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<Original Article>

Detection and molecular characterization of *Hepatozoon canis, Babesia vogeli*, *Ehrlichia canis*, and *Anaplasma platys* in dogs from Metro Manila, Philippines

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Abstract: The study of canine vector-borne diseases in the Philippines started in the 1970s but only gained interest in the past decade. Characterization of such diseases in the Philippines remains incomplete, thus, it is necessary to obtain additional information on the prevalence and diversity of canine tick-borne diseases in the country. In this study, blood samples were obtained at two veterinary clinics in Metro Manila, Philippines from 114 dogs suspected of having canine tick-borne pathogens. Polymerase chain reaction (PCR) was performed on whole blood DNA extracts followed by sequencing, and the following pathogens were detected: *Hepatozoon* (*H.*) *canis* (5.26%), *Babesia* (*B.*) *vogeli* (5.26%), *Ehrlichia* (*E.*) *canis* (4.39%), and *Anaplasma platys* (3.51%). Additionally, a set of multiplex PCR primers were developed to detect *H. canis, Babesia* spp. (*B. canis* and *B. vogeli*), and *E. canis* in canine blood. Multiplex and conventional single-reaction PCR results for the 114 dog blood samples were similar, except for one *H. canis* sample. Multiplex PCR is, therefore, a useful tool in screening infected dogs in veterinary clinics. This study's results, together with those of previous studies in the country, show that canine vector-borne pathogens are an emerging veterinary concern in the Philippines.

Keywords: Philippines, canine vector-borne diseases, dogs, molecular detection, multiplex polymerase chain reaction

Introduction

Canine vector-borne diseases (CVBDs) caused by the protozoan parasites *Hepatozoon* spp. and *Babesia* spp. as well as members of the prokaryotic group Anaplasmataceae (*e.g.*, *Ehrlichia* spp. and *Anaplasma* spp.) have been the subject of studies over the years due to their impact to both companion and working dogs [25]. Once canine vector-borne (CVB) infections manifest in canine blood cells, subclinical (*e.g.*, lethargy, depression) to potentially fatal (*e.g.*, multiple organ failure) symptoms can occur. The causative agents of CVBDs are transferred via tick vectors, hence, the travel and settlement of domestic dogs to areas where these vectors and pathogens are endemic (*e.g.* near wilderness areas) have contributed to their spread [25, 29]. In the Philippines, there were already reports of CVBDs in humans and dogs in the 1970s. The presence of *Hepatozoon* sp. in a human patient [7] and in canines [24] as well as *Babesia* sp. in canines [6] has been reported. However, follow-up studies were only available decades later with the advent of molecular methods leaving a large gap on surveillance of CVB infections for roughly three decades. These recent studies used polymerase chain reaction (PCR) detection to identify *Hepatozoon* (*H.*) *canis*, *Anaplasma* (*A.*) *platys*, *Ehrlichia* (*E.*) *canis*, and *Babesia* spp. in dogs [2, 8, 36, 37, 39] and in brown ticks (*Rhipicephalus* [*R.*] *sanguineus*) isolated from dogs [38]. While it is difficult to determine whether CVBDs were already endemic to the Philippines or brought by infected ticks or dogs from other countries, these studies proved that they are indeed present in domestic dogs and ticks, and that there is a need for additional prevalence data on CVBDs in the country.

This study aimed to survey the presence of *Babesia* spp., *Hepatozoon* spp., and Anaplasmataceae infecting domestic dogs in Metro Manila, Philippines and develop a multiplex PCR protocol that can be used to detect the presence of *H. canis, Babesia* spp. (*Babesia* [*B.*] *canis* and *B. vogeli*), and *E. canis* in dogs. The current conventional method of detection of CVBDs is blood smear examination (with Giemsa staining) together with observation of clinical signs [1, 15]. However, this method poses several disadvantages. First, microscopy

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is both labor-intensive and time-consuming. It also requires trained microscopists since infected cells can be confused with other blood cells such as platelets and white blood cells with phagocytosed materials [23]. Second, several clinical signs – such as fever, anorexia, depression, thrombocytopenia, and anemia – are common to CVB infections, and may vary among hosts [32]. Finally, concurrent infections – which are not easily detected in microscopy – are known to occur as some of these pathogens have the same tick vector (*e.g. E. canis, H. canis,* and *B. vogeli* are all carried by *R. sanguineus*) [29]. If these difficulties are ignored, misdiagnosis of the disease can occur. Thus, multiplex PCR is an affordable, accurate, and rapid method of detection that can be used for proper detection of CVBDs. Ultimately, investigation of prevalence and efficient screening of the pathogens are important first steps in controlling the spread of CVBDs, most especially in the Philippines where inter-island transmission can lead to infection of local populations lacking immunity against CVBDs.

Materials and Methods

Sample collection

The research protocol used in this study was reviewed and approved by the Institutional Animal Care and Use Committee of the University of the Philippines Los Baños under assigned protocol no. 2012-35. A total of 114 canine blood samples were obtained from the Makati Dog and Cat Hospital in Makati City, Philippines and the Carlos Veterinary Clinic in Parañaque City, Philippines from 2013-2014. All of these dogs were suspected to have CVBDs due to presence of signs of infection such as high fever (> 39ºC), low platelet count (< 200,000 platelets/mm^3), low white blood cell count (< 6,000 white blood cells/mm^3), presence of clinical signs (*e.g.*, lethargy), presence of ticks, and positive serology results for *Ehrlichia* sp. DNA was extracted using DNAzol BD reagent (Life Technologies; USA) following the manufacturer's protocol, and extracts were stored at −4ºC until PCR amplification.

Molecular detection of *H. canis*, *Babesia* spp., and Anaplasmataceae

Conventional PCR and DNA sequencing using primers that are specific for target CVB pathogens were done. *H. canis* infections were identified by amplification and sequencing of 660 bp PCR products using Hep-F/Hep-R [13]. *Babesia* spp. (*B. canis, B. vogeli,* and *B. rossi*) infections were identified by amplification and sequencing of PCR products using the primers PIRO-A1/PIRO-B, which produce a 450 bp PCR product [9]. Anaplasmataceae were detected and identified by amplification and sequencing of the 345 bp products of the primers EHR16SD/EHR16SR [12, 26]. Further identification of *Anaplasma* spp. was conducted by sequencing larger parts of the16S rRNA gene using the primer pairs fD1/EHR16SR and EHR16SD/Rp2 [12]. PCR amplification was performed using previously published protocols for Hep-F/Hep-R [16], PIRO-A1/PIRO-B [5], and EHR16SD/EHR16SR [34]. Full 16S rRNA gene amplification of *Anaplasma* spp. was performed according to the protocol of Inokuma *et al*. [12]. The primers Hep-F/Hep-R and PIRO-A1/PIRO-B target the18S rRNA genes of their respective CVB pathogens while primers EHR16SD, EHR16SR, fD1, and Rp2 amplify the 16S rRNA gene of Anaplasmataceae. PCR was performed thrice on positive samples and sent to the Philippine Genome Center for sequencing to rule out false positives. Sequences were aligned using the Clustal W feature of BioEdit 7.2.0 [11]. Sequences were then uploaded onto the nucleotide BLAST website (National Center for Biotechnology Information, USA) to determine the most similar sequences and confirm their species identity.

Phylogenetic analysis

Phylogenetic trees were constructed to confirm species identification of the samples. Neighbor joining (NJ) and maximum parsimony (MP) phylogenetic trees were constructed using either PAUP* 4.01b [31] or MEGA 6 software [33]. Maximum likelihood (ML) trees were constructed by uploading the aligned sequences to the PHYML website [10].

The phylogenetic tree of partial *H. canis* 18S rRNA gene was constructed with sequences from *H. canis* (GenBank accession nos. EU289222 and KJ634654; National Center for Biotechnology Information), *H. americanum* (GenBank accession no. AF176836), *H. catesbianae* (GenBank accession nos. AF176837 and AF130361), *H. felis* (GenBank accession nos. KM435071, KC138533, and KC138534), and *Sarcocystis arctosi* (GenBank accession no. EF564590) as outgroup. The phylogenetic tree of *Babesia* spp. partial 18S rRNA gene was constructed with sequences from *B. canis* (GenBank accession nos. AY072926, AY270247, AY527063, AY649326, AY962186, AY962187, HQ662634, KC593877-KC593879, KF499115, KM111282, and KM111283), *B. rossi* (GenBank accession nos. AB303071-AB303075, AB935163-AB935166, DQ111760, JQ613104, JQ613105, and L19079), *B. vogeli* (GenBank accession nos. AB303076, AY072925, AY07771, DQ297390, HM590440, HQ148663, HQ148664, HQ662635, JN713120-JN713122, JX304677, JX304679-JX304683, JX112785, JX871891, KF970926, and KF970929), and *Babesia* sp. 'Spanish dog' (GenBank accession no. EU583387) with *Babesia* sp. 'Oklahoma dog' (GenBank accession no. AF20563) and *B. gibsoni* (GenBank accession no. KJ142323) as outgroups. The phylogenetic tree of *Anaplasma* sp. 16S rRNA gene was constructed with the sequences of *A. bovis* (GenBank accession nos. AB196475 and AB211163), *A. centrale* (GenBank accession nos. AB211164 and AF309869), *A. marginale* (GenBank accession nos. AF309867, AF309868, KU686788-KU686793, and NR074556), *A. odocoilei* (GenBank accession no. JX876644), *A. ovis* (GenBank accession no. AY262124), *A. phagocytophilum* (GenBank accession nos. AB196720, AB196721, DQ458805, and DQ458808), *A. platys* (GenBank accession nos. AF536828 and EU439943), *Anaplasma* sp.

(GenBank accession nos. KR261620-KR261622), and uncultured *Anaplasma* sp. (GenBank accession nos. JN862824 and JX402624) with *E. canis* (GenBank accession no. U26740) as an outgroup.

Multiplex primer set design

Several gene sequences of *E. canis, B. canis, B. vogeli, B. rossi,* and *H. canis* were obtained from the GenBank Nucleotide Database for multiplex primer design. These primers were designed from *E. canis* 16S rRNA gene sequences (GenBank accession nos. AB723707, AB723708, AB723709, AB723710, AB723711, AB723712, JX261981, and JX893522), *B. canis, B. rossi,* and *B. vogeli* 18S rRNA gene sequences (GenBank accession nos. *B. canis canis* EU622792, *B. canis canis* AY072926, *B. canis* JF461263, *B. canis rossi* JQ613104, *B. canis rossi* JQ613105, *B. canis canis* JX227980, *B. canis* JX678979, *B. canis vogeli* JX871889, and *B. canis vogeli* JX871891), and *H. canis* 18S rRNA gene sequences (GenBank accession nos. AF176835, AY461378, JX441117, JX466885, KC138531, and KC138540). Sets of sequences for each organism were aligned using the Clustal W feature of BioEdit 7.2.0 to determine regions specific for each of the target species. Gene sequences that had these conserved regions for each species were imported to PrimerPlex 2.6.2 (PREMIER Biosoft, USA). Multiplex primer sets specific for the gene sequences were then designed with primer pairs having melting temperatures within 55 to 65°C. The designed primers targeted conserved regions of *E. canis* 16S rRNA gene (Ehr16S_multiF and Ehr16S_multiR), *H. canis* 18S rRNA gene (Hep18S_multiF and Hep18S_multiF), and *Babesia* spp. (*B. canis* and *B. vogeli*) 18S rRNA gene (Bab18S_multiF and Bab18S_multiR) (Table 1). Amplicon sizes were restricted to 200 to 700 bp in size at 100 to 150 bp intervals. Default software specifications to avoid formation of secondary structures such as hairpin loops and primer dimers were used. Primer sequences were tested for specificity using both nucleotide BLAST (blastn) and primer BLAST features available at the National Center for Biotechnology Information (NCBI) website. The optimal multiplex primer set was then picked based on similarities in melting temperature, low tendency of secondary structure formation, and high specificity for target gene sequence.

Optimum annealing temperature and analytical sensitivity and specificity

Multiplex PCR was initially performed on positive control DNA extracts of *E. canis, B. canis,* and *H. canis* to obtain the optimum annealing conditions as well as analytical specificity and sensitivity of the designed primers. DNA extracts of positive controls were requested from the following universities: *E. canis* from the Koret School of Veterinary Medicine of the Hebrew University of Jerusalem (Israel), *B. canis* from the Department of Parasitology and Zoology of the Szent Istvan University (Godollo, Hungary), and *H. canis* from the Dipartimento di Medicina Veterinaria of the Universita degli Studi di Bari Aldo Moro (Bari, Italy). *A. platys* and *B. vogeli* DNA extracts were obtained during sampling of canine blood in this study. All multiplex PCR experiments were performed with KAPA2G Fast multiplex kit according to the manufacturer's protocol with 0.25 μM of each multiplex primer. The optimum annealing temperature was obtained using gradient PCR (58, 59, 60, 61, and 62°C in each) on DNA extracts of *E. canis, B. canis,* and *H. canis*. Single reaction PCR was performed on DNA extracts of *E. canis, B. canis, H. canis, B. vogeli* and *H. canis* mixture, and *A. platys* to determine possible cross-reactions of primers with other canine blood-borne pathogens. Multiplex PCR was also performed on DNA extracts of *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis,* and *Staphylococcus aureus* to determine if the primers would cross-react to any of these four common bacteria that may contaminate blood samples during blood or DNA extraction [3, 28]. Analytical sensitivity was determined by performing multiplex PCR on 1:10, 1:100, and 1:1,000 dilutions of available *E. canis* (24.4 ng/μL), *B. canis* (19.1 ng/ μL), and *H. canis* (45 ng/μL) DNA. Single or multiplex PCR mixes containing only the respective primers and no DNA template served as negative controls for all reactions.

Results

Symptoms of CVBD in dogs sampled

Of the 114 dogs sampled, 72 (63.16%) presented only one sign of infection, 37 (32.46%) presented a combination of any two signs of infection, while the five remaining dogs presented three signs of infection (4.39%). The most com-

Table 1. Multiplex primers designed using PrimerPlex 2.6.2 (PREMIER Biosoft) that target the 18S rRNA gene regions of *Hepatozoon canis* (Hep) and *Babesia* spp. (*B. canis* and *B. vogeli*; Bab) and the 16S rRNA gene region of *Ehrlichia canis* (Ehr)

Primer name	Sequence (5' to 3')	T_m (°C)	Target	Product size
Ehr16S_multiF	5'–GCCTAACACATGCAAGTCGAACGGACAATT-3'	65.3	*Ehrlichia canis*	664 bp
Ehr16S_multiR	5'–AACCAGATAGCCGCCTTCGCCACT-3'	64.8		
Bab18S_multiF	5'–ACCCAAACCCTCACCAGAGTAGCAATT–3'	63	*Babesia* spp. (*B. canis* and *B. vogeli*)	491 bp
Bab18S_multiR	5'–GACGACCTCCAATCACTAGTCGGCATAG–3'	63.4		
Hep18S_multiF	5'–AACGACTCCTTCAGCACCTTACGAGAA–3'	62.7	*Hepatozoon canis*	376 bp
Hep18S_multiR	5'–TCACAGACCTGTTATTGCCTCAAACTTCCT–3'	63.4		

Table 2. Prevalence of *Hepatozoon canis, Babesia vogeli, Ehrlichia canis,* and *Anaplasma platys* infections and the type and number of symptoms found in hosts

	Hepatozoon canis	*Babesia vogeli*	*Ehrlichia canis*	*Anaplasma platys*
Prevalence	6/114 (5.26%)	6/114 (5.26%)	6/114 (5.26%)	4/114 (3.51%)
Symptoms				
High fever (> 39ºC)	0/6	0/6	0/6	0/4
Low platelet count (< 200,000 platelets/mm^3)	1/6	4/6	4/6	4/4
Low white blood cell count (< 6,000 WBC/mm^3)	0/6	0/6	0/6	0/4
Presence of clinical signs (*e.g.*, lethargy)	0/6	0/6	0/6	0/4
Presence of ticks	2/6	0/6	0/6	0/4
Positive serology results for *Ehrlichia* sp.	4/6	6/6	6/6	1/4
Number of symptoms				
1 symptom	5/6	2/6	2/6	3/4
2 symptoms	1/6	4/6	4/6	1/4
3 symptoms	0/6	0/6	0/6	0/4

Table 3. DNA sequences of amplicons deposited in GenBank for primers Hep-F/Hep-R (*Hepatozoon canis*), PIRO-A1/PIRO-B (*Babesia vogeli*), EHR16SD/EHR16SR (Anaplasmataceae), and fD1/EHR16SR and EHR16SD/Rp2 (*Anaplasma platys*)

Sample	Percent identification	Percent coverage	GenBank accession number of most similar sequence	GenBank accession number
EEEE_HepF	99	99	*Hepatozoon canis* clone 7243 (KC138535)	KP182929
JJJ_HepF	99	100	*Hepatozoon canis* clone 7243 (KC138535)	KP182930
LLL_ HepF	100	100	*Hepatozoon canis* clone 7243 (KC138535)	KP182931
M_ HepF	99	100	*Hepatozoon canis* clone 7243 (KC138535)	KP182932
UUUU_ HepF	99	100	*Hepatozoon canis* clone 7243 (KC138535)	KP182933
VVVV_ HepF	99	100	*Hepatozoon canis* clone 7243 (KC138535)	KP182934
C_PIRO	100	100	*Babesia canis vogeli* SK-011 (JX112785)	KP182935
EEEE_PIRO	100	99	*Babesia canis vogeli* SK-011 (JX112785)	KP182936
FFFF_ PIRO	99	99	*Babesia canis vogeli* SK-011 (JX112785)	KP182937
GGGG_ PIRO	100	100	*Babesia canis vogeli* SK-011 (JX112785)	KP182938
JJJ_ PIRO	100	100	*Babesia canis vogeli* SK-011 (JX112785)	KP182939
SSS_ PIRO	99	98	*Babesia canis vogeli* SK-011 (JX112785)	KP182940
EEE_Ecanis	99	99	*Ehrlichia canis* isolate TrKysEcan3 (KJ513197)	KP182941
HHH_ Ecanis	99	99	*Ehrlichia canis* isolate TrKysEcan3 (KJ513197)	KP182942
LLL_Ecanis	99	100	*Ehrlichia canis* isolate TrKysEcan3 (KJ513197)	KP182947
T_ Ecanis	97	97	*Ehrlichia canis* isolate S3b (KJ659037)	KP182948
UU_ Ecanis	99	99	*Ehrlichia canis* isolate TrKysEcan3 (KJ513197)	KP182949
YY_ Ecanis	99	99	*Ehrlichia canis* isolate TrKysEcan3 (KJ513197)	KP182950
HHHH_Anaplasma	99	99	Uncultured *Anaplasma* sp. (FJ943580)	KP182943
IIII_ Anaplasma	99	99	Uncultured *Anaplasma* sp. (FJ943580)	KP182944
QQQ_Anaplasma	99	99	Uncultured *Anaplasma* sp. (FJ943580)	KP182945
RRR_Aphagocytophilum	99	97	*Anaplasma phagocytophilum* (HG916767)	KP182946
Isolate_RRR	99	97	*Anaplasma platys* (AY530806)	KX447502
Isolate_HHHH	100	99	*Anaplasma platys* Gigio (EU439943)	KX447503
Isolate_IIII	99	99	*Anaplasma platys* isolate A.pl.#87 (JQ396431)	KX447504
Isolate_QQQ	100	99	*Anaplasma platys* isolate A.pl.#87 (JQ396431)	KX447505

mon signs of infection observed were presence of anti-*Ehrlichia* IgG antibodies in serum (71.05%), low platelet count (47.37%), and presence of ticks (14.91%). The following signs of infection were found in less than 5% of the dogs:

presence of clinical signs (3.51%), high fever (2.63%), and low white blood cell count (1.75%).

PCR detection and DNA sequencing

PCR and sequencing detected *E. canis* in 5.26% (6/114), *B. vogeli* in 5.26% (6/114), *H. canis* in 5.26% (6/114), and *A. platys* in 3.51% (4/114) out of 114 dogs. All dogs positive for CVBDs exhibited either one or two symptoms (Table 2) whereas all dogs positive for any of the four have either or both low platelet counts and presence of anti-*Ehrlichia* sp. IgG. Besides, none of the dogs that showed positive infection in PCR and multiplex PCR manifested clinical signs of the disease, high fever, or low white blood cell counts. Lastly, two dogs infected with *H. canis* have ticks found in their fur. All sequences were 97-100% similar to their respective species identity (Table 3). Whilst *H. canis, B. vogeli,* and *E. canis* sequences were 99–100% similar to their respective identified species. For the *Anaplasma* sp., the use of EHR16SD/EHR16SR set of primers exhibited that three of the four samples were 99% similar to uncultured *Anaplasma* sp. while the remaining was 97% similar to *A. phagocytophilum*. Subsequent PCR using fD1/EHR6R and EHR16SD/Rp2 sets of primers to amplify larger parts of the 16S rRNA gene, and sequence analysis of products confirmed that the isolates were 97–99% similar to *A. platys*.

Phylogenetic trees were constructed to confirm species identity of *H. canis, B. vogeli,* and *A. platys.* Samples clustered with their respective species identity with high bootstrap support from the ML, NJ, and MP phylogenetic trees further confirmed the identity of the samples as *H. canis* (Fig. 1)*, B. vogeli* (Fig. 2)*,* and *A. platys* (Fig. 3). The sequences obtained in this study were stored in GenBank with accession nos. KP182929-KP182934 for partial 18S rRNA gene sequences of *H. canis*, KP182935-KP182940 for partial 18S rRNA gene sequences of *B. vogeli,* KP182941-KP182950 for partial 16S rRNA gene sequences of all Anaplasmataceae, and KX447502-KX447505 for full 16S rRNA gene sequences of *A. platys*.

Multiplex PCR

The multiplex PCR primers were able to amplify their respective targets at all temperatures used in the gradient PCR experiment with lower limits of detection of 4.5 ng/μL, 19.1 ng/μL, and 2.4 ng/μL for *H. canis, B. canis,* and *E. canis* DNA, respectively (data not shown). To standardize, an annealing temperature of 60°C was used for all experiments. The three primer sets amplified their intended target CVB pathogen in both single reaction and multiplex PCR without cross-reacting with any of the other bacterial and CVB pathogen DNA used in the analytical specificity study (Fig. 4). Multiplex PCR detected 4.39% (5/114) *H. canis* infections, 5.26% (6/114) *B. vogeli* infections, and 5.26% (6/114) *E. canis* infections from the 114 dogs. All samples positive for single reaction PCR and sequencing were also positive for multiplex PCR except for one *H. canis* infection. Moreover, multiplex PCR also detected one case of *E. canis* and *H. canis* co-infection and two cases of *B. vogeli* and *H. canis* co-infections.

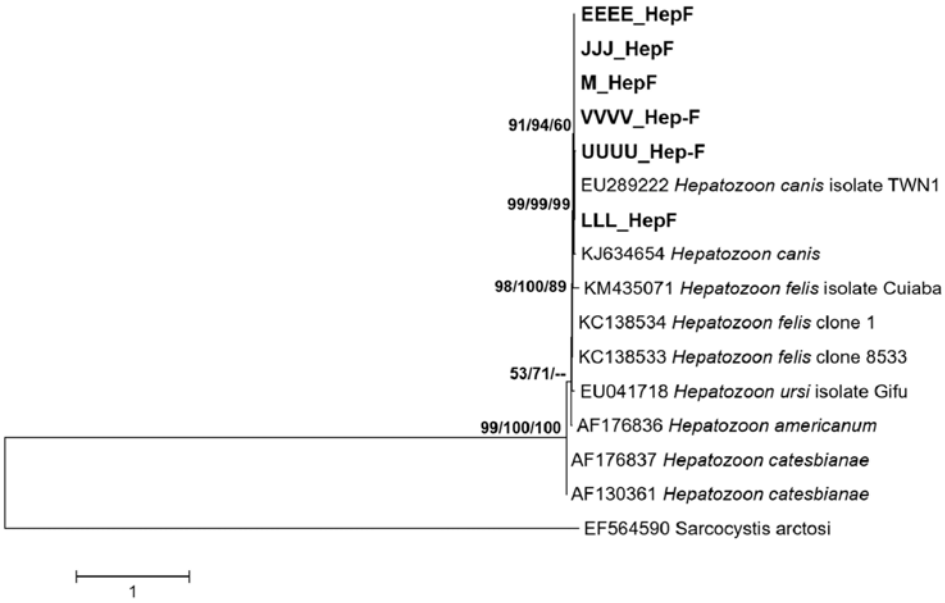

Fig. 1. Consensus phylogenetic tree based on the maximum likelihood (ML) tree using partial 18S rRNA gene sequences (517 unambiguously aligned nucleotide positions) of 15 *Hepatozoon* specimens and 1 outgroup species (*Sarcocystis arctosi*). The bootstrap consensus tree was constructed using the HKY model and inferred from 1,000 replicates. The bootstrap values of the three phylogenetic methods used are shown in the order ML/neighbor joining (NJ) and maximum parsimony (MP). The interior branches are marked with – if the bootstrap value is less than 50%.

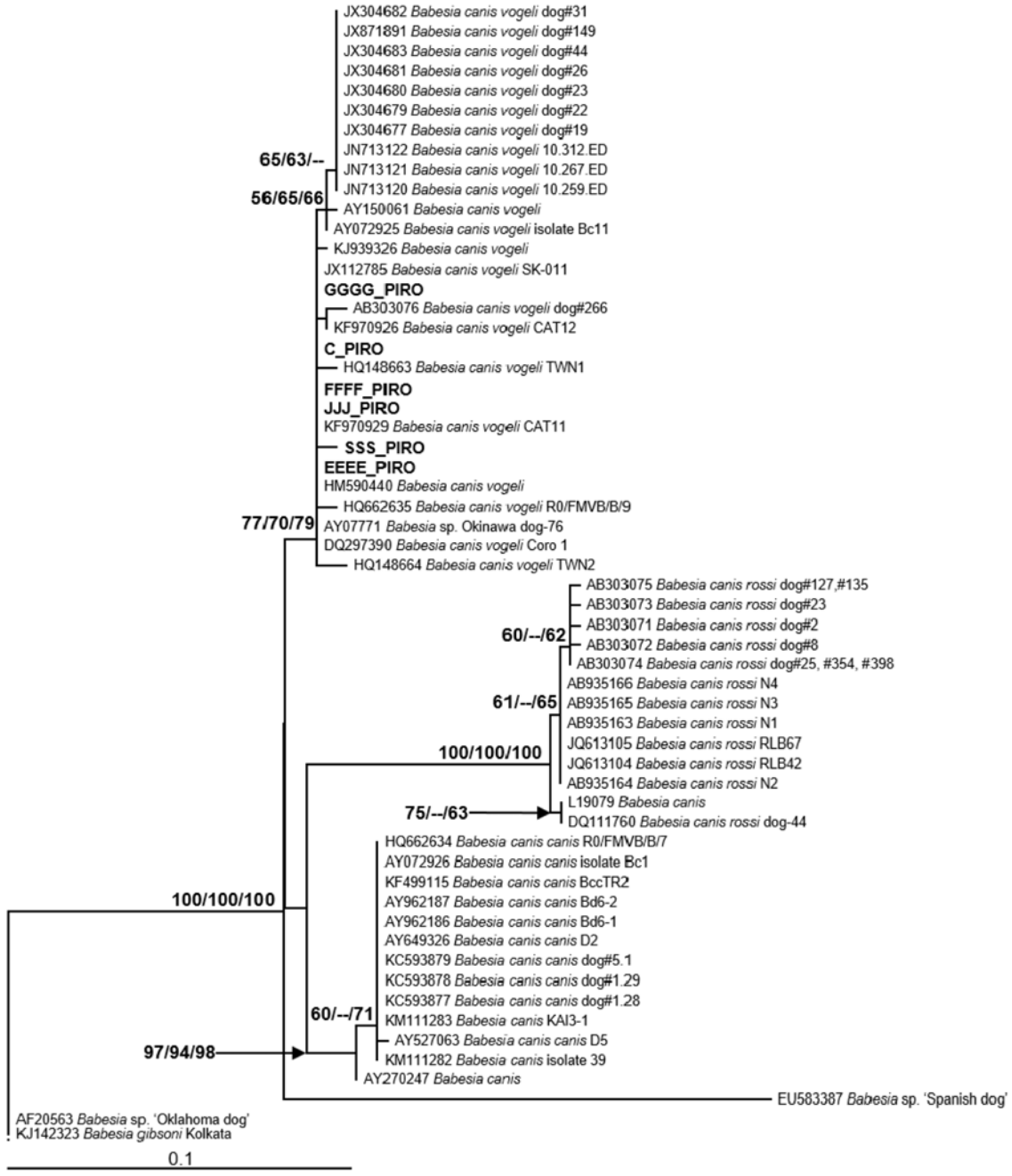

Fig. 2. Consensus phylogenetic tree based on the maximum likelihood (ML) tree using partial 18S rRNA gene sequences (343 unambiguously aligned nucleotide positions) of 56 *Babesia* specimens and 2 outgroup species (*Babesia* sp. Oklahoma dog and *Babesia gibsoni*). The bootstrap consensus tree was constructed using the GTR+G model and inferred from 1,000 replicates. The bootstrap values of the three phylogenetic methods used are shown in the order ML/neighbor joining (NJ) and maximum parsimony (MP). The interior branches are marked with – if the bootstrap value is less than 50%.

Discussion

H. canis, B. vogeli, E. canis, and *A. platys* infections in dogs in Metro Manila, Philippines are present in low prevalence. In this study, *H. canis, B. vogeli, E. canis* and *A. platys* were found in 5.26%, 5.26%, 5.26%, and 3.51%, respectively, of 114 dogs admitted at the Makati Dog and Cat Hospital and the Carlos Veterinary Clinic. Phylogenetic analyses and BLAST results confirm the identification of these with high bootstrap support. Notably, the detected *Anaplasma* infections were identified as *A. platys* after full 16S rRNA gene sequencing and phylogenetic analyses. Results show that CVB pathogens in dogs from Metro Manila are more diverse than previously reported. In fact, a previous study

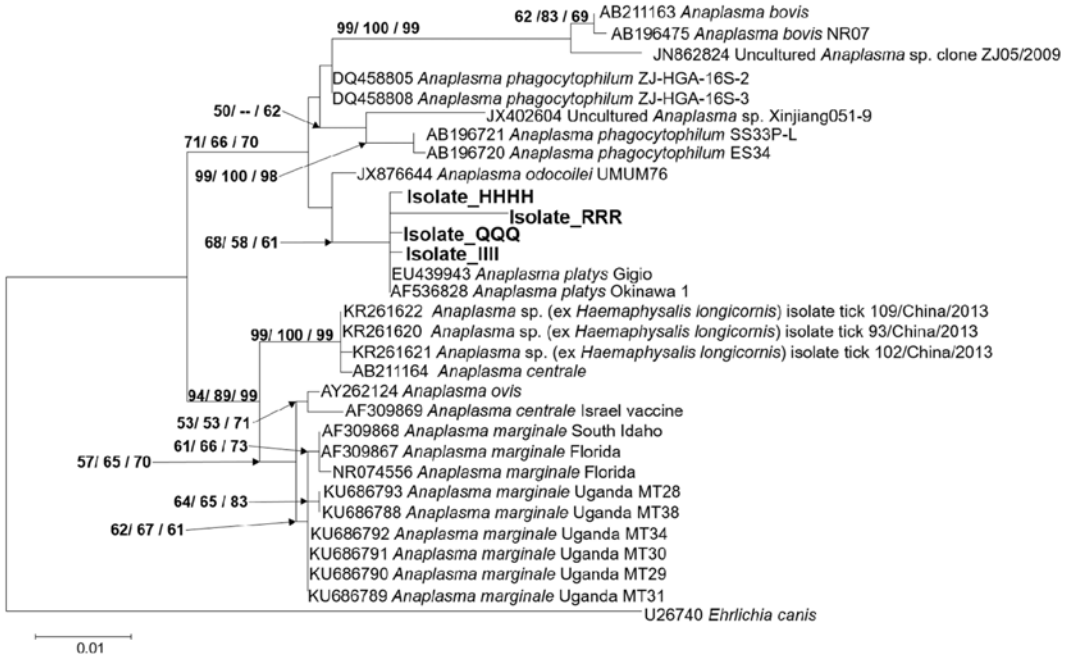

Fig. 3. Consensus phylogenetic tree based on the maximum likelihood (ML) tree using full 16S rRNA gene sequences (819 unambiguously aligned nucleotide positions) of 30 *Anaplasma* specimens and 1 outgroup species (*Ehrlichia canis*). The bootstrap consensus tree was constructed using the TrN model and inferred from 1,000 replicates. The bootstrap values of the three phylogenetic methods used are shown in the order ML/neighbor joining (NJ) and maximum parsimony (MP). The interior branches are marked with – if the bootstrap value is less than 50%.

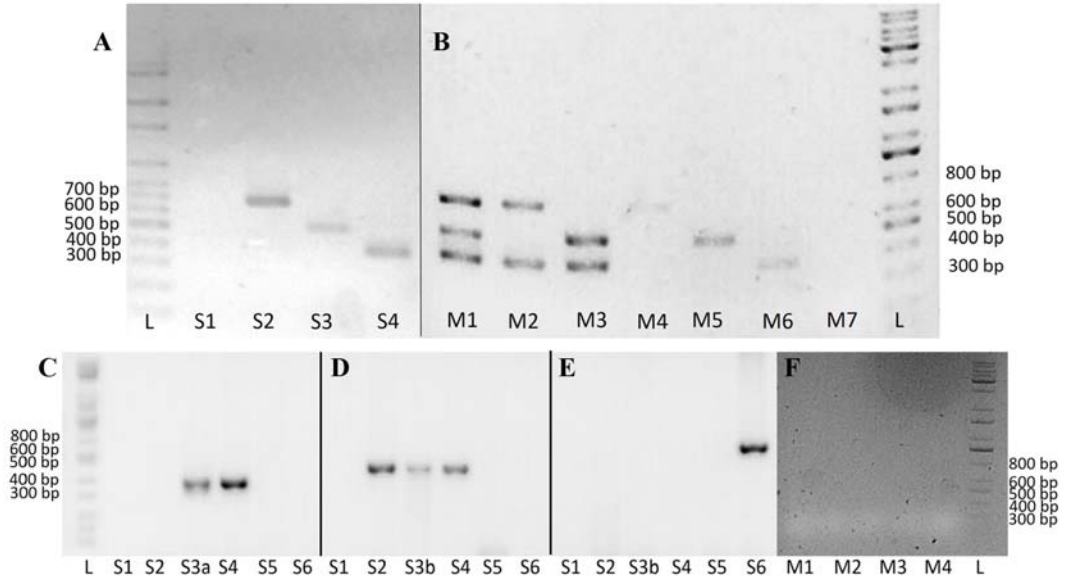

Fig. 4. Results of single reaction and multiplex polymerase chain reaction (PCR) in 1.5% agarose gel on positive and negative control DNA using the designed multiplex PCR primers. Positive bands are indicated by 376 bp (*Hepatozoon* [*H.*] *canis*), 491 bp (*Babesia* sp.), and 664 bp (*Ehrlichia* [*E.*] *canis*). (A) Single reaction PCR. L, molecular weight ladder; S1, negative control; S2, *E. canis* with Ehr16S_multi primer pair; S3, *Babesia* (*B.*) *canis* with Bab18S_multi primer pair; S4, *H. canis* with Hep18S_multi primer pair. (B) Multiplex PCR. M1, positive control DNA of *E. canis, B. canis,* and *H. canis*; M2, mixed DNA sample of *E. canis* and *H. canis*; M3, mixed DNA sample of *B. vogeli* and *H. canis*; M4, DNA sample of *E. canis*; M5, DNA sample of *B. vogeli*; M6, DNA sample of *H. canis*; M7, negative control; L, molecular weight ladder. Single reaction PCR using primers (C) Hep18S-multi primer pair, (D) Bab18S_multi primer pair, and (E) Ehr16s_multi primer pair: S1, negative control; S2, *B. canis*; S3a, *H. canis*; S3b, *B. vogeli*; S4, *B. vogeli* and *H. canis*; S5, *A. platys*; S6, *E. canis*; L, molecular weight ladder. (F) Multiplex PCR: M1, negative control; M2, *Bacillus subtilis*; M3, *Staphylococcus aureus*; M4, *Pseudomonas aeruginosa*; M5, *Escherichia coli*; L, molecular weight ladder.

only detected one case of canine *H. canis* infection out of 168 blood samples from dogs around Metro Manila using the primers Babesia-F/Babesia-R and blood smear examination [2]. PCR detection has been instrumental in detection of CVB pathogens in the country including published accounts of canine infections of *E. canis* [36], *A. platys* [37], and *B. vogeli* [39] in Cebu City and *Babesia* sp. and *E. canis* in the province of Nueva Ecija [8]. In addition, dogs seropositive for *B. gibsoni* infections have also been reported in the province of Cavite [12]. More surveys in stray and pet dogs can clarify the prevalence and diversity of CVB pathogens in the country as well as the inherent risks. In neighboring Southeast Asian countries, more than 10% of sampled population of dogs can be infected with CVB pathogens. For example, a survey of CVBDs out of 101 free-roaming dogs in Cambodia reported prevalences of up to 32.7% for *Babesia* spp., 10.9% for *H. canis,* and 21.8% for *E. canis* [14]. Similarly, *E. canis* prevalence can be as high as 25.5% of stray dogs in Malaysia [19] and 20.4% of pet dogs [30] and 21.5% of stray dogs [27] in Thailand. Moreover, surveys of CVBDs in the Philippines are limited to detection of *Hepatozoon* spp., *Babesia* spp., *Anaplasma* spp., and *Ehrlichia* spp. Recent studies have already shown presence of *Dirofilaria immitis* and *Mycoplasma* spp. in dogs from Cambodia [14] and *Mycoplasma* spp. in dogs from Thailand [20].

Multiplex PCR combines the sensitivity and specificity of PCR with the added advantage of simultaneous detection of multiple targets in one reaction. The current standard for CVBD detection – blood smear examination with Giemsa staining – has been demonstrated to achieve only up to 74% sensitivity in detecting *E. canis* after examination of 1,000 oil immersion field slides of both canine buffy coats and lymph nodes [23]. Moreover, detection by blood smear examination becomes difficult in subclinical cases where parasitemia is low [15]. PCR detection in canine blood samples has higher sensitivity compared to blood smear examination which is conventionally used for routine detection of CVBDs [5]. Moreover, multiplex PCR has been proven useful in simultaneous detection of CVBDs since current results show that reliance only on serology and presence of symptoms may not be accurate for diagnosis [18]. Presence of anti-*Ehrlichia* IgG and low platelet count were the two most commonly observed symptoms in this group of dogs. There were 81 out of 114 dogs positive for serology but only 6 out of the 81 were actually infected with *E. canis* indicating more cases of past infections rather than current ones. Poor correlation or concordance between PCR prevalence and seroprevalence has been recorded before [22, 30, 35] since presence of antibody does not necessarily mean presence of the parasite in blood samples. Low platelet count is a symptom of canine monocytic ehrlichiosis [29] and canine babesiosis [15] and this symptom has been suggested as a screening test for canine monocytic ehrlichiosis to further improve reliability of diagnosis [4]. However, later studies show no correlation with *E. canis, A. platys,* and/or *Babesia* spp. infections [17, 21]. In this study, there were 54 out of the 114 dogs surveyed that had low platelet counts but only 13 of 54 were infected with any of the four causative agents of CVBDs indicating other causes of thrombocytopenia. Results of conventional PCR and sequencing confirm the results of multiplex PCR. All samples detected by the former methods were also detected using multiplex PCR except for one case of *H. canis* infection. This may be due to a lower limit of detection of Hep-F/Hep-R compared to Hep18S_multiF/Hep18S_mutiR. Nevertheless, results still indicate the utility of the designed multiplex PCR primers in screening for CVBDs and as a possible aid in diagnosis. The high analytical sensitivity and specificity of the primers are a good compliment for other diagnostic methods such as observation of symptoms, serology, and microscopy since these have known issues in these areas. However, additional tests are necessary to determine the clinical sensitivity and specificity of the primers in larger sample populations due to the low prevalence of CVBDs in the current sample population.

In summary, this study gives crucial information on the underreported diversity and prevalence of CVBDs in the Philippines which have been lacking from 1977 to 2008. In addition, the multiplex PCR protocol developed in this study shows promise in clinical application for screening of CVBDs in canine blood. A proper screening method is necessary for both clinical use and prevalence studies in the Philippines. Future prevalence studies can address the current extent and effect of CVBD as well as determine which species are already present in local dog populations from other parts of the country. The multiplex PCR protocol offers a quick and simultaneous method for screening of CVBDs in local dog populations as well as pets travelling from foreign countries. It is recommended that future studies also add *B. gibsoni* and *A. platys* to the multiplex detection protocol since these are also present in local canine populations.

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