EURASIAN
JOURNAL OF VETERINARY SCIENCES
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RESEARCH ARTICLE

PCR assay and microscopy for examination of mixed *Ehrlichia canis* and *Babesia* spp. infection in Bomb-sniffing dogs and other canines in National Capital Region, Philippines

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Özet

Baticados AM, Baticados WN, Villarba LA, Carlos ET, Carlos SMEAS, Fajardo PW. Filipinler'de Ulusal Başkent Bölgesi'ndeki Bomb Sniffing cinsi ve diğer köpeklerde *Ehrlichia canis ve Babesia spp.* mix enfeksiyonunun PZR ve mikroskobik muayene ile incelenmesi. **Eurasian J Vet Sci, 2011, 27, 2, 111-116.**

Amaç: Filipinler'de köpeklerdeki *Babesia* ve *Ehrlichia* türlerinin moleküler identifikasyonu ile ilgili yayınlanmış detaylı bilgi henüz bulunmamaktadır. Bu nedenle Ulusal Başkent Bölgesi (Metropolitan Manila)'nde seçilen belediye köpeklerindeki miks *Ehrlichia canis* ve *Babesia* spp. enfeksiyonunun PZR kullanılarak belirlenmesine çalışıldı. Ayrıca farklı belediyelerde melez ve saf kan köpek gruplarının yanı sıra, yaş ve cinsiyet gruplarındaki etkenleri de tespit etmek amaçlandı.

Gereç ve Yöntem: Metropolitan Manila'dan seçilen belediyelerdeki toplam 168 adet köpek kan örneği kan parazit muayenesi (BPE) ve polimeraz zincir reaksiyon(PZR) yöntemleri kullanılarak *E. canis* ve *Babesia* spp. enfeksiyonları yönünden incelendi.

Bulgular: BPE ve PZR yöntemlerine tabi tutulan bütün kan örnekleri *E. canis* ve *Babesia* spp. yönünden negatif bulundu. Veriler, farklı parametreler açısından (klinik belirti), dişilerde (% 60.11), saf kan köpeklerde (%99.10) ve 0-2 yaş gruplarında (%32.94) olarak belirlendi.

Öneri: Negatif sonuçlar, etkenin yokluğunu göstermemektedir. Etkenlerin vücudun diğer organlarında lokalize olması, enfeksiyonun kronik veya latent dönemde bulunması, örnek toplama zamanı, devam eden veya geçmişte uygulanan tedavi, diğer hastalıkların varlığı gibi durumlarda yanlış negatif sonuçlar ortaya çıkabilir.

Abstract

Baticados AM, Baticados WN, Villarba LA, Carlos ET, Carlos SMEAS, Fajardo PW. PCR assay and microscopy for examination of mixed *Ehrlichia canis and Babesia* spp. infection in Bomb-sniffing dogs and other canines in National Capital Region, Philippines. **Eurasian J Vet Sci, 2011, 27, 2, 111-116.**

Aim: There are yet no published records detailing molecular identification of *Babesia* and *Ehrlichia* species in dogs in the Philippines. The detection of mixed *Ehrlichia canis* and *Babesia* spp. infection in canines of selected municipalities of the National Capital Region (Metropolitan Manila) using PCR was therefore endeavored. In addition, the research further intended to detect the individual organisms in different municipalities, age and sex categories as well as between groups of mixed and pure breed dogs.

Materials and Methods: A total of 168 canine blood samples from selected municipalities of Metropolitan Manila were examined for *E. canis* and *Babesia* spp. infection using blood parasite examination (BPE) and polymerase chain reaction (PCR) assay methods.

Results: All the blood samples subjected to BPE and PCR assay were found negative for *E. canis* and *Babesia* spp. The data showed that in terms of the different parameters there were more females (60.11%); purebred (88.10%) and dogs aged <1-2 years (32.94%).

Conclusion: Negative results do not automatically dictate the absence of the pathogens. This is because false-negative results may occur in the following conditions; during subdetectable quantities of pathogens in the blood due to localization of the pathogen in other organs of the body, stages of chronic and asymptomatic carriers of infection; timing of collection; ongoing or past treatments administered and presence of other diseases.

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Received: 18.12.2010, Accepted: 14.01.2011
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Anahtar kelimeler: PZR, mikroskop, *Ehrlichia canis, Babesia* spp., Filipinler
Keywords: PCR, microscopy, *Ehrlichia canis, Babesia* spp., Philippines

► Introduction

Ehrlichia canis and *Babesia* spp. are important tick-borne pathogens transmitted by the brown dog tick *Rhipicephalus sanguineus.* Both organisms are distributed worldwide, particularly in tropical and subtropical regions (Iqbal et al 1994, Mc Bride et al 1996, Wen et al 1997). *E. canis* causes canine monocytic ehrlichiosis (CME) (Helestine et al 2003) while *Babesia* spp. causes canine babesiosis (Sobczyk et al 2005).

The most common clinical manifestations of canine babesiosis are fever, hemoglubinuria, hemolytic anemia, which occasionally results to death (Ulutas et al 2005) and jaundice (Homer et al 2000). Clinical features of CME on the other hand, include non-regenerative anemia, pronounced hyperglobulinemia, thrombocytopenia and thrombocytopathy (Blagburn 2006). Both infections are fatal to dogs. It has been reported that *E. canis* and *Babesia* spp. can exist as concurrent infections in dogs (Kordick et al 1999, Suksawat et al 2001, Harikrishnan et al 2005). These simultaneous infections can bring about potentiation of the manifested infection (Kordick et al 1999) which may lead to a more serious condition in canines, thus early detection of organisms is essential.

Diagnosis of both infections can be confirmed by direct examination of peripheral blood smears; cell culture reisolation; detection of specific antibodies using enzyme-linked immunosorbent assay (ELISA) and immuno-fluorescent antibody (IFA) testing; and amplification of specific genes from the organism by polymerase chain reaction (PCR) (Iqbal et al 1994).

To date, the latest published report on clinical observation of canine *Babesia* species (i.e. *B. canis*) in the Philippines was made more than three decades ago (Carlos et al 1972). Likewise, in the case of the rickettsial organism (*Ehrlichia* spp.), the only published report in the Philippines dealt with the hematology and cytopathology of the bacteria in canines (Morales and Baticados 2007). In addition, there are yet no published records detailing molecular detection of *Babesia* and *Ehrlichia* species in dogs in the Philippines. The study therefore aimed to identify mixed *Ehrlichia canis* and *Babesia* spp. infection in canines of selected municipalities of Metro Manila using PCR and BPE. The research further intended to categorize each individual parasite's profile according to different localities, age and sex categories as well as between groups of mixed and purebred dogs.

A total of 168 canine blood samples from selected municipalities of the National Capital Region (NCR) also known as Metropolitan Manila, were found negative for *E. canis* and *Babesia* spp. using BPE and PCR assay methods. Majority of the samples (40.48%) came from Makati City. In addition, the data showed that in terms of the different parameters (signalment), there were more females (60.11%); majority were purebred dogs (88.10%) and mostly animals aged <1-2 yrs old (32.94%). However, negative results do not automatically dictate the absence of the pathogens. This is because false-negative results may occur in the following conditions; during sub-detectable quantities of pathogens in the blood due to localization of the pathogen in other organs of the body, stages of chronic and asymptomatic carriers of infection (Iqbal et al 1994, Skotarczak 2003, Goodfellow and Shaw 2005); timing of collection (Viljoen et al 2005) and ongoing or past treatments administered (Morgan 1997).

To our knowledge, there are no published records detailing molecular identification of *Babesia* and *Ehrlichia* species in dogs in the Philippines. The aim of this research was to detect *Ehrlichia canis* and *Babesia* spp. infection in canines from selected municipalities of the National Capital Region (Metropolitan Manila) using BPE and PCR.

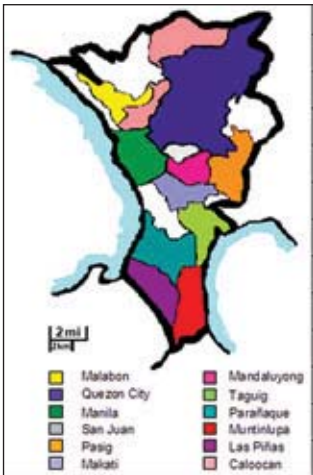
► Materials and Methods

- *Dogs*

A total of 168 dogs from randomly selected municipalities of Metropolitan Manila (Metro Manila) were used in the study. Samples came from different municipalities including Makati, Quezon City, Muntinlupa, Parañaque, Manila, Mandaluyong, Caloocan, San Juan, Taguig, Pasig, Malabon and Las Piñas (Figure 1). Dogs sampled either had a history of tick exposure or observed presence of the ticks during collection. Moreover, the animals were categorized according to sex (male or female); breed (purebred or mixed breed) and age groups (<1–2 yrs; 2.1–4 yrs; 4.1–6 yrs; 6.1–8 yrs and above 8 yrs) (Figure 2B). Samples were gathered during the summer months (March to May) wherein ticks were most prevalent in the country.

- *Blood collection*

Canine whole blood samples (3 to 5 mL) were collected from the cephalic vein. A portion of the blood was



Locality	N	%
Malabon	1	0.6
Quezon City	21	12.5
Manila	39	23.21
San Juan	5	2.98
Pasig	3	1.79
Makati	68	40.48
Mandaluyong	6	3.57
Taguig	4	2.38
Parañaque	7	4.17
Muntinlupa	8	4.76
Las Piñas	1	0.6
Caloocan	5	2.98

A B

Figure 1. Map of sampling sites (A). (B) Total number and percentage distribution of samples per locality are shown.

Primers	Sequence 5' to 3'	Reference
Ehrlichia EHR-OUT1	(5'-CTG-GCA-AGC-TTA-ACA-CATGCC-AAC-ATC-TCA-CGA-C-3')*	Breitschwerdt *et al.*, 1998; Dawson *et al.*, 1992
HER-OUT2	(5'-GCT-CGT-TGC-GGG-ACT-TAACCC-AAC-ATC-TCA-CGA-C-3')*	
HE3-R	(5'-CTT-CTA-TAG-GTA-CCG-TCA-TTA-TCT-TCC-CTAPT)*	
E.canis	(5'-CAA-TTA-TTT-ATA-GCC-TCT-GGC-TAT-AGG-AA)*	
Babesia Babesia-F	(5'-GTGAAACTGCGAATGGCTCA-3')*	Oyamada *et al.*, 2005; Inokuma *et al.*, 2003
Babesia-R	(5'-CCATGCTGAAGTATTCAAGAC-3')*	

A

Parameters	N	%
Sex		
Male	67	39.88
Female	101	60.11
Breed		
Mixed	20	11.90
Purebreed	148	88.10
Age		
<1 - 2 yrs	56	33.33
2.1 - 4 yrs	31	18.45
4.1 - 6 yrs	43	25.60
6.1 - 8 yrs	7	4.17
Above 8 yrs	31	18.45

B

Figure 2. Primers for the amplification of Ehrlichia canis and Babesia spp. The primers were manufactured by Eurogentec AIT, Singapore (A). Signalment of the randomly sampled canines. The number and percentage distribution of canines for each parameter are displayed.

used for blood smear examination and the remaining blood samples were stored in ethylenediaminetetraacetic acid (EDTA)-coated vacutainers and freezed at -40 ^{0}C to be later processed for DNA isolation and PCR.

- *Blood smear preparation*

An aliquot of blood (5-10 µL) was used to prepare the blood smear stained with Giemsa® (Gurr, BDH Chemicals Ltd., England) for blood parasite examination. Peripheral blood smears were stained for 30 minutes, washed and then air dried in a vertical position. Afterwards, each smear was systematically viewed in an effort to pinpoint morulae of *Ehrlichia canis* and trophozoites of *Babesia spp.* Both high power objective (HPO) and oil immersion objective (OIO) were used to examine the whole slide. Suspected parasites were photographed by Canon Digital IXUS I 5.0 megapixels (Canon, USA) and were re-examined for confirmation.

- *DNA extraction*

A total of 100 µL of blood sample was pipetted off and transferred to a 1.5 mL microcentrifuge tube. Nine volumes of extraction buffer and 10 µL of proteinase K (100 µg/mL) was added to the blood sample then incubated at 55 ^{0}C for 12 hrs. Phenol-chloroform-isoamyl alcohol (PCI) at pH 8.0 was mixed with the incubated samples and centrifuged at 14000 rpm at room temperature for 10 min. Aqueous layer was transferred to a new 1.5 mL microcentrifuge tube and the same amount of chloroform was added and the mixture centrifuged as performed previously. The aqueous layer was transferred to another tube and mixed with 1 mL of 99.5% ethanol and 10-15 µL of 3M NaCOOH and centrifuged at 14000 rpm for 10 min. Centrifuged solution was decanted and 1 mL of 70% ethanol was added and centrifuged again at 14000 rpm for 10min. The extracted pellet was air-dried at room temperature. Fifty microliters of Tris-EDTA buffer was mixed with the pellet. Tubes were stored at -40 ^{0}C until further processing (Sambrook and Russell 2001, Baticados 2005, 2010).

- *PCR amplification*

Ehrlichia canis: Amplification was done with a 50 µL reaction mixture with 1 µL of DNA template, 200 µM of dNTP mixture; 0.1 pmol of EHR-OUT1 and EHR-OUT2 primers (Figure 2A); 25 pmoles of HE3-R and *E. canis* primers; and 2.5 U of *Taq* DNA polymerase in a 10x reaction buffer. First round of amplification was repeated 20 times and included denaturation at 94 ^{0}C for 45 seconds (s) and annealing and chain extension at 72 ^{0}C for 1.5 min. Second round of amplification included denaturation at 94 ^{0}C for 45 s, annealing at 55 ^{0}C for 45 s and chain extension at 72 ^{0}C for 1 min. This cycle was repeated for 50 times and was followed by a final chain extension of 72 ^{0}C for 5 min. Amplification was done using Thermal Cycler (Touchgene Gradient, TECHNE, Cambridge, UK) (Dawson et al 1992, Breitschwerdt 1998).

Babesia spp.: The PCR primers (Figure 2A) used were based on previous reports (Inokuma et al 2003, Oyomada et al 2005). Polymerase chain reaction amplification commenced with a 25 µL reaction mixture containing; 1 µL of each DNA template, 200 µM dNTP mixture, 20 pmoles of each primers (Table 2), distilled water and 1.5 U of Taq DNA polymerase in a 10x PCR reaction buffer. Amplification was carried out with the following conditions: initial denaturation at 94 ^{0}C for 5 min; 40 repeated cycles of denaturation at 94 ^{0}C for 30 s, annealing at 45 ^{0}C for 30 s and chain extension at 72 ^{0}C for 90 s. A final extension at 72 ^{0}C for 5 min completed the last step (Inokuma et al 2000). Autoclaved distilled water was used as negative control.

- *Electrophoresis*

Amplicons were electrophoresed through 1.8% agarose gel in an electrophoresis chamber (BIO-RAD, USA) and subsequently stained with ethidium bromide. Observed results were documented with Digi-Doc-It Imaging System (UVP, USA). Negative control and a molecular weight marker served as indicators of the product size and were used in the proper identification and estimation of PCR bands.

► Results

Parasitological examination using high power and oil immersion objectives showed that morulae of *Ehrlichia canis* and *Babesia* spp. tropozoite forms were not detected in 168 canine blood samples, despite the presence and/or history of tick infestation of the entire sampled population of canines (Figure 1A, 1B). In addition, no *E. canis* and *Babesia* spp. amplicons were obtained after performing polymerase chain reaction assay using the reported primer-pairs (Figure 2A) for each organism under study. The highest numbers of blood samples obtained per category were as follows; females (60.11%), purebred dogs (88.10%), Makati

City (40.48%) and age group ranging from <1-2 yrs (32.94%) (Figure 2B).

► Discussion

Blood smear microscopy revealed that the processed blood smears were negative for *Babesia* spp. and *E. canis* parasites. The works of Alleman and Couto (2006) stated that the parasitemia of *Babesia* spp. in most dogs ranges from 2-6% of the infected red blood cells. Conversely, in the study of Water et al (1999) as cited by Pantoja (2006), only a small percentage (4%) was found positive for *E. canis.* Moreover, Iqbal et al (1994) also stated that direct examination and identification of organisms in the peripheral blood smear is not a reliable method because only a few cells are infected and that they are not usually detected. Therefore, factors that may contribute to the difficulty of diagnosing blood parasites through direct microscopical examination are low parasitemia levels and the stage of infection. The trophozoites of *B. canis* and morulae of *E. canis* are easy to find in acutely infected animals but are rarely evident in the chronic and subclinical stages of infection (Pantoja 2006).

Results of polymerase chain reaction assays were likewise negative for both *E. canis* and *Babesia* spp. genomic DNA. According to Viljoen et al (2005) and Wardrop et al (2005), negative results may either be due to absence of the pathogen in the sample at the time of collection or presence of concentrations of DNA samples in sub-detectable quantities. Thus, false-negative results may arise from low numbers of circulating organisms and these are usually seen in chronically infected carriers and in animals that had undergone antimicrobial therapy (Alleman and Couto 2006). In a study cited by Birkenheuer et al (2003), false negative PCR tests were obtained in 30% of the samples from *Babesia* spp. chronically infected cattle due to low percent of parasitemia. In addition, De Barros Macieira et al (2005) specified that *E. canis* negative results in PCR assays of peripheral blood may actually be PCR positive in splenic tissue samples. This suggests that spleen is probably the last organ to harbor *E. canis* parasites during recovery. Furthermore, false-negative results may occur in cases of platelet congestion disorders due to vaccines and drugs like quinidine, acetaminophen, trimethoprim-sulfamethoxazole, gold compounds and antibiotics (penicillin) (Morgan 1997).

The study was hampered by several limitations namely; time and financial constraints, as well as client compliance for repeated blood sample collection. Since generally the stage of *Ehrlichia* and *Babesia* parasite infection could greatly affect successful identification of the organism in both molecular and parasitiological tests, repeated or serial blood collections are strongly recommended. Moreover, the use of various primers for the amplification of both *Ehrlichia canis* and *Babesia* spp. may likewise improve the sensitivity of parasite detection in field samples.

► Conclusions

Localization of the pathogen in other organs of the body, existence of chronic stage and/or asymptomatic carriers of infection, timing of collection and given treatments may cause sub-detectable quantities of pathogens in the blood. Hence, PCR studies may give false-negative results.

► Acknowledgement

The authors would like to express their sincere appreciation to Ms. Julie Hufano and all the Makati Dog and Cat Hospital (MDCH) staffs as well as Dr. Sherlyn G. Subiaga and Dr. Jomarte M. Magcalas for their assistance during the survey and blood collection of samples.

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