UNIVERSITI PUTRA MALAYSIA

MOLECULAR IDENTIFICATION AND PREVALENCE OF EHRLICHIA CANIS AND ANAPLASMA PLATYS IN DOGS IN SELECTED AREAS OF MALAYSIA

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MOLECULAR IDENTIFICATION AND PREVALENCE OF *EHRLICHIA CANIS* AND *ANAPLASMA PLATYS* IN DOGS IN SELECTED AREAS OF MALAYSIA

By

MOJGAN NAZARI

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

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DEDICATION

I dedicate this thesis to my lovely family and my faithful husband, without their patience, understanding, support, and most of all love, the completion of this work would not have been possible.

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

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Chairman: **Malaika Watanabe, PhD**

Faculty: **Faculty of Veterinary Medicine**

Ehrlichia species are the etiological agents of emerging and life-threatening tick-borne diseases of both humans and animals. They have been implicated in serious and fatal infections in companion animals and livestock and the potential to cause severe life-threatening diseases emphasizes the need for early diagnosis of ehrlichiosis. Limited studies have been conducted to investigate tick-borne diseases in Malaysia, and only two studies have been published on *Ehrlichia canis* thus far, the first one relied solely on light microscopic detection techniques and the other on immunofluorescence antibody test (IFA) test for diagnosis. The objective of this study was to carry out a molecular study of the 16S rRNA gene of *Ehrlichia canis* (*E. canis*) and *Anaplasma platys (A. platys*) infections in dogs in Malaysia using the polymerase chain reaction (PCR) technique which is known to be the most sensitive and specific method for the diagnosis of canine ehrlichiosis worldwide. For this purpose, canine blood samples were collected from pet dogs (n=323) presented to

veterinary clinics and stray dogs (n=177) from February 2009 to February 2010. After DNA extraction, standard PCR was performed with a genus-specific set of primers EHR16SD, and EHR16SR followed by *E. canis* spices-specific PCR (CANIS/ GA1UR), and *A. platys* species-specific PCR (PlatysF/ PlatysR). Analysis of PCR products revealed that 2.0% (n=10) of dogs were positive for *E. canis*, (1.2% among the clinic group, and 3.4% among the stray dogs), and 4.6% (n=23) were positive for *A. platys* (1.2% and 10.73% among clinic group and stray dogs respectively). The sex, age, and breed of the clinical cases were noted and hematological and serum biochemical results were also obtained for the clinic group. Statistical analysis showed no consistent significant differences between *E.canis* or *A.platys* infection status, sex, age, breed, and clinical or hematological abnormalities. In order to achieve a larger size for sequence analysis, all positive samples were amplified with another set of primers (FD1/RP2). Sequence analysis of positive samples for both *E.canis* and *A.platys* showed 100% identity to a number of registered strains in NCBI GenBank. In conclusion the present study revealed for the first time the presence of genetically confirmed *E. canis* and *A. platys* pathogens with an overall prevalence rate of 2.0% and 4.6%, respectively in naturally infected dogs in Malaysia.

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

PENGENALAN MOLEKUL DAN KELAZIMAN EHRLICHIA CANIS DAN ANAPLASMA PLATYS PADA ANJING DI KAWASAN TERPILIH MALAYSIA

Oleh

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Spesies *Ehrlichia* ialah ejen etiologi bagi penyakit bawaan kutu berbangkit dan mengancam nyawa manusia dan haiwan. Mereka ini telah diimplikasikan dengan jangkitan yang serius dan membawa maut kepada haiwan peliharaan dan ternakan, dan ini menekankan keperluan untuk diagnosis ehrlichiosis di peringkat awal. Kajian untuk menyiasat penyakit bawaan kutu di Malaysia adalah terhad dan setakat ini hanya dua kajian telah diterbitkan mengenai *Ehrlichia canis,* yang pertama diagnosis bergantung kepada ujian mikroskopik dan yang lagi satu ialah teknik immunofluorescene antibody test(IFA). Objektif kajian ini adalah untuk menjalankan ujian secara molekular dengan menggunakan gen 16S rRNA *Ehrlicia canis* (*E. Canis*) dan *Anaplasma platys* (*A. Platys*) dalam teknik reaksi rantai polymerase (PCR), kaedah yang paling sensitif dan spesifik pada masa kini untuk diagnosis penyakit ehrlichiosis di Malaysia. Untuk tujuan ini, sampel darah anjing

dari anjing kesayangan (n = 323) daripada anjing peliharaan yang dibawa ke klinik veterinar dan anjing terbiar (n = 177) telah dikumpulkan dari Februari 2009 hingga Februari 2010. Selepas pengekstrakan DNA, PCR standard yang menggunakan satu set primer genus-khusus EHR16SD dan EHR16SR telah dijalankan dan dikuti dengan PCR khusus *E. canis* (CANIS/GA1UR) dan PCR khusus *A. platys* (PlatysF/PlatysR). Analisis produk PCR menunjukkan bahawa 2% (n = 10) positif untuk *E. canis* dalam anjing (1.2% dari kumpulan klinik dan 3.4% dari kumpulan anjing terbiar), dan 4.6% (n = 23) positif untuk *A. platys* (1.2% dari kumpulan anjing klinik dan 10.73% dari kumpulan anjing terbiar). Jantina, umur dan baka bagi kes – kes klinikal dikenalpasti dan keputusan untuk ujian haematologikal dan biokimia serum untuk anjing klinik juga diperolehi. Analisis statistik menunjukkan tiada perbezaan signifikan yang konsisten di antara status jangkitan *E. canis* atau *A. platys*, jantina, umur, baka dan keputusan haemotologikal dan biokimia serum yang abnormal. Untuk mendapatkan jumlah sampel yang lebih besar untuk jujukan yang lebih panjang untuk tujuan analisis jujukan, semua sampel yang positif telah diamplikasi dengan satu lagi set primer (FD1/RP2). Analisis jujukan bagi semua sampel yang positif untuk *E.canis* dan *A.platys* menunjukkan identiti 100% homologi dengan beberapa strain berdaftar di NCBI Bank Gen. Kesimpulannya, kajian ini adalah yang pertama mengesahkan kewujudan pathogen *E. canis* dan *A. platys* secara genetik dengan kadar prevalens keseluruhan sebanyak 2.0% untuk *E. canis* dan 4.6% untuk *A. platys* untuk jangkitan secara semulajadi pada anjing di Malaysia.

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I certify that an Examination Committee has met on December, 06, 2011 to conduct the final examination of Mojgan Nazari on his MVSc thesis entitled " MOLECULAR IDENTIFICATION AND PREVALENCE OF EHRLICHIA CANIS AND ANAPLASMA PLATYS IN DOGS IN SELECTED AREAS OF MALAYSIA" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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

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DECLARATION

I declare that the thesis is based on 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 other institutions.

MOJGAN NAZARI

Date: December 2011

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LIST OF ABBREVIATIONS/NOTATIONS/GLOSSARY OF TERMS

A. platys	*Anaplasma platys*
CICT	Canine infectious cyclic thrombocytopenia
CME	Canine monocytic ehrlichiosis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide-5′-triphosphate
E value	Expected value
E. canis	*Ehrlichia canis*
EDTA	Ethylene diaminete traacetic acid
ELISA	Enzyme-linked Immunosorbent assay
HGE	Human Granulocytic Ehrlichiosis
IFA	Immunofluorescence antibody test
µl	Microliter
$MgCl_2$	Magnesium chloride
P value	Probability of obtaining a test statistic at least as extreme as the one that was actually observed
PCR	Polymerase chain reaction
SDW	Sterile distilled water
SID	Once a Day
Taq	Thermus aquaticus
UPM	Universiti Putra Malaaysia

CHAPTER 1

INTRODUCTION

Tick-borne diseases represent a problem of growing importance for public health. The multiple outbreaks of different tick transmitted diseases has increased public awareness about these emerging and reemerging zoonotic diseases (Parola, *et al*., 2000).

Ehrlichiosis and anaplasmosis are tick-borne diseases that have gained attention lately as they are responsible for causing disease of varying severity in both humans, domestic and wild animals. These diseases are caused by rickettsial organisms in the family *Anaplasmataceae*, of the genera *Ehrlichia* and *Anaplasma* (Skotarczak, 2003). Rickettsiales are able to infect a broad range of hosts, and multiple pathogens can co-exist in both vertebrate and invertebrate hosts, necessitating extensive research on these pathogens. Canine infection with *Ehrlichia spp*. and *Anaplasma spp*. is common throughout the world. And infections are more common in areas where tick infestation is high.

E. canis is the etiologic agent of canine monocytic ehrlichiosis (CME), a severe and clinically significant disease of dogs. Ehrlichiosis in dogs caused by *E. canis* was first reported in 1935 in Algeria and later in southern India and other parts of Africa in the 1940s (Harrus, *et al*., 1999). The importance of *E. canis* as a canine pathogen went relatively unrecognized until it was associated with outbreaks of canine tropical

pancytopenia in Singapore and Malaysia from 1963 to 1968 and was identified as being the cause of an epizootic of canine tropical pancytopenia in U.S. military dogs stationed in Vietnam that resulted in approximately 200 deaths over a 4-year period in late 1968 (Huxsoll, *et al*., 1969; Mavromatis, *et al*., 2006).

Anaplasma platys; formerly known as *Ehrlichia platys;* is the etiological agent of canine infectious cyclic thrombocytopenia (CICT), which was first reported in the USA in 1978 (Harvey, *et al*., 1978). *A. platys* develops within the platelets resulting in a thrombocytopenia. Infected dogs however are usually asymptomatic (De La Fuente, *et al*., 2006). *A. platys* infection has been reported throughout the world, but because of the low bacteremia it is often hard to detect the infection in vivo (De La Fuente, *et al*., 2006).

Although ehrlichiosis and anaplasmosis were previously only considered important diseases of animals, they are now known to be important emerging zoonoses in people as well. People contract these infections the same way as animals: via the bite of an infected tick acquired from a tick-infested environment. Although direct transmission from an infected animal to a person is possible through accidental inoculation of contaminated blood or tissue, animals are not an immediate source of infection to humans but may act as reservoirs or sentinels of infection (Dumler, *et al*., 2001; Dumler & Walker, 2001).

Ehrlichia canis and *Anaplasma platys* can be fatal to dogs, therefore accurate and quick diagnosis followed by appropriate therapy is crucial for a positive clinical

outcome. Unfortunately, diagnosis is not straight forward and cannot be made based on clinical signs or serological results alone (Vinasco, *et al*., 2007).

Traditionally, microscopic and serological evaluations have been used in combination with clinical signs for the diagnosis of ehrlichiosis and anaplasmosis. Due to the limitations of light microscopic examination for the detection of *E .canis*, it was imperative to study the prevalence using more reliable diagnostic methods. Recently, the development of molecular technology has provided useful, reliable, sensitive, specific and rapid tools for the detection and identification of tick-borne pathogeneses (Sparagano, *et al*., 1999; Vinasco, *et al*., 2007; Warner & Dawson, 1996). Furthermore there are only few documented studies about tick-borne diseases in Malaysia, though many clinics claim to have seen a significant number of cases diagnosed based on clinical signs and sometimes identification of morulae under the light microscope (Rahman, *et al*., 2010; Rajamanickam, *et al*., 1985).

Due to high *E. canis* prevalence rates of even up to 30% around the world, and because *Ehrlichia* species are the etiological agents of emerging and life-threatening tick-borne disease in humans and domestic animals, there was a pressing need to determine actual prevalence rates in Malaysia (Dagnone *et al*., 2003; Bulla *et al*., 2004; Ndip *et al*., 2005; Carvalho *et al*., 2008; De Barros *et al*., 2008; Yabsley *et al*., 2008; Tzipory *et al*., 2010).

This information can help clinicians diagnose their cases more accurately and without over or misdiagnosis of diseases, as well as help in following treatment

success. This knowledge will also lead to greater awareness and vigilance with increased travel and movement of pets in and out of a known endemic area. Therefore in this study the prevalence and molecular characterization of *Ehrlichia* and *Anaplasma* detected from canine blood was carried out for the first time in Malaysia.

1.1 Hypothesis

The current study hypothesizes that

1- *Anaplasma platys* and *E. canis* are present in dogs in Malaysia, since this pathogens are common rickettsial pathogen of dogs found worldwide.

2- There would be large numbers of infected dogs in Malaysia because of the presence of a conductive environment for the tick vectors.

3- It is expected that a new strain of *E. canis* or *A. platys* may be identified in Malaysia.

1.2 Objectives

1- To determine the molecular prevalence of *Ehrlichia canis* and *Anaplasma platys* in dogs in Malaysia.

2- To study the relationship between signalment and clinical findings and infective status of the dogs.

3- To use molecular methods (PCR) to detect *Ehrlichia* and *Anaplasma* from blood.

4- To identify and compare the detected strains with other strains in NCBI GenBank to determine whether the Malaysian strains are unique.

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