Studies on Canine Babesiosis in Okinawa Island

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ABSTRACT. In fifty three mongrel dogs transferred from the Okinawa animal control center to our laboratory, *Babesia* organisms were tempted out for detection by splenectomy and dexamethazone administration. As a result, 11 strains of *Babesia* organisms were detected in 10 dogs, 7 strains were large and resembled *B. canis*, and 4 were small resembling *B. gibsoni*. The blood drawn from the dog most heavily infected with the large parasites was inoculated into 3 healthy mongrel dogs, and the heaviest parasitemia drawn from these three dogs was prepared in liquid nitrogen. This prepared blood was inoculated into a beagle designated C-11. In turn its blood, once confirmed to be infected, was inoculated into beagle C-12. Infected blood drawn from C-12 was passaged to a third beagle designated as C-13. C-12 was subsequently inoculated with *B. gibsoni* after the large parasites had disappeared from its peripheral blood. Serial examinations were continued until the dog's death. IFA testing was done on the sera of the inoculated beagles both prior to inoculation, and after the appearance of parasites in the peripheral blood. Sera from C-11 and blood smears containing large parasites were sent to Dr. Ristic, for identification. From data obtained in this research, and Dr. Ristic's independent results, the large parasites detected by us from dogs on Okinawa Island were concluded to *B. canis*.—**Key words:** babesiosis, canine, Okinawa.

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INTRODUCTION

Three species of *Babesia* are known as dog parasites. These species are *Babesia canis*, *B. vogeli*, and the smaller *B. gibsoni*. Only the small type is reported to have been found in Japan [8]. Izeki [5] reported a high incidence of canine babesiosis in Ohita prefecture and assumed that it was caused by *B. gibsoni* or a closely related parasite. Canine piroplasmosis was later reported in Miyazaki, Tokyo, Hyogo, Osaka, Tokushima, Kyoto, Nagoya, and Kagoshima prefectures and cities. All outbreaks were considered to be caused by *B. gibsoni*. Inakaba [2] reported a case of canine piroplasmosis in 1957, and Ogura suspected it to be caused by *B. canis* from the physical characteristics of the para-

site, in his explanation in the text book of Veterinary Microbiology [9]. However, the mode of infection was not clear, and similar cases have not been reported thereafter. In Japan, *B. gibsoni* has been recognized as the cause of canine babesiosis, whereas infections with *B. canis* or other parasites have not yet been confirmed.

As we have often encountered patients suspected of canine babesiosis in the clinic, the distribution of *Babesia* species causing this disease in Okinawa Island in 1979~1981 was investigated. Mongrel dogs transferred from the Okinawa animal control center were used in the study. Large and small types of *Babesia* organisms were isolated from these dogs, and the large type was passaged to healthy canines. Serological testing was

Table 1. Experimental animals and detected *Babesia* spp.

Area	Examined dogs	Dogs infected with <i>Babesia</i> spp.	<i>Babesia</i> spp.	
			Small type	Large type
north	8 (M5, F3)	1 (M)		1
middle	7 (M4, F3)	1 (M)		1
south	9 (M4, F5)	3 (M1, F2)	1	3
unknown	29 (M15, F14)	5 (M3, F2)	3	2
Total	53 (M28, F25)	10 (M6, F4)	4	7

Okinawa island

Table 2. Experimental dogs used in passage of large parasites

No.	Strain	Age	Sex	Body weight
54	Mongrel	2.0 year	M	10.0 kg
55	"	2.0	F	10.5
56	"	2.0	M	10.0
C-11	Beagle	1.0	M	11.0
C-12	"	1.0	M	13.0
C-13	"	1.0	M	11.0

done on each infected animal.

MATERIALS AND METHODS

Experimental animals: Fifty three mongrel dogs (No. 1~53) assumed to be approximately 1 year old by dentition and weighing about 10 kg, were obtained from the Okinawa animal control center and used for the detection of *Babesia* species (Table 1). Dogs introduced into our laboratory were preliminarily fed for 7~10 days, and treated to eliminate ectoparasites such as ticks and fleas. During this time, the dogs' general conditions were observed.

Detection of *Babesia* spp.: Estimations of red blood cell count (RBCC), packed cell volume (PCV), hemoglobin (Hb), white blood cell count (WBCC), and leukocyte percentages, as well as blood smear examinations for parasites, were carried out in blood samples drawn from the above-mentioned dogs. Sera were stored at -20°C . Following these tests, splenectomy was performed under general anesthesia and 0.1 mg/kg of dex-

amethazone (DM) was injected subcutaneously for 4 days post-surgically to induce a parasitemia. After the operation, blood samples were drawn 7 times every other day. The smears were stained with Giemsa stain and examined microscopically.

In detection of *Babesia* species by blood smears, 360 microscopic fields were examined per sample. Samples exhibiting any number of parasites were considered to be positive.

Experimental animals: Three non-infected mongrel dogs obtained from the Okinawa animal control center and 3 beagles which were homebred, healthy, and had no history of babesiosis were used in the passage of the large parasites (Table 2).

Infected blood and method of passage: Infected blood was drawn from dog No. 26, an animal severely infected with large parasites. This blood, and blood infected with *B. gibsoni* presented by Professor Naoyoshi Suzuki (Obihiro Univ.), were stored in liquid nitrogen prior to being used for passage [3]. Blood samples were drawn from dogs used in the passage, smeared for microscopic examination, and centrifuged to obtain sera. The dogs were then splenectomized and inoculated with infected blood from dog No. 26. Additionally DM was injected subcutaneously for 5 days to accelerate the infection.

Ten ml of the infectious blood was injected subcutaneously into the 3 mongrel dogs. Subsequently, 250ml of blood was drawn

Fig. 1 Small type parasites (*B. gibsoni*) found in the blood of dog No. 31. Giemsa stain. $\times 1000$.

Fig. 2 Large type parasites (*B. canis*) found in the blood of dog No. 26. Giemsa stain. $\times 1000$.

from the dog showing a very heavy parasitemia, and stored in liquid nitrogen. 4.5 ml of this blood was injected subcutaneously into beagle C-11, and 50 ml of its blood confirmed to be parasitemic was inoculated intravenously to beagle C-12. 50 ml of infected blood from beagle C-12 was inoculated to C-13 intravenously. Furthermore, 20 ml inoculation of *B. gibsoni* was given to C-12, after the large parasites had disappeared from its peripheral blood spontaneously.

The method of parasites detection: Every day, from day 2~14 post inoculation, and every other day thereafter for days 63 through 330, 360 fields of Giemsa stained blood samples were examined microscopically as in the above-mentioned survey. The criteria for severity of infection were as followed: No parasite found should be considered negative, more than 1 parasitized erythrocyte found as +, more than 1 found in every 10 fields as ++, more than 1 found in every field as +++, and more than 10 found in every field as ++++.

Seroreactions of dogs passaged with parasites: Seroreactions of dogs passaged with

parasites were tested by the indirect fluorescent antibody technique (IFA). FITC labeled anti-dog IgG rabbit serum (Biochemical Industry, Tokyo) was used for IFA, and blood infected with large parasites drawn from C-11 and blood samples of *B. gibsoni* were used as antigens. The methods of antigen preparation and estimating antibody titers were based upon the research of Ristic *et al.* [12]. Sera drawn from C-11, before and 330 days post inoculation, and blood smears containing the large parasites were sent to Dr. Ristic (Univ. of Illinois), for identification.

RESULTS

Detection of Babesia spp.: Ten of fifty three dogs (18.9%) were found to have parasites within their erythrocytes. Three of the 10 dogs were infected with small type of *Babesia*. Parasites found in the three were circular or oval in the shape, and closely resembled *B. gibsoni* (Fig. 1). Six of the ten were infected with larger parasites, and found to be pearled, double pearled, triple pearled, quartered, ring shaped, or amoeboid, exhibiting a close similarity to *B. canis* (Figs. 2-4).

Fig. 3. Large type parasites (*B. canis*) found in the blood of beagle C-11. Giemsa stain. $\times 1000$.

The other one was infected with both types.

Measurement of large parasites: We classified the large parasites into 5 groups by shape, and estimated the size by measuring their length and width (Table 3).

The severity of infection with large parasites in dogs No. 54, 55, and 56, from samples drawn 10 days post inoculation with blood from dog No. 26, were evaluated as +, +, and ++ respectively. Specific changes in general conditions such as body temperature, appetite, and vigor were not noted in these three dogs. 250 ml of blood was drawn from dog No. 56 according to the severity of the parasitemia 11th days after inoculation and preserved in liquid nitrogen.

Clinical observations and results of parasite detection: Dog C-11 showed an increased body temperature of 40.3°C , and parasitemia with large parasites valued ++ on the 8th day after inoculation with 4.5 ml of preserved blood from dog No. 56. A fever of about 40°C was persistent for 7 days, and parasitemia valued ++ continued for the same amount of time. After this period, the body temperature decreased to normal and stayed within

Fig. 4. Large type parasites (*B. canis*) found in the blood of beagle C-12. Giemsa stain. $\times 10000$. Abbreviation G: *B. gibsoni*, P: Peared, DP: Double peared, TP: Triple peared, Q: Quartered, R: Ring shaped, A: Amoeboid

the normal range until the experiment's termination. A parasitemia valued + or ++ continued until the 52nd day post inoculation, and thereafter no parasitemia was apparent with the exception of 4 blood smears valued + during the period 52~120 days after inoculation. The initial RBCC was 565×10^4 . This value decreased comparatively rapidly as a parasitemia appeared, and was lowest at 235×10^4 on the 17th day. Thereafter, the number of RBC's increased gradually and recovered to the initial value over 7 weeks. As expected, the PCV also showed changes proportional to the RBC values. The minimum PCV was 22.5%. The Hb values showed the same tendency as the PCV, while the WBCC kept within almost normal ranges throughout the period of observation. Dog C-11 is presently alive, 2 years after the beginning of the experiment.

Fifty ml of blood drawn from C-11, 13 days after inoculation with parasites was injected intravenously to C-12. An elevation in body temperature to 40.2°C and parasite-

Table 3. Size of large type parasites (μm)

Shape	Peared	Double peared	Quartered	Ring shaped	Amoeboid
Length	$4.19 \pm 0.85^*$	3.45 ± 0.52	3.30 ± 0.33	3.66 ± 0.70	4.09 ± 0.91
Width	2.67 ± 0.41	2.40 ± 0.54	2.18 ± 0.4	2.97 ± 0.43	3.25 ± 0.78

*Mean $\pm$ SDTable 4. Antibody titer in dogs used for *Babesia* spp. passage

No.	Pre-passaged serum		Post-passaged serum	
	<i>Babesia</i> large type	<i>B. gibsoni</i>	<i>Babesia</i> large type	<i>B. gibsoni</i>
C-11	<40	<40	1280	<40
C-12	<40	<40	320	320
C-13	<40	<40	160	<40

mia valued ++ were noted on the 3rd day after inoculation. A ++ parasitemia continued for 9 days; thereafter, parasites were detected only sporadically. 63 days after the initial inoculation, the large parasites could no longer be detected in the peripheral blood. At that time, C-12 was given an additional inoculation of 20 ml of *B. gibsoni* infected blood. A parasitemia valued +, caused by *B. gibsoni*, was found on the 3rd and 4th days after inoculation. The parasitemia increased to ++ on day 7 and 8, +++ on day 11, and ++++ on day 14, 17, and 21. The dog died on the 21st day. Body temperature was elevated to above 40°C on the 3rd, 4th and 5th days after the initial inoculation with large parasites, thereafter body temperature was within the normal range except sporadic elevations on a few days when the dog was parasitemic. However, it was again elevated to above 40°C on the 8th~11th days after inoculation with *B. gibsoni*. Initial values of RBCC and PCV were 795×10^4 and 44% respectively. These values decreased rapidly once parasitemia was detected, and reached a minimum of 200×10^4 and 20% on the 39th day after the initial inoculation. At the time of the second inoculation, the RBCC and PCV were 354×10^4 and 21.5%, and on the day of death, they were 260×10^4 and 17%, respectively. Appetite and vigor remained almost normal

except a slight decrease during the first febrile episode. However, 19 days after inoculation with *B. gibsoni*, the dog became lethargic and anorectic.

Fifty ml of blood drawn from C-12, 23 days after inoculation with parasitemic blood from C-11, was injected intravenously to C-13. A parasitemia valued ++ was detected on the 2nd~7th days post inoculation. After day 7, parasitemia was detected only sporadically until the final inspection. Changes in body temperature, appetite, vigor, etc. showed to be very similar to C-11. RBCC and PCV were estimated as 695×10^4 and 45% before inoculation, and the lowest values were found to be 198×10^4 and 11.5% on the 68th day after inoculation. These values then recovered gradually and showed to be almost normal on the final inspection. This dog is still alive, 2 years after the beginning of the experiment.

Results of seroreactions of dogs passaged with Babesia spp.: Blood samples were drawn from beagles C-11, C-12, and C-13 before inoculation with *Babesia* organisms. Post-inoculation titers were drawn from C-11 on 330th day, from C-12 on 63rd day, and from C-13 on 136th day after inoculation. Using sera and IFA antigen prepared from the infected blood of C-11, IFA testing was carried out. As indicated in Table 4, anti-

body titers were lower than 1:40 in the 3 dogs before inoculation. Post-inoculation titers were elevated as 1:1280 (C-11), 1:320 (C-12), and 1:160 (C-13). A second round of IFA testing was carried out using sera obtained from C-12, 21 days after the additional inoculation with *B. gibsoni*, the previously drawn sera of dogs C-11~C-13, and IFA antigen prepared from *B. gibsoni* infected blood. As indicated in Table 4, only the serum from C-12 showed a high antibody titer, elevated as 1:320 against *B. gibsoni*. The titers of C-11 and C-13 were lower than 1:40.

The sera from C-11, and the blood smears containing large parasites found in our studies were sent to Dr. Ristic, for identification of the parasites. According to his investigations, serum antibody titers, before inoculation of dog No. 56, were negative to both *B. gibsoni* and *B. canis*. Serum taken on the 330th day after inoculation showed titers of 1:10240 to *B. canis* and none to *B. gibsoni* by IFA testing. Parasites seen in the blood smears were all identified as *B. canis* (Ritic, M. 1982: Personal communication).

DISCUSSION

Etiologic organisms causing canine babesiosis are now classified into 3 species, *B. gibsoni*, *B. canis*, and *B. vogeli* [8]. Canine babesiosis reported in Japan has been considered to be caused primarily by *B. gibsoni*. One case was suspected as being caused by *B. canis* according to the parasite's morphology, but it had not been confirmed before this study. There are no reported cases of *B. vogeli* in Japan. We attempted to isolate *Babesia* species in 53 mongrel dogs on Okinawa Island, by splenectomy and DM administration. As a result, 10 of 53 dogs were found to be positive for parasites. One of the 10 exhibited two different species. The parasites were classified into 2 groups, large and small. Six dogs exhibited large parasites, 3 exhibited small, and one exhibited both. The

morphology of the large parasites was examined in peripheral blood smears, and it was found that the sizes varied according to the shape. Generally, they were found to be 3.3~4.2 μm in length and 2.2~3.3 μm in width (Table 3). These sizes were somewhat smaller than in previously reported cases of *B. canis* (4.5~5.0 μm by 2.5~3.0 μm) [7].

Iwahashi [4] reported that the primary signs of canine babesiosis were hemolytic anemia and splenomegaly. It is reported that, with *B. canis*, anemia, splenomegaly, and jaundice are consistent signs. Partial or total anorexia and a high fever have also been observed. Hemoglobinuria was a rare finding [1]. Purnell [11] stated that acute symptoms characteristic of dogs infected with *B. canis* were hemoglobinuria, hemoglobinemia, jaundice and an elevated body temperature. In dogs passaged with large parasites in this study, decreases in RBCC and PCV were observed; however, neither hemoglobinuria, hemoglobinemia, jaundice, nor marked changes of appetite and/or vigor were noted. Moreover, the changes in blood properties gradually recovered and the dogs tolerated the disease.

Appearances of parasitemia in dogs given passaged blood varied according to the route and dosage of inoculation. Parasitemia appeared within 10 days in dog No. 56, 8 days in C-11, 3 days in C-12, and 2 days in C-13. In C-12 and C-13, 50 ml of infected blood was injected intravenously. Although a more severe infection was expected, no specific differences were noted in the course of the disease. Parasitemias in these 4 dogs were noted at varying intervals after inoculation. Furthermore, the parasitemia was noted only sporadically throughout the course of the disease, and no tendency of increased severity was found. Phillips *et al.* [10] reported that inoculation with *B. gibsoni* after splenectomy caused a fatal disease in dogs. It was also reported that when dogs infected with *B. gibsoni* were splenectomized during conva-

lescent stages of the disease, they relapsed into an acute stage [10]. This information corresponds to findings in case C-12 after the dog was additionally inoculated with *B. gibsoni*.

By comparison with *B. gibsoni*, the large parasites detected in this study are weak in both proliferation and pathogenicity. These facts, as well as a difference in morphology, suggest that this parasite is a different species to *B. gibsoni*.

Using the blood infected with large parasites from C-11 as antigen, IFA testing with sera of C-11, C-12, and C-13 taken before inoculation showed to be negative for the antigen. Antibody titers after inoculation were as high as 1:1280, 1:320, and 1:160, respectively. These sera all showed negative reactions to *B. gibsoni*. According to these results, the large parasites have no cross-reaction with *B. gibsoni*, and were shown to belong to a different species.

The results of IFA tests, using post inoculation serum from C-11, carried out by Dr. Ristic, showed results similar to our own. The difference between the titers, 1:10240 estimated by him and 1:1280 by us, is thought to be due to the difference of antigens used in the respective IFA tests. Furthermore, Dr. Ristic identified the parasites found in blood smears from C-11 as *B. canis* by morphology.

Among the ticks carrying *B. canis*, *Rhipicephalus sanguineus* is thought to be most important, and it can effect transovarial transmission [8]. The distribution of this tick was reported by the 406th Medical General Laboratory Camp Zama in 1957 to be very limited in Japan, the Ryukyu Islands, and Korea; however it is quite possible that the ticks have been brought into Okinawa by military working dogs and pets accompanying U.S. forces here [13]. Kitaoka [6] stated in 1977 that this species is being found on dogs in Okinawa with increased frequency. Ticks gathered from many of 53 mongrel dogs used in our experiment also belonged to

the species *R. sanguineus*.

From the above-mentioned experimental results, Dr. Ristic's IFA results using the serum of C-11 and his identification of large parasites found in the blood smears, and the fact that *R. sanguineus* can be considered endemic in Okinawa, the large parasites detected in this study were concluded to be *B. canis*.

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要 約

沖縄本島における犬バベシア症に関する研究: 与那嶺久雄・一木彦三・浜川昌敬²⁾・島袋 哲²⁾・杉山公宏¹⁾・磯田政恵¹⁾ (日本獣医畜産大学獣医学科獣医第二外科学教室, ¹⁾同獣医病理学教室, ²⁾沖縄県家畜衛生試験場) —著者らは沖縄本島における犬バベシア症の実態を知る目的でこの研究を行なった。沖縄県動物管理所から譲渡された雑種犬53頭について, 脾摘除術および dexamethazone の投与によりバベシア属原虫の誘出をはかったところ, 10頭から11株のバベシア属原虫を検出した。そのうち大型の7株は *Babesia canis* に, 他の小型の4株は *B. gibsoni* に近似していた。大型原虫が検出された血液を健康雑種犬3頭に接種し, 最も強い原虫血症を示した1頭から血液 250 ml を採取して液体窒素に保存後, 健康なビーグル1頭に接種し, その感染血液を順次継代して原虫血症および臨床症状を観察した。また原虫の自然消失後に *B. gibsoni* を追接種して死亡まで観察した。いっぽう, 感染血液の接種前および原虫血症確認後の血清について間接蛍光抗体法による抗体検出を行なった。以上の実験成績および *Rhipicephalus sanguineus* が常在する環境から, 著者らが沖縄本島の犬から検出した大型原虫は *B. canis* であることが明らかになった。