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Short communication

Molecular detection and phylogenetic analysis of *Ehrlichia canis* in a Philippine dog

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ABSTRACT

Canine monocytic ehrlichiosis (CME), caused by a rickettsial bacterium, *Ehrlichia canis*, is distributed worldwide, particularly in tropical and subtropical regions. Transmission of *E. canis* is primarily mediated by the vector tick, *Rhipicephalus sanguineus* sensu lato and the bacteria then infect and replicate in monocytes and macrophages. Many cases are seen in veterinary hospitals and treated routinely; however, the genetic variation of *E. canis* strains found in the Philippines has been poorly investigated to date. In this study, the *16S* rRNA gene and the *gp200* gene of *E. canis* were detected by polymerase chain reaction from an infected dog in the Philippines, and the deduced amino acid sequence of the *gp200* gene was subjected to a phylogenetic analysis. The Philippine genotype formed a cluster with the Taiwan genotype, and was somewhat divergent from the USA and Brazil strains. This suggested that *E. canis* underwent evolution in East and Southeast Asia, confirming the utility of the *gp200* gene for the assessment of genetic relationships among strains.

1. Introduction

Canine monocytic ehrlichiosis (CME) is a systemic infection in dogs caused by a rickettsial bacterium *Ehrlichia canis*, an obligate intracellular pathogen that infects and replicates in canine monocytes and macrophages (Huxsoll et al., 1970). The clinical symptoms can vary but include fever, weight loss, lethargy, lymphadenopathy, thrombocytopenia, and bleeding disorders (Harrus et al., 1999). The diagnosis is dependent on clinical manifestations, microscopic observation, and/or serologic as well as molecular tests (Allison and Little, 2013). CME is a tick-borne disease which is distributed worldwide, and the pathogen is primarily transmitted by the brown dog tick (*Rhipicephalus sanguineus* sensu lato) (Groves et al., 1975; Nava et al., 2015). Although the prevalence of CME is quite low in some countries and the disease is not endemic in Japan (Inokuma et al., 2003), the prevalence is much higher in many countries in Asia, especially in tropical and subtropical regions, reflecting the distribution of the vector tick (Neer et al., 2002). Many cases of *E. canis* infection occur each year in the Philippines; however, the genetic characteristics of *E. canis* strains found in the Philippines are poorly understood to date.

Although many studies have used the *16S* ribosomal RNA (rRNA) gene to analyze the molecular epidemiology of *E. canis*, the *16S* rRNA gene alone might not be sufficient for analyzing the molecular diversity of *E. canis* because it is highly conserved among strains (Siarkou et al., 2007; Vinasco et al., 2007). Recently, Zhang et al. (2008) proposed the use of the *gp200* gene for this purpose because its gene sequence is highly conserved among the strains from the USA and Brazil but substantially divergent in the Israel strain. In addition, Huang et al. (2010) reported the *gp200* gene sequence from the Taiwan genotype, confirming the divergence in this gene between strains with different geographical distributions. The *gp200* gene encodes a major immunoreactive protein, which provides the species specificity (McBride et al., 2003). Because the divergence of the *gp200* gene may influence the efficacy of vaccines, monitoring this divergence may be helpful for future studies that aim to develop effective vaccines against *E. canis* infection.

In this study, *E. canis* was detected in a dog in the Philippines by molecular detection of the *16S* rRNA gene, and the sequence of the *gp200* gene was subjected to a phylogenetic analysis to assess the genetic characteristics of the strain of *E. canis* found in the Philippines.

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2. Materials and methods

2.1. Animals samples

Non-infected peripheral blood samples were collected from a clinically healthy 3-year-old beagle kept at the Experimental Animal Facility, Graduate School of Veterinary Medicine, Hokkaido University. Peripheral blood samples from two *E. canis*-infected dogs were collected at a veterinary hospital in Laguna, the Philippines. Dog #1 was a 1-year-old male Chow Chow and dog #2 was a 4-year-old male Shih-tzu. The *E. canis* infection was serologically confirmed by a rapid diagnosis kit, WITNESS Ehrlichia (Zoetis, Parsippany, NJ, USA).

2.2. Polymerase chain reaction (PCR) analyses and sequencing

DNA was extracted from 100 to 200 µL of blood samples using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) according to the manufacturer's instructions, and reconstituted in 50 µL of DNA Rehydration Solution. Conventional and nested PCR analyses were performed using ExTaq (Takara, Shiga, Japan) according to the manufacturer's instructions. Briefly, 0.5 µM of each gene-specific primer and 1 µL of the DNA template were incorporated in a final volume of 20 µL of PCR mixture. The primer sets for canine *ACTB* (internal control), *E. canis 16S* rRNA, and *E. canis gp200* genes (EC200-F3 and EC200-R3) were described previously by other groups (Chuang et al., 2009; Inokuma et al., 2003; Huang et al., 2010). For nested PCR to amplify the *16S* rRNA gene, 1 µL of the product from the first PCR was used as a template. The amplicons were visualized by ethidium bromide in a 2% agarose gel after electrophoresis.

For the sequencing analysis, the amplicon was purified using FastGene gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan), cloned into pGEM-T Easy vector (Promega), and sequenced using GenomeLab GeXP Genetic Analysis System (Beckman Coulter, Fullerton, CA, USA).

2.3. Phylogenetic analysis

The resulting nucleotide sequence of the *gp200* gene was converted into a deduced amino acid sequence using MEGA version 6 software (Tamura et al., 2013), and aligned using BioEdit version 7 software (Hall, 1999). A phylogenetic tree was constructed with MEGA version 6 software, using the neighbor-joining method (Saitou and Nei, 1987).

2.4. Nucleotide sequence accession number

The *gp200* gene sequence from the Laguna Philippines genotype was submitted to the DDBJ/GenBank database, with accession number LC269347.

3. Results

3.1. Detection of the 16S rRNA gene in blood samples obtained from the E. canis-infected dogs

Both dogs from the veterinary hospital in Laguna showed clinical symptoms of CME, including fever, lethargy, and epistaxis. Leukopenia and thrombocytopenia were seen in the complete blood count and both dogs were positive for antibodies against *E. canis*, as detected by a serological assay kit (WITNESS Ehrlichia). Dog #1 had been treated with doxycycline (approximately 10 mg/kg, oral administration) once the day before the blood sampling, whereas dog #2 had received no treatment. The nested PCR to detect the *16S* rRNA gene of *E. canis* was performed using species-specific primer sets and DNA samples extracted from the lysed blood, showing a specific band (409 bp) in the sample from dog #2; however, no band was detected in the sample of dog #1 (Fig. 1A and B).

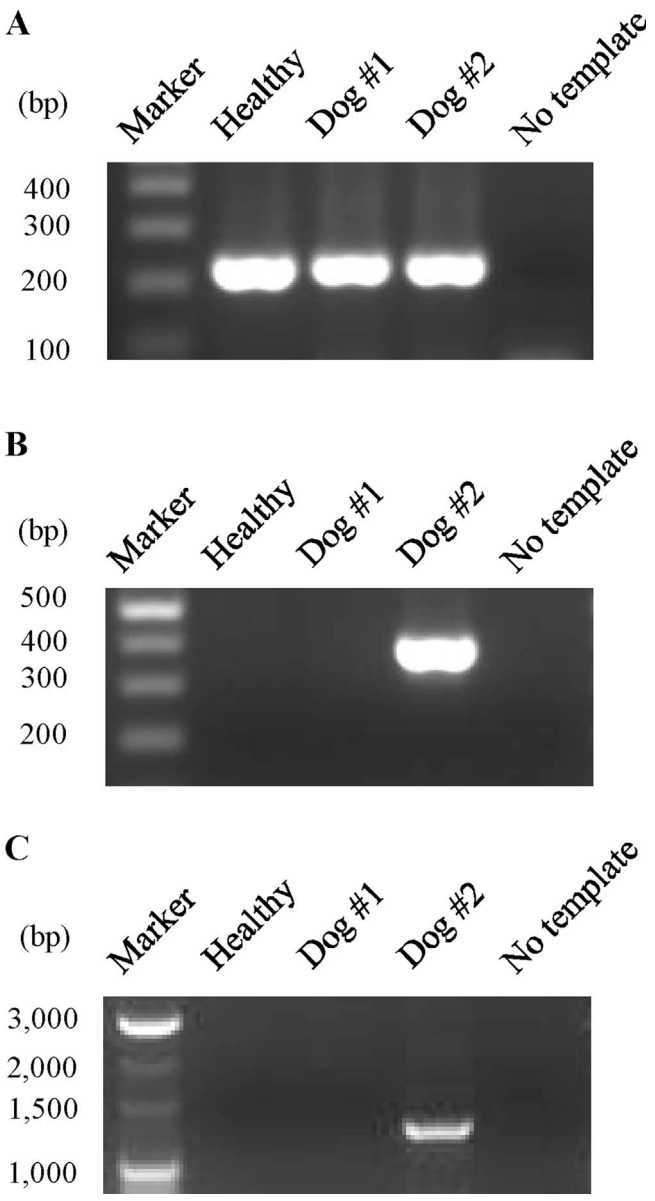

Fig. 1. Detection of the *16S* rRNA and the *gp200* genes in peripheral blood samples from an *E. canis*-infected dog. Conventional or nested PCR analysis was performed using species-specific primers. The DNA extracted from peripheral blood of a healthy dog was used as a control template. (A) The amplification of a housekeeping gene (*ACTB*, 237 bp). (B) The amplification of the *16S* rRNA gene (409 bp). (C) The amplification of the *gp200* gene (1286 bp). Distilled water was used as a no template control.

3.2. Phylogenetic analysis of the gp200 of an E. canis Philippine genotype

A part of the *gp200* gene of *E. canis* was amplified by PCR using the DNA sample from dog #2 (Fig. 1C). The deduced amino acid sequence obtained for the *gp200* gene showed 93.7% amino acid identity with the Taiwan genotype (ADF30849), and 92.5%, 92.1%, and 91.8% identity with the Israel (ABV02079), Brazil (ABV02080), and USA (AAK01145, AAZ68408) strains, respectively (Fig. 2A). The phylogenetic tree was inferred using neighbor-joining method of Saitou and Nei (1987), comparing the *gp200* amino acid sequences of the strains and genotypes mentioned above. The Philippine genotype formed a cluster with the Taiwan genotype (Fig. 2B).

4. Discussion

Although CME is considered a major bacterial infection in dogs in