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RNA polymerase sub-unit β (*rpoB*) characterization of *Ehrlichia canis* detected from dogs and *Rhipicephalus sanguineus* ticks in Cebu, Philippines

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ABSTRACT

Ehrlichia canis, a canine tick-borne pathogen with wide geographic distribution, has been serologically and molecularly detected in the Philippines. The present study aimed to characterize *E. canis* detected from *Rhipicephalus sanguineus* ticks and dogs in Cebu, Philippines, using the RNA polymerase sub-unit Beta (*rpoB*), a gene that has been used for disease diagnosis and resolution of phylogenetic relationships between closely-related species. Using a 16S rRNA gene-based PCR that screens *Ehrlichia* spp., DNA samples obtained from the blood of 10 dogs, confirmed to be serologically positive for *E. canis,* were tested and found positive for *E. canis* after subsequent DNA sequencing. DNA from infected ticks and the 16S rRNA-*E. canis*-positive canine blood samples from the present study were further analyzed using the *rpoB* gene. All registered *Ehrlichia* spp. *rpoB* gene sequences were aligned to design specific primers that can amplify a partial 1572-bp length sequence of *E. canis*. The obtained sequences revealed 99.8-100 % identities with each other, and 99.8-100 % and 87.8-89.1 % identities with registered *E. canis* and *E. chaffeensis* sequences from the USA, respectively. Phylogenetic analysis revealed that the obtained partial *rpOB* sequences formed a clade with *E. canis* strains from the USA. The present study is the first *rpoB* characterization of *E. canis* in the Philippines, and apparently in Asia, and provides additional evidence of the presence of the pathogen in the country. It also adds information on the high conservation of the *rpoB* gene in *E. canis*.

Key words: *Ehrlichia canis*, *rpoB*, dogs, *Rhipicephalus sanguineus*, Philippines

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Introduction

Ehrlichia canis is a tick-borne pathogen affecting dogs worldwide (HARRUS and WANER, 2011). It is a Gram-negative, coccoid to ellipsoidal, often pleomorphic, intracytoplasmic bacteria, belonging to the family *Anaplasmataceae* (DUMLER et al., 2001). It is transmitted by the ubiquitous brown dog tick, *Rhipicephalus sanguineus* complex (YBAÑEZ et al., 2012). The disease it causes, Canine Monocytic Ehrlichiosis (CME), is manifested by various hematological signs, with thrombocytopenia as the most common finding (HARRUS et al., 1996; WANER et al., 2000). CME can be a multisystemic disease (WANER and HARRUS, 2013), and has three recognized phases: acute, subclinical and chronic (RISTIC and HOLLAND, 1993). *E. canis* strains found in the non-myelosuppressive and myelosuppressive CME shared an identical 16S rRNA genotype (SIARKOU et al., 2007).

E. canis is usually detected and characterized based on the 16S rRNA gene (SIARKOU et al., 2007; YBAÑEZ et al., 2012). In the Philippines, the presence of the pathogen has already been confirmed using peripheral blood smear examination, serological tests and molecular tests (BATICADOS and BATICADOS, 2011; MORALES and BATICADOS, 2007; YBAÑEZ 2014). It has already been characterized using the 16S rRNA (YBAÑEZ et al., 2012; YBAÑEZ 2014), heat shock operon (*groESL*) and citrate synthase (*gltA*) genes (YBAÑEZ et al., 2012), but not yet for the *rpoB* gene. Until this study, only strains from the USA had been characterized using this gene (TAILLARDAT-BISCH et al., 2003; MAVROMATIS et al., 2006). The *rpoB* gene has already been used to validate the results of phylogenetic analysis of *Ehrlichia* species from other genes (TAILLARDAT-BISCH et al., 2003; CASE et al., 2007), and to classify genotypes in other species (KO et al., 2002). Thus, the present study aimed to obtain information on the *rpoB* gene of *E. canis* from infected ticks and dogs in the Philippines.

Materials and methods

DNA samples from dogs and infected ticks. DNA samples were obtained from the blood of 10 dogs that were presented at the GPY Veterinare Animale Veterinary Clinic, Cebu City, Philippines, and found serologically positive for *E. canis* using an Immunocomb® (Biogal, Israel), a commercial test kit with a reported sensitivity and specificity of 86 % and 98 %, respectively (HARRUS et al., 2002). In addition, DNA from 4 *Rhipicephalus sanguineus* ticks, previously confirmed to be infected with *E. canis* (YBAÑEZ et al., 2012), was also used. DNA extraction, elution and storage were performed as previously described (YBAÑEZ et al., 2012).

16S rRNA PCR assay. For the 16S rRNA PCR, only canine DNA samples were tested using a previously described method (YBAÑEZ et al., 2012), with slight modification to confirm infection. Briefly, primer pairs fD1/Rp2 and EHR16SD/EHR16SR were

respectively used for the 1st and 2nd round PCR to amplify a final 345-bp-target of *Ehrlichia* spp.. Final volumes of 10 µL and 25 µL were used for the first and second round PCRs, respectively, using a similar method by YBAÑEZ et al. (2013). The negative and positive controls used were double distilled water, and *A. bovis* (YBAÑEZ et al., 2014), respectively.

RpoB-based PCR Assay. All DNA samples were tested by nested PCR using newly designed primers (Table 1). In the first round PCR, the primers ECRPOBF1 and ECRPOBR1 were used to amplify a 1572 bp DNA fragment. A final volume of 10 µL was set, comprised of 4.9 µL of DDW, 1 µL of 2 mM dNTP,1 µL 10x PCR buffer, 1 µL of 10 pmol of each primer, 0.1 µL of 5 units AmpliTaq Gold polymerase (Roche Molecular Systems, Inc., USA), and 1 µL of DNA template. The step-down cycling conditions were the following: initial denaturation at 95 for 9 minutes, followed by 40 cycles of 95 for 30 seconds, 65 ºC (with 2 ºC incremental decrease until reaching a final annealing temperature at 55 ºC) for 30 seconds and 72 ºC for 1.5 minutes, and final extension at 72 ºC for 5 minutes. In the second round PCR, the primers ECRPOBF2 and ECRPOBR2 were paired with the first round PCR primers to amplify DNA targets of 1241 bp and 954 bp in length. A final volume of 25 µL was set, comprised of 16.35 µL distilled water, 2.5 µL of dNTP (2 mM), 2.5 µL 10x PCR buffer, 1.25 µL of each primer at 10 pmol each, 0.15 µL of 5 units AmpliTaq Gold polymerase (Roche Molecular Systems, Inc.) and 1 µL of the 1st PCR amplicon. Similar cycling conditions to the 1st PCR were employed, except for the starting annealing and final annealing of 60 ºC and 55 ºC, respectively. Final amplicons were visualized under UV light using 1.5 % agar gel after electrophoresis.

Table 1. Oligonucleotide sequence of *rpoB* primers used in the present study

Primer name	*rpoB* nucleotide position at *E. canis* reference strain (CP000107)	Oligonucleotide Sequence (5` to 3`)	Length	Ref.
ECRPOBF1	148-177	GTAGGTCAGAATACTGAGAGTGGTATAAAG	30	This study
ECRPOBR1	1691-1719	ATCTCTAACTTCAAACCCTGCTCTTTCTC	29	
ECRPOBF2	766-789	TGTGAGAAAGGGTGGATAGTACCT	24	
ECRPOBR2	1364-1388	TCACCATCCCTTAACAGAACTAACT	25	

Cloning and sequencing. Final amplicons were purified using a Gel Extraction Kit (QIAGEN, Valencia, CA) and were cloned using a TA cloning kit (Invitrogen, USA). DNA sequencing was performed as described previously (YBAÑEZ et al., 2012). The

obtained DNA sequences were aligned as previously described (YBAÑEZ et al., 2012) to obtain partial but longer DNA fragments (1572 bp). Sequence comparison was also performed as previously described (YBAÑEZ et al., 2012). All sequences obtained in this study were registered with the DNA Database of Japan (DDBJ), and are also accessible in GenBank.

Phylogenetic analysis. The *rpoB* sequences were translated into deduced amino acids, and were manually trimmed to include only the sequence of interest. Percent identities were computed and multiple sequence alignments were performed as previously described (YBAÑEZ et al., 2012). Analyses by ML with prior best model testing using MEGA 5 (TAMURA et al., 2011) and by BI using MrBayes 3.2 (RONQUIST et al., 2012) guided by the prior best model test results from MEGA 5 were employed. A total of 1000 bootstrap replications and 1,000,000 generations were used in the analyses using MEGA 5 and MrBayes 3.2, respectively. Tree results from MrBayes 3.2 were viewed using the FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/).

Results

Using the 16S rRNA screening PCR, all dogs were found positive for *Ehrlichia* spp. DNA sequences from the 10 positive dogs were found 100 % identical to registered *E. canis* sequences in the Genbank database. On the other hand, all samples showed positive bands after the 2nd round PCRs in the partial *rpoB* gene amplification. DNA sequencing from each positive sample revealed final lengths of 1572 bp after alignment, which were found to be 99.8-100 % identical with each other, and 99.8-100 %, 87.8-89.1 % and 87.0-87.1 % identical to registered *E. canis* (CP000107 and AF401091), *E. chaffeensis* (CP000236) and *E. ruminantium* (CR925677 and CR925678) sequences from the USA, respectively. Phylogenetic analyses revealed (Fig. 1) that the *E. canis* strains from the Philippines formed a clade with that from the USA. The clade was also strongly supported either by bootstrap values (Fig. 1) or posterior probabilities. Partial *rpoB* gene sequences obtained in the present study were registered with the DNA Database of Japan with the accession numbers AB859624-37. The sequences are also accessible at GenBank.

Discussion

In the present study, new *rpoB* primers were developed by aligning registered *E. canis* sequences. A final partial DNA fragment of 1572 bp in length was obtained. DNA sequencing results revealed that while the partial sequences of both *16S rRNA* and *rpoB* genes are highly conserved among *E. canis* strains, the *rpoB* gene appears to exhibit a higher degree of variation between *Ehrlichia* species level. In several studies, the *rpoB* gene has been shown to be very useful in resolving species identities (MOLLET et al., 1997; TAILLARDAT-BISCH et al., 2003). While the target DNA fragment may appear to

be short in relation to the entire gene length, partial and much shorter sequences of *rpoB* are shown by other studies to be informative enough to allow species identification and differentiation (BONDOSO et al., 2013; KO et al., 2002; KORCZAK et al., 2004).

The *rpoB* gene was utilized in the present study because of its suitability for phylogenetic studies of the erhlichieae (MOLLET et al., 1997; TAILLARDAT-BISCH et al., 2003). With a higher discriminatory power than the 16S rRNA, this gene has been used in several phylogenetic studies (MOLLET et al., 1997; DAHLLÖF et al., 2000). On the other hand, the *rpoB*-based topologies that were produced using ML (Fig. 1) and BI (figure not shown) were found identical. The trees also showed that *E. canis* in the Philippines is phylogenetically close to the USA strains. This observation was well supported by high bootstrap and posterior probability values.

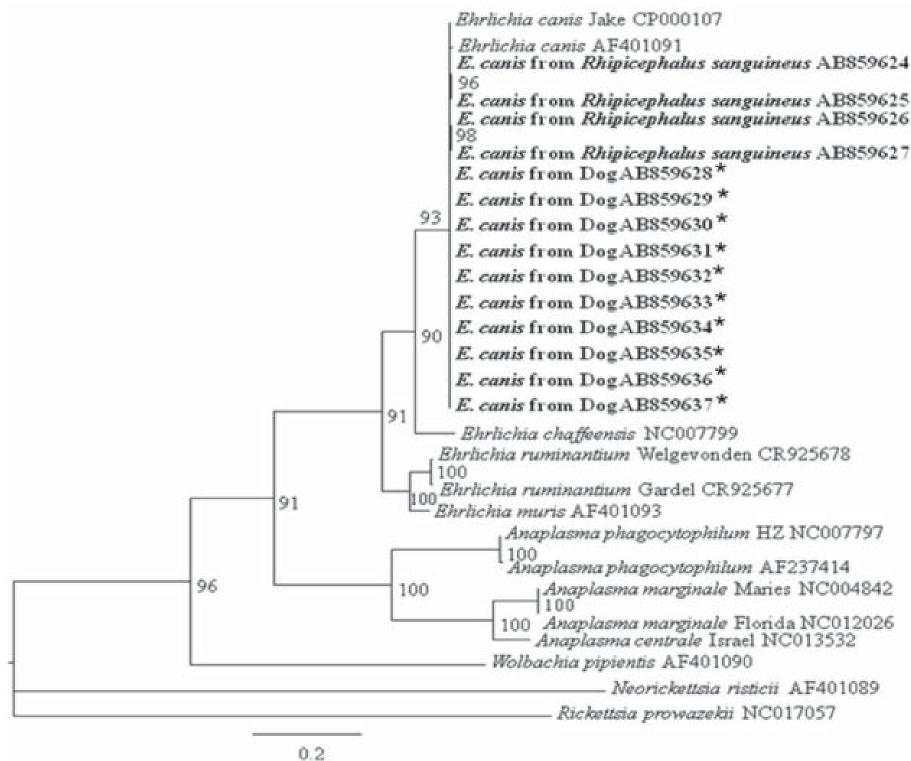

Fig. 1. Phylogenetic analyses of *Ehrlichia canis* based on the RNA polymerase sub-unit Beta (*rpoB*) gene. The tree was inferred by the Bayesian method using Mr.Bayes3.2. Values on the nodes represent posterior probability values expressed in percent. *Rickettsia prowazekii* was used as the outgroup. Sequences obtained in the present study are indicated with asterisk (*).

The *rpoB* characterization of *E. canis* in the present study is apparently the first in Asia. Before this study, there were only 2 registered *rpoB* sequences of *E. canis* (USA strains only), and the registration of new sequences from the present study is a valuable addition. It will be interesting to compare the *rpoB* sequences of other countries to further evaluate the intra-species conservation of this gene in *E. canis* of different geographic areas.

The *rpoB* gene of the Philippine *E. canis* strain was found to be highly conserved at the intra-species level, but a higher degree of variation was observed at the inter-species level (87.0-89.1 %). The results of this study are in accordance with other *rpoB*-based studies, and support the usefulness of the *rpoB* gene in identifying and/or delineating species. With high inter-species sequence variation, new *rpoB*-based diagnostic tools that are specific to *E. canis* may be developed. The *rpoB* gene has already been applied in pathogen diagnostics (DRANCOURT and RAOULT, 2002; KO et al., 2002; RENESTO et al., 2001). On the other hand, the obtained sequences provided additional molecular evidence to previously conducted studies on the presence of *E. canis* in the Philippines (YBAÑEZ et al., 2012, YBAÑEZ et al., 2014).

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YBAÑEZ, A. P., R. H. D. YBAÑEZ, N. YOKOYAMA, H. INOKUMA: Karakterizacija podjedinice β (*rpoB*) RNA polimeraze vrste *Ehrlichia canis* izdvojene iz pasa i krpelja *Rhipicephalus sanguineus* u području Cebu na Filipinima. Vet. arhiv 85, 601-608, 2015.

SAŽETAK

Ehrlichia canis, geografski vrlo proširena rikecija u pasa, dokazana je serološki i molekularno na Filipinima. Određena su obilježja *E. canis* dokazane u krpelja *Rhipicephalus sanguineus* i pasa u pokrajini Cebu na Filipinima. To je učinjeno analizom podjedinice β (*rpoB*) RNA polimeraze, gena koji je rabljen za dijagnosticiranje bolesti i otkrivanje filogenetske srodnosti između usko srodnih vrsta. Upotrebom lančane reakcije polimerazom temeljene na genu 16S rRNA pomoću kojeg se razlikuju vrste roda *Ehrlichia*, u krvi 10 pasa serološki pozitivnih na *E. canis* dokazana je DNA specifična za *E. canis*. DNA iz zaraženih krpelja i uzorci krvi pasa pozitivnih na 16S rRNA-*E. canis* bili su dalje analizirani na osnovi gena *rpoB*. Sve dokazane sekvencije gena *rpoB* rikecija roda *Ehrlichia* bile su poravnate radi sinteze specifičnih početnica s kojima se može umnožiti slijed specifičan za *E. canis* dužine 1572 bp. Umnožene sekvencije pokazivale su 99,8-100 % identičnosti međusobno, 99,8-100 % identičnosti s vrstom *E. canis* iz SAD-a i 87,8-89,1 % identičnosti sa slijedovima *E. chaffeensis* iz SAD-a. Filogenetska analiza je pokazala da se sekvencije *rpoB* nalaze u skupini sa sojevima *E. canis* iz SAD-a. U istraživanju je prvi put analiziran *rpoB* vrste *E. canis* na Filipinima i u Aziji. Ono pruža dodatni dokaz prisutnosti te vrste na tom području. Također pruža informaciju o visokoj konzerviranosti gena *rpoB* vrste *E. canis*.

Ključne riječi: *Ehrlichia canis*, *rpoB*, psi, *Rhipicephalus sanguineus*, Filipini