Detection of Ehrlichial Infection by PCR in Dogs from Yamaguchi and Okinawa Prefectures, Japan

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ABSTRACT. Species-specific nested polymerase chain reaction (PCR) was used to detect the presence of possible canine ehrlichial agents (*Ehrlichia canis*, *E. chaffeensis*, *E. ewingii*, *E. equi* and *E. platys*) and monocytic ehrlichial agents found in Japan (*E. muris* and a recently discovered *Ehrlichia* species detected from *Ixodes ovatus*) in blood samples from dogs in Yamaguchi and Okinawa Prefecture, Japan. Partial sequence of *E. platys* was detected from 1 of 67 dogs (1.5%) tested from Yamaguchi Prefecture and 24 out of 87 (27.6%) in the subtropical Okinawa Prefecture. Dogs in Okinawa and Miyako Islands had a higher positive rate (69.2 and 45.0%, respectively) than Ishigaki Island (11.1%). Another dog in Yamaguchi Prefecture had a positive PCR reaction to the *Ehrlichia* sp. detected from *I. ovatus*. No other *Ehrlichia* were found in these samples.

KEY WORDS: canine, *Ehrlichia*, PCR.

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Ehrlichioses are important emerging tick-borne diseases in both humans and animals and has worldwide distribution. Ehrlichial agents such as *Ehrlichia canis*, *E. ewingii* and *E. platys* are well known to be pathogenic to dogs. *Ehrlichia chaffeensis* and *E. phagocytophila* geno-group *Ehrlichia* (*E. equi*, *E. phagocytophila* and human granulocytic ehrlichia) have also been reported to be infectious for canidae [1, 2, 18]. These *Ehrlichia* have been reported mainly from USA and Europe and little information are available on canine ehrlichiosis in Japan. Only one confirmed case of *E. canis* infection in a dog transferred from Indonesia has been recently reported [20]. Positive antibodies against *E. canis* were found in dogs from Okinawa and Yamaguchi prefectures [7, 8, 12]. *Rhipicephalus sanguineus*, which is a well-recognized vector for *E. canis*, is established in Japan [6]. Many dogs have been introduced into Japan from USA, where canine ehrlichial agents such as *E. canis*, *E. ewingii*, *E. chaffeensis*, *E. platys* and *E. equi* are found [1, 13, 16]. Recently partial 16S rRNA of *E. platys* were found in ticks and free-roaming dogs in Okinawa Island, which is 1,000 km southwest of the main land [9, 15].

Today, 4 *Ehrlichia* species, *E. sennetsu* [14], the *Stellantis falcatus* (SF) agent [3], *Ehrlichia muris* [10], and a recently discovered *Ehrlichia* species originally isolated from *Ixodes ovatus* (*I. ovatus*) [19] have been reported in the main land of Japan. The suspected vectors of *E. muris* and the recently discovered *Ehrlichia* species are *Haemaphysalis flava* (*H. flava*) and *I. ovatus* tick, respectively [11, 19]. These arthropods are widely distributed in mainland of Japan [23]. Although both *H. flava* and *I. ovatus* often bite dogs [5], these ehrlichial agents have not been reported in dogs yet. The SF agent and supposedly *E. sennetsu* are transmitted by helminths [3], which may explain why these agents have been found localized in only Kyushu island. The fact that antibodies reactive against *E. canis* antigens

were found in dogs suggests that *E. canis* or other serologically related agents like *E. muris* and the recently discovered *Ehrlichia* species are potential infectious agents of dogs. The aim of this study was to evaluate the prevalence of canine ehrlichiosis including *E. canis*, *E. ewingii*, *E. chaffeensis*, *E. platys*, *E. equi*, *E. muris*, and recently discovered *Ehrlichia* species originally isolated from *I. ovatus*, in dogs from Yamaguchi and Okinawa Prefectures by molecular method.

Blood samples were collected from 67 randomly selected dogs that presented for illness or vaccination at the Animal Hospital in Yamaguchi University between December 1998 and June 1999 in the main land of Japan. The dogs originated from Yamaguchi Prefecture and neighboring sites. The history of tick infestation was not recorded. In Okinawa Prefecture, samples were taken from 87 free-roaming dogs that were captured on Okinawa, Miyako and Ishigaki Islands, and transferred to an animal shelter on Okinawa Island in August 1997 and October 1998. Various degrees of infestation with *R. sanguineus* and small numbers of *H. longicornis* were observed in dogs from Okinawa Prefecture by physical examination at the time of bleeding. EDTA-anticoagulated blood was collected for PCR. Heparinized blood was occasionally submitted as a clinical specimen.

DNA was extracted from whole blood (200 µl) following the QIAamp Tissue Kit procedure (QIAGEN GmbH, Hilden, Germany), adjusted in 200 µl of Tris-EDTA buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) and stored at –20°C until used. A species-specific nested PCR was used to amplify ehrlichial DNA. The first PCR amplification was performed in a 25 µl reaction containing 5 µl (approximately 50 to 200 ng) of DNA template with the primer set of universal fD1 (5'-AGA-GTT-TGA-TCC-TGG-CTC-AG-3', 22) and *Ehrlichia* genus-specific EHR16SR (5'-TAG-CAC-TCA-TCG-TTT-ACA-GC-3') [17]. These primers

can amplify the ehrlichial 760 bp partial sequence of 16S rRNA gene. The amplification was performed with a method described previously [9]. Briefly, each reaction mixture contained 12.5 pmol of each primer, 0.75 U of Taq DNA polymerase, 20 mM of each dNTP, 10 mM Tris-HCl, 50 mM KCl, 1.6 mM MgCl₂ and 5 μ l of template dog genomic DNA in a final volume of 25 μ l. The amplification was performed in a Peltier model PTC-200 thermal cycler (MJ Research, Inc., Watertown, MA, U.S.A.) with the following program: an initial 5 min denaturation at 95°C; 34 repeated cycles of denaturation (95°C for 30 s), annealing (53°C for 30s), and extension (72°C for 90 s); followed by a 5 min extension at 72°C. Distilled water and DNA of the human granulocytic ehrlichiosis agent were included as a negative and a positive control, respectively. The amplification products were visualized on a 2% agarose gel after electrophoretic migration of 8 μ l of amplified material. As several non-specific bands were observed in the first PCR, it was difficult to evaluate the results. Then the first PCR product from each sample was diluted 1 to 10 with distilled water and 5 μ l of the diluted solution was used as the template DNA for the second PCR in a final volume of 25 μ l. The primers for the second step PCR were as follows. Species-specific forward primers CANIS [21], CHAFFEENSIS [21], EWINGII [21], EQUI [21], MURIS (5'-GAT-AGC-TAC-CCA-TAG-CTT-TTT-TAG-C-3', this study) and OVATUS (5'-GAC-AAT-TAC-CTA-TAG-CCT-TTT-TGG-TTA-C-3', this study) were used with the *Ehrlichia* genus-specific reverse primer of GA1UR [21] to amplify *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. equi*, *E. muris* and the recently discovered *Ehrlichia* species isolated from *I. ovatus*, respectively. For the species-specific amplification of *E. platys*, PLATYS-F (5'-AAG-TCG-AAC-GGA-TTT-TTG-TC-3') and PLATYS-R (5'-CTT-TAA-CTT-ACC-GAA-CC-3') were designed. The annealing temperature of the second PCR was performed at 55°C. The other PCR conditions were the same as in the first PCR. Distilled water and DNA of each agent were included as a negative and a positive control in each PCR reaction with the exception of

E. ewingii DNA that was not available for this study. The amplification products were visualized on a 2% agarose gel after migration.

Randomly selected positive amplification products were then extracted with QIAPCR purification kit (QIAGEN) for sequence analysis to confirm the results obtained by nested PCR. Fluorescence-labeled dideoxynucleotide technology was used for DNA sequencing reactions (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, U.S.A.). The sequencing fragments were separated, and data were collected on an ABI PRISM™ 310 Genetic Analyzer (Perkin-Elmer). The collected sequences were assembled and edited with the AutoAssembler version 1.4 (Perkin-Elmer). The sequence data of the PCR products were analyzed by BLAST for the homology search.

In Yamaguchi Prefecture, one of the 67 dogs tested yielded a 504 bp fragment with *E. platys* specific primers, while another dog showed a 412 bp fragment with specific primers to a recently discovered *Ehrlichia* species isolated from *I. ovatus* (Table 1). No dogs were PCR positive for the other tested *Ehrlichia*.

Sequence analysis of the 466 bp fragment of the positive *E. platys* PCR excluding the primer regions showed that the *Ehrlichia* had 99.8% homology (1 nucleotide absent) with the registered *E. platys* sequence (AF156784) in GenBank. The medical history of this dog (7 years old male) noted that it was presented to the hospital for demodecosis. Hematological examination was not performed in the patient, because the dog appeared otherwise healthy. This is the first detection of *E. platys* DNA from dog in mainland of Japan. The dog did not show any signs of *E. platys* infection such as fever, anorexia or lethargy, although the agent usually does not cause clinical illness in dogs [4]. Information about the tick infestation was not available for the patient. *Rhipicephalus sanguineus*, the likely vector tick of *E. platys* has never been recorded in Yamaguchi Prefecture, although it occasionally found on dogs in main land of Japan [5]. Yamaguchi is one of Prefectures where US army base exists. More epidemiological studies are required to clarify

Table 1. PCR results for dogs in Yamaguchi and Okinawa Prefecture

Species	Yamaguchi Prefecture		Okinawa Prefecture					
	Numbers of dogs tested	Numbers reactive (%)	Okinawa island		Miyako island		Ishigaki island	
			Numbers of dogs tested	Numbers reactive (%)	Numbers of dogs tested	Numbers reactive (%)	Numbers of dogs tested	Numbers reactive (%)
<i>E. canis</i>	67	0 (0)	13	0 (0)	20	0 (0)	54	0 (0)
<i>E. chaffeensis</i>	67	0 (0)	13	0 (0)	20	0 (0)	54	0 (0)
<i>E. ewingii</i>	67	0 (0)	13	0 (0)	20	0 (0)	54	0 (0)
<i>E. muris</i>	67	0 (0)	13	0 (0)	20	0 (0)	54	0 (0)
<i>Ehrlichia</i> from <i>I. ovatus</i> #	67	1 (1.5)	13	0 (0)	20	0 (0)	54	0 (0)
<i>E. equi</i>	67	0 (0)	13	0 (0)	20	0 (0)	54	0 (0)
<i>E. platys</i>	67	1 (1.5)	13	9 (69.2)*	20	9 (45.0)**	54	6 (11.1)

#: A recently discovered *Ehrlichia* species originally isolated from *I. ovatus* in Japan.

*: Significant differences against Yamaguchi Prefecture and Ishigaki Island (Exact Fisher's Test, $P < 0.0001$).

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the origin of the infection and demonstrate evidence of *E. platys* infection in Yamaguchi Prefecture.

The PCR fragment positive for *Ehrlichia* sp. originally detected from *I. ovatus*, was also analyzed and found 100% identical to the registered sequence of the agent (AF260591). The dog (11 years old female) was diagnosed with immune-mediated skin disease, and had been treated with prednisolone (1 mg/kg, per oral administration once a day) to control its clinical signs. Anorexia, depression and weight loss were recorded at the time of bleeding. Hematological examination revealed moderate thrombocytopenia ($7.4 \times 10^4/\mu\text{l}$), and anemia (RBC: $402 \times 10^4/\mu\text{l}$, Hb: 6.6 g/dl, PCV: 27%), but no morulae were reported. Although this *Ehrlichia* can cause severe illness in immunocompetent laboratory mice, the susceptibility of and pathogenesis to domestic animals or human are still unknown (19). The relationship of the immune-mediated skin disease, clinical signs, hematological abnormalities found in this case and the presence of the *Ehrlichia* have not been established. Because *I. ovatus* is one of the most common tick species of dogs in mainland Japan [5, 23] and the dog had a history of tick exposure, it may have acquired the infection through the bite of *I. ovatus*. We demonstrated here in that the recently discovered *Ehrlichia* species originally isolated from *I. ovatus* could be found in dogs. More epidemiological evidences are required to confirm the infectivity of the agent to the dogs.

In Okinawa Prefecture, 24 of the 87 examined dogs (27.6%) were PCR positive for *E. platys*. The positive rate was significantly higher than that of Yamaguchi ($P < 0.0001$, Fisher's exact test). This difference between Yamaguchi and Okinawa Prefectures is likely due to the fact that *R. sanguineus* has not yet been recovered in Yamaguchi Prefecture while it is the most dominant tick species of dogs in Okinawa Prefecture [7]. Sequence analysis of the PCR products of 5 randomly selected dogs among the 24 positive showed that the fragments had 99.8 (1 nucleotide absent) to 100% homology with the published *E. platys* sequence of GenBank (AF156784). Dogs in Okinawa and Miyako Islands had a higher positive PCR rate (69.2 and 45.0%, respectively) than Ishigaki Island (11.1%) (Table 1). Physical and hematological examinations were not performed on these dogs studied in Okinawa Prefecture. More clinical and hematological evidences are required to confirm the existence of *E. platys* infection. All samples were negative for other tested *Ehrlichia*. Although *R. sanguineus* is the main vector of *E. canis* and canine antibodies against *E. canis* have been reported in the Okinawa Prefecture [7, 12], no DNA of *E. canis* were detected in the present study. Serological cross reactivity can be occurred among the same *E. canis* geno-group pathogens, such as *E. muris*, *E. chaffeensis* and *E. ewingii*; however, the DNA of these *Ehrlichia* was not detected. Additional epidemiological study is needed to clarify the phenomenon.

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