UNIVERSITI PUTRA MALAYSIA

MOLECULAR INVESTIGATION OF FELINE CORONAVIRUS (FCoV) IN LOCAL PET CATS

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MOLECULAR INVESTIGATION OF FELINE CORONAVIRUS (FCoV) IN LOCAL PET CATS

LIEW WUAN HOONG

A Project Paper Submitted to the
Faculty of Veterinary Medicine, Universiti Putra Malaysia
In Partial Fulfillment to the Requirement for the
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MARCH 2017

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Molecular Investigation of Feline Coronavirus (FCoV) in Local Pet Cats", by Liew Wuan Hoong and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD 4999- Final Year Project

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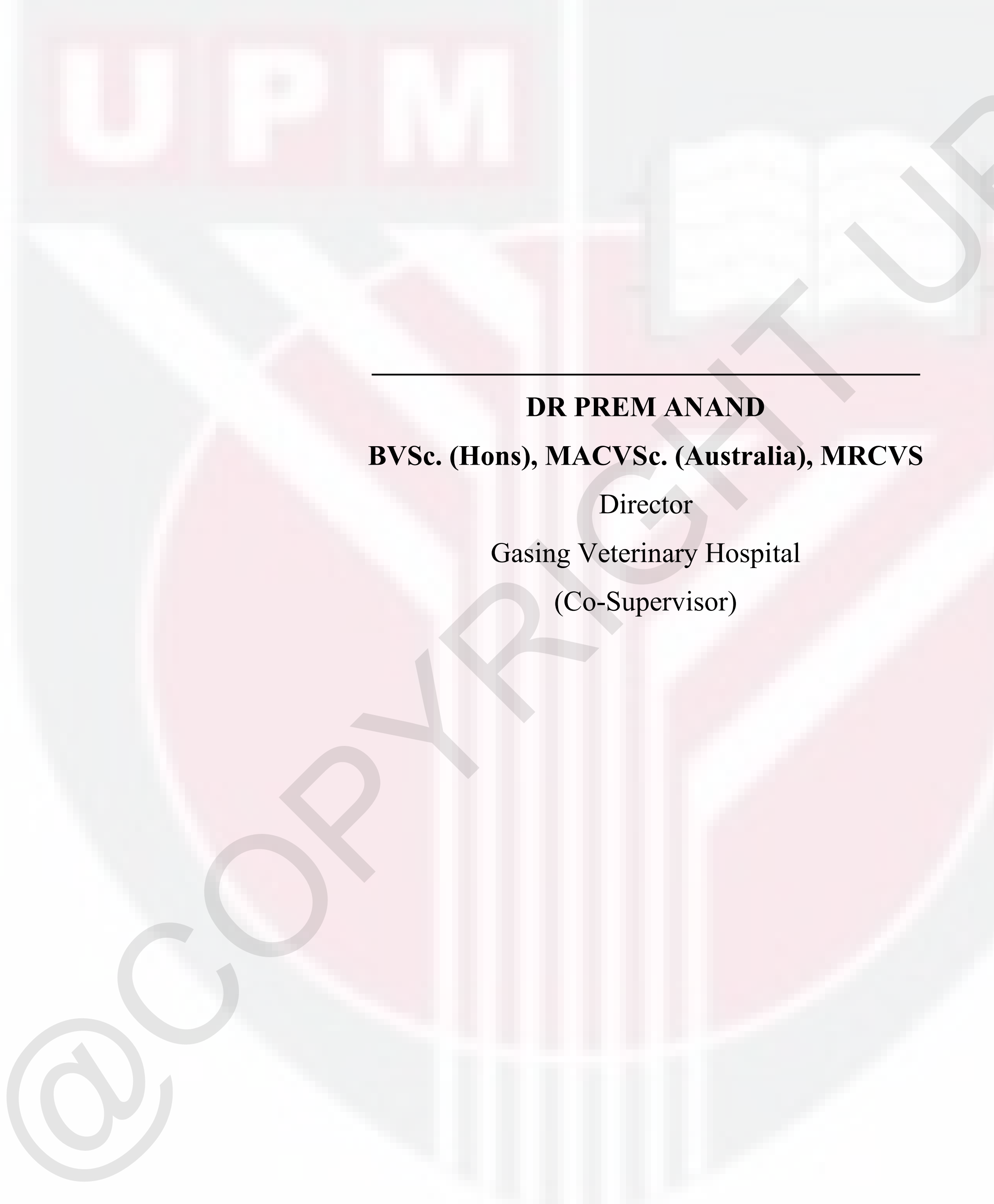
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DEDICATIONS

I would like to dedicate my project paper to my family and friends who are always there in my journey.

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It has been an honour to be able to complete this project paper, which at the very same time, it also means that the road to completion of Degree of Doctor of Veterinary Medicine is getting really near. Along the way, there are so many kind souls who have been there, giving out their best to help and make this project as great as it can be.

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
µL	Microliter
µM	Micromolar
3' UTR	3' Untranslated region
5' UTR	5' Untranslated region
AMV	Avian Myeloblastosis Virus
APN	Aminopeptidase-N
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
bp	Basepair
CCoV	Canine coronavirus
cDNA	Complementary deoxyribonucleic acid
CNS	Central Nervous System
CoV	Coronavirus
DNA	Deoxyribonucleic acid
Dntp	Deoxynucleotide
E	Envelope
EDTA	Ethylenediaminetetraacetic acid
ERGIC	Endoplasmic reticulum-to-Golgi intermediate compartment

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FCoV	Feline coronavirus
FECV	Feline enteric coronavirus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonis virus
FPV	Fakulti Perubatan Veterinar
FYP	Final Year Project
G	Gauge
g	Relative Centrifugal Force
HCoV	Human coronavirus
HE	Hemagglutinin-esterase
IACUC	Institutional Animal Care and Use Committee
IFN	Interferon
kb	Kilobase
kDa	Kilodalton
M	Membrane
$MgSO_4$	Magnesium Sulphate
mL	Milliliter
mm	Millimetre
mM	Millimolar
mRNA	Messenger ribonucleic acid
N	Nucleocapsid

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NCBI	National Centre for Biotechnology Information
nm	Nanometer
ORF	Open reading frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
pH	Potential of Hydrogen
POL	Polymerase gene
RER	Rough endoplasmic reticulum
RNA	Ribonucleic acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S gene	Spike protein gene
TAE	Tris-acetate-ethylenediaminetetraacetic acid
TGEV	Transmissible gastroenteritis virus
UPM	Universiti Putra Malaysia
V	Voltage
w/v	Weight/volume percent

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ABSTRAK

Abstrak dari kertas projek dikemukakan kepada Fakulti PErubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999- Projek Ilmiah Tahun Akhir

PENYIASATAN MOLEKUL *FELINE CORONAVIRUS* (FCoV) DALAM KUCING KESAYANGAN TEMPATAN

oleh

Liew Wuan Hoong

2017

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Jangkitan *feline coronavirus* (FCoV) adalah sangat biasa di dalam populasi kucing. FCoV diklasifikasikan kepada dua biotip iaitu *feline enteric coronavirus* (FECV) dan *feline infectious peritonitis virus* (FIPV), di mana FIPV menyebabkan penyakit kompleks imun yang membawa maut dengan menukarkan tropisme dari enterosit kepada monosit. Kajian-kajian sebelum ini pada pengesanan molekul FCoV di dalam kucing dijalankan di kateri, tetapi kajian tentang penyiasatan

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kehadiran antigen FCoV di dalam kucing kesayangan tempatan agak terhad. Dengan mengambil kira fakta ini, kajian ini bertujuan untuk mengesan antigen FCoV melalui assai RT-PCR di dalam kucing tempatan dan membandingkan persamaan strain FCoV yang dikenalpasti dengan virus terdahulu melalui analisis filogenetik. Dengan menggunakan pensampelan mudah, swab rektum dan lapisan buf dikumpulkan daripada 16 ekor kucing kesayangan yang sakit secara klinikal dan 5 ekor kucing kesayangan yang sihat. RNA diekstrak dan tertakluk kepada *one-step RT-PCR,* yang menyasarkan gen polymerase. Daripada 21 sampel najis hanya terdapat satu yang positif bagi FCoV dan tidak ada lapisan buf yang positif. Analisis filogenetik mendedahkan bahawa sample yang dikenalpasti positif adalah sangat homologi, sehingga 95%, dengan strain FCoV dari Netherlands pada penjujuk separa gen polymerase. Kesimpulannya, kajian ini telah mengesan antigen FCoV dalam kucing kesyangan tempatan dan pengesanan negatif tidak boleh secara keseluruhannya menolak kemungkinan jangkitan FCoV kerana diagnosis virus ini sangat kompleks yang memerlukan beberapa siri analisis.

Kata kunci: feline coronavirus, feline enteric coronavirus, feline infectious peritonitis virus, RT-PCR, analisis filogenetik

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ABSTRACT

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

MOLECULAR INVESTIGATION OF FELINE CORONAVIRUS (FCoV) IN LOCAL PET CATS

by

Liew Wuan Hoong

2017

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Feline coronavirus (FCoV) infection is very common in cat population. FCoV is further classified into two biotypes namely feline enteric coronavirus (FECV) and mutated feline infectious peritonitis virus (FIPV), in which FIPV causes a fatal immune complex disease by changing the tropism from enterocytes to monocytes. Previous studies on molecular detection of FCoV in cats were carried out in catteries but there is limited study on investigation of the presence of FCoV antigen in local pet cats. By considering this fact, this study aims to detect FCoV antigen via RT-

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PCR assay in local pet cats and to compare the similarity of the identified FCoV strain with previous related virus by phylogenetic analysis. By using convenience sampling, rectal swabs and buffy coat were collected from 16 clinically ill pet cats and 5 healthy pet cats. Viral RNA was extracted and subjected to one-step RT-PCR, targeting polymerase gene. Only 1 out of 21 fecal samples was positive for FCoV and none for buffy coat. Phylogenetic analysis revealed that the identified positive sample was highly homologous, up to 95%, to a FCoV strain from Netherlands on partial sequence of polymerase gene. In conclusion, this study detected FCoV antigen in local pet cats and negative detection could not completely rule out the possibilities of FCoV infection due to the complexity of the virus diagnosis that require multiple series of analysis.

Keywords: feline coronavirus, feline enteric coronavirus, feline infectious peritonitis virus, RT-PCR, phylogenetic analysis

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1.0 INTRODUCTION

Feline coronavirus (FCoV) is a subspecies of *Alphacoronavirus 1*, from genus *Alphacoronavirus* classified within the subfamily of *Coronavirinae*. (Kipar & Meli, 2014). FCoV infection is ubiquitous and distributed worldwide in household and wild cats especially in crowded environment like catteries and shelters.

There are two biotypes of FCoV in cats, namely Feline Enteric Coronavirus (FECV) and Feline Infectious Peritonitis Virus (FIPV) (Addie *et al.,* 2009; Sharif *et al.,* 2010a). Each biotype has two serotypes, I and II mainly based on their antigenic relationship to canine coronavirus. FIPV is believed to be mutated from FECV within the body of a persistently FCoV-infected cat (Pedersen, 2014a). These two biotypes are morphologically and serologically similar, but causing different clinical signs, with FECV causes a transient gastroenteritis or asymptomatic infection whereas FIPV causes a fatal immune-mediated disease, feline infectious peritonitis (FIP). The peak age for FIP development is between 6 months to 2 years old (Hartmann, 2005). FIP is categorized into wet and dry forms accordingly to the clinical signs manifested. Wet form is characterized by peritonitis and/or pleuritis caused by complement-mediated vasculitis, leading the inflammatory fluid leaking into body cavities whereas dry form is involved with partial cell-mediated immunity, characterized by granuloma formation in various organs like central nervous system and ocular system (Pedersen, 2009)

FCoV is transmitted through fecal-oral route. Virus shedding occurs intermittently and some cats can shed virus up to 10 months (Hartmann, 2005; Sharif *et al.,* 2009b).

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Thus, reverse-transcriptase polymerase chain reaction (RT-PCR) can be used to detect FCoV antigen in the feces as a part of multi-cat household management (Herrewegh *et al.,* 1995b). After being infected by FCoV, monocyte-associated viraemia occurs. Thus, buffy coat which is rich with leukocytes can be used to check for viraemia due to FCoV using RT-PCR (Kipar *et al.,* 2010).

There are numerous studies have been carried on molecular detection of FCoV antigen in cats from catteries and shelters, but there is limited study on investigation of the presence of FCoV antigen in local pet cats. The hypothesis of the study proposes that FCoV antigen will be detected via RT-PCR in local pet cats. This study will add up the information on FCoV for the future. Other than that, FCoV causes FIP which is a very fatal disease in cat, even though there have been lots of studies on FCoV, there are still issues related to FCoV waiting to be solved.

In Malaysia, the phylogenetic analysis of FCoVs on partial sequence of 3'UTR had been done around 7 years back (Sharif *et al.,*2009b; Sharif *et al.,* 2010a). Thus, this study will again use phylogenetic analysis to get a glimpse into the current status of FCoVs in Malaysia.

Thus, the objectives of the study are:

1. To detect FCoV antigen via RT-PCR assay in local pet cats.

2. To compare the similarity of identified positive samples with the previous related virus by phylogenetic analysis.

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2.0 LITERATURE REVIEW

2.1 Classification of Feline Coronavirus

Feline coronavirus (FCoV) is a subspecies of *Alphacoronavirus 1* from genus *Alphacoronavirus* classified within the subfamily of *Coronavirinae*. Under species *Alphacoronavirus 1,* there are other subspecies such as canine coronavirus (CCoV) type I and type II and transmissible gastroenteritis virus (TGEV) (Kipar & Meli, 2014).

There are two biotypes of FCoV in cats, namely Feline Enteric Coronavirus (FECV) and Feline Infectious Peritonitis Virus (FIPV) (Addie *et al.,* 2009; Sharif *et al.,* 2010a). These two biotypes are morphologically and serologically similar, but causing different clinical signs, with FECV causes a transient gastroenteritis or asymptomatic infection whereas FIPV causes a fatal immune-mediated disease, feline infectious peritonitis (FIP).

Each biotype is subdivided into two serotypes, I and II based on their ability to culture *in vitro*, their antigenic relationship to CCoV, neutralization reactivity with the spike protein specific monoclonal antibodies and also amino acid sequences of the spike protein gene (Sharif *et al.,* 2010b; Pedersen, 2014a). FCoV serotype I is wholly feline strain, whereas FCoV serotype II is the result of double recombination of FCoV serotype I and CCoV (Fiscus & Teramoto, 1987; Addie *et al.,* 2003; Kummrow *et al.,* 2005). Serotype I is difficult to grow in cell culture while serotype II strains are appeared to be more adaptable to cell culture and lead to a lytic cytopathic effect (Addie *et al.,* 2003; Kummrow *et al.,* 2005).

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There have been few postulations on the development of feline infectious peritonitis: i) 'internal mutation' theory whereby FIPVs arise from FECVs in the same environment, most of the time is the individual, persistently infected host, due to the mutations in any of the genes, ORF 3c accessory gene, S gene or ORF 7b accessory gene that switches the tropism of intestinal epithelium of FECVs to monocyte/macrophage of FIPVs; ii) studies by Brown *et al.* (2009) and Licitra *et al.* (2013) on 'two viruses' theory that suggested that there is existence of two distinct types of feline coronaviruses circulating in the population at which one causes FIP and the other does not, but both studies couldn't prove the theory (Pedersen, 2014a).

2.2 Properties of Feline Coronavirus

The schematic diagram of the structure of coronavirion is shown in Figure 2.1. It is an enveloped particle, spherical to pleomorphic in shape and the diameter is around 75-150nm (average 100nm) (Lai, 2007). FCoVs are linear single-stranded positive sense RNA virus with an almost 30kb non segmented genome and 11 putative open reading frames (ORFs) (Kipar & Meli, 2014). In fact, coronavirus is the RNA virus that has the largest genome.

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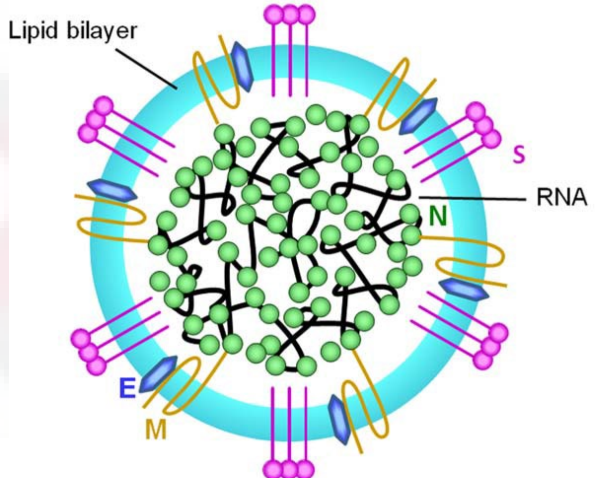

Figure 2.1: A schematic diagram of a typical structure of FCoV**.** S, spike (peplomer); M, membrane; E, envelope; N, nucleocapsid (Kipar & Meli, 2014).

Starting at the 5' end of FCoV genome, there is a 'leader sequence' of 65-98 nucleotides. It is then followed by an untranslated region, called 5' UTR of about 200-400 nucleotides. It is then followed by two large open reading frames (ORFs) 1a and 1b measured up to about 20 kilobases encoding for the non-structural replicase proteins (POL1a and POL1b) which are involved in the synthesis of RNA. The remaining 9 open reading frames (ORFs) in the genome encode for 4 structural proteins: spike (S); envelope (E); membrane (M); nucleocapsid (N); and for 5 accessory proteins: 3a, b and 3c, 7a and 7b respectively. All these are expressed individually from a nested set of subgenomic messenger RNAs (mRNAs) at which each of them has a 'leader sequence' derived from the 5' end of the genome (Shieh *et al*., 1987; Kipar & Meli, 2014). Besides, at the 3' end of the genome, there is another untranslated region of approximately of 200-500 nucleotides, called 3' UTR with a pol (A) tail of varied length follows by. The 5' and 3' UTRs are both very important for RNA replication and transcription (Tekes *et al.,* 2008, Pedersen, 2009).

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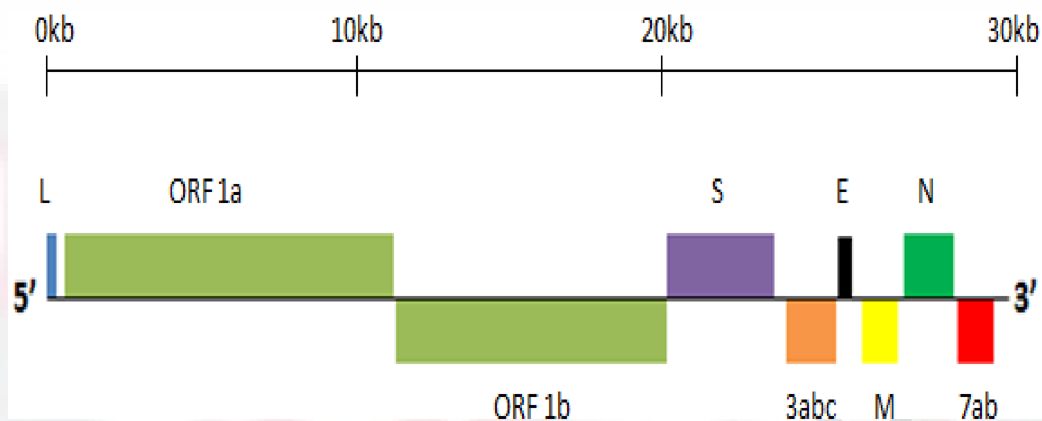

Figure 2.2: A schematic diagram of a typical genomic organization of FCoV. kb, kilobases; L, leader sequence; ORF, open reading frame; S, spike; E, envelope; M, membrane; N, nucleocapsid.

Spike protein that forms part of the envelope of coronavirus, is a 180 to 200 kDa glycoprotein arranged in peplomers, 12 – 24 nm long, which is important to induce antibody response and cell-mediated immunity in the host. The S peplomers are dome-shaped and arranged like a crown, at the same time, they determine the cell tropism of the coronavirus (Belouzard *et al.,* 2012). The M and E proteins found as smaller surface glycoproteins are crucial for virus maturation, assembly, budding and interaction with the host cell. M protein penetrates the envelope of the coronavirus and connects it to the nucleocapsid and involves in the RNA packaging; while E protein interacts with the M protein in the budding compartment of the host cell. The N protein interacts with the viral genome RNA, forming the nucleocapsid that is the core of the coronavirus, which is also flexible and helical. It is critical for viral transcription, translation and virus budding (Olsen, 1999; Kipar & Meli, 2014).

For the accessory proteins (3a, 3b, 3c, 7a and 7b), no specific function has thus far been defined. 3a, 3b and 3c proteins are well conserved among genus

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Alphacoronavirus, especially 3a and 3b among subspecies of species alphacoronavirus 1 and 3c gene has recently been shown to be necessary for the replication of enteric FCoV. For 7a protein, it is shown to act as a type 1 interferon (IFN) antagonist and protects the virus from antiviral state, while ORF 7b is found only in FCoV, CCoV, and ferret CoV which encodes for a soluble glycoprotein that can induce antibodies in naturally infected cats (Herrewegh *et al.,* 1995a; Kennedy *et al.,* 2008; Kipar & Meli, 2014).

2.3 Viral Replication of Feline Coronavirus

Viral replication is firstly initiated by the binding of the spike proteins in the envelope of the virions to the host cells by through the cell-surface receptors which are aminopeptidase-N (APN). FCoV replicates in the cytoplasm. Upon entering to the host cell, the virus undergoes disassembly, and the genomic RNA is being released. Then, the ORF 1a and 1b genes are firstly translated into two large precursor polyproteins which are further processed to produce an RNA-dependent RNA polymerase and several non-structural proteins which then facilitate the functional replication-transcription complex.

Next, the positive sense genomic RNA acts as a template to synthesize a complementary strand of antisense RNA. These complementary RNAs are then used to produce more genomic and subgenomic mRNAs which are important to be translated to produce all the structural and non-structural proteins of the virions. Each mRNA has a leader sequence at its 5’ end, and then each of them is translated

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to produce one or more protein. Once translated, the N protein interacts with the newly produced genomic RNA to form the core of FCoV, the helical nucleocapsid.

The structural proteins S, M and E are inserted into the membrane of the rough endoplasmic reticulum (RER), and then from there, there are being transported to the ER-to-Golgi intermediate compartment (ERGIC) to join the nucleocapsid. All these are then assembled into particles by budding. The virions are then transported out of the cell through an exocytosis-like fusion of the smooth-walled vesicles that contain the virion particles with the plasma membrane of the host cell. All in all, M proteins play an indispensable role in the assembly process of the virion by interacting with all viral assembly partners, including the other M molecules to form the basic matrix of the viral envelope, as well as the structural proteins E, S and hemagglutinin-esterase (HE) (if present), and also the very important core of FCoV, the nucleocapsid, which has contributed directly to the assembly of the virion. Some of the S proteins are transported and embedded on the surface of the cell membrane which may play a role in mediating cell-to-cell fusion of the uninfected cells (De Han & Rottier, 2005).

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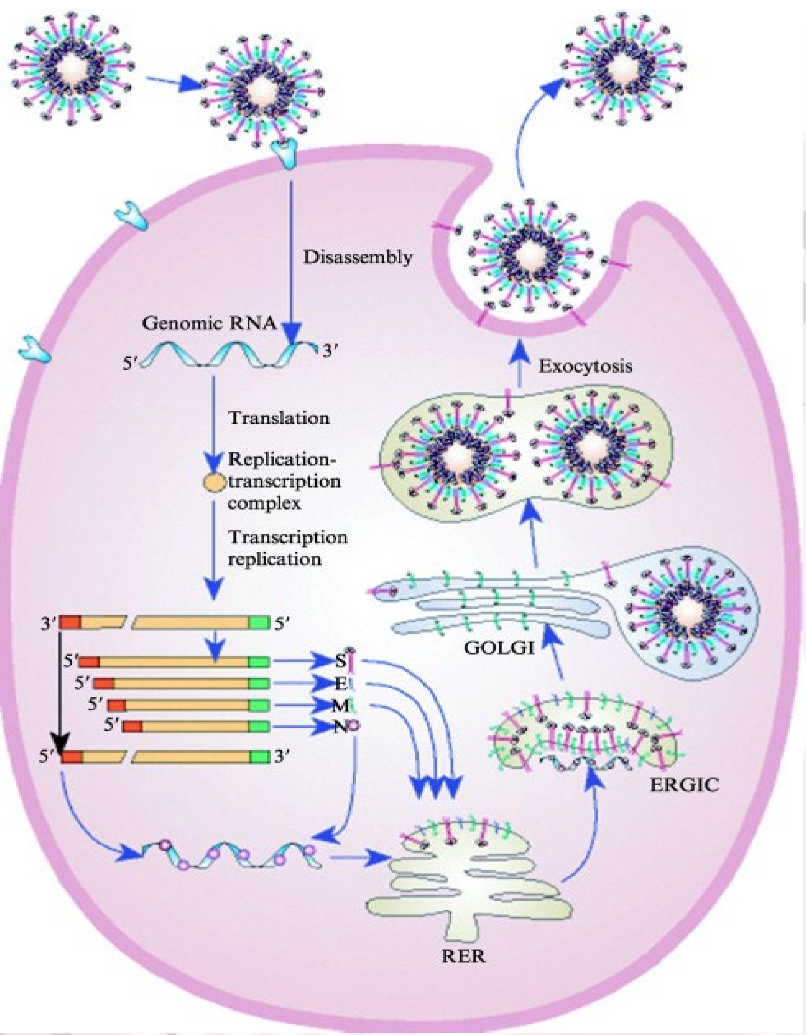

Figure 2.3: A schematic diagram shows the complete transcription and replication of FCoV (De Han & Rottier, 2005). It starts with fusion of the virus envelope with the cellular membrane, followed by disassembly of the virus particle. Then ORF 1a and 1b are translated into polyproteins constitute replication-transcription complex. Negative-strand RNAs and subgenomic mRNAs are synthesized. Each subgenomic mRNA is translated into one protein. Then, assembly of virion takes place in ERGIC. Virion is then released by an exocytosis-like fusion of the smooth-walled vesicles with the plasma membrane.

2.4 Epidemiology of Feline Coronavirus

Feline coronavirus infection is very common in cat population. Almost 25% of cats in single-cat household (Pederson, 1976, Rohrbach *et al.,* 2001) and 25-40% of pet cats and up to 75-90% of cats in catteries, shelters or multi-cat households have antibodies against FCoV and the virus is present virtually in all catteries and shelters with more than six cats and is shed by 60% or more of pet cats from multi-cat household (Arshad *et al.,* 2004; Pedersen, 2009; Sharif *et al.,* 2009a). As comparison, this virus is considered to be rare in free-roaming ownerless cats or those cats with a more extensive lifestyle due to lesser close contact with each other, or not sharing the same litter trays for defecating that reduce the chances of Coronavirus transmission (Hartmann, 2005; Bell *et al.,* 2006).

As mentioned earlier, the FCoV infection is spread by the fecal-oral route and the virus mainly infects enterocytes with viral shedding in feces occurs within a week of exposure from the ileum, the colon and the rectum (Herrewegh *et al.,* 1997; Pedersen *et al.,* 2008; Kipar & Meli, 2014). If given a dry environment, the virus can survive up to 7 weeks in the environment (Hartmann, 2005). In early infection, the virus may also be found in the saliva, respiratory discharge and urine (Stoddart *et al.,* 1988; Herrewegh *et al.,* 1997). According to a study by Pedersen *et al.* (2008), the shedding of FECV can be persistent for up to 18 months or more, be persistent for 4-6 months and intermittent for months thereafter, or cleared within 6-8 months.

The prevalence of FECV infection in multi-cat household is generally high which can exceed 90%, but only 7.8-12% of all FCoV-infected cats will develop feline

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infectious peritonitis (FIP) (Hartmann, 2005). There are several risk factors for both the FCoV infection and the development of the fatal form of FIP. One of them is multi-cat household, which increases the chance of FCoV transmission through faecal-oral route by sharing of litter tray. Besides, age is another important risk factor, at which 70 % positive FCoV infections are less than 1 year old (Rohrer *et al.,* 1994; Hartmann, 2005). According to Sharif *et al.* (2009a), breed of cats has a significant association with FCoV infection, at which purebred Persian cats are more susceptible to being FCoV-positive than mixed-breed cats. According to a study by Foley *et al.* (1997), the risk factors give rise to FIP in catteries include cats with age between 6 months old to 2 years old, cats with higher coronavirus titre, higher overall frequency of faecal coronavirus shedding and if a cattery has high number of chronic FECV shedders. All these are associated with increased risk of FIP.

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2.5 Pathogenesis and Clinical Signs of Feline Coronavirus

After a cat is infected with FCoVs via fecal-oral route, the main site of viral replication is the intestinal epithelium by binding to the plasma membrane of the target host cells, the enterocytes. The binding involves the spike protein of the virions and the corresponding cell-surface receptors which are aminopeptidase-N (APN). These cellular receptors are found distributing widely on many cell types including respiratory and enteric epithelial cells, and also in blood cells (Takano, 2007; Dye *et al.,* 2008).

Normally, following ingestion, the virus enters the enterocytes and replicates rapidly within the cells, destructing the intestinal epithelium. Virus particles being released from the infected cells will continue to infect the neighbouring non-infected cells. If sufficient amount of enterocytes being destroyed, resulting in villous atrophy, diarrhoea can occur. Yet, most FECV infections are asymptomatic or resulting in mild gastroenteritis. Mild gastroenteritis only occurs in 1-5% of the all the FCoV-infected cats, mostly kittens. Those asymptomatic cats with persistent infection can shed FCoVs intermittently or continuously, acting as source of infection to other cats in the household (Wentworth and Holmes, 2001; Hartmann, 2005; Tusell *et al.,* 2007). In addition to that, during course of natural infection of FECVs, it also causes systemic infection via monocyte-associated viraemia, but the exact pathogenesis is unknown. Yet, as compared to FIPV infection, the levels detected in blood are much lower for FECVs (Meli, 2004; Simon *et al.,* 2005; Can-Sahna *et al.,* 2007).

On the other hand, FIP is an immune complex disease involving FIPVs, antiviral antibodies and complement. The complex immune reactions then cause disseminated vasculitis which is the hallmark of FIP (Hartmann, 2005; Pedersen, 2009). FIP occurs when FECVs mutate to FIPVs, gaining an enhanced monocyte/macrophage tropism (Stoddart & Scott, 1989; Rottier *et al.,* 2005), infecting the circulating monocytes. Subsequently, the infected circulating monocytes facilitate the distribution of the virus to various organs and tissues within the body such as mesenteric lymph nodes, serosal surfaces of the intestines, kidneys, meninges, spinal cord and retina of the eyes (Pedersen, 2009). Then, the virus attracts more antibodies, complement is fixed, macrophages and neutrophils concentrate at the site, and granulomatous lesions develop. Apart from that, when the circulating antigen-antibody complexes deposit in the endothelium of blood vessels, the complement will be fixed and granulomatous lesions will start to develop. As the inflammatory reaction progresses, more chemotactic substances and vasoactive amines are produced. All these lead to increased vascular permeability which subsequently gives rise to protein-rich exudation (Levy & Hutsell, 2016).

FIP has two forms of clinical manifestation which are the effusive or wet form and non-effusive or dry form. Cats with a poor cell-mediated immune response usually end up having wet form FIP. It is characterized by peritoneal effusion (pot-bellied) and/or less commonly pleural effusion or pericardial effusion and dehydrated cats; while cats with partial protection against the infection by cell-mediated immunity usually end up having dry form FIP. It is characterized by the inflammatory pyogranulomatous lesions found in almost any organs including kidneys, liver,

mesenteric lymph nodes, spleen and omentum, causing more diverse and vague clinical signs.

Normally, dry form also involves central nervous system (CNS) and ocular system. Wet form is more common as compared to dry form. Besides, wet form has a more rapidly progressive course with death in weeks to several months while dry form may continue for a period of many months especially in cats with only ocular system involvement (Pedersen, 2009; Diaz & Proma, 2009). Dry form may develop to effusive form at the end stage when the immune system finally collapses (Goodson *et al.,* 2009; Patel & Heldens, 2009).

2.6 Diagnosis of Feline Coronavirus

Infection of FECVs rarely causes severe clinical signs that require specific diagnosis of the causing agent. FCoV can be demonstrated in the faeces of infected cats by electron-microscopic examination and by reverse transcriptase polymerase chain reaction (RT-PCR), indicating viral shedding and carrier status of the cats (Sparkes, 2004). Whereas for ante-mortem diagnosis of FIP, it requires multiple tests interpreted with history, signalment and clinical signs of the FIP-suspected cats (Pedersen, 2014).

There are both indirect and direct tests in diagnosing FIP, at which direct tests might provide a definitive diagnosis, while indirect tests may further increase the difficulty to determine if the clinical signs are indeed caused by FIP. The classic indirect tests include complete blood count (CBC), total serum protein, albumin and globulin levels, A:G ratio and serum biochemistry (Addie *et al.,* 2009; Pedersen, 2009;

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Drechsler *et al.,* 2011). Normally, the CBC reveals non-regenerative anemia, leucocytosis with an absolute increase in neutrophils and an absolute decrease in lymphocytes, high serum globulin and low albumin with a low A:G ratio. Besides, cats with FIP are common to have hyperbilirubinemia and hyperbilirubinuria that are not normally associated with elevations of liver enzymes (Pedersen, 2014).

In addition to that, analysis of effusion of peritoneal and/or pleural cavity in wet-form FIP is another useful ancillary diagnostic test. The fluid usually appears yellow-tinged due to bilirubin, clear to moderately cloudy, viscous and high in protein, and may contain a fair number of cells (500-5000/µL) like macropahges, neutrophils and some lymphocytes. At the same time, the effusion can be further used in Rivalta test which has a sensitivity of 91% and a specificity of 66% (Fischer *et al.,* 2012). A few drops of the aspirated effusion are mixed with a weak acetic acid solution in a tube, and the appearance of white flocculent clump represents a positive result.

Measurement of feline coronavirus antibody titers is another indirect diagnostic test of FIP. Both FECVs and FIPs evoke the same antibody responses, thus it cannot be used to definitively diagnose FIP, but the likelihood of a titer being associated with FIP increases with its magnitude, thus it may give a postulation of FIP in cats. Yet, the presence of low coronavirus titers in cats with confirmed FIP has posed problem in interpreting the result. In the case of a cat dies with differential diagnosis of FIP, a thorough necropsy with adequate histopathologic examination of diseased organs and tissues is an accurate way to confirm a diagnosis (Pedersen, 2014).

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All in all, a definitive diagnosis of FIP will need the detection and identification of viral RNA or proteins within macrophages in typical lesions or fluids from diseased tissues. These direct tests include identification of viral proteins by immunohistochemistry which is also a gold standard and identification of viral RNA by PCR-based tests especially from blood, diseased tissues and fluids (Sharif *et al.,* 2010b ; Pedersen, 2014).

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3.0 MATERIALS AND METHODS

3.1 Animals

The research project was firstly approved by the Institutional Animal Care and Use Committee (IACUC), with reference number: UPM/IACUC/AUP – FYP.2016/FPV (26,56) (as attached Appendix A). A total of 21 pet cats were identified in Gasing Veterinary Hospital, Petaling Jaya, Selangor. 16 of them were clinically ill with various clinical signs, including gastrointestinal signs, respiratory signs, renal disease, hepatobiliary disease whereas the other 5 cats were healthy. The signalment was categorized as shown in Table 3.1.

Table 3.1: Signalment of the pet cats, classified based on different categories.

Signalment		No. of Cats
a) Health Status	Healthy	5
	Clinically Ill	16
b) Age	<2 years old	4
	≥2 years old	17
c) Gender	Intact Male	6
	Intact Female	2
	Castrated Male	9
	Spayed Female	4
d) No. of Cats in the household	Single & indoor	10
	Single & semi-roamer	4
	*Multi & indoor	4
	Multi & semi-roamer	3
e) **Vaccination Status	Up-to-date	14
	Not-up-to-date	7

Note: The asterisk represents *Multi-cat household: ≥ 2; **Not vaccinated against FCoV respectively.

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3.2 Samples Collection

The cats were identified, and owners were approached for their consent with the samples collection from their cats (as attached Appendix B). Approximately 1-3 mL of blood from jugular, cephalic or saphenous vein and rectal swab were collected. Blood was collected by the attending veterinarians of the Hospital while rectal swab was collected by the student with the supervision from the attending veterinarians with the cats being properly restrained. A total of 42 samples were collected, consisting of 21 blood samples and 21 rectal swabs, from 21 pet cats. The sampling was carried out by using convenience sampling method at which only first 21 pet cats that visited to Gasing Veterinary Hospital within the first week (8 Jan – 15 Jan 2017) were selected.

3.3 Samples Transportation, Processing and Storage

The blood was kept in ethylenediaminetetraacetic acid (EDTA) collection tube (BD Franklin, USA), while the rectal swab was kept in a 2.0 mL microcentrifuge tube containing 0.5 mL of Phosphate Buffer Saline (PBS) of pH 7.2. The rectal swab was kept in -80 °C until further analysis while blood samples were stored temporarily at 4 °C and processed within 12 hours. The blood was centrifuged at 450 x *g* for 5 minutes to separate out the red blood cells, buffy coat and plasma. The buffy coat was then carefully aspirated with 23 G needle of 1.5 inches attached to a 1 mL syringe and kept in a 2.0 mL microcentrifuge tube, stored in -80 °C until further used.

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3.4 RNA Extraction

3.4.1 Rectal Swab Samples

The RNA from rectal swab was extracted by using FavorPrep™ Viral Nucleic Acid Extraction Kit I (Favorgen®, Taiwan) according to the manufacturer's instructions. About 150 µL of rectal swab sample was pipetted into a 2.0 mL microcentrifuge tube (Eppendorf, Germany), then 570 µL of VNE buffer was added, the sample mixture was mixed well by vortexing and it was allowed to stand at room temperature (15-25 °C) for 10 minutes. Next, 570 µL of 100 % ethanol was added to the sample mixture, it was mixed well by pulse vortexing. Then a VNE column was combined with a collection tube provided in the kit. It was followed by transferring 700 µL of sample mixture by pipetting to the VNE column and centrifuged at 8,000 x *g* for 1 minute. The flow-through was discarded and the VNE column was combined back again to the used collection tube. The step was repeated with the rest of sample mixture. The collection tube with the flow through was discarded and the VNE column was transferred to a new collection tube. Then 500 µL of wash buffer 1 was added to the VNE column, and centrifuged at 8,000 x *g* for 1 minute. The flow-through was discarded and the VNE column was combined back again to the same collection tube. It was then followed by adding in 750 µL of wash buffer 2 to the VNE column and it was centrifuged at 8,000 x *g* for 1 minute. The flow-through was discarded and the VNE column was combined back again to the same collection tube. This step was repeated once. In order to dry the VNE column, the VNE column was again centrifuged at approximately 18,000 x *g* for an additional 3 minutes. The collection tube with the flow through was discarded.

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The VNE column was then combined with an elution tube. Next, 50 µL of preheated RNase-free water was added to the membrane center of the VNE column, and it was allowed to stand for 2 minutes and centrifuged at 18,000 *g* for 2 minutes to elute the nucleic acid. The eluted RNA was then aliquoted into 3 parts and stored at -80 °C until being used.

3.4.2 Buffy Coat Samples

The RNA from each buffy coat sample was extracted using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions. About 250 µL of buffy coat sample was pipetted into a 1.5 mL microcentrifuge tube (Eppendorf, Germany), then about 700 µL of TRIzol® Reagent (Invitrogen, USA) was added, the sample mixture was then allowed to stand at room temperature (15-25 °C) for 5 minutes. Then 200 µL of chloroform was added and mixed thoroughly by vortexing for 15 seconds. The sample mixture was then allowed to stand at room temperature for 15 minutes.

Next, it was centrifuged at 12,000 x *g* at 4 °C for 15 minutes (Centrifuge 5417 R, Eppendorf, Germany). The sample would appear separated into three phases, which were lower red organic phenol-chloroform phase, interphase and a colourless upper aqueous phase. The aqueous phase of about 500 µL was pipetted out by angling the tube at 45 °C and placed into a new 1.5 mL microcentrifuge tube.

Next, 800 µL of isopropanol was added to precipitate the RNA. The mixture was gently mixed and allowed to stand at room temperature for 30 minutes. Next, it was centrifuged at 12,000 x *g* at 4 °C for 20 minutes. The supernatant was removed from

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the tube. Next, 1 mL of 100% ethanol (WVR Chemicals, France) was added by pipetting to wash the RNA pellet, the mixture was then centrifuged at 12,000 x *g* for 20 minutes at 4 °C. The ethanol was then discarded. The step was repeated once. Then, the RNA pellet on the wall of the tube was marked, and the RNA pellet was being air-dried for about 3 hours. The RNA pellet was then dissolved with 15-20 µL of RNase-free water, depending on the size of the RNA pellet. The solution was then aliquoted into 2 parts and stored at -80 °C until further used.

3.5 Measurement of RNA Concentration

The total RNA concentration was determined by using spectrophotometer (BiophotometerPlus, Eppendorf, Germany). Firstly, 100 µL of deionized water was placed in a cuvette (Uvette®, Eppendorf, Germany) and placed in the spectrophotometer for blank. Next, 1 µL of extracted RNA was diluted with 99 µL of deionized water in a new cuvette and placed again in the spectrophotometer. The absorbance was measured at A260/A280 (ratio 1.8-2.1) and A260/A230 (ratio 2.0-2.2). The concentration of the extracted RNA was measured in µg/ml unit.

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3.6 Primers Selection

The primer pair was designed by using NCBI Primer-Blast tools (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (Bande *et al.,* unpublished). The primer pair targets the conserved polymerase gene (RNA-dependent RNA polymerase) of genus *Alphacoronavirus*. The primer sequences are shown in Table 3.6.

Table 3.6: The sequences of forward and reverse primers target the conserved polymerase gene.

Primer	Primer Sequence	Expected RT-PCR Product Size (bp)
Forward	5'- GTCTGGGACTATCCTAAGTGTGA-3'	420
Reverse	5'-CCATCATCAGATAGAATCATCATA-3'	

3.7 One Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

One Step RT-PCR was performed by using a commercial kit, Access RT-PCR System (Promega, USA). The RT-PCR was carried out as a single-tube assay with a final reaction mixture of 25 µL. First, the RNA template, forward and reverse primers and RT-PCR reagents (AMV/T*fl* Reaction Buffer (5x), 25mM $MgSO_4$, dNTPs Mix, AMV Reverse Transcriptase (5U/µL) and T*fl* DNA Polymerase (5U/µL)) were thawed and stored on ice to keep them chilled. Then the RT-PCR reagents were mixed by vortexing and centrifuged briefly. Next, the reaction mixture was prepared in each PCR tube as in Table 3.7.

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Table 3.7: The reaction mixture of 25 μL prepared for RT-PCR.

Reagent		Volume/reaction (μL)
1. AMV/T*fl* Reaction Buffer (5x)		5
2. $MgSO_4$ (25mM)		2
3. dNTPs Mix (10mM each dNTP)		0.5
4. Forward Primer (10μM)		0.5
5. Reverse Primer (10μM)		0.5
6. AMV Reverse Transcriptase (5U/μL)		0.5
7. T*fl* DNA Polymerase (5U/μL)		0.5
8. RNA Template		5
9. Nuclease-free water		10.5
	Total	**25**

The reaction mixture was then spun shortly for few seconds and the RT-PCR amplification was performed on Mastercycler Gradient Thermal cycler (Eppendorf, Germany). The cycling protocols were carried as shown in Table 3.8.

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Table 3.8: Optimized cycling conditions of conventional RT-PCR assay for detection of Feline Coronavirus (FCoV).

Step	Temperature	Time	Cycle
Reverse Transcription	45 °C	45 min	1
AMV RT Inactivation & RNA/cDNA/Primer Denaturation	95 °C	1 min	1
Denaturation	94 °C	30 sec	35
Annealing	54 °C	30 sec	
Extension	72 °C	2 min	
Final Extension	72 °C	10 min	1
Hold	12 °C	∞	1

3.8 Separation of PCR Products by Agarose Gel Electrophoresis

Agarose (Vivantis, Malaysia) was used to prepare 1.5 % (w/v) agarose gel for electrophoresis by adding 1.5 grams of agarose powder into 100 mL of 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer. Next, the mixture was heated in a microwave (Panasonic, Malaysia) until the powder was totally dissolved. Then, 4 µL of nucleic acid staining solution (Redsafe^{TM}, iNtRON, Korea) was added to the agarose solution and mixed thoroughly by gently swirling the 500 mL glass bottle (Schott, Germany).

The agarose solution was first being cooled to around 60 °C. Then it was poured into a gel casting tray with well-forming comb nicely fitted. The agarose solution was allowed to solidify at room temperature around 20-30 minutes. The whole gel casting tray was covered with aluminium foil to reduce the exposure to light that can cause reaction to the staining solution added earlier. The comb was then removed, and the gel together with the tray was was placed into the electrophoresis tank filled with 1X TAE buffer (Medigene, Malaysia).

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Then, 5µL of negative control was pipetted out and mixed well with 1µL of DNA Blue/Orange Loading Dye, 6x (Promega, USA) before being loaded into the first well. It was then followed by 5 µL of PCR product from each sample being pipetted out and mixed individually with 1 µL DNA Blue/Orange Loading Dye, 6x and lastly being loaded into the wells. Positive control was being prepared with the same manner as the negative control and sample, and being loaded into the third well. Lastly, 3 µL of VC 100 bp Plus DNA ladder (Vivantis, Malaysia) was added into the second well as comparison for the RT-PCR products.

Electrophoresis was carried out at 80V for approximately 45 minutes by using PowerPAC™ Basic (Bio-Rad, USA) or until the DNA ladder reached the opposite end of the gel. After that, the gel was placed in GeneGenius Gel Light Imaging System/Gel Imager (Syngene, UK) for viewing. The image was captured and analysed by using GeneDirectory software (Syngene, UK).

3.9 DNA Purification and Sequencing

The unpurified PCR products in the form of DNA were sent for purification and sequencing at First Base Laboratories Sdn. Bhd. (Malaysia) by using Sanger sequencing method.

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3.10 Bioinformatics Analysis of Feline Coronavirus Polymerase Gene Sequence

3.10.1 Basic Local Alignment Search Tool (BLAST)

The Basic Local Alignment Search Tool (BLAST) is a tool under the National Centre for Biotechnology Information (NCBI) database which enables the users to search nucleotide sequences to find the regions of similarity between biological sequences, normally is the query and the target sequences.

Edited partial polymerase gene sequences were subjected to BLAST to confirm that they were similar to other FCoV isolates or even coronavirus of other species of animals, and to identify the percentage of sequence homology. Those related sequences were downloaded from the GenBank® sequence database by NCBI and were analysed with the software of Molecular Evolutionary Genetics Analysis V7 (MEGA, USA).

3.10.3 Multiple Alignments

The partial polymerase gene sequence of the related reference isolates downloaded and one from local FCoV strain (Cat-18) were aligned by using MEGA V7 software. Multiple alignments were carried out on the sequences to align them in the best match using built-in MUSLCE program. Once the alignment was performed, the sequences were trimmed to have the base pairs to be in the same length in order to generate an accurate phylogenetic tree. Besides, a pairwise sequence identity matrix was created to identify pairwise nucleotide identity for the reference isolates and the local FCoV strain by using CLC Main Workbench V9 software (Qiagen, Germany).

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3.10.4 Construction of Molecular Phylogenetic Tree

An unrooted phylogenetic tree was constructed by using maximum-likelihood method after multiple alignments. MEGA V7 software was used to generate a phylogenetic tree. Tree reliability was accessed by using 1000 bootstrap replications. The sequence of the local FCoV strain (from the positive sample) was compared with the isolates of FCoV, CCoV, TGEV, Human Coronavirus (HCoV) and FIPV from different countries including Unite States of America, Netherlands, China, Taiwan, Australia, Japan and Korea.

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4.0 RESULTS

4.1 RT-PCR Amplification

4.1.1 Rectal Swab Samples

Based on the conventional RT-PCR assay using forward primer of 5'-GTCTGGGACTATCCTAAGTGTGA-3' and reverse primer of 5'-CCATCATCAGATAGAATCATCATA-3', only 1 out of 21 samples was positive for feline coronavirus (FCoV), which was 4.76% of all cases. Positive result was defined as band being expressed at the 420 bp region after running electrophoresis on 1.5% (w/v) agarose gel. This specific band at 420 bp is the amplification products of the RNA-dependent RNA polymerase gene region of FCoV. For positive control, FECV 79-1683 from American Type Culture Collection (ATCC) with No. VR-989™ was used, whereas for negative control, only buffer reaction without RNA template after RT-PCR was used. Figure 4.1.1 showed an example of the RT-PCR results of the rectal swab samples from first 10 cats after gel electrophoresis. Figure 4.1.2 showed RT-PCR results of the rectal swabs from the remaining 11 cats after gel electrophoresis.

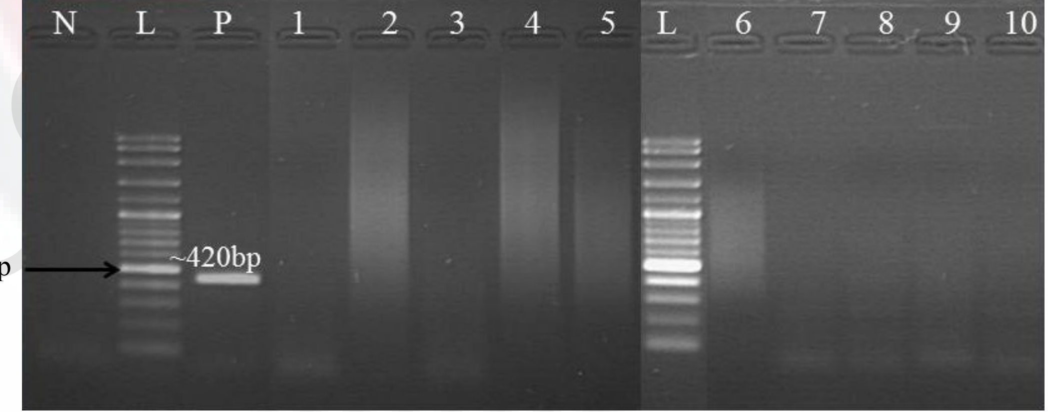

Figure 4.1.1: RT-PCR results of rectal swabs of first 10 cats using primers targeting the conserved polymerase gene of FCoV. Lane N: Negative control; Lane L: 100 bp DNA marker; Lane P: Positive control (FECV 79-1683); Lane 1-10: Negative amplification.

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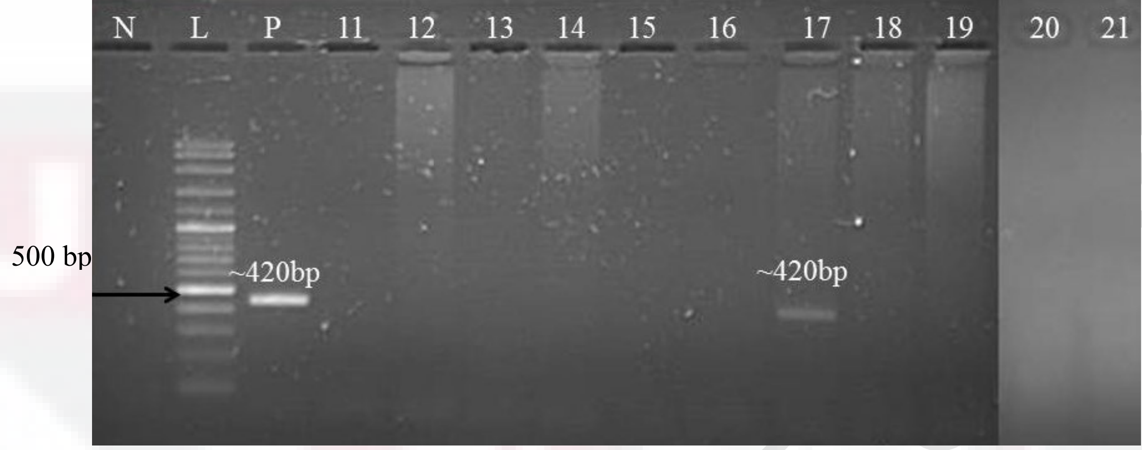

Figure 4.1.2: RT-PCR results of rectal swabs of the remaining 11 cats using primers targeting the conserved polymerase gene of FCoV. Lane N: Negative control; Lane L: 100 bp DNA marker; Lane P: Positive control (FECV 79-1683); Lane 11-16 and 18-21: Negative amplification; Lane 17: Positive amplification.

4.1.2 Buffy Coat Samples

Based on the conventional RT-PCR assay, none of the buffy coat samples tested was positive for feline coronavirus (FCoV). For positive control, FECV 79-1683 from American Type Culture Collection (ATCC) with No. VR-989™ was used, whereas for negative control, only buffer reaction without RNA template after RT-PCR was used. Figure 4.1.3 showed an example of the RT-PCR results of the buffy coat samples from first 15 cats after gel electrophoresis. Figure 4.1.4 showed another example of the RT-PCR results of the buffy coat samples from the remaining 6 cats after gel electrophoresis.

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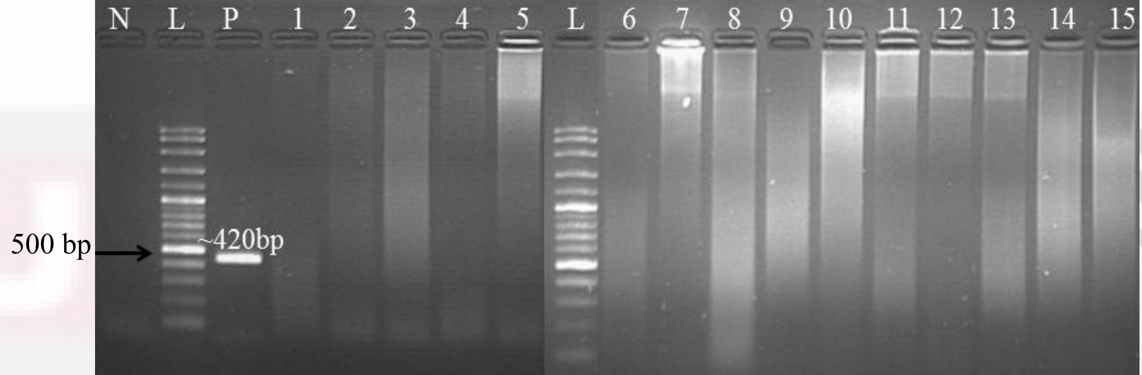

Figure 4.1.3: RT-PCR results of buffy coat samples of first 15 cats using primers targeting the conserved polymerase gene of FCoV. Lane N: Negative control; Lane L: 100 bp DNA marker; Lane P: Positive control (FECV 79-1683); Lane 1-15: Negative amplification.

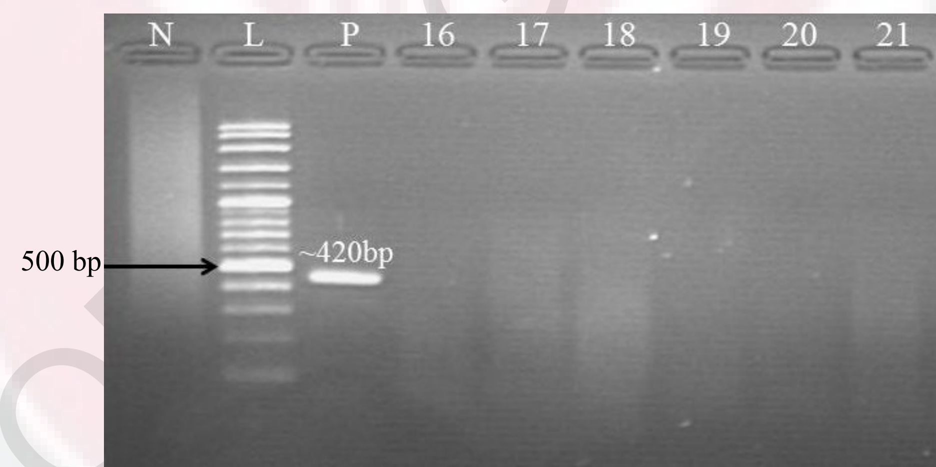

Figure 4.1.4: RT-PCR results of buffy coat samples of the remaining 6 cats using primers targeting the conserved polymerase gene of FCoV. Lane N: Negative control; Lane L: 100 bp DNA marker; Lane P: Positive control; Lane 16-21: Negative amplification.

4.2 Bioinformatics Analysis of Local FCoV Strain

4.2.1 Sequence Editing and Assembly

Only 1 PCR product of FCoV from Cat-18 was positive, and it was sent for sequencing. Sequence outputs in form of electropherogram were edited and assembled using bioinformatics software. Sequence fragment of FCoV was of length of 395 bp. The sequence was assigned with an individual identification of FCoV_Malaysia_C18.

4.2.2 Basic Local Alignment Search Tool (BLAST)

The results of BLAST search showed that the partial sequence of polymerase gene of the local FCoV strain (Cat-18) was highly similar (92-98%) to the reference isolates found in the GenBank® of NCBI. At the same time, the coronavirus isolates of other animal species were found to have high similarities too, including CCoV and TGEV. The results suggested that the partial sequence of polymerase gene was conserved region in different coronavirus, but mostly were those under species alphacoronavirus 1.

4.2.3 Multiple Alignment and Pairwise Comparison

The sequence of the local FCoV strain (Cat-18) was aligned with other reference isolates downloaded from Genbank® based on their sequence identity and reliability in publications (**Table 4.2.3**). The alignment was done using MEGA V7.

Table 4.2.3: Reference isolates of coronavirus downloaded from GenBank®.

No.	Accession No.	Country	Source	Designation
1.	FJ938062.1	Netherlands	Spiro *et al.*, 2009	FCoV_UU9_Utrecht_ Netherlands
2.	EU186072.1	USA	Tekes *et al.*, 2008	FCoV_Black_USA (FIP)
3.	FJ938053.1	Netherlands	Spiro *et al.*, 2009	FCoV_UU7_Utrecht_ Netherlands
4.	FJ938051.1	USA	Spiro *et al.*, 2009	FCoV_RM_California_USA
5.	AB781793.1	Japan	Terada *et al.*, 2014	FCoV_KUK-H/L_Japan (FIP-Serotype I)

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No.	Accession No.	Country	Source	Designation
6.	AB781796.1	Japan	Terada *et al.*, 2014	FCoV_C3678_Japan
7.	AB781794.1	Japan	Terada *et al.*, 2014	FCoV_C3663_Japan (Serotype I)
8.	AB907634.1	Japan	Terada *et al.*, 2014	FCoV_Tokyo/cat/130627_Japan (Serotype II)
9.	AB781795.1	Japan	Terada *et al.*, 2014	FCoV_Yayoi_Japan (Serotype I)
10.	AF516906.1	Australia	Naylor *et al.*, 2002	CCoV_UWSMN-1_Sydney_Australia
11.	JQ404410.1	USA	Thor *et al.*, 2012	CCoV_TN-449_Georgia_USA
12.	AF124986.1	USA	Stephensen *et al.*, 1999	CCoV _California_USA
13.	GQ477367.1	Taiwan	Chuang *et al.*, 2009	CCoV_NTU336/F/2008_Taipei_Taiwan
14.	JN856008.2	USA	Town *et al.*, 2011	CCoV_A76_NewYork_USA
15.	JN234464.1	South Korea	Lee *et al.*, 2013	HCoV_HKU1_KNIH-2K85305_Korea
16.	HM130814	China	Wang *et al.*, 2011	HCoV_229E_PUMCH8425_Beijing_China
17.	AF124992.1	USA	Stephensen *et al.*, 1999	TGEV_California_USA
18.	AY437877.1	China	Yu *et al.*, 2003	TGEV_TH-98_Heilongjiang_China

Pairwise nucleotide comparison was done using CLC Genomic Workbench V9 (Qiagen, Germany) comparing the sequence of the local FCoV strain and the reference isolates to determine the sequence identity (Figure 4.2.3) The comparison revealed that the partial sequence of polymerase gene of the local strain was 84-95% in term of homology with the reference isolates of FCoV.

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4.2.4 Construction of Phylogenetic Tree

The unrooted phylogenetic tree depicting the relationship between the local strain (Cat-18) with the reference isolates available in GenBank® that included the coronavirus from different animal species was generated using the maximum-likelihood method with 1000 bootstrap replicates (Figure 4.2.4). MEGA V7 software was used to construct the tree.

Based on pairwise comparison of the sequence identity matrix, the reference isolate with the closest sequence identity to local FCoV strain was the FCoV_UU9_Utrecht_Netherlands (GenBank® accession number of FJ938062.1) from Netherlands. The same similarity was observed on the phylogenetic tree with the FCoV_UU9 was in the same clade as the local FCoV strain (FCoV_Malaysia_C18), and under the clustering of all the feline coronavirus (FCoV) isolates. Yet, this phylogenetic tree cannot differentiate its serotype as serotype I or II or biotype as feline enteric coronavirus (FECV) or feline infectious peritonitis virus (FIPV).

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		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
FCoV_Malaysia_C18	1		94.51	94.26	86.12	84.21	93.52	93.52	83.49	83.97	83.97	83.25	83.25	83.25	85.29	86.78	86.28	81.10	65.59	23.74
FCoV_UU9_Utrecht_Netherlands	2	0.04		96.96	86.09	84.41	95.70	96.96	82.97	84.17	84.17	83.73	83.73	83.25	87.37	88.89	87.88	82.25	65.57	24.27
FCoV_Black_USA (FIP)	3	0.04	0.03		87.53	86.33	97.22	97.22	84.89	85.61	85.61	83.73	83.73	83.25	87.37	89.39	88.38	81.77	66.33	23.79
FCoV_KUK-H_L_Japan (FIP-Serotype II)	4	0.04	0.04	0.02		96.19	85.85	86.81	94.92	94.92	94.92	82.78	82.78	82.30	77.51	79.19	78.47	84.11	60.78	23.96
FCoV_Tokyo_cat_130627_Japan(Serotype II)	5	0.07	0.06	0.03	0.04		84.41	85.85	94.16	94.92	94.92	83.01	83.01	83.49	78.95	79.19	78.95	85.09	61.27	25.18
FCoV_RM_California_USA	6	0.05	0.04	0.03	0.04	0.06		96.46	83.45	84.89	84.89	82.78	82.78	82.54	86.62	87.88	86.87	80.58	64.81	23.79
FCoV_UU7_Utrecht_Netherlands	7	0.05	0.03	0.03	0.03	0.04	0.04		84.65	85.13	85.13	84.21	84.21	83.97	88.13	88.89	88.38	81.53	66.84	24.03
FCoV_Yayoi_Japan(Serotype I)	8	0.08	0.07	0.05	0.05	0.06	0.07	0.05		92.89	92.89	83.01	83.01	82.30	78.47	79.19	78.95	84.35	61.76	24.45
FCoV_C3663_Japan (Serotype I)	9	0.07	0.06	0.04	0.05	0.05	0.05	0.05	0.07		100.00	83.73	83.73	83.25	78.23	80.38	78.95	84.60	59.56	24.69
FCoV_C3678_Japan	10	0.07	0.06	0.04	0.05	0.05	0.05	0.05	0.07	0.00		83.73	83.73	83.25	78.23	80.38	78.95	84.60	59.56	24.69
TGEV_California_USA	11	0.14	0.12	0.12	0.13	0.13	0.14	0.12	0.13	0.12	0.12		100.00	95.69	90.43	91.87	91.15	93.30	63.40	25.30
TGEV_TH-98_Heilongjiang_China	12	0.14	0.12	0.12	0.13	0.13	0.14	0.12	0.13	0.12	0.12	0.00		95.69	90.43	91.87	91.15	93.30	63.40	25.30
CCoV_California_USA	13	0.14	0.13	0.13	0.14	0.12	0.14	0.12	0.14	0.13	0.13	0.04	0.04		91.39	89.95	90.91	94.02	63.64	26.71
CCoV_NTU336_F_2008_Taipei_Taiwan	14	0.15	0.13	0.13	0.14	0.12	0.14	0.12	0.13	0.13	0.13	0.04	0.04	0.03		94.43	95.70	88.49	66.58	26.70
CCoV_A76_NewYork_USA	15	0.13	0.11	0.11	0.12	0.12	0.13	0.11	0.12	0.10	0.10	0.03	0.03	0.05	0.06		97.22	87.53	67.59	25.97
CCoV_TN-449_Georgia_USA	16	0.13	0.13	0.12	0.13	0.12	0.14	0.12	0.12	0.12	0.12	0.04	0.04	0.04	0.04	0.03		88.49	68.61	25.97
CCoV_UWSMN-1_Sydney_Australia	17	0.14	0.12	0.13	0.14	0.12	0.14	0.13	0.13	0.13	0.13	0.05	0.05	0.04	0.05	0.06	0.05		63.97	24.82
HCoV_229E_PUMCH8425_Beijing_China	18	0.39	0.41	0.39	0.41	0.41	0.42	0.39	0.40	0.44	0.44	0.38	0.38	0.38	0.39	0.37	0.36	0.40		23.77
HCoV_HKU1_KNIH-2K85305_Korea	19	2.82	2.71	2.94	3.02	2.50	2.94	2.82	2.76	2.66	2.66	2.41	2.41	2.10	2.07	2.21	2.21	2.36	2.66	

Figure 4.2.3: Sequence identity matrix with pairwise comparison to compare the sequence identity of the nucleotides of the local isolate with reference isolates of FCoV.

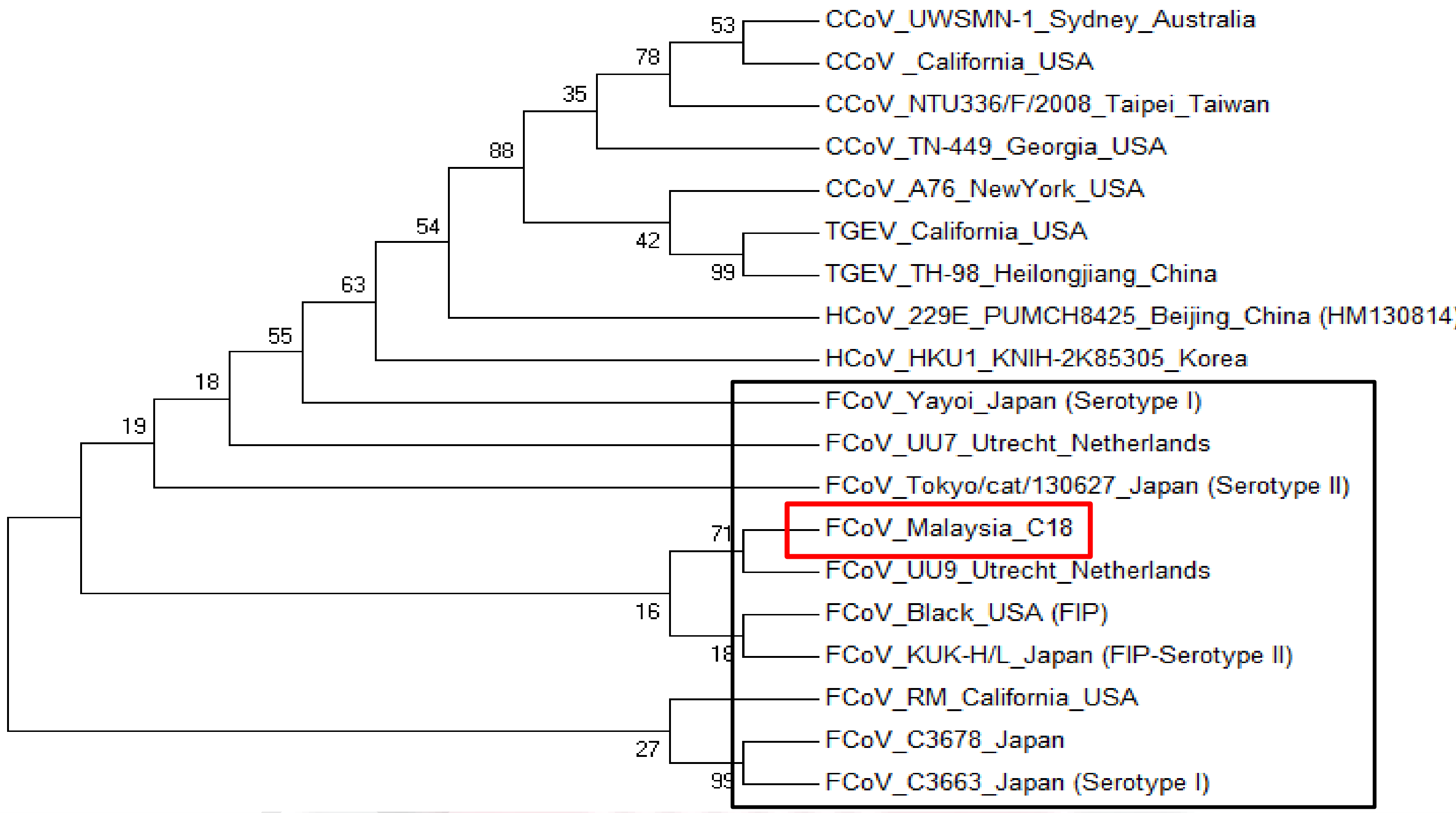

Figure 4.2.4: The unrooted phylogenetic tree depicting the relationship between the local isolate (FCoV_Malaysia_C18) with the reference isolates. The phylogenetic tree was constructed using the maximum-likelihood method with 1000 bootstrap replicates.

5.0 DISCUSSIONS

RT-PCR is a sensitive molecular detection method for viral RNA or DNA for various viruses. Rectal swabs sample collection was chose for FCoV detection because it shed mainly in faeces (Hartmann, 2005). The primer pair used is targeting Pol (polymerase gene) region which is a conserved region for FCoV. Thus it can be used to detect the circulating FCoV in the cat population (Sharif *et al.,* 2010a). Yet, it cannot differentiate biotypes or serotypes of FCoV. Besides, it can also detect other coronavirus like CCoV and TGEV.

From the RT-PCR results, only one out of 21 cats was tested positive, which was 4.76%. A previous study done by Sharif *et al.* (2009a) involved 44 cats in two catteries in Malaysia revealed that viral shedding by 84% of the cats, whereas this study utilized pet cats and at the same time 10 out of 21 of them were from single-cat household and kept strictly indoor. This group of cats had no contact with other cats, and there was no sharing of litter tray with other cats, it would definitely reduce the chances of fecal-oral transmission of FCoV. There were 4 out of 21 cats were from single-cat household but they were also semi-roamers. Semi-roaming cats used to bury feces in distinct territories which would also reduce the fecal-oral transmission of FCoV, yet there were limitations to know if the cats were in contact with other cats in the area, and the overall cat population densities in the area which could affect the virus transmission (Hartmann, 2005; Cave, 2004)

Besides, there were 7 cats from multi-cat household and the positive sample was detected from this group of cats. Multi-cat household has always been known to be a risk factor causing FCoV transmission. Most of the time, pet cats are better taken

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care of, with separate litter trays, and constant clean-up by the owners, this can definitely reduce the fecal-oral route transmission of FCoV (Hartmann, 2005).

As compared to serological test, RT-PCR assay has the advantage of directly detecting FCoV viral genome which indicates current infection or shedding (if it is detected in faeces), whereas detection of antibodies indicates past infection (Sharif *et al.,* 2009a). A single negative result is not conclusive to rule out FCoV infection because a FCoV-infected cat might shed the virus intermittently towards the end of infection (Herrewegh, 1997). According to Addie & Jarret (2001), a cat has to be tested negative monthly over a 5-month period in order to be considered a non-shedder.

Originally, it was shown that FECV was only localized in enterocytes, and FIPV is mutated from FECV based on the theory of internal mutations happen at certain genes in the persistently infected cats that switches the tropism of intestinal epithelium to monocyte/macrophage (Pedersen, 2014a), thus systemic infection was a defining moment for the development of feline infectious peritonitis. But the theory was proven to be incorrect, because regardless of development of FIP, monocyte-associated viremia does occur in FECV infection. Thus, in this study, buffy coat which is rich with leukocytes was taken for the FCoV detection indicating viraemia. Normally, viraemia occurs one week after FCoV infection, then it may not be detected afterwards, but it can recur along the course of infection in some cats (Kipar *et al.,* 2010).

In this study, there was no FCoV detected in all the buffy coat samples, indicating there was no viremia in all 21 cats, including Cat-18 with rectal swab being positive

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and Cat-21 with suspected-FIP sign of recurrent peritoneal effusion. Based on RT-PCR result for Cat-18, it was postulated that cat was only affected by FECV, less likely to be FIPV, or the viremia had subsided at the later stage of infection, even with viral shedding, as supported by study done by Kipar *et al.* (2010). Yet, even Cat-18 had no typical FIP signs, it cannot be confidently ruled out that FIPV may be present in the faeces, which was supported by a recent study by Wang *et al.* (2013) in Taiwan proving a horizontal transmission of FIPV via faeces, even though the earlier studies revealed that the horizontal transmission of FIPV would be very unlikely.

Based on RT-PCR result for Cat-21 with the absence of detectable viraemia or viral genome in buffy coat, it was postulated that FCoV may reside within parenchymal cells and/or resident macrophages. (Kipar *et al.,* 2010). According to Hartmann (2005), most cats with FIP will also shed virus in faeces, yet the virus load seems to reduce after a cat has developed FIP, which may explain why Cat-21 in this study showed a negative RT-PCR result for FCoV from faecal sample.

RT-PCR is a very sensitive method to detect FCoV antigen, with sensitivity and specificity are more than 90%, but the definitive diagnosis of FIP requires multiple tests due to the complexity of the diagnosis of FCoV. The examples of indirect tests that are normally used including complete blood count, with albumin, globulin and bilirubin checked, analysis of effusions, feline coronavirus antibody titers and Rivalta test. The gold standard for FIP diagnosis is immunohistochemistry on the biopsied or necropsied tissue (Pedersen, 2014b).

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From the phylogenetic tree constructed based on partial polymerase gene, the local FCoV strain formed a clade with a FCoV strain from Netherlands, and from the calculation of pairwise distances, it showed similarity of sequence identity of up to 95%. It suggested that they might be from the common ancestor. However, this result was not conclusive and definitive as the sequence used for the phylogenetic analysis was only limited to partial sequence of the RNA-dependent RNA polymerase gene, which was about 395 bp. For more accurate phylogenetic analysis and identification of the local FCoV strain, a full genome of the local strain should be obtained (Sharif *et al.,* 2009b). The phylogenetic analysis of FCoV in Malaysia done back in year 2010 using primer targeted 3'UTR showed that the homology of 3'UTR sequences of Malaysia FCoV strains had a homology of about 96% and the homology decreased to 93% when compared to published sequences of FCoV from USA, Netherlands, Taiwan and UK, which was still considered to be highly homologous (Sharif *et al.,* 2010a).

The local FCoV strain was in the same cluster with other FCoV isolates, thus the local FCoV strain was more closely related to FCoVs as compared to other coronavirus such as CCoVs, TGEVs, and human coronaviruses, it may suggest that there was no recombination with other species that might occured in the sequence, which was very common in coronavirus (Fehr & Perlman, 2015).

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CONCLUSION

This study detected feline coronavirus (FCoV) in local pet cats. Although most of the samples were negative, a single negative result could not completely rule out the possibilities of FCoV infection in the cats due to the complexity of the pathogenesis and diagnosis of the virus which requires multiple series of analysis. Phylogenetic analysis on the partial polymerase gene showed that the Malaysia FCoV strain was highly homologous to a FCoV strain from Netherlands.

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RECOMMENDATIONS

A larger sample size should be considered with the samples from high-risk group like multi-cat household as this study was on detection of FCoV, thus collecting more samples, which are also from high-risk group will increase the chance of FCoV detection and subsequently provides a better overview on the phylogenetic relationships of the FCoV strains in Malaysia and expand the existing genetic databases.

In this study, the primer targeted the RNA-dependent RNA polymerase gene, a conserved region for coronavirus, however it cannot differentiate between FCoV serotype I and II. In order to differentiate serotype I and II, the primer that targets the spike-protein gene should be used.

The genomes of FECV and FIPV have a homology of up to 99.5%. In order to differentiate them, full genome sequencing should be done and with special emphasis on the genes of 3c, 7b as well as spike-protein gene as few studies have shown that mutations in these genes are probably the cause of FIP development (Hartmann, 2005; Pedersen, 2014a). In addition to that, since the gene mutation that leads to FIP development is yet to be determined, experimental transmission of the FIPV isolate to specific pathogen free cats, followed by observation of development of clinical signs of FIP and lastly re-isolation of the virus from cats with FIP signs with sequencing can facilitate the collection of bioinformatics data of FCoV.

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APPENDIX A: APPROVAL LETTER FROM IACUC

PEJABAT TIMBALAN NAIB CANSELOR (PENYELIDIKAN DAN INOVASI)
OFFICE OF THE DEPUTY VICE CHANCELLOR (RESEARCH AND INNOVATION)

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE

Date:	30 December 2016
AUP No.:	FYP.2016/FPV (26, 56)
Project Titles:	(i) Molecular investigation of enteric and mutated forms of feline coronavirus (FCoV) in clinically Ill cats. (ii) Molecular detection of feline leukemia virus (FeLV) genome with correspond to clinically Ill cats associated with FeLV-related disorders.
Principal Investigator:	Dr. Nor Yasmin Bt Abd Rahaman
Associates:	Prof. Dr. Abd Rahman Omar; Assoc. Prof. Dr. Siti Suri Arshad; Dr. Prem Anand; Dr. Farina Mustaffa Kamal; Dr. Nur Fazila Saulol.
Students:	Liew Wuan Hoong; Kunambiga A/P Mummoorthy
Committee Decision:	The committee has reviewed and approved the proposed animal utilisation protocol, subject to relevant permit and/ or owner's consent.
Project Classification:	Acute
Category of Invasiveness:	B
Source of Animals:	Gasing Veterinary Hospital, 53 & 55, Jalan 5/58, Gasing Indah, 46000 Petaling Jaya, Selangor
Number of Animals Approved:	20 Cats
Duration:	9 January, 2017 – 12 February, 2017

(Prof. Dr. Mohd Hair Bejo)
Chairman,
Institutional Animal Care and Use Committee
Universiti Putra Malaysia

Pejabat Timbalan Naib Canselor (Penyelidikan dan Inovasi), Universiti Putra Malaysia, 43400 UPM Serdang, Selangor Darul Ehsan, Malaysia
Pejabat Timbalan Naib Canselor (P&I) 603-8947 1002 603-8945 1646, Pejabat Pentadbiran TNCPI 603-8947 1608 603-8945 1673, Pejabat Pengarah, Pusat Pengurusan Penyelidikan (RMC) 603-8947 1601 603-8945 1596, Pejabat Pengarah, Putra Science Park (PSP) 603-8947 1291 603-8946 4121 http://www.tncpi.upm.edu.my

APPENDIX B: CLIENT CONSENT FORM

Front

Informed Owner Consent Form

Department of Veterinary Laboratory Diagnosis,
Faculty of Veterinary Medicine, Universiti Putra Malaysia (UPM),
43400 UPM Serdang, Selangor.

Title of Study:

1. Molecular Investigation of Enteric and Mutated Forms of Feline Coronavirus (FCoV) in Clinically Ill Cats

2. Molecular Detection of Feline Leukemia Virus (FeLV) Genome with Correspond to Clinically Ill Cats Associated with FeLV-related Disorders

Involvement: These studies will be carried out by Liew Wuan Hoong with matric no.: 168469 and Kunambiga Mummoorthy with matric no.: 170006, both are final year students of the course Doctor of Veterinary Medicine (DVM), under the supervision of the principal investigator, Dr. Nor Yasmin Bt. Abd Rahaman, the senior lecturer from Faculty of Veterinary Medicine, UPM as well as the attending veterinarian, Dr. Prem Anand, the Director of Gasing Veterinary Hospital.

Period of Study: 19 December 2016 – 12 February 2016

Purpose of the Study:

i. To study the relationship between the presence of the FCoV and FeLV antigen detected in blood, faeces and saliva via PCR with the history & clinical signs presented in the clinically ill cats.
ii. Early identification of the infection through clinical signs may lead to the undertaking of informed action by veterinarians or owners to isolate the suspected sick cats, preventing the viruses or disease from further transmitting and spread the virus to other cats, especially in multi-cat household.

Procedures:
1. The clinically ill cat is presented and restrained.
2. The cat will be examined by the veterinarian attending to the case, and history of the cat will also be taken.
3. Cat's owner will be briefed on the study and if the owner agrees, owner consent form will be given and filled in.
4. Cat will be restrained on the table for blood collection through either through cephalic vein or jugular vein and transferred in an EDTA tube, at the same time, oral swab and rectal swab will be taken from the same animal.
5. After other diagnostic tests related to the case are done, cat will be returned to the owner.

Back

Possible Risk(s): In terms of invasiveness, little discomfort will be incurred on the animals upon the procedure of restraining for blood collection, taking oral swab and rectal swab. The procedures will be done either by the trained veterinarians or the students under the supervision of the attending veterinarian.

Voluntary Participation: The participation in this study is voluntary and withdrawal from the study is permitted at any time requested without any repercussions.

Confidentiality: Information from this study may be used in a published media and/or used for educational purposes but the status of disease in the animal; name of owner; animal name will remain confidential. If the owner is interested, results of his/her cat will be informed.

Financial Compensation: The will be no cost incurred upon the owner who participates, and at the same time, the owner will not get any compensation from the procedures done or problems that may be resulted in these studies.

Declaration

I have read and understand the above explanation in regard to my participation in this study and have been given an opportunity to ask any questions about the study.

Name of Owner :______________________ Signature :__________________
Phone No. :______________________
Email Address :______________________ Date :__________________

Signature of the FYP's Student :______________________ Date :__________________

For further enquiries or concerns, do contact:

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