Tropical Biomedicine 34(3): 570–575 (2017)

Molecular and serological prevalence of *Anaplasma* and *Ehrlichia* sp. among stray dogs in East Malaysia

Konto, M.[1,4*], Tukur, S.M.[1,4], Watanabe, M., Abd-Rani, P.A.M.[1], Sharma, R.S.K.[2], Fong, L.S.[3] and Watanabe, M.[1]

[1]Department of Companion Animal Medicine and Surgery, Faculty of Veterinary Medicine, Universiti Putra Malaysia

[2]Department of Veterinary Laboratory Diagnostics, Faculty of Veterinary Medicine, Universiti Putra Malaysia

[3]Department of Veterinary Clinical Studies, Faculty of Veterinary Medicine, Universiti Putra Malaysia

[4]Department of Veterinary Parasitology and Entomology, University of Maiduguri, Nigeria

[*]Corresponding author e-mail: maraika@upm.edu.my

Received 25 February 2016; received in revised form 7 May 2017; accepted 19 May 2017

Abstract. *Anaplasma* and *Ehrlichia* species are important bacterial pathogens of various animal species including dogs and humans. They constitute a major health problem worldwide, especially in the tropics and sub-tropics. In order to identify the prevalence of *Anaplasma* and *Ehrlichia* sp. from dogs in the selected area, a total of 100 randomly selected clinically healthy rescued dogs from animal shelters in Sarawak and Sabah, East Malaysia (50 dogs per State) were used for this study. Whole blood was collected from these dogs and screened for presence of *Anaplasma* and *Ehrlichia* spp. using polymerase chain reaction (PCR) and commercial ELISA test (SNAP 4Dx® Plus) for detection of antigen and antibody against the two pathogens respectively. 27% (*A. platys*) and 33% (*E. canis*) of the sampled population were positive using PCR; while the serological prevalence was 29% for *Anaplasma* sp. and 55% for *Ehrlichia* sp. The results of this investigation revealed that stray dogs are potential reservoir hosts of *Anaplasma* and *Ehrlichia* sp. infection with a high prevalence in the area. The high prevalence rates detected highlights the urgent need to address the growing population of possible vectors and reservoirs for transmission in the region and also to look into the public health aspect of the disease.

INTRODUCTION

Anaplasmosis and ehrlichiosis are important tick-borne diseases of dogs that pose a threat not only to the canine population, but also to humans worldwide due to their close contact with dogs as companions. Among the microorganisms infecting dogs, *Anaplasma platys* and *Ehrlichia canis* are of major concern in the tropics and subtropics as aetiological agents of canine cyclic thrombocytopenia and canine monocytic ehrlichiosis respectively. Both species have been reported to be cosmopolitan in distribution due to the ubiquitous nature and distribution of their tick vector *Rhipicephalus sanguineus* (Woody & Hoskins, 1991). They are associated with varying degrees of clinical signs ranging from mild asymptomatic infection to acute severe disease depending on the host immunity, pathogen virulence, infective dose, the route of infection and environmental factors (Irwin and Jefferies, 2004; Watanabe, 2012; Mokhtar *et al.*, 2013; Nazari *et al.*, 2013; Sykes and Foley, 2014).

Polymerase chain reaction (PCR) is the most sensitive and specific diagnostic method for epidemiological studies on canine anaplasmosis and ehrlichial infections worldwide (Weisburg *et al.*, 1991; Warner and Dawson, 1996; Parola *et al.*, 1998; Parola *et al.*, 2000; Inokuma *et al.*, 2000, 2001a, b; Shaw *et al.*, 2001). Recent studies using PCR

and sequence analysis for the detection of *Anaplasma* and *Ehrlichia* infections among dogs in Peninsular Malaysia have been conducted from whole blood samples (Mokhtar *et al.*, 2013; Nazari *et al.*, 2013; Koh *et al.*, 2015). However, even though the presence of these two bacterial infections in dogs has been reported in Peninsular (West) Malaysia, there is as of yet no available data to demonstrate their presence in East Malaysia (Borneo Island) using molecular methods. This study attempted for the first time to confirm the existence of *Anaplasma platys* and *Ehrlichia canis* in blood samples collected from stray dogs in East Malaysia, using PCR.

METHODOLOGY

Ethics statement

This research was conducted in line with the guidelines of the Animal Care and Use Committee, Faculty of Veterinary Medicine, Universiti Putra Malaysia with approval code: R074/2013. Informed consent was obtained from the managers of the animal shelters where sampling was conducted and assurance of anonymity, prior to sampling.

Sampling method

A total of 100 randomly selected clinically healthy rescued dogs from animal shelters from Sarawak (50 dogs) and Sabah (50 dogs), East Malaysia were included in the study. The sex, age and breed of the dogs were noted. 200 µl of whole blood was collected in EDTA tubes.

PCR

DNA was extracted from whole blood using the Qiagen® Blood and Tissue Kit according to manufacturer's guidelines. All DNA samples were screened by standard PCR using genus-specific primers that amplify a 360bp fragment of the 16S rRNA gene of members of the family *Anaplasmataceae* (Dawson *et al.*, 1996). A second PCR was conducted with species-specific primers for the detection of *A. platys* (Criado-Fornelio *et al.*, 2003) and *E. canis* (Dawson *et al.*, 1996) as shown in Table 1. The PCR amplification protocol was set up for each hemopathogen (Table 2) within a 25 µl reaction mixture containing 4 µl of DNA template and 21 µl of master mix (Promega®) (5µl of 25x buffer without $MgCl_2$, 1 µl of 10mM dNTP, 5 µl of 25x$MgCl_2$) 1 µl of 20 pmol primer (NHK-Bioscience), 0.3µl of 20 units of Taq polymerase and sterile distilled water to a final volume of 21 µl. The thermal cycling parameters were set for each pathogen (Table 1) using a BioRad Mycler® thermal cycler. The amplification products were visualized on a 1.5% – 2% agarose gel (depending on the molecular weight) after electrophoretic migration for 45 minutes at 100V. The gels were stained with ethidium bromide for 15 minutes and visualized under UV trans-illumination (BioRad Alpa Imager^R, USA). Amplicons obtained by PCR were then purified and sequenced. For serology, SNAP 4Dx® Plus test kit (IDEXX, USA) was used and all the test procedures were conducted according to the manufacturers guidelines.

Table 1. Primer sequences and thermal cycling parameters used for PCR

Pathogen	Primers	Sequence	Expected size
Anaplasmataceae	fD1 5' Rp2 5'	AGAGTTTGATCCTGGCTCAG 3' ACGGCTACCTTGTTACGACTT 3'	360bp
Ehrlichia canis	CANIS 5' GA1UR 5'	CAATTATTTATAGCCTCTGGCTATAGGA 3' GAGTTTGCCGGGACTTCTTCT 3'	400bp
Anaplasma platys	PlatysF 5' PlatysR 5'	AAGTCGAACGGATTTTTGTC 3' CTTTAACTTACCGAACC 3'	500bp

Table 2. Thermal cycling parameters set for each pathogen

Pathogen	Annealing T°C	Extension	No. of cycles
Anaplasmataceae	62 for 30 sec	72 for 1 min 30 sec	40 cycles
A. platys	64.5 for 30 sec	72 for 1 min	40 cycles
E. canis	60 for 30 sec	72 for 1 min 30 sec	40 cycles

Statistical analysis

Statistical analysis was performed using Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL). Relation between categorical outcomes, i.e. absence or presence of specific infection within gender was compared using the chi-square test. Prevalence rates were calculated at 95% confidence interval and statistical significance was set at $p \leq 0.05$.

RESULTS AND DISCUSSION

PCR revealed that 27% of the sampled population were infected with *A. platys* and 33% were infected with *E. canis* with 99% query cover and identity to sequences KT357373.1 and KJ095115.1 respectively, deposited in the GenBank. In contrast to the result obtained from PCR, the serological prevalence was 29% for *Anaplasma* sp. and 55% for *Ehrlichia* sp. (Figure 1).

As expected, the serological prevalence was higher than the molecular prevalence as serology detects the presence of antibodies. It is important to note that the presence of antibodies only reflects exposure to the pathogens and not necessarily an active infection. Thus, with the ubiquitous presence of ticks in our environment, it is only reasonable to assume that most of the stray dog population has been exposed to *Ehrlihia canis* and *Anaplasma platys* at some point in time and these antibodies persist even though the infection has been cleared (Palmer *et al.*, 2000, Harrus and Waner, 2011). This is reiterated by the fact that the stray dogs sampled were all healthy and clinical signs of disease were absent. Furthermore cross-reactivity is inherent in the serological test used whereby it not only detects *A. platys* antibodies, but also *A. phagocytophilum* antibodies (Rikihisa, 1991; Waner *et al.*, 2001; Cardenas *et al.*, 2007). Similarly the test detects antibodies to *Ehrlichia* sp. which includes not only *E. canis* but *E. ewingii* as well. Though, neither *A. phagocytophilum* nor *E. ewingii* has been detected as of yet in Malaysia, it would be interesting to investigate the presence of these two pathogens. Although the SNAP 4Dx® Plus test is a very convenient and useful diagnostic tool, a diagnosis of canine anaplasmosis or ehrlichiosis should be based on a combination of compatible clinical and laboratory findings (Harrus *et al.*, 2002; Hegarty *et al.*, 2009; Harrus and Waner, 2011).

The PCR technique is considered the definitive confirmatory test for *A. platys* and *E. canis* infection. However, though PCR is very sensitive, when the infection is subclinical or in the chronic phase, the antigen may not be as readily detected in the blood and it has been suggested that splenic samples will increase sensitivity in subclincal and chronic ehrlichiosis (Codner and Farris-Smith, 1986; Iqbal *et al.*, 1994; Skotarczak, 2003; Nakaghi *et al.*, 2008; Harrus and Waner, 2011).

Demographic distribution of the hemopathogens showed that dogs in Sarawak had a significantly higher ($p=0.04$ and 0.001) infection rate for both *A. platys* and *E. canis* (respectively) using PCR than those in Sabah. Local breeds showed a significantly ($p=0.01$) higher *E. canis* prevalence using PCR than pedigree dogs (Table 3).

State-wise distribution of the two hemopathogens based on sex, age and breed showed that adult dogs had a significantly ($p < 0.05$) higher *Ehrlichia* sp. infection rate using both PCR and serology than younger dogs (Table 4).

The high prevalence of the hemopathogens among dogs in the area agrees with earlier reports by Irwin and Jefferies,

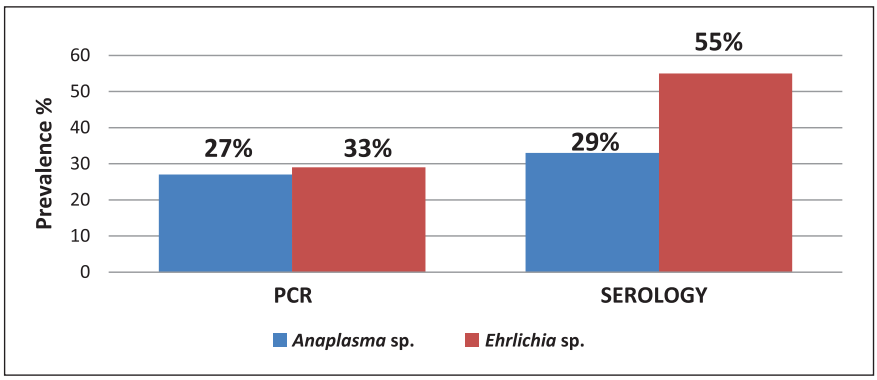

Figure 1. Molecular and seroprevalence of *Anaplasma* and *Ehrlichia* sp. among stray dogs in East Malaysia.

Table 3. Location, sex, age and breed-wise molecular and seroprevalence of *Anaplasma platys* and *Ehrlichia canis* among stray dogs in East Malaysia

Factor	Category (n=100)	Frequency (PCR/Serology)		Prevalence % (PCR/Serology)	
		Anaplasma	*Ehrlichia*	*Anaplasma*	*Ehrlichia*
State	Sabah n=50	9 / 12	10 / 28	18 / 24	20 / 56
	Sarawak n=50	18 / 17	23 / 27	36* / 34	46* / 54
Sex	Male n=41	7 / 13	18 / 23	17 / 32	44 / 56
	Female n=59	20 / 16	15 / 32	33 / 27	25 / 54
Age	Young n=20	3 / 2	1 / 3	15 / 10	5 / 15
	Adult n=80	24 / 27	32 / 52	30 / 34*	40* / 65*
Breed	Local n=83	23 / 26	32 / 46	28 / 31	39* / 55
	Pedigree n=17	4 / 3	1 / 9	24 / 18	6 / 53

NB *: Each subscript denotes a subset of variables whose column proportion differs significantly from each other at 0.05 levels, n = 100.

Table 4. State-wise distribution of the two hemopathogens based on sex, age and breed

Factor	Category	Frequency Sab/Sar n=50	Prevalence % (no. positive)							
			Sabah State				Sarawak State			
			Anaplasma sp.		*Ehrlichia* sp.		*Anaplasma* sp.		*Ehrlichia* sp.	
			PCR	Serology	PCR	Serolo	PCR	Serology	PCR	Serol
Sex	Male	15/26	13(2)	40(6)	27(4)	53(8)	19(5)	27(7)	54(14)	58(15)
	Female	35/24	20(7)	17(6)	17(6)	57(20)	54(13)	42(10)	38(9)	50(12)
Age	Young	20/0	15(3)	10(2)	5(1)	15(3)	–	–	–	–
	Adult	30/50	20(6)	33(10)	30(9)*	83(25)*	36(18)	34(17)	46(23)	54(27)
Breed	Local	33/50	15(5)	27(9)	27(9)	58(19)	36(18)	34(17)	46(23)	54(27)
	Pedigree	17/0	24(4)	18(3)	6(1)	52(9)	–	–	–	–

(2004) and Watanabe (2012), which is most likely due to Malaysia's climatic condition that favours the growth and proliferation of the vectors and their associated pathogens. Limited veterinary care services and facilities are other compounding factors that contribute to the high infection rates among dogs in the area. This study revealed a high

prevalence of *Anaplasma* and *Ehrlichia* sp. infection among stray dogs in the area, at the same time their potential role as reservoir hosts of these hemopathogens in the area. This further necessitates conscientious efforts to identify and curb the tick vectors and control the spread of infection to naive dogs in the region. Further investigation into the public health aspect of the pathogens is also recommended due to the close proximity between dogs and humans in the study area.

Acknowledgements. The research was supported in part by the Universiti Putra Malaysia, Research University Grant Scheme (Project no: 01-01-09-0662RU). The SNAP 4Dx® kits (IDEXX, USA) were sponsored by Arachem Sdn Bhd. Our profound gratitude to Dr. Randolf from Sabah, Dr. Davies Banda from Sarawak, staff of the parasitology laboratory (Faculty of Veterinary Medicine, UPM) for their kind assistance.

Competing interest

All authors read and approved the final manuscript and declared that they have no competing interests.

REFERENCES

Cardenas, A.M., Doyle, C.K., Zhang, X., Nethery, K., Corstvet, R.E., Walker, D.H. & McBride, J.W. (2007). Enzyme-linked immunosorbent assay with conserved immunoreactive glycoproteins gp36 and gp19 has enhanced sensitivity and provides species-specific immunodiagnosis of *Ehrlichia canis* infection. *Clinical and Vaccine Immunology* **14**: 123-128.

Codner, E.C. & Farris-Smith, L.L. (1986). Characterization of the subclinical phase of ehrlichiosis in dogs. *Journal of the American Veterinary Medical Association* **189(1)**: 47-50.

Criado-Fornelio, A., Martinez-Marcos, A., Buling-Sarana, A. & Barba-Carretero, J.C. (2003). Molecular studies on *Babesia*, *Theileria* and *Hepatozoon* in southern Europe: part I. Epizootiological aspects. *Veterinary Parasitology* **113(3)**: 189-201.

Dawson, J.E., Biggie, K.L., Warner, C.K., Cookson, K., Jenkins, S., Levine, J.F. & Olson, J.G. (1996). Polymerase chain reaction evidence of *Ehrlichia chaffeensis*, an etiologic agent of human ehrlichiosis, in dogs from southeast Virginia. *American Journal of Veterinary Research* **57(8)**: 1175-1179.

Harrus, S., Alleman, A.R., Bark, H., Mahan, S.M. & Waner, T. (2002). Comparison of three enzyme-linked immunosorbant assays with the indirect immunofluorescent antibody test for the diagnosis of canine infection with *Ehrlichia canis*. *Veterinary Microbiology* **86**: 361-368.

Harrus, S. & Waner, T. (2011). Diagnosis of canine monocytotropic ehrlichiosis (*Ehrlichia canis*): an overview. *The Veterinary Journal* **187(3)**: 292-296.

Hegarty, B.C., de Paiva Diniz, P.P., Bradley, J.M., Lorentzen, L. & Breitschwerdt, E., (2009). Clinical relevance of annual screening using a commercial enzyme-linked immunosorbent assay (SNAP 3Dx) for canine ehrlichiosis. *Journal of the American Animal Hospital Association* **45**: 118-124.

Inokuma, H., Yamamoto, S., Tanahara, N., Kiyuna, T. & Ohshiro, S. (1998). Tick infestation and tick-borne diseases infection of dogs in Okinawa Island. *Journal of Japan Vet. Med. Assoc.* **51**: 361–364 (in Japanese with English summary).

Inokuma, H., Raoult, D. & Brouqui, P. (2000). Detection of *Ehrlichia platys* DNA in brown dog ticks (*Rhipicephalus sanguineus*) in Okinawa Island, Japan. *Journal of Clinical Microbiology* **38**: 4219-4221.

Inokuma, H., Ohno, K., Onishi, T., Raoult, D. & Brouqui, P. (2001a). Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. *Journal of Vet. Med. Sci.* **63**: 815-817.

Inokuma, H., Parola, P., Raoult, D. & Brouqui, P. (2001b). Molecular survey of *Ehrlichia* infection in ticks from

animals in Yamaguchi Prefecture, Japan. *Veterinary Parasitology* **99**: 335-339.

Inokuma, H., Fujii, K., Matsumoto, K., Okuda, M., Nakagome, K., Kosugi, R., Hirakawa, M. & Onishi, T. (2002). Demonstration of *Anaplasma (Ehrlichia) platys* inclusion in peripheral blood platelets of a dog in Japan. *Veterinary Parasitology* **110**: 145-152.

Irwin, P.J. & Jeffries, R. (2004). Arthropod-transmitted diseases of companion animals in Southeast Asia. *Trends Parasitology* **20**: 27-34.

Iqbal, Z., Chaichanasiriwithaya, W. & Rikihisa, Y. (1994). Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. *Journal of Clinical Microbiology* **32(7)**: 1658-1662.

Koh, F.X., Panchadcharam, C. & Tay, S.T. (2015). Vector-Borne Diseases in Stray Dogs in Peninsular Malaysia and Molecular Detection of *Anaplasma* and *Ehrlichia* spp. from *Rhipicephalus sanguineus* (Acari: *Ixodidae*) Ticks. *Journal of Medical Entomology*, tjv153.

Mokhtar, A.S., Lim, S.F. & Tay, S.T. (2013). Molecular detection of *Anaplasma platys* and *Babesia gibsoni* in dogs in Malaysia. *Tropical Biomedicine* **30(2)**: 345-348.

Nakaghi, A.C.H., Machado, R.Z., Costa, M.T., André, M.R. & Baldani, C.D. (2008). Canine ehrlichiosis: clinical, hematological, serological and molecular aspects. *Ciência Rural* **38(3)**: 766-770.

Nazari, M., Lim, S.Y., Watanabe, M., Sharma, R.S.K., Nadzariah, Cheng, N.A.B.Y. & Watanabe, M. (2013). Molecular detection of *Ehrlichia canis* in dogs in Malaysia **7(1)**: e1982.

Palmer, G.H., Brown, W.C. & Rurangirwa, F.R. (2000). Antigenic variation in the persistence and transmission of the *ehrlichia Anaplasma marginale*. *Microbes and Infection* **2(2)**: 167-176.

Parola, P., Beati, L., Cambon, M., Brouqui, P. & Raoult, D. (1998). Ehrlichial DNA amplified from Ixodes ricinus (Acari: Ixodidae) in France. *Journal of Medical Entomology* **35**: 180-183.

Parola, P., Roux, V., Camicas, J.-L., Baradji, I., Brouqui, P. & Raoult, D. (2000). Detection of *Ehrlichiae* in African ticks by PCR. *Trans. R. Soc. Trop. Med. Hyg.* **94**: 707-708.

Rikihisa, Y. (1991). Cross-reacting antigens between Neorickettsia helminthoeca and *Ehrlichia* species, shown by immunofluorescence and Western immunoblotting. *Journal of Clinical Microbiology* **29**: 2024-2029.

Shaw, S.E., Day, M.J., Birtles, R.J. & Breitswerdt, E.B. (2001). Tick-borne infectious diseases of dogs. *Trends Parasitology* **17**: 74-80.

Skotarczak, B. (2003). Canine ehrlichiosis. *Annals of Agricultural and Environmental Medicine* **10(2)**: 137-142.

Sykes, J.E. & Foley, J.E. (2014). Anaplasmosis: A textbook on canine and feline infectious diseases. Chapter **29**: Page 290-299.

Warner, C.K. & Dawson, J.E. (1996). Genus- and species-level identification of *Ehrlichia* species by PCR and sequencing. In: Persing, D.H. (Ed.), *PCR Protocols for Emerging Infectious Diseases*. ASM Press, Washington, DC, pp. 100-105.

Waner, T., Harrus, S., Jongejan, F., Bark, H., Keysary, A. & Cornelissen, A.W. (2001). Significance of serological testing for ehrlichial diseases in dogs with special emphasis on the diagnosis of canine monocytic ehrlichiosis caused by *Ehrlichia canis*. *Veterinary Parasitology* **95**: 1-15.

Watanabe, M. (2012). Main pet Arthropod-borne diseases in Asia. *9th Merial Symposium on Parasitosis and Arthropod-borne Diseases*. Tokyo 6th – 9th November 2012. Pp 18-21.

Weisburg, W.G., Barns, S.M., Pelletier, D.A. & Lane, D.L. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Journal of Bacteriology* **173**: 697-703.