



UNIVERSITI PUTRA MALAYSIA

***PREVALENCE, MOLECULAR DETECTION AND PHYLOGENETIC
ANALYSIS OF FELINE LEUKAEMIA VIRUS IN PENINSULAR MALAYSIA***

FARUKU BANDE

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PENINSULAR MALAYSIA**



FARUKU BANDE

**MASTER OF SCIENCE
UNIVERSITI PUTRA MALAYSIA**

2012



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PENINSULAR MALAYSIA**



By

FARUKU BANDE

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfilment of the Requirements for the Degree of Masters of Science**

January 2012

DEDICATION

This thesis work is dedicated to my parents and family for their unrelenting support.



Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfilment of the requirement for the degree of Master of Science

**PREVALENCE, MOLECULAR DETECTION AND PHYLOGENETIC
ANALYSIS OF FELINE LEUKAEMIA VIRUS IN
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FARUKU BANDE

January 2012

Chairperson: Siti Suri Arshad, PhD

Faculty: Veterinary Medicine

Feline leukaemia virus (FeLV) is a gammaretrovirus typically associated with the development of lymphoma, anaemia and immunodeficiency in cats. A cross sectional survey was carried out from January 2010 to December 2010 to determine the prevalence and risk factors associated with FeLV infection in Peninsular Malaysia. A total of 368 cats were screened for the presence of FeLV p27 antigen and information on cat's demography as well as health status were obtained and analysed by logistic regression for their association with the risk of FeLV infection. The overall prevalence of FeLV was found to be 12.2% (45/368). Prevalence was higher among sick cats (18.9%) compared to healthy cats (5.1%) and in client-owned cats (13.1%) compared to shelter cats (10.3%). Logistic regression analysis revealed that male gender, young age, aggressive behaviour, sickness, living in multi-cat household or shelter environment significantly increased the risk of FeLV positivity. Thus, cats under these categories are likely at higher risk of developing FeLV associated conditions and possibly play a role in shedding the virus to other naïve cats. Higher prevalence of FeLV observed in this

study suggests the need for specific control measures such as screening and vaccination to ensure that FeLV infection is controlled among cats in Peninsular Malaysia. In a follow-up study, a highly sensitive nested PCR assay was optimized and used to detect the presence of FeLV viral RNA and provirus DNA from the plasma and blood samples respectively. Comparison was made between p27 antigen test and nested RT-PCR assay. In FeLV viral RNA detection, a total of 78 cats comprising up of an equal number of p27 antigen positive and p27 antigen negative cats were evaluated. Viral RNA was detected in 87.2% (35/39) p27 antigen positive and 12.8% p27 seronegative cats respectively. A substantial agreement was observed between p27 antigen detection test and nested RT-PCR assay ($\kappa = 0.74$). On the other hand, when nested PCR assay was used to determine FeLV proviral DNA status of 39 p27 antigen negative cats, about 97.4% (38/39) of p27 antigen negative cats were found positive for FeLV proviral DNA. Detection of FeLV viral RNA and Proviral DNA among antigen positive and antigen negative cats suggest that both persistent and latent FeLV infections are prevalent among cats in Peninsular Malaysia. Although substantial agreement was recorded between p27 antigen test and nested RT-PCR assay, nested RT-PCR was shown to offer additional advantage as it was able to detect viral RNA in 12.8% antigen negative cats. Cats that were tested negative for FeLV antigen but provirus DNA positive are likely to go undetected when only antigen detection assays are considered for the diagnosis of FeLV. Thus, combination of both p27 antigen detection test and PCR assays will provide better understanding of FeLV infection status of cats. To gain further insight into Malaysian FeLV genotypic variation, 29 nested PCR positive samples were selected, sequenced and subjected to multiple sequence alignment and phylogenetic analysis. Malaysian FeLV samples were shown to exhibit higher sequence homology ranging from 91-100%. When compared with the reference FeLV isolates,

Malaysian FeLV had highly conserved sequence variation that reduces up to 84%. Phylogenetic analysis further revealed that local FeLV sequences were group in two distinct clusters. The majority of local FeLV samples (86%) clustered together with FeLV-K01803 which is a member of FeLV-B subgroup from UK, while the remaining samples (14%) were grouped with FeLV-GM1 isolate which is also a member of subgroup B. This might suggest that FeLV-B is likely the most predominant sub-group occurring in Peninsular Malaysia. In overall, this study has provided valuable information on the epidemiology of FeLV in both client-owned and shelter cat population in Malaysia. Additionally, the study provided the first molecular-based FeLV study in Malaysia; highlighted the significance of using PCR assay as a diagnostic tool for evaluating FeLV infection status and one of the few Genebank representations of FeLV sequences from South East Asian region. Future studies should be directed toward isolation and adaptation of FeLV in cell culture, whole genome sequencing and vaccine efficacy studies so as to further understand the molecular characteristics, immune response and pathogenesis of local FeLV isolates.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Sarjana Sains

**PREVALEN, PENGESANAN MOLEKUL DAN ANALISIS FILOGENETIK
VIRUS FELIN LEUKAEMIA VIRIS DI SEMENANJUNG MALAYSIA**

Oleh

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Januari 2012

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Virus leukemia felin (FeLV) adalah gammaretrovirus yang biasanya dikaitkan dengan kejadian limfoma, anemia dan keimmunodefisienan dalam kucing. Satu kajian keratan rentas telah dijalankan dari Januari 2010 hingga Disember 2010 untuk menentukan faktor prevalen dan risiko yang dikaitkan dengan jangkitan FeLV di Semenanjung Malaysia. Sebanyak 368 kucing telah diuji untuk kehadiran antigen FeLV p27 dan maklumat mengenai demografi serta status kesihatan kucing telah diperolehi dan dianalisis oleh regresi logistik untuk mengesan kaitannya dengan risiko jangkitan FeLV. Keseluruhan prevalen FeLV didapati 12.2%. Prevalen adalah tinggi di kalangan kucing sakit (18.9%) berbanding kucing sihat (5.1%) dan kucing milik pelanggan (13.1%) berbanding dengan kucing tempat perlindungan (10.3%). Analisis regresi logistik mendedahkan bahawa jantina jantan, usia muda, agresif, penyakit, tinggal di persekitaran pelbagai kucing atau tempat berlindung dengan ketara meningkatkan risiko positifiti FeLV. Oleh itu, kucing di bawah kategori ini adalah pada risiko yang lebih tinggi dikaitkan dengan FeLV dan mungkin memainkan peranan dalam menyebarkan

virus kepada kucing lain. Ketinggian prevalen FeLV positif yang di diperhatikan dalam kajian ini mencadangkan keperluan bagi langkah-langkah kawalan yang spesifik seperti saringan dan vaksinasi untuk memastikan bahawa jangkitan FeLV dibendung pada kucing di Semenanjung Malaysia. Untuk menentukan kehadiran viral RNA dan provirus DNA FeLV, bersarang PCR esei yang sangat sensitif dioptimumkan dan digunakan untuk mengesan kehadiran virus RNA dan DNA provirus FeLV daripada plasma dan sampel darah, masing-masing. Perbandingan telah dibuat antara ujian p27 antigen dan bersarang RT-PCR esei. Dalam pengesanan RNA FeLV, sebanyak 78 kucing yang terdiri daripada bilangan sama p27 kucing antigen antigen positif dan negatif p27 dinilai. RNA virus dikesan dalam 87.2% (35/39) kucing antigen positif dan 12.8% antigen negatif. Kaitan yang besar yang diperhatikan di antara ujian p27 pengesanan antigen dan esei bersarang RT-PCR assay ($\kappa = 0.74$). Sebaliknya, esei bersarang PCR telah digunakan untuk menentukan status DNA proviral FeLV pada 39 kucing p27 negatif antigen. Hasil kajian menunjukkan bahawa 97.4% (38/39) kucing p27 antigen negatif adalah positif untuk DNA proviral FeLV. Penemuan ini menunjukkan bahawa kedua-dua jangkitan FeLV berterusan dan pendam adalah prevalen di kalangan kucing di Semenanjung Malaysia. Walaupun kaitan yang besar direkodkan di antara ujian p27 antigen dan esei bersarang RT-PCR esei, esei bersarang RT-PCR menunjukkan banyak kelebihan kerana ia dapat mengesan virus RNA pada 12.8% kucing antigen negatif. Kajian ini juga menunjukkan bahawa proviral DNA FeLV adalah prevalen di kalangan kucing yang antigen negatif. Kucing ini tidak dapat dikesan apabila ujian pengesanan antigen hanya digunakan dalam diagnosis FeLV. Oleh itu, gabungan kedua-dua p27 ujian pengesanan antigen dan ujian molekul akan memberikan pemahaman yang lebih baik tentang status jangkitan kucing. Untuk mendapatkan maklumat lanjut mengenai ciri-ciri genetik strain FeLV Malaysia, 29

sampel positif sarang PCR dipilih, mengikut urutan dan tertakluk kepada penjajaran urutan pelbagai dan analisis filogenetik. FeLV tempatan menunjukkan urutan homologi 91-100% yang lebih tinggi, tetapi homologinya menurun kepada 84% apabila sampel FeLV tempatan dibandingkan dengan FeLV rujukan. Filogenetik analisis selanjutnya mendedahkan bahawa FeLV tempatan tergolong dalam dua kelompok yang berbeza. Majoriti sampel FeLV tempatan (86%) tergolong dalam kelompok sama dengan FeLV-K01803 iaitu sub-kumpulan FeLV-B dari UK. Manakala baki sampel (14%) tergolong dengan isolat FeLV-GM1 iaitu anggota sub-kumpulan B berasal dari UK. Penemuan ini mungkin mencadangkan bahawa FeLV-B mungkin yang paling utama berlaku di Semenanjung Malaysia. Secara keseluruhan, kajian ini telah memberikan maklumat yang berharga pada epidemiologi FeLV dalam populasi kucing milik pelanggan dan perlindungan. Selain itu, kajian ini adalah kajian pertama berasaskan molekul pada FeLV di Malaysia dan telah menekankan kepentingan menggunakan cerakin PCR sebagai alat diagnostik untuk menilai status jangkitan FeLV kucing. Kajian ini juga telah menyumbangkan urutan FeLV yang pertama kedalam Genebank dari rantau selatan Asia Timur. Kajian seterusnya harus diarahkan ke arah pengasingan dan penyesuaian FeLV dalam kultur sel, penjujukan genom keseluruhan dan kajian keberkesanan vaksin untuk memahami ciri-ciri molekul, tindak balas imun dan patogenesis isolat FeLV Malaysia.

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I certify that a Thesis Examination Committee has met on 12TH January 2012 to conduct the final examination of FARUKU BANDE on his thesis entitled “Prevalence, Molecular Detection and Phylogenetic Analysis of Feline Leukaemia Virus in Peninsular Malaysia” in accordance with the Universities and University colleges Act 1971 and Constitution of Universiti Putra Malaysia [P.U.(A)106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously, and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institutions.

FARUKU BANDE

Date:



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LIST OF ABBREVIATIONS

AAFP	American Association of feline practitioners
Amp	Amperage
AMV	Avian Myeloblastosis Virus
ATCC	American Type-culture Collections
BaEV	Baboons endogeneous retrovirus
BIC	Bayesian information center
Bp	Base pair
CA	Capsid associated
CD 4+	Cluster of differentiation 4
CD8+	Cluster of differentiation 8
cDNA	Complementary DNA
CI	Confidence interval
Cm ³	Centimeter cube
CO ₂	Carbon di-oxide
CrFK	Crandell Feline kidney
DNA	Deoxyribonucleic Acid
DSH	Domestic Short Hair
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzymes linked immunosorbent Assay
EM	Electron microscopy
enFeLV	Endogenous feline leukaemia virus
ER	Endoplasmic reticulum
exFeLV	Exogenous feline leukaemia virus

FAIDS	Feline acquired immunodeficiency
FBS	Fetal Bovine Serum
FeLV	Feline leukaemia virus
Fg	Femtogram
FIPV	Feline infectious peritonitis Virus
FIV	Feline immunodeficiency virus
FLVCR	Feline leukaemia virus –c receptors
FPV	Feline panleukopenia virus
G	Gravity
γ	Gamma
HIV	Human Immunodeficiency Virus
HTLV	Human T-cell leukaemia virus
ICTV	International Committee on Taxonomy of Viruses
IN	Integrase
Kb	Kilobase
LTRs	Long Terminal repeats
MA	Matrix Associated
MDA	Maternally derived Antibodies
Mg	Magnesium
Min	Minute
mL	Mililitre
ML	Maximum likelihood
mRNA	Messenger RNA
NA	Not applicable

NC	Nucleocapsid
NCBI	National Center for Biotechnology Information
Nef	Negative replicating factor
Ng	Nanogram
NJ	Neighbour Joining
Nm	Nanometer
no. or n	Number
NTC	No Template Control
°C	Degree Celsius
OD	Optical Density
OR	Odds Ratior
P value	Probability
PAWS	Paws Animal Welfare Society
PB	Primer Binding
PBS	Posphate buffer saline
PCR	Polymerase Chain Reaction
Pg	Pictogram
Pit	Phosphate transporter
Pol	Polymerase
Ppt	Polypurine tract
RBC	Red blood Cells
RNA	Ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction

S	Sedimentation rate
SE	Standard Error
siRNA	Short interfering ribonucleic acid
SPCA	Society for Prevention of Cruelty to Animal
sqKm	Square kilometre
SU	Surface protein
TAE	Tris-acetate EDTA
Tat	Trans- activator of transcription
TEM	Transmission Electron Microscopy
TM	Transmembrane
™	Registered Sign
tRNA	Transfer RNA
UPM	Universiti Putra Malaysia
UV	Ultraviolet
UVH	University Veterinary Hospital
V	Volt
VI	Virus isolation
Vif	Viral infectivity factor
VNA	Virus neutralizing Antibodies
Vpu	Viral protein U
V.S.	Versus
W/V	Weight per volume
A	Alpha
B	Beta

Δ	Delta
ϵ	Epsilon
$\text{\textcircled{R}}$	Registered sign
μg	Microgram



CHAPTER 1

INTRODUCTION

The domestic cat (*Felis catus*) is considered the most socialised carnivore due to its long companionship to humans (Case, 2003). Cat is becoming the most populous pet with increasing population over 500 million worldwide. Recently, the number of cats is estimated to supersede the number of dogs in many countries, with many people favouring cat as a pet compared to dog (Wise et al., 2002; APPMA, 2008; Shepherd, 2008). The growing number of pet cats has been attributed to several benefits including their role in improving psychological health and self esteem of pet owners especially children and old people (Beck et al., 1996; Melson, 2003; Walsh, 2009). In addition, cat is considered a good model for the understanding of hereditary and infectious diseases of humans (Dunham, 2006; Kristina et al., 2011). On the other hand, cats are important sources of veterinary and public health important diseases that could adversely affect the health of animals and humans (Angulo et al., 1994; Akhtardanesh et al., 2010). Thus, considerable researches have focused mainly towards the improvement of both welfare and health conditions of this important specie (Slater, 2004; Farnworth et al., 2010).

Of all the diseases affecting domestic cat, FeLV is the most important cause of morbidity and mortality with death rate reaching almost 50% within 2 years and 80% following 3years following persistent infection (Hardy et al., 1976a; Hoover and Mullin 1991; Hatmann et al., 2011). Infection with FeLV is mainly characterized by anaemia, lymphosarcoma, leukaemia and immunodeficiency (Rojko and Kociba,

1991). Recent studies have shown that, almost 80% of feline lymphosarcoma are associated with FeLV (Gabor et al., 2001; Jackson et al., 1993; Suntz et al., 2010; Weiss et al., 2010). Approximately, 70-90% of persistently FeLV infected cats died within a period of 18 months to 4 years after the infection (Hardy et al., 1976a; McClelland et al., 1980). The mortality occurring in FeLV is largely associated with severe defects in both the humoral and cellular immune system which results in lymphopenia, neutrophil abnormalities and reduction in the number of CD4+ and CD8+ cell, eventually ending in severe immunodeficiency known as feline AIDS (Ogilvie et al., 1988).

The genome of FeLV is made up of single stranded positive sensed enveloped polyadenylated RNA classified under gammaretroviridae (Goff, 2007; Dunham and Graham, 2008). FeLV viral RNA is diploid in nature and possessed 3 coding gene structure; the *gag*, *pol* and *env* (Coffin, 1979; Leis et al., 1988). Flanking the 5' and 3' regions of FeLV provirus are long terminal repeat structures (LTRs) which functions in regulating the expression of viral proteins and aid in viral tumorigenicity (Matsumoto et al., 1992).

Based on genome characteristic and tissue tropism, four distinct subgroups of FeLV have been recognised. These subgroups are designated FeLV-A, B, C and T (Sarma and Log, 1973; Anderson et al., 2000). FeLV-A is the most common subgroup and serves as a prototype virus from which other subgroups evolved (Donahue et al., 1988). FeLV-B arises *de novo* following recombination of envelope sequences of FeLV-A with endogenous feline leukaemia virus present in cat. FeLV-B is the most common subgroup isolated from sick cats (Takeuchi et al., 1992; Anderson et al.,

2001). The last two subgroups, FeLV-C and T arises from mutation of FeLV-A. FeLV-C and FeLV-T subgroups are associated with aplastic anaemia and T-cell leukaemia respectively (Quigley et al., 2000; Cheng et al., 2007). Apart from the exogenous retroviruses horizontally transmitted among domestic cats, endogenous retroviruses such as RD114 and enFeLV are vertically transmitted in the cat's germ cells. These viruses are believed to evolve from ancient cats that preyed on rats infected with murine leukaemia virus (Benvenisti et al., 1974; Weiss, 2006; Okada et al., 2011). So far, enFeLV have not been reported to cause natural infection in cat, (Pandey et al., 1991). Naturally, transmission of FeLV occur oronasally following direct contact of naïve cats with the infected saliva, feaces, milk and other body secretions of infected cat (Hardy et al., 1973a; Jarrett et al., 1973a).

Other sources of infection include bite, blood transfusion and contaminated objects (Pedersen, 1987). An *in utero* infection has also been described to account for foetal death in 80% of FeLV cases (Hoover and Mullin, 1991).

Diagnosis of FeLV is carried out by different methods such as: - virus isolation (VI) which involved the propagation and isolation of the virus in cell culture (Jarrett et al., 1964a; Kawakami et al., 1967); immunoflourescent antibody test (IFA), which detects the presence of FeLV gag protein (Hardy and Zuckerman, 1991); Enzyme-linked immunosorbent assay (ELISA) and immunochromatographic methods which detects the presence of FeLV p27 antigen (Tonelli, 1991; Eto et al., 2003). The long duration needed in VI as well as the non-cytopathiceffect of FeLV makes virus isolation a procedure that is rarely carried out in routine practice (Hardy, 1991). On the other hand, serological assays such as ELISA and immunochromatography are

less sensitive in detecting FeLV p27 antigen during early phase of viraemia and when the virus becomes latently integrated in the bone marrow or other tissues (Hardy and Zukerman, 1991). In addition, single ELISA based assays have been reported less sensitive in regions of low prevalence rate as well as in cat with low infection risk (Beatty et al., 2011). Thus, a highly sensitive test is necessary for understanding cat's FeLV infection status as well as FeLV epidemiology. Recently polymerase chain reaction (PCR) assays have been developed and used in the detection of FeLV viral RNA and proviral DNA. PCR assay was found to be highly sensitive than the traditional diagnostic methods and capable of detecting both viral RNA and FeLV proviral DNA (Jackson et al., 1996; Miyazawa and Jarrett, 1997; Hofmann-Lehman et al., 2001; Tandon et al., 2005). Epidemiological studies have shown that, FeLV infection is widely distributed among cats (Gleich et al., 2009; Little et al., 2011). However, prevalence varies considerably with geographical location, cat's lifestyle as well as population density (Hoover and Mullin., 1991; O'Connor et al., 1991; Gleich et al., 2009).

Although several studies have been carried out on important viral diseases of cats in Malaysia (Rahman et al., 1984; Arshad et al., 2004; Sharif et al., 2009), little information is available on FeLV as the available studies were documented more than 2 decades ago. These studies considered only client-owned cats tested at the University Veterinary Hospital, Universiti Putra Malaysia (UPM-UVH). Thus, occurrence of FeLV in the population of stray or un-owned cats remained un-investigated (Noor and Cheng 1983; Cheng 1990). Moreover, previous studies used only p27 antigen detection test as method which has been reported less sensitive especially in the detection of early and latent FeLV infections, thus, prevalence of

FeLV might be underestimated since cats are not routinely tested for retrovirus infections in Malaysia.

With the advent of highly sensitive molecular techniques such as PCR, it is desirable to explore the potentials of these methods to determine the FeLV infection status of cats in Malaysia as well as to explore the genetic characteristic and phylogeny of local FeLV isolates since no similar study have been reported. The result of this study will provide useful information to cat owners, veterinarians and policy makers in designing an effective control and prevention programme against FeLV.

Objectives

The objectives of this study are:-

- i.** To determine the prevalence and risk factors associated with FeLV infection in Peninsular Malaysia.
- ii.** To determine the occurrence of FeLV viral RNA and proviral DNA among cats in Peninsular Malaysia
- iii.** To compare and contrast between FeLVp27 antigen detection test and nested RT-PCR assay.
- iv.** To compare the Malaysian FeLV with reference FeLV isolates using phylogenetic analysis.

Hypotheses

HA1: FeLV is prevalent among cats in Peninsular Malaysia

HA2: Specific risk factors significantly influence the occurrence of FeLV among cats in Peninsular Malaysia.

HA3: FeLV RNA and proviral DNA could be detected from p27 antigen positive and p27 antigen negative cats respectively

HA4: There is significant difference between p27 antigen detection test and nested RT- PCR assay.

HA4: There is genetic variation between Malaysian FeLV and other reference FeLV isolates.

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