UNIVERSITI PUTRA MALAYSIA

DETECTION OF PORCINE BOCAVIRUS IN MALAYSIAN SWINE HERDS

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DETECTION OF PORCINE BOCAVIRUS IN MALAYSIAN SWINE HERDS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Detection of Porcine Bocavirus in Malaysian Swine Herds", by Daniel Mohan Jacob and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirement for the course VPD 4999 – Project

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DEDICATIONS

This project paper is dedicated to the One Almighty God, who had created me and made all things possible,

To my family,

Grandmother

Father

Mother

Brother, Sister

Vivian Tan

& my late mother

And to all my teachers who have committed themselves towards the noble cause of education.

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LIST OF ABBREVIATIONS

%	Percent
µl	Microliter
µM	Micromolar
°C	Degree Celsius
ARD	Acute Respiratory Disease
ATPase	Adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BPV	Bovine bocavirus
Csl BoV	California sea lion bocavirus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EtBr	Ethidium bromide
FBoV	Feline bocavirus
g	Gram
GBoV	Gorilla bocavirus
HBoV	Human bocavirus
HK	Hong Kong
ICTV	International Committee on Taxonomy of Viruses
ILN	Inguinal lymph node
KDN	Kidney
mA	Milliampere
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Milliliter

MLN	Mesenteric lymph node
MVC	Minute virus of canines
NCBI	National Centre for Biotechnology Information
ng	Nanogram
no.	Number
NP1	Nuclear phosphoprotein
NS1	Nonstructural protein 1
NTC	No template control
ORF	Open reading frame
PBoV	Porcine bocavirus
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PCV2	Porcine circovirus type-2
Pg	Pig
PMWS	Post Weaning Multisystemic Wasting Syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
PTTV2	Porcine torque teno virus species 2
RTU	Ready-to-use
SPL	Spleen
sPLA	Secretory phospholipase
TAE	Tris-acetate-EDTA
UK	United Kingdom
USA	United States of America
V	Volt
v	Version
VP1	Viral protein 1
VP2	Viral protein 2
x g	Relative centrifugal force

ABSTRAK

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

PENGESANAN PORSIN BOCAVIRUS DALAN KAWANAN PORSIN MALAYSIA

Oleh

Daniel Mohan Jacob

2015

Penyelia: Assoc. Prof. Dr Siti Suri Arshad

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Sejak penemuan porsin bocavirus (PBoV) di Sweden pada tahun 2009, sebanyak 11 negara lain telah melaporkan. Oleh sebab Malaysia mempunyai industri ternakan porsin yang signifikan, projek ini bermatlamat untuk mengesan PBoV dalam kawanan porsin tempatan. Menggunakan teknik persampelan mudah, 11 ekor porsin telah disampel dari tiga buah ladang yang terletak di negeri Perak dan Selangor. Setiap sampel tisu yang diperoleh daripada pembedahan karkas telah diuji dengan kaedah PCR konvensional menggunakan primer spesifik yang direka untuk menyasar gen *nonstructural* (NS) 1. Berdasarkan keputusan PCR, 10 daripada 11 porsin positif untuk PBoV. Untuk

pengesahan lanjut, penjujukan nukleotid NS1 telah dilakukan, dan jujukannya dibandingkan dengan pencilan rujukan. Tambahan pula, analisa filogenetik telah dilakukan untuk mengenal pasti hubungan antara strain Malaysia dengan pencilan rujukan. Analisa bioinformatik menunjukkan bahawa pencilan Malaysia hampir sama dengan PBoV3 dari USA. Untuk lebih memahami PBoV di Malaysia, kajian prevalens, pengasingan virus, dan penjujukan genom lengkap perlu dilaksanakan. Kesimpulannya, negara Malaysia merupakan negara ketiga belas mengesan PBoV dalan kawanan porsinnya melaui kaedah PCR dan penjujukan separa gen NS1.

Kata kunci: Porsin bocavirus, PCR, penjujukan, porsin, analisa filogenetik

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial requirement for the course VPD 4999 - Project

DETECTION OF PORCINE BOCAVIRUS IN MALAYSIAN SWINE HERDS

By

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2015

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Porcine bocavirus (PBoV), which was first discovered in Swedish pigs in 2009, has since been detected in 11 other countries at the time of writing. As Malaysia is host to a significant swine livestock industry, this study aimed to describe PBoV in local swine herds. Using the convenience sampling method, 11 pigs were sampled from three farms located in the states of Perak and Selangor. Each tissue sample obtained from necropsy were subjected to conventional PCR assay using specific primers designed to target the conserved nonstructural protein 1 (NS1) gene. Based on PCR assay, 10 out of 11 pigs were positive for PBoV. For further confirmation, partial nucleotide sequencing of the NS1 gene was performed, and the sequence compared to reference isolates. In addition,

phylogenetic analysis was performed to determine the genetic relationship of Malaysian PBoV strains to reference isolates. Bioinformatics analysis determined that the Malaysian strain was highly similar (95%) to PBoV3 isolated in USA. To further understand PBoV in Malaysia, prevalence study, virus isolation and complete genome sequencing should be performed. In conclusion, Malaysia is the 13th country in the world that has described PBoV in its swine herds by PCR assay and partial sequencing of the NS1 gene.

Key words: Porcine bocavirus, PCR, sequencing, swine, phylogenetic analysis

1.0 INTRODUCTION

1.1 *Bocavirus*

Bocavirus is a genus of the family *Parvoviridae*, subfamily *Parvovirinae* which has been recognized in veterinary medicine since the early 1960's. To date, bocaviruses which have been discovered include bovine parvovirus (BPV) (Chen *et al*., 1986), minute virus of canine (MVC) (Binn *et al*., 1970), porcine bocavirus (PBoV) (Blomström *et al*., 2009), gorilla bocavirus (GBoV) (Kapoor *et al*., 2010a), feline bocavirus (FBoV) (Lau *et al*., 2012), and California sea lion bocavirus (Csl BoV) (Li *et al*., 2011). As studies continue to be carried out, it is possible that bocaviruses will be detected in more animal species.

Bocaviruses have been of increasing importance in recent years as studies have shown human infections with human bocavirus (HBoV), which may have had a zoonotic origin (Schildgen *et al*., 2012). HBoV was first identified in Sweden from nasopharyngeal aspirate samples in children with respiratory infections (Allander *et al*., 2005). Since then, it has been associated with acute lower respiratory tract disease in children (Fry *et al*., 2007), and adults, especially in elderly patients (Liu *et al*., 2011). In addition, HBoV has also been detected from stool samples in children with gastroenteritis (Arthur *et al*., 2009).

In Malaysia, HBoV was first detected in a 13-month old boy with pneumonia and underlying asthma (Etemadi *et al*., 2012). Subsequently, a study of 125 hospitalized children with Acute Respiratory Disease (ARD) were analyzed for the presence of HBoV. The study showed that 5.6% (7/125) of the cases were positive for HBoV (Tg Rogayah *et al*., 2014).

Because HBoV and PBoV both belong to the genus *Bocavirus* and family *Parvoviridae*, they share many similarities in terms of their virus characteristics. At the amino acid sequence level, the predicted nonstructural protein 1 (NS1) gene of PBoV exhibited sequence identities of 38.0 – 54.7% to HBoV (Zeng *et al*., 2011). Hence, experience from HBoV research may be applied to PBoV, especially since there is an increasing number of studies involving the pathogenicity of HBoV.

1.2 Porcine Bocavirus

Porcine bocavirus (PBoV) is a recently discovered virus that infects pigs and is classified within the *Bocavirus* genus (family *Parvoviridae*, subfamily *Parvovirinae*). It was first discovered in 2009 in Sweden, when a novel porcine parvovirus with genetic relationship to bocaviruses was incidentally found in pigs with post-weaning multisystemic wasting syndrome (PMWS) (Blomström *et al*., 2009). Since its discovery, PBoV has been detected in 12 countries. Those countries are Sweden, China, USA, Canada, Mexico, Romania, Hungary, Uganda, Korea, Cameroon, UK (Zhou *et al*., 2014), and Thailand (Saekhow & Ikeda, 2014).

As more countries detect PBoV, more strains are being discovered. At the time of writing, nine genotypes of PBoV had been identified and sequenced. However, an agreeable method of classifying these strains has yet to be established. Currently, the two methods of classification that are widely accepted are based on the sequencing of the NS1 and viral protein 1 gene (VP1) of PBoV (Zhou *et al*., 2014). Based on these methods of classification, PBoV's are named based on their order of discovery and subgroup. Presently, known genotypes of PBoV's are: PBoV1, PBoV2, PBoV3, PBoV4, PBoV5, PBoV3C, PBoV-6V, PBoV-7V, and swBoV CH437.

To date, the significance of PBoV in pig production, pig health, and public health has yet to be determined. Studies have shown that the detection rate of PBoV is significantly higher in sick pigs compared to healthy pigs. Moreover, positive samples for PBoV are also significantly higher in pigs co-infected with porcine circovirus type-2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine torque teno virus species 2 (PTTV2), thus suggesting that PBoV may be involved with PMWS in post-weaning piglets (Zhai *et al*., 2010). It has also been indicated that PBoV may be associated with diarrhoea and respiratory disease. (Zhang *et al*., 2015).

Despite the significant contribution of swine farming to the Malaysian livestock industry, no study has been carried out to determine the presence of PBoV in Malaysia. Hence, this study was undertaken to fulfill the following objectives:

i. To detect the presence of porcine bocavirus in pigs in Malaysia using the conventional PCR method.

ii. To confirm PCR detection of porcine bocavirus by partial DNA sequencing of the NS1 gene.

For this research, the following hypotheses were proposed:

i. Porcine bocavirus is detected in tissue samples of local pigs by conventional PCR.

ii. Partial DNA sequence of the NS1 gene is similar to that of reference isolates.

2.0 LITERATURE REVIEW

2.1 Virus Structure

Being a new member of the *Bocavirus* genus, belonging to the family *Parvoviridae* and subfamily *Parvovirinae*, porcine bocavirus (PBoV) is a non-lipid enveloped, autonomously replicating virus that exhibits icosahedral symmetry and is 25 – 30 nm in diameter. Their linear single-stranded genome is approximately 5 kb in length and contains terminal palindromic sequences (Zeng *et al*., 2011).

Through complete coding of the PBoV genome, three primary open reading frames (ORFs) that were identified are ORF1, ORF2, and ORF3. ORF1 codes a non-structural protein (NS1) and is located at the 5' - end of the genome. ORF1 has been demonstrated to contain conserved motifs that are associated with rolling-circle replication as well as helicase and ATPase activities (Lau *et al*., 2011). In addition, the NS1 has been shown to be vital for viral DNA replication in canine minute virus (MVC), which may be the same case for PBoV (Sun *et al*., 2009).

ORF2 encodes the viral protein 1 (VP1) and viral protein 2 (VP2) capsid proteins. At the amino acid sequence level of the VP1 protein, a highly conserved "HDXXY" and "YXGXG" motif of secretory phospholipase ($sPLA_2$) is found in most parvoviruses. These motifs indicate sPLA2 activity that is vital for the infectivity of parvoviruses. However, bocaviruses have shown an atypical "YXGXF" motif instead of "YXGXG"

(Cheng *et al*., 2010). Nevertheless, a study has shown that the "YXGXF" motif in human bocavirus (HBoV) also possesses $sPLA_2$ activity (Qu *et al*., 2008). Due to the similarities between HBoV and PBoV, it may be proposed that PBoV could also exhibit $sPLA_2$ activity which would indicate its infectivity (Cheng *et al*., 2010).

ORF3 encodes the nuclear phosphoprotein 1 gene (NP1) and is located in between the ORF1 and ORF 2 expressions. This formation is a structural characteristic of the *Bocavirus* genus. The function of the NP1 gene has been found to be important for MVC DNA replication (Sun *et al*., 2009) as well as blocking interferon production in HBoV which suggests that it may have a mechanism to evade the immune system (Zhang *et al*., 2012).

2.2 Taxonomy and Nomenclature

Using the International Committee on Taxonomy of Viruses (ICTV) criteria which states that a virus must exhibit less than 95% homology of the nonstructural gene to be considered a new species, PBoV's have been defined as a new species in the *Bocavirus* genus (Zeng *et al*., 2011). However, PBoV has yet to be added into the ICTV database. Instead, the ICTV has recently (2013) renamed the *Bocavirus* genus to *Bocaparvovirus* even though subsequent published journals still refer to the genus as *Bocavirus*. Previously in 2012, the ICTV database contained only the canine minute virus (MVC) and bovine parvovirus (BPV) under the *Bocavirus* genus (International Committee on Taxonomy of Viruses, 2012). The latest database updated in 2013 has

renamed MVC to carnivore bocaparvovirus, BPV to ungulate bocaparvovirus, as well as added in pinniped bocaparvovirus (California sea lion bocavirus), and primate bocaparvovirus (gorilla bocavirus) into the genus (ICTV Virus Taxonomy, 2014). As PBoV has yet to be classified by the ICTV, researchers continue to report their findings as porcine bocavirus and not porcine bocaparvovirus.

Currently, new strains of PBoV's are named based on chronological order of their discovery and subgroups. To date, there have been nine PBoV strains identified, which are: PBoV1, PBoV2, PBoV3, PBoV4, PBoV5, PBoV3C, PBoV-6V, PBoV-7V, and swBoV CH437 (Zhou *et al*., 2014).

Presently, the most widely accepted method of classification for PBoV is based on the complete sequencing of the VP1 gene. If the nucleotide difference is larger than 40%, it is classified as a member of a different group; whereas, if the nucleotide difference is more than 10%, it is considered as a different subgroup (Yang *et al*., 2012). Based on this method, PBoV's are currently classified into three groups, with the third group further divided into five subgroups: PBoV3A, PBoV3B, PBoV3C, PBoV3D, and PBoV3E (Zhou *et al*., 2014). Group 1 consists of PBo-likeV (FJ872544), PBoV-SX (HQ223038), and PBoV1-H18 (HQ291308). Group 2 includes PBoV1/2-CHN (HM053693 / HM053694), and PBoV2-A6 (HQ291309). Group 3 contains PBoV3/4-UK (JF512472 / JF512473), PBoV3/4-HK (JF429834 / JF429835), and PBoV3C (JN681175) (Zhou *et al*., 2014).

However, as there is currently no definitive standard to classify PBoV, classification based on the complete sequencing of the NS1 gene is also accepted.

According to the criteria of the ICTV, NS1 gene DNA sequence homology is defined as an important marker for the classification of different bocavirus species.

2.3 Epidemiology

Around the world, more countries are detecting PBoV within their swine herds. To date, 12 countries have detected PBoV within their swine populations. Those countries are: Sweden, China, USA, Canada, Mexico, Romania, Hungary, Uganda, Korea, Cameroon, UK (Zhou *et al*., 2014), and Thailand (Saekhow & Ikeda, 2014). As a point of interest, the Romanian study was conducted on wild boars (*sus scrofa*) with a detection rate of 12.9% (Cadar *et al*., 2011). In general, detection rates vary among countries ranging from 1.5% up to 88% (Zhou *et al*., 2014). However, these figures are not representative of the true prevalence, as sampling methods and health status of pigs differ between studies. Countries such as China and USA have detected all three groups of PBoV genotypes with USA having a much higher detection rate of 42% compared to China's 11% (Zhang *et al*., 2015).

It has been found that age is a variable factor for PBoV infections. Weaners show a significantly higher infection rate (69.7%) compared to piglets (13.6%), boars (0%), sows (7.7%), and aborted fetuses (0%) (Zhai *et al*., 2010). It has been suggested in various literature that lower detection rates in suckling piglets may be due to maternal antibody protection although no research has been carried out (Cadar *et al*., 2011).

2.4 Detection Methods of Porcine Bocavirus

The clinical specimens used to detect PBoV include sera, lungs, lymph nodes, tonsil, liver, nasopharyngeal swabs, fecal samples (Zhou *et al*., 2014), and saliva (Choi *et al*., 2014). From these samples, detection methods for PBoV involve cell culture, immunofluorescence assay, and sequence detection assays such as PCR and real time PCR. PBoV has been successfully cultured in primary pig kidney cells and subsequently identified via electron microscopy, immunofluorescence assays, and PCR (McKillen *et al*., 2011). In the same study, monoclonal antibodies against PBoV were produced, thus allowing the possibility of development of an ELISA detection method. The PCR detection method favours targeting the NS1, VP1, and VP2 gene for primer design. In addition, a real time PCR method has also been developed targeting the NP1 gene (Li *et al*., 2011).

For tissue samples, highest detection rates for PBoV are found in the lymph nodes, spleen, and tonsil. In one study, the positive rates of spleen (20.75%) and inguinal lymph node (27.18%) were shown to be higher than other organs (Liu *et al*., 2014). These results was replicated in another study where the spleen (20.8%) and inguinal lymph node (27.2%) also showed higher positive rates (Zhang *et al*., 2015). These results suggest that these organs may be sites of active replication of PBoV.

2.5 Pathology

Not much is understood about the pathogenicity of PBoV. However, there is a consistency in findings that PBoV has a significantly higher infection rate in diseased pigs as compared to healthy pigs. Higher PBoV detection rates have been associated with co-infections in post-weaning piglets with porcine circovirus type-2 (PCV2), porcine reproductive and respiratory syndrome virus (PRRSV), and porcine torque teno virus species 2 (PTTV2) (Zhai *et al*., 2010). The highest detection rate to date was 88% in pigs with PMWS (Blomström *et al*., 2010). Additional studies showed that pigs with clinical signs such as trembling, fever, testicular atrophy, abortion, or death appear to be more likely to be infected with PBoV (Zhou *et al*., 2014). There has also been association between PBoV with respiratory and gastrointestinal disease where 29.1% and 48.4% of PBoV infected pigs showed respiratory and gastrointestinal signs respectively (Zhang *et al*., 2015).

2.6 Genetic Sequencing and Phylogenetic Analysis of PBoV

Phylogenetic analysis of PBoV with other bocaviruses at the amino acid sequence level, the NS1 gene of PBoV showed sequence identity with MVC of 42 – 43%, BPV of 30 – 35%, GBoV of 40%, and HBoV of 38 – 42%; the NP1 gene showed sequence identity with MVC of 37 - 40%, BPV of 35 – 36%, GBoV of 32%, and HBoV of 38.8 – 42% (Zeng *et al*., 2011).

Sequencing technology is now actively used to identify the strains of PBoV though partial sequencing of the NS1, VP1, VP2 or NP1 gene as well as complete sequencing of the PBoV genome. Despite the wealth of sequence data in the databases, there has yet to be a satisfactory classification system to cover the diversity of all available PBoV sequences (Zhang *et al*., 2015).

2.7 Bocavirus in Other Animal Species

Within the veterinary field, the earliest identification of *Bocavirus* was discovered in the canine species in 1970 which was the canine minute virus (MVC), or also known as canine parvovirus type-1 (Binn *et al.*, 1970). In dogs, the MVC is considered to be less pathogenic than canine parvovirus type-2 which causes severe enteritis. MVC has been established to cause illness such as bronchitis and interstitial pneumonia in puppies as well as transplacental infections with embryo resorption. The prevalence of MVC in USA was obtained to be approximately 50% by detection of hemagglutination-inhibiting antibodies (Carmichael *et al.*, 1994).

Since then further detection studies have discovered bocaviruses in several other animal species. The following detection was in 1970 which was the bovine parvovirus (BPV) (Chen *et al.*, 1986).

In 2010, a study on Western Gorillas identified a novel non-human primate bocavirus isolated from four stool samples of Western Gorillas with acute enteritis. It was

subsequently named gorilla bocavirus species 1 (GBoV). Phylogenetic analysis indicated that GBoV is most closely related to HBoV (Kapoor *et al*., 2010a).

The next *Bocavirus* to be identified is the California sea lion bocavirus (Csl BoV). From that study, a total of four *Bocavirus* species were identified and subsequently named Csl BoV 1 to Csl BoV 4 respectively. Based on phylogenetic analysis, Csl BoVs were most closely related to MVC, showing 54%, 60 to 64%, and 64 to 67% amino acid similarities in the NS1, NP1, and VP1 regions respectively (Li *et al*., 2011).

Following that, feline bocavirus (FBoV) was discovered in Hong Kong from feline fecal, nasal, urine, kidney and blood samples. With ≤ 5.7% nucleotide identities to the genome of MVC, phylogenetic analysis showed that FBoV is distantly related to other bocaviruses (Lau *et al*., 2012). The pathogenicity of the FBoV is still not completely understood.

2.8 Bocavirus in Humans

Since 2005, studies on bocaviruses have expanded from the veterinary field to the field of human medicine through the discovery of human bocavirus (HBoV). It was first isolated in Sweden using for the first time a procedure called "molecular virus screening" (Allander *et al*., 2005). This method of molecular detection involves DNase treatment of samples, random nucleic acid amplification and cloning, followed by large scale sequencing and bioinformatics analyses (Jartti *et al*., 2012). Between 2009 and 2010,

three new strains were discovered. They are, HBoV2 (Kapoor *et al*., 2009), HBoV3 (Arthur *et al*., 2009), and HBoV4 (Kapoor *et al*., 2010b). Although the pathogenesis of HBoV is not fully understood, it has been extensively researched and described in lower respiratory tract illness (Fry *et al*., 2007) and gastroenteritis in children (Arthur *et al*., 2009), as well as respiratory disease in elderly patients (Liu *et al*., 2011). Clinical signs associated with HBoV infections are rhinitis, pharyngitis, cough, dyspnoea, wheezing, pneumonia, acute otitis media, fever, nausea, vomiting, and diarrhoea. Phylogenetically, HBoV is most closely related to gorilla bocavirus (GBV), followed by bovine parvovirus (BPV), and subsequently porcine bocavirus (PBoV) (Jartti *et al*., 2012).

In Malaysia, HBoV was first detected in a 13-month old boy who was hospitalized with pneumonia and underlying asthma. HBoV was detected using the PCR method (Etemadi *et al*., 2012). Subsequently, a study of 125 hospitalized children with Acute Respiratory Disease (ARD) was carried out to detect the presence of HBoV from tracheal and nasal aspirates. The study showed that 5.6% (7/125) of the cases were positive for HBoV, with 57.1% of the cases detected from Johor. Based on sequencing of the NP1 protein, it was determined that the Malaysian virus was the HBoV1 strain, and it had no significant difference compared to reference isolates. Phylogenetic analysis showed that the Malaysian isolates were highly homologous to isolates from Thailand, Taiwan, Germany, Japan, Hong Kong, and Sweden (Tg. Rogayah *et al*., 2014).

3.0 MATERIALS AND METHODS

3.1 Sample Selection

A total of 11 pigs were sampled from three local pig farms located in Perak and Selangor using the convenience sampling method. Among the selected pigs, eight were sick and runt while the other three were healthy. Selected pigs were euthanized and necropsy was performed on-site. The following tissue samples were collected: mesenteric lymph node, submandibular lymph node, inguinal lymph node, spleen, tonsil, lung, kidney, and liver. During necropsy, abnormal lesions were recorded.

This research project was approved by the Institutional Animal Care and Use Committee (IACUC), with reference number: UPM/IACUC/AUP – R063/2014.

3.2 DNA Extraction

The DNA from each sample was extracted using a commercial DNA extraction kit with methods described by the manufacturer (DNeasy® Blood & Tissue Kit 250, Qiagen®, Germany). In summary, 1 g of each sample was minced using a pair of scissors and then transferred into individual 15 ml centrifuge tube (Corning®, USA) containing 5 ml of phosphate buffer saline (PBS). The suspension was then homogenized using a homogenizer (Ultra-Turrax, IKA, China) and centrifuged at 500 x g for 1 min (Universal 32 R, Hettich Zentrifugen, Germany). Next, 1 ml of supernatant from each sample was

pipetted into individual 2 ml microcentrifuge tubes (Eppendorf, Germany). For DNA extraction, 200 µl of supernatant was pipetted into a 2 ml microcentrifuge tube and centrifuged at 300 x g for 5 min. The sample was then resuspended with 200 µl of PBS. 20 µl of proteinase K (Qiagen®, Germany) and 200 µl of Buffer AL was added into the tube and mixed thoroughly by vortexing (Finevortex, Korea). The mixture was then incubated (WiseTherm, Korea) at 56 ºC for 10 min. Next, 200 µl of 96% ethanol (Essen-Haus, Malaysia) was added into the lysate and mixed by vortexing. The mixture was pipetted into a DNeasy spin column placed in a 2 ml collection tube, and centrifuged at 6000 x g for 1 min (Mikro 22 R, Hettich Zentrifugen, Germany). The collection tube and flow through were then discarded. Next, the spin column was placed in a new 2 ml collection tube. About 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 were discarded. The spin column was placed in a new 2 ml collection tube and 500 µl of Buffer AW2 was added. It was then centrifuged at 20000 x g for 5 min and the collection tube and flow through were again discarded. Following that, the spin column was placed in a new 2 ml Eppendorf tube. Elution was carried out by adding 200 µl of Buffer AE into the center of the spin column membrane. It was incubated at room temperature for 1 min, followed by centrifuging at 6000 x g for 1 min. The collected DNA elution was pipetted back into the spin column to be centrifuged again for increased DNA yield.

3.3 Measurement of DNA Concentration

The final DNA concentration was obtained by performing spectrophotometry. After the extraction process, 2 ml of the DNA extract was diluted in 98 ml of nuclease free water (Qiagen®, Germany) in a cuvette and placed in a BioPhotometer Plus photometer (Eppendorf, Germany).

3.4 Primer Selection

PCR amplification relies on two primers that determine the region of sequence to amplify in the forward and reverse direction. The forward primer (F) is designed to amplify the sequence towards the reverse primer (R); while the reverse primer is designed for the complementary strand in the opposing direction. Therefore, primer design is an important consideration for specific amplification with high yield.

To design a primer for this study, several published journals were reviewed. It was determined that one of the three open reading frames (ORF) of PBoV, ORF 1, codes a non-structural protein (NS1) at the 5' – end of the genome (Lau *et al*., 2011), which serves as a highly conserved splice site (Zeng *et al*., 2011). Subsequent studies also showed that primers designed to target the NS1 gene showed high probability of detection from porcine tissue samples of up to 42 % (Zhang *et al*., 2014). The primer set and the journal sources are stated in Table 3.1.

Table 3.1: Primer set for detection of the conserved NS1 gene of porcine bocavirus by conventional PCR assay

Reference	Primer	Sequence 5' → 3'
1. Zeng *et al*., 2011 2. Liu *et al*., 2014 3. Zhang *et al*., 2014 4. Huang *et al*., 2014	Forward	5' – ACAGGCAGCCGATCACTCACTAT - 3'
	Reverse	5' - CTCGTTCCTCCCATCAGACACTT - 3'

3.5 Polymerase Chain Reaction

PCR reaction was carried out using HotStarTaq® Plus Master Mix Kit, (Qiagen®, Germany). The prepared master mix, HotStarTaq® Plus Master Mix, 2x (5 U/µl), contains HotStarTaq Plus DNA Polymerase, PCR Buffer (with 3 mM $MgCl_2$), and 200 µM each dNTP.

The PCR reaction was set up in each tube by adding 10 µl of HotStarTaq® Plus Master Mix, 2x, 1 µl of forward primer, 1 µl of reverse primer, and 3-5 µl of DNA template depending on the DNA concentration (<200 ng/reaction). RNase-Free water was added to make the final volume in each tube 20 µl.

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 3.2.

Table 3.2: Optimized cycling conditions of conventional PCR assay for detection of porcine bocavirus

Step	Time	Temperature
Initial heat activation	5 min	95 ºC
Denaturation	1 min	94 ºC
Annealing	30 sec	54 ºC
Extension	1 min	72 ºC
Number of cycles	35 cycles	
Final extension	10 min	72 ºC

3.6 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 in a microwave (NN-SM332M, Panasonic, Malaysia) until all the powder had dissolved and the agarose solution was homogenous. The agarose solution was then cooled to about 60 ºC and poured into a gel mold with a well forming comb. After the gel solidified, it was removed from the mold 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 PCR product from each tube was pipetted out and mixed individually with 1 µl of DNA loading dye (CoralLoad Concentrate, 10x, Qiagen®) and loaded into the wells. Subsequently, 100bp DNA Ladder RTU (GeneDireX, Taiwan) DNA marker

was added into the first well as comparison for the PCR products. Electrophoresis was carried out using PowerPac™ Basic (Bio-Rad, USA) power supply at 80 V and 400 mA for approximately 35 min or until the DNA ladder (yellow dye) reached the opposite end of the gel. After electrophoresis was complete, the gel was transferred into ethidium bromide (EtBr) staining solution (Amresco, USA) for 30 min. Next, the gel was removed and immersed in dH_20 for 10 min for destaining. Lastly, the gel was removed from the destaining 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).

3.7 DNA Purification

To increase DNA yield, PCR was repeated for all positive samples using four PCR tubes per known positive sample. Each tube contained 10 µl of HotStarTaq® Plus Master Mix, 2x, 1 µl of forward primer, 1 µl of reverse primer, and 3-5 µl of DNA template depending on the DNA concentration (<200 ng/reaction). RNase-Free water was added to make the final volume in each tube 20 µl. After PCR, gel electrophoresis was performed to confirm PCR amplification of the target DNA.

DNA purification was carried out using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) using methods described by the manufacturer. All four PCR tubes containing the PCR product were combined into one PCR tube for purification. Then, an equal volume (80 µl) of Membrane Binding Solution was added into the PCR

tube. The solution was then transferred into a Minicolumn assembly which consists of a SV Minicolumn inserted into a collection tube. The solution was incubated at room temperature for 1 min and then centrifuged at 16000 x g for 1 min. The flow through was discarded and the Minicolumn was reinserted into a new collection tube. Then, 700 µl of Membrane Wash Solution was added into the Minicolumn assembly and centrifuged at 16000 x g for 1 min. Again, the flow through was discarded and the Minicolumn was reinserted into a new collection tube. Subsequently, 500 µl of Membrane Wash Solution was added into the Minicolumn, and centrifuged at 16000 x g for 5 min. Next, the collection tube was emptied and reattached to the Minicolumn. The Minicolumn assembly was then centrifuged at 16000 x g for 1 min with the microcentrifuge lid open to allow evaporation of any residual ethanol. For DNA elution, the Minicolumn was transferred into a 2 ml microcentrifuge tube, and 50 µl of RNase-Free water was added into the Minicolumn. The column was incubated at room temperature for 1 min, then centrifuged at 16000 x g for 1 min. Lastly, the Minicolumn was discarded, and the purified DNA contained in the 2 ml microcentrifuge tube was stored at – 20 ^{o}C until sent for sequencing.

3.8 DNA Sequencing

The purified PCR products and primers were sent to an external commercial sequencing service provider (Bioneer, South Korea). There, each purified PCR product was sequenced using the Sanger's method with the same forward and reverse primer. A

sequencing kit was used (BigDye® Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher Scientific Inc., USA) as well as an automated sequencing analyzer (3730xl DNA Analyzer, Applied Biosystems®, USA).

3.9 Bioinformatics Analysis of Porcine Bocavirus Partial NS1 Gene Sequence

3.9.1 Sequence Editing and Assembly

The sequencing results in the form of chromatogram were edited using bioinformatics software CLC Main Workbench version 7.5.1 (Qiagen®, Germany) in order to perform sequence trimming, reverse complement sequence, and assembly of forward and reverse sequences.

3.9.2 Basic Local Alignment Search Tool (BLAST)

The National Centre for Biotechnology Information's (NCBI) introduction of the Basic Local Alignment Search Tool (BLAST) in 1990 has been vital to research as it allowed users to scan huge databases of sequences to locate regions of local similarities (Altschul *et al.*, 1990).

Edited partial NS1 sequences were subjected to BLAST searches to confirm that they are similar to other PBoV isolates, and identify the percentage of sequence homology. In addition, the related PBoV sequences from the database were downloaded into the CLC Main Workbench software for comparison with local isolates of partial NS1 sequences.

3.9.3 Multiple Alignments

Downloaded reference sequence isolates and local sequence isolates were aligned using CLC Main Workbench software. Multiple alignment was carried out on the sequences to align them to the best match. Once the sequences were aligned, the sequences were trimmed to ensure that they were the same base pair length in order to generate a more accurate phylogenetic tree.

In addition to multiple alignment, CLC Main Workbench software was also used to create a pairwise sequence identity matrix to identify pairwise nucleotide identity.

3.9.4 Construction of Molecular Phylogenetic Tree

An unrooted phylogenetic tree was constructed using the distance-based neighbour-joining method after multiple alignment. CLC Main Workbench software was used to generate the phylogenetic tree, and tree reliability was accessed using 1000 bootstrap replications. The sequences of local PBoV were compared with isolates from USA, China, United Kingdom, and Hong Kong.

4.0 RESULTS

4.1 Organ Sampling

In this experiment, three separate farms located in Perak and Selangor were visited for sampling. From these three farms, a total of 11 pigs were obtained for necropsy, and 73 tissue samples were obtained.

Tissues harvested include mesenteric lymph node, submandibular lymph node, inguinal lymph node, spleen, tonsil, lung kidney and liver. Of those 11 pigs, six pigs originated from Farm X (Selangor), two pigs from Farm Y (Perak), and three pigs from Farm Z (Perak). In addition, 3 out of the 11 pigs were clinically healthy, and the remaining 8 were sick and runt. The data of sampled pigs is shown in Table 4.1.

Table 4.1: Data of sampled pigs indicating farm source, pig ID, age and clinical status

No.	Farm	Pig ID	Age (days)	Clinical Status
1.	Farm X	Pg 1	60	Sick
		Pg 2	60	Sick
		Pg 3	60	Sick
		Pg 9	40	Sick
		Pg 10	40	Healthy
		Pg 11	40	Sick
2.	Farm Y	Pg 4	80	Healthy
		Pg 5	80	Healthy
		Pg 6	7	Sick
3.	Farm Z	Pg 7	21	Sick
		Pg 8	60	Sick

4.2 Amplification of Partial Nonstructural Protein 1 (NS1) Gene of PBoV by PCR Assay

Based on conventional PCR results, 10 out of 11 pigs selected for this study were found to be positive for porcine bocavirus (PBoV) in one or more tissue samples. The PCR assay was performed using specific primers targeting the nonstructural protein 1 gene (NS1), and positive results were defined as a band expression at the 690 bp region after electrophoresis on 1.5% agarose gel. The forward primer used was PBoV- F: 5'-ACAGGCAGCCGATCACTCACTAT-3', and reverse primer was PBoV – R: 5'-CTCGTTCCTCCCATCAGACACTT-3'. For positive control, DNA of pig 9 mesenteric lymph node was used; whereas for negative control, DNA of pig 6 liver was selected. Gel electrophoresis exhibiting bands at the 690 bp region is the specific amplification product of the partial NS1 region. An example of gel electrophoresis is shown for pig 11 with PBoV positive bands of 690 bp shown in wells 2, 4, and 5 (Figure 4.1). Electrophoreses result for other pigs with their selected organs are in shown in the appendices (Appendix 8.1). Pigs 1,2, and 3 are shown in Figure 8.1.1, pigs 4 and 5 in Figure 8.1.2, as well as pigs 7 and 8 in Figure 8.1.3. Control positive and control negative were obtained from mesenteric lymph node pig 9 and liver pig 6, respectively.

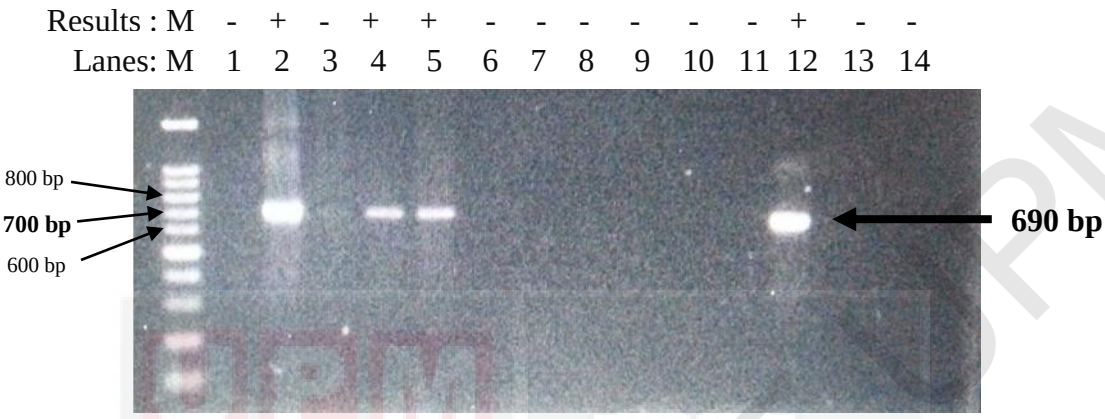

Figure 4.1: PCR assay for pig 11 using specific primers targeting the conserved NS1 gene of porcine bocavirus to produce 690 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. The target bands are observed in organs from pig 11. Lane 2 (mesenteric lymph node), lane 4 (tonsil), lane 5 (lung) and lane 14 (positive control). No bands were observed on lanes 1 (NTC), lane 3 (spleen), and lane 14 (negative control). 100bp DNA Ladder RTU (GeneDireX, Taiwan) DNA marker was used as reference in lane M.

Out of the 11 pigs selected for this study, 10 were found to be positive for PBoV by conventional PCR (90.9%) for any organ tested positive. The only pig to be tested negative was pig 6, which was the only piglet in this study.

From the total of 11 pigs sacrificed for this study, 73 tissue samples were harvested for PCR assay. PCR study showed that 17 out of the 73 tissue samples were positive for PBoV (23.3%) (Table 4.2). The detection rates of PBoV according to organ sample showed that mesenteric lymph node, tonsil, liver, and lung had the highest detection rates with 45.5%, 30%, 25% and 22.2% respectively. The only organ sample with no PBoV detected was the submandibular lymph node (0%).

Table 4.2: Detection rates of PBoV antigen in various organs of 11 pigs by PCR test by amplifying the conserved region of NS1 gene

No.	Organ	Number of positive organ(s)/number organs tested (%)		Total number sequenced
1.	Mesenteric lymph node	5/11	(45.5)	3
2.	Submandibular lymph node	0/4	(0.0)	-
3.	Inguinal lymph node	2/10	(20.0)	-
4.	Spleen	1/11	(9.1)	1
5.	Tonsil	3/10	(30.0)	1
6.	Lung	2/9	(22.2)	-
7.	Kidney	2/10	(20.0)	1
8.	Liver	2/8	(25.0)	-
	Total	**17/73**	**(23.3)**	**6**

4.3 Bioinformatics Analysis of Malaysian PBoV Isolates

4.3.1 Sequence Editing and Assembly

Sequencing outputs in the form of chromatograms were edited and assembled using bioinformatics software. It was determined that only six out of the 17 sequences were of satisfactory quality and length which is approximately 690 bp (Appendix 8.2). The remaining sequences were not acceptable due to numerous ambiguous nucleotides. The six sequences were from the mesenteric lymph node, spleen, and kidney of pig 9, kidney from pig 1, inguinal lymph node from pig 2, and mesenteric lymph node from pig 8. Each sequence was assigned individual identifications (Table 4.3).

Table 4.3: Sequences obtained from sequencing of partial NS1 gene from local PBoV isolates from four pigs with designated identifications

Farm	Pig ID	Organ	Sequence ID	Figure (Appendix)
	Pg 9	Mesenteric lymph node	PBoV_MYS_9_MLN	8.2.4
	Pg 9	Spleen	PBoV_MYS_9_SPL	8.2.5
Farm X	Pg 9	Kidney	PBoV_MYS_9_KDN	8.2.6
	Pg 1	Kidney	PBoV_MYS_1_KDN	8.2.1
	Pg 2	Inguinal lymph node	PBoV_MYS_2_ILN	8.2.2
Farm Z	Pg 8	Mesenteric lymph node	PBoV_MYS_8_ILN	8.2.3

4.3.2 Basic Local Alignment Search Tool (BLAST)

The results of the BLAST search confirmed that the sequences of the conserved NS1 gene from local isolates were highly similar to reference isolates within the GeneBank. High similarities were found with sequences from the Chinese provinces of Jiangsu, and Shandong, Hunan as well as the American states of Minnesota, and Iowa. These reference isolates were downloaded from the GeneBank based on their sequence identity and reliability in publications (Table 4.4). The BLAST results also suggested that local PBoV isolates were most closely related to PBoV3 and PBOV5 strains. Results of the BLAST search also suggested that the partial NS1 gene that was sequenced is highly conserved due to a large number of PBoV hits.

Table 4.4: Reference isolates of PBoV3 and PBoV5 downloaded from GeneBank

No.	Accession no.	Country	Source	Designation
Genotype: Porcine bocavirus 3				
1.	JX944651.1	China	Liu *et al*., 2014	PBoV3_Hunan_China
2.	JX944660.1	China		PBoV3_Shandong_China
3.	KF025519.1	USA	Jiang *et al*., 2014	PBoV3_Iowa_USA
4.	KC514535.1	USA	Huang *et al*., 2014	PBoV3_Minnesota(33)_USA
5.	KC514531.1	USA		PBoV3_Minnesota(34)_USA
6.	KC514540.1	USA		PBoV3_Minnesota(14)_USA
7.	KC514561.1	USA		PBoV3_Minnesota(76)_USA
8.	KC514542.1	USA		PBoV3_Minnesota(39)_USA
9.	JF512472.1	UK	McKillen *et al*., 2011	PBoV3V_Northern Ireland
10.	JF429834.1	Hong Kong	Lau *et al*., 2011	PboV3_HK
Genotype: Porcine bocavirus 5				
11.	JN831651.1	China	Li *et al*., 2012	PBoV5_Jiangsu_China

4.3.3 Multiple Alignment and Pairwise Comparison

The six sequences in this study were subjected to multiple alignment to align the sequences in order to better spot sequence identities and create a phylogenetic tree.

Sequence identity matrix with pairwise nucleotide comparison was performed comparing the six sequences from local isolates and downloaded reference isolates to

determine sequence identity (Figure 4.2). The comparison revealed that the three sequences from pig 9 were highly homologous with sequence identities between 98.79% - 99.24%. The other three sequences from pig 1, 2, and 8 showed less sequence identities between 80.76% - 90.61%.

In addition, high sequence identities were observed between pig 9 sequences and the sequence from Minnesota, USA (97.27% – 97.88%).

		1	2	3	4	5	6	7	8	9	10
PBoV_MYS_Pg1_KDN	1	(2)	87.27	83.64	90.15	90.61	89.85	91.67	92.42	91.21	84.09
PBoV_MYS_Pg2_ILN	2	87.27		84.24	81.06	81.52	80.76	81.52	82.27	81.82	85.15
PBoV_MYS_Pg8_MLN	3	83.64	84.24		82.58	83.03	82.27	81.67	81.67	82.73	94.09
PBoV_MYS_Pg9_MLN	4	90.15	81.06	82.58	(1)	99.24	99.24	93.94	94.39	97.73	82.27
PBoV_MYS_Pg9_SPL	5	90.61	81.52	83.03	99.24		98.79	94.09	94.55	97.88	82.42
PBoV_MYS_Pg9_KDN	6	89.85	80.76	82.27	99.24	98.79		93.64	94.09	97.27	81.97
PBoV3_Minnesota(14)_USA	7	91.67	81.52	81.67	93.94	94.09	93.64		96.52	93.48	81.97
PBoV3_Minnesota(33)_USA	8	92.42	82.27	81.67	94.39	94.55	94.09	96.52		94.55	82.12
PBoV3_Minnesota(34)_USA	9	91.21	81.82	82.73	(3) 97.73	97.88	97.27	93.48	94.55		83.18
PBoV3_Minnesota(39)_USA	10	84.09	85.15	94.09	82.27	82.42	81.97	81.97	82.12	83.18	
PBoV3_Minnesota(76)_USA	11	84.09	93.18	82.73	80.15	80.61	79.85	80.76	81.06	81.21	83.94
PBoV3_Hunan_China	12	82.88	91.52	81.97	78.48	78.64	78.18	79.24	79.39	79.55	82.73
PBoV3_Shandong_China	13	87.88	91.82	87.27	83.94	84.09	83.64	83.94	84.85	85.00	88.48
PBoV3_HK	14	83.64	84.24	(5) 95.15	81.82	82.58	81.52	81.52	80.76	83.03	95.45
PboV3V_UK	15	82.27	83.03	93.33	81.52	81.67	81.21	81.97	80.45	81.82	94.55
PBoV5_Jiangsu_China	16	84.09	(4) 94.70	83.64	79.24	79.70	78.94	80.00	79.70	80.30	84.24
PBoV3_Iowa_USA	17	51.82	44.85	44.70	53.79	53.94	53.48	56.67	55.30	53.87	45.15

Figure 4.2: Sequence identity matrix with pairwise comparison to compare sequence identity of the six nucleotide sequences derived from the partial NS1 gene of local PBoV isolates with reference isolates. The results of this pairwise comparison shows that the three sequences obtained from pig 9 are highly homologous (1) (98.79% - 99.24%). Sequences from pigs 1, 2, and 8 show lower sequence identity (2) (80.76% - 90.61%). In comparison to reference isolates, high identity is observed between sequences from pig 9 and the sequence from Minnesota, USA (3) (97.27% – 97.88%), pig 2 and the sequence from Jiangsu, China (4) (94.70%), as well as pig 8 with the sequence from Hong Kong(5) (95.15%).

4.3.4 Construction of Phylogenetic Tree

The unrooted phylogenetic tree showing relationship between Malaysian porcine bocaviruses with reference isolates was generated using the neighbour-joining (NJ) method with 1000 bootstrap replicates (Figure 4.3). CLC Main Workbench software was used to construct the tree.

The tree is divided into two clusters, group A and group B. Sequences from pig 2 (PBoV_MYS_Pg2_ILN) and pig 8 (PBoV_MYS_Pg8_MLN) are grouped in group A where its members are more distantly related compared to members in group B. The PBoV isolate from pig 2 is most closely related to PBoV3 strain from Minnesota, USA and PBoV5 strain from Jiangsu, China. Subsequently, the PBoV isolate from pig 8 is most closely related to PBoV3 strains from Hong Kong, Northern Ireland, and Minnesota, USA. Group A shows discrepancies in PBoV strains as it contains PBoV3 and PBoV5 strains.

Group B contains sequences from pig 1 (PBoV_MYS_Pg1_KDN) and pig 9 (PBoV_MYS_Pg9). Only one sequence from pig 9 was used in the construction of the phylogenetic tree due to the high level of identity among the three sequences from pig 9.

The PBoV isolate from pig 1 is the most distantly related PBoV isolate as it forms its own clade. The PBoV isolate from pig 9 is most closely related to three PBoV3 isolates from Minnesota, USA. Therefore, it is highly suggestive that the PBoV isolate from pig 9 is of the PBoV3 strain.

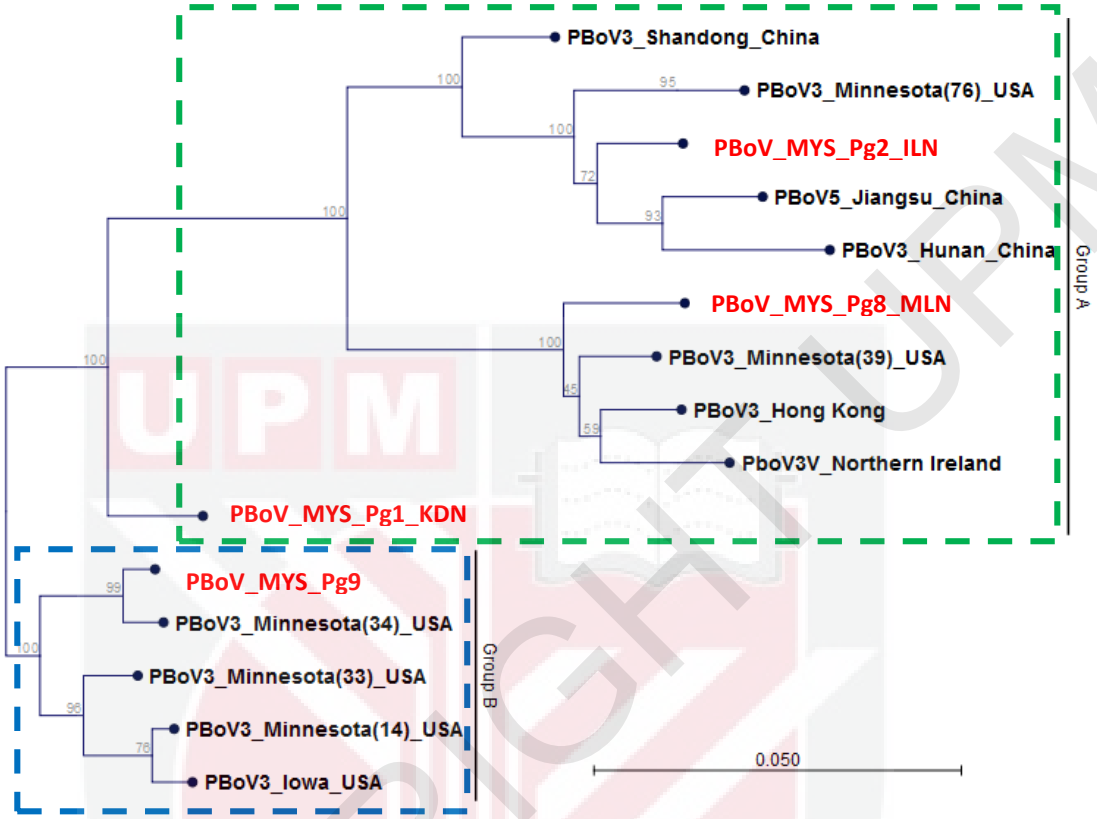

Figure 4.3: Unrooted phylogenetic tree of Malaysian PBoV isolates and reference isolates. The phylogenetic tree was constructed using the neighbour-joining (NJ) method with 1000 bootstrap replicates. The tree is divided into two clusters, group A (green) and group B (blue). Malaysian isolates are denoted in red. PBoV isolate of pig 9 (PBoV_MYS_Pg9) shows close relationship with PBoV3 isolates from Minnesota, USA.

5.0 DISCUSSION

5.1 Positive Detection of Porcine Bocavirus (PBoV) in Malaysia by PCR Assay

This study has successfully described porcine bocavirus in swine herds for the first time in Malaysia. Using the convenience sampling method to select sick and runt pigs based on availability, 10 out of 11 of the pigs were positive for PBoV by PCR assay, which means a positive detection rate of 90.9%

Overall, this positive detection rate is consistent with other publications which report detections up to 88% in sick pigs (Zhou *et al*., 2014). As this study had a much smaller sample size with seven out of the 11 pigs sampled being sick and runt, a high detection rate is consistent with other reported findings. The only pig that tested negative was the only piglet in the study which was 21 days old. This result is supported by reported findings which state that piglets have a significantly lower detection rate which may be attributed to maternal antibodies, but has yet to be researched (Cadar *et al*., 2011).

In terms of tissue detection rates, the findings from this study are not significant due to a small sample size and poor understanding of the viruses' pathology. However, results showing high positive PBoV detections in the mesenteric lymph node and tonsil are consistent with other reported findings (Liu *et al*., 2014).

On another note, based on current knowledge of reviewed articles, this is the first study in the world to specifically detect PBoV from kidney tissue samples. Kidney

samples from pig 1 and pig 9 were positive for PBoV based on PCR results, and were successfully sequenced. Although some studies have reported positive PBoV results for pooled organs which includes kidney tissue (Choi *et al*., 2014), no report has done so specifically from kidney tissue. The legitimacy of this novel finding is supported by a previous study which discovered that PBoV can be cultured in primary pig kidney cell lines (McKillen *et al*., 2011). The detection of PBoV is kidney tissue suggests that the virus is able to replicate within kidney cells thus causing renal pathology. This finding may be of importance towards the understanding of the complete pathology of this virus.

5.2 Partial Nonstructural Protein 1 (NS1) Gene Sequencing and Bioinformatics Analysis of Malaysian Isolated PBoV

This aspect of the research is also a novel study in Malaysia. Using the PCR amplified region of the highly conserved NS1 gene of PBoV, bioinformatics analyses were able to be performed on local PBoV isolates to compare them with other PBoV reference isolates in the GeneBank.

Firstly, the primers used are suitable for detection of PBoV by amplification of the 1000 bp – 1700 bp region of the NS1 gene, and are commonly used in other recent studies worldwide (Zhang *et al*., 2015). The amplified region is approximately 690 bp in length and is highly conserved as proven by accurate and specific BLAST search results.

Next, BLAST searches and pairwise comparison highly suggest that the sequences obtained are specifically partial sequences of the NS1 gene due to high sequence identities of more than 90% to most reference isolates.

The PBoV isolate from pig 9 (PBoV_MYS_Pg9) has the highest sequence identity to PBoV3 reference isolates from Minnesota (97.27% – 97.88%), which also suggests that the PBoV isolated from pig 9 is a PBoV3 genotype. The phylogenetic tree also supports this finding as pig 9 PBoV isolate forms a distinct group (group B) with Minnesota PBoV3 isolates. In addition, the PBoV isolate from pig 8 mesenteric lymph node (PBoV_MYS_Pg8_MLN) also forms a distinct group with PBoV3 reference isolates from Minnesota, Hong Kong and Northern Ireland. Therefore, it is suggested as well that pig 8 PBoV isolate is the PBoV3 genotype.

PBoV isolated from pig 1 kidney (PBoV_MYS_Pg1_KDN) does share high nucleotide sequence identity with Minnesota isolates, but fails to form a distinct group in the phylogenetic tree. Therefore, more study is required for this sequence for genotyping. Similarly, pig 2 inguinal lymph node isolate (PBoV_MYS_Pg2_ILN) requires further study in order to be genotyped. Although it exhibits high nucleotide identity to PBoV3 Minnesota, Hunan, and Shandong isolates, it shares it highest nucleotide identity with a PBoV5 strain from Jiangsu, China thus causing discrepancies in its genotyping.

Further studies that involve identifying the complete genomes of Malaysian isolates will prove more reliable in genotyping these local PBoV isolates.

6.0 CONCLUSION

In conclusion, Malaysia is the 13th country in the world that has described porcine bocavirus (PBoV) in its swine herds. PBoV was successfully detected using PCR assay and partial sequencing of the NS1 gene. Bioinformatics analyses of the partial sequence of the highly conserved NS1 gene have shown that Malaysian isolates of PBoV share high sequence identities with PBoV3 strains from Minnesota, USA.

7.0 RECOMMENDATIONS

Firstly, as porcine bocavirus (PBoV) has been detected in Malaysia, a prevalence study should be carried out in order to better understand the distribution of this virus in Malaysian swine herds. Optimally, more farms from more states should be involved. In addition, pigs from different age and gender groups should be included to cover all pigs in a farm such as piglets, weaners, growers, finishers, boars, gilts, and sows. Finishing pigs may also be sampled at the slaughter house level to determine if the virus is potentially entering the human food chain.

Secondly, the virus should also be isolated and cultured in a suitable cell culture for confirmatory description of the virus through methods such as electron microscopy and immunohistochemistry. Furthermore, cell cultures would also allow study into the cytopathic effects of PBoV virus which may increase understanding of its pathogenesis. To take a leap further, once a susceptible cell culture is established and the virus is adequately adapted in the cell culture for cloning, virus inoculation into animal models such as healthy pigs may be performed for transmission studies

Lastly, identification of complete genomes of local isolates through next generation sequencing (NGS) would be ideal for genotyping and further understanding protein expressions of PBoV.

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8.0 APPENDICES

8.1 Gel Electrophoresis Results of PCR Assay

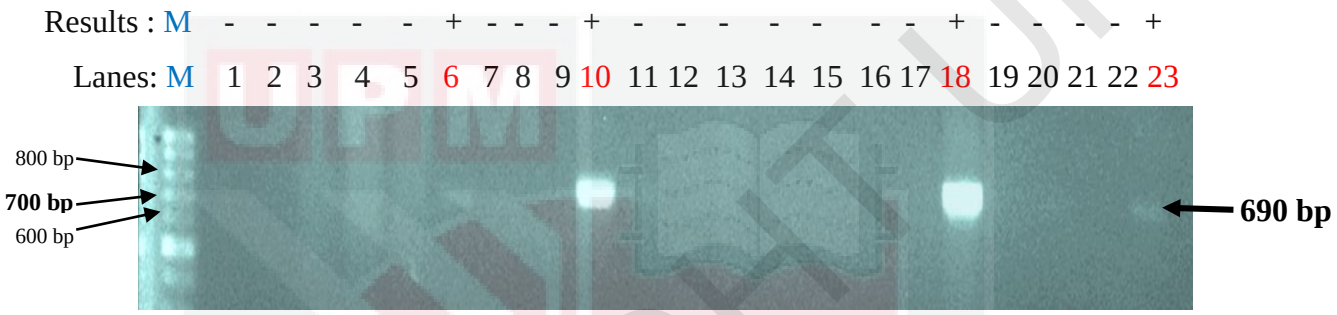

Figure 8.1.1: Pig 1, 2, and 3 PCR assay carried out using specific primers targeting the NS1 gene to produce 690 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. Positive samples are observed in lane 6 (pig 1, kidney), lane 10 (pig 3, mesenteric lymph node), lane 18 (pig 2, inguinal lymph node), and lane 23 (pig 2, liver). 100bp DNA Ladder RTU (GeneDireX, Taiwan) DNA marker was used as reference in lane M.

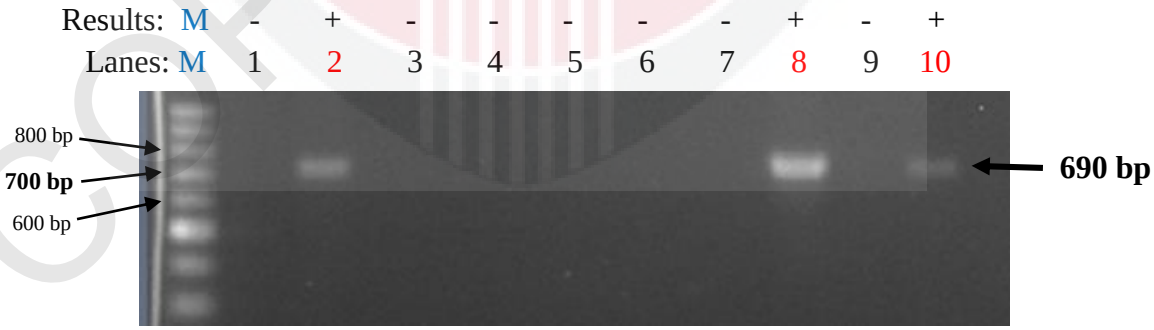

Figure 8.1.2: Pig 4 and 5 PCR assay carried out using specific primers targeting the NS1 gene to produce 690 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. Positive samples are observed in lane 2 (pig 5, tonsil), lane 8 (pig 4, lung), and lane 5 (pig 4, liver). 100bp DNA Ladder RTU (GeneDireX, Taiwan) DNA marker was used as reference in lane M.

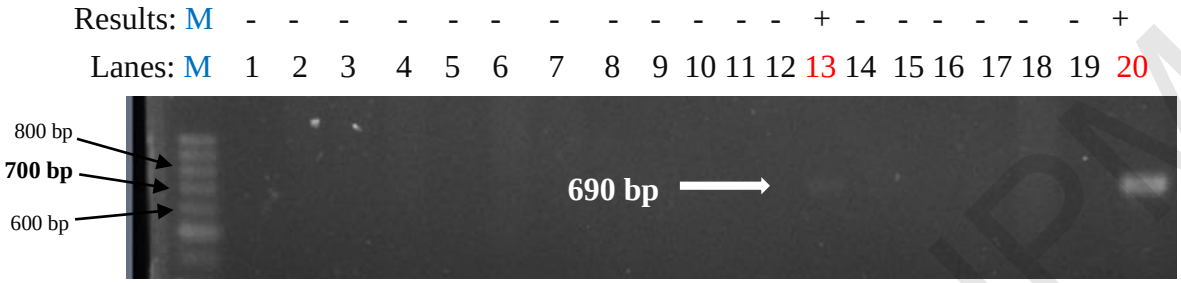

Figure 8.1.3: Pig 7 and 8 PCR assay carried out using specific primers targeting the NS1 gene to produce 690 bp PCR products. Electrophoresis was carried out on 1.5% agarose gel for 45 min. Positive samples are observed in lane 13 (pig 7, tonsil), and lane 20 (pig 8, MLN). 100bp DNA Ladder RTU (GeneDireX, Taiwan) DNA marker was used as reference in lane M.

8.2 Partial Nonstructural Protein 1 (NS1) Gene Sequence of Malaysian PBoV Isolates

```
LOCUS       PBoV_MYS_Pg1_MLN       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 GCCGTAAGGG TCTTGATTGA CCGGTCTGGT CATGAACAGC ACCGAGGATA CCGGGGAGCC
       61 GGGCATAAGA TGGCTCGCTG GGGAGATCTG CCTCAGGTGG GTGAGAATTC TCTGGCTAGA
      121 GAGAACACTC GGACCAGACC GACTAAGATT ACCAAGAAAC AGCGTGTGAT GTTAGATACC
      181 TTGCAGAGAT GCGAGGATCA ATTCATCTGC ACCAAGGAGG AGCTGACTAT GCTCCATCCT
      241 GACATGGTGA TCATGTTCGA GAGCTCTCCT GGCGGGTCTA GAACGCTCGA CGAGATGCTC
      301 GAGATGCACC GGGTGAGGGT CACGCGCTCT TACACGGCTC TGGGGTACAT CCTGAAACAG
      361 TTTCCGGAGA ATAGGTTCAT CAAGCCGGAC AACAAGGTGG TCAGGCTTTT GAACATCCAG
      421 GGCTACAACC CTATTCAGGT CGGCCACTGG GTGGCGACCG TGCTCTACAA AAAGGCCGGG
      481 AAACAAAACA CGCTCTGCTT CTTTGGACCG GCCAGTACCG GGAAGACCAA TCTGGCTAAG
      541 GCCATCGCTC ATGCGGTGAA GGTGTATGGT TGTGTGAATC ATCTCAACAA GAGCTTCGTC
      601 TTTAACGATT GCCAGAACAA GCTCTTGTGC TGGTGGGAGG AGTGCGTGAT GCACAACGAC
      661 TGGGTGGAGC CGGCCAAGTG TCTGATGGGA
//
```

Figure 8.2.1: Partial NS1 sequence of Malaysian PBoV isolate from pig 1 kidney

```
LOCUS       PBoV_MYS_Pg2_ILN       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 CCGTAGGGTC TCATGACCGG CTGGTCATGA ACAGCAGTGA GGAGACAGGA GAACCTGTCC
       61 ATAAGATGGC GCGCTGGGGA GAGCTTCCTC AGGTGGGTGA GAACTCGCTA GCTAGAGAGA
      121 ACACTCGGCC TAGGCCTGCC AAGATCACCA AGAAACAGTG TGTGATGATA GATACACTCC
      181 AGAGATGCGA GGATGAACAC ATCTGCACTA AAGAGGAGCT GACTATGCTA CATCCAGATC
      241 TGGTGATTAT GTTTGAGAGC TCTCCGGGCG GCTCTCGAAC GCTCGATGAG GTGCTCGAGA
      301 TGCATCGCGT GAGAGTCACT CGCTCTTACA CGGCTCTGGG CTATATTCTC AAACAGTTTC
      361 CAGAGAACAA GTTCATTAAG CCGGACAACA AAGTGGTCAG GCTTTTGAAC ATCCAGGGCT
      421 ACAATCCTAT TCAGGTCGGC CACTGGGTGG CGACCGTGCT CTCTAAAAAG GCTGGAAAAC
      481 AGAACACACT CTGTTTCTAT GGTCCGGCCA GTACAGGGAA GACCAATCTA GCTAAGGCGA
      541 TCGCGAACGC GGTGAAGGTG TATGGTTGTG TGAACCATCT CAACAAGAAT TTTGTCTTTA
      601 ACGATTGCCA GAGCAAACTC TTGGCCTGGT GGGAAGAGTG TGTGATGCAC AACGATTGGG
      661 TCGAGCCGGC CAAGTGTCTG TGAAAAGAGA
//
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Figure 8.2.2: Partial NS1 sequence of Malaysian PBoV isolate from pig 2 inguinal lymph node

```
LOCUS       PBoV_MYS_Pg8_MLN       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 CCCTAGGCCT CTCCTATGAC CGGCTGGTCA TGAACAGCGC CGAGGAGACA GGGGAGCCTG
       61 TCCATAAGGC GGCGCGCTGG AGCGATCTGC CTGAGGTGGG TGAGAACTCG CTGGCTAGAC
      121 AGAACACTCA GACGAGGCCA ACGAAGCTCA CTAGAAAGCA GCATGTGATG TTAGATACAC
      181 TGCAGAGATG TCAGGAGGAG TTTATCTGTA CCAAGGAGGA GTTAACGATG CTGCATCCAG
      241 ATCTAGTCAT TATGTTTGAG AGTACTCCGA GCGGGTCTCG AACACTCGAA GAGGTGCTCG
      301 AGATGCATCG GGTGAGAGTA ACTAGAACAG AGACAGCTCT GAGTTACATT AGGCGGCAGT
      361 TTCCAGACAG CAAGTTTATC AAGCCGGAGA ATAAGGTGGT CAGGCTTTTG AACATCCAGG
      421 GCTACAATCC TATACAGGCC GGCCACTGGG TGGCGACCGT GCTCTCTAAA AAGGCCGGGA
      481 AACAGAACAC GATCTGTTTC TTTGGTCCGG CTAGTACGGG GAAGACCAAT CTGGCTAAAG
      541 CTATCGCGAA CGCGGTGAAG GTGTATGGTT GTGTCAATCA CCTCAACAAG AGCTTTGTCT
      601 TTAACGATTG CCAGAACAAG CTCATATGCT GGTGGGAGGA GGCGGTGATG CACAACGATT
      661 GGGTAGAGCC GGCCAAGTGT CTGATGGGGG
//
```

Figure 8.2.3: Partial NS1 sequence of Malaysian PBoV isolate from pig 8 mesenteric lymph node

```
LOCUS       PBoV_MYS_Pg9_MLN       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 CCCGTAGGTT GCATGACGTC TGGTCATGAA CAGCAGCGAG GATACCGGGG AGCCGGTACA
       61 CAAATGGCTC GCTGGGGAGA TCTGCCTCAG GTCAGTGAGA ATTCTCTGGC TAGACAGAAC
      121 ACTCAGACCA GGCCTACCAA GATTAATAAG AAGCAGCACC TGATGTTAGA TACCTTGCAG
      181 AGGTGCGAGG AGCAGTTCAT CTGCACCAAG GAGGAGCTGA CTATGCTCCA TCCTGATGTG
      241 GTGATCATGT TCGAGAGCAC GCCTAGCGGG TCGAGGACGC TAGACGAGAT CTTGGACATG
      301 CACCGGGTGA GGATCACGCG CTCGTACACG GCGCTGGGGT ACATCCTCAA GCAGTTTCCG
      361 GAGAGTAGGT TCATCAAGCC GGAGAATAAG GTGGTCAGAC TCTTGAACAT CCAGGGCTAC
      421 AACGCGATGC AGGTCGGCCA CTGGGTGGCG ACCGTGCTAG ACAAGAAGGC CGGGAAACAG
      481 AACACGATCT CGTTCTTTGG TCCGGCCAGC ACGGGCAAGA CCAATCTGGC TAAGGCCATC
      541 GCTCAGGCGG TGAAGGTGTA CGGTTGTGTC AATCACCTCA ACAAGAGCTT CGTCTTTAAC
      601 GATTGCCAGA ACAAGCTCTT GTGCTGGTGG GAGGAGGCGG TGATGCACAA CGACTGGGTG
      661 GAGCCGGCCA AGTGTCTGTG GGGAAAAAAA
//
```

Figure 8.2.4: Partial NS1 sequence of Malaysian PBoV isolate from pig 9 mesenteric lymph node

```
LOCUS       PBoV_MYS_Pg9_SPL       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 AACTTGGAGT TGCATGACGT CTGGTCATGA ACAGCAGCGA GGATACCGGG GAGCCGGTAC
       61 ACAGATGGCT CGCTGGGGAG ATCTGCCTCA GGTCAGTGAG AATTCTCTGG CTAGACAGAA
      121 CACTCAGACC AGGCCTACCA AGATTAATAA GAAACAGCAC CTGATGTTAG ATACCTTGCA
      181 GAGGTGCGAG GAGCAGTTTA TCTGCACCAA GGAGGAGCTG ACTATGCTCC ATCCTGATGT
      241 GGTGATCATG TTCGAGAGCA CGCCTAGCGG GTCGAGGACG CTAGACGAGA TCTTGGACAT
      301 GCACCGGGTG AGGATCACGC GCTCGTACAC GGCGCTGGGG TACATCCTCA AGCAGTTTCC
      361 GGAGAGTAGG TTCATCAAGC CGGAGAATAA GGTGGTCAGG CTCTTGAACA TCCAGGGCTA
      421 CAACGCGATG CAGGTCGGCC ACTGGGTGGC GACCGTGCTA GACAAAAAGG CCGGGAAACA
      481 GAACACGATC TCGTTCTTTG GTCCGGCCAG CACGGGCAAG ACCAATCTGG CTAAGGCCAT
      541 CGCTCAGGCG GTGAAGGTGT ACGGTTGTGT CAATCACCTC AACAAGAGCT TCGTCTTTAA
      601 CGATTGCCAG AACAAGCTCT TGTGCTGGTG GGAGGAGGCG GTGATGCACA ACGACTGGGT
      661 GGAGCCGGCC AAGTGTCTGA TGAGAGAAAC
//
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Figure 8.2.5: Partial NS1 sequence of Malaysian PBoV isolate from pig 9 spleen

```
LOCUS       PBoV_MYS_Pg9_KDN       690 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 TCCCCGAGGT TGGCATGACT GTCTTGGTCA TGAACAGCAG CGAGGATACC GGGGAGCCGG
       61 TAACAAGATG GCTCGCTGGG GAGATCTGCC TCAGGTCAGT GAGAATTCTC TGGCTAGACA
      121 GAACACTCAG ACCAGGCCTA CCAAGATTAA TAAGAAGCAG CACCTGATGT TAGATACCTT
      181 GCAGAGGTGC GAGGAGCAGT TCATCTGCAC CAAGGAGGAG CTGACTATGC TCCATCCTGA
      241 TGTGGTGATC ATGTTCGAGA GCACGCCTAG CGGGTCGAGG ACGCTAGACG AGATCTTGGA
      301 CATGCACCGG GTGAGGATCA CGCGCTCGTA CACGGCGCTG GGGTACATCC TCAAGCAGTT
      361 TCCGGAGAGT AGGTTCATCA AGCCGGAGAA TAAGGTGGTC AGACTCTTGA ACATCCAGGG
      421 CTACAACGCG ATGCAGGTCG GCCACTGGGT GGCGACCGTG CTAGACAAGA AGGCCGGGAA
      481 ACAGAACACG ATCTCGTTCT TTGGTCCGGC CAGCACGGGC AAGACCAATC TGGCTAAGGC
      541 CATCGCTCAG GCGGTGAAGG TGTACGGTTG TGTCAATCAC CTCAACAAGA GCTTCGTCTT
      601 TAACGATTGC CAGAACAAGC TCTTGTGCTG GTGGGAGGAG GCGGTGATGC ACAACGACTG
      661 GGTGGAGCCG GCCAAGTGTC TGATGGGAGG
//
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Figure 8.2.6: Partial NS1 sequence of Malaysian PBoV isolate from pig 9 kidney