UNIVERSITI PUTRA MALAYSIA

DETECTION OF BOCAVIRUS IN MALAYSIAN CATS AND DOGS

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DETECTION OF BOCAVIRUS IN MALAYSIAN CATS AND DOGS

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CERTIFICATION

It is hereby certified that we have read this project paper entitled “Detection of Bocavirus in Malaysian Cats and Dogs”, by Lee Chee Yien 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

My family,

Grandfather

Grandmother

Father

Mother

Brother, Sister

Friends

And to all those who have made me who I am today.

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LIST OF ABBREVIATIONS

%	Percent
µl	Microliter
µM	Micromolar
℃	Degree Celsius
aa	Amino acid
ATPase	Adenosine triphosphatase
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BPV	Bovine parvovirus
CBoV	Canine bocavirus
CBoV2	Canine bocavirus 2
CBoV3	Canine bocavirus 3
CMV	Canine minute virus
Csl BoV	California sea lion bocavirus
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FBoV	Feline bocavirus
FBoV2	Feline bocavirus 2
g	Gram
GBoV	Gorilla bocavirus
HBoV	Human bocavirus

HI	Hemagglutination-inhibition
IACUC	Institutional Animal Care and Use Committee
ICTV	International Committee on Taxonomy of Viruses
kbp	Kilobase pairs
mg	Milligram
mg/kg	Milligram per kilogram
$MgCl_2$	Magnesium chloride
min	Minutes
ml	Milliliter
mm	Millimeter
mM	Millimolar
MVC	Minute virus of canine
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
NP1	Nuclear phosphoprotein
NS1	Nonstructural protein
nt	Nucleotide
NTC	No template control
ORF	Open reading frame
PBoV	Porcine bocavirus
PCR	Polymerase Chain Reaction
RNase	Ribonuclease
TAE	Tris-acetate-ethylenediaminetetraacetic acid
U	Unit
UPM	Universiti Putra Malaysia

USA	United States of America
UV	Ultraviolet
v	Version
V	Volts
VP1	Viral protein 1
VP1u	Viral protein 1-unique
VP2	Viral protein 2
WRCC	Walter Reed canine cell
$\times g$	Relative centrifugal force

ABSTRAK

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

PENGESANAN BOCAVIRUS DALAM KUCING DAN ANJING MALAYSIA

Oleh

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2016

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Sejak kebelakangan ini, terdapat penemuan novel kumpulan felin bocavirus (FBoV) dan kanin bocavirus (CBoV) yang semakin meningkat di seluruh dunia. Kucing dan anjing mempunyai kaitan yang rapat dalam kehidupan manusia. Namun, tiada penyelidikan berkenaan pengesanan FBoV dan CBoV di Malaysia. Oleh itu, projek ini bermatlamat untuk mengesan bocavirus dalam kucing dan anjing Lembah Klang. Menggunakan teknik persampelan mudah, 4 kucing dan 37 anjing disampel daripada 2 pusat perlindungan haiwan yang berlainan. Sampel tisu merangkumi nod limfa submandibular, paru-paru, buah pinggang, nod limfa mesenteric, usus dan nod limfa inguinal diperoleh daripada nekropsi haiwan diproses dan diuji dengan kaedah PCR konvensional menggunakan primer spesifik menyasar kepada gen *nonstructural* 1 (NS1). Berdasarkan analisa PCR, 100% (4/4) kucing dan 24.3% (9/37) anjing masing-masing positif untuk FBoV dan CBoV.

Penjujukan nukleotid separa NS1 telah dilaksanakan untuk 2 produk PCR FBoV dan dibandingkan dengan pencilan rujukan. Analisa filogenetik awal menunjukkan bahawa pencilan FBoV Malaysia adalah berbeza dengan pencilan rujukan. Penyelidikan lanjut berkenaaan prevalens dan patologi harus dilakukan untuk memahami sepenuhnya patogenesis FBoV dan CBoV ke atas hos. Kesimpulannya, Malaysia merupakan negara yang kelima dalam pengesanan FBoV dan CBoV.

Kata kunci : felin bocavirus, kanin bocavirus, PCR, penjujukan, 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 BOCAVIRUS IN MALAYSIAN CATS AND DOGS

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2016

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Recently, there have been an increasing number of novel groups of feline bocavirus (FBoV) and canine bocavirus (CBoV) discoveries around the world. Despite that cats and dogs are highly associated in human living environment, no study has been done to determine the presence of FBoV and CBoV in Malaysia. Thus, this study aimed to detect bocavirus in local cats and dogs in Klang Valley. Using convenience sampling method, 4 cats and 37 dogs were chosen from animal shelters. Tissue samples consisted of submandibular lymph node, lung, kidney, mesenteric lymph node, intestine, and inguinal lymph node obtained from necropsies were processed and subjected to conventional PCR using specific primers targeting the conserved nonstructural protein 1 (NS1) gene. Based on PCR analyses, 100% (4/4) and 24.3% (9/37) of cats and dogs were positive for FBoV and CBoV, respectively. Partial nucleotide sequencing of the NS1 gene were performed on 2 PCR products of FBoV and comparison of sequences were performed with published isolates. Preliminary phylogenetic analysis revealed that Malaysian FBoV isolates are distinct from reference isolates. Further study on the prevalence and pathology of FBoV and CBoV should be carried out on cats and dogs to fully understand the pathogenesis of FBoV and CBoV in the host. In conclusion, Malaysia is the fifth country to detect FBoV and CBoV.

Key words: feline bocavirus, canine bocavirus, PCR, sequencing, phylogenetic analysis

1.0 INTRODUCTION

Bocavirus is a genus classified within the family of *Parvoviridae*, subfamily of *Parvovirinae*. The name of the genus Bocavirus is a combination of the initials of the two earliest bocavirus discovered; which are the bovine parvovirus (BPV) and canine minute virus (CMV). To date, bocaviruses that have been discovered include canine minute virus (CMV) (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*., 2010a), feline bocavirus (FBoV) (Lau *et al*., 2012), and California sea lion bocavirus (Csl BoV) (Li *et al*., 2011).

The genus Bocavirus came to significant when a group of Swedish scientist identified human bocavirus (HBoV) in pooled samples from the respiratory tract causing respiratory disease in children (Allander *et al*., 2005). Over the decade, HBoV have been reported worldwide including Malaysia, when it was first detected in a 13-month-old boy suffering from pneumonia and underlying asthma (Etemadi *et al*., 2012). Some of the bocaviruses have been associated with diarrheal and respiratory illness in human and other mammals such as dogs, cattle, and pigs (Allander *et al*., 2005; Kapoor *et al*., 2012; King *et al.,* 2011) even though pathogenicity is unknown (Martin *et al*., 2009).

Phylogenetic analyses show that HBoV groups have CMV and BPV in clade, in which it shares a sequence identity of 44.1% and 42.6% respectively (Manteufel & Truyen, 2008). According to Zeng *et al*. (2011), PBoV exhibited sequence identities of 38.0 – 54.7% to HBoV. Since both HBoV and animal bocavirus are closely related, studies on canine bocavirus or other animal bocaviruses could possibly emulate studies that had been conducted for HBoV; whereby in turn potentially serves as an animal study model for further HBoV research.

Canine minute virus (CMV) or also referred as minute virus of canine (MVC) for certain journals, was the first known bocavirus infecting dogs isolated from fecal sample of a clinically healthy dog (Binn *et al*., 1970). It has been proven to infect fetuses and cause reproductive disorders. Young animals may suffer severe respiratory infection (Carmichael *et al*., 1994); while adults may be subclinically infected or show mild diarrhea (Manteufel and Truyen, 2008). Studies on the seroprevalence of CMV suggest that this virus is widespread in many countries (Manteufel and Truyen, 2008).

The second species of dog bocavirus or known as canine bocavirus (CBoV) was identified in 2012 in respiratory samples from diseased and healthy dogs (Kapoor *et al.*, 2012). Three distinctly different strains of CBoV have been identified, provisionally named CBoV (Kapoor *et al*., 2012), CBoV2 (Bodewes *et al.,* 2014) and CBoV3 (Li *et al*., 2013). Unlike CMV, pathogenic potential of CBoV remains unknown but it is possibly associated to respiratory disease (Kapoor *et. al*., 2012) and gastrointestinal disease (Bodewes *et al.,* 2014).

Feline bocavirus (FBoV) is a newly discovered bocavirus that infects domestic cats at was first described in Hong Kong in 2012 (Lau *et al*., 2012). Later, another feline bocavirus denoted as FBoV2 was identified in Portugal (Ng *et al*., 2014), which is distinctly different from FBoV found in Hong Kong. Since then, no other study that demonstrates the presence of bocavirus in cats have been carried out and the pathogenicity of feline bocaviruses remains unknown. The clade of feline bocavirus (FBoV and FBoV 2) is most closely related to CMV, CBoV and CsL BoV (Ng *et al*. 2014).

In year 2015, with the positive detection of PBoV in pig population using conventional polymerase chain reaction (PCR) method, this marks the first report of animal bocavirus in Malaysia (Daniel, 2015; Daniel *et al*., 2015); hence, proving the potential presence of other animal bocaviruses in Malaysia. Despite cats and dogs are the most common domestic pets worldwide that share close habitat with human; no study has been carried out to determine the presence of bocavirus in cats and dogs in Malaysia. The hypotheses for this study is feline and canine bocaviruses are present in Malaysia and could be detected in their tissue samples. Therefore, the main objective of this study is to determine the presence of bocavirus in cats and dogs in Malaysia using conventional PCR method.

2.0 LITERATURE REVIEW

2.1 Virus Structure

The genus Bocavirus within the subfamily Parvovirinae is a small non-enveloped, autonomously replicating, single-stranded, deoxyribonucleic acid (DNA) virus with an icosahedral capsid. The genome length is about 5.4 kbp (Manteufel and Truyen, 2008). Bocaviruses are unique among parvoviruses as they contain a third open reading frame (ORF) between the non-structural and structural-coding regions (Kapoor *et al*., 2010b; Manteufel and Truyen, 2008; Qiu *et al*., 2008).

Bocaviruses generally consist of three open reading frames (ORFs), which are ORF1, ORF2 and ORF3. ORF1 encodes a 644 - 778 amino acid (aa) and predicted 804 aa nonstructural protein 1 (NS1) for canine bocavirus and feline bocavirus respectively (Kapoor *et al*., 2012; Lau *et al*., 2012, Li *et al.*, 2013). Within NS1 of both feline bocavirus and canine bocavirus, conserved motifs associated with rolling-circle replication, helicase and adenosine triphosphatase (ATPase) were identified (Lau *et al*., 2012).

ORF2 encodes 689 - 712 aa overlapping the viral protein (VP1/VP2) capsid ORFs of canine bocavirus and feline bocavirus respectively (Kapoor *et al*., 2012; Lau *et al*., 2012; Li *et al.*, 2013). The VP1-unique (VP1u) regions of both feline bocavirus and canine bocavirus also contained phospholipase A_2 motifs required for parvovirus infectivity, with the presence of a calcium-binding loop and catalytic residues (Lau *et al*., 2012). As has been shown for human parvovirus B19 and several other parvoviruses, phospholipase A_2 activity is of crucial importance for virus release from lysosomes after cell entry and therefore important for initiation of virus replication within the nucleus (Zádori *et al*., 2001).

ORF3 encodes a 194 - 195 aa and 218 aa nuclear phosphoprotein 1 (NP1) in canine bocavirus and feline bocavirus respectively (Kapoor *et al*., 2012; Lau *et al*., 2012; Li *et al*., 2013). The function of the unique bocavirus protein, NP1, remains unclear, although it has been found to be essential for virus replication in CMV (Sun *et al*., 2009).

In addition to the three ORFs, a strain of CBoV genomes found in Hong Kong encode an additional ORF, ORF4, immediately downstream of ORF1, encodes a 144 aa protein, which overlaps with the predicted second exon of NS1 (Lau *et al*., 2012). This ORF was not found in other known bocaviruses, including CBoV strain Con-161 (Lau *et al*., 2012). Figure 2.1 shows the comparative genome organization of FBoV, CBoV strain Con-161 and CBoV strain HK882F.

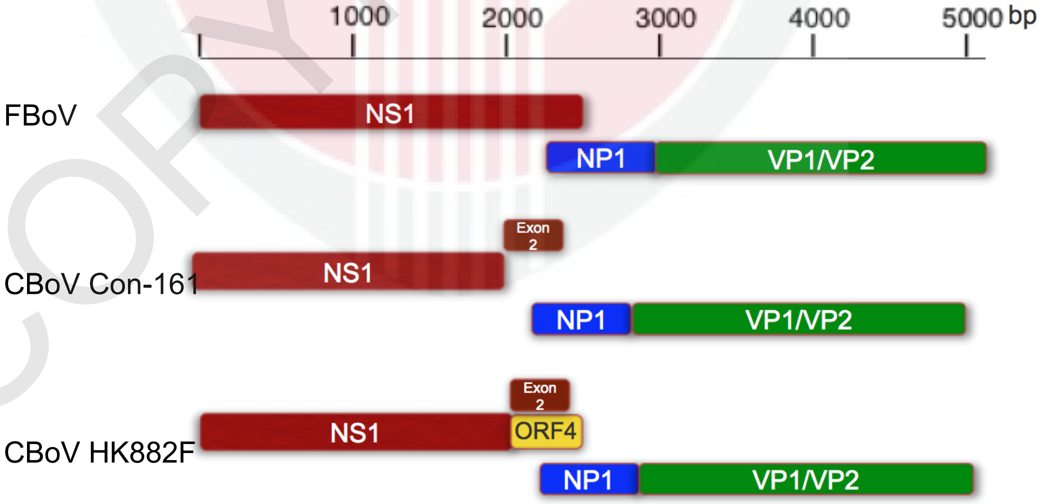


Figure 2.1 : Comparative genome organization of FBoV, CBoV strain Con-161 and CBoV strain HK882F. Box labeled ‘Exon 2’ represent the second exon of the NS1 protein. Red boxes represent the NS1 protein, blue boxes represent NP1 protein and green boxes represent the overlapping VP1 and VP2 protein. Yellow box represent a unique additional ORF found in CBoV isolate of Hong Kong.

2.2 Taxonomy and Nomenclature

According to the latest database updated in 2013 of International Committee on Taxonomy of Viruses (ICTV), the genus Bocavirus was renamed to Bocaparvovirus even though subsequent published journals still refer the genus as Bocavirus. Following that, canine minute virus (CMV) was renamed to carnivore bocaparvovirus 1; and added to the list was canine bocavirus 1 (CBoV) assigned as carnivore bocaparvovirus 2; and feline bocavirus (FBoV) assigned as carnivore bocaparvovirus 3. 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

Studies on the seroprevalence of CMV suggest that these viruses are widespread in canine populations in many countries (Manteufel and Truyen, 2008). As to 2008, countries with seroprevalence of CMV are USA, Japan, Korea, Turkey and Italy (Manteufel and Truyen, 2008). The seroprevalence of CMV vary among countries ranges from 5% - 89.7%. However, these figures are not representative of the true prevalence, as sampling methods and health status of dogs differ between studies. In Italy the seroprevalence differs from region to region, for example in Palermo 30.5% of investigated dogs had CMV-specific antibodies (Pratelli *et al.,* 1999) but in Naples only 9.5% of the dogs were seropositive (Rinaldo *et al*., 2000).

To date, CBoV are only reported in Hong Kong (Lau *et al*., 2012), United States of America (USA) (Kapoor *et al*., 2012; Li *et al*., 2013), Germany (Bodewes *et al*., 2014) and Korea (Choi *et al*., 2015). The prevalence of CBoV is yet to be determined as only two countries have studied using a population of dogs; showing prevalence ranges from 4.1% - 23%; which is not representative to true prevalence at all.

For FBoV, only 4 countries reporting the presence of virus namely Hong Kong (Lau *et al*., 2012), Portugal (Ng *et al*., 2014), USA (Zhang *et al.,* 2014) and Belgium (Garigliany *et al.*, 2016). In Portugal and Hong Kong, both countries were able to detect the virus from fecal sample with positive rates of 6% (Ng *et al*., 2014) and 7.2% (Lau *et al*., 2012) respectively.

2.4 Detection Methods

Canine minute virus was first diagnosed in fecal and blood samples of dogs by observing cytopathic effects characterised by large intranuclear inclusions bodies in CMV-infected Walter Reed canine cell (WRCC) line and subjected to serum neutralization test as well as hemagglutination-inhibition test (HI) (Binn *et al*., 1970). Diagnosis of CMV infection can be confirmed by various methods including virus isolation, polymerase chain reaction (PCR), immunofluorescence and hemagglutination (Manteufel and Truyen, 2008). Indirect hemagglutination assays and indirect immunofluorescence assays are still considered the gold standard for antibody detection of CMV (Manteufel and Truyen, 2008).

In a study conducted in Hong Kong, CBoV was detected in fecal samples, nasal samples, urine samples and blood samples using nested PCR. Same goes for FBoV, the virus was isolated from fecal samples, nasal samples, urine samples, blood samples, and an additional organ of kidney, using nested PCR (Lau *et al*., 2012). The latter study uses three sets of primer pairs. Forward primer 5'–GCCAGCACNGGNAARACMAA-3' and reverse primer 5'-CATNAGNCAYTCYTCCCACCA-3' targeted to a 141 bp fragment of the NS1 gene of known bocavirus including HBoV, BPV and CMV. Specific primers included forward primer 5'-TCTACAAGTGGGACATTGGA-3' and reverse primer 5'-GAGCTTGATTGCATTCACGA-3' targeted at 133bp fragment of NS1 to detect FBoV while forward primer 5'-AGGTCGGCCACTGGCTGT-3' and reverse primer 5'-CAGCTTAACGGCATTCACTA-3' targeted a 128bp fragment of NS1 to detect CBoV (Lau *et al*., 2012). In this study, highest detection rate for CBoV were found in the urine samples (6.3%), followed by nasal samples (5.1%), fecal samples (4.6%) and blood samples (0.3%). As for FBoV, highest detection rate was found in fecal samples (7.2%), followed by kidney samples (2.0%), urine samples (1.6%), blood samples (0.8%) and nasal samples (0.3%) (Lau *et al.*, 2012).

In recent study conducted on the detection of PBoV in Malaysia, specific primers targeting 690bp fragment of NS1 successfully detect PBoV in pig population (Daniel, 2015; Daniel *et. al.,* 2015). The highest detection rate according to organ samples for PCoV were found in mesenteric lymph node (45.5%), followed by tonsil (30%), liver (25%), and lung (22.2%) (Daniel, 2015; Daniel *et al*., 2015).

2.5 Clinical Manifestations and Pathology

Clinical signs of CMV are clearly understood as compared to CBoV and FBoV. This virus causes abortions in bitches and severe respiratory infections in newborn puppies, but infections are mostly subclinical in adult animals (Carmichael *et al*., 1994). Clinical signs associated with CMV infection depend on the time of infection during pregnancy and may represent embryonal resorption, abortion, birth deformities, still birth and birth of weak puppies (Carmichael *et al*., 1994). Older dogs remain subclinical or show mild diarrhea (Manteufel and Truyen, 2008).

As for CBoV, the pathogenicity is not clearly understood, however, it has been associated with respiratory infection. Study also demonstrated higher prevalence in sick animals suggesting that these viruses are more likely to infect diseased animals as opportunistic infections or that they can cause or enhance the pathology of other infections (Kapoor *et al*., 2012). It is also associated with enteric infection (Bodewes *et al.,* 2014).

So far there is no study demonstrating the pathogenicity of FBoV and is yet to be discovered. However, as FBoV could be isolated together with several viruses, it may be associated with co-infections (Ng *et a.l*, 2014). FBoV and CBoV were detected in different organ samples from the respective animals, suggesting a wide tissue tropism. While FBoV was mainly detected in fecal samples in cats, CBoV demonstrated the highest detection rate in urine samples, suggesting that the latter virus may cause more systemic infections in dogs rather than respiratory disease alone (Lau *et al*., 2012).

3.0 MATERIALS AND METHODS

3.1 Animals

A total of 37 dogs were identified at animal shelter located in Selangor area. They consisted of female and male adult, local breed dogs. 26 of the dogs had diarrhea while 11 of them are clinically healthy.

A total of 4 cats were identified at a different animal shelter located in Selangor area. They consist of all adult, local breed cats. All of the cats were clinically ill, and only one of them had diarrhea.

3.2 Sample Selection

The dogs were euthanized using pentobarbitone (Dolethal®, France) at 100 mg/kg, intravenously performed by a veterinarian of the animal shelter. Necropsy was performed and tissue sampling was done at the post mortem laboratory of Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM). Tissues samples collected consisted of mesenteric lymph node, submandibular lymph node, inguinal lymph node, and lungs.

The cats were euthanized using pentobarbitone at 100 mg/kg, intravenously performed by a veterinarian of the animal shelter. Necropsy was performed and tissue sampling was done on site. Tissue samples collected include mesenteric lymph node, submandibular lymph node, kidney, lungs and intestine.

Both dogs and cats sampling were done using convenience sampling method, which depends on the availability of euthanized animals in the animal shelter. This research project was approved by the Institutional Animal Care and Use Committee (IACUC), with reference number : UPM/IACUC/AUP – R063/2014 dated 7th January 2015.

3.3 Tissue Processing and Sample Pooling

One gram of each tissue sample was minced using a sterile pair of scissors and then transferred into 15 ml centrifuge tube (Corning, USA) containing 5 ml of phosphate buffer solution (PBS) pH 7.2. The suspension was then homogenized (PRO250, PRO Scientific Inc., USA) and centrifuged at 500 × *g* for 5 min (Universal 32 R, Hettich Zentrifugen, Germany). About 200 µl of the supernatant was pipetted into 1.5ml microcentrifuge tube (Eppendorf, Germany). To pool samples, 200 µl of each tissue sample of the same animal was pipetted into a 1.5 ml microcentrifuge tube and mixed through vortexing (Finevortex, Korea).

3.4 DNA Extraction

The DNA from each sample was extracted using a commercial DNA extraction kit according to the manufacturer's instruction (DNeasy® Blood and Tissue Kit 250, Qiagen, Germany). About 200 µl of sample was pipetted into a 1.5 ml microcentrifuge tube 20 µl of proteinase K (Qiagen, Germany) and 200 µl of Buffer AL was added into each microcentrifuge tube and mixed thoroughly by vortexing. The mixture was incubated in an incubator (WiseTherm, Korea) at 56 ℃ for 10 min. Next, 200 µl of 96% ethanol (EssenHaus, 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 × *g* for 1 min (Mikro 22 R, Hettich Zentrifugen, Germany). The collected tube and flow through was discarded. The spin column was then put in a new 2 ml collection tube. 500 µl Buffer AW1 was added into the middle of spin column and centrifuged at 6000 × *g* for 1 min. The collection tube and flow through was discarded and spin column was placed into a new collection tube. 500 µl Buffer AW2 was added into the center of spin column and centrifuged at 20,000 × *g* for 4 min and the collection tube and flow through was discarded. Next, the spin column was placed into a new 1.5 ml microcentrifuge tube. Elution was performed 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.

3.5 Measurement of DNA Concentration

The final DNA concentration was determined by spectrophotometer. Firstly, 100 µl of deionized water was placed in a cuvette and placed in photometer for blank. Next, 2 µl of extracted DNA was diluted with 98 µl of deionized water in a new cuvette and placed in a photometer (BiophotometerPlus, Eppendorf, Germany).

3.6 Primer Selection

Primers were selected from several published journals. Within NS1 of both CBoV and FBoV, conserved motifs associated with rolling-circle replication, helicase and ATPase were identified. It had been proven that the detection of bocavirus sequences in various tissue samples of cats and dogs could be done by using specific primers designed to target NS1 gene of feline bocavirus and canine bocavirus (Mochizuki *et al*., 2002; Lau *et al.*, 2012). The primer sets are listed in Table 3.1.

Table 3.1 : Primer set for detection of bocavirus by conventional PCR assay

Virus	Primer	Sequence 5' → 3'	Detection region	Product size	Reference
CMV	Forward	5'-GCGAATTCGTGGTATGCACCTATATACAACGGAC-3'	VP1/ VP2 gene, nucleotide 3506-3539	1266bp	Mochizuki *et al*., 2002
	Reverse	5'-CGGGATCCGGATGCGACATAGGCAGAGTTCCATC-3'			
CBoV	Forward	5'-AGGTCGGCCACTGGCTGT-3'	NS1 gene, nucleotide1528-1655	128bp	Lau *et al*., 2012
	Reverse	5'-CAGCTTAACGGCATTCACTA-3'			
FBoV	Forward	5'-TCTACAAGTGGGACATTGGA-3'	NS1 gene, nucleotide1568-1700	133bp	Lau *et al*., 2012
	Reverse	5'-GAGCTTGATTGCATTCACGA-3'			

3.7 Polymerase Chain Reaction

PCR reaction was performed using HotStarTaq® *Plus* Master Mix Kit (Qiagen, Germany). HotStarTaq® *Plus* Master Mix, 2× contains HotstarTaq Plus DNA Polymerase (5 U/µl), PCR Buffer with 1.5mM magnesium chloride ($MgCl_2$), and 200 µM of each deoxynucleotide triphosphates (dNTP).

The PCR reaction was set up in each PCR tube by adding 10 µl of HotStarTaq® *Plus* Master Mix, 1 µl of forward primer, 1 µl of reverse primer, and 2-3 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 PCR amplification was performed using Mastercycler Gradient thermal cycler (Eppendorf, Germany). The cycling conditions were carried out as described in Table 3.2 and Table 3.3.

Table 3.2 : Optimized cycling conditions of conventional PCR assay for detection of canine bocavirus and feline bocavirus.

Step	Time	Temperature
Initial heat activation	5 min	95 ℃
Denaturation	1 min	94 ℃
Annealing	1 min	50 ℃
Extension	1 min	72 ℃
Number of cycles	40 cycles	-
Final extension	10 min	72 ℃

Table 3.3 : Optimized cycling conditions of conventional PCR assay for detection of canine minute virus.

Step	Time	Temperature
Initial heat activation	5 min	94 ℃
Denaturation	30 sec	94 ℃
Annealing	30 sec	55 ℃
Extension	30 sec	72 ℃
Number of cycles	30 cycles	-
Final extension	5 min	72 ℃

3.8 Agarose Gel Electrophoresis and Photography

Agarose (Vivantis, Malaysia) was used to prepare 2.0% (w/v) agarose gel for electrophoresis. 50 ml of tris-acetate-ethylenediaminetetraacetic acid buffer (TAE buffer) and 1.0 g of agarose powder was mixed thoroughly and dissolved in a microwave (Panasonic, Malaysia). The agarose solution was cooled about 60℃ and 2.5 µl of nuclei acid staining solution (Redsafe™, Intron, Korea) was added and mixed thoroughly by swirling the glass bottle (Schott, Germany). Then, the solution was poured into a gel mold with well-forming comb and let the solution to solidify, forming a gel. The gel was the removed from the mold carefully and placed into an electrophoresis tank (Bio-Rad, USA). TAE buffer was added into the tank until the buffer immersed the surface of the gel by approximately 5 mm. Subsequently, 5 µl of PCR product from each PCR tube was pipetted out and mixed individually with 1 µl of DNA loading dye (Intron, Korea) and loaded into the wells. Next, 5 µl 100bp BenchTop DNA ladder (Promega, USA) DNA marker was added into the first well as comparison for the PCR products. Electrophoresis was carried out at 80V for approximately 35 min using PowerPac™ Basic (Bio-Rad, USA) or until the DNA ladder reached the opposite end of the gel. After electrophoresis, the gel was placed in a GelDoc XR + UV transilluminator (Bio-rad, USA) for viewing. The DNA bands were photographed using Quantity One Basic software (Bio-rad, USA).

3.9 DNA Purification

PCR amplification was repeated for all positive samples using four reaction PCR tubes. Each tube consisted of 10 µl of HotStarTaq® Plus Master Mix, 1 µl of forward primer, 1 µl of reverse primer, 2-3 µl of DNA template depending on the DNA concentration (<200 ng/reaction) and 5-6 µl RNase-Free water which made the final volume in each tube 20 µl. Subsequently, PCR and gel electrophoresis was performed.

DNA purification was carried out using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instruction. DNA purification can be done using gel slice or from PCR product. For purification using PCR product, four PCR tubes containing PCR product of the sample were combined in one PCR tube. Then, an equal volume of Membrane Binding Solution was added into the PCR tube. For purification following gel electrophoresis, DNA band was excised from gel and placed in a 1.5 ml microcentrifuge tube. 10 µl Membrane Binding Solution per 10 mg of gel slice were added and vortexed. Next, The gel with solution was incubated at 60℃ until gel slice was completely dissolved.

Following adding Membrane Binding Solution, dissolved gel mixture or prepared PCR product was transferred to the Minicolumn (Promega, USA) inserted into a collection tube and incubated at room temperature for 1 min. Then, 700 µl

Membrane Wash Solution was added into the minicolumn and centrifuged at 16000 × *g* for I min. Flow through was discarded and minicolumn was reinserted back to the collection tube. 400 μl Membrane Wash Solution was added into the minicolumn and centrifuged at 16000 × g for 5 min. The flow through and collection tube was discarded. Minicolumn was placed in a 1.5 ml microcentrifuge tube and centrifuged at 16000 × *g* for 1 min with lid open to allow evaporation of any residual ethanol. For DNA elution, the minicolumn was transferred into a new 1.5 ml microcentrifuge tube. 50 μl of RNase-Free water was added into the minicolumn and incubated at room temperature for 1 min. The minicolumn assemble was centrifuged at 16000 × *g* for I min. The minicolumn was discarded and purified DNA contained in the 1.5 ml microcentrifuge tube was stored at -20 ℃ (Acson, Malaysia) until further sequencing.

3.10 DNA Sequencing

The purified PCR products were sent for sequencing (Bioneer, South Korea) using the Sanger's method with the same forward and reverse primer. A sequencing kit (BigDye® Terminator v3.1 Cycle Sequencing Kit, Thermo Fisher Scientific Inc., USA) and an automated sequencing analyzer (3730x1 DNA Analyzer, Applied Biosystems®, USA) were used for the analysis.

3.11 Bioinformatics Analysis of Feline Bocavirus Partial NS1 Gene Sequence

3.11.1 Sequence Editing and Assembly

The sequencing results in the form of chromatogram were edited using bioinformatics software CLC Main Workbench version 8.5.1 (Qiagen, Germany) in order to perform sequence trimming, reverse complement sequence, and assembly of forward and reverse sequences.

3.11.2 Basic Local Alignment Search Tool (BLAST)

The Basic Local Alignment Search Tool (BLAST) is a tool introduced by the National Centre for Biotechnology Information (NCBI) to allow user to scan huge database of sequences to local genomic 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 FBoV isolates, and to identify the percentage of sequence homology. Besides, any related FBoV sequences from the database were downloaded into the CLC Main Workbench v8.5.1 software for comparison with local isolates of partial NS1 sequences.

3.11.3 Multiple Alignments

Reference sequence isolates and local sequence isolates were aligned using CLC Main Workbench v8.5.1 software. Multiple alignments were 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 an accurate phylogenetic tree. In addition to multiple alignments, CLC Main Workbench v8.5.1 software was also used to create a pairwise sequence identity matrix to identify pairwise nucleotide identity.

3.11.4 Construction of Molecular Phylogenetic Tree

An unrooted phylogenetic tree was constructed using the distance – based neighbour-joining method after multiple alignments. CLC Main Workbench software v8.5.1 was used to generate a phylogenetic tree. Tree reliability was accessed using 1000 bootstrap replications. The sequences of local FBoV were compared with isolates from Hong Kong, United States of America and Belgium.

4.0 RESULTS

4.1 PCR Amplification

Based on conventional PCR using forward primer 5’-TCTACAAGTGG GACATTGGA-3’ and reverse primer 5’-GAGCTTGATTGCATTCACGA-3’, 4 out of 4 (100%) cats were positive for feline bocavirus (FBoV) in one or more tissue samples. Positive results were defined as band being expressed at the 133 bp region after electrophoresis on 2.0% (w/v) agarose gel. Specific band at the 133 bp region is the specific amplification product of the partial NS1 region of FBoV. For positive control, DNA of cat-3 mesenteric lymph node was used; whereas for negative control, DNA of cat-2 intestine was used. An example of the gel electrophoresis for cat with FBoV positive bands of 133 bp is shown in lanes 4, 5, 6 and 7 (Figure 4.1).

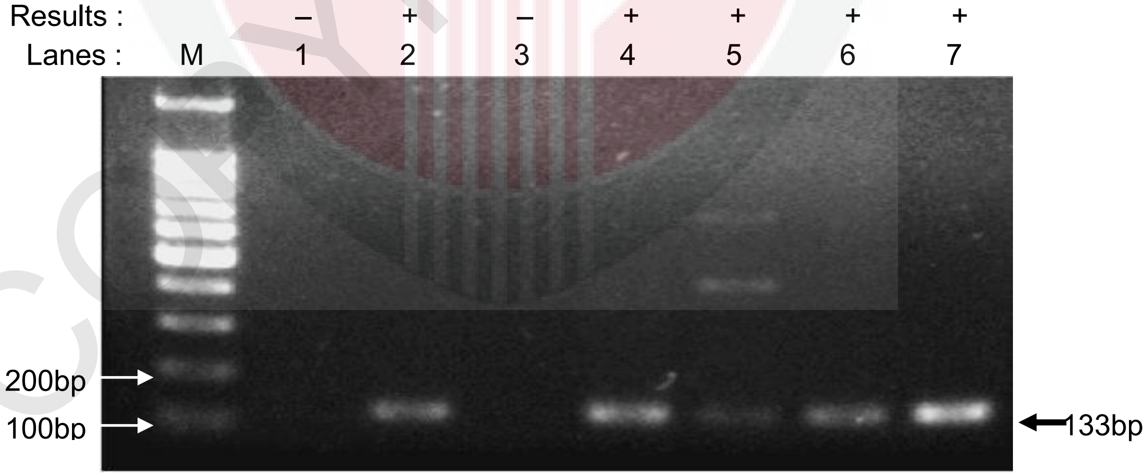


Figure 4.1 : PCR assay of pool samples for all cats using specific primers targeting the conserved NS1 gene of feline bocavirus to produce 133 bp PCR products. Electrophoresis was carried out on 2.0% (w/v) agarose gel. The target bands were observed in all cats as show in lane 4 (cat-1), lane 5 (cat-2), lane 6 (cat-3) and lane 7 (cat-4). Lane 1: control negative (NTC). Lane 2: positive control. Lane M: 100bp DNA marker

Legends : – negative PCR; + positive PCR

Based on conventional PCR results, 9 out of 37 (24.3%) dogs selected for this study were tested to be positive for canine bocavirus (CBoV) in one or more tissues samples. Primer used were forward primer 5'-AGGTCGGCCACTGGCTGT-3' and reverse primer 5'-CAGCTTAACGGCATTCACTA-3' targeting conserved region of the NS1 gene of CBoV. Positive results were defined as band being expressed at the 128 bp region after electrophoresis on 2.0% agarose gel. In this study, 128 bp region is the specific amplification product of the partial NS1 region of CBoV. For positive control, DNA of dog-11 mesenteric lymph node was used; whereas for negative control, DNA of dog-13 was used. Positive samples of dogs with CBoV revealed the bands of 128 bp is shown in lanes 4, 11 and 12 (Figure 4.2).

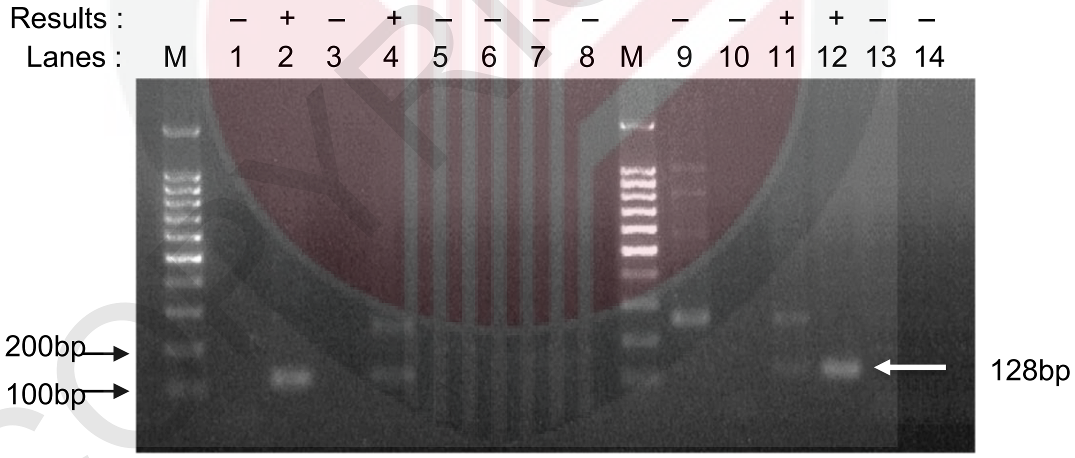


Figure 4.2 : PCR assay of pool samples for dog 1 to dog 11 using specific primers targeting the conserved NS1 gene of canine bocavirus to produce 128 bp PCR products. Electrophoresis was carried out on 2.0% (w/v) agarose gel. The target bands were observed in lane 4 (dog-1), lane 11 (dog-8) and lane 12 (dog-9). Negative dogs are in lanes 3, 5, 6, 7, 8, 9, 10, 13, and 14. Lane 1: control negative (NTC). Lane 2: positive control. Lane M: 100bp DNA marker
Legends : – negative PCR; + positive PCR

PCR assay was further conducted with various tissue samples individually for all positive animals. Tissue samples collected from cat include mesenteric lymph node, submandibular lymph node, kidney, lung and intestine. The detection percentage of FBoV according to organ sample showed that mesenteric lymph node had the highest detection with 100% (4/4) detection percentage. This followed by submandibular lymph node, kidney and lung with detection percentage of 25% (1/4) for each tissue. The only organ sample with no FBoV detected was the intestine (0%). Electrophoresis results of various tissues (mesenteric lymph node, submandibular lymph node, kidney, lungs and intestine) of cat-1 and cat-2 are shown in Figure 4.3. All the PCR results for individual tissue samples for cats are summarized in Figure 4.4.

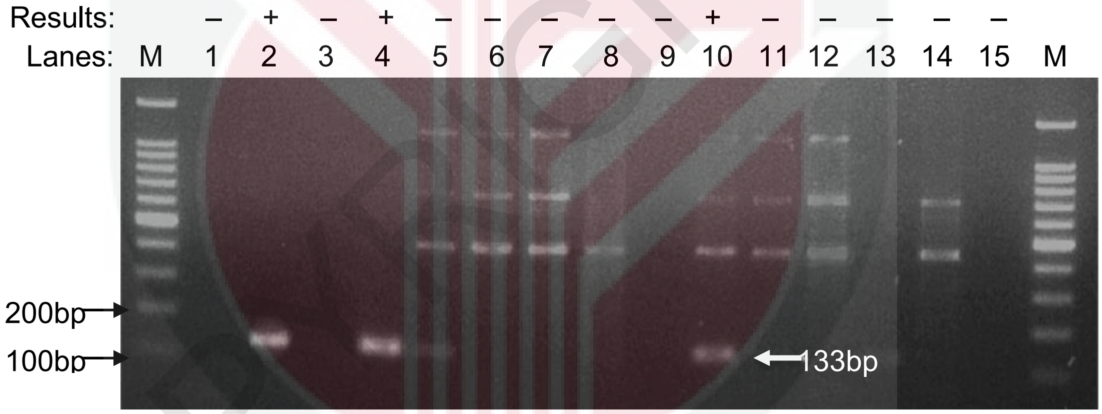


Figure 4.3 : PCR assay of individual tissue samples (mesenteric lymph node, submandibular lymph node, kidney, lungs and intestine) for cat-1 and cat-2 using specific primers targeting the conserved NS1 gene of feline bocavirus to produce 133 bp PCR products. Electrophoresis was carried out on 2.0% (w/v) agarose gel. The target bands were observed in lane 4 (cat-1 mesenteric lymph node), lane 5 (cat-1 submandibular lymph node), and lane 10 (cat-2 mesenteric lymph node). Lane 1: control negative (NTC). Lane 2: positive control. Lane M: 100bp DNA marker

Legends : – negative PCR; + positive PCR

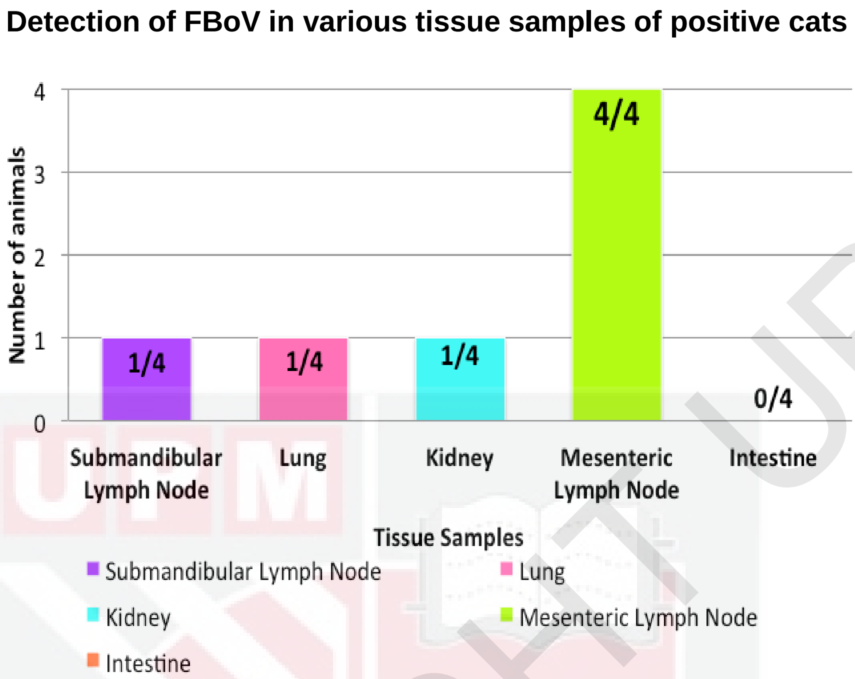


Figure 4.4 : Bar chart showing the frequency of positive FBoV detection in individual tissue samples of positive cats. The mesenteric lymph node contributed to the highest detection with all (4/4) cats tested positive. This is followed by submandibular lymph node (1/4), lung (1/4) and kidney (1/4), whereby all contributed from different cats. FBoV is negative in all intestine samples.

On the other hand, mesenteric lymph node, submandibular lymph node, inguinal lymph node, lung and intestine were sampled from dogs and PCR assay was performed on various tissues individually. The detection percentage of CBoV according to organ sample showed that mesenteric lymph node had the highest detection with 88.8% (8/9) detection percentage. This is followed by submandibular lymph node, lung, intestine and inguinal lymph node with detection percentage of 66.7% (6/9), 55.5% (5/9), 55.5% (5/9) and 44.4% (4/9) respectively. Electrophoresis result for various tissue samples of dog-11 is shown in Figure 4.5. All the results for individual tissue samples of positive dogs were summarized in Figure 4.6.

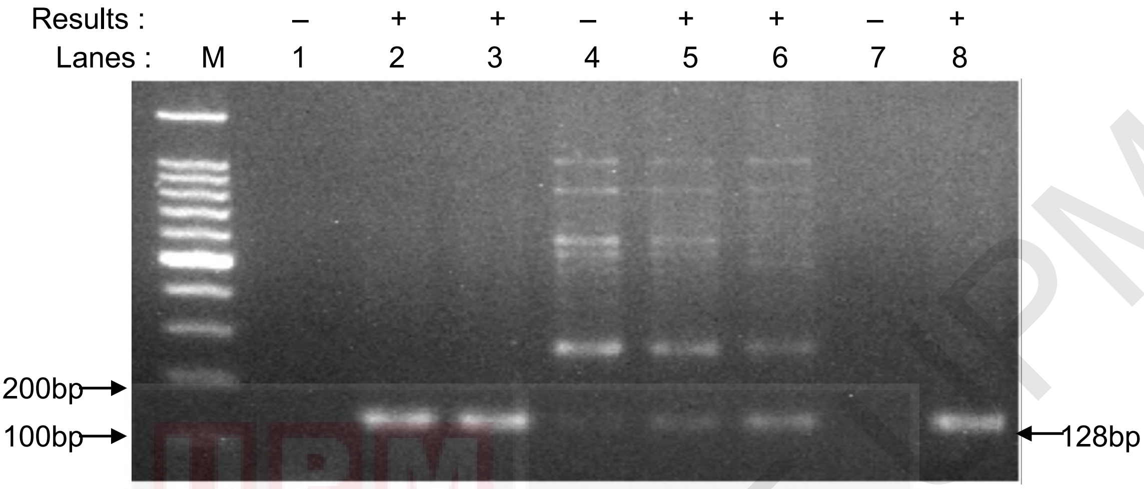


Figure 4.5 : PCR assay of individual tissue samples (mesenteric lymph node, submandibular lymph node, inguinal lymph node, lungs and intestine) for dog-11 using specific primers targeting the conserved NS1 gene of canine bocavirus to produce 128 bp PCR products. Electrophoresis was carried out on 2.0% (w/v) agarose gel. The target bands were observed in lanes 2 (mesenteric lymph node), 3 (submandibular lymph node), 5 (lung), and 6 (intestine) band being expressed at the 128 bp region. Lane 1: control negative (NTC). Lane 8: positive control. Lane M: 100bp DNA marker

Legends : – negative PCR; + positive PCR

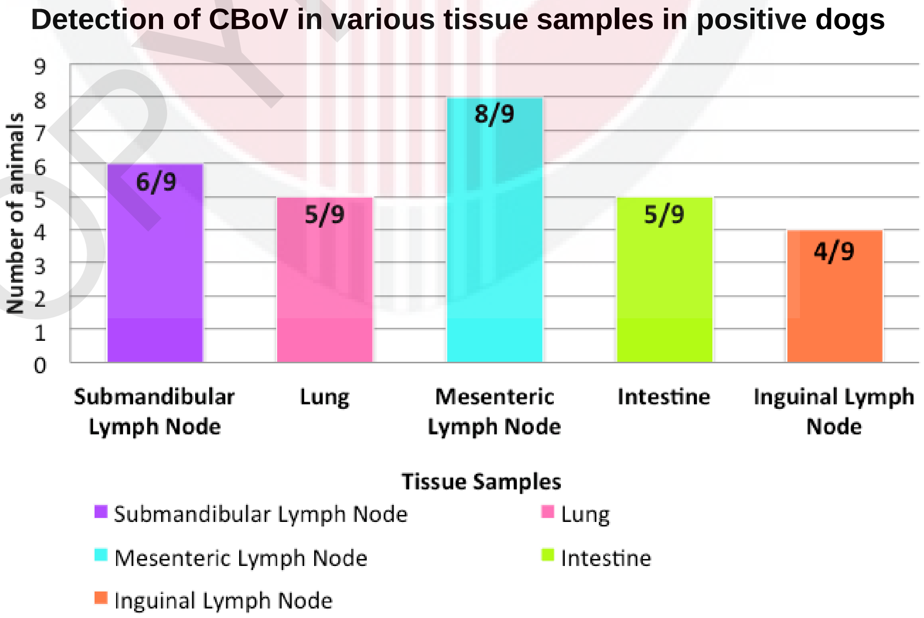


Figure 4.6 : Bar chart showing the frequency of positive CBoV detection in individual tissue samples of positive dogs. The mesenteric lymph node contributed to the highest detection with 8 out of 9 dogs tested positive. This is followed by

submandibular lymph node (6/9), lung (5/9), intestine (5/9) and inguinal lymph node (4/9).

However, conventional PCR on dog pooled tissue sample using forward primer 5’-GCGAATTCGTGGTATGCACCTATATACAACGGAC-3’ and reverse primer 5’-CGGGATCCGGATGCGACATAGGCAGAGTTCCATC-3’ targeting canine minute virus (CMV) yield no result with no band expression on the 2.0% (w/v) agarose gel.

4.2 Bioinformatics Analysis of Malaysian FBoV Isolates

4.2.1. Sequence Editing and Assembly

Only 2 PCR products of FBoV, which are from cat 2 and cat 3 were of satisfactory quality to be sent for sequencing. Sequence outputs in form of electropherogram were edited and assembled using bioinformatics software. Sequence fragment of FBoV cat-2 and cat-3 are of length 101 bp and 110 bp length respectively (Figure 4.7 and 4.8). Each sequence was assigned with different individual identifications (Table 4.1).

Table 4.1 : Sequences obtained from sequencing of the partial NS1 gene from local FBoV isolates from 2 cats with designated identifications.

Cat ID	Sequence ID	Figure
Cat-2	FBoV_Malaysia_C2	4.7
Cat-3	FBoV_Malaysia_C1	4.8

```
LOCUS       FBoV_Malaysia_C1       110 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 CGGCGTTTCC TATTAAAAGG CGGGAAACAA AATACATAAA ATTTCTTTGG ACCGGCTAGC
       61 ACCGGTAAAA CAAATCTGGC CAAGGCAATC GTGAATGCAA TCAAGCTCAA
//
```

Figure 4.7 : Partial NS1 sequence of FBoV_Malaysia _C1 with length 110 bp.

```
LOCUS       FBoV_Malaysia_C2       101 bp    DNA     linear   UNA
FEATURES             Location/Qualifiers
ORIGIN
        1 TAAAAATTGT CGCTCGCTGG GTACACTCAT ACATTAAATT TCTTTGGACC GGCTAGCACC
       61 GGTAAACAAA TCTGGGGAGG CGTCTGGGCG ATCAGGCAAA T
//
```

Figure 4.8 : Partial NS1 sequence of FBoV_Malaysia _C2 with length 101 bp.

4.2.2 Basic Local Alignment Search Tool (BLAST)

The results of BLAST search revealed that the partial sequence from local isolates were highly similar (98%) to the conserved NS1 gene reference isolates within the Genbank® (National Center for Biotechnology Information, USA). High similarities were found with sequences from USA, Belgium and Hong Kong. Results of BLAST search also suggested that the partial NS1 gene sequenced is highly conserved due to search result only shown matches with FBoV.

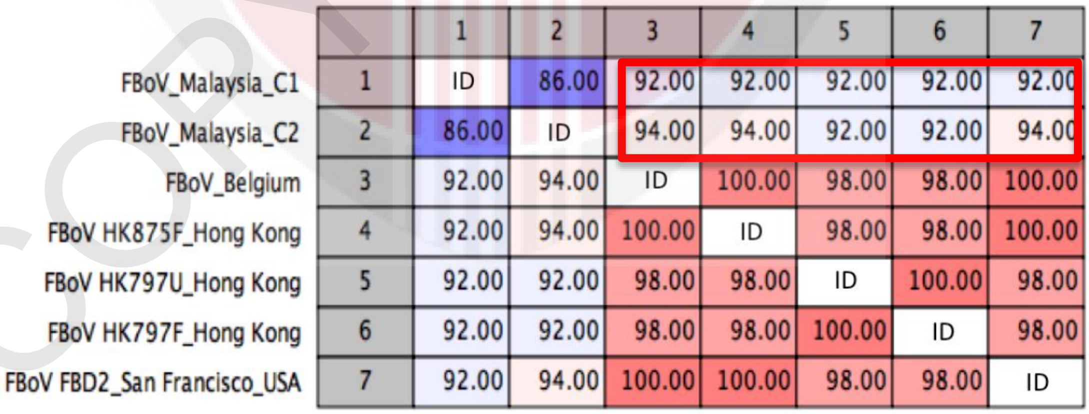
4.2.3 Multiple Alignment and Pairwise Comparison

The two sequences in this study were aligned with other reference isolates from Genbank® using CLC Genomic Workbench v8.5.1. These reference isolates were downloaded from Genbank® based on their sequence identity and reliability in publications (Table 4.2). This is done to identify the location of the sequence identities.

Table 4.2 : Reference isolates of FBoV downloaded from Genbank®

No.	Accession No.	Country	Source	Designation
1.	KP769860	Belgium	Garigliany *et al*., 2016	FBoV_Belgium
2.	KM017745	USA	Zhang *et al*., 2014	FBoV FBD2_San Francisco_USA
3.	JQ692587	Hong Kong	Lau *et al.,* 2012	FBoV HK875F_Hong Kong
4.	JQ692586	Hong Kong	Lau *et al*., 2012	FBoV HK797U_Hong Kong
5.	JQ692585	Hong Kong	Lau *et al*., 2012	FBoV HK797F_Hong Kong

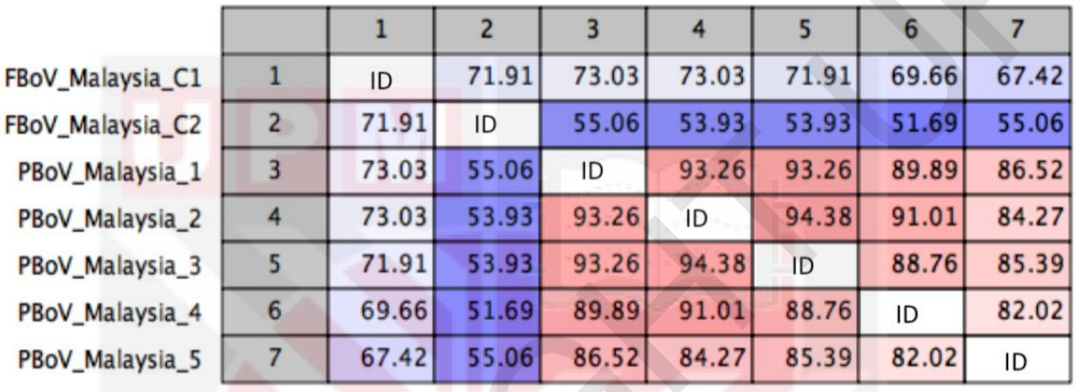
Pairwise nucleotide comparison was performed comparing the two sequences from local isolates and reference isolates to determine sequence identity (Figure 4.9). The comparison revealed that the partial NS1 gene of the two sequences were only homologous with only 92% and 94%.



		1	2	3	4	5	6	7
FBoV_Malaysia_C1	1	ID	86.00	92.00	92.00	92.00	92.00	92.00
FBoV_Malaysia_C2	2	86.00	ID	94.00	94.00	92.00	92.00	94.00
FBoV_Belgium	3	92.00	94.00	ID	100.00	98.00	98.00	100.00
FBoV HK875F_Hong Kong	4	92.00	94.00	100.00	ID	98.00	98.00	100.00
FBoV HK797U_Hong Kong	5	92.00	92.00	98.00	98.00	ID	100.00	98.00
FBoV HK797F_Hong Kong	6	92.00	92.00	98.00	98.00	100.00	ID	98.00
FBoV FBD2_San Francisco_USA	7	92.00	94.00	100.00	100.00	98.00	98.00	ID

Figure 4.9 : Sequence identity matrix with pairwise comparison to compare sequence identity of the two nucleotides derived from the partial NS1 gene of local FBoV isolates with reference isolates. ID: Identical.

Pairwise nucleotide comparison was also performed comparing the two sequences from local isolates and five PBoV of local isolates to determine sequence identity (Figure 4.10). The comparison revealed that there was low sequence homology of the partial NS1 gene with only 51.69% to 73.03% sequence identity. Hence, it is highly suggestive that PBoV and FBoV of Malaysian isolates are virus of different species.



		1	2	3	4	5	6	7
FBoV_Malaysia_C1	1	ID	71.91	73.03	73.03	71.91	69.66	67.42
FBoV_Malaysia_C2	2	71.91	ID	55.06	53.93	53.93	51.69	55.06
PBoV_Malaysia_1	3	73.03	55.06	ID	93.26	93.26	89.89	86.52
PBoV_Malaysia_2	4	73.03	53.93	93.26	ID	94.38	91.01	84.27
PBoV_Malaysia_3	5	71.91	53.93	93.26	94.38	ID	88.76	85.39
PBoV_Malaysia_4	6	69.66	51.69	89.89	91.01	88.76	ID	82.02
PBoV_Malaysia_5	7	67.42	55.06	86.52	84.27	85.39	82.02	ID

Figure 4.10 : Sequence identity matrix with pairwise comparison to compare sequence identity of the two nucleotides derived from the partial NS1 gene of local FBoV isolates with five local PBoV isolates. ID: Identical.

4.2.4 Construction of Phylogenetic Tree

The unrooted phylogenetic tree showing relationship between Malaysian feline bocaviruses with all isolates available in Genbank® was generated using the neighbour-joining (NJ) method with 1000 bootstrap replicates (Figure 4.11). CLC Main Workbench version 8.5.1 software was used to construct the tree.

The tree includes sequences from cat-3 (FBoV_Malaysia_C1), and cat-2 (FBoV_Malaysia_C2), as well as 19 other reference isolates downloaded from GenBank®, which is every other known FBoV sequence in the world. The reference sequences originate from three countries; which are USA, Hong Kong, and Belgium.

Based on pairwise comparison of the sequence identity matrix, the reference isolate with the closest sequence identity to local Malaysian FBoV isolates was the FBoV isolate FBD2 (Genbank® accession number KM017745) from San Francisco USA. This similarity is observed on the phylogenetic tree as the FBoV isolate FBD2 is the closest related references isolate to the Malaysian FBoV clade.

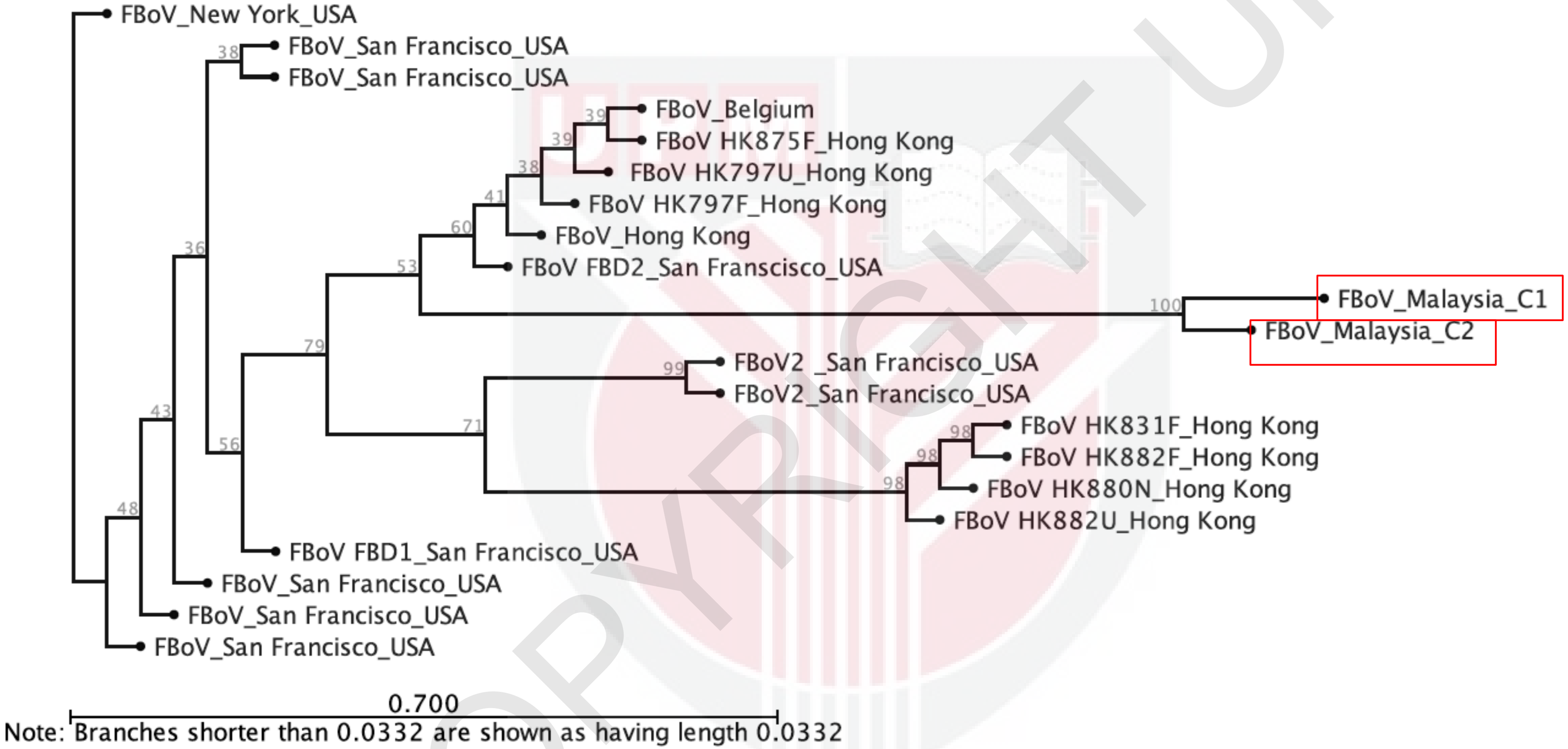


Figure 4.11 : Unrooted phylogenetic tree of two Malaysian FBoV (boxes) and reference isolates. The phylogenetic tree was constructed using the neighbor-joining method with 1000 boostrap replicates. Malaysia isolates are denoted as FBoV_Malaysia_C1 and FBoV_Malaysia_C2, forming a distinct clade among themselves.

5.0 DISCUSSION

5.1 Detection of Canine Bocavirus (CBoV) in Malaysia

This study is the first study in Malaysia to have described the existence of canine bocavirus (CBoV). This also makes Malaysia the fifth country in the world to have described CBoV in dog population. Out of 37 dogs, 9 dogs (24.3%) were detected positive with CBoV using conventional PCR assay. This is consistent with the finding in San Francisco where 23% (36 out of 158) of the dogs were tested positive using nested PCR (Kapoor *et al*., 2012). However, the incidence is lower in other studies with detection rate of 6.3% (Lau *et al.*, 2012) and 9.6% (Choi *et al*., 2015) respectively. The higher detection rate in this study could be explained by the fact that shelter animals are kept under crowded condition and they might have a greater risk of exposure to numerous potential pathogens (Pesavento and Murphy, 2014); which may contribute to the predisposing factors towards CBoV. However, more studies including a larger sample size should be done in order to fully understand the prevalence of CBoV in Malaysia.

To date, CBoV has been detected in fecal, nasal, urine, liver, and blood, intestine, lungs samples of dogs with or without clinical signs (Kapoor *et al.*, 2012; Bodewes *et al.,* 2014; Choi *et al*., 2015). This study describes the detection of CBoV in mesenteric lymph node, submandibular lymph node, inguinal lymph node, lung and intestine.

5.2 Detection of Feline Bocavirus (FBoV) in Malaysia

This is the first study that describes the presence of feline bocavirus (FBoV) in Malaysia, and fifth country in the world. All four cats (100%) tested were positive with FBoV. However, this does not reflect the true prevalence of FBoV in Malaysia as the sample size is too small and sampling was bias as all cats sampled were sick prior euthanasia. Study had done in Hong Kong reveal 7.2% (26/363) prevalence, with detection in fecal, nasal, urine, kidney, and blood samples (Lau *et al*., 2012) and 6% of fecal sample from 55 cats from Portugal (Ng *et al.,* 2014). To fully understand the prevalence of FBoV, a larger sample size should be undertaken.

Using PCR assay, FBoV is detected in mesenteric lymph node, submandibular lymph node, kidney, lungs and intestine. Previously, FBoV had been detected in fecal, nasal, urine, kidney, blood, and brain samples (Lau *et al*., 2012; Ng *et al.,* 2014; Zhang *et al*., 2014; Garigliany *et al*., 2016). This study revealed that FBoV has wide tissue tropism; which include the hematopoietic organs.

5.3 Bocavirus Detection in Mesenteric Lymph Node

It is found that in the highest detection of bocavirus among all tissues samples in all cats and dogs in Malaysia was in mesenteric lymph node. This finding is also supported by the findings of Daniel *et al.* (2015); where the study also provide evidences of mesenteric lymph node contributing to the highest detection of PBoV among other tissues. With the consistent findings of bocavirus detection in mesenteric lymph node of cats, dogs and pigs, this suggests that mesenteric lymph node might play an important role in the pathogenicity of bocavirus. This also suggests to us that gastrointestinal system may be involved in the pathophysiology of Bocavirus infection in animal. It is found that CBoV infect only low numbers of epithelial cells of the intestinal tract but may have play a role in intestinal disease due to infection and partial destruction of the intestinal immune system (Bodewes *et* *al*., 2014). This probably explains the higher detection of bocavirus antigen in mesenteric lymph nodes as compared to intestine.

To date, the pathogenicity of FBoV and CBoV remain to be determined (Lau *et al.*, 2012). In this study, both FBoV and CBoV were detected in different samples from cats and dogs respectively. Therefore, this study supports the previous findings that bocavirus could be detected in different tissue samples from respective animals; suggesting a wide tissue tropism (Lau *et al*., 2012, Kapoor *et al.,* 2012; Zhang *et al.,* 2014, Ng *et al*., 2014; Garigliany *et al*., 2016). Although research have shown association of CBoV in respiratory and gastrointestinal disease, the ability of this virus to cause respiratory and gastrointestinal disease remains debatable as it is detected in healthy animal and also co-detected with other disease causing pathogens (Kapoor *et al*., 2012; Bodewes *et al.*, 2014).

5.4 Partial Nonstructural Protein 1 (NS1) Gene Sequencing and Bioinformatics Analysis of Malaysian Isolated FBoV

Sequencing analysis of two cat samples (FBoV_Malaysia_C1 and FBoV_Malaysia_C2) revealed that the sequences are highly conserved as search results only limited to feline bocavirus. The partial NS1 sequence of local isolates matches 98% sequence identities of partial NS1 gene of the reference isolates (Genbank® accession number KP769860, KM017745, JQ692587, JQ692586 and JQ692585).

From the phylogenetic tree, it is apparent that the Malaysian isolates form their own clade that is distantly related from other reference isolates. This suggests that local FBoV isolates may be of a variant strain. However, this result is not definitive as the sequence used for the phylogenetic analysis is limited to only a partial sequence, which are about 101 bp (FBoV_Malaysia_C2) and 110 bp (FBoV_Malaysia_C1) long. For more accurate phylogenetic analysis and identification of new strains, a complete genome of the local FBoV isolates should be obtained.

Calculation of pair-wise distances using the 101 nt and 110 nt long NS1 region of 2 FBoV local isolates between reference isolates resulted in up to 92% to 94% sequence identity. To be classified a member of the species of the genus Bocavirus, the ICTV criterion should meet the following criteria: (i) probably antigenically distinct; (ii) natural infection is confined to a single host species; and (iii) having 95% homologous NS gene DNA sequence (http://www.ictvdb.org/). While the antigenic properties of FBoV yet to be explored and FBoV Malaysian isolates matches less than 95% homologous partial NS1 gene sequence compared with known feline bocaviruses, this suggests that FBoV Malaysian isolates may represent one or more novel species within the genus Bocavirus. However, since this study uses partial NS1 gene (133bp out of 2415bp of the NS1 gene) for comparison, it cannot be ruled out that there is possibilty that FBoV Malaysia isolates are indeed related to other isolates.

CONCLUSION

Bocavirus is prevalent in cats and dogs in Malaysia. Malaysia is the 5th country in the world that has described feline bocavirus (FBoV) in cats and canine bocavirus (CBoV) in dogs. CBoV was detected in mesenteric lymph node, submandibular lymph node, inguinal lymph node, lung and intestine of dogs. FBoV was detected in mesenteric lymph node, submandibular lymph node, kidney and lung of cats. Phylogenetic analysis on the partial NS1 gene showed that Malaysian FBoV are distantly related to FBoV FBD2 isolate of San Francisco, USA and forms a distinct clade among themselves.

RECOMMENDATIONS

The primers used in this study for detection of FBoV and CBoV by amplification of the partial NS1 gene targeted on a short amplicon. This resulted in a short sequence enable genetic comparison to be done limited to only one gene; which is less accurate and less specific if comparison is done using a longer sequence. Using a primer that targets a short amplicon also increase the possibility of amplifying non-specific DNA. Therefore, its is suggested using primers that flank longer base pair of bocavirus for better phylogenetic analysis.

A larger sample size should be considered, as the sample size for this study is too small, especially for cats' samples. It does not truly reflect the true prevalence of FBoV and CBoV in Malaysia. To further understand the distribution of FBoV and CBoV, optimally, animals from more shelters from more states should be involved. Other than that, animals sampling should include adult and young animals, with or without clinical signs. This project can also extend to pet animals.

In addition to that, since the pathology of FBoV and CBoV is still poorly understood, it will be useful if more organs should be examined to investigate the tissue tropism of bocavirus. This will subsequently able to suggest the possible pathogenesis of bocaviruses. Histologic examination of the bocavirus positive samples will give insights on cytopathology induced by bocavirus.

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