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Research Article

Molecular and Serological Detection of Tick-Borne Hemopathogensamong Stray Dogs in East Malaysia

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Abstract

The current study focused on some of the most important common tick-borne hemopathogens of dogs in East Malaysian region with the aim of providing information regarding their prevalence and distribution among sex, age and breed. Blood samples were collected from 104 clinically healthy dogs, randomly selected from animal shelters located within Sarawak (50) and Sabah (54). Total DNA was extracted from blood samples and amplified by polymerase chain reaction (PCR) for the detection of *Ehrlichia, Anaplasma*, *Babesia* and *Hepatozoon* species. Of the samples tested, 86.5% were infected with at least one of the four hemopathogens; of which *Babesia* pp. predominated with a prevalence of 65.4%, followed by *Ehrlichia canis, Anaplasma platys* and *Hepatozoon canis* with prevalence rates of 47.1%, 38.5% and 28.8% respectively. The prevalence rates of *Babesia* spp. and *Ehrlichia canis* were significantly ($p<0.05$) higher in Sarawak compared to Sabah ($p = 0.001$ each). Male dogs showed a significantly ($p < 0.05$) higher *E. canis* infection rate than their female counterparts and adult dogs had a higher infection rate than younger dogs. There was no significant difference among sex, age and breed for the other hemopathogens. Co-infections were common and most dogs were infected with at least two pathogens (44.2%). *Babesia* spp and *E. canis* were most often seen to co-infect (35.6%). The study revealed high molecular and serological prevalence of tick-borne hemopathogens as well as mixed infections among stray dogs in East Malaysia.

INTRODUCTION

Tick-borne diseases are a problem worldwide, especially in the warm humid climates of the tropics and subtropics [1-6]. The humid warm climatic conditions of the tropics favour the growth and proliferation of tick vectors and other reservoir hosts, at the same time shortening their generation interval over time [7-9]. Some common tick-borne hemopathogens of veterinary significance in tropical regions include *Babesia, Hepatozoon, Ehrlichia* and *Anaplasma* species [10-14]. Clinically, they are the most significant hemopathogens of dogs and are associated with varying severity of clinical signs and a major health concern to dogs [15,16]. An increase in the stray dog population poses a potential threat to the naïve dog population as they may act as reservoirs of infection [17].

Despite an increase in the stray dog population in the area, coupled with the abundance of reservoir hosts and favourable climatic conditions for the survival of both the ticks and their reservoirs in the region; there is relatively low awareness regarding tick-borne disease among dog owners in the area. No information could be obtained regarding the prevalence of tick-borne pathogens of dogs in East Malaysia. Hence, this study was designed to determine the prevalence of some common tick-borne hemopathogens of dogs in the study area using serology and PCR and establish their co-infectivity status.

MATERIALS AND METHODS

Ethics statement

Approval was obtained from the Institutional Animal Care and Use Committee Universiti Putra Malaysia (IACUC) (Approval code: R074/2013). Consent was obtained from the shelters prior to sampling.

Sampling

A total of 104 stray dogs of different sex, breed and age were randomly sampled from animal shelters in Sabah and Sarawak between May 2013 and June 2014 (Figure 1). The age groups were stratified into young (0 - 12 months of age) and adult (12 months and above), while the breeds were broadly classified into local

Figure 1 Map of East Malaysia.
Topographic map of East Malaysia. The map shows the two central locations where samples were collected in Sabah and Sarawak. Other locations (indicated as small stars) are focal points where the dogs were rescued and brought in to the central locations for each state.

and pedigree. Three ml of blood was drawn from the cephalic vein out of which 1ml was aliquoted into ethylene diamine tetra acetic acid (EDTA) tubes for microscopy and PCR, and the remaining 2ml was aliquoted into plain tubes for serology.

Sampling site

Sampling was carried out in Kuching, Sarawak and Kota Kinabalu, Sabah, East Malaysia (Figure 1).

Sampling sites inclusion criteria

The animal shelters must have had a population of more than 100 dogs at the point of sampling, no recent treatment against tick-borne pathogens should have been carried out, they must have adequate records concerning the source, treatment and management status of the dogs in the shelter and the shelters must cater for dogs from a large perimeter across the state.

Animal inclusion criteria

Dogs from the quarantine units of the shelters were used for this study, because they were newly brought-in with no treatment against any ectoparasites or diseases by the animal shelter prior to sample collection. Therefore, the dogs screened in the study were stray dogs for a range of 3 to 4 weeks before initiation of the study.

DNA extraction and PCR amplification

DNA was extracted from whole blood (200 µl) following the-QIAamp blood and tissue kit protocol (QIAGEN GmbH, Hilden, Germany) and stored at -20^{0}C for further use. Initially, all DNA samples were screened by PCR amplification for the presence of pathogens' DNA at genus level using genus-specific primers (Table 1). Positive samples were then further screened using species-specific primers (Table 1). PCR amplification was carried out in final volumes of 25 ml reaction mixtures containing 4µl of DNA template, 5µl of25X buffer without MgCl2, 1µl of10mM dNTP, 5µl of25X MgCl2, 1µl of 20pmol of each primer [18-22], 1.5U of Taq polymerase and 7.7µl of sterile distilled water. PCR was carried out in a thermal cycler (BioRad, MyCycler^R USA) with the cycling conditions indicated in Table 2. The amplified products were electrophoresed on a 1.5% - 2% agarose gel (depending on the size of the PCR product) Positive controls were obtained from our previous work confirmed via sequencing. After electrophoresis the gels were stained with ethidium bromide and visualized under a UV transilluminator (BioRadAlpaImager^R, USA).

Serological analysis

The SNAP® 4DX® PLUS in-vitro serological diagnostic test was employed for the detection of antibodies to *Anaplasma phagocytophillum, A. platys, Ehrlichia canis* and *E. ewingii* inserum samples. All test procedures and interpretation of results were conducted according to the manufacturer's specifications contained in the user manual's guide (IDEXX Laboratories, Inc.).

NB: As indicated by the manufacturer, the test procedure cannot differentiate between the results of *A. phagocytophillum* and *A. platys.* Which means a positive result simply indicates the presence of antibodies to *A. phagocytophilum and / or A. platys. The* same applies for *E. canis and/ or E. ewingii.* Serological detection of *Babesia* and *Hepatozoon* spp. was not conducted in this study.

Sequencing

Amplicons obtained from the PCR reactions were extracted using the Wizard® SV Gel and PCR Clean-Up System purification kit (Promega, USA) for direct sequence analysis using ABI prism™ Bigdye™ terminator cycle sequencing Ready reaction kit V.3.1. All sequences were aligned manually using the ClustalWprogram ww.ebi.ac.uk/clustalw). Sequences obtained were compared with thoseavailable in GenBank using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences version 20.0 (SPSS Inc., Chicago, IL). Relation between categorical outcomes, i.e. absence or

Table 1: Primer sets used for PCR amplification.

Target sequence	Primer sequence (5' - 3')	Length (bp)	References
Anaplasmataceae 16S rRNA	fD1 5'AGAGTTTGATCCTGGCTCAG 3' Rp2 5' ACGGCTACCTTGTTACGACTT 3'	350bp	[22]
Ehrlichiacanis 16S rRNA	CANIS 5' CAATTATTTATAGCCTCTGGCTATAGGA 3' GA1UR 5' GAGTTTGCCGGGACTTCTTCT 3'	409bp	[22]
Anaplasma platys 16S rRNA	PlatysF 5' AAGTCGAACGGATTTTTGTC 3' PlatysR 5' CTTTAACTTACCGAACC 3'	500bp	[23]
Babesiadae 18S rRNA	BT-F1 5' GGTTGATCCTGCCAGTAGT 3' BT-R1 5' GCCTGCTGCCTTCCTTA 3'	350bp	[24]
Babesiavogel 18S rRNA	C172F 5' GTTTATTAGTTTGAAACCCGC 3' C626R 5' GAACTCGAAAAAGCCAAACGA 3'	450bp	[25]
Babesia gibsoni 18S rRNA	Gib599F5' CTCGGCTACTTGCCTTGTC 3' Gib1270R5' GCCGAAACTGAAATAACGGC 3'	690bp	[25]
Hepatozooncanis 18S rRNA	HEPF 5' ATACATGAGCAAAATCTCAAC 3' HEPR 5' CTTATTATTCCATGCTGCAG 3'	696bp	[26]

Table 2: Thermal cycling parameters for each pathogen An initial denaturation was set at 95^{0}C for 5min, the denaturation cycle was set at 95^{0}C for 30sec, a final extension at 72^{0}C for 5min and holding at 10^{0}C were set for all pathogens.

Pathogen	Annealing T0C	Extension T0C	Number of cycles
Anaplasmataceae	62 for 30sec	72 for 1min30sec	40 cycles
A. platys	64.5 for 30sec	72 for 1min	40 cycles
Babesia genus	64 for 30sec	72 for 1min	40 cycles
B. vogeli	57.5 for 30sec	72 for 1min	40 cycles
B. gibsoni	62 for 30sec	72 for 1min	40 cycles
E. canis	60 for 30sec	72 for 1min30sec	40 cycles
H. canis	60 for 30sec	72 for 1min30sec	40 cycles

presence of specific infection, gender and age were compared using the chi-square test and the Fisher's exact test was employed for age and breed.Differences between sampling locations and parasites prevalence rates were analysed at 95% confidence interval. Statistical significancewas set at $p \leq 0.05$.

RESULTS

The results of this investigation showed that 86.5% of the stray dog population sampled were infected with at least one of the four hemopathogens identified, with the genus *Babesia* predominating (65.4%), followed by *E. canis* (47.1% for PCR and 56.7% for serology) and *Anaplasma platys* (38.5% for PCR and 31.7% for serology); while *Hepatozooncanis* recorded the least prevalence of 28.8% (Table 3). However, demographic distribution of the individual pathogens among stray dogs between the two states (Sarawak and Sabah) showed that *Anaplasma platys* predominated (46.3%) in Sabah, while *Babesia vogeli* predominated in Sabah (92.9%). Significant ($p < 0.05$) differences were recorded for *B. canis, B.gibsoni* and *E. canis* from dogs between Sarawak and Sabah (Table 4).

Sex, age and breed-wise prevalence of the hemopathogens among stray dogs in the area using PCR showed that male and adult dogs have a significantly ($p < 0.05$) higher *E. canis* infection rate compared to females and younger dogs respectively. The same result was recorded using serology in adult dogs than young dogs for *Ehrlichia canis;* in addition, *Anaplasma* spp. infection appeared to be higher ($p < 0.05$) among adults than young dogs. No significant differences ($p > 0.05$) were found among sex, age and breed for the hemopathogens using both PCR and serology in the area (Table 5).

Co-infection status among stray dogs for the various hemopathogens in the study area showed that 44.2% were harbouring two hemopathogens concurrently. Further analysis to investigate which of the two hemopathogens concurrently infects the dogs revealed that *Babesia* spp. and *E. canis* were most often seen to co-infect (Table 6).

DISCUSSION

To the best of our knowledge, this study was the first to report the prevalence of tick-borne hemopathogens of dogs in East Malaysia, using both molecular and serological techniques for detection.

The current study was strictly conducted on rescued stray dogs from various animal shelters and dog pounds within the study area and the results of this investigation cannot be applied to the general dog population in the area, however the high prevalence rates of the various hemopathogens is of concern as the stray dogs are a constant reservoirs of infection. This is worrying especially as East Malaysia is less developed compared to West Malaysia and veterinary care is not readily available for dog owners. The reason for the high *Babesia* spp. and *E. canis* prevalence rates compared to other hemopathogens and between the two states could not be fully ascertained.

In contrast to the high prevalence among dogs infected with two hemopathogens simultaneously, only 5.8% of the total population was infected with all four hemopathogens. This finding might be due to the vectoral capacity of a common tick-vector (like *Rhipicephalus sanguineus* and *Haemaphysallis* spp.) in transmitting all the four hemopathogens [23,24]. At

Table 3: Prevalence of tick-borne hemopathogens identified from stray dogs in East Malaysia.

Pathogen	PCR		Serology	
	Frequency (n = 104)	Prevalence (%)	Frequency (n = 104)	Prevalence (%)
A. platys	40	38.5	33	31.7
E. canis	49	47.1	59	56.7
Babesia spp.	68	65.4	_	_
H. canis	30	28.8	_	

Table 4: Demographic/Prevalence and distribution of tick-borne hemopathogens in dogs identified from Sabah and Sarawak, Malaysia.

Pathogen	PCR			Serology		
	Frequency Sabah/ Sarawak (n = 54 / 50)	Prevalence (%)	p-value	Frequency Sabah/ Sarawak	Prevalence (%)	p-value
A. platys	25 / 15	46.3[a] / 30.0[a]	0.088	16 / 17	29.6 / 34.0	0.394
E. canis	15 / 34	27.8[a] / 68.0[b]	0.001	32 / 37	59.3 / 54.0	0.366
Babesia spp.	26/ 42	48.1[a] / 84.0[b]	0.001	_	_	_
H. canis	20 / 10	37.0[a] / 20.0[a]	0.055	_	_	_

NB: Row values with different superscript between comparative groups (Sabah / Sarawak) are statistically significant (p≤0.05). n = 54 for Sabah and n = 50 for Sarawak.

Table 5: Age, sex and breed-wise prevalence of the various hemopathogens in stray dogs in East Malaysia.

Factor Category	Frequency (n = 104)	PCR / Serological Prevalence (%)		
		Anaplasma spp	*Ehrlichiaspp.H.*	*canisBabesia spp.*
Sex:				
Male	45	16(35.6)/17(37.8)	28(62.2)[a]/27(60.0)	13(28.9) / - 26(57.8) / -
Female	59	24(40.7)/16(27.1)	21(35.6)[b]/32(54.2)	17(28.8) / - 42(71.2) / -
Age:				
Young	20	9(45)/2(10)[a]	1(5)[a]/3(15)[a]	8(40) / - 12(60) / -
Adult	84	31(36.9)/31(36.9)[b]	48(57.1)[a]/56(66.7)[b]	22(26.2) / - 56(66.7) / -
Breed:				
Local	87	33(37.9)/30(34.5)	48(55.2)/50(57.5)	22(25.3) / - 57(65.5)/ -
Pedigree	17	7(41.2)/3(17.6)	1(5.9)/9(52.9)	8(47.1) / - 11(64.7) / -

NB: Row values with different superscript between comparative groups (eg. Male and female) are statistically significant (p≤0.05) for each of the diagnostic tests.

Table 6: Mixed infections among dogs in East Malaysia.

Pathogen	Frequency (n = 104)	Prevalence (%)
No infection	14	13.5
Single infection	22	21.2
Co-infections	46	44.2
Mixed infection	16	15.4
(3 pathogens)		
Mixed infection	6	5.8
(4 pathogens)		

this point in time, no conclusions can be made concerning the relationship between *Babesia* and *Ehrlichia* spp for being the two most commonly related hemopathogens in terms of co-infection. However, the results of this study serve as an important reminder for veterinarians to be aware of the possibility of mixed infections.

As this study was the first of its kind to investigate canine tick-borne hemopathogens in Sabah and Sarawak, the need to further investigate and characterize the different strains of these hemopathogens for a better understanding of their epidemiology is paramount. At the same time it necessitates a conscientious effort to further investigate other pathogens of possible zoonotic significance [25-27] in order to provide adequate information on the zoo epidemiology or epizootiology of these hemopathogens.

CONCLUSION

In conclusion, this study confirmed for the first time, the presence of tick-borne hemopathogens (*Anaplasma platys, Ehrlichia canis, Babesia vogeli, Babesia gibsoni* and *Hepatozoon canis*) in Sarawak and Sabah States of East Malaysia. *Babesia* spp. predominated as the most common hemopathogen in both the two States of East Malaysia. Age and sex affects the prevalence of *Ehrlichia canis* and *Anaplasma platys.* The presence of mixed infection was also established.

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