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MOLECULAR PREVALENCE AND GENOME ANALYSIS OF DOMESTIC CAT HEPADNAVIRUS IN MALAYSIA

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By

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

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

MOLECULAR PREVALENCE AND GENOME ANALYSIS OF DOMESTIC CAT HEPADNAVIRUS IN MALAYSIA

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July 2021

Chair : Associate Professor Gayathri Thevi Selvarajah, PhD
Faculty : Veterinary Medicine

Domestic cat hepadnavirus (DCH, genus *Orthohepadnavirus,* family *Hepadnaviridae*) was first reported from whole blood samples of domestic cats in Sydney, Australia in 2018. Following that, DCH have been detected from various tissues including serum, formalin-fixed, paraffin embedded liver tissues, kidney, lung, heart, intestine, brain and lymph nodes. The pathogenesis of DCH is still unknown, but it was reported in cats with viraemia (6.5-12.4%), hepatocellular carcinoma (28%) and chronic hepatitis (43%). To date, there are no data regarding molecular prevalence of DCH among domestic cats in Malaysia. Moreover, detection of DCH in paired blood and liver samples of individual cats are not documented. In this study, the objectives are to determine the molecular prevalence of DCH from blood and liver tissues using conventional PCR method and evaluate the association of DCH with common feline viral diseases and hepatopathies. In addition, complete genome sequence (CGS) and phylogenetic analyses of Malaysian DCH compared to known DCH isolates and other hepadnaviruses were also included as in the objective. A cross-sectional study was done using 253 whole blood, 87 fresh liver tissues and 20 archived liver samples which includes domestic cats from animal shelters and patients of University Veterinary Hospital (UVH). The risk factors associated with DCH were analysed by using IBM SPSS 22 which included *Chi*-square and Fisher's exact test method. Survey criteria included origin, sex, age, status of common feline viral diseases, serum biochemistry level and hepatic lesions of the cats. Phylogenetic tree using CGS was plotted by using maximum likelihood approach available in Molecular Evolutionary Genetics Analysis (MEGA7). Histological slides of liver tissues were evaluated based on World Small Animal Veterinary Association (WSAVA) standards and graded using modified Ishak score method. From whole blood samples, 12.3% (n = 32/253) were tested DCH-positive. Prevalence of DCH was significantly higher in pet cats (16.6%, n = 24/145) compared to shelter cats (6.5% (n = 7/108). Among the DCH positives, 62.5% (n = 20/32) were cats more than 2 years old. Liver samples showed higher DCH detection rate (14.9%, n = 13/87) compared to blood samples. Five out of

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these 13 cats tested positive for DCH in their paired liver and blood samples. Serum alanine transaminase (ALT) level was elevated (> 95 U/L) in 12 out of the 23 DCH-positive cats (52.2%, p = 0.012). Upon histopathology, 3 out of 4 liver tissue with lymphoid aggregates were also positive for DCH (p = 0.001). From CGS analysis Malaysian DCH strain, with a genome size of 3184 bp was obtained with lowest genetic variability to Thailand DCH strain. The phylogenetic analysis demonstrated that the Malaysian DCH strain clustered together into same branch with Australian strain showing that the Malaysian and Australian DCH strains have a common ancestor. The present study reports DCH as a common feline virus among domestic cats of Malaysia with risk factors associated with origin, and age of the cat. This study provides insights on a new variant of DCH detected and sequenced for the first time from fresh liver tissue sample. The impact of this virus on inducing liver-related diseases in felines warrants further investigation.

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PREVALENS MOLEKULAR DAN ANALISIS GENOM HEPADNAVIRUS KUCING DOMESTIK DI MALAYSIA

Oleh

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Hepadnavirus kucing domestik (DCH, genus *Orthohepadnavirus*, famili *Hepadnaviridae*) pertama kali dilaporkan daripada sampel darah kucing domestik dari Sydney, Australia pada tahun 2018. Berikutan itu, DCH telah dikesan dari pelbagai tisu termasuk serum serta sampel hati, ginjal, paru-paru, jantung, usus, otak dan nodus limfa yang terawet formalin dan terbenam parafin (FFPE). Patogenesis DCH masih tidak diketahui tetapi ianya telah dilaporkan dalam kucing dengan viremia (6.5 - 12.4%), karsinoma hepatoselular (28%) dan hepatitis kronik (43%). Sehingga kini, tiada data berkenaan dengan prevalens molekular DCH dalam kalangan kucing di Malaysia. Tambahan pula, pengesanan DCH daripada sampel berpasangan dari darah dan hati setiap individu kucing juga tidak direkodkan. Oleh itu, tujuan kajian ini adalah untuk menentukan prevalens molekular DCH daripada tisu darah dan hati menggunakan kaedah PCR dan menilai hubungan DCH dengan penyakit virus kucing yang lazim dan penemuan hepatopati. Selain daripada itu, objektif lain kajian ini adalah untuk mengkaji penjujukan genom lengkap (CGS) dan perbandingan analisis filogenetik DCH Malaysia dengan isolat DCH yang diketahui dan hepadnavirus lain. Satu kajian keratan lintang telah dibuat menggunakan 253 sampel darah, 87 sampel tisu hati segar dan 20 sampel hati yang telah diarkibkan yang merangkumi kucing peliharaan dari pusat perlindungan haiwan dan pesakit dari Hospital Veterinar Universiti (UVH). Analisis faktor risiko yang berkaitan dengan prevalens molekular DCH pada kucing telah dibuat menggunakan IBM SPSS 22 yang merangkumi ujian *Chi*-square dan 'Fisher's exact'. Kriteria tinjauan termasuklah asal usul, jantina, usia, status penyakit virus kucing yang lazim, penemuan biokimia serum dan lesi hepatopati kucing yang dikaji. CGS dan analisis filogenetik diplot dengan menggunakan pendekatan kemungkinan maksimum yang terdapat dalam Analisis Genetik Molekul Evolusi (MEGA7). Slaid histologi tisu hati telah dinilai dan diskor berdasarkan standard Persatuan Veterinar Haiwan Kecil Sedunia (WSAVA) dan kaedah 'Modified Ishak'. Daripada sampel darah lengkap, 12.3% (n = 32/253) didapati positif untuk DCH. Prevalens DCH adalah jauh lebih tinggi

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dalam kalangan kucing peliharaan (16.6%, n = 24/145) berbanding kucing dari pusat pelindungan haiwan (6.5% (n = 7/108). Di antara yang didapati positif DCH, 62.5% (n = 20/32) adalah kucing yang berumur lebih daripada 2 tahun. Sampel hati menunjukkan kadar pengesanan DCH yang lebih tinggi (14.9%, n = 13/87) berbanding dengan sampel darah. Lima daripada 13 ekor kucing diuji positif daripada sampel hati dan darah yang berpasangan. Dua belas daripada 23 (52.2%, p = 0.012) ekor kucing yang didapati positif untuk DCH mempunyai bacaan aminotransferase alanina (ALT) serum yang tinggi (> 95 U / L). Evaluasi histopatologi menunjukkan 3 daripada 4 tisu hati dengan lesi agregat limfoid juga positif untuk DCH (p = 0.001). Analisis CGS menunjukkan bahawa strain DCH Malaysia dengan saiz genom 3184 bp mempunyai kepelbagaian genetik yang rendah berbanding dengan strain Thailand. Analisis filogenetik mendapati strain DCH Malaysia berada dalam gugusan dengan satu cabangan yang sama dengan strain Australia yang menunjukkan bahawa strain Malaysia berasal dari keturunan yang sama dengan strain DCH Australia. Kajian ini menunjukkan DCH adalah satu lagi virus kucing yang lazim di kalangan kucing domestik Malaysia dengan faktor risiko yang berkaitan dengan asal usul dan usia kucing. Kajian ini telah menemukan varian baharu DCH yang berjaya dikesan dan untuk pertama kalinya analisis penjujukan genom lengkap telah dibuat daripada sampel tisu hati. Kajian lanjut perlu dilaksanakan.bagi menyelidik kesan virus ini dalam mengakibatkan penyakit hati pada kucing.

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moderate to marked portal inflammation surrounding bile duct (B) and hepatic vein (V) (400X); [d] lymphocytes are clustered near the portal tract within sinusoids (S) (400X). Scale bar: 50 microns

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LIST OF ABBREVIATIONS

ALP	Alkaline phosphatase
ALT	Alanine transaminase
Anti-DHBs	Antibody to duck hepatitis B virus surface antigen
Anti-HBc	Antibody to human hepatitis B virus core antigen
Anti-HBs	Antibody to human hepatitis B virus surface antigen
ASHV	Arctic squirrel hepatitis virus
AST	Aspartate aminotransferase
bp	Base pair
BSA	Bovine serum albumin
BtHV	Bat hepadnavirus
CaCl2	Calcium chloride
cDNA	Complementary DNA
CGS	Complete genome sequence
ChHBV	Chimpanzee hepatitis B virus
CI	Confidence interval
cPCR	Conventional PCR
CRFK	Crandell Rees feline kidney cells
Cryo-EM	Cryogenic electron microscopy
DCH	Domestic cat hepadnavirus
DHBcAg	Duck hepatitis B virus core antigen
DHBeAg	Duck hepatitis B virus e antigen
DHBsAg	Duck hepatitis B virus surface antigen
DHBV	Duck hepatitis B virus

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DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer xylene
EDTA	Ethylenediaminetetraacetic acid
FCoV	Feline coronavirus
FeLV	Feline leukemia virus
FFPE	Formalin-fixed paraffin-embedded
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus
FIV	Feline immunodeficiency virus
g	gram
GiHBV	Gibbon hepatitis B virus
GoHBV	Gorillas hepatitis B virus
GSHV	Ground squirrel hepatitis virus
H&E	Hematoxylin and eosin stain
HBcAg	Hepatitis B virus core antigen
HBeAg	Hepatitis B virus e antigen
HBsAg	Hepatitis B virus surface antigen
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HepG2	Human hepatoma cells
HIV	Human immunodeficiency virus
ID	Identity document
IPTG	Isopropyl β-d-1-thiogalactopyranoside
ISH	In-situ hybridization
kb	Kilo bases

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m	Meter
M	Marker
MCL	Maximum composite likelihood
MEGA	Molecular evolutionary genetics analysis
MgCl2	Magnesium chloride
mL	Milliliter
µL	Microliter
n	Sub-total population
N	Total number of population
NA	Nucleotide analogues
NCBI	National Centre for Biotechnology Information
OR	Odds ratio
ORFs	Open reading frames
ORO	Oil red O stain
OuHBV	Orangutans hepatitis B virus
%	Percentage
P	Polymerase
PCR	Polymerase chain reaction
PegIFNα	Pegylated interferon α
pgRNA	Pregenomic RNA
pH	Power of Hydrogen
preC/C	Precore/core
preS/S	Presurface/surface
qPCR	Quantitative PCR
Ref	Reference
RNA	Ribonucleic acid

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rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
SEC	Size exclusion chromatography
Ser	Serine
SPSS	Statistical Package for the Social Sciences
UK	United Kingdom
UPM	Universiti Putra Malaysia
USA	United State of America
UV	Ultraviolet
UVH	University Veterinary Hospital
v/v	Volume per volume
VLP	Virus-like particle
w/v	Weight per volume
WHV	Woodchuck hepatitis virus
WMHBV	Woolly monkey hepatitis B virus
X	Times

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CHAPTER 1

INTRODUCTION

Hepadnavirus has been detected in many species especially in avian and mammals. This partially double-stranded DNA has strong hepatotropism with a small range of host which are vertebrates (Urban *et al*., 2010). A well-known member of *Hepadnaviridae* is human hepatitis B virus (HBV) which is known for causing chronic hepatitis leading to cirrhosis and hepatocellular carcinoma (HCC) (Karayiannis, 2017; Seeger & Mason, 2015).

Domestic cat hepadnavirus (DCH) is a new member of hepatotropic virus under the genus *Orthohepadnavirus* (mammal) and family *Hepadnaviridae*. It was first detected from a tom cat diagnosed with large cell lymphoma in 2018, Australia *(*Aghazadeh *et al*., 2018). Following that, DCH screening has been done in cats from Sydney, Italy and Thailand using whole blood, serum, liver, heart, lung, intestine, kidney, urinary bladder and spleen tissue samples (Aghazadeh *et al*., 2018; Lanave *et al*., 2019; Piewbang *et al*., 2020).

In 2018, up to 6.5% (n = 8/123) of blood samples from cats in Sydney, Australia were tested positive for DCH by using conventional PCR assay method (Aghazadeh *et al*., 2018). In 2019, up to 33.3% (n = 14/42) of feline sera from cats in Italy with retroviral infection was found positive for DCH where the viral DNA was quantified using the quantitative real-time PCR assay (Lanave *et al*., 2019). In a study by Pesavento *et al*. (2019), screening for DCH on diseased and normal feline liver from stored formalin-fixed, paraffin embedded (FFPE) liver tissues showed 17.07% (n = 14/82) of detection.

There have been no studies to compare detections in both blood and liver samples from the same cat. This information will potentially increase our understanding of DCH infection at different clinical phases as observed in HBV-infected patients such as carrier, immune tolerant, active and chronic phases (Trépo *et al*., 2014). For centuries, blood-transfused human recipients had the risk of suffering from post-transfusion hepatitis from an unknown cause, until subsequent discovery of HBV. Therefore, if DCH is detectable in whole blood and sera, it would potentially be transmitted through blood transfusion. However, it is yet to be proven.

The discovery of DCH at three different geographical locations have demonstrated unalike DCH strains which are DCH, Sydney (3187 bp), DCH, Italy (3184 bp) and DCH, Thailand (3184 bp). To date this virus has not been reported in Malaysian domestic cat population. There are still questions on risk factors associated with other common feline virus infections that can cause immunosuppression such as feline immunodeficiency virus (FIV), feline leukaemia virus (FeLV) and feline infectious peritonitis (FIP). The complete

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genome sequence (CGS) of Malaysian DCH is necessary to add on to the pool of information on the genetic variability that exists for this virus. To increase our understanding on the risks for diseases and how this new feline hepadnavirus can impact feline health, further molecular and genetic characterization of this virus among Malaysian domestic cat population is necessary.

In this study, molecular prevalence of DCH will be studied using conventional PCR assay in paired whole blood and liver tissue samples from both shelter and pet owned domestic cats. For survey criteria, age (less than vs more than 2 years old), sex (male vs female), type of ownership (shelter vs pet cats), elevation in serum alanine transaminase (ALT) level and status of viral co-infections with FIV, FeLV and FIP will be studied. Additionally, complete genome sequence of the local strain will be sequenced and aligned to the published DCH strains from Sydney, Italy and Thailand. Phylogenetic analysis will also be done to relate the evolution of DCH with other hepadnaviruses.

1.1 Research Hypothesis

The hypotheses were:

1. DCH is present in whole blood and liver tissues of domestic cats in Malaysia. Higher prevalence is detected in liver tissues (hepatotropic) compared to circulating blood (viraemia).
2. There is a strong positive association between DCH detection and chronic hepatitis in affected cats, however no associations to other common feline viruses.
3. Genetically, the Malaysian DCH strain is similar to Sydney, Italy and Thailand strains.

1.2 Objectives

The objectives of this study are:

1. To detect and determine the molecular prevalence of DCH from blood and liver tissues using conventional PCR assay method.
2. To evaluate the association of positive DCH cases with the presence of immunosuppressive viral diseases (FIV, FeLV and FIP) and pathological findings of the liver.
3. To determine the complete genome sequence and phylogenetic analyses of Malaysian DCH strain compared to the known DCH isolates and other hepadnaviruses.

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