimulated interests in the development of a rapid detection method for influenza A viruses. In this study, four diagnostic methods for the detection of type A influenza viruses were explored. A conventional one-tube nucleoprotein reverse transcriptase polymerase chain reaction (NP RT-PCR) was developed for rapid detection of avian influenza A viruses. This method successfully detected 14 different haemagglutinin (HA) subtypes of different origins.

A multiplex RT-PCR that successfully amplified three RNA templates of H5, H7 and H9 in one tube was also developed. The designed primers were specific in amplification of the HA genes of H5, H7 and H9 of type A influenza viruses. No amplification was observed with other avian infectious viruses such as Newcastle disease virus, infectious bronchitis virus and infectious

bursal disease virus. An enzyme-linked immunosorbent assay (ELISA) detection method was then developed to detect the amplified PCR products. This method was 10 times more sensitive than the detection of PCR product using agarose gel electrophoresis. This method (RT-PCR-ELISA) was as sensitive as virus isolation in specific-pathogen-free (SPF) embryonated eggs. The detection limit of the RT-PCR-ELISA was compared with agarose gel electrophoresis and one-step SYBR Green I real time PCR. The RT-PCR-ELISA was 10 times less sensitive than SYBR Green I real time PCR.

The whole process for the detection of type A influenza virus and the avian H5, H7 and H9 subtypes, from extraction of RNA to analysis of PCR product by agarose gel electrophoresis or colorimetric assay can be completed within 6 h. It provides a rapid means of identification of the type and subtypes of influenza viruses and would be very useful for their surveillance. The advantage of using an ELISA reader is in removing any element of subjective interpretation as a source of error. The methods developed in this study, were tested on suspected cases. The finding indicated that the methods are rapid, sensitive and specific, and thus would be a method of choice for the surveillance of avian influenza virus. Moreover, the RT-PCR-ELISA method allows handling of a large number of samples and can be used in many diagnostic laboratories. Among the four methods developed, the SYBR Green I real time PCR was the best method in terms of sensitivity and specificity. This is followed by RT-PCR-ELISA, multiplex and conventional RT-PCR assays.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

KAEDAH PENGESANAN PANTAS DAN PENGELASAN VIRUS INFLUENZA TIP A BERDASARKAN TINDAK BALAS RANTAI POLIMERASE

Oleh

BROOMAND CHAHARAEIN

February 2006

Pengerusi: **Profesor Aini Ideris, PhD**

Fakulti: **Perubatan Veterinar**

Virus influenza avian (AIV) yang berpatogenik tinggi menyebabkan kadar morbiditi serta kadar kematian yang tinggi dan masih merupakan ancaman besar terhadap industri poltri. Kewujudan kesedaran terhadap merebaknya ancaman penyebaran virus influenza baru pada masa kebelakangan ini telah menimbulkan minat terhadap pembangunan kaedah pengesanan pantas virus influenza A. Dalam kajian ini, empat kaedah diagnosis telah dibangunkan dengan jayanya. Kaedah satu tiub tindak balas rantai polimerase transkripsi berbalik nukleoprotein (NP RT-PCR) konvensional telah dibangunkan untuk pengesanan pantas virus influenza A avian. Kaedah ini berjaya mengesan 14 subtip hemaglutinin HA berlainan dari pelbagai sumber asal.

Kaedah RT-PCR "multipleks" juga dibangunkan dan berjaya mengamplifikasi 3 templat asid ribonuklei (RNA) subtip H5, H7 dan H9 dalam satu tiub. Primer-primer yang direka adalah spesifik dalam amplifikasi gen HA untuk

H5, H7 dan H9 virus influenza jenis A. Amplifikasi tidak didapati bagi virus berjangkit lain seperti virus penyakit Newcastle, virus bronkitis berjankit dan virus penyakit bursa berjankit. Kemudian, suatu kaedah pengesanan menggunakan kaedah ELISA telah dibangunkan untuk mengesan produk PCR yang telah diamplifikasi. Kaedah ini didapati 10 kali ganda lebih sensitif berbanding pengesanan produk PCR dengan menggunakan kaedah elektroforesis gel agaros. Kaedah ini (RT-PCR-ELISA), adalah sama sensitif dengan kaedah pemencilan virus dalam telur berembrio bebas patogen spesifik (SPF). Kaedah PCR masa-nyata SYBR Green 1 satu langkah juga telah dibangunkan dan dibandingkan dengan elektroforesis gel serta PCR-ELISA. PCR masa-nyata SYBR Green 1 adalah 10 kali ganda lebih sensitif berbanding RT-PCR-ELISA.

Keseluruhan proses pengesanan virus influenza jenis A dan subtip H5, H7 serta H9, bermula daripada pengekstrakan RNA sehingga menganalisis produk PCR secara elektroforesis gel atau kaedah kolorimetri boleh disempurnakan dalam masa 6 jam. Ini memberikan cara yang lebih pantas untuk pengenalpastian tip dan subtip virus influenza dan sangat berguna untuk pemantauan virus influenza. Kelebihan penggunaan pembaca ELISA adalah dalam penyingkiran sebarang elemen penafsiran subjektif yang merupakan sumber kesilapan.

Empat kaedah yang dibangunkan dalam kajian ini telah diuji keatas kes yang disyaki. Penemuan menunjukkan kaedah ini adalah pantas, sensitif, dan spesifik, oleh itu merupakan kaedah pilihan mengesan virus influenza avian.

Malahan, kaedah-kaedah tersebut boleh mengendalikan kuantiti sampel yang banyak serta boleh digunakan dalam banyak makmal-makmal diagnosis. Diantara empat kaedah yang dibangunkan, kaedah PCR masa-nyata SYBR Green 1 adalah yang terbaik dari segi sensitiviti dan spesifisiti. Ini diikuti dengan kaedah RT-PCR-ELISA, multiplex dan konvensional RT-PCR.

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LIST OF ABBREVIATIONS

AF	Allantoic fluid
AC	Antigen capture
AGDP	Agar gel diffusion precipitation
AIV	Avian Influenza Virus
bp	Base pair
C	Cytosine
Ca	Calcium
CAM	Chorioallantoic membrane
cDNA	Complementary deoxyribonucleic acid
CEF	Chicken embryo fibroblast
CEK	Chicken embryo kidney
cm	Centimetres
CPE	Cytopathic effect
°C	Degree Celsius
d-	Deoxy
DAS-ELISA	Double antibody sandwich-ELISA
dd	Dideoxy
DNA	Deoxyribonucleic acid
ds	Double stranded
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra acetic acid
ELD_{50}	Embryo lethal dose fifty percent
ELISA	Enzyme-linked immunosorbent assay

Fig	Figure
FMDV	Foot and mouth disease virus
HA	Haemagglutinin
HAU	Haemagglutination Unit
HCl	Hydrochloric acid
HI	Haemagglutination Inhibition
HIV	Human immunodeficiency virus
HPAI	Highly pathogenic avian influenza
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
kb	kilobase
kDa	kilodalton
kV	kilovolt
LPAI	Low pathogenic avian influenza
M	Molar
Mab	Monoclonal antibody
Mg	Magnesium
ml	Millilitre
M1	Matrix one
MVP	Malaysian Vaccine and Pharmaceutical Sdn Bhd
mM	Millimolar
µm	Micrometre
µg	Microgram
NA	Neuraminidase
NaCl	Sodium chloride
NCR	Non coding region

ng	Nanograme
nm	Nanometre
NO2	Nitrogen dioxide
NP	Nucleoprotein
NS1	Non Structural Protein 1
NS2	Non Structural Protein 2
ORF	Open reading frame
PA	Polymerase A
PB1	Polymerase B1
PB2	Polymerase B2
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
p.i	Post-inoculation
RFLP	Restriction fragment length polymorphism
RT- PCR	Reverse-transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RdRp	RNA dependent –RNA polymerase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SN	Serum neutralisation test
SPF	Specific-pathogen-free
SPSS	Statistical package for social science
ss	Single stranded
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA

TE	Tris-EDTA
TEM	Transmission electron microscopy
Tm	Melting temperature
Tris	2-amino-2-(hydroxymethyl)-1, 3 propandiol
U	Uracyl
UPM	Universiti Putra Malaysia
VRI	Veterinary Research Institute
vRNPs	Viral ribonucleoprotein particles
vv	Very virulent
w/v	Weight/volume