UNIVERSITI PUTRA MALAYSIA

MOLECULAR DETECTION OF FELINE LEUKEMIA VIRUS (FeLV) IN CLINICALLY ILL LOCAL CATS

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MOLECULAR DETECTION OF FELINE LEUKEMIA VIRUS (FeLV)

IN CLINICALLY ILL LOCAL CATS

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In partial Fulfilment of the Requirement for the

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Universiti Putra Malaysia,

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CERTIFICATION

It is hereby certified that we have read this project entitled ‘‘Molecular detection of Feline leukemia virus (FeLV) in clinically ill local cats”, by Kunambiga Mummoorthy and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course of VPD 4999-Final Year Project

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DEDICATIONS

This project paper is dedicated to

My family

My friends

My coffee

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LIST OF ABBREVIATIONS

%	Percent
µL	Microliter
µM	Micromolar
°C	Degree Celsius
ATCC	American Type-culture Collections
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CD4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
Dntp	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent Assay
FeLV	Feline leukemia virus
g	Gram
IACUC	Institutional Animal Care and Use Committee
IN	Integrase
Kb	Kilobase
LTRs	Long Terminal repeats
MEGA	Molecular Evolutionary Genetics Analysis

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mL	Milliliter
mg	Milligram
mg/kg	Milligram per kilogram
MgSO4	Magnesium Sulphate
Mm	Millimeter
NCBI	National Center for Biotechnology Information
NJ	Neighbour-joining
PBS	Phosphate buffer solution
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
SU	Surface protein
RT-PCT	Reverse Transcription Polymerase Chain Reaction
TAE	Tris-acetate-ethylenediaminetetraacetic acid
UPM	Universiti Putra Malaysia
USA	United States of America
UK	United Kingdom
UV	Ultraviolet
w/v	Weight per volume
x*g*	Relative centrifugal force

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ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinanar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek IlmiahTahun Akhir

PENGESANAN MOLEKUL FELINE LEUKEMIA VIRUS (FeLV) DALAM KUCING TEMPATAN YANG SAKIT SECARA KLINIKAL

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2017

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Feline leukemia virus (FeLV) tergolong di bawah genus Gammaretrovirus dan dikaitkan dengan pelbagai tanda-tanda klinikal di seluruh dunia. Setakat ini, pencirian molekul FeLV tempatan yang pertama dan satu-satunya dilakukan pada tahun 2014 telah

mendedahkan bahawa pencilan tempatan berkait rapat dengan pencilan UK. Oleh kerana liputan terhad kajian dan sifat semula jadi virus yang biasanya mengintegrasikan DNA ke dalam genom dan menjalani mutasi, kajian mengenai status semasa jangkitan FeLV adalah diperlukan. Oleh itu, kajian ini bertujuan untuk mengesan antigen FeLV pada kucing sakit secara klinikal dengan mengunakan kaedah RT-PCR dan untuk membandingkan persamaan varian yang dikenalpasti pada masa ini dengan pencilan virus terdahulu dari Malaysia dan taburan geografi yang lain. Dengan menggunakan kaedah pensampelan mudah, plasma dan air liur dikumpulkan dari 15 kucing sakit secara klinikal dan 5 kucing sihat dari Hospital Gasing Veterinar. Nukleik asid virus telah diasingkan dan tertakluk kepada One-Step RT-PCR dengan primer khusus yang mensasarkan kawasan U3LTR dan gag separa yang sangat terpelihara. Dua kucing telah diuji positif untuk antigen dari kumpulan sakit secara klinikal. Separa nukleotida penjujuk and analisis filogenetik mendedahkan bahawa varian semasa didapati 93-99% homolog kepada pencilan Malaysia sebelum ini dan masih berkait rapat dengan varian daripada UK. Menariknya, ia juga didapati berkait rapat dengan varian yang diasingkan dari Jepun. Kesimpulannya, kajian ini menonjolkan kemungkinan hubungan evolusi di antara FeLV dari Malaysia dengan FeLV dari UK dan Jepun.

Kata kunci: *Feline Leukemia Virus (FeLV), RT-PCR, Separa nukleotida, penjujukan, analisis filogenetik, varian*

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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 DETECTION OF FELINE LEUKEMIA VIRUS IN CLINICALLY ILL IN LOCAL CATS

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2017

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Feline leukemia virus (FeLV) belongs under the genus Gammaretrovirus and is associated with a wide range of clinical signs worldwide. Sofar, the first and only molecular characterization of local FeLV isolates performed in 2014 revealed that local isolates to be closely related to UK isolate. Due to limited coverage of the study and the nature of the virus that typically integrates the DNA into the host genome and undergoes mutation, study on the current status of FeLV infection is necessary. Therefore, this study aim to detect FeLV antigen in clinically ill cats by RT-PCR and to compare the currently

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identified variant similarity with previous related virus isolates from Malaysia and other geographical distribution. By using convenience sampling method, plasma and saliva were collected from 15 clinically ill cats and 5 healthy cats from Gasing Veterinary Hospital. Viral nucleic acid was extracted and subjected to One-Step RT-PCR with specific primer targeting the highly conserved U3LTR and partial gag regions. Two cats were tested positive for the antigen from the clinically ill group. Partial nucleotide sequencing and phylogenetic analysis revealed that the current variant are found to be 93-99% homologous to the previous Malaysian isolates and is still closely related to UK isolate. Interestingly, they were also found to be closely related to isolates from Japan. In conclusion, this study highlights the possibilities of evolutionary relations between FeLV from Malaysia with FeLV of UK and Japan.

Keywords: *Feline leukemia virus, RT-PCR, partial nucleotide sequencing, phylogenetic analysis*

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1.0 INTRODUCTION

Feline leukaemia virus (FeLV) is belongs to Gammaretrovirus genus from the family of Retroviridae. It was first described in the year 1964 by Jarrett *et al*., and is a naturally occurring gammaretrovirus that infects domestic catsand sporadically wild cats (Arjona *et al.,* 2007; Torres *et al.,* 2008; Bolin *et al.,*2011). It is an enveloped, positive sense, single-stranded RNA virus (Boeke *et al*., 2011). There are currently four FeLV subgroups of clinical importance namely FeLV-A, FeLV-B, FeLV-C or FeLV-T (Chandhasin *et al*., 2005; Levy, 2008). Subgroup FeLV-A is found in all naturally infected cats meanwhile subgroup B arises from the recombination of endogenous FeLV with FeLV-A whereas FeLV subgroup C and T are the mutated forms of FeLV-A. (McCaw,2010; Eiden *et al.,* 2010). FeLV is mostly transmitted via oro-nasal route via saliva and nasal secretions through sharing of food and water dishes as well as the cat's grooming or aggressive behaviour. Occasionally, vertical transmissions could occur but is of little relative significance (Gomes-Keller *et al.,* 2006; McCaw, 2010).

FeLV related disorders are associated with the manifestation of immunosuppression, lymphoid or myeloid tumors, anaemia, reproductive problems, immune complexes, enteritis and certain other disorders (McCaw, 2010). FeLV infections are divided into four stages namely the abortive infection (regressor cats), regressive infection (transient viraemia followed by latent infection), progressive infection (persistent viraemia) and focal or atypical infection (Torres *et al*., 2005, Hofmann-Lehmann *et al*., 2007; 2008, Levy *et al*., 2008). The disease is distributed worldwide in the feline population and the

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prevalence varies greatly with geographical location and risk factors such as health status, age and population density (Gleich *et al*., 2009, Bande *et al*., 2012).

In most clinics and hospitals, the diagnosis of FeLV is usually done with the detection of p27 antigen with rapid test kits (Hartmann *et al*., 2007). Conversely, the demonstration of p27 antigen is relatively difficult during the early viraemia and latent infections and studies conducted showed that FeLV viral RNA and provirus DNA are better predictors of progressive and latent infections respectively (Cattori *et al*., 2009).

To date, the first and only molecular assay investigation of the clinical status of Malaysian cats was carried out in 2014 by Bande *et al*., together with the first sequencing and phylogenetic characterizations of the Malaysian FeLV isolates that revealed Malaysian FeLV to be highly homologoues to each other and showed a possible evolutionary relationship with FeLV in UK. The hypothesis for this study proposes that the current local FeLV variant would be highly homologous to previous Malaysian isolates and would likely be closely related to the UK strain. This study was undertaken to fulfil these following objectives:

1. To detect FeLV antigen in clinically ill cats by using RT-PCR method.
2. To compare currently detected FeLV with previously detected FeLV from Malaysia and other geographical distribution

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2.0 LITERATURE REVIEW

2.1 Genomic structure

Feline Leukemia virus (FeLV) resides in the Gammaretrovirus genus and family of Retroviridae. Generally Retroviruses are made up of single stranded, positive sense RNA genome and the matured virion comprises of an enveloped structure of about 100 nm in diameter with a spherical conical core and dimericpolyadenylated RNA genome of about 7-8 kilobases (kb) length(Boeke *et al.,*2011)as shown in Figure 2.1FeLV has a diameter of 100-115 millimicron and a simple genomic structure of approximately from 8.5 to 8.7 Kb (Soe *et al*., 1983) as shown in figure 2.1 Retrovirus replicates by an RNA-dependant DNA polymerase (Reverse transcriptase) which transcribes viral RNA to double stranded DNA for integration into the host chromosome (Bannert *et al.,* 2010). Retroviruses essentially possess both RNA as genetic material while in the virion form and in DNA when integrated with the host genome as proviruses(Hartmann, 2012).

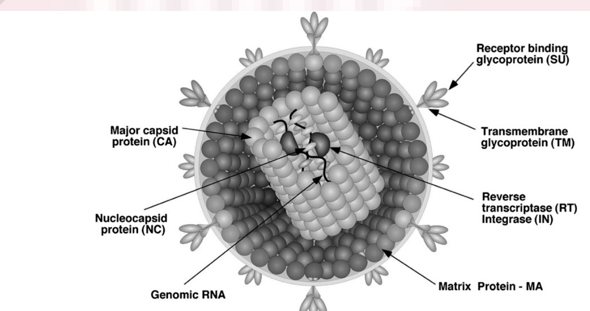

Figure 2.1: Structure of Retrovirus particle (Acquired from Virus *Taxonomy: Ninth Report of the International Committee on Taxonomy of Viruses*, 2012)

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The coding regions of FeLV genome are sequentially arranged in a *gag-pol-env* direction, starting from 5' to 3' direction as shown in figure 2.1.2.The *gag* gene protein encoded within the coding regions functions in the DNA synthesis as well as viral integration in early infection whereas the *pol* gene codes for the enzymes Reverse Transcriptase (RT) and integrase (IN).The RT enzyme reverse transcribes viral RNA to DNA and directs DNA polymerase as well as endonucleolytic activities whereas the IN enzyme aids in the integration of viral DNA into the cellular DNA target and formation of provirus(Bannert *et al.,* 2010).

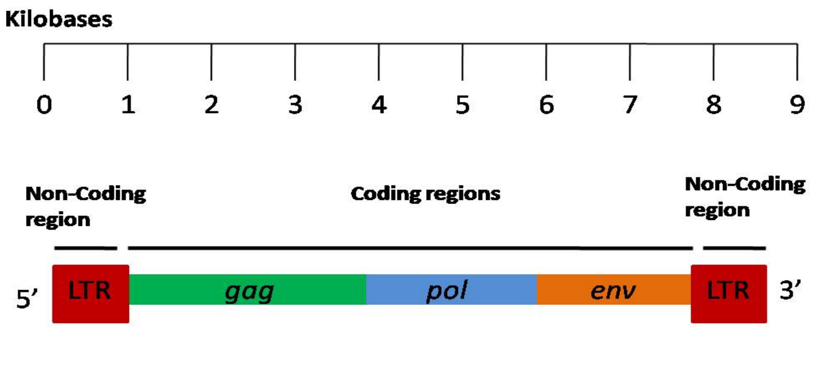

Figure 2.2: Linear arrangement of FeLV coding and non-coding regions (Adapted from *Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses*, 2012)

The *env*protein within the coding region acts as the cellular precursor and functions in mediating the interaction between the host cell and virion, in order to facilitate the entry of the virus into the host cell (Bolin *et al.,* 2011). The LTR (long terminal) region contains the U3 segment that is highly conserved within the region and serves as a differentiating feature between endogenous and exogenous FeLV(Bande et al., 2014).

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2.2 Stages of FeLV Infection

FeLV infections could be classified into several stages based on the clinical signs and presence of either free antigen in the form of RNA in the blood and saliva or in the form of provirus DNA. The stages are namely divided into the abortive infection, regressive infection, progressive infection and atypical infection (Hartmann, 2012).

The abortive stage arises following an encounter of the virus with the immunocompetent cats (formerly called “regressor cats)” that may terminate viral replication via an excellent humoral and cell-mediated immune response and the cat may never become viraemic (Major *et al*., 2010) These cats would have high levels of neutralizing antibodies and neither viral RNA or proviral DNA of FeLV can be detected in the blood at any time. However, with the usage of sensitive detection methods such as PCR, the virus actually can still be found in tissue samples. It indicates that the cat could not completely clear off the FeLV antigen from all of its bodily cells (Hartmann, 2012).

The regressive stage of infection develops following an effective immune response, whereby in this stage, the virus replication and viraemia are contained prior to or shortly after bone marrow infection. After the initial infection, replicating FeLV spreads systemically through infected mononuclear cells (lymphocytes and monocytes). During this stage, cats are likely to have positive results on tests that detect free antigen in plasma (e.g., ELISA, PCR). They shed the virus in this stage. However, the viraemia is terminated within weeks or months (therefore formerly called “transient viraemia). In the later stage of regressive infection following the transient viraemia, the virus would integrate of a

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copy ofits viral genome into cellular chromosomal DNA(bone marrow stem cells) and will undergo latent infection whereby the host cells now carry the part of the virus in the form of provirus DNA. At this stage however, no virus is actively produced (Hofmann-Lehmann*et al.,* 2001). Generally cats with regressive infection will show negative results in all tests that detect FeLV antigen. Cats in this stage of infection have a potential of reverting into a viraemic stage again due to the fact that the information for producing complete viral particles is present and can potentially be reused when antibody production decreases especially after immunosuppression (Hartmann, 2012).

Progressive infection occurs when then FeLV infection is not contained at an early stage by the host immune system. Thus, extensive virus replication occurs starting from the lymphoid tissues, followed by the bone marrow, mucosal and glandular epithelial tissues causing the infected cats to be persistently viraemic, making it infectious to other cats for the remainder of their life (Gomes-Keller *et al*., 2006). This condition was previously identified as "persistent viraemia stage" and is now classified as progressive infection. Cats with progressive infection develop FeLV-associated diseases and condition such as lymphoma, leukemia, anaemia, enteritis and Feline acquired immunodeficiency syndrome (FAIDS)due to decrease CD4+ and CD8+ T lymphocytes production and therefore most of them will die within a few years mostly 8 months to 3 years following infection(Hartmann, 2012). In this stage of infection, the cats will be tested positive for both the free RNA in the blood and saliva as well as the provirus DNA (Torres *et al*., 2005; Pepin *et al*., 2007).

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Atypical infections which are also known as focal infections have been reported in up to 10% of the experimentally infected cats but considerably rare in field cases. It develops following the partial clearance of the virus form the cat body and a persistent atypical local viral replication will develop in tissues such as in the mammary glands, bladder or eyes(Hartmann, 2012). In this stage of infection,cat may or may not be tested positive when tested for antigen detection due to intermittent or low-grade production of antigen(Levy*et al*., 2008).

2.3 Epidemiology

Feline leukemia virus affects domestic cats worldwide and causes considerable immunosuppression in cats (Sapian,2011). A recent FeLVprevalence study conducted in Malaysia in 2012 reported that the prevalence of FeLVin healthy and sick cat population is 5.1% and 18.9%, respectively (Bande *et al*., 2012). While studies carried out in the Asian region reported that the FeLV prevalence among cats to be 14.7% in Singapore (Chew-Lim *et al*., 1989) ,0% in Vietnam (Miyazawa *et al*., 1998), 2.9% in Japan (Maruyama *et al*., 2003)and 6% in Taiwan(Lin *et a*l., 1995). Meanwhile, in non-Asian countries, the prevalence is reported to be 3.4 % in Canada (Little *et al*., 2009), 5.3% and 3.7% in both Raleigh and Gainesville, USA, (Lee *et al*., 2002) and 4.6 % in Egypt (Al-Kappany *et al*., 2011). The variable prevalence rate of FeLV may have arisen from the FeLV vaccination practices in different countries as well as the difference in the lifestyle of the cats (Bande *et al*.,2012).

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FeLV is mostly transmitted via oro-nasal route via saliva and nasal secretions through sharing of food and water dishes as well as the cat's grooming or aggressive behaviour. (Gomes-Keller*et al*.,2006; McCaw, 2010). In some cases, vertical transmission is observed whereby it could lead to abortion and foetal death in-utero associated cases and kittens may become infected through suckling from infected queens (McCaw, 2010). Fleas are experimentally proven to be the vector for FeLV transmission (Vobias *et al*., 2003).

In addition, one of the risk factors associated with the FeLV infectionispopulation density whereby it is more commonly seen in multi-cat households and shelters or catteries due to stress, poor hygiene and increased direct contact among cats (Bande *et al*., 2012). Health status of the cat also determines the susceptibility towards FeLV(Levy *et al*., 2006). A study conducted in Malaysia states that sick cats are five times more likely to test for FeLV p27 antigen (Bande *et al.,* 2012). FeLV infection is generally considered to be a disease of the friendly or socialized cats (Hardy, 1973), but another contradicting study conducted by Bande *et al*., in 2012, observed a higher prevalence amongst aggressive cats. Young kittens are reported to be more susceptible to FeLV infections as compared to older cats (Levy *et al*., 2006).

2.4 Clinical findings associated with FeLV

Clinical signs of FeLV infection are usually non-specific, but anaemia or immunosuppression are more commonly seen in most cases but can also be classified into hematologic disorders, immunosuppression, tumors, immune-mediated diseasesand other

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syndromes including neuropathy, reproductive disorders and fading kitten(McCaw, 2010; Hartmann,2012).

2.5 DiagnosisofFeLV

The most widely employed method for FeLV detection is the enzyme-linked immunosorbent assay (ELISA) technique available in commercial kits that detect the FeLV p27 antigen(Hartmann *et al*., 2007). Similar to the principle of the ELISA, the immunochromatography is becoming more popular that also detects the FeLV p27 antigen (Hartmann *et al.,* 2007).

Virus isolation could be considered to be the gold standard for the diagnosis of FeLV(Hartmann *et al.,* 2007) and the samples used in FeLV isolation include the plasma, bone marrow and FeLV infected tissues (Gomes-Keller *et al.,* 2006). Electron microscopy could also be used for the identification of the morphological structure of FeLV(Jarrett *et al.,* 1964). Besides that, immunofluorescent antibody test(IFAT) that detects the *gag* antigen from the peripheral blood leukocytes and plateletsis also a relatively reliable test(Hartmann *et al.,* 2007).

Polymerase Chain Reaction (PCR) has contributed significantly to the current understanding of FeLV epidemiology, pathogenesis, transmission potentials and vaccination outcome(Hoffman-Lehmann*et al*., 2007). Samples frequently used in PCR detection of FeLV include the serum, plasma, whole blood, saliva, feces, urine, bone marrow and organ tissues (Lutz *et al*., 2009).

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3.0 MATERIALS AND METHODS

3.1 Animals

Prior to proceeding with sample collection, approval from the Institutional Animal Care and Use committee was obtained with the AUP No: /IACUC/AUP-FYP.2016/FPV (26,25) dated 30th December 2016(Appendix A). A sum of 20 animals was identified at the Gasing Veterinary Hospital, Bukit Gasing, Petaling Jaya, Selangor. Of those, 15 of the cats were owned by clients and exhibited a wide variety of clinical signs such as respiratory signs, renal disease, gastrointestinal signs and etc.The remaining 5 cats used for this study are cats found to be clinically healthy with no apparent illness.

3.2 Sample Selection

After the clinically ill cats were identified, owners were approached to a briefing regarding the study and were presented the consent forms. Once consent was obtained(attached in appendix B), sample collection was precededwith. After restraining the cat, 2 mL of blood (either via cephalic, saphenous or jugular vein) and oral swab were collected. The attending veterinarians at the Hospital withdrew the blood whilst the student under the supervision of the veterinarian obtained the oral swab. Further related information regarding the cats was also obtained for each individual client.

The samples for the healthy cats were collected from the resident cats of the Hospital that are clinically healthy and undergo regular screening tests as they are kept for the purpose of donating blood. Convenience sampling method were performed whereby any clinically ill cats were targeted and obtained samples from only after the consent of the owner was

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obtained. The clinically healthy cats were conveniently chosen from the resident cats of the hospital.

3.3 Sample Transportation, Processing and Storage

The blood was kept in the Ethylenediaminetetraacetic acid (EDTA) blood collection tube (BD Franklin, USA). While the oral swab was kept in a 2.0 mL microcentrifuge tube (Eppendorf, Germany) containing 0.5 mL of PBS (Phosphate Buffer Solution) of pH 7.2. The samples were then stored temporarily in an ice box with approximate temperature of 4 °C and processed in the Virology Laboratory in Faculty Perubatan Veterinar, UPM within 6 hours.

The blood was centrifuged at 450 x *g* for 5 minutes to separate the plasma, buffy coat and red blood cells. The plasma was then carefully pipetted and transferred into a 1.5 mL microcentrifuge tube (Eppendorf, Germany) and stored at -80°C and subjected to further analysis. The microcentrifuge tube containing the oral swab was also kept in the same -80°C condition without any processing until further used.

3.4 Total DNA/RNA extraction

The nucleic acid from each plasma sample was extracted by using FavorPrep™ Viral Nucleic Acid Extraction Kit 1(Favorgen, Taiwan) according to the manufacturer's instructions. 150 µL of plasma was transferred into a 1.5 ml microcentrifuge tube (Eppendorf,Germany), then 570 µL of VNE buffer containing the carrier RNA was added to the plasma sample and subjected to vortexing. The mixture was then incubated

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for 10 minutes at room temperature. Following that, about 570 µL of 100 % ethanol (WVR Chemicals, France) was then added into the sample mix and mixed well by process of plus-vortexing. A VNE column was combined with the provided collection tube and 700 µL of the sample mixture was transferred to the VNE column and centrifuged at 8000 x *g* for 1 minute and the flow-through was discarded. The VNE column was once again combined with the used collection tube. The rest of the sample mixture was then transferred into the VNE column and again centrifuged at 8000 x *g*for 1 minute. The flow-through was discarded along with the collection tube, while the VNE column was combined with a new collection tube. 500 µL of wash buffer1 was added to the VNE column and centrifuged at 8000 x*g* for 1 minute. The flow-through was discarded while the VNE column was combined with the used collection tube. 750µL of wash buffer 2 was then added to the VNE column and again centrifuged at 8000 x *g* for 1 minute.The flow-through was discarded and the VNE column was combined with the used collection tube. The previous step was repeated once. The VNE column with the used collection tube was then centrifuged at full speed (~18,000 x *g*) for an additional 3 minutes to dry the VNE column. The flow through was discarded together with the collection tube. The VNE column was combined with a provided elution tube and 500 µL of pre-heated RNase-free water was added to the membrane centre of the VNE Column. The VNE column was allowed to stand for 2 minutes in room temperature. It was then centrifuged at ~18,000 x *g* for 2 minutes to elute the nucleic acid. The eluted nucleic acid was then stored at -80° C until further processing.

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The nucleic acid extraction from the oral swab was extracted using innuPrep Virus DNA/RNA kit (Analytik Jena, Germany) according to the manufacturer's instructions. 200µL of lysis solution CBV/Carrier mix was pipetted into a 1.5 mL microcentrifuge tube (Eppendorf, Germany). 200 µL of sample (Saliva mixed with PBS) and 20 µL of Proteinase K were added into the tube and were mixed vigorously by pulsed vortexing for 10 seconds. The sample was then incubated at 70°C for 10 minutes. Then, 400µL of binding solution SBS was added to the lysed sample and mixed by vortexing. The sample was then applied to the provided spin filter located in a provided 2.0 mL receiver tube. The cap was closed and the sample was centrifuged at ~16,000 x *g* for 1 minute. The receiver tube was then discarded together with the filtrate. The spin filter was then placed into a new 2.0 mL receiver tube. 500µL of washing solution HS was then added into the spin filter and was then centrifuged at ~16,000 x*g* for 1 minute. The receiver tube was again discarded with the filtrate. The spin filter was again placed into a new 2.0 mL receiver tube. Next, 650µL of washing solution LS was added into the spin filter and again centrifuged at ~16,000 x *g* for 1 minute.

The receiver tube was again discarded with the filtrate and the spin filter was placed into a new 2.0 mL receiver tube. The previous step was repeated and the filtrate was discarded. The spin filter with a new 2.0mLreceiver tube was then centrifuged at ~16,000 x *g* for 5 minutes to remove all traces of ethanol. The 2.0 mL receiver tube was discarded and the spin filter was placed into a provided 1.5 mL elution tube. 60 µL of pre-heated RNase-free Water (70°C) was added into the spin filter and was incubated at room temperature

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for 2 minutes. The sample was then centrifuged at ~14000 x *g* for 1 minute. The eluted nucleic acid was then kept at -80°C.

3.5 Primer selection

Primers were selected based on several published journals to carry out RT-PCR. The primer pair selected was U3-F and G-R(Miyazawa &Jarrett, 1997; Bande *et al*.,2012). Primers were synthesized (First Base, Malaysia) and used to amplify target DNA of 770 bp as seen in table 3.1

Table 3.1: Oligonucleotides primers used in RT-PCT assay for the detection of exogenous FeLV

Primer	Target Region	Primer Sequence	Product Size (bp)
Forward: U3F	U3LTR	5’-ACAGCAGAAGTTTCAAGGCC-3’	770bp
Reverse: GR	*gag* region	5’-GACCAGTGATCAAGGGTGAG-3’	

3.6 Measurement of DNA/RNA Concentration

The final DNA/RNA concentration was obtained by performing spectrophotometry. After the extraction process had been completed, 1µL of the extracted nucleic acid was diluted in 98µL of deionized water in a cuvette and placed in a Biophotometer Plus photometer(Eppendorf, Germany) followed by reading of the concentration.

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3.7 Reverse Transcription-Polymerase Chain Reaction

One Step RT-PCR amplification

Amplification of nucleic acid from extracted plasma and saliva sample was carried out with the RT-PCR step by using an Access RT-PCR® system according to the manufacturer's instructions (Promega, USA). The reaction volume was prepared in 25µL thin wall micro tubes containing PCR reaction mixtures as seen in table 3.2

Table 3.2: Reaction mixture used in One-Step RT-PCT assay to detect RNA of the exogenous FeLV

No.	Reagent	Volume/ 1 reaction
1	Tfl 5X Reaction Buffer	5µL
2	$MgSO_4$(25mM)	2 µL
3	dNTP mix	0.5 µL
4	Foward Primer(U3F)10µM	0.5 µL
5	Reverse Primer(GR)10µM	0.5 µL
6	Rnase Inhibitor	0.5 µL
7	AMV Reverse Transcriptase	0.5 µL
8	Tfl DNA Polymerase	0.5 µL
9	RNA Template	5 µL
10	Nuclease-Free Water	10 µL
	Total=	25µL

The One-Step RT-PCR amplification was performed on Mastercycler Gradient thermal cycler (Eppendorf, Germany). The temperature and other programme conditions used in the One-Step RT-PCR step is described in Table 3.3

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Table 3.3: Amplification programme used in One-Step RT-PCR for the detection of exogenous FeLV

No.	Process	Temperature	Time	Cycle
1	Reverse Transcription	45° C	45 min	1x
2	AMV RT inactivation & RNA/cDna/Primer denaturation	95° C	1 min	1x
3	Denaturation	94° C	30 s	35 x
4	Annealing	58° C	30 s	
5	Extension	72° C	2 min	
6	Final Extension	72° C	10 min	1x
7	Hold/Soak	12° C	∞	1 x

Next, the amplification of DNA from the extracted saliva sample was carried out. The PCR mixture and amplification conditions were similar to the previous One-Step RT-PCR assay used for the RNA extraction from plasma carried according to the manufacturer's instructions (Promega, USA) except for the reverse transcription which was skipped in the conventional PCR programme. In addition, the RT-enzymes (AMV) as well as the RNase inhibitor were not included in the reaction mixture as seen in table 3.4

Table 3.4: Reaction mixture used in performing RT-PCR assay to determine the presence of FeLV virus DNA in cat saliva sample

No.	Reagent	Volume /1 reaction
1	Tfl 5X Reaction Buffer	5µL
2	$MgSO_4$(25 mM)	2 µL
3	dNTP	0.5 µL
4	Foward Primer (U3F) 10µM	0.5 µL
5	Reverse Primer(GR) 10µM	0.5 µL
6	Tfl DNA Polymerase	0.5 µL
7	RNA Template	5 µL
8	Nuclease-Free Water	11 µL
	Total=	25 µL

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The RT-PCR amplification was performed on Mastercycler Gradient thermal cycler (Eppendorf, Germany). The temperature and other programme conditions used in the RT-PCR step is described in Table 3.5

Table 3.5: Amplification protocol used in RT-PCR for the detection of DNA of exogenous FeLV

No.	Process	Temperature	Time	Cycle
1	Denaturation	94°C	30 seconds	35 x
2	Annealing	58°C	30 seconds	
3	Extension	72°C	2 minutes	
4	Final Extension	72°C	10 minutes	1 x
5	Hold/Soak	12°C	∞	1 x

3.8 Agarose Gel Electrophoresis

The amplified PCR products were analysed by gel electrophoresis using 1.5% (w/v) agarose gel (Vivantis, Malaysia). 100 mL of 1X tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer was added into a 500 ml Schottbottle(Schott, Germany). 1.5 g of the agarose powder is then dissolved in the 1X TAE solution to obtain 1.5% solution. The mixture was heated in a microwave (Panasonic, Malaysia) for about 1 minute or until the powder has been completely dissolved. Before the gelis solidified,4 µL of nucleic acid staining solution (Redsafe™, Intron, Korea) was pipette and mixed well into the solution. The agarosewas allowed to cooluntil about 60°C before poured on the gel casting tray (Medigene, Malaysia). The casted gel was allowed to solidify before removing the comb. The casted gel was then submerged in an electrophoresis tank(Medigene, Malaysia) containing 1 X TAE buffer. About 5µL of negative control was mixed with 1 µL of 6x Blue Green loading dye®(Promega, USA) on a clean parafilm® M(Chicago, Illinois) and

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loaded into the first well. The PCR products were also mixed and loaded in similar manner. The positive control was loaded into the third well whilst the 3µL of 100 bp BenchTop DNA ladder (Vivantis,USA) was loaded into the second well. Electrophoresis was run at 80 volt and 400 ampere for 45 minutes(Bio-RAD,USA). The gel was removed at the end of the run and viewed under the Geldoc XR+ UV transilluminator (BioRAD,USA). The image was then photographed using software Quantity One Basic (BioRAD,USA)

3.9 DNA/RNA sequencing

Un-purified positive samples were sent for purification and sequencing (First Base, Malaysia) using the Sanger sequencing method with the similar forward and reverse primer.

3.10 Bioinformatics Analysis of Feline Leukemia Virus Gene Sequence

3.10.1 Basic Local Alignment Search Tool (BLAST)

Edited partial U3LTR-*gag* region were subjected to BLAST (National Center for Biotechnology Information, U.S) searches in order to confirm the similarity to other FeLV isolates and to identify the percentage of sequence homology. Related FeLV sequences from the database were then downloaded into the CLC Main Workbench v 8.5.1 software (Qiagen, Germany) for comparisons with the current FeLV variant.

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3.10.2 Multiple Alignments and pairwise comparison

The current variant was aligned with the local sequence isolates and reference sequence isolates using the CLC Main Workbench v 8.5.1 software (Qiagen, Germany). Multiple alignments were then carried out with the sequences to align them to the best match. Once the sequences were aligned, the sequences were then trimmed to ensure that they were the same base pair length with each other in order to construct an accurate phylognetic tree. The software was also used to create pairwise sequence identity matrix to identify pairwise nucleotide identity.

3.10.3 Construction of Molecular Phylogenetic Tree

An unrooted phylognetic tree was constructed using Neighbour Joining method with the aid of Molecular Evolutionary Genetics Analysis (MEGA)7 software using distance based and topology pattern. Tree reliability was assed using 1000 bootstrap replications.

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4.0 RESULTS

4.1 RT-PCR Amplification

Based on the reverse transcriptase PCR using forward primer 5'-ACAGCAGAAGTTTCAAGGCC-3' that targeted the U3 segment of LTR region and reverse primer5'-GACCAGTGATCAAGGGTGAG-3' that targeted the gag region, none of the 5 healthy cats tested positive for the virus. However, 2 out of 15 of the clinically ill cats were positive for the Feline leukemia virus (FeLV) in both the plasma and saliva. Positive results were defined as band being expressed at 770 bp region after electrophoresis on 1.5% (w/v) agarose gel. The specific band of 770 bp is the specific expected amplification size of the partial U3LTR-gag region of the FeLV. For the positive control DNA from feline lymphoblastic cell line (FL24) persistently infected with FeLV-UCDI strain was obtained from ATCC. An example for the gel electrophoresis for the cats with FeLV positive bands of 770 bp is shown in lane 5 and 17Figure 4.1 for saliva &Figure 4.2 for plasma

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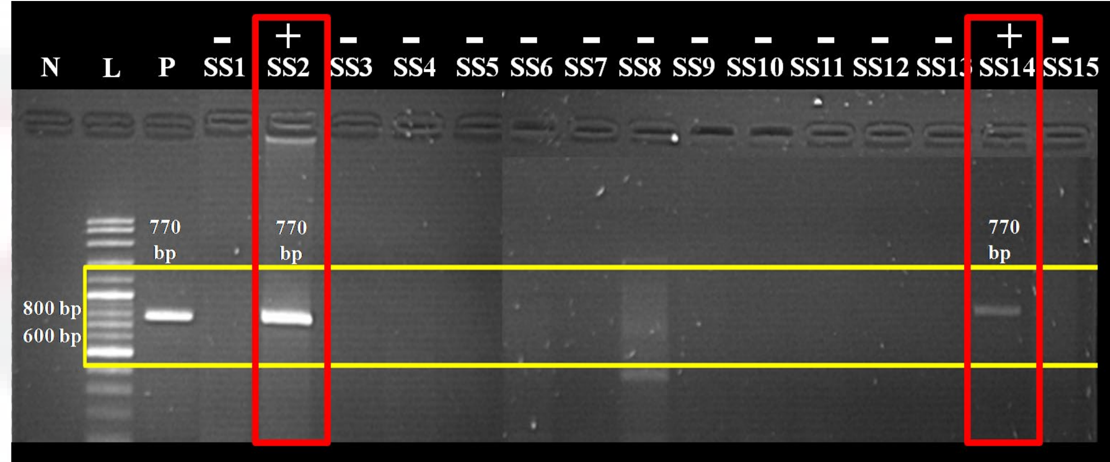

Figure 4.1: RT-PCR assay of saliva of clinically ill cats using specific primer targeting U3LRT-gag region of Feline leukemia virus to produce 770 bp PCR products. Electrophoresis was carried out on 1.5 % (w/v) agarose gel.The target bands were conserved for all cats as shown in lane 5(Cat-2) and lane 17(Cat-14). Lane 1: Control negative (N), Lane 2: Ladder(L), Lane 3: Positive control(P). Note: - negative PCR; + positive PCR

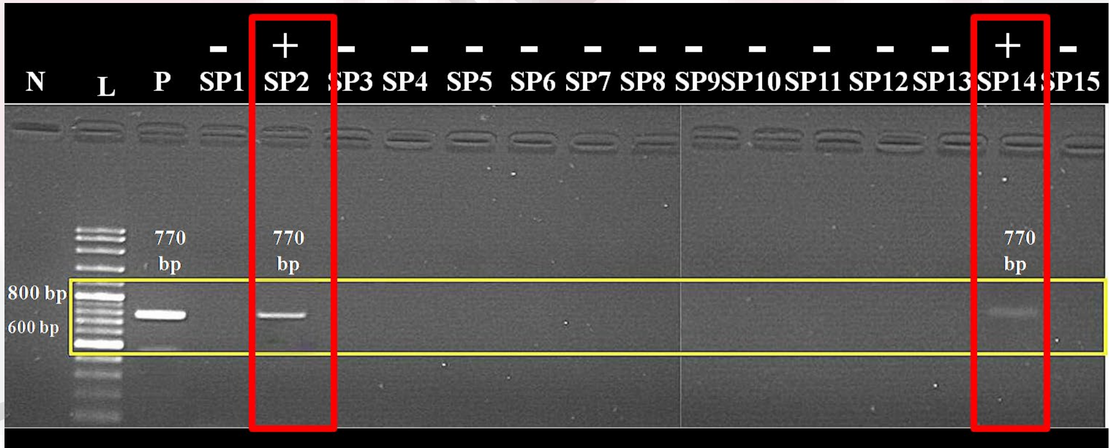

Figure 4.2: RT-PCR assay of plasma of clinically ill cats using specific primer targeting U3LRT-gag region of Feline leukemia virus to produce 770 bp PCR products. Electrophoresis was carried out on 1.5 % (w/v) agarose gel. The target bands were conserved for all cats as shown in lane 5(Cat-2) and lane 17(Cat-14). Lane 1: Control negative (N), Lane 2: Ladder (L), Lane 3: Positive control (P). Note: - negative PCR; + positive PCR

4.2 Bioinformatics Analysis of Malaysian FeLV isolates

4.2.1 Basic Local Alignment Search Tool (BLAST)

The results from BLAST revealed that the partial sequence from the current variant were highly similar (>90%)at the conserved U3LTR-gag regions reference isolates within the Genbank®(National Center for Biotechnology Information, USA). High similarities were found with sequences from Malaysia, USA, UK and Japan.

Table 4.1: Reference isolates of FeLV downloaded from Genbank®

No.	Strain	Accession No.	Country
1	FeLV-UPM01	HQ197367	Malaysia
2	FeLV-UPM02	HQ197368	Malaysia
3	FeLV-UPM03	HQ197369	Malaysia
4	FeLV-UPM07	HQ197373	Malaysia
5	FeLV-UPM11	HQ197377	Malaysia
6	FeLV-UPM19	JF815542	Malaysia
7	FeLV-UPM20	JF815543	Malaysia
8	FeLV-UPM29	JF815552	Malaysia
9	FeLVRicard	AF052723	USA
10	FeLV-FAIDS	M18247	USA
11	FeLV-K01803	D13922	UK
12	FeLV-GM1	D13922	UK
13	FeLV-TWK25	GQ465833	Taiwan
14	FeLV-TWK30	GQ327961	Taiwan
15	FeLV-Glasgow	KP728112	Scotland
16	FeLV-ON33	AB847229	Japan
17	FeLV-AT34	AB847164	Japan
18	FeLV-IT38	AB847183	Japan

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4.2.2 Multiple Alignment and Pairwise Comparison

Pairwise nucleotide comparison was performed comparing the two variants isolated from this study with local isolates and reference isolates to identify the sequence identity (Figure 4.3& Figure 4.4). The comparison revealed that the partial U3LTR-gag regions of the two sequences were homologues with from 94%-99% with the previous local isolates and homologous from 87.7%-98% with the reference isolates from overseas.

		1	2	3	4	5	6	7	8	9	10	11
FeLV_BROWNIE_Gasing_Malaysia	1	ID	98.31	99.44	99.44	99.25	99.06	99.62	99.44	98.49	93.23	94.36
FeLV_BOBOI_Gasing_Malaysia	2	0.02	ID	98.87	98.87	98.68	98.49	98.68	98.49	97.55	92.48	93.42
FeLVUPM01_MALAYSIA	3	0.01	0.01	ID	100.00	99.81	99.62	99.81	99.62	98.68	93.42	94.55
FeLVUPM02_MALAYSIA	4	0.01	0.01	0.00	ID	99.81	99.62	99.81	99.62	98.68	93.42	94.55
FeLVUPM07_MALAYSIA	5	0.01	0.01	0.00	0.00	ID	99.44	99.62	99.44	98.49	93.23	94.36
FeLVUPM28_MALAYSIA	6	0.01	0.02	0.00	0.00	0.00	ID	99.44	99.25	98.31	93.42	94.55
FeLVUPM11_MALAYSIA	7	0.00	0.01	0.00	0.00	0.00	0.01	ID	99.81	98.87	93.61	94.74
FeLVUPM26_MALAYSIA	8	0.01	0.02	0.00	0.00	0.00	0.01	0.00	ID	99.06	93.42	94.55
FeLVUPM19_MALAYSIA	9	0.02	0.02	0.01	0.01	0.01	0.02	0.01	0.01	ID	92.48	93.61
FeLVUPM20_MALAYSIA	10	0.07	0.08	0.07	0.07	0.07	0.07	0.06	0.07	0.08	ID	98.49
FeLVUPM03_MALAYSIA	11	0.05	0.07	0.05	0.05	0.05	0.05	0.05	0.05	0.06	0.02	ID

Figure 4.3: Sequence identity matrix with pairwise comparison to compare sequence identity of the two nucleotides derived from the U3LTR-gag region of current variant with local isolates. Note : ID: Identical

		1	2	3	4	5	6	7	8	9	10	11	12
FeLV_BROWNIE_Gasing_Malaysia	1	ID	98.31	97.18	95.68	96.43	96.80	96.05	94.36	93.98	93.98	91.73	87.78
FeLV_BOBOI_Gasing_Malaysia	2	0.02	ID	96.42	94.92	95.86	95.86	95.11	93.42	93.05	93.42	91.54	87.41
FeLV_ON33_JAPAN	3	0.03	0.04	ID	94.74	95.68	95.86	95.49	93.61	93.61	93.42	90.98	86.84
FeLV-K01803	4	0.04	0.05	0.05	ID	94.93	95.68	95.31	93.43	93.43	94.00	90.24	86.12
FeLV_Rickard_USA	5	0.03	0.04	0.04	0.04	ID	98.87	98.12	94.92	94.54	94.73	92.09	87.95
FeLV_Glasgow 1_Scotland	6	0.03	0.04	0.04	0.03	0.01	ID	98.49	96.05	95.67	95.48	92.47	88.32
FeLV_FAIDS_USA	7	0.04	0.05	0.04	0.04	0.02	0.02	ID	95.30	94.92	94.73	91.53	87.38
FeLV GM1_UK	8	0.05	0.07	0.06	0.06	0.05	0.04	0.04	ID	97.37	96.80	94.16	89.64
FeLV IT38_JAPAN	9	0.06	0.07	0.06	0.06	0.05	0.04	0.05	0.02	ID	98.12	94.16	89.64
FeLV AT34_JAPAN	10	0.06	0.07	0.07	0.05	0.05	0.05	0.05	0.03	0.02	ID	93.79	89.27
FeLV-TWK25_TAIWAN	11	0.06	0.07	0.07	0.08	0.06	0.06	0.07	0.04	0.04	0.05	ID	94.63
FeLV-TWK30_TAIWAN	12	0.11	0.11	0.12	0.12	0.11	0.11	0.12	0.09	0.09	0.09	0.05	ID

Figure 4.4: Sequence identity matrix with pairwise comparison to compare sequence identity of the two nucleotides derived from the U3LTR-gag region of current variant with overseas isolates. Note ID: Identical

4.2.3 Construction of Phylognenetic tree

The unrooted phylognetic tree showing relationship between Malaysian Feline leukemia virus isolate with all isolates obtained from the Genbank® was generated using Neighbour Joining (NJ) method as seen in Figure 4.5. The tree includes sequences from cat-2(FeLV-BOBOI_Gasing 1_MALAYSIA) and cat-14 (FeLV-BROWNIE_Gasing 2_MALAYSIA) as well as 18 other reference isolates downloaded from Genbank®. The reference sequences originate from four countries; UK, USA, Japan and Taiwan.

Based on the pairwise comparison of the sequence identity matrix, the current variants were closest in homology to local Malaysian isolates and some Japanese strain. This similarity is observed on the phylogenetic tree as the current FeLV variants were found to be within the same cluster as majority of previously isolated Malaysian FeLV isolate.

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This cluster was closely related to an isolate from FeLV_ON33_Japan(Genbank®accession numberAB847229).

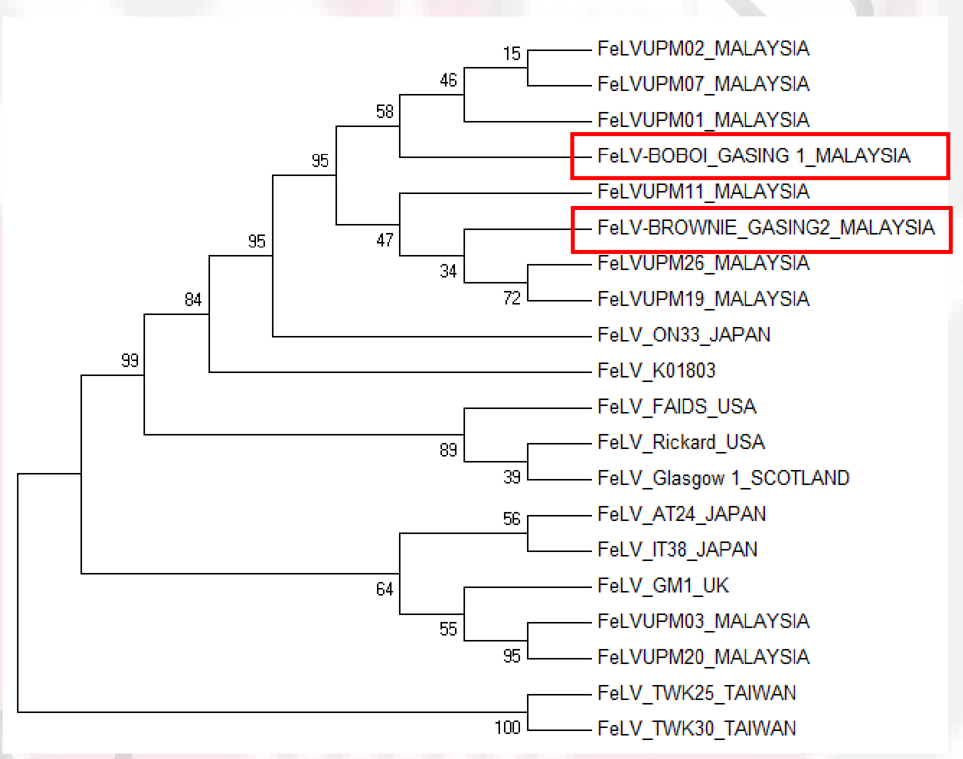

Figure 4.5: Unrooted phylogenetic tree of two current FeLV variant (boxes) and reference isolates. The phylogenetic tree was constructed using Neighbour-Joining (NJ) method with 1000 bootstrap replicates. Current variants are denoted as FeLV-BOBOI_Gasing1_ Malaysia and FeLV-BROWNIE_Gasing 2_Malaysia, found within the cluster group with majority of the previous Malaysian isolate.

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5.0 DISCUSSION

Clinical signs related with FeLV are generally non-specific and early detection of FeLV infected cats is most crucial. Detection of nucleic acid in the plasma gives the indication that the cat might be in the early stage of infection (regressive infection) or chronic stage of infection (progressive infection) as the presence indicates signs of viraemia (Hofmann-Lehmann *et al.,* 2001) as seen in this study. In order to distinguish regressive and progressive infections, peripheral blood should be tested at least 16 weeks after the first antigen testing, as regressively infected cats will turn negative at latest 16 weeks after infection, while progressively infected cats will remain positive(Hartmann, 2012). The detection of the viral nucleic acid in the saliva indicates the cats to be in the progressive stage of infection due to shedding of the virus in the saliva (Hartmann, 2012).As in this study, the viral nucleic acid was detected in both the saliva and the plasma, both the cats are likely to be in the progressive stage of infection (a.k.a. persistent viraemia) and are likely to develop FeLV related diseases/conditions along with being infectious to other cats due to the viral shedding.

Risk factors associated with the development of FeLV in host is if the cat is from a multi-cat household and is a semi-roamer or free roamer (Bande *et al.,* 2012). The risk is also higher for sick cats to be infected by FeLV as compared to healthy cats (Little *et al.,* 2009; Bande *et al.,* 2012). Other risk factor may include the neutered status of the animal whereby intact males are more prone to FeLV infection as compared to castrated males (Miyazawa *et al.,* 2002; Bande *et al.,* 2012). In this study one of the cat was a male,

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castrated, stray (free-roamer cat) whereas the other cat was an un-castrated male from a multi-cat household. Both the cats were clinically ill and seem to fit the criteria for the increased risk factor for FeLV infection development.

FeLV, true to its nature as a retrovirus is subjected to genetic variation due to error prone replication and recombination processes (Chandahasin *et al*., 2005; Dunham &Graham, 2008). In this study, the U3LTR-*gag* region of naturally occurring exogenous FeLV (based on the primer) was partially sequenced and subjected to phylogenetic analysis. The multiple sequence alignment and sequence alignment revealed 94%-99% of the current variant from this study when compared with the previous Malaysian isolates. However, the homology decreased to 87.7% when the current variant was compared with reference isolates from overseas. Hence, it is highly suggestive that theU3LTR-gag regions of the variant from this study are highly homologous with previous local isolates and still somewhat homologous to the reference isolates from overseas.

There are high sequence homology observed among the FeLV isolates derived from different geographic and temporal clusters, as reported by Donahue *et al.,* 1988, Chandahasin *et al*., 2005 and Bande *et al*., 2014 that reported findings of strong sequence conservation (>97%) amongst these FeLV isolates due to the conserved nature of the U3 segment of the LTR region and also the *gag* region. However, in this study, the homology did reduce to 87.7 %, because although the U3 segment is the conserved region of the LTR segment in the FeLV, natural isolates have been reported to exhibit genetic variation within the terminally repeated structures of the LTRs as stated in Bannert *et al.,* 2010.

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This might be due to the error prone replication and recombination process commonly seen occurring in retroviruses (Mansky, 1998).

In this study, the variants isolated were seen to be within the same cluster in the phylogenetic tree as most of the previous Malaysian FeLV isolates which could be due to their similar ancestral lineage. In the study conducted by Bande *et al.,* in the year 2014 it was reported that most of the Malaysian FeLV exhibited a closer relationship with an isolate from UK(Genbank®accession numberD13922) which was suggested could be associated with translocation of animals between these two countries given the long historical/colonial relations between UK and Malaysia. In contrast to that, this study have provided new evidence suggesting the existence of an even closer relation between the current variant isolated from this studyand majority of the previous Malaysian FeLV isolate with FeLV_ON33_Japan (Genbank® accession number AB847229) an isolate from Japan which might indicate an evolutionary relationship between the FeLV from these two countries. It is possible that these isolates might have originated from one of these two regions or evolved independently.

The FeLV_ON33_Japan (Genbank® accession number AB847229) was one of many isolates from a study conducted by Kawamura *et al*., in the year 2015 and the sequences from this study was not submitted to Genbank® until later in that year. This could be the fact on why this relation between the Japanese FeLV and Malaysian FeLV was not observed at the study by Bande et al., in the year 2014 as it was done a year prior to the existence of the Japanese FeLV sequence in the Genbank® records. Due to the somewhat

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conserved nature of the U3LTRregion, conclusion about the FeLV subgroup requires further investigations of FeLV envelope protein gene to further characterize the nature of the FeLV found in Malaysia (Bande *et al*., 2014).

CONCLUSION

In conclusion, based on the partial sequence of U3LTR-*gag* region, this study has demonstrated that the current Malaysian FeLV variant was highly homologous with previous Malaysian FeLV isolates suggesting possible circulation of the similar FeLV strain amongst Malaysian local cats. Phylogenetic analysis on the U3LTR-*gag* region revealed that Malaysian FeLV isolates might have close evolutionary relationship with Japanese FeLV and UK FeLV

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RECOMMENDATION

Based on this study, it is recommended to consider a larger sample size for future endeavours in order to increase the number of isolated variant sequences to be catalogued for future references. The samples should also be obtained from distribution that covers all of the Malaysian state range in order to further understand the current sequence conservation.

Besides that, further update on the sequence analysis and phylogenetic tree as frequent as possible is also recommended in order to reveal any relations with new FeLV isolated from all over the world. The positive samples should also be further characterised in the cell culture (Crandell Reese Feline Kidney) and sent for full genome sequencing via Next Generation Sequencing (NGS).

In addition, genetic characterization of the *env* or full genome should be performed as it would give definitive sub-grouping of the virus. This will be useful for better comparison via bio informatics analysis.

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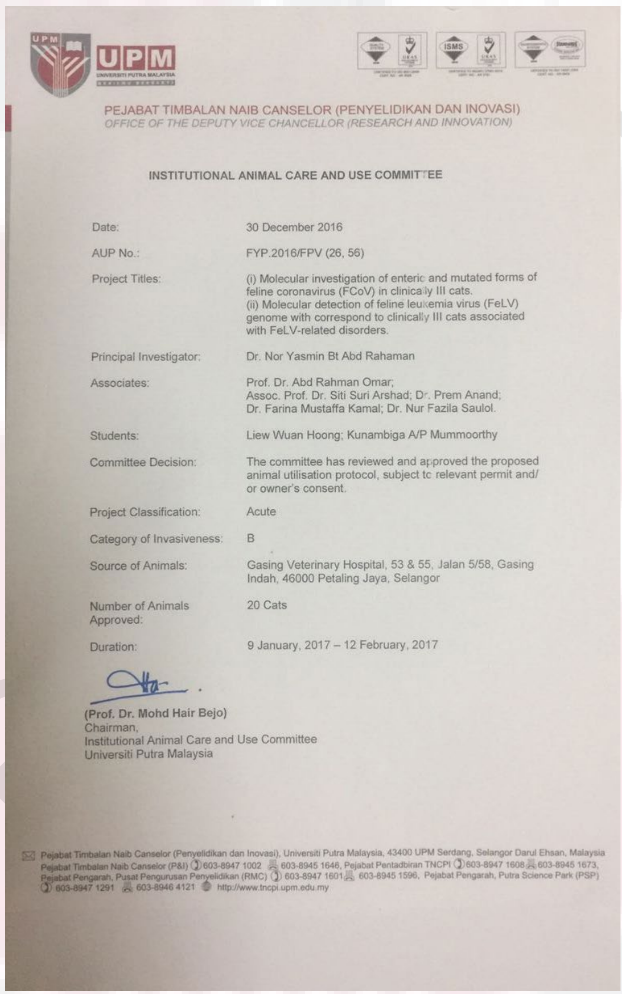
APPENDICES

APPENDIX A: APPROVAL LETTER FROM IACUC



UPM
UNIVERSITI PUTRA MALAYSIA

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: INFORMED OWNER CONSENT FORM

FRONT

BACK

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.

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:

1. Principal Investigator:
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