Epidemiological survey of Babesia gibsoni infection in dogs in eastern Japan

Full Paper [Parasitology] **JVMS No. 4163**

1st Revised MS on 22 November 2004

Epidemiological Survey of Babesia gibsoni Infection in Dogs in Eastern Japan

Takako MIYAMA[1)], Yoshimi SAKATA[2)], Yojiro SHIMADA[3)], Shoji OGINO[3)], Malaika WATANABE[1)], Kazuhito ITAMOTO[1)], Masaru OKUDA[1)], Rodolfo A. Verdida [4)] Xuenan XUAN[4)], Hideyuki NAGASAWA[4)] and Hisashi INOKUMA*[1)]

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

*Correspondence to: H. INOKUMA, Fax. +81 (83) 933-5895

E-mail address: inokuma@yamaguchi-u.ac.jp

Running head: CANINE BABESIA GIBSONI INFECTION IN EASTERN JAPAN

関東以北の犬における Babesia gibsoni 感染状況調査--見山孝子[1)]・坂田義美[2)]・島田洋二郎[3)]・荻野祥樹[3)]・渡辺麻麗香[1)]・板本和仁[1)]・奥田 優[1)]・Rodolfo A. Verdida[4)]・玄 学南[4)]・長澤秀行[4)]・猪熊 壽[1)]

([1)]山口大, [2)]メリアル・ジャパン(株), [3)]日本全薬工業(株), [4)]帯畜大・原虫病研究センター)

関東以北の犬の Babesia gibsoni 感染状況を調べる目的で、2003 年 4 月から 10 月までの間、関東以北の 13 都県の動物病院を対象として調査を行った。B. gibsoni 感染症を疑った犬 115 頭の末梢血、および血清または血漿を採取し、B. gibsoni 特異的 PCR および ELISA を用いて感染の有無を確認した。115 頭のうち青森、福島、茨城、群馬、千葉、東京、神奈川、長野 8 都県の 35 頭が、PCR もしくは ELISA で陽性を示した。陽性犬 35 頭の品種は、土佐犬 28 頭、アメリカン・ピット・ブル・テリア 4 頭、雑種 3 頭であった。陽性犬におけるマダニ寄生歴が明らかなものは 3 例だけであり、感染経路としてマダニ以外の要因が関与することが考えられた。陽性犬 35 頭のうち、22 頭で hemoplasma の感染が確認され、この割合は B. gibsoni 陰性犬よりも有意に高かった。

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

INTRODUCTION

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℃ 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℃. Genus-specific PCR for Ehrlichia and Babesia [5, 27], and screening PCR for hemoplasmas [14] (including 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 detected by B. gibsoni -specific PCR and ELISA are shown in 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 15dogs) 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 (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 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 ($\times 10^4$μl), and this was significantly lower than that in PCR- and ELISA-negative dogs [29.97±17.38 ($\times 10^4$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 on 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 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 in fighting dogs may be bite wounds rather than tick bites. 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 Haemobartonella spp. have been reclassified as hemotorophic 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

1. Farwell, G.E., LeGrand, E.K.and Cobb, C.C. 1982. Clinical observations on Babesia gibsoni and Babesia canis infections in dogs. J. Am. Vet. Med. Assoc. **180**: 507-511.
2. Fukumoto, S., Sekine, Y., Xuan, X., Igarashi, I., Sugimoto, C., Nagasawa, H., Fujisaki, K., Mikami, T. and Suzuki, H. 2004. Serodiagnosis of canine Babesia gibsoni infection by enzyme-linked immunosorbent assay with recombinant P50 expressed in Escherichia coli. J. Parasitol. **90**: 387-391.
3. Ikadai, H., Tanaka, H., Shibahara, N., Matsuu, A., Uechi, M., Itoh, N., Oshiro, S., Kudo, N., Igarashi, I. and Oyamada, T. 2004. Molecular evidence of infections with Babesia gibsoni parasites in Japan and evaluation of the diagnostic potential of a loop-mediated isothermal amplification method. J. Clin. Microbiol. **42**: 2465-2469.
4. Inokuma, H., Tamura, K. and Onishi, T. 1995. Incidence of brown dog ticks, Rhipicephalus sanguineus, at a kennel in Okayama Prefecture. J. Vet. Med. Sci. **57**: 567-568.
5. Inokuma, H., Ohno, K., Onishi, T., Raoult, D. and Brouqui, P. 2001. Detection of ehrlichial infection by PCR in dogs from Yamaguchi and Okinawa Prefectures, Japan. J. Vet. Med. Sci. **63**: 815-817.

6. Inokuma, H., Fujii, K., Matsumoto, K., Okuda, M., Nakagome, K., Kosugi, R., Hirakawa, M. and Onishi, T. 2002. Demonstration of Anaplasma (Ehrlichia) platys inclusions in peripheral blood platelets of a dog in Japan. Vet. Parasitol. **110**: 145-152.

7. Inokuma, H., Yoshizaki, Y., Shimada, Y., Sakata, Y., Okuda, M. and Onishi, T. 2003. Epidemiological survey of Babesia species in Japan performed with specimens from ticks collected from dogs and detection of new Babesia DNA closely related to Babesia odocoilei and Babesia divergens DNA. J. Clin. Microbiol. **41**: 3494-3498.

8. Inokuma, H., Yoshizaki, Y., Matsumoto, K., Okuda, M., Onishi, T., Nakagome, K., Kosugi, R. and Hirakawa, M. 2004. Molecular survey of Babesia infection in dogs in Okinawa, Japan. Vet. Parasitol. **121**: 341-346.

9. Inokuma, H., Okuda, M., Yoshizaki, Y., Hiraoka, H., Miyama, T., Itamoto, K., Une, S., Nakaichi, M. and Taura, Y. 2004. Clinical observation of Babesia gibsoni infection with low parasitemia confirmed by PCR. Vet. Rec. (in press).

10. Itoh, N., Higuchi, S., Ogasawara, T., Ogasawara, A. and Kawamura, S. 1987. An out break of canine Babesiosis in Aomori Prefecture. J. Jpn. Vet. Med. Assoc. **40**: 167-171 (in Japanese with English summary).

11. Higuchi, S., Simomura, S., Yoshida, H., Hoshi, F., Kawamura, S. and Yasuda, Y. 1991. Development of Babesia gibsoni in the gut epithelium of the tick, Haemaphysalis longicornis. J. Vet. Med. Sci. **53**: 129-131.

12. Higuchi, S., Fujimori, M., Hoshi, F., Kawamura, S. and Yasuda, Y. 1995. Development of Babesia gibsoni in the salivary glands of the larval tick, Rhipicephalus sanguineus. J. Vet. Med. Sci. **57**: 117-119.

13. Jefferies, R., Ryan, U.M., Muhlnickel, C.J. and Irwin, P.J. 2003. Two species of canine Babesia in Australia: detection and characterization by PCR. J. Parasitol. **89**: 409-412.

14. Jensen, W.A., Lappin, M.R., Kamkar, S. and Reagan, W.J. 2001. Use of a polymerase chain

reaction assay to detect and differentiate two strains of Haemobartonella felis in naturally infected cats. Am. J. Vet. Res. **62**: 604-608.

15. Kettner, F., Reyers, F. and Miller, D. 2003. Thrombocytopaenia in canine babesiosis and its clinical usefulness. J. S. Afr. Vet. Assoc. **74**: 63-68.

16. Lobetti, R.G. 1998. Canine Babesiosis. Comp. Cont. Educ. Pract. **20:** 418-430.

17. Matsuu, A., Kawabe, A., Koshida, Y., Ikadai, H., Okano, S., and Higuchi, S. 2004. Incidence of canine Babesia gibsoni infection and subclinical infection among Tosa dogs in Aomori Prefecture, Japan. J. Vet. Med. Sci. **66**: 893-897.

18. Messick, J. B. 2004. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. Vet. Clin. Pathol. **33**:2-13.

19. Shimada, Y., Beppu, T., Inokuma, H., Okuda, M. and Onishi, T. 2003. Ixodid tick species recovered from domestic dogs in Japan. Med. Vet. Entomol. **17**: 38-45.

20. Verdida, R.A., Hara, O.A., Xuan, X., Fukumoto, S., Igarashi, I., Zhang, S., Dong, Junyan, Inokuma, H., Kabeya, H., Sato, Y., Morimoto, T., Maruyama, S., Claveria, D. and Nagasawa, H. Serodiagnosis of Babesia gibsoni infection in dogs by an improved enzyme-linked immunosorbent assay with recombinant truncated P50.J. Vet. Med. Sci. (in press).

21. Zahler, M., Rinder, H., Zweygarth, E., Fukata, T., Maede, Y., Schein, E. and Gothe, R. 2000. 'Babesia gibsoni' of dogs from North America and Asia belong to different species. Parasitology **120**: 365-369.

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

Primer name	Oligonucleotide sequence (5'-3')
B.gibsoni-specific primer	
Gib599F	CTC-GGC-TAC-TTG-CCT-TGT-C
Gib1270R	GCC-GAA-ACT-GAA-ATA-ACG-GC
Babesia genus-specific primer	
RIB19	CGG-GAT-CCA-ACC-TGG-TTG-ATG-CGT-C
RIB20	CCG-AAT-TCC-TTG-TTA-CGA-CTC
Hemoplasma-specific primer	
F2	ACG-AAA-GTC-TGA-TGG-AGC-AAT-A
R2	ACG-CCC-AAT-AAA-TCC-G(A/G)A-TAA-T
Ehrlichia genus-specific primer	
EHR16SD	GGT-ACC-(C/T)AC-AGA-AGA-AGT-CC
EHR16SR	TAG-CAC-TCA-TCG-TTT-ACA-GC

Table 2 Results of PCR assay and ELISA in the 115 dogs with suspected B. gibsoni infection

		PCR		
		Numbers of positive dogs (%)	Numbers of negative dogs (%)	Total
ELISA	Numbers of positive dogs (%)	21 (18.3)	6 (5.2)	27 (23.5)
	Numbers of negative dogs (%)	8 (6.9)	80 (69.6)	88 (76.5)
	Total	29 (25.2)	86 (74.8)	115

Table 3 Comparison of breed, sex, tick exposure, bites by other dogs and infection with hemoplasma between dogs positive in PCR and/or ELISA for *B. gibsoni* and dogs negative in both tests

	PCR- and/or ELISA-positive N=35	PCR- and ELISA-negative N=80
Breed	Fighting dogs: 32*	Fighting dogs: 15
	Tosa: 28	Tosa : 7
	American Pit Bull Terrier: 4	American Pit Bull Terrier: 8
	Others: 3*	Others: 65
	Mongrel :3	Purebreds: 51
		Mongrel :14
Sex	Male: 29*	Male: 41
	Female: 6	Female: 39
Tick exposure	3*	38
Bites by other dogs	26*	7
Infection with hemoplasma	22*	14

*Significant difference compared with PCR- and ELISA-negative dogs ($P<0.05$)

Fig.1. Distributions of B. gibsoni-infected dogs in the eastern part of Japan. A) Result of B. gibsoni-specific PCR. B) Result of ELISA with GST-P50t..

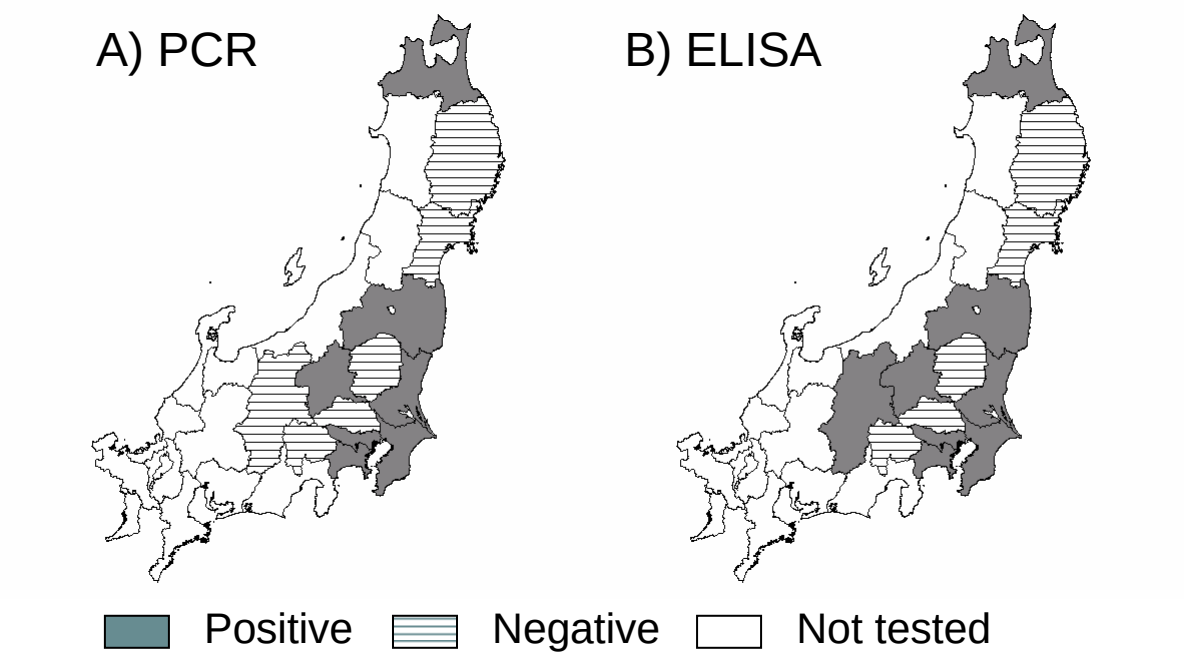

Fig.1