Epidemiological Survey of Babesia gibsoni Infection in Dogs in Eastern Japan

Takako MIYAMA1), Yoshimi SAKATA2), Yojiro SHIMADA3), Shoji OGINO3), Malaika WATANABE3), Kazuhiro ITAMOTO5), Masaru OKUDA3), Rodolfo A. VERDIDA4), Xuenan XUAN4), Hideyuki NAGASAWA4) and Hisashi INOKUMA1)*

1)Faculty of Agriculture, Yamaguchi University, Yamaguchi 753–8515, 2)Merial Japan, Ltd., Tokyo 100–0014, 3)Nippon Zenyaku Kogyo Co., Ltd., Koriyama, Fukushima 963–0196 and 4)National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan

(Received 1 October 2004/Accepted 5 January 2005)

ABSTRACT. To determine the distribution of Babesia gibsoni infection in dogs in the eastern part of Japan, an epidemiological survey of dogs suspected of having B. gibsoni infection was attempted using the polymerase chain reaction (PCR) and enzyme-linked immunosorbent assay (ELISA). Thirty-five of 115 such dogs (30.4%) were positive by PCR and/or ELISA. The 35 positive dogs consisted of 28 Tosa dogs, 4 American Pit Bull Terriers, and 3 mongrel dogs in Aomori, Fukushima, Ibaraki, Gunma, Chiba, Tokyo, Kanagawa, and Nagano Prefectures. The positive dogs had a significantly lower rate of tick exposure and a higher rate of bites by other dogs. Twenty-two of 35 B. gibsoni-positive dogs were infected with hemoplasma, and the rate of infection was significantly higher than that of B. gibsoni-negative dogs.

KEY WORDS: Babesia gibsoni, eastern Japan, enzyme-linked immunosorbent assay, hemoplasma, polymerase chain reaction

Canine babesiosis is a tick-borne disease caused by protozoal parasites, Babesia gibsoni (B. gibsoni) and Babesia canis (B. canis). They infect the red blood cells of dogs and typically cause hemolytic anemia. Infection with B. gibsoni can generally result in more severe clinical manifestations than infection with B. canis, and may cause multiple organ dysfunctions. Therefore, B. gibsoni is clinically more important than B. canis in Japan. B. gibsoni is distributed in many regions throughout the world, including Asia, Africa, Europe, America and Australia [1, 13, 16]. In Japan, it is distributed mainly in the western part [3, 4, 7, 8] and only a few epidemiological and clinical studies have been reported on canine B. gibsoni infection in eastern Japan. All of the confirmed cases of B. gibsoni infection in the eastern part of Japan were found among Tosa dogs, a fighting breed raised only in Aomori Prefecture [3, 10, 17]. Transmission of B. gibsoni in this area was thought not to occur via ticks [3], although the vector tick species, Haemaphysalis longicornis, is distributed throughout Japan [19]. Thus, the actual distribution of B. gibsoni in dogs in eastern Japan is unknown.

In general, Babesia infections are diagnosed based on the observation of a thin blood film stained with Giemsa. However, this method is affected by the subjectivity of the observers and is limited in its sensitivity. Recently, molecular methods, including polymerase chain reaction (PCR), have proven effective in some epidemiological studies of Babesia infection in dogs [3, 8]. Meanwhile, an enzyme-linked immunosorbent assay (ELISA) with immunodominant antigen P50 of B. gibsoni has been developed as a serodiagnostic method [2]. The advantages of PCR and ELISA over other techniques are their sensitivity and specificity for diagnosing canine babesiosis caused by B. gibsoni. Using the combination of these two methods, not only current infection but also past infections can be detected.

In the present study, an epidemiological survey of dogs suspected of having B. gibsoni infection was attempted using PCR and ELISA to determine the distribution of B. gibsoni infection in dogs in the eastern part of Japan.

MATERIALS AND METHODS

Sampling: For PCR and ELISA, EDTA-anticoagulated blood samples and sera were collected from 115 dogs examined at the animal hospitals located in 13 prefectures (Aomori, Iwate, Miyagi, Fukushima, Ibaraki, Tochigi, Gunma, Saitama, Chiba, Tokyo, Kanagawa, Yamanashi and Nagano) from February to October, 2003. The dogs covered in this study showed symptoms suggestive of B. gibsoni infection, including anorexia, anemia, icterus, hemoglobinuria, or fever, or showed B. gibsoni-like protozoa in thin blood films. Clinical status and epidemiological information were collected from the veterinarians treating these dogs at the same time.

DNA extraction and PCR: Total DNA was extracted from each canine sample with a QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany), adjusted to a total volume of 200 µl in TE buffer and stored at −20°C until use. For the screening of B. gibsoni, PCR amplification was performed in a 25-µl reaction mixture containing 5 µl of each DNA template with the primer set Gib599F and Gib1270R, which was designed based on the 18S rRNA gene sequence [8]. The amplification procedure was reported previously [6], but the annealing temperature in this study was always 55°C. Genus-specific PCR for Ehrlichia and Babesia [5, 27], and screening PCR for hemoplasmas [14] (including

*Present Address: INOKUMA, H., Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080–8555, Japan.
the organisms formerly known as *Haemobartonella* spp.) were also performed on all samples by using the primers listed in Table 1.

**ELISA:** ELISA with GST-P50t was essentially carried out according to the protocol of Verdida et al. [20]. Briefly, 96-well microplates were coated with the antigens, GST-P50t and GST (negative control), at a concentration of 250 ng per well. If the difference between the absorbance of the antigen (GST-P50t)-containing well and that of the control antigen (GST)-containing well was equal to or greater than 0.1, the reaction was considered positive. The ELISA titer was expressed as the reciprocal of the maximum dilution that showed a positive reaction.

**Statistical analyses:** To compare the results for different breed, sex, history of tick exposure and bites by other dogs, and the co-infection rate between PCR- and/or ELISA-positive dogs and the respective negative dogs, chi-square tests were performed. The results of the complete blood count were also compared between two groups using Mann-Whitney tests. Stat View Ver 5.0 (Hulinks) was used to analyze both tests, and values of P<0.05 were considered significant.

**RESULTS**

The geographic distributions of positive dogs detecte by *B. gibsoni*-specific PCR and ELISA are shown Fig. 1. Twenty-nine of 115 dogs (25.2%) in 7 prefectures were positive in the *B. gibsoni*-specific PCR. These dogs were from Aomori (13 of 14 dogs), Fukushima (1 of 3 dogs), Ibaraki (3 of 9 dogs), Gunma (2 of 17 dogs), Chiba (4 of 20 dogs), Tokyo (4 of 15 dogs) and Kanagawa (2 of 12 dogs) Prefectures. Twenty-seven of 115 dogs (23.5%) in 8 prefectures were seropositive in the ELISA with GST-P50t. Nagano Prefecture was newly added to the area of positive in the ELISA with GST-P50t. Nagano Prefecture was newly added to the area of positive in the ELISA (Fig. 1). Twenty-one of 29 dogs that were positive by PCR were also positive by ELISA. However, 8 of 29 dogs that were positive by

---

### Table 1. Oligonucleotide sequences of primers used to detect pathogens in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Oligonucleotide sequence (5′–3″)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. gibsoni</em>-specific primer</td>
<td></td>
</tr>
<tr>
<td>Gib599F</td>
<td>CTC-GGC-TAC-TTG-CCT-TGT-C</td>
</tr>
<tr>
<td>Gib1270R</td>
<td>GCC-GAA-ACT-GAA-ATA-ACG-GC</td>
</tr>
<tr>
<td>Babesia genus-specific primer</td>
<td></td>
</tr>
<tr>
<td>RIB19</td>
<td>CGG-GAT-CCA-ACC-TGG-ATG-CGT-C</td>
</tr>
<tr>
<td>RIB20</td>
<td>CCG-AAT-TCC-TGG-TTA-GCA-CTC</td>
</tr>
<tr>
<td>Hemoplasma-specific primer</td>
<td></td>
</tr>
<tr>
<td>F2</td>
<td>ACG-AAA-GTC-TGG-AGC-AAT-A</td>
</tr>
<tr>
<td>R2</td>
<td>ACG-CCC-AAT-AAA-TCC-G(A/G)A-TAA-T</td>
</tr>
<tr>
<td><em>Ehrlichia</em> genus-specific primer</td>
<td></td>
</tr>
<tr>
<td>EHR16SD</td>
<td>GGT-ACC-ACG-AGA-AGT-CC</td>
</tr>
<tr>
<td>EHR16SR</td>
<td>TAG-CAC-TCA-TCG-TTC-ACA-GC</td>
</tr>
</tbody>
</table>
PCR were negative by ELISA. Six dogs were negative by PCR and positive by ELISA. Thirty-five of 115 dogs (30.4%) were positive by PCR and/or ELISA. The remaining 80 dogs were negative (69.6%) by both PCR and ELISA (Table 2).

*Babesia* genus-specific PCR did not detect any new positive results. All samples were negative for *Ehrlichia*. However, 36 of 115 dogs were positive by screening PCR for hemoplasmas.

Breed, sex, history of tick exposure and bites by other dogs, and hemoplasma infection rate of PCR- and/or ELISA-positive dogs were compared with those of dogs negative in both tests. The results are shown in Table 3. The PCR- and/or ELISA-positive dogs consisted of 28 Tosa dogs, 4 American Pit Bull Terriers and 3 mongrels. All of these Tosa dogs and 1 of the 3 mongrel dogs were male, while the other 6 dogs were female. Only 3 of the 35 positive dogs had confirmed history of tick exposure, and the other dogs either had no history of tick exposure or their exposure history was unknown. Twenty-six of the 35 positive dogs had bites by other dogs and the rate of bites was significantly higher than that of negative dogs.

Twenty-two of 35 PCR- and/or ELISA-positive dogs (62.9%) were positive for hemoplasma infection by PCR, while the rate of hemoplasma infection in *B. gibsoni*-negative dogs was 17.5%. The difference was significant.

The mean ± standard deviation of the platelet counts in dogs that were PCR- and/or ELISA-positive was 16.31 ± 15.39 (× 10^4 µl), and this was significantly lower than that in PCR- and ELISA-negative dogs [29.97 ± 17.38 (× 10^4 µl)]. However, there were no significant differences in the percentage of animals with fever and jaundice, or in RBC counts, PCV or hemoglobin concentration between dogs that were PCR- and/or ELISA-positive and dogs that were PCR- and ELISA-negative.

**DISCUSSION**

In the present study, PCR and ELISA were used to perform an epidemiological study of *B. gibsoni* infection in dogs in eastern Japan. Both the PCR and ELISA with GST-P50t tests used in this study have been reported to be sensitive and specific methods for the detection of *B. gibsoni* infection in dogs [8, 20]. Using these tests, we detected *B. gibsoni* infection in 35 dogs from 8 prefectures in the eastern part of Japan.

Positive results in the PCR test imply the existence of *B. gibsoni* protozoa in the peripheral blood, namely current
infection. On the other hand, positive results in ELISA imply the existence of antibody to *B. gibsoni*, and thus this method can detect infection in the past as well as current infection. Thus, dogs with positive results in PCR and/or ELISA are considered to have experienced *B. gibsoni* infection. Conversely, dogs with negative results in both PCR and ELISA have a low likelihood of having experienced *B. gibsoni* infection. Eight of 29 dogs were PCR positive but negative by ELISA. This result may have occurred in the following situations: in the early stage of *B. gibsoni* infection before antibody production, or in dogs with low antibody production owing to immunosuppressant therapy or underlying diseases inducing immunosuppressive conditions. Six dogs were PCR negative and ELISA positive. This indicates that these dogs had infection of *B. gibsoni* in the past, and antibody production against *B. gibsoni* was long-lasting, although *B. gibsoni* antigens in the peripheral blood were currently under the limit of detection by PCR. Another possibility is that the parasites were distributed mainly in other organs such as the spleen, although they were low in peripheral blood.

Thirty-two of 35 dogs that were positive by PCR and/or ELISA were traditional fighting breeds: Tosa dogs (28 dogs) and American Pit Bull Terriers (4 dogs). They had a significantly lower rate of tick exposure and higher rate of bites by other dogs compared to dogs negative in both tests. This result suggests that the main route of transmission of *B. gibsoni* is generally transmitted by ticks such as *Haemaphysalis longicornis* or *Rhipicephalus sanguineus* [11, 12]; however, it can also be transmitted by direct blood contamination, such as through fighting or blood transfusions.

Three mongrel dogs that were PCR and/or ELISA positive had no history of dog bites. One of them had a prior history of tick exposure in Hyogo Prefecture and subsequently showed clinical manifestations including lethargy, anorexia, anemia, and fever, suggesting *B. gibsoni* infection. It is possible that the dog was infected with *B. gibsoni* via ticks in western Japan. The other two dogs had no apparent tick exposure, and their routes of infection were uncertain.

The present study revealed that most of the dogs positive for *B. gibsoni* showed thrombocytopenia. This finding is similar to those of previous studies [9, 15]. However, there were no other significant differences of clinical manifestations between the PCR- and/or ELISA-positive dogs and the dogs negative for both tests.

Recently the red cell parasites formerly known as *Hae-mobartonella* spp. have been reclassified as hemotropic mycoplasmas (hemoplasmas) [18]. In our study, the rate of co-infection of hemoplasma in the PCR- and/or ELISA-positive group was significantly higher than that of *B. gibsoni*-negative dogs, but there were no significant differences of clinical manifestations in dogs with versus without co-infection. This finding suggests that the route of transmission of *B. gibsoni* and hemoplasma was the same. However, there have been no studies reported on this issue. Further studies will be necessary to clarify the relationship between *B. gibsoni* and hemoplasma infection in dogs. Fourteen among 80 dogs that were negative by *B. gibsoni* PCR and ELISA were hemoplasma positive. Thus, the clinical manifestations among these dogs may possibly be caused by hemoplasmas; however, the rest of 66 dogs were detected no pathogens in this study. The causes of the clinical symptoms found in these dogs were not determined in this study.

To date no ticks in the eastern part of Japan have been found to be positive for *B. gibsoni* [7]. The present study revealed that *B. gibsoni*-infected dogs were widely distributed in the eastern part of Japan and most of them were fighting dogs and had a low rate of exposure to ticks. It is possible that *B. gibsoni* infection is prevalent only among fighting dogs in the eastern part of Japan. However, *Haemaphysalis longicornis*, a predominant tick species in dogs, is known to transmit *B. gibsoni* [11]. It is widely distributed throughout Japan, including eastern Japan, the area of this study [17]. Thus, it is also possible that *B. gibsoni* is transmitted through blood from infected dogs to ticks and may be established among ticks in the eastern part of Japan. Surveys of ticks as spontaneous vectors of *B. gibsoni* and dogs other than fighting dogs will be needed to evaluate the actual distribution of *B. gibsoni* in eastern Japan.

ACKNOWLEDGMENTS. The authors would like to acknowledge the assistance of the participating veterinary practitioners and their staffs. This work was supported in part by Merial Japan Ltd. and a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science.

REFERENCES

7. Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda,


