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MOLECULAR DETECTION AND CHARACTERIZATION OF TICK-BORNE HEMOPATHOGENS IN STRAY DOGS IN MALAYSIA

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By

KONTO MOHAMMED

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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DEDICATION

This thesis is dedicated to my beloved mother Late Hajiya Hadiza Mohammed, my father and teachers for their guidance, devotion and relentless patience.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

MOLECULAR DETECTION AND CHARACTERIZATION OF TICK-BORNE HEMOPATHOGENS IN STRAY DOGS IN MALAYSIA

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October 2016

Chairman : Associate Professor Malaika Watanabe, PhD
Faculty : Veterinary Medicine

Tick-borne hemopathogens like *Babesia, Ehrlichia, Anaplasma* and *Hepatozoon* species pose significant health threats to dogs worldwide. Southeast Asia is one of the few regions of the world with inadequate records concerning vector-borne diseases of dogs and cats, yet it happens to be among the most heavily populated with stray dogs and cats and a climate that is ideal for the tick vectors. In an attempt to achieve a comprehensive scenario for Malaysia, molecular screening and characterization of five major canine tick-borne hemopathogens (namely: *Anaplasma platys, Babesia vogeli, Babesia gibsoni, Ehrlichia canis* and *Hepatozoon canis*) was carried out.

West (Peninsular) Malaysia was divided into four quadrants; North, East, South and West Peninsular, while the East (Island) Malaysia was divided into North and South. Eight out of the 12 States of Malaysia were selected for this study which comprises: Penang, Kedah, Pahang, Selangor, Kuala Lumpur and Johor from West (Peninsular) Malaysia and Sarawak and Sabah from East (Island) Malaysia. A total of 436 stray dogs from 9 animal shelters were selected for this study comprising seven animal shelters from West Malaysia and two animal shelters from East Malaysia. Age-groups were stratified into young and adult, while the breeds were broadly classified into pedigree and local. Whole blood was collected from each dog and DNA extraction was carried out. All DNA samples were screened via standard PCR using family-specific primers that amplify the 360bp fragment of the 16S rRNA gene for *Anaplasma and Ehrlichia* and a genus specific primer that amplify the 350bp fragment of the 18S rRNA gene for *Babesia and Hepatozoon.* Amplicons obtained were extracted, purified and confirmed by sequencing. For characterization, DNA from the purified PCR product from each representative samples were cloned into a suitable bacterial cell (*Escherichia coli*). Relations between categorical outcomes were compared using the chi-square test and prevalence rates were calculated at 95% confidence interval. For the characterization study, sequences were aligned and edited using the BioEdit

ClustalW program. For comparing and analyzing the nucleotide sequences, the BLAST program was used. The phylogenetic trees were constructed using the Neighbor-Joining method.

This study confirmed the molecular prevalence of five tick-borne hemopathogens namely: *A. platys, B. vogeli, B. gibsoni, E. canis* and *H. canis* in Malaysia with *A. platys* being the most prevalent hemopathogen (36.5%), followed by *B. gibsoni* (27.6%), *B. vogeli* (22.5%), *E. canis* (18.8%) and *H. canis* (15.8%). Presence of co-infection was also established with a concurrent infection with *A. platys* and *Babesia* being the most prevalent (18.8%); followed by *Babesia* and *E. canis* (10.8%), *A. platys* and *E. canis* (8.4%), *H. canis* and *Babesia* (6.4%), *H. canis* and *E. canis* (4.6%) and *H. canis* and *A. platys* (2.7%). Triple infection combination of *Babesia-Anaplasma-Ehrlichia* predominated (61.3%, 19 dogs). However, local breeds showed a significantly ($p > 0.05$) higher infection rate than the pedigree breed for *H. canis, Babesia* and *A. platys* ($p= 0.03$, 0.001 and 0.04 respectively).

Spatial distribution based on individual pathogens per region showed that Northern East Malaysia (Sabah) had the highest prevalence for *H. canis* (37%); *B. vogeli* (35.7%) and *A. platys* (64.9%) predominated in West Peninsular, while *B. gibsoni* (76%) and *E. canis* (46%) predominated in Southern East Malaysia (Sarawak). Distribution of hemopathogens based on geographic locations in Malaysia revealed that Southern East Malaysia (Sarawak) had the highest prevalence for tick-borne hemopathogens in Malaysia.

Infectivity status to hemopathogens among stray dogs revealed that the Eastern Peninsular predominated for infectivity with at least one hemopathogen (35.4%), while the Southern East Malaysia (Sarawak) predominated for dogs infected with 2, 3, 4, and all the 5 pathogens under investigation with prevalence of 44%, 20%, 6% and 2% respectively.

This study reported for the first time the genetic diversity of tick-borne hemopathogens among stray dogs in Malaysia and indicated the presence of both indigenous and foreign genotypes in Malaysia. West Peninsular Malaysia showed the highest number of genotypes, followed by the Southern East Malaysia (Sarawak).

The findings from this study provided a comprehensive data on the etiologies of canine tick-borne hemopathogens, their distribution and genetic diversities in Malaysia. This study can also be used for future epidemiological studies and or intervention programme. However, the zoonotic aspects of this hemopathogens as well as the reservoirs of infection have not been looked into in this study; thus, there is the need to further expand our knowledge on that aspect.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

PENGESANAN MOLEKULAR DAN PENCIRIAN HEMOPATOGEN BAWAAN SENGKENIT PADA ANJING TERBIAR DI MALAYSIA

Oleh

KONTO MOHAMMED

Oktober 2016

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Beberapa patogen darah bawaan hama kecil contohnya sengkenit, seperti spesies *Babesia*, *Ehrlichia, Anaplasma* dan *Hepatozoon* telah menimbulkan ancaman kesihatan yang ketara kepada anjing seluruh dunia. Asia Tenggara adalah antara beberapa kawasan di dunia yang tidak mempunyai rekod mencukupi mengenai penyakit bawaan vektor bagi anjing dan kucing. Namun, kawasan ini adalah antara kawasan yang mempunyai taburan anjing dan kucing terbiar yang tinggi dan cuaca yang paling ideal untuk kelansungan hidup vektor seperti sengkenit. Dalam usaha untuk mengetahui senario yang lebih menyeluruh di Malaysia, saringan molekular dan penjujukan lima patogen darah bawaan sengkenit (seperti: *Anaplasma platys, Babesia vogeli, Babesia gibsoni, Ehrlichia canis* dan *Hepatozoon canis*) telah dijalankan.

Semenanjung Malaysia telah dibahagikan kepada empat kuadran; Utara, Timur, Selatan dan Barat, manakala Malaysia Timur terbahagi kepada Utara dan Selatan. Lapan daripada 12 negeri di Malaysia telah dipilih untuk kajian ini, dimana meliputi: Pulau Pinang, Pahang, Selangor, Kuala Lumpur, dan Johor untuk bahagian Semenanjung Malaysia dan termasuk juga Sabah dan Sarawak daripada Malaysia Timur. Sebanyak 436 anjing terbiar dari 9 tempat perlindungan haiwan di Semenanjung Malaysia dan 2 pusat perlindungan dari Malaysia Timur telah dipilih untuk menjalankan kajian ini. Stratifikasi dikelaskan mengikut kumpulan umur dan baka. Kumpulan umur distratakan kepada muda dan dewasa manakalah kumpulan baka telah dikelaskan secara umum kepada baka tulen dan tempatan. Sampel darah diambil dari setiap anjing dan pengestrakan DNA dilakukan. Semua sampel DNA disaring melalui 'Standard PCR' menggunakan primer dari famili-spesifik yang mengamplifikasi 360 pasangan bes daripada gen 16S rRNA untuk *Anaplasma* dan *Ehrlichia* dan pimer gen-spesifik yang mengamplifikasi 350 pasangan bes daripada gen 18s rRNA untuk *Babesia* dan *Hepatozoon*. Amplikon yang terhasil diestrak, ditulenkan dan disahkan oleh penjujukan. Untuk pencirian, DNA daripada produk PCR yang tulen dari setiap sampel wakil telah diklon ke

dalam sel bakteria yang sesuai (*Escherichia coli*). Perhubungan antara hasil mutlak dibandingkan menggunakan ujian dan kelaziman kadar chi-square dikira pada 95% selang keyakinan. Untuk kajian pencirian, urutan jujukan telah disusun dan disunting menggunakan program BioEdit ClustalW. Bagi proses perbandingan dan analisa urutan jujukan, program BLAST telah digunakan. Pokok filogenetik dibangunkan mengunakan kaedah Neighbor-Joining.

Kajian ini mengesahkan prevalen molekular bagi 5 patogen darah bawaan sengkenit iaitu: *A. platys, B. vogeli, B. gibsoni, E. canis* dan *H. canis* di Malaysia dan *A. platys* (36.5%) mencatat prevalen tertinggi, diikuti *B. gibsoni* (27.6%), *B. vogeli* (22.5%), *E. canis* (18.8%) dan *H. canis* (15.8%). Kehadiran ko-infeksi antara *A. platys* dan *Babesia* juga dapat dirungkai dengan jumlah prevalen tinggi sebanyak 18.8%, diikuti ko-infeksi antara *Babesia* dan *E. canis* (10.8%), *A. platys* dan *E. canis* (8.4%), *H. canis* dan *Babesi*a sp. (6.4%), *H. canis* dan *E. canis* (4.6%), dan *H. canis* dan *A. platys* (2.7%). Kombinasi 3 infeksi serentak antara *Babesia-Anaplasma-Ehrlichia* mendominasi (61.3%, n=9). Walau bagaimanapun, baka anjing tempatan menunjukkan kadar jangkitan yang ketara ($p > 0.05$) lebih tinggi daripada baka anjing asli bagi *H. canis*, *Babesia* dan *A. platys* ($p = 0.03, 0.001$ dan 0.04) masing-masing.

Distribusi spatial berdasarkan patogen individual mengikut setiap rantau menunjukkan bahawa bahagian Utara Malaysia Timur (Sabah) mempunyai prevalen tertinggi bagi *H. canis* (37%); *B. vogeli* (35.7%) dan *A. platys* (64.9%) mendominasi Barat Semenanjung, manakala *B. gibsoni* (76%) dan *E. canis* (46%) mendominasi Selatan Malaysia Timur (Sarawak). Distribusi patogen darah berdasarkan faktor lokasi geografi di Malaysia menunjukkan bahawa Selatan Malaysia Timur (Sarawak) mencatat prevalen tertinggi untuk patogen darah bawaan sengkenit di Malaysia.

Status jangkitan untuk patogen darah dalam kalangan anjing terbiar mendedahkan bahawa Timur Semenanjung mendominasi sekurang-kurangnya jangkitan satu patogen darah (35.4%), manakala Selatan Malaysia Timur (Sarawak) didominasi oleh anjing yang dijangkiti dengan 2, 3, 4, dan kesemua 5 patogen dengan prevalen sebanyak 44%, 20%, 6% dan 2% masing-masing.

Kajian ini adalah yang pertama melaporkan kepelbagaian genetik patogen darah dalam kalangan anjing terbiar di Malaysia dan menunjukkan kehadiran kedua-dua genotip asli dan asing di Malaysia. Barat Semenanjung Malaysia menunjukkan jumlah tertinggi genotip, diikuti oleh Selatan Malaysia Timur (Sarawak).

Penemuan daripada kajian ini menyumbangkan data yang komprehensif mengenai etiologi patogen darah anjing bawaan sengkenit, distribusinya dan kepelbagaian genetiknya di Malaysia. Kajian ini juga boleh digunakan untuk kajian epidemiologi masa depan dan atau program intervensi. Walau bagaimanapun, aspek-aspek zoonotik patogen darah ini serta reservoir jangkitan masih belum diteliti dalam kajian ini; dengan itu, terdapat keperluan untuk mengembangkan lagi pengetahuan kita mengenai aspek tersebut.

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

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LIST OF SYMBOLS AND ABBREVIATIONS

α	Alpha
µm	Micro meter
µg	Microgram
µl	Microliter
Cm^2	Square centimetre
ml	Millilitre
ANOVA	Analysis of variance
CO_2	Carbon dioxide
dH_2O	Deionized distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid

CHAPTER 1

INTRODUCTION

1.1 Brief Overview

Tick-borne hemopathogens are aetiological agents of a spectrum of emerging and re-emerging vector borne diseases of dogs that include Babesia, Theileria, Ehrlichia, Anaplasma and Hepatozoon species. These pathogens have been reported worldwide and exert impact in the tropics and subtropics (Watanabe et al., 2004; Matjila et al., 2008; Cardoso et al., 2010; Chomel, 2011; Chandrawathani et al., 2014) where they pose a significant health threat to dogs (Irwin and Jefferies, 2004; Mariana et al., 2011).

1.2 Diseases induced and threat posed by tick-borne hemopathogens

A wide variety of tick-borne pathogens have been reported to infect dogs in Malaysia, notable among them are protozoa, bacteria and rickettsiae species that cause diseases such as babesiosis, theileriosis, hepatozoonosis, anaplasmosis, ehrlichiosis and rickettsiosis (Rajamanickam, 1985; Watanabe 2012; Nazari *et al*., 2013; Mokhtar 2013, Chandrawathani, 2014).

Tick and other vector-borne diseases pose an increasingly big threat to dogs and other animals in Malaysia. The recent increase in pet ownership, a high influx of tourists and other exotic pet varieties into the country necessitates for an urgent control measures due to the health and economic impact associated with these pathogens.

International standards for animal health and hygiene for the quality control of pets and other animals' disease prevention and control are essential to sustain the Malaysian expanding economy. The large populations of stray dogs and the increasing popularity of pet ownership in Southeast Asian countries including Malaysia coupled with the ideal environment for the sustainability of vectors helps in transmission of vector-borne disease to susceptible hosts (Irwin and Jefferies, 2004).

In similitude with other developing countries, stray dogs remain a serious public health issue. Priority for controlling stray animal population includes an effective, sustainable and practical approach, but this has always been a problem due to the limitation in financial support and lack of public awareness. With the overpopulation of the stray animals, animal shelters are normally operating with insufficient funding, staffs, and expertise in managing the shelters.

1.3 Factors predisposing stray dogs to tick-borne diseases

Increased vector population densities due to ecological changes is one of the most important factor that leads to the emergence of new or undiscovered diseases and the resurgence of the old quiescent ones. Additionally, unrestricted movements of infected animals from one location to another have also led to the spread of vector-borne pathogens from an endemic region to non-endemic areas (Wilson, 1995; Gratz, 1999; Irwin and Jefferies, 2004; Watanabe, 2012).

Climate, immune status, availability of vertebrate animals and vector populations' densities, and abundance of suitable reservoir hosts, among other factors, exerts influence on disease transmission and establishment (Irwin and Jefferies, 2004; Salman, 2012). However, availability of suitable tick populations is the most important factors that determine the establishment of infection in a new environment (Emmons, 1988).

The three most important factors that moderate the transmission and infection intensity of tick-borne diseases worldwide are climate change, changes in land use or ecological influence and movements of animals through importation of exotic and wildlife species, migratory birds and movement of domestic animals (Gratz, 1999; Robson and Allen, 2000; Gubler *et al.,* 2001; Cumming and Van-Vuuren, 2006; Ergönül, 2006; Nijhof *et al*., 2007; Tack *et al.,* 2010; Molin *et al.,* 2011; Salman 2012; Madder and Pascucci, 2012; Low *et al*., 2014). These factors influence the prevalence and proliferation of both the ticks and the pathogens they transmit by moderating the survival and fecundity rate of the ticks, time of the year and level of tick activity; specifically the blood sucking rate and time taken for a complete lifecycle of the tick-borne pathogens within the ticks (Gratz, 1999; Salman, 2012).

Interactions between these three factors are important for forecasting how the prevalence and distribution of ticks and tick-borne diseases may appear in an area. Risk assessments should focus on looking for combinations of factors that may directly or indirectly affect these three factors. A risk assessment module can be proposed based on these factors and a framework designed for this purpose could be used to screen for the emergence of unexpected disease events (Gale *et al.,* 2009).

1.4 Prevention and control of tick-borne diseases

Good quality and proper prevention and control measures can best be achieved by an accurate disease diagnosis method. Newer disease diagnosis method like polymerase chain reaction (PCR) are more accurate and reliable than other conventional techniques like Serology and microscopy that are less accurate especially when there is a case of low level infection and lack of morphological distinguishing features during microscopy and in a case of

cross-reactivity and latent infection as in the case of serology, making detection and identification to specie and sub-specie levels very difficult.

With the advent of newer molecular diagnostic techniques, all anomalies and difficulties associated with accurate pathogen detection and identification of tick-borne hemopathogens were solved; and many new species of pathogens were discovered and many were re-classified.

1.5 Efforts toward tackling companion animal's diseases in Malaysia

The current joint efforts by the Division of Veterinary Services Malaysia and various non-governmental organisations (NGO's) in providing veterinary services for companion animals have yielded tremendous results by creating awareness among animal owners, preventing cruelty to animals by establishing animal welfare facilities, especially in rural areas, and infrastructure for diagnostic support (such as regional veterinary pathology laboratories) in small animal practices. Limited surveillance for the accumulation of information on canine and feline diseases has been put in place in each region. The pet owners on their part have also started to maintain high standards of hygiene in the cities. In recent years, researchers in the field of Veterinary and Animal Sciences have achieved tremendously in their research findings towards providing other specialist in the field with updated and relevant information.

Modern diagnostic tests and epidemiological surveys suitable for diagnosis of infections have been going on to differentiate between a new and previous infections, and or diagnosis in individuals with high antibody levels living in endemic areas. To overcome these limitations, and those associated with traditional pathogen-detection systems such as microscopy, highly sensitive DNA-based methods have been developed. Unfortunately, this new technology is not cost effective and will take many years before it is readily available in all localities. However, it is now apparent from the studies in South East Asia which have applied these molecular tools to companion animal blood or to their arthropod parasites that there are intriguing, and maybe even surprising, discoveries to be made soon.

1.6 Justification of the study

Southeast Asia is one of the few regions of the world with inadequate records concerning diseases of pet animals, yet they happen to be among the most heavily populated with stray dogs and cats (Irwin and Jefferies, 2004). The combination of the tropical climate, large population of stray dogs and cats, and an increase in pet ownership in the region provides favorable conditions for the survival and maintenance of both the tick vector and the pathogens that they harbor. The stray animals not only serve as reservoirs of infection to other animals but also increase the risk of zoonotic disease transmission. Basic

demographics for pet populations are scarce to non-existent in most countries of the region (Peters and Pasvol, 2002; Batson, 2008).

Despite the large stray dog population and an ever increasing pet population in Malaysia, there is limited published data on the molecular prevalence, to non concerning thier epidemiology and phylogenetics of canine vector-borne diseases. Since early 80s, researchers have been striving to report the prevalence of ticks and tick-borne pathogens of dogs in Malaysia and its neighboring countries (Rajamanickan *et al*., 1985, Inokuma *et al.,* 2003, Irwin and Jefferies, 2004, Rahman *et al*., 2010, Mariana *et al*., 2011, Nazari *et al*., 2013; Mokhtar *et al.,* 2013, Chandrawathani *et al.,* 2014). However, there is no adequate, specific and detailed information regarding the prevalence, spatial distribution and phylogenetic analyses of tick-borne hemopathogens of dogs in the country. Therefore, the needs to further investigate, using more sensitive techniques to determine the prevalence, spatial distribution and phylogenetics of these pathogens infecting dogs in Malaysia.

1. Early reports suggested that tick-borne transmitted infections associated with protozoa, rickettsia and bacteria are prevalent in Southeast Asia including Malaysia (Rajamanickan *et al*., 1985, Suksawat *et al.,* 2001a; Irwin and Jefferies, 2004, Rahman *et al*., 2010, Mariana *et al*., 2011, Chandrawathani *et al.,* 2014). Therefore, for further studies are essential for us to obtain a clear picture of their diversity and distribution.
2. Currently, there is a paucity of information regarding the molecular prevalence, spatial distribution and phylogenetic of the various tick-borne hemopathogens of dogs in Malaysia.
3. The potential risk posed by the increasing number of stray dogs in the country and the pathogens that they harbor requires investigation.

1.7 Aim and objectives of the study

Aim : The aim of this study was to determine the prevalence and spatial distribution as well as to carryout molecular characterization of the various tick-borne hemopathogens of stray dogs in Malaysia to provide a more accurate picture of the current situation of these etiological agents of disease in the country.

Objectives : The objectives of this study are to:

1. Determine the molecular prevalence of common tick-borne hemopathogens of stray dogs in Malaysia.
2. Determine the spatial distribution of the various hemopathogens in Malaysia.
3. Further characterize the hemopathogens identified during the course of the study by polymerase chain reaction.

1.8 Research questions and Hypothesis

1.8.1 Research questions

1. What are the prevalences of the common tick-borne pathogens of stray dogs in Malaysia?
2. What is their distribution pattern among region, gender, age and breeds?
3. What are the genetic similarities of the Malaysian strains of the hemopathogens identified from this study from other geographically dispersed strains?

1.8.2 Hypothesis

H_1 = Prevalence of tick-borne hemopathogens among stray dogs in Malaysia is high at $\alpha \leq 0.05$ ($H_o = \mu_{prev} > \mu_o$).

H_1 = Prevalence of tick-borne hemopathogens among stray dogs in Malaysia is low at $\alpha \leq 0.05$ ($H_A = \mu_{prev} < \mu_o$).

H_2 = There is equal distribution of canine tick-borne hemopathogens among region, gender, age and breeds in Malaysia at $\alpha \leq 0.05$ ($H_o = \mu_{distribution\ (West,\ male,\ young,\ locals)} = \mu_{distribution\ (East,\ female,\ adult,\ pedigree)}$).

H_2 = There is no equal distribution of canine tick-borne hemopathogens among region, gender, age and breeds in Malaysia at $\alpha \leq 0.05$ ($H_A = \mu_{distribution\ (West,\ male,\ young,\ locals)} \neq \mu_{distribution\ (East,\ female,\ adult,\ pedigree)}$).

H_3 = The Malasian isolates for the various hemopathogens are genetically similar to other geographically dispersed strains at $\alpha \leq 0.05$ ($H_o = \mu_{Malaysian\ strain} = \mu_{other\ strains}$).

H_3 = The Malasian isolates for the various hemopathogens are not genetically similar to other geographically dispersed strains at $\alpha \leq 0.05$ ($H_o = \mu_{Malaysian\ strain} \neq \mu_{other\ strains}$).

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