UNIVERSITI PUTRA MALAYSIA

DEVELOPMENT OF POLYMERASE CHAIN REACTION (PCR) TECHNIQUE FOR DETECTION OF *MYCOPLASMA HYOPNEUMONIAE* AND PSEUDORABIES VIRUS IN PORCINE CLINICAL SAMPLES

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TAN SHIN – YI

A project paper submitted to the

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In partial fulfilment of the requirement for the

DEGREE OF DOCTOR OF VETERINARY MEDICINE

Universiti Putra Malaysia,

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Development of Polymerase Chain Reaction (PCR) Technique for Detection of *Mycoplasma hyopneumoniae* And Pseudorabies Virus in Porcine Clinical Samples", by Tan Shin-Yi and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999 – Final Year Project

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DEDICATIONS

This project paper is dedicated to God, whom I was created and guided by Him

To my dearest family,

My late grandparents

Father

Mother

Sisters

& Kwang Yan

And to all my teachers and lecturers whom contributed to who am I now and who I will be in future.

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CONTENTS

	Page
TITLE	i
CERTIFICATION	ii
DEDICATION	iii
ACKNOWLEDGEMENTS	iv
CONTENTS	vi
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	x
ABSTRAK	xi
ABSTRACT	xiii
1.0 INTRODUCTION	1
1.1 *Mycoplasma hyopneumoniae*	1
1.2 Pseudorabies Virus	2
2.0 LITERATURE REVIEW	4
2.1 *Mycoplasma hyopneumoniae*	4
2.1.1 Overview of Mycoplasmas	4
2.1.2 Epidemiology	4
2.1.3 Diagnostic methods	6
2.1.4 Pathology	8

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2.2 Pseudorabies Virus 10

2.2.1 Overview of Pseudorabies Virus 10

2.2.2 Epidemiology 10

2.2.3 Diagnostic methods 11

2.2.4 Pathology 13

3.0 MATERIALS AND METHODS 15

3.1 Samples Collection 15

3.2 DNA Extraction 15

3.3 Preparation of Positive control 16

3.4 Measurement of DNA Concentration 17

3.5 Primer Selection 17

3.6 Polymerase Chain Reaction 20

3.7 Agarose Gel Electrophoresis and Photography 22

4.0 RESULTS AND DISCUSSION 24

4.1 Amplification of 16S rRNA, H1 and p36 genes of Mhyo by PCR Assay 24

4.2 Amplification of gD, gB and gC genes of PRV by PCR Assay 26

5.0 CONCLUSION 31

6.0 RECOMMENDATIONS 32

REFERENCES 33

APPENDICES 37

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LIST OF TABLES

		Page
TABLE 3.1:	Primers set for detection of Mhyo by conventional PCR assay………………………………………………………...	19
TABLE 3.2:	Primers set for detection of PRV by conventional PCR assay………………………………………………………...	19
TABLE 3.3:	Optimized cycling conditions of conventional PCR assay for detection of Mhyo………………………………………	21
TABLE 3.4:	Optimized cycling conditions of conventional PCR assay for detection of PRV………………………………………..	21

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LIST OF FIGURES

		Page
FIGURE 4.1:	PCR assay using specific primers targeting the conserved 16S rRNA gene of *Mycoplasma hyopneumoniae* to produce 649 bp PCR products……………………………....	24
FIGURE 4.2:	PCR assay using specific primers targeting the conserved oligonucleotide H1 gene of *Mycoplasma hyopneumoniae* to produce 853 bp PCR products…………………………..	25
FIGURE 4.3:	PCR assay using specific primers targeting the conserved immunodominant protein p36 gene of *Mycoplasma hyopneumoniae* to produce 948 bp PCR products…………	25
FIGURE 4.4:	PCR assay using specific primers targeting the conserved gD gene of Pseudorabies virus to produce 455 bp PCR products…………………………………………………….....	26
FIGURE 4.5:	PCR assay using specific primers targeting the conserved gB gene of Pseudorabies virus to produce 334 bp PCR products…………………………………………….................	27
FIGURE 4.6:	PCR assay using specific primers targeting the gC gene of Pseudorabies virus to produce 788 bp PCR products……...	27

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LIST OF ABBREAVIATIONS

%	Percent
µl	Microliter
µm	Micrometer
µM	Micromolar
ºC	Degree Celsius
AD	Aujeszky's Disease
bp	Base pairs
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
g	Gram
kb	Kilobase
km	Kilometer
mA	Milliampere
MgCl2	Magnesium chloride
Mhyo	*Mycoplasma hyopneumoniae*
min	Minutes
ml	Milliliter
PCR	Polymerase Chain Reaction
PRV	Pseudorabies Virus
SEP	Swine Enzootic Pneumonia
PRDC	Porcine Respiratory Disease Complex
PRRSV	Porcine Reproductive and Respiratory Syndrome Virus
PCV2	Porcine Circovirus 2
RNA	Ribonucleic acid
rpm	Revolutions per minute

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999 – Projek Ilmiah Tahun Akhir.

KEMAJUAN TEKNIK RANTAIAN REAKSI POLIMERASE DALAM PENGESANAN *MYCOPLASMA HYOPNEUMONIAE* DANPSEUDORABIES VIRUS DARIPADA SAMPEL KLINIKALPORSIN

Oleh

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2016

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Penyelia bersama: Dr. Nor Yasmin Abd. Rahaman

Dengan kemajuan terkini dalam biologi molekul, pengesanan molekul telah menjadi antara satu teknik diagnosis alternatif. Oleh sebab *Aujeszky's Disease* dan *Enzootic Pneumonia* menyumbang kepada kerugian ekonomi yang besar dalam industri ternakan porsin, kajian ini bertujuan untuk menentukan set primer yang sesuai untuk pengesanan *Pseudorabies virus* (PRV) dan *Mycoplasma hyopneumoniae* (Mhyo) daripada sampel klinikal menggunakan PCR. 15 ekor porsin berumur lebih kurang 3 bulan yang mempunyai masalah sistem penafasan telah dikorbankan untuk memperolehi sampel tisu. Vaksin komersial dan sampel klinikal positif telah

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digunakan sebagai kawalan positif. Setiap sampel tisu peparu dan tonsil telah diuji dengan kaedah PCR konvensional menggunakan 3 set primer yang berlainan yang direka untuk menyasar kawasan dipulihara DNA genomik untuk Mhyo dan PRV bermasing-masing. Berdasarkan keputusan PCR untuk sampel tisu peparu, antara 3 daripada 15 ekor porsin memperolehi keputusan positif untuk Mhyo dengan menggunakan ketiga-tiga primer tersebut manakala kesemua 15 ekor porsin memperolehi keputusan negative untuk PRV dalam sampel tisu tonsil. Untuk mengoptimumkan lagi protokol ini, penambahan sampel saiz dan perbezaan suhu ketara harus dilakukan. Kesimpulannya, semua set primers yang dipilih adalah sesuai untuk pengesanan Mhyo dan PRV menggunakan teknik PCR.

Kata kunci: *porsin, PCR, primer, Pseudorabies virus, Mycoplasma hyopneumoniae*

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ABSTRACT

Abstract of a project paper submitted to the Faculty of Veterinary Medicine, Universiti Putra Malaysia in partial fulfilment of the requirement for the course VPD 4999 – Final Year Project.

DEVELOPMENT OF POLYMERASE CHAIN REACTION TECHNIQUE FOR DETECTION OF *MYCOPLASMA HYOPNEUMONIAE* AND PSEUDORABIES VIRUS IN PORCINE CLINICAL SAMPLES

by

Tan Shin - Yi

2016

Supervisor: Dr. Ooi Peck Toung

Co-supervisor: Dr. Nor Yasmin Abd. Rahaman

With recent advancement in molecular biology, molecular detection has become an alternative diagnostic technique. As Aujeszky's disease and Enzootic Pneumonia caused great economic losses in swine livestock industry, this study aimed to determine suitable primers sets for Pseudorabies virus (PRV) and *Mycoplasma hyopneumoniae* (Mhyo) detection in clinical samples using PCR. 15 pigs aged approximately 3 months old showing clinical signs of respiratory distress were sampled. Commercial vaccine and positive clinical samples were used as positive control. Each lung and tonsil tissue samples were subjected to conventional PCR assay

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using 3 different sets of primers designed to target conserved regions of genomic DNA for Mhyo and PRV respectively. Based on PCR assay for lung tissue samples, 3 out of 15 pigs were positive for Mhyo with all 3 primers sets whereas for tonsil tissue samples, all 15 pigs were negative for PRV. To further optimize the current protocol, more sample size and gradient PCR assay should be performed. In conclusion, all the primers sets chosen were suitable to be used for Mhyo and PRV detection using PCR.

Keywords: *porcine, PCR, primer, Pseudorabies virus, Mycoplasma hyopneumoniae*

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1.0 INTRODUCTION

1.1 *Mycoplasma hyopneumoniae*

Mycoplasma hyopneumoniae is the aetiological agent of swine enzootic pneumonia (SEP) which is one of the important diseases in the swine production industry. Besides, Mhyo also contributes to Porcine Respiratory Disease Complex (PRDC) by interacting with respiratory viral pathogens such as PRRSV, PCV2 and PRV. Consequently, both SEP and PRDC cause significant economic losses in swine production industry by affecting feed conversion ratio (FCR) and average daily weight gain (ADG). It causes decreased growth rate, increased feed conversion ratio, increased treatment costs and increased mortality (Iris, 2010). Affected pigs with SEP will show a gradual onset of a chronic, dry, non-productive cough that is most evident when animals are aroused, particularly in pigs at the finishing stage of the production cycle (Thacker and Minion, 2012).

Although bacteriological culture is considered the gold standard among diagnostic technique to isolate Mhyo from clinical samples, it requires a special medium known as the Friis medium (Sibila *et al.*, 2009) and the isolation is very laborious, time-consuming as it needs at least 15 days for colony to grow and the culture can easily overgrown by other Mycoplasmas such as *M. hyorhinis* and *M. flocculare*. Serological tests such as ELISA can be performed especially for large amount of samples from pig herds but interpretation of serological results is difficult

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and challenging (Erlandson *et al.*, 2005). Besides, in comparison with PCR, serological tests have lower sensitivity may be due to the organism normally colonizes the airways, resulting in minimal interaction with the systemic immune system and causing variable serological results.

With the recent advancement in molecular biology, PCR is an alternative of diagnostic method. It is more rapid, relatively inexpensive compared to bacteriological culture and is highly sensitive and specific. Hence, this study was undertaken to fulfil the following objectives:

i. To determine suitable primers set for detection of *Mycoplasma hyopneumoniae*.
ii. To detect *Mycoplasma hyopneumoniae* from clinically affected animals using the selected primers with PCR technique.

1.2 Pseudorabies Virus

Pseudorabies virus, also known as as Suid Herpesvirus type 1 (SuHV-1), belongs to the genus *Varicellovirus* in the subfamily *Alphaherpesviriae* of the family *Herpesviridae* (Kluge *et al.*, 1999). Pigs are the only natural host for PRV although the virus is capable of infecting a wide range of hosts. It is the aetiological agent of Aujeszky's disease that causes severe economical and production losses to the swine industry worldwide by fatal infection of piglets and abortions in pregnant sow. Fatal infections are predominant in younger pigs with the exhibition of neurological signs such as ataxia, convulsions, and sudden death whereas respiratory distress and

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subclinical infection is primarily present in adult pigs. In pregnant sow, PRV results in infection of fetuses which lead to resorption, mummification, or abortion (Mettenleiter *et al.*, 2012). PRV is able to establish latent infection after primary replication and reactivation of the virus may take place following stressful events (Pomeranz *et al.*, 2005) which poses a threat to the health status of pigs in the herds.

Virus isolation (VA) is the commonly performed diagnostic technique to detect PRV from clinical samples followed by confirmation with immunofluorescence, immunoperoxidase or neutralization tests (OIE, 2004). However, with the recent advancement in molecular biology, PCR is an alternative to VA which can be used to detect PRV genomes with designed primers that is able to target conserved regions of PRV strains such as gB, gC, gD and gE. In comparison with VA, PCR can yield result in a shorter amount of time, is highly specific and sensitive and has the ability to detect dormant virus in latency (Ayala *et al.*, 2012). As a result, a better control and prevention of AD can be achieved.

Although there are several conventional PCRs targeting genes encoding gB, gC, gD and gE have been established (Yoon *et al.*, 2005; Perez and Arce, 2009; Huang *et al.*, 2004), but there is still no internationally agreed standard. Hence, this study was undertaken to fulfil the following objectives:

i. To determine suitable primers set for detection of Pseudorabies Virus.
ii. To detect Pseudorabies Virus from clinically affected animals using the selected primers with PCR technique.

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2.0 LITERATURE REVIEW

2.1 *Mycoplasma hyopneumoniae*

2.1.1 Overview of Mycoplasmas

Mycoplasmas belong to the class "Mollicutes" (mollis, soft; cutis, skin, in Latin) as taxonomically they lack of cell walls and are very small in size. It infects a wide variety of plants and animals including human (Thacker and Minion, 2012). The cells are predominantly spherical and the size are 0.3 – 0.8 µm in diameter. They are the smallest self-replicating organisms known (Maniloff, McElhaney, Finch and Baseman, 1992; Razin, 2006). Mycoplasmas in pigs can be divided into pathogenic which includes *Mycoplasma hyopneumoniae, M. hyorhinis, M. hyosynoviae* and *M. suis*; and nonpathogenic which includes *M. flocculare, M. sualvi* and *M. hyopharyngis.*

2.1.2 Epidemiology

Mycoplasma hyopneumoniae is the aetiological agent of swine enzootic pneumonia (SEP) which is one of the important diseases in the swine production industry. This agent has the ability to suppress both the innate and acquired immunity at the lung thereby allowing proliferation of upper respiratory commensal bacteria such as *Pasteurella multocida, Streptococcus suis, Haemophilus parasuis,* and/or A*ctinobacillus pleuropneumoniae* in the lungs which result in chronic bronchopneumonia (Thacker and Minion, 2012). Not only that, Mhyo also plays a part in Porcine Respiratory Disease Complex (PRDC) together with other viral pathogens

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such as PRRSV and PCV2. EP and PRDC contribute to significant economic losses in swine production industry due to decreased growth rate, increased feed conversion ratio, increased treatment costs and increased mortality (Iris, 2010).

Transmission of Mhyo under field conditions may occur in two ways which are direct and indirect transmission. Direct transmission includes vertical and/or horizontal transmission in which sow transmits the disease to piglets and/or infected pigs to susceptible pigs. Indirect transmission is via airborne transmission and/or mechanical vector such as fomites.

Vertical transmission occurs via nose-to-nose contact between infected sow and her suckling piglets (Rautiainen and Wallgren, 2001; Thacker *et al.*, 2006). Several studies have shown that there is an increased risk of infection with a lower parity in the sows (Fano *et al.*, 2006; Sibila *et al.*, 2007; grosse Beilage *et al.*, 2009). However, there is still more room of improvement on the research of relationship between sow parity and disease development in their piglets.

Horizontal transmission may occur by direct contact with penmates via respiratory secretions. Mhyo infects individual for long periods whereby it has been isolated from the respiratory tract of individual pigs for up to 214 days (Pieters *et al.*, 2009). However, the spread is generally slow and the transmission is inefficient as indicated by the experimental transmission study done by Meyns *et al.* (2004) that only one penmate is infected during the nursery period. However, once the infection is established, it will spread to other age groups and maintained within the farm (Iris,

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2010).Infected pigs may also remain clinically symptomless as a carrier which posed a threat to other susceptible pigs.

Indirect transmission by airborne is becoming more important recently as stated by (Desrosiers, 2004). According to an experiment by Dee *et al.* (2009), the furthest distance that Mhyo can be transmitted in long distances via airborne route from one source to another is 4.7km.Although there is a possibility of mechanical transmission of Mhyo via personnel or fomites, it is considered of limited importance but further study is required for further understanding.

There are several risk factors for Mhyo infection in pig farms such as larger herd size, multi-source purchase policy, continuous flow production systems, high stocking density and poorer biosecurity measures which contribute to increased risks of Mhyo infection in pig herds (Iris, 2010).

2.1.3 Diagnostic methods

Currently there are various methods to diagnose SEP. The main clinical sign of SEP is gradual onset of a chronic, dry, non-productive cough that is most evident when animals are aroused, particularly in pigs at the finishing stage of the production cycle (Thacker and Minion, 2012). Abattoir surveillance can be performed at the level of abattoir to score the lung lesion as an estimation of SEP incidence. However, the downside of this diagnosis is the lesion is not pathognomonic of Mhyo infection. Next, isolation of Mhyo from affected lungs by bacteriological culture is considered as the gold standard among diagnostic techniques (Thacker, 2006) but it requires a special

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medium known as the Friis medium (Sibila, 2009). It is very sensitive especially at the later stages of SEP but the culture is very laborious, time-consuming as it needs at least 15 days for colony growth and the culture can easily overgrown by other Mycoplasmas such as *M. hyorhinis* and *M. flocculare* (Thacker and Minion, 2012). Serological tests such as ELISA and complement fixation test are commonly used also to detect antibody titres against Mhyo in the pig herds. It is relatively rapid, inexpensive and is easily performed to yield useful information on the presence of maternally-derived and acquired antibodies, as well as on the time required for animals to seroconvert (Sibila, 2007). With the recent advancement in molecular biology, molecular detection is an alternative which can be achieved by several options comprised of DNA or RNA hybridization and Polymerase Chain Reaction (PCR). According to a study done by Cai *et al.* (2007), PCR has a diagnostic sensitivity of 97.3% and specificity of 93.0%. Besides, PCR method is more rapid and relatively inexpensive compared to bacteriological culture. There are several types of porcine clinical samples being reported to be used for PCR including tracheal washings nasal swabs, lung fragments and bronchoalveolar lavage fluids (Cai *et al.*, 2007; Yamaguti *et al.*, 2008).

2.1.4 Pathology

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Mhyo is able to colonize the respiratory tract epithelium, stimulate a prolonged inflammatory reaction, suppress the host's immune responses as well as interact with other infectious agents which lead to pathology in the host (Thacker and Minion, 2012). Mhyo is host specific as it establishes infection only in pigs, as supported by Razin (2006) that Mycoplasmas usually exhibit a rather strict host and tissue specificity, probably reflecting their nutritionally exacting nature and obligate parasitic mode of life. Mhyo colonizes the airway by binding to the cilia of epithelial cells in pigs facilitated by protein P97 and some other glycoproteins (Minion *et al.*, 2004). This colonization then leads to negative impact on the function of epithelial cells to "filter" the invading pathogens and debris, which consequently provide opportunity to the upper respiratory commensal bacteria such as *P. multocida, S. suis, H. parasuis, A. pleuropneumoniae* and others to establish secondary infections. As a result, bronchopneumonia is developed from the primary infection by Mhyo together with co-infection by secondary pathogens, in which the disease is known as enzootic pneumonia (Thacker and Minion, 2012). However, not all infections caused by Mhyo result in clinical pneumonia as it depends on various factors such as the organism load, virulence of infecting strain(s) and presence of secondary bacterial and/or bacterial infections. Clinical disease usually begins at the age of 2 to 6 months old in pigs and spread slowly in the herd (Thacker and Minion, 2012). Other than enzootic pneumonia, Mhyo also can cause PRDC by interacting with respiratory viral pathogens such as PRRSV, PCV2 and PRV.

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The common gross lesions seen in chronic cases of enzootic pneumonia are purple to gray rubbery consolidation of the cranial ventral portions of the lungs in a lobular pattern. Exudate is present and can be expressed from the airway which is consist of catarrhal exudate in uncomplicated cases, and mucopurulent exudate in complicated cases by secondary pyogenic bacterial infections (Thacker and Minion, 2012).

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2.2 Pseudorabies Virus

2.2.1 Overview of Pseudorabies Virus

The aetiology of Aujeszky's disease is pseudorabies virus (PRV), also known as as Suid Herpesvirus type 1 (SuHV-1), belongs to the genus *Varicellovirus* in the subfamily *Alphaherpesvirinae* of the family *Herpesviridae* (Kluge *et al.*, 1999). Pigs are the only natural host for PRV although the virus is capable of infecting a wide range of hosts, basically all mammals except higher primates, including cattle, sheep, cats, dogs and rats. This is because pigs are able to survive a productive infection which accounts for their ability to remain latently infected following clinical recovery, except in piglets under 2 weeks old, which die from encephalitis (Mettenleiter *et al.*, 2012; OIE, 2012).The genome of the virus is a double-stranded linear DNA molecule of 143 kb in length and it is estimated to contain 72 genes that encode 70 different proteins (Klupp *et al.*, 2004).PRV contributes in the economic losses and production losses in the swine industry (Pomeranz *et al.*, 2005) by causing predominantly neurological disorders in piglets, respiratory infections in adult pigs and reproductive disorder in sows (Mettenleiter *et al.*, 2012).

2.2.2 Epidemiology

AD occurs worldwide including most of the Southeast Asian countries except some countries have successfully eradicated the disease such as the United States of America, Canada, New Zealand and many Member States of the European Union (OIE, 2012).

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Direct transmission occurs by direct contact between pigs via mucosae of the nasal, oral cavities, conjunctiva, and also via vaginal mucosa or semen in breeding pigs. Vertical transmission can occur as well transplacentally especially in the last third of gestation and from sow to their piglets via colustrum.

Indirect transmission is another route of transmission by contact with fomites such as contaminated bedding and water and carcasses of rats, swine and other infected animals. Airborne transmission is possible as well in short-distances (Mettenleiter *et al.*, 2012).

Besides, there is an increasing in interest about the role of feral swine in transmission of AD to domestic pigs recently. Extensive serological studies have demonstrated that they are reservoirs for PRV and there are increased in numbers of infected pigs with the spread of feral swine in the southern United States (Beran, 2002). However, the findings are debatable (Ruiz-Fons *et al.*, 2008; Pannwitz *et al.*, 2012).

2.2.3 Diagnostic methods

The presence of clinical signs and severity depends on the age and immunological status of the pig (Nauwynck, 1997). Neurological signs are more predominant in juvenile animals whereas in adult swine, there is predominantly respiratory involvement such as coughing, sneezing, dypsnea with high morbidity. Sudden death with few clinical signs is usually observed in neonatal pigs of less than 7 days old. In piglet of 2 to 3 weeks old, there are severe signs of central nervous system involvement comprised of trembling, ataxia, convulsion, tremor, paralysis

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with mortality rate up to100%. Older pigs may show neurological signs but decreased in incidence as age-dependent resistance is developed. In gilt n sow, depending on the phase of gestation, embroyonic death, resorption of fetuses, mummified fetuses, abortion, stillbirth, respiratory signs and fever may be seen (Mettenleiter *et al.*, 2012).

Suspicion of AD must be followed by laboratory confirmation as the infection produces no pathognomonic clinical signs or gross postmortem lesions (Mettenleiter *et al.*, 2012). Diagnosis of AD can be achieved by identification of the agent including virus isolation and PCR. As suggested by OIE (2012) and Mettenleiter *et al.* (2012), samples should be collected from the oro-pharyngeal fluid, nasal swabs or tonsil swabs from living pigs, or brain, tonsil, lung and spleen from dead pigs. Generally, porcine kidney cell line (PK-15) is generally employed as the cell line to recover PRV in virus isolation. In a positive sample, cytopathic effect (CPE) usually will appear within 24 to 72 hours which is development of a monolayer with accumulations of birefringent cells, followed by complete detachment of the cell sheet as well as syncytial development (OIE, 2012).

PCR has the advantages of highly specific and sensitive, can be performed quickly and has the ability to detect dormant virus when viral antigens are not expressed (Ayala *et al.*, 2012). Primers to be used should target regions of the genome conserved among PRV strains (Mettenleiter *et al.*, 2012). There are several conventional PCRs targeting genes encoding gB, gC, gD and gE have been established

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(Yoon *et al.*, 2005; Perez and Arce, 2009; Huang *et al.*, 2004), but there is still no internationally agreed standard.

Another diagnostic technique is serological tests. Serological tests that are commonly used now is enzyme-linked immunosorbent assay (ELISA) although virus neutralization test (VNT) was once the reference diagnostic method as ELISA is able to process large amount of samples from farms. Serum is the preferred matrix to perform the test (OIE, 2012). Latex agglutination test can also be performed as it is able to differentiate natural infections from vaccination with gE deleted vaccines (Yong *et al.*, 2005).

2.2.4 Pathology

Following oronasal cavity infection in the natural host which is swine, primary replication takes place in the epithelial cells of the upper respiratory tract. After that, the virus then enter the sensory nerve endings that innervate the site of infection which usually around the facial and oropharyngeal area. It then spreads centripetally by fast axonal retrograde transport and reaches the cell bodies of infected neurons (Pomeranz *et al.*, 2005; Mettenleiter, 2012). This result in a latent infection which is a long-term infection where the PRV becomes dormant and the virions cannot be recovered. Latency usually occurs at the trigeminal ganglia in domestic swine. Stressful events in future may reactivate the virus and cause shedding of virus such as poor animal husbandry, farrowing, concurrent disease conditions and transportation (Pomeranz *et al.*, 2005).

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The most common gross lesions take place in the upper respiratory tract, including rhinitis with patchy epithelial necrosis and necrotizing laryngotracheitis. Lower respiratory tract may exhibit inconsistent lesions which are consolidation and reddening that scattered throughout the lungs, especially focused on the cranioventral lung lobes (Mettenleiter, 2012).

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3.0 MATERIALS AND METHODS

3.1 Samples Collection

A total of 15 pigs were sampled from 4 local pig farms located in Perak and Selangor using the convenience sampling method. All the pigs selected had clinical signs of respiratory distress problem. They were humanely euthanized and necropsy was performed on-site. Lung and tonsil tissue samples were collected. During necropsy, abnormal lesions were recorded.

3.2 DNA Extraction

The DNA was extracted from each lung and tonsil tissue sample using a commercial DNA extraction kit (DNeasy® Blood & Tissue Kit 250, Qiagen®, Germany) with methods described by the manufacturer. In summary, 1 g of each sample was minced using a pair of scissors and then pounded in a mortar using pestle with addition of some sterile sands and phosphate buffer saline (PBS) to help in homogenizing the tissue sample. The homogenized sample was then transferred into a 15ml centrifuge tube (Vivantis®, USA) containing 10 ml of phosphate buffer saline (PBS). The suspension was centrifuged at 10,000 rpm for 10 min (Universal 32 R, Hettich Zentrifugen, Germany). Next, 1 ml of supernatant from each sample was pipette into individual 1.5 ml microcentrifuge tubes (Eppendorf, Germany).For DNA extraction, 200 µl of supernatant was pipetted into a 1.5 ml microcentrifuge tube and centrifuged at 300 x g for 5 min. 20µl of Proteinase K (Qiagen®, Germany) and 200 µl of Buffer AL was added into the tube and mixed thoroughly by vortexing

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(Finevortex, Korea). The mixture was then incubated at 56ºC for 10 min. Next, 200 µl of 96% ethanol (Essen-Haus, Malaysia) was added into the lysate and mixed thoroughly by vortexing. The mixture was then pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube (Qiagen®, Germany) and centrifuged at 6000 x g for 1 min (Mikro 22 R, Hettich Zentrifugen, Germany). The collection tube and flow-through was discarded and the spin column was placed in a new 2 ml collection tube. 500 µl of Buffer AW1 was added into the spin column and centrifuged at 6000 x g for 1 min. Again, the collection tube and flow-through was discarded. The spin column was placed in a new 2 ml collection tube followed by addition of 500 µl of Buffer AW2. It was centrifuged at 20,000 x g for 3 min and the flow-through and collection tube was discarded. The spin column was transferred to a new 1.5ml microcentrifuge tube (Eppendorf, Germany). 200 µl of Buffer AE was added to the centre of the spin column membrane for DNA elution. This was followed by incubation at room temperature (25ºC) for 1 min and centrifuging at 6000 x g for 1 min. The collected DNA elution was pipetted back into the spin column to be centrifuged again to increase DNA yield.

3.3 Preparation of Positive control

Commercial vaccines (Ingelvac® Aujeszky MLV and Ingelvac® M. hyo, Gernany) were used in attempt to obtain positive control for PRV and Mhyo respectively, following DNA extraction and procedures as described for clinical samples. However, the attempt was unsuccessful for Mhyo, hence; clinical samples

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that were positive for Mhyo after PCR assay were used as positive control for Mhyo instead.

3.4 Measurement of DNA Concentration

The final DNA concentration was obtained by performing spectrophotometry. After the extraction process, 2 µl of the extracted DNA was diluted in 98 µl of deionized water in a cuvette and placed in a BioPhotometer Plus photometer (Eppendorf, Germany) followed by reading of the concentration.

3.5 Primer Selection

Primers selection for PCR assay is important as a pair of primers play an important role in the success of DNA amplification of a particular target region. Primers are short sequences of complementary DNA which bind to certain nucleotide sequences along the DNA strand. It is necessary before a DNA polymerase can add a nucleotide onto the -3' end.

To select 3 primers sets for this study, several published journals were reviewed. First set of primers for Mhyo detection is suggested by Cai *et al.*, (2007) from Animal Health Laboratory, Laboratory Services Division, University of Guelph, Canada targeting 16SrRNA genes with a 649-bp product using lung tissue sample of pig. Second primer set was suggested by Baumeister *et al.*, (1998) from Institutfür Mikobiologie und Tierseuchen, Tierärztliche Hochschule Hannover, Denmark to detect oligonucleotides H1, an 853-bp fragment specific for the Mhyo genome in

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bronchoalveolar lavage fluid (BALF) of pigs. Third set is suggested by Caron *et al.*, (2000) from from Institut Armand-Frappier, Centre de Microbiologieet Biotechnologie, Canada, designed to target a 948-bp genes encoding species-specific immunodominant proteins p36.

On the other hand, 3 sets of primers were selected for PRV amplification using PCR assay based on reviews of several published journals as well. The first primer set suggested by Perez and Arce (2009) from Grupo de Virología, Centro Nacional de Sanidad Agropecuaria. San José de las Lajas, La Habana, Cuba were designed to target on highly conserved nucleotide region of the viral gD glycoprotein. The second set of primer suggested by Ayala *et al.* (2012) from Instituto Nacional de Investigaciones Forestales, Mexico targeting a 334 bp fragment of the gB glyocoprotein gene. Lastly, the third set of primers was suggested by Fonseca *et al.* (2010) from Brazil targeting the viral gC glycoprotein with a 788 bp. The primer set and the journal sources are stated in Table 3.1 and Table 3.2.

Table 3.1: Primers set for detection of Mhyo by conventional PCR assay

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Reference	Primer	Nucleotide Sequence 5' → 3'	Expected product (b.p.)
1. Cai *et al.* (2007), Canada	Forward	5'- GAGCCTTCAAGCTTCACCAAGA-3'	649
	Reverse	5'- TGTGTTAGTGACTTTTGCCACC-3'	
2. Baumeister *et al.* (1998), Denmark	Forward	5'- TAGAAATGACTGGCAGACAA -3'	853
	Reverse	5'- GAGGCTTGATTTTGGAGTC -3'	
3. Caron *et al.* (2000), Canada	Forward	5'- GGGCCGATGAAACCTATTAAAATAGCT - 3'	948
	Reverse	5'- GCCGCGAAATTAAATATTTTTAATTGCA TCCTG-3'	

Table 3.2: Primers set for detection of PRV by conventional PCR assay

Reference	Primer	Nucleotide Sequence 5' → 3'	Expected product (b.p.)
1. Perez & Arce (2009), Cuba	Forward	5'- GGTGGACCGGCTGCTGAACGA -3'	455
	Reverse	5'- GCTGCTGGTAGAACGGCGTCA-3'	
2. Ayala *et al.* (2012), Mexico	Forward	5'- ATGGCCATCTCGCGGTGC – 3'	334
	Reverse	5'- ACTCGCGGTCCTCCAGCA – 3'	
3. Fonseca Jr. *et al.* (2010), Brazil	Forward	5'- GTTTCCTGATTCACGCCCACGC – 3'	788
	Reverse	5'- GAAGGGCTCACCGAAGAGGAC – 3'	

3.6 Polymerase Chain Reaction

PCR reaction was carried out using HotStarTaq® Plus Master Mix Kit, (Qiagen®, Germany). The prepared master mix, HotStarTaq® Plus Master Mix contains HotStarTaq Plus DNA Polymerase, PCR Buffer with 3 mM MgCl and 400 µM each dNTP and RNase-Free Water. The PCR reaction was amplified in a 20 µl reaction containing 10 µl of HotStarTaq® Plus Master Mix, 1 µl of forward primer, 1 µl of reverse primer, 3-5 µl of DNA template depending on the DNA concentration (<200 ng/reaction) and RNase-Free water. The amplification was performed using a Mastercycler Gradient thermal cycler (Eppendorf, Germany). The cycling conditions were carried out as recommended by the manufacturer as described in Table 3.3 and Table 3.4.

Table 3.3: Optimized cycling conditions of conventional PCR assay for detection of Mhyo

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Reference	Initial heat activation	Denaturation	Annealing	Extension	Number of Cycles	Final Extension
1. Cai *et al.* (2007)	95C for 5min	94ºC for 20s	55ºC for 30s	72ºC for 40s	35 cycles	72C for 10min
2. Baumeister *et al.* (1998)	95C for 5min	93ºC for 30s	55ºC for 30s	72ºC for 90s	50 cycles	72C for 10min
3. Caron *et al.* (2000)	95C for 5min	94ºC for 1min	50ºC for 1min	72ºC for 90s	35 cycles	72C for 10min

Table 3.4: Optimized cycling conditions of conventional PCR assay for detection of PRV

Reference	Initial heat activation	Denaturation	Annealing	Extension	Number of Cycles	Final Extension
1. Perez &arce (2009)	95ºC for 5min	94ºC for 15s	68ºC for 1 min	68ºC for 1 min	35 cycles	72ºC for 10min
2. Ayala *et al.* (2012)	95ºC for 5min	95ºC for 1min	62ºC for 1 min	72ºC for 1min	35 cycles	72ºC for 10min

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3. Fonseca Jr. *et al.* (2010)	95ºC for 5min	8 cycles at 95ºC for 50s; 67ºC for 50s; 7º2C for 50s; 27 cycles at 95ºC for 50s; 64ºC for 50s; 72ºC for 50s	72ºC for 10min

3.7 Agarose Gel Electrophoresis and Photography

A commercially prepared agarose (Vivantis, #PC0701 – 500 g) was used to prepare 1.5 % (w/v) agarose gel for electrophoresis. A mixture of 40 ml of electrophoresis buffer (TAE buffer) was mixed with 0.6 g of agarose powder and boiled using a hot plate (Cimarec™ Stirring Hot Plates, USA) until the solution is completely clear and no small floating particles are visible. 4µlof RedSafe™ Nucleic Acid Staining Solution (20,000x) was added to agarose solution and the flask was swirled gently to mix the solution. The agarose solution was then cooled to about 60ºC and poured into a gel tray until the comb teeth are immersed about ¼ to ½ into the agarose. After the gel solidified, it was removed from the gel tray and placed into an electrophoresis tank (Bio-Rad, USA). TAE buffer was added into the tank until the buffer immerses the surface of the gel by approximately 5 mm. Next, 5 µl of BenchTop 100bp DNA Ladder (Promega, USA) DNA marker was added into the first well as comparison for the PCR products. 5 µl of PCR product from each tube was pipetted out and mixed individually with 1 µl of DNA loading dye (6X Loading Dye Solution (XO), iNtRON-biotechnology) and loaded into the wells. Electrophoresis was carried out using PowerPac™ Basic (Bio-Rad, USA) power supply at 80 V and

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300 mA for approximately 35 min or until the DNA ladder (yellow dye) reached the opposite end of the gel. After electrophoresis was completed, the gel was removed from the tank and placed in a Gel Doc XR+ UV transilluminator (Bio-Rad, USA) for viewing. The DNA bands were photographed using Quantity One Basic software (Bio-Rad, USA).

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4.0 RESULTS AND DISCUSSION

4.1 Amplification of 16S rRNA, H1 and p36 genes of Mhyo by PCR Assay

Based on the results obtained, 3 out of 15 pigs (pig no. 5, 8 and 13) selected for this study were found to be positive for *Mycoplasma hyopneumoniae* with first primer set (Figure 4.1). Positive results were defined as a band expression at the 649 bp region which is partial of 16s rRNA region after electrophoresis on 1.5% agarose gel (Cai *et al.,* 2007). During the early phase of experiment, positive lung tissue sample was served as positive control .Both second and third set of primers also produced successful band at the targeted region of 853 bp and 948 bp respectively (Figure 4.2 and Figure 4.3).

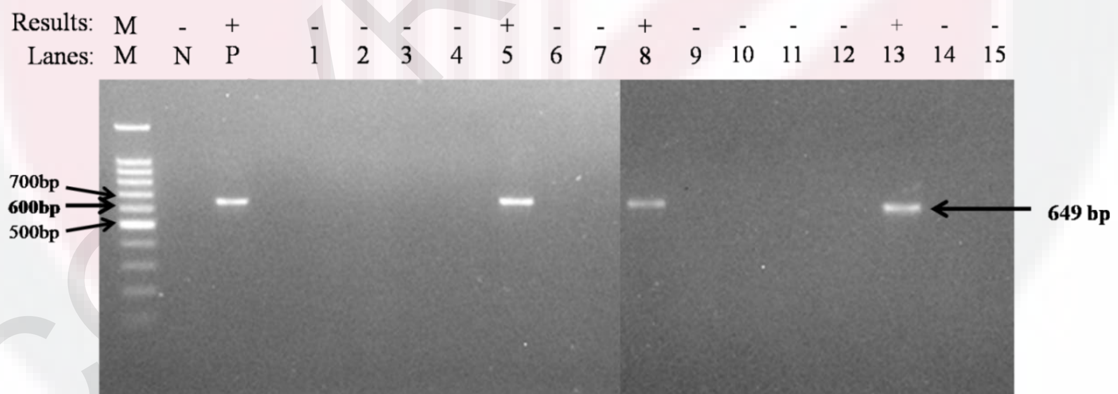

Figure 4.1: PCR assay using specific primers targeting the conserved 16S rRNA gene of *Mycoplasma hyopneumoniae* to produce 649 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. The target bands are observed in lung tissue samples from pig 5, 8 and 13. N (No template control), P (Positive control) and Lane 1 to 15 (lung tissue samples from pig 1 to 15 respectively). M, as DNA marker (100bp DNA Ladder, Promega, USA).

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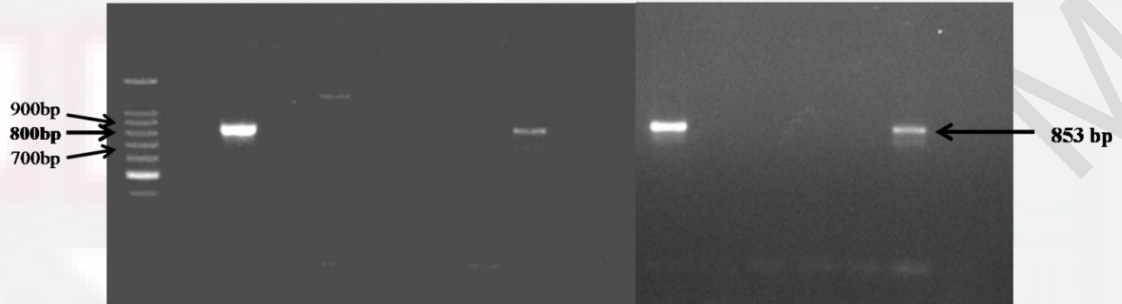

Figure 4.2: PCR assay using specific primers targeting the conserved oligonucleotide H1 gene of *Mycoplasma hyopneumoniae* to produce 853bp PCR products. The text abbreviation same with Figure 4.1

Figure 4.3: PCR assay using specific primers targeting the conserved immunodominant protein p36 gene of *Mycoplasma hyopneumoniae* to produce 948bp PCR products. The text abbreviation same with Figure 4.1

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4.2 Amplification of gD, gB and gC genes of PRV by PCR Assay

Based on conventional PCR results for the first set of primer suggested by Perez and Arce (2009), positive control was formed at the targeted region, which is 455bpregion. However, there was no positive clinical sample detected from all 15 pigs (Figure 4.4). For positive control, DNA extracted from commercial vaccine (Ingelvac® Aujeszky MLV, Boehringer Ingelheim) was used. The result is the same for the second and third set of primer in which bands were only expressed at positive control lane at the targeted region of 334bp and 788bp respectively (Figure 4.5 and Figure 4.6).

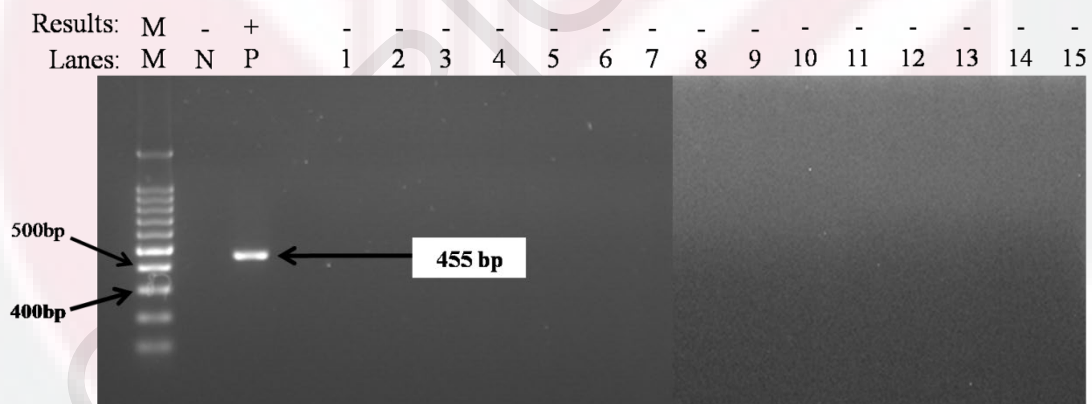

Figure 4.4: PCR assay using specific primers targeting the conserved gD gene of Pseudorabies virus to produce 455 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. N (No template control), P (Positive control) and Lane 1 to 15 (tonsil tissue samples from pig 1 to 15 respectively). Band was observed only on positive control lane. No positive clinical sample was detected. M, as DNA marker (100bp DNA Ladder, Promega, USA).

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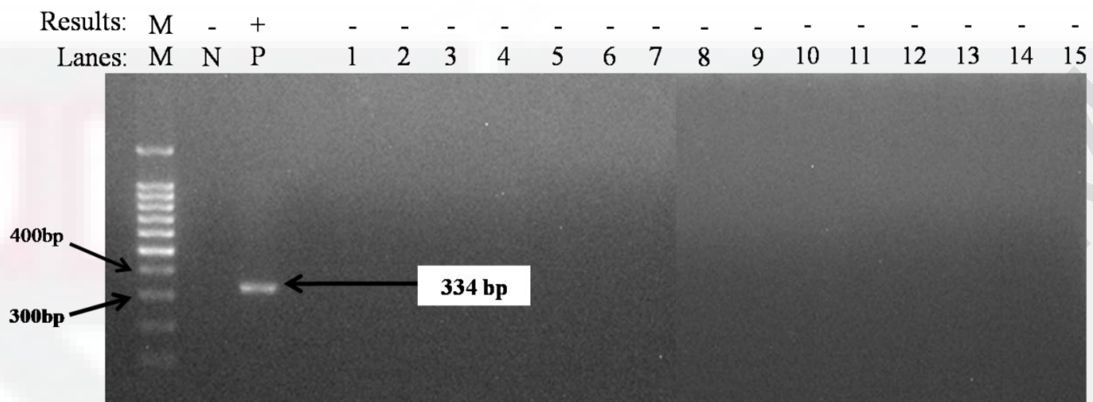

Figure 4.5: PCR assay using specific primers targeting the conserved gB gene of Pseudorabies virus to produce 334bp PCR products. The text abbreviation same with Figure 4.4

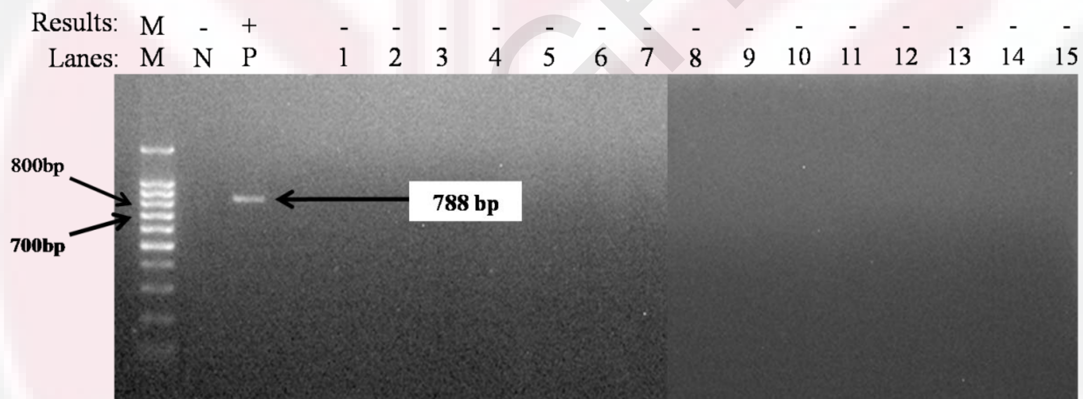

Figure 4.6: PCR assay using specific primers targeting the gC gene of Pseudorabies virus to produce 788bp PCR products. The text abbreviation same with Figure 4.4

In this study, 3 sets of primers were selected for Mhyo and PRV based on reviews from several published journals were selected. According to Chen and Janes (2002), the primers length should be in between 17 to 28 bases for good specificity to obtain a unique target sequence. Most of the primers selected in this study have length between these bases. Hence, the primers are able to target the sequence more specifically. Besides, another criterion is that the primers must be designed to amplify

a sequence conserved among PRV strains, for example parts of the gB or gD genes, which code for essential glycoproteins (OIE, 2002). Primers suggested by Perez and Arce (2009) and Ayala *et al.* (2012) target partial of gD and gB genes, respectively. gD gene encodes for glycoprotein D which is a type I membrane protein critical for virus replication in different organs as it plays important role in viral entry whereas gB gene encodes for glycoprotein B which is also a type I membrane protein that facilitates viral entry via fusion and cell-to-cell spread (Klupp *et al.*, 2004; Perez and Arce, 2009).

Other than that, the primers suggested by Cai *et al.* (2007) which targets 16S rRNA were selected as this gene has been the most widely applied in PCR assay to detect Mhyo. As rRNA is a part of the ribosome, the changes in this gene is relatively slow, hence, there is a less chance of mutation that target mismatches can be excluded (Mattsson, Bergström, Wallgren and Johansson, 1995). As for the second set of primers which designed to target the oligonucleotides H1, an 853 bp fragment that is specific for the Mhyo genome (Baumeister *et al.*, 1999). Caron *et al.* (2000) states that p36 gene is an immunodominant protein which is highly conserved among different strains of Mhyo as it carries antigenic determinants that are species-specific. It is important to select the primers which target a conserved region which is species-specific to prevent amplification of incorrect DNAs which can lead to a false positive result.

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Several studies have shown different methods of sample collection for Mhyo detection using PCR assays, such as nasal swabs, bronchoalveolar lavage fluids, tonsil tissue and tracheobronchial brush samples (Baumeister *et al.*, 1999; Kurth *et al.*, 2002). From this study, lung tissue sample is a suitable sample to be collected for PCR assay which is consistent with the study done by Cai *et al.* (2007). However, there are other studies that showed bronchoalveolar lavage fluid (BALF) or trachea-bronchial swabs are more effective samples to detect Mhyo compared to nasal or tonsillar swabs as related to the pathogenesis of Mhyo in which it attaches to cilia of epithelial cells of airways during colonization (Sibila *et al.*, 2008; Marois *et al.*, 2007). Kurth *et al.* (2002) also showed that tracheobronchial brushes and BALF were more sensitive than lung tissue for sampling for Mhyo detection. However, there is still indefinite conclusion on the use of lung tissue for PCR as Moorkamp *et al.* (2008) suggested that lung samples are more appropriate than BALF in cases of moderate to severe EP. On the other hand, obtaining of lung tissue sample unfortunately has to be from dead animals unlike BALF can be collected from live animals. Lung tissue sample is relatively easy to perform after the pigs were euthanized in comparison with BALF that the collection requires anaesthesia and is technically challenging. On the other hand, tonsils, brain and lungs are the preferred sample for PRV detection (OIE, 2002); hence tonsil is used as the sample in this study for PCR assays. Besides, tonsils and trigeminal ganglia are the ideal sample for virus detection in latently infected animals (Mettenleiter *et al.*, 2012).

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PCR is a diagnostic technique which has higher sensitivity than the traditional method using fluorescent antibody test (Cai *et al.*, 2007). There were several types of PCR assays being studied, single-stage PCR, nested-PCR, real-time PCR and multiplex PCR (Huang *et al.*, 2004; Yoon *et al.*, 2005; Ma *et al.*, 2008).As nested-PCR is more sensitive than single-stage PCR (Kurth *et al.*, 2002), we can recommend use of nested-PCR to detect negative samples as the organisms present in the sample may be too low for single-stage PCR to detect. Huang *et al.* (2004) developed the use of multiplex PCR to detect multiple viral infections of swine including PRV and other important viral pathogens such as porcine circovirus (PCV) and porcine parvovirus (PPV). The ability to detect and differentiate field and vaccine strains of PRV was demonstrated by Ma *et al.* (2008) using real-time PCR with primers designed for gB and gE genes. Therefore, there is still a lot of room of improvements in the development of PCR assay in swine diseases. Nevertheless, each diagnostic technique has their advantages and limitations; hence, it is important to combine various techniques for better diagnostic accuracy (Sibila *et al.*, 2007).

All three sets of primers for each Mhyo and PRV can be considered suitable to be used for PCR assays as positive result(s) was obtained after PCR was run using the different primers. Detection of PRV in clinical samples was unsuccessful could be due to the pigs were not suffering from PRV infections but other diseases such as porcine pleuropneumonia and Glasser's disease. Pigs 5, 8 and 13 were very likely to be suffering from Mhyo infections based on the positive result obtained from the PCR assays.

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5.0 CONCLUSION

In conclusion, all three sets of primers are suitable for detection of *Mycoplasma hyopneumoniae* and Pseudorabies virus with PCR technique. *Mycoplasma hyopneumoniae* is successfully detected from porcine clinical samples but not Pseudorabies virus. With the development of these primers, it is able to support better the field veterinarian on their diagnosis.

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6.0 RECOMMENDATIONS

Firstly, as there was no positive clinical sample detected for PRV using PCR assays, more porcine clinical samples should be obtained for PRV detection. The clinical samples can be obtained from different age group of pigs suspected with Aujeszky's disease infection including piglets that show neurological signs such as trembling, ataxia, convulsion and tremor, and from gilt and sow that have reproductive problems such as abortion, stillbirth and mummified fetuses. Secondly, since there was positive clinical samples detected for Mhyo, these samples can be sent for genotype sequencing to understand the situation of Malaysia as currently there are limited data on our country's isolate genotype. This would be helpful in SEP control and prevention in pig farms. With the knowledge of Malaysia genotype sequence, more efficient vaccines can be used to control this disease. Thirdly, other types of clinical samples other than lung tissues and tonsils can be collected and tested for PCR assays with the primers selected in this study to compare the efficacy of different porcine clinical samples and to determine the optimal sampling site in pigs for Mhyo and PRV detection.

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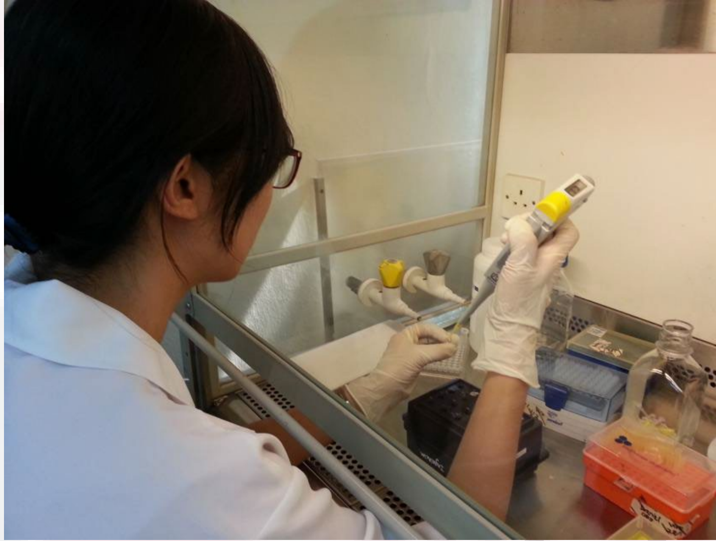
Appendix I

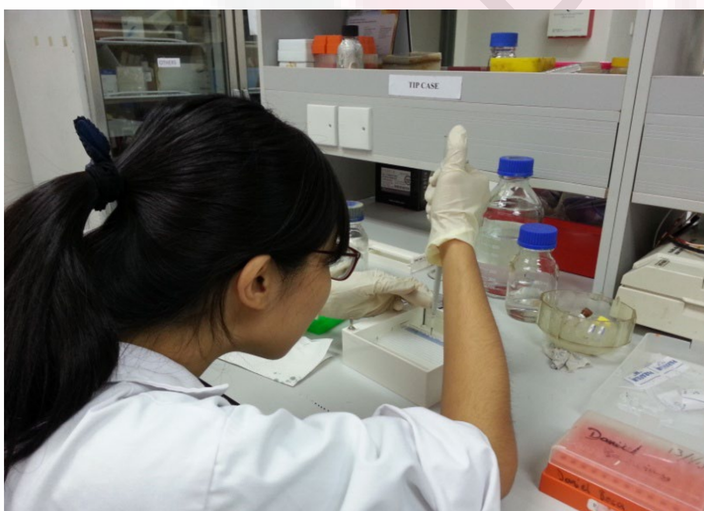
(Top left) One of the selected pigs; (top right) Humane euthanasia of pig; (Bottom left) Postmortem performed on-site; (Middle right) tonsil; (Bottom right) Lungs

Appendix II

DNA extraction performed for the tissue samples



agarose gel to run electrophoresis



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