UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF A SOLID – BASED PAPER STRIP ASSAY FOR RAPID DIAGNOSIS OF PSEUDORABIES

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By

TAM YEW JOON

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

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This thesis is especially dedicated to my beloved "Ah Ma", family and friends.……

May all of your hopes, remembrance and memories live on forever.……

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

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May 2004

Chairman: Professor Abdul Rani Bahaman, Ph.D.

Faculty: Veterinary Medicine

Pseudorabies (Aujeszky's disease) is an economically significant disease of swine known to cause central nervous disorders, respiratory disease, reproductive failure and mortality in infected pigs. In attempts to eradicate the disease from becoming endemic, early detection is important to prevent further economic losses and to allow for detection and removal of infected pigs in domestic herds. Thus, a rapid and sensitive technique is necessary for the detection of the virus. For rapid and simple examination, an immuno – chromatographic lateral – flow assay system based on immunologic recognition of specific pseudorabies virus antigen was developed by utilising, as signal generator, colloidal gold conjugated to secondary antibody to detect primary or sample antibody in the sera of pseudorabies infected animals. The pseudorabies virus used as a capture antigen in the test strip was first cultivated in VERO cell culture and then purified by sucrose gradient separation to produce the viral protein concentration of 3.8 mg/ml. A sample of the antigen stock was then subjected to SDS PAGE protein analysis. Minor differences were noted between

the sample proteins and reported protein profile of pseudorabies virus. The standard pseudorabies antigens reacted well with the hyperimmune serum (HIS).

The antibody detection system is basically composed of colloidal gold – labelled antibodies fixed on a conjugate pad, and the complementary pseudorabies antigen immobilised onto a nitrocellulose membrane forming capture zone. If the target antibody is present in a specimen, the colloidal gold-labelled antibody will form a complex with the antibody sample. Subsequently, the formed complex will migrate to the capture zone and is then bound to the solid phase via antigen – antibody interaction. As a result, a signal marker is generated by the accumulation of colloidal gold for detection confirmation.

The results obtained demonstrated that the optimum combination of pseudorabies antigen needed as the capture reagent and gold conjugate as secondary antibody recognition marker was at a concentration of 0.38mg/ml and at 1:10 dilution factor respectively. The sensitivity of the solid – based test strip towards pseudorabies antibodies was high with a detection limit of 1 to 10,000 – dilution factor. The specificity of the assay was 100% with no cross – reaction being observed with other sera or antibodies. Accurate reading time needed for confirmation of the assay can be completed in 5 min with a whole blood sample of 25 µl. The colloidal gold – labelled antibody is stable at room temperature for 6 months or more.

Findings from this study indicated that the solid – based test strip assay system provided high sensitivity and specificity for the detection of pseudorabies at low levels of antibody concentration. The assay was rapid, simple, cheap, and does not require any sophisticated equipment. Thus, the solid based test strip will be a useful serological screening technique or for rapid diagnosis of an infectious disease in target populations of animals characterised by heterogeneous antibody responses.

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

PEMBINAAN ESEI JALUR KERTAS BERASAS PEPEJAL DIAGNOSIS YANG PANTAS UNTUK PENGESANAN PSEUDORABIES

Oleh

TAM YEW JOON

Mei 2004

Pengerusi: **Profesor Abdul Rani Bahaman, Ph.D.**

Fakulti: **Perubatan Veterinar**

Pseudorabies (penyakit Aujeszky's) adalah penyakit yang amat penting dari segi ekonomi di dalam industri babi. Sebagaimana yang dilapori, penyakit ini mengakibatkan masalah saraf pusat, penyakit respirasi, kegagalan reproduksi dan kematian di kalangan haiwan yang dijangkiti. Dalam cubaan menghalang penyakit ini daripada menjadi endemik, pengesanan awal mesti diperolehi untuk mengenalpasti dan mengasingkan haiwan berjangkit daripada kelompok peliharaan. Dengan itu, satu teknik diagnosis yang pantas, ringkas dan peka adalah amat diperlukan untuk pengesanan virus ini.

Bagi mendapatkan pengesanan yang pantas dan mudah, satu teknik immunokromatograf sistem esei aliran lateral berpandukan pengecaman immunologi secara spesifik kepada pseudorabies dikaji dengan menggunakan zarah emas sebagai pembekal isyarat, yang berkonjugat kepada anti tikus untuk mengesan antibodi yang terdapat pada serum haiwan yang telah dijangkiti virus pseudorabies.

Antigen yang digunakan sebagai bahan penangkapan, terdahulu dibiak dan ditulenkan dengan pisahan kecerunan sukrose untuk pengeluaran protin pseudorabies yang berkepekatan 3.8 mg/ml. Daripada stok protin ini, sampel diambil dan diuji dengan kaedah SDS PAGE dan kandungan profilnya dibandingkan untuk pengesahan virus. Berpandukan kepada keputusan yang didapati, tiada perbezaan besar dijumpai diantara sampel yang diuji berbanding profil yang telah dilaporkan. Antigen pseudorabies menunjukkan tindakbalas imunogen yang baik kepada serum hiperimun (HIS) yang diperolehi.

Peranti ini pada asasnya terdiri daripada zarah emas yang berkonjugat kepada antibodi dipasang pada lapisan konjugat, dan antigen pseudorabies digerakan pada membran nitroselulosa untuk membentuk zon penangkapan. Jika antibodi yang dihendaki berada di dalam sampel, zarah emas berkonjugat akan membentuk kompleks dengannya dan akan berpindah kepada bahagian zon penangkapan. Di sini, kompleks emas berkonjugat dan antibodi daripada sampel akan diikat pada antigen pseudorabies melalui interaksi antigen – antibodi. Sebagai keputusan, satu isyarat akan dapat dilihat hasil daripada pengumpulan zarah – zarah emas tersebut menunjukkan pengesahan pengesanan.

Keputusan yang didapati menunjukkan bahawa kombinasi yang optimum diantara konsentrasi antigen pseudorabies yang diperlukan sebagai bahan penangkapan dan zarah emas berkonjugat sebagai pembekal isyarat adalah pada kepekatan 0.38mg/ml dan 1:10 faktor pencairan. Kepekaan jalur ujian berasas pepejal ini terhadap antibodi pseudorabies adalah tinggi dengan had pengesanan

pada 1:10,000 faktor pencairan. Spesifikasi esei ini adalah 100% dengan ketiadaan perentasan reaksi diantara sera atau antibodi yang lain. Masa yang diperlukan untuk pengesanan yang tepat untuk peranti ini adalah 5 minit dengan sampel darah minima berjumlah 25 µl. Zarah emas berkonjugat yang digunakan dapat berada di dalam keadaan stabil pada suhu bilik pada sekurang – kurang 6 bulan atau lebih.

Kesimpulannya, keputusan yang didapati menunjukkan sistem jalur ujian berasa pepejal esei ini mempunyai kepekaan yang tinggi dan spesifikasi yang baik untuk pengesanan penyakit Pseudorabies. Peranti esei ini adalah pantas, senang dan murah untuk digunakan, dan juga tidak memerlukan sebarang perkakas atau alat tambahan. Oleh itu, sistem jalur ujian berasas pepejal adalah lebih berguna untuk mendiagnosis serologi penyaringan jangkitan penyakit dalam populasi haiwan sasaran berciri daripada respon antibodi heterogenus.

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I certify that an Examination Committee met on 12th May 2004 to conduct the final examination of Tam Yew Joon on his Master of Science thesis entitled "Development of a Solid – Based Paper Strip Assay for Rapid Diagnosis of Pseudorabies" in accordance with Universiti Pertanian (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are 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 or concurrently submitted for any other degree at UPM or other institutions.

TAM YEW JOON

Date:

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LIST OF ABBREVIATIONS

ABTS	Azino – bis (3 – Ethylbenzthiazoline sulphonic acid)
Ag	antigen
Ab	antibody
AP	alkaline phophatase
ATV	antibiotic trypsin versene
BCIP	Bromochloroindolyl Phosphate. Disodium salt
BSA	bovine serum albumin
CMC	carboxyl methyl cellulose
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
cm	centre meter
°C	degree Celsius
DAB	3, 3’ Diaminobenzidine
DNA	deoxyribonucleic acid
ddH_2O	distilled and deionised water
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme linked immunosorbent assay
EIA	enzyme immunoassay
FBS	foetal bovine serum
g	gram
HCl	hydrochloric acid
HIS	hyper immune serum

h	hour
HA	haemaagglutination
HRP	horseradish peroxidase
H_2O_2	hydrogen peroxide
kbp	kilo base pair
KCl	potassium chloride
kD	kilo Dalton
KH_2PO_4	di – potassium hydrogen phosphate anhydrous
L15M	Leibovitz – 15 media
L	litre
lb/sq	pounds per square cubic
M	molar
mM	millimolar
mg	milligram
ml	millilitre
M_r	molecular weight
µl	microlitre
min	minute
NaCl	natrium chloride
Na_2HPO_4	di – natrium hydrogen phosphate
NBT	Nitro Blue Tetra sodium
NC	nitrocellulose
nm	nanometer
OD	optical density

PRV	pseudorabies virus
PBS	phosphate – buffered saline
PBS –T	phosphate – buffered saline Tween – 20
PCR	polymerase chain reaction
p.f.u	plaque forming assay
pH	hydrogen ion exponent
%	percentage
rpm	revolutions per minute
SDS PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
sec	second
TNE	Tris NaCl EDTA
U	unit
UPM	Universiti Putra Malaysia
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER 1

INTRODUCTION

Pseudorabies (Aujeszky's disease) is a highly contagious, widespread and economically significant disease of swine characterised by a range of clinical signs, including central nervous system disorders, respiratory diseases, reproductive failure and death, depending on age, reproductive status and immune status of the infected swine (Kluge *et al*., 1992). Following a primary replication, pseudorabies virus can establish latent infection in swine. Under certain stress factors, latent virus may reactivate, which results in reshedding and transmission of the virus to susceptible animals (Ros Bascunana *et al.*, 1997). In geographic areas where pseudorabies virus (PRV) infection is enzootic, control and eradication programs often employed the use of vaccines and diagnostic tools as aids in the attempt to control the economic losses.

However, attempts for total eradication of the virus are still unsuccessful as carrier animals spread the infective virus without showing any signs of the disease. Van Nes *et al.* (2001) reported that transmission of pseudorabies was still detectable among groups of conventional pigs even though being vaccinated (Van Nes *et al.*, 2001). In Malaysia, despite vaccination, outbreak of the disease has been reported frequently in different parts of the country (Too, 1995). Due to the expanding population, the demand and consumption of swine products are expected to increase substantially. From these views, the disease has emerged to

be one of the most important problems affecting the swine industries and serious steps must be taken to prevent severe losses.

Rapid and accurate diagnosis of pseudorabies would definitely pave for a higher effectiveness in eradication of the disease. The classical, early direct diagnosis method for pseudorabies detection would have been the use of serum neutralizing test (SNT). This test was able to perform pseudorabies antibody serum titration and was generally used as comparison with newly developed methods. However, it becomes redundant due to complication in sample processing and time consuming. During the past decades, another widely used approach was the enzyme – linked immunosorbent assay (ELISA) which replaced SNT in pseudorabies detection. It offers high specificity, sensitivity and ease of operation over other standard laboratory procedures. Not to mention its ability to process large amount of samples at one time. Current diagnosis of pseudorabies detection utilises ELISA with gene deleted markers which have additional advantage of differentiating between vaccinated and infected swine (Kinker *et al.*, 1997).

Nevertheless, some of the disadvantages of the ELISA format which necessitates further improvement of the methodology include the lengthy time required for antigen – antibody reaction, reagent additions, enzymatic conversion of substrate and several washing steps between various operations. Many of the available immuno diagnostic tools were also not easy to apply in the field, since these techniques require special equipment and reagents, and performing any of

the tests even in the laboratory takes time, sometimes needing overnight incubation steps.

As an alternative to the use of current diagnostic tools, the immuno – chromatography test strip assay has become a new approach for detecting many veterinary diseases. With the advent in immuno – chromatography based techniques, numerous reports describing the advantages and functions in diagnosing diseases were published attesting to the perceived importance of this new diagnostic tool (Eliades *et al*., 1998; Kim and Choi, 2000). Thus, the employment of immuno – chromatographic test strip will provide an easy mean for detection of pseudorabies virus. The use of labelling substances like gold and the immobilisation of biological components (antigen / antibody) makes it possible to facilitate a convenient and relatively inexpensive approach to obtain rapid analytical results due to the elimination of washing steps and faster antigen – antibody interaction.

The development of an on – site test strip that uses visual identification provide many advantages such as safety, rapidity, simplicity, easy handling, economic and high sensitivity. With the development of immuno – chromatography principles proceeding towards the test strips, the ability to perform diagnostic tests at a location remote from the laboratory would be highly desirable for speed and economy.