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REGULAR ARTICLES

First molecular detection of porcine bocavirus in Malaysia

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Abstract

Several strains of porcine bocaviruses have been reported worldwide since their first detection in Sweden in 2009. Subsequently, the virus has been reported to be associated with gastrointestinal and respiratory signs in weaner and grower pigs. Although Malaysia is host to a self-sufficient swine livestock industry, there is no study that describes porcine bocavirus in the country. This report is the first to describe porcine bocavirus (PBoV) in Malaysian swine herds. PBoV was identified in various tissues from sick and runt pigs using the conventional PCR method with primers targeting conserved regions encoding for the nonstructural protein (NS1) gene. Out of 103 samples tested from 17 pigs, 32 samples from 15 pigs were positive for porcine bocavirus. In addition, a higher detection rate was identified from mesenteric lymph nodes (52.9%), followed by tonsil (37.0%), and lungs (33.3%). Pairwise comparison and phylogenetic analyses based on a 658-bp fragment of NS1 gene revealed that the Malaysian PBoV strains are highly similar to PBoV3 isolated in Minnesota, USA. The presence of porcine bocavirus in Malaysia and their phylogenetic bond was marked for the first time by this study. Further studies will establish the molecular epidemiology of PBoV in Malaysia and clarify pathogenicity of the local isolates.

Keywords Porcine bocavirus · Swine · Detection · Sequencing · Phylogenetic analysis

Introduction

Bocavirus is a genus of the family *Parvoviridae*, subfamily *Parvovirinae*, which has been recognized in veterinary medicine since the early 1960s. Up to date, several bocaviruses have been discovered including the minute virus of canine (MVC) (Binn et al. 1970), bovine parvovirus (BPV) (Chen et al. 1986), human bocavirus (HBoV) (Allander et al. 2005), porcine bocavirus (PBoV) (Blomström et al. 2009), gorilla bocavirus (GBoV) (Kapoor et al. 2010), California sea lion bocavirus (Csl BoV) (Li et al. 2011b), feline bocavirus (FBoV) (Lau et al. 2012), lapine bocaparvovirus (Lanave et al. 2015), bat bocaparvovirus (BtBoV) (Lau et al. 2016), and rat bocaparvovirus (RBoV) (Lau et al. 2017).

The genus *Bocavirus* has gained attention in recent years as studies have linked pathogenic human infections to HBoV (Allander et al. 2005), which may have a zoonotic origin (Schildgen 2013). In Malaysia, HBoV was first detected in a 13-month-old boy with pneumonia and underlying asthma (Etemadi et al. 2012). Subsequently, a study to detect HBoV was conducted locally involving 125 hospitalized children with acute respiratory disease (ARD). The study showed that 5.6% (7/125) of the cases were positive for HBoV, after being tested negative for adenovirus, influenza A and B viruses, parainfluenza viruses (type 1–3), and respiratory syncytial virus by viral isolation and immunofluorescence antibody technique (IFAT). (Tg Rogayah et al. 2014). At a molecular level, the open reading frame (ORF2) of PBoV encodes the viral protein 1 (VP1) and viral protein 2 (VP2) capsid proteins. These viral proteins of PBoV have shown similarities to HBoV in terms of secretory phospholipase (sPLA2) motifs which indicate the infectivity of the virus (Cheng et al. 2010).

PBoV is a recently discovered virus that infects pigs, and based on its molecular biology, the virus was classified within

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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. These countries are Sweden, China, USA, Canada, Mexico, Romania, Hungary, Uganda, Korea, Cameroon, UK (Zhou et al. 2014), and Thailand (Saekhow and Ikeda 2014).

In Malaysia, the swine industry is fairly developed. With a standing pig population of close to 1.4 million heads in 544 farms, the country produces 96% of its pork supply. As of 2013, the entire industry was valued at an estimated RM 1.6 billion (USD 360 million) (Department of Veterinary Services Malaysia 2013).

The clinical specimens used for the detection of 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). The same study shows that monoclonal antibodies against PBoV were produced, thus allowing the possibility of development of an ELISA detection method. The PCR detection method favors targeting the NS1, VP1, and VP2 genes for primer design. In addition, a more sensitive real-time PCR method has also been developed targeting the NP1 gene (Li et al. 2011a).

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

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 studies that lower detection rates in suckling piglets may be due to maternal antibody protection although it has yet to be verified through research (Cadar et al. 2011).

Pathogenically, the significance of PBoV in pig production, pig health, and public health has yet to be determined. However, there is a consistency in findings that PBoV has a significantly higher infection rate in ill 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) (Blomström et al. 2010; Shan et al. 2011; Zhai et al. 2010). The highest detection rate up to date was 88% in pigs with PMWS (Blomström et al. 2010). Additional studies have shown 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). More recent studies suggest the association between PBoV infections with gastrointestinal and respiratory signs. A study carried out found that 29.1 and 48.4% of PBoV-infected pigs have developed respiratory and gastrointestinal signs (Zhang et al. 2015). In 2016, evidence that PBoV infections may be associated with encephalomyelitis in weaner pigs was discovered in Germany when next-generation sequencing (NGS) was used to detect PBoV from the brain tissue of a weaner. Although the possibility of co-infections with *Mycoplasma hyorhinis* or PMWS could not be ruled out, PBoV-specific signals were identified in neurons of the spinal cord using fluorescent in situ hybridization (FISH) (Pfankuche et al. 2016).

Despite the importance of the pig industry in Malaysia in addition to the increasing evidence of the importance of PBoV towards porcine health, there is no information upon the presence of PBoV in Malaysia. Therefore, this study was conducted to describe PBoV in Malaysian swine herds using molecular assay and genetic sequencing methods.

Materials and methods

Sample collection

A total of 103 tissue samples from 17 pigs were used in this study. The samples were sourced from a tissue archive used in a previous study for PCV2/PMWS detections. The samples originated from three local pig farms situated in Perak and Selangor. Also, the convenience sampling method was used. The following tissue samples were used: mesenteric lymph node, submandibular lymph node, inguinal lymph node, spleen, tonsil, lung, kidney, and liver. Details of samples collected are summarized in Table 1.

DNA extraction

The DNA was extracted from each tissue sample using a commercial DNA extraction kit with methods described by the manufacturer (DNeasy® Blood & Tissue Kit 250, Qiagen®, Germany).

Table 1 Data of sampled pigs indicating farm source, pig ID, and age

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

PBoV PCR assay

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

Specific forward 5′–ACAGGCAGCCGATCACTCAC TAT-3′ and reverse 5′-CTCGTTCCTCCCATCAGACACTT-3′ primers were selected based on the conserved region of the PBoV nonstructural protein 1 (NS1) region (Huang et al. 2014; Liu et al. 2014; Zeng et al. 2011; Zhang et al. 2015). The PCR reaction was set up in each tube by adding 10 μl of HotStarTaq® Plus Master Mix, 2×, 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 obtain a final volume of 20 μl for each tube.

Cycling conditions included an initial heat activation at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 30 s, and extension at 72 °C for 1 min. Final extension was at 72 °C for 10 min. PCR products were separated by electrophoresis on 1.5% agarose gel, and a band at the 690-bp position suggested the presence of PBoV.

Sample analysis

PCR samples determined as positive were tabulated according to individual tissues and results expressed as percentage of positive samples out of the total number tested.

Sequencing and phylogenetic analyses

PCR products from six positive tissue samples were conveniently selected and were purified using a commercial PCR purification kit, Wizard® SV Gel, and PCR Clean-Up System (Promega, USA) with methods as described by the manufacturer. Molecular sequencing was performed using the Sanger sequencing method by Bioneer (South Korea). Both forward and reverse primers were used for the sequencing. Partial NS1 strands from the positive PCR products were uploaded into the GenBank database with the following accession numbers (Table 2).

The assembled partial NS1 sequences were subjected to basic local alignment search tool (BLAST) searches to confirm that they are similar to other PBoV isolates, and identify the percentage of sequence homology. A total of 10 reference sequences from the GenBank with high sequence similarities were downloaded for use in phylogenetic analysis (Table 3).

An unrooted phylogenetic tree was constructed using the maximum probability in the MEGA7 software after multiple alignments (Kumar et al. 2016). The tree reliability was accessed using 1000 bootstrap replicates and sequences of local PBoV were compared with PBoV isolates from USA, China, UK, and Hong Kong. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Pairwise comparison was performed between 5 local PBoV isolates and 10 reference isolates. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were deleted. There was a total of 657 positions in the final dataset.

Results

PBoV detection by PCR assay

Out of the 103 tissue samples tested from 17 pigs, 32 were found to be positive for PBoV based on molecular PCR screening (Table 4). This shows a considerable prevalence of porcine bocavirus in Malaysian swine herds. An example of positive results is shown in Fig. 1.

It was observed that the mesenteric lymph node showed the highest positive detection rate of 45.5% (Table 5), followed by tonsils, 30.0%, and lungs 25.0%. It was also a notable finding that PBoV was detected from kidney tissues of two pigs.

Table 2 Partial NS1 gene accession numbers of Malaysian PBoV isolates

Pig ID	Organ	Sequence ID	Accession no.
Pg 9	Mesenteric lymph node	PBoV_MY/UPM001	KX686996
Pg 9	Spleen	PBoV_MY/UPM002	KX686997
Pg 9	Kidney	PBoV_MY/UPM003	KX686998
Pg 2	Inguinal lymph node	PBoV_MY/UPM004	KX686999
Pg 8	Mesenteric lymph node	PBoV_MY/UPM005	KX687000

Table 3 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.	KC514535.1	USA	Huang et al. 2014	PBoV3_Minnesota(33)_USA
4.	KC514531.1	USA		PBoV3_Minnesota(34)_USA
5.	KC514540.1	USA		PBoV3_Minnesota(14)_USA
6.	KC514561.1	USA		PBoV3_Minnesota(76)_USA
7.	KC514542.1	USA		PBoV3_Minnesota(39)_USA
8.	JF512472.1	UK	McKillen et al. 2011	PBoV3V_Northern Ireland
9.	JF429834.1	Hong Kong	Lau et al. 2011	PboV3_HK
Genotype: porcine bocavirus 5				
10.	JN831651.1	China	Li et al. 2012	PBoV5_Jiangsu_China

Table 4 Data of sampled pigs indicating farm source, pig ID, age, and positive PBoV detection

No.	Farm	Age (days)	PBoV detection
1.	Farm X	60	+
		60	+
		60	+
		40	+
		40	+
		40	+
2.	Farm Y	80	+
		80	+
		7	–
3.	Farm Z	21	+
		60	+

Phylogenetic analysis of Malaysian isolate of PBoV

The unrooted phylogenetic tree showing relationship between Malaysian porcine bocaviruses with reference isolates was generated using the neighbor-joining (NJ) method (Fig. 2).

Malaysian PBoV strains were shown to be divided into three different clusters. Sequences from pig 2 (PBoV MY/UPM004) are grouped with PBoV3 and PBoV5 strains from China and USA. This group shows discrepancies in PBoV strains as it contains PBoV3 and PBoV5 strains. Pig 8 (PBoV MY/UPM005) is grouped with PBoV3 strain from USA, Hong Kong, and Northern Ireland. Three sequences obtained from pig 9 (PBoV MY/UPM001-003) form a closely related group among themselves as well as with PBoV3 strains from Minnesota, USA. Therefore, it is highly suggestive that the PBoV isolate from pig 9 is of the PBoV3 strain.

Pairwise comparison shows nucleotide difference ranging from 0.8 to 19.2% among local PBoV isolates. However, between local PBoV and other PBoV isolates, the highest nucleotide difference is 21.8% which is between PBoV_MY/UPM003 and the PBoV3 isolate from Hunan, China. The closest sequence similarity was observed between local PBoV sequence PBoV_MY/UPM002 and PBoV isolate from Minnesota, USA (PBoV_Minnesota(34)_USA), with sequence similarity of 98.2% (Fig. 3).

Discussion

This study has described for the first time porcine bocavirus in swine herds in Malaysia. Albeit a small sample size was obtained through the convenience sampling method, a high prevalence of 90.9% was observed.

Fig. 1 Pigs 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

Table 5 Difference in prevalence between tissue samples

No.	Organ	Number of positive organ(s)/number of organs tested (%)	
1.	Mesenteric lymph node	9/17	(52.9)
2.	Submandibular lymph node	0/4	(0.0)
3.	Inguinal lymph node	4/16	(25.0)
4.	Spleen	4/17	(23.5)
5.	Tonsil	6/16	(37.5)
6.	Lung	5/15	(33.3)
7.	Kidney	2/10	(20.0)
8.	Liver	2/8	(25.0)
	Total	32/103 (31.1)	

Overall, this positive detection rate is consistent with other publications which report detections up to 88% in sick pigs (Zhou et al. 2014). This result is also supported by reported discoveries 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 mesenteric lymph node had the highest detection rate (45.5%) followed by the tonsil (30%). This is also consistent with other reported discoveries (Liu et al. 2014). This may suggest that the pathogenesis of porcine bocavirus infections may involve the lymphoid tissues and gastrointestinal system (Gunn et al. 2015).

On another note, based on current knowledge of reviewed articles, this is the first study to specifically detect PBoV from kidney tissue samples. Kidney samples from two pigs were positive for PBoV based on PCR results and were successfully sequenced. Although some studies have reported positive PBoV results for pooled organs which include kidney tissue (Choi et al. 2014), no report has been specifically done upon kidney tissue. This finding is supported by a previous study which suggested that PBoV can be cultured in primary pig kidney cell lines (McKillen et al. 2011). The detection of PBoV in kidney tissue suggests that the virus is able to replicate within kidney cells, thus causing renal pathology.

The PCR assay used in this study will count for rapid screening and monitoring of pig heard for bocavirus; moreover, the NS1 gene-related PCR is commonly used in other recent studies worldwide due to the conserve nature of the gene segment (Zeng et al. 2011; Huang et al. 2014; Liu et al. 2014; 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.

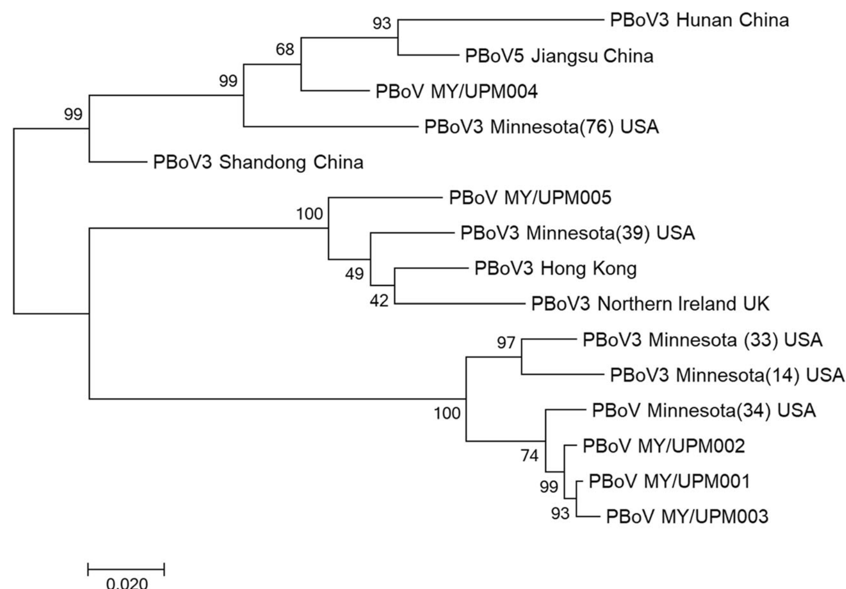

Fig. 2 Molecular phylogenetic analysis by the maximum probability method. The evolutionary history was inferred by using the maximum probability method based on the Jukes-Cantor model (Jukes and Cantor 1969). The tree with the highest log probability (− 3135.6450) is shown. The percentage of trees in which the associated taxa are clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite probability approach, and then selecting the topology with superior log probability value

Fig. 3 Appraisals of evolutionary divergence between sequences. The number of base differences between sequences is shown. The analysis involved 15 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding. All positions containing gaps and missing data were eliminated. There was a total of 658 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016)

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	12	14
1 PBoV_MY/UPM004														
2 PBoV_MY/UPM005	0.158													
3 PBoV_MY/UPM003	0.191	0.176												
4 PBoV_MY/UPM001	0.188	0.173	0.008											
5 PBoV_MY/UPM002	0.184	0.169	0.012	0.008										
6 PBoV3_Hong_Kong	0.158	0.049	0.184	0.181	0.173									
7 PBoV3_Northern_Ireland_UK	0.170	0.067	0.187	0.184	0.182	0.052								
8 PBoV3_Hunan_China	0.085	0.181	0.217	0.214	0.213	0.176	0.195							
9 PBoV3_Shandong_China	0.082	0.128	0.163	0.160	0.158	0.126	0.144	0.099						
10 PBoV3_Minnesota_(33)_USA	0.178	0.184	0.058	0.055	0.053	0.193	0.196	0.207	0.152					
11 PBoV3_Minnesota(39)_USA	0.149	0.059	0.179	0.176	0.175	0.046	0.055	0.173	0.116	0.179				
12 PBoV3_Minnesota(76)_USA	0.068	0.173	0.201	0.198	0.193	0.169	0.181	0.117	0.090	0.190	0.161			
13 PBoV5_Jiangsu_China	0.053	0.164	0.210	0.207	0.202	0.161	0.176	0.065	0.091	0.204	0.158	0.090		
14 PBoV3_Minnesota(14)_USA	0.185	0.184	0.062	0.059	0.058	0.185	0.181	0.208	0.161	0.035	0.181	0.193	0.201	
15 PBoV_Minnesota(34)_USA	0.181	0.172	0.024	0.020	0.018	0.169	0.181	0.204	0.149	0.053	0.167	0.187	0.196	0.064

Phylogenetic analysis revealed that the PBoV isolate from pig 9 (PBoV MY/UPM001-003) has the highest sequence identity to PBoV3 reference isolates from Minnesota (79.9–98.2%), which also suggests that the PBoV isolated from pig 9 is a PBoV3 genotype. Interestingly, sequence data from pig 9 forms a distinct group with Minnesota PBoV3 isolates. In addition, the PBoV isolate from pig 8 mesenteric lymph node (PBoV MY/UPM005) also forms a distinct group with PBoV3 reference isolates from Minnesota, Hong Kong, and Northern Ireland. Therefore, it is also suggested that pig 8 PBoV isolate is likely of PBoV3 genotype. Close identity between American and Malaysia isolates might be associated with translocation of breeding pigs from the USA into Malaysia, considering that trade exists between the two countries. Pig 2 inguinal lymph node isolate (PBoV MY/UPM004) requires further study in order to be genotyped. Although it exhibits high nucleotide identity to PBoV3 Minnesota, Hunan, and Shandong isolates, it shares high nucleotide identity to 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 reliability in genotyping and grouping of local PBoV isolates. It is also important to conduct a nationwide survey in order to establish the molecular epidemiology and understand the genetic diversity of local PBoV in Malaysia to guide control and preventions.

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Compliance with ethical standards

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

Conflict of interest The authors declare that they have no conflict of interests.

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