Survey of Tick Infestation and Tick-Borne Ehrlichial Infection of Dogs in Ishigaki Island, Japan

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ABSTRACT. Twelve (54.5%) of 22 free-roaming dogs in Ishigaki Island had tick infestation identified as *Rhipicephalus sanguineus*. There were 121 ticks recovered and consisted of 28 females, 58 males, 22 nymphs and 3 larvae. Infection of dogs possibly with canine ehrlichial pathogens was examined by both indirect immunofluorescence assay and polymerase chain reaction (PCR). Two dogs of the 13 examined were sero-positive for the human granulocytic ehrlichia agent, and one of two dogs was PCR positive for *Ehrlichia platys*. This dog had platelet numbers slightly lower than normal value, however, no morulae were found within platelet on peripheral blood smear stained with Giemsa.

KEY WORDS: *Ehrlichia platys*, Ishigaki Island, *Rhipicephalus sanguineus*

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Ishigaki Island is one of the Nansei islands of Japan and is located 2000 km southwest of the capital Tokyo, and 200 km east of Taiwan. The island has a subtropical climate and different tick fauna from main land Japan. While *Boophilus microplus* has been recognized as the principal tick species that infest cattle in Ishigaki Island [6, 23]. Only recently *Amblyomma testudinarium*, *A. geoemydae*, and *A. nitidum* from wild pigs [23], *Ixodes granulatus* from rats [23], *Ixodes* sp. from a bird [6], and *Dermacentor* sp. from a dog [6] have been reported. Recently tick-borne rickettsial diseases have been documented as emerging pathogens of animals and humans. Ehrlichial agents such as *Ehrlichia canis*, *E. ewingii* and *E. platys* are well known to be pathogenic to dogs. *E. chaffeensis* and *E. phagocytophila* geno-group *Ehrlichia* (*E. equi*, *E. phagocytophila* and the human granulocytic ehrlichia agent (HGE)) have also been reported to be infectious to canids [4, 14]. Reports of tick-borne ehrlichial infection are mainly from the U.S.A. and Europe, and little information is available on canine ehrlichiosis in Japan. There is one confirmed case of *E. canis* infection in a dog that originated from Indonesia [19]. More recently, partial sequence of *E. platys* DNA was detected from ticks and dogs in Okinawa Prefecture [9, 11]. Furthermore ticks may transmit spotted fever group (SFG) of rickettsiae while feeding on humans or animals [15]. Only *Rickettsia japonica* is known to be endemic in mainland Japan, and its vectors are local ticks such as *Haemaphysalis longicornis* and *I. ovatus* [20]. Dogs are known to be positive for the antibodies against *R. japonica* and are possible reservoirs of the infection [7]. To date little information is available on rickettsial infection of dogs in Ishigaki Island. In the present study, we examined tick infestation in dogs in Ishigaki Island. We also examined blood samples for both ehrlichial and rickettsial infections, and performed serological test and polymerase chain reaction (PCR) assay.

Twenty-two free-roaming dogs in Ishigaki Island were examined for tick infestation in July, 1998. They were captured and kept in a dog shelter on the day or one day prior to examination. Ticks were collected by hand from all the dogs under sedation and preserved in 70 % ethanol at room temperature until microscopic identification. A total of 121 ticks were recovered from 12 dogs (54.5%).

Sera were collected from randomly selected 13 dogs. EDTA-anticoagulated blood was also collected from the same dogs for complete blood count, smear observation and PCR to detect rickettsial pathogen. *E. canis*, Oklahoma strain [22] and the HGE agent, Webster strain [1] and *R. conori*, Moroccan strain (ATCC VR-14) were used as antigens in immunofluorescence assay (IFA) as previously reported [2]. *E. canis* and the HGE agent that were selected to detect antibodies for ehrlichial agents belong to each geno-group *Ehrlichiae*. *R. conori* was used to detect antibodies against SFG *Rickettsia*. Antigens were applied to each well of 18-well slides with a pen at different position around the edge of the wells and fixed in acetone for 10 min. Serial twofold dilutions of test sera were reacted with fluorescence isothiocyanate (FITC) conjugated affinity purified rabbit anti-dog IgG (Jackson Immuno Research Laboratories, Inc., PA, U.S.A.). End point titers were determined as the last dilution at which brightly stained organisms could be detected on a fluorescence microscope. Serum from a naturally infected dog was used for *E. canis* positive controls. Negative dog serum was obtained from laboratory dogs kept in Yamaguchi University. Sera from mouse experimentally infected with the HGE agent and *R. conori* respectively, were used as positive controls for these agents. Anti-mouse IgG conjugated with FITC (Jackson Immuno Research Laboratories, Inc., PA, U.S.A.) was used as the second antibodies for the positive sera of the HGE and *R. conori*. All the serological tests were performed at Unité

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DNA was extracted from whole blood (200 μ l) following the QIAamp Tissue Kit procedure (QIAGEN GmbH, Hilden), adjusted in 200 μ l of TE buffer and stored at -20°C until used. A species-specific PCR based on 16S rRNA gene sequence was used to amplify ehrlichial DNA as described elsewhere [9, 21]. Species-specific forward primers CANIS, CHAFFEENSIS, EWINGII and EQUI were used with the *Ehrlichia* genus-specific reverse primer of GA1UR to amplify *E. canis*, *E. chaffeensis*, *E. ewingii* and *E. equi*, respectively [21]. For the species-specific amplification of *E. platys*, PLATYS-F and PLATYS-R were used [9]. Five μ l of DNA extract from each sample was used as the template DNA for the PCR in a final volume of 25 μ l. The amplification of 381 bp fragment of the gene encoding for the citrate synthase was performed using primer pairs Rp CS.877p and Rp CS.1258n, which can amplify most of the rickettsial species [17]. The annealing temperature for all the PCR was 55°C . Distilled water and DNA of each agent was included as a negative and a positive control in each PCR reaction. For the rickettsial PCR, DNA of *R. sibirica* was included as a positive control. Positive amplification products were then extracted with QIAPCR purification kit (QIAGEN) for direct sequence analysis to confirm the results obtained by PCR with the method described previously [9]. The sequence data of the PCR products were analyzed by BLAST for the homology search.

All the tick species examined were identified as brown dog tick (*Rhipicephalus sanguineus*), and consisted of 28 females, 58 males, 22 nymphs and 3 larvae. *Rhipicephalus sanguineus* has a worldwide distribution and among the

most prevalent tick species of dogs in Okinawa Island [8]. This tick is also easily found in Taiwan [3]. As Ishigaki Island has a similar fauna as Okinawa Island, one may easily expect that the tick exists in Ishigaki Island, which is located between Okinawa Island and Taiwan.

The results of serological test and PCR are summarized in Table 1. Of the blood samples obtained from 13 dogs, two dogs (Nos. 1 and 2) possessed antibodies against the HGE agent with titer of 1:64. One dog (No. 1) of the two showed positive PCR result for *E. platys* specific PCR (Fig. 1). Sequence analysis of the 469 bp fragment of the positive PCR excluding the primer regions showed that the *Ehrlichia* had 100% homology with the registered *E. platys* sequence in GenBank; AF286699 and AF303467. The partial 16S rRNA gene sequence of *E. platys* determined in this study has been registered in the GenBank under accession number AY029158. The HGE antigen was used to detect antibodies against *E. phagocytophila* geno-group species including *E. platys*, *E. equi*, *E. phagocytophila*, and the HGE agent. Members of the genus *Ehrlichia* share numerous common antigens, especially among the same geno-group of ehrlichiae [5]. Vectors of three granulocytic *Ehrlichia* species (*E. equi*, *E. phagocytophila*, and the HGE agent), *I. ricinus*, *I. scapularis* and *I. pacificus* have never been recorded in Japan [12, 13, 16]. Although the cross-reactivity between the *E. platys* and the HGE agent have yet been proven, the positive antibodies against the HGE agent found in the present study may actually due to the *E. platys* infection.

While females, males and nymphs of *R. sanguineus* were recovered from dog No.1, its blood samples were PCR negative for all other *Ehrlichia* or *Rickettsia* tested. The platelet

Table 1. Results of serological test and PCR assay in blood samples of free-roaming dogs in Ishigaki Island

Dog No.	Serological titers			PCR	
	<i>E. canis</i>	the HGE agent	<i>R. conori</i>	<i>Ehrlichia</i>	<i>Rickettsia</i>
1	<25	64	<25	<i>E. platys</i>	–
2	<25	64	<25	–	–
3–13	<25	<25	<25	–	–

Fig. 1. Dog No.1 showed a 506 bp positive band (arrow) for *Ehrlichia platys* specific PCR with primers PLATYS-F and PLATYS-R (lane 1), but other dogs showed negative (lane 2–13). P: Positive control, N:negative control, M: 100 bp DNA ladder.

count of the dog was $8.2 \times 10^4/\mu\text{l}$, which was slightly lower than normal value ($20\text{--}50 \times 10^4/\mu\text{l}$) [10], however, no morulae were found within the platelets on peripheral blood smear stained with Giemsa. The PCR results of other 12 dogs were negative for all tested pathogens. Dog No. 2 was also infested with *R. sanguineus* showed normal value in RBC, WBC and platelet counts and negative PCR for all *Ehrlichia* tested. The reactivity of the dog serum with the HGE agent indicates previous ehrlichial infection. There were no detectable antibodies to *E. canis* at all.

Other than a registered platelet count in one dog slightly lower than that of normal and one of the dogs being PCR positive for *E. platys*, there were no other findings to support *E. platys* infection in *R. sanguineus*, a suspected vector of *E. platys*. While, *R. sanguineus* is also a well-known vector of *E. canis*, we did not find any indications of such in the present study. The blood samples were PCR negative for *E. ewingii*, *E. chaffeensis* and *E. equi*. We also did not detect rickettsia DNA in dogs' blood samples, and serological tests were all negative in the present study. In the light of earlier reports by Satoh *et al.* [18] of seroprevalence of antibodies against SFG rickettsia among dogs and humans in Okinawa, more epidemiological studies are necessary to establish existence of SFG rickettsial infection in Ishigaki Island.

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