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MOLECULAR PREVALENCE AND HAPLOTYPE DIVERSITY OF TICK-BORNE HAEMOPATHOGENS IN SHELTER DOGS IN PENINSULAR 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 Science

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MOLECULAR PREVALENCE AND HAPLOTYPE DIVERSITY OF TICK-BORNE HAEMOPATHOGENS IN SHELTER DOGS IN PENINSULAR MALAYSIA

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Canine tick-borne haemopathogens (TBH) constitute a significant concern worldwide and warrant closer surveillance due to their zoonotic potential. Infection in dog is a reliable indicator for infection risk in a certain geographical area. The detection of these TBHs is mainly achieved by microscopic evaluation, seroprevalence, and molecular detection. Although molecular detection was used in recent studies, the studies were limited to the central region and certain main cities in Peninsular Malaysia. Additionally, there is scarce information on molecular detection of the haemopathogens in the vector infesting dogs in Peninsular Malaysia. Detailed research is imperative to demonstrate the host-vector interaction between the animal host (dog) and arthropod vector *(Rhipicephalus sanguineus* (*R. sanguineus* tick)) for the transmission of TBHs of dogs in Peninsular Malaysia. Therefore, the present study was designed to investigate the molecular detection of TBHs in shelter dogs and their ticks in Peninsular Malaysia, as well as to determine the pattern of haplotype separation between TBHs in dogs and their ticks. A total of 220 dogs' blood and 140 *R. sanguineus* ticks were collected from animal shelters in Peninsular Malaysia during the study. The presence of haemopathogens in blood and tick samples was detected using conventional PCR, sequenced, and identified at the species level. *Ehrlichia canis* (*E. canis*) was detected from dogs and ticks with detection rates of 20% (n=43) and 1.43% (n=2), respectively. On the other hand, 12% (n=26) of the dogs and 1.43% (n=2) of the ticks were tested positive for *Anaplasma platys* (*A. platys*). *Babesia gibsoni* (*B. gibsoni*) was only detected in dogs (7%; n = 16), whereas *Babesia vogeli* (*B. vogeli*) was detected in both tick (0.71%; n=1) and dogs (7%; n=16). Male and young dogs showed significantly higher *A. platys* and *B. vogeli* infection rates ($p<0.05$), respectively. Up to triple infections of haemopathogens observed in the sampled dogs but no co-infection of TBHs in ticks obtained in the present study. The haplotype network analysis results revealed no specific pattern or separation between isolates of TBHs in dogs and ticks from different region,

since samples collected from different region were observed clustered together in the same haplotypes, thus were not able to demonstrate the pattern of canine TBHs transmission in Peninsular Malaysia. However, the study revealed a higher haplotype and nucleotide diversities when using mitochondrial gene marker compared to nuclear gene marker, which will be useful in exploring variation in population of parasites. The findings obtained in the present study contribute to a more comprehensive information on the prevalence and distribution of TBHs in shelter dogs and their ticks in Peninsular Malaysia, as well as providing basic knowledge on the molecular variation of TBHs isolates in Peninsular Malaysia as baseline for future molecular characterisation studies.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia Sebagai memenuhi keperluan untuk Ijazah Sarjana Sains

PREVALENS MOLEKULAR DAN KEPELBAGAIAN HAPLOTIP HEMOPATOGEN BAWAAN SENGKENIT PADA ANJING DI PUSAT PERLINDUNGAN DI SEMENANJUNG MALAYSIA

Oleh

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Hemopatogen bawaan sengkenit pada anjing menjadi kebimbangan seluruh dunia dan memerlukan pengawasan yang lebih dekat kerana potensi zoonosis mereka. Jangkitan pada anjing merupakan petunjuk yang boleh dipercayai bagi risiko jangkitan di sesebuah kawasan geografi. Pengesanan hemopatogen bawaan sengkenit ini selalunya dilakukan melalui kaedah penilaian mikroskopik, prevalens serologi, dan pengesanan molekular. Walaupun pengesanan menggunakan kaedah molekular telah digunakan dalam kajian terbaru, kajian yang telah dilakukan hanya tertumpu kepada kawasan tengah dan bandar-bandar tertentu di Semenanjung Malaysia. Tambahan pula, terdapat sedikit maklumat mengenai pengesanan molekular hemopatogen ini pada vektor yang ada pada anjing di Semenanjung Malaysia. Kajian terperinci adalah perlu bagi menunjukkan interaksi antara perumah (anjing) dan vektor (sengkenit *R. sanguineus*) dalam penyebaran hemopatogen bawaan sengkenit pada anjing di Semenanjung Malaysia. Oleh itu, kajian ini dijalankan untuk menyiasat prevalens molekular bagi hemopatogen bawaan sengkenit pada anjing di pusat perlindungan dan sengkenit anjing di Semenanjung Malaysia, serta menyiasat corak pemisahan haplotip antara hemopatogen bawaan sengkenit pada kedua-dua anjing dan sengkenit. Sebanyak 220 sampel darah anjing dan 140 sengkenit (*R. sanguineus*) telah diperolehi dari pusat perlindungan haiwan di Semenanjung Malaysia bagi kajian ini. Kehadiran hemopatogen dalam sampel darah dan sengkenit telah dikesan menggunakan kaedah PCR konvensional, dijujukan, dan di identifikasi mengikut spesis. *E. canis* telah dikesan pada anjing dan sengkenit, masing-masing dengan kekerapan 20% (n=43), dan 1.43% (n=2). Dalam masa yang sama, 12% (n=26) ekor anjing dan 1.42% (n=2) sengkenit telah diuji positif bagi *A. platys*. *B. gibsoni* hanya dikesan dalam anjing (7%; n=16), manakala *B. vogeli* dikesan pada kedua-dua sengkenit (0.71%; n=1) dan anjing (7%; n=16). Anjing jantan dan anjing muda masing-masing menunjukkan ketinggian jangkitan *A. platys* dan *B. vogeli* yang ketara ($p<0.05$). Sehingga tiga hemopatogen menjangkiti seekor anjing pada masa yang sama dikesan, tetapi tiada

ko-infeksi hemopatogen dalam sengkenit ditemui dalam kajian ini. Keputusan analisis jaringan haplotip menunjukkan ketiadaan corak spesifik diantara hemopatogen bawaan sengkenit pada anjing dan sengkenit daripada kawasan yang berbeza, kerana sampel yang diperolehi dari kawasan yang berbeza didapati berkongsi haplotip yang sama, yang dengan demikian tidak dapat menunjukkan corak penularan hemopatogen bawaan sengkenit di Semenanjung Malaysia. Walau bagaimanapun, kajian ini menunjukkan kepelbagaian haplotip dan nukleotid yang lebih tinggi apabila menggunakan penanda gen mitokondria berbanding dengan penanda gen nuklear, yang berguna dalam meneroka variasi dalam populasi parasit. Penemuan yang diperoleh dalam kajian ini menyumbang kepada maklumat yang lebih komprehensif mengenai penyebaran dan prevalens hemopatogen bawaan sengkenit pada anjing dan sengkenit anjing di Semenanjung Malaysia, serta pengetahuan asas mengenai variasi molekular hemopatogen bawaan sengkenit di Semenanjung Malaysia sebagai garis pangkal bagi pencirian molekular bagi kajian pada masa hadapan.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
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LIST OF ABBREVIATIONS

AMOVA	Analysis of molecular variance
A. platys	*Anaplasma platys*
B. canis	*Babesia canis*
B. gibsoni	*Babesia gibsoni*
BLAST	Basic Local Alignment Search Tool
BPE	Blood parasite examination
B. rossi	*Babesia rossi*
B. vogeli	*Babesia vogeli*
bp	Base pair
p	Calculated probability
CME	Canine Monocytic Ehrlichiosis
$\chi 2$	Chi-square
CI	Confidence Interval
mm3	Cubic millimetre
°C	Degree Celsius
dH2O	Deionised distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E.	*Ehrlichia*
E. canis	*Ehrlichia canis*
EDTA	Ethylenediaminetetraacetic acid
H.	*Haemaphysalis*
h	Haplotype diversity
ICCT	Infectious Canine Cyclic Thrombocytopenia
IACUC	Institutional Animal Care and Use Committee
kg	kilogram
MgCl2	Magnesium chloride
ML	Maximum Likelihood
µm	Micrometre
µl	Microliter
mM	milliMolar
ml	milligram
mm	millimetre
NCBI	National Center for Biotechnology Information
-	Negative
π	Nucleotide diversity
pi	Pairwise differences
PCR	Polymerase chain reaction
%	Percentage
R.	*Rhipicephalus*
R. sanguineus	*Rhipicephalus sanguineus*
rRNA	Ribosomal ribonucleic acid
SEA	Southeast Asia
spp.	Species
SPSS	Statistical Package for the Social Sciences

n	Sub-total population
TBD	Tick-borne diseases
TBH	Tick-borne haemopathogens
N	Total number of populations
TAE	Tris-acetic acid-EDTA
UV	Ultraviolet
U	Unit
USA	United States of America
UPM	Universiti Putra Malaysia
V	Volt
W	Watt

CHAPTER 1

INTRODUCTION

Tick-borne haemopathogens can contribute to the emergence of infection in animals worldwide. The reports on new tick-borne haemopathogens and the spread of tick-borne pathogens to new locations over the decades have highlighted the importance of these tick-borne pathogens. The spread of tick-borne haemopathogens in a new region, where they were not reported before, was mainly due to the movement of animals, mostly companion animals (e.g. dog and cat), which were brought during travel and infested by ticks abroad. These travelling companion animals brought the tick vector and the haemopathogens in these ticks with them, thus contribute to the circulation of ticks and tick-borne haemopathogens globally (Beugnet and Marie, 2009; Otranto and Dantas-Torres, 2010; Skotarczak, 2018). The capability of these tick vector to transmit these pathogens to a wide range of animal hosts demand the need for surveillance, especially in companion animals.

Companion animals such as cats and dogs, which usually adopted as pets and lived together with humans, could be considered as an important source of zoonotic tick-borne haemopathogen. Therefore, the tick-borne haemopathogens potential to infect companion animals warrant closer surveillance due to their zoonotic potential (Shaw et al., 2001; Hamer et al., 2009; Little, 2010; Maia et al., 2014; Skotarczak, 2018). Dogs particularly have a higher susceptibility to tick-borne haemopathogens due to their greater exposure to the tick vector (living habitat, less protection (e.g. clothing), and inability to remove ticks on their own) in which can be a sensitive indicator for the infection risk in a geographical area or could act as a baseline prevalence of TBD spatial distribution. The detection of tick-borne haemopathogens in dogs were useful to indicate the risk of tick-borne haemopathogens infection to human (Perez-Vera et al., 2014; Andersson et al., 2017).

These tick vectors carry diseases that cause problems worldwide and usually high in tropical and subtropical countries (Irwin and Jefferies, 2004; Mariana et al., 2011). Malaysia, like the other South-East Asian countries, experiences a tropical climate; high temperature and humidity, which is suitable for the growth and proliferation of tick vectors. The large stray dogs' population in Malaysia acts as a readily available host for these tick vectors. These stray dog populations may harbour these arthropod vectors, and due to the lack of provision of care in terms of medication and hygiene, causing them to be susceptible to tick-borne diseases. There are numerous tick-borne diseases with different causative agents in the dogs, and the most common are namely babesiosis, ehrlichiosis, and anaplasmosis (Irwin and Jefferies, 2004; Mariana et al., 2011; Nazari et al., 2013). The ability of dogs to travel from one region to another while acts as a sentinel to these arthropod vectors, and the pathogens in them warrant the need for detailed and thorough information regarding the prevalence of tick-borne haemopathogens of dogs in Malaysia.

1.1 Problem Statement

The available data on tick-borne haemopathogens prevalence in Malaysia were not comprehensive and only focusing on certain regions of Malaysia. In addition, most of the work used conventional methods rather than the highly sensitive molecular detection technique. Moreover, very little works using molecular techniques are available for the detection of these tick-borne haemopathogens in a tick as vector considering this tick vector importance in tick-borne haemopathogens transmission. There is no available study on the relationship between tick-borne haemopathogens in dogs and their ticks in Malaysia to date. Therefore, the present study aims to provide an extensive study for tick-borne haemopathogens screening in dogs and their tick vector in Peninsular Malaysia by the mean of a molecular method and to determine the phylogenetic relationship of various tick-borne haemopathogens isolates in dogs and ticks, with the focus on interpreting the pattern of distribution of these tick-borne haemopathogens from different regions of Peninsular Malaysia. The findings of this study might have implications for the epidemiology, management, and diseases representation.

1.2 Research Objectives

The objectives of this study are:

i. To determine the molecular prevalence of important tick-borne haemopathogens in shelter dogs in Peninsular Malaysia.
ii. To determine the molecular prevalence of important tick-borne haemopathogens in ticks infesting shelter dogs in Peninsular Malaysia.
iii. To determine the pattern of haplotype separation between tick-borne haemopathogens in dogs and their ticks from the different region using haplotype network analysis.

1.3 Hypothesis

i. The true prevalence of tick-borne haemopathogens in shelter dogs in Peninsular Malaysia will be revealed.
ii. The true prevalence of tick-borne haemopathogens in ticks infesting shelter dogs in Peninsular Malaysia will be revealed.
iii. The pattern of haplotype separation of tick-borne haemopathogens in Peninsular Malaysia will reveal that the tick-borne haemopathogens isolates obtained from the same region are sharing the same haplotype.

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