Short Communication

Epidemiological Survey of Babesia divergens Asia Lineage in Wild Sika Deer (Cervus nippon) by Using Direct PCR in Japan

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SUMMARY: Babesia divergens is the major causal agent of zoonotic human babesiosis across Europe. Previously, we reported the detection of a B. divergens Asia lineage in wild sika deer (Cervus nippon) in Japan which was genetically closely related to the European B. divergens. To further elucidate its etiology, we conducted a large epidemiological survey by combining lineage-specific PCR system and blood direct PCR. The infection rate of the Asia lineage was 6.6% (116/1,747) throughout Japan, where Hokkaido (45%), Nagano (17%), Iwate (12%), Gunma (11%), and Yamagishi (11%) were highly enzootic (>10%) among the 30 prefectures examined. European B. divergens was not detected. A geographical information system (GIS) map revealed dense populations of PCR-positive deer in the mountains including the Japanese Alps in eastern Honshu, and Hokkaido. These areas markedly overlapped with the major habitats of Ixodes persulcatus, a principal tick vector responsible for the transmission of the Asia lineage. Other areas in southern Japan including Miyazaki, Kagoshima, and Shimane Prefectures, where positive sika deer were sporadically detected, may be habitats for other tick species involved in the enzootic cycle as I. persulcatus were scarce. The rise in human babesiosis cases is occasionally attributed to healthy blood donors who were unaware of tick bites and Babesia infection. Therefore, there is an urgent need to investigate whether infections in humans have occurred in Japan.

Human babesiosis is an emerging tick-borne disease caused mainly by a zoonotic pathogen Babesia divergens (B. divergens EU lineage) across Europe. In Japan, we previously detected B. divergens which was closely related to the European B. divergens in sika deer (Cervus nippon) (B. divergens Asia lineage) (1,2). However, due to insufficient statistical data, it was impossible to draw definite conclusions regarding the geographical distribution of the pathogen.

To investigate deer in large numbers, we first developed a rapid detection system by applying the blood direct PCR (Phusion Blood Direct PCR kit, Thermo Fisher Scientific, Waltham, MA, USA) to the lineage-specific nested PCR methodology (2). To evaluate the effectiveness of the direct PCR methodology for detecting intra-erythrocytic Babesia parasites, direct PCR was performed on freshly prepared hamster’s erythrocytes infected with B. microti IpSG 13-1-2 strain (infection rate of 5.7%) (National Institute of Infectious Diseases approval no.114014), according to the manufacturer’s instructions. B. microti was used for this test as isolation of B. divergens was unsuccessful. Desired amplicons were obtained by using primers targeting 18S rRNA of Babesia, Piro0F, and Piro6R (3). Hamster’s red blood cells (RBCs) were used as template at 2–5% of total volumes (Fig. 1A). Based on this result, deer screening was performed as follows: For the 1st PCR, 0.4 µl of RBCs were added directly to a 20 µl mixture. Next, 1 µl of the 1st PCR amplified product was used in a 20 µl mixture (ExTaq, Takara Bio Inc., Shiga, Japan) (2). To detect B. divergens Asia lineage, primers targeting 18S rRNA, div101F/div1353R and divJA/div1296R were used for the 1st and 2nd PCR, respectively (2). Fig. 1B shows that the direct nested PCR method was highly effective for analyzing the field samples. After screening sika deer by the direct nested PCR, DNA was extracted randomly from the PCR-positive RBCs (50 samples) and further used for performing general nested PCR (2).

Blood samples from wild sika deer, which were hunted from 2012–2018 in Japan, were collected from hunters. With each blood sample, information such as the deer’s age, sex, body weight, and geographical location where the deer was hunted were noted. Geographical location details for the 42 deer samples collected from Hokkaido (including 18 of the total positives) were not available. Blood was collected in heparin-coated tubes and sent to the laboratory for further analysis. Erythrocytes were separated by centrifugation and were then used in the direct nested PCR. A total of 1,609 samples were examined and their results are listed in Table 1. Table 1 also includes the result of our previous studies conducted in 2007 and 2008 (1). Based on the results of both the studies, we calculated the overall infection rate as 6.6% (116/1,747). Among 30 prefectures examined, positive
sika deer were found at 15 prefectures where infection rates varied from 44.6% (Hokkaido) to 1.1% (Shimane) (Fig. 2A). Throughout this study, other lineages of B. divergens including EU and US (1) were not detected. To precisely identify the endemic area, the geographic information system (GIS) map was generated based on the geographical distribution data of the hunted PCR-positive deer (2007, 2008, and 2012–2018) (Fig. 2B left). The deer infected with the Asia lineage appeared to be widely distributed throughout Hokkaido even after excluding the 18 PCR-positive cases whose geographical location data were unavailable. In contrast, the positive

| Prefecture | Total 2012-2018 | 2007-2008<sup>1)</sup> |
|------------|----------------|------------------------|
|            | no. examined  | no. positive | % positive | no. examined | no. positive | no. examined | no. positive |
| Hokkaido   | 74            | 33          | 44.6%      | 56          | 27          | 18          | 6           |
| Aomori     | 1             | 0           | 0          | 1           | 0           | –           | –           |
| Iwate      | 77            | 10          | 13         | 66          | 9           | 11          | 1           |
| Miyagi     | 105           | 3           | 2.9        | 105         | 3           | –           | –           |
| Fukushima  | 4             | 0           | 0          | 4           | 0           | –           | –           |
| Tochigi    | 90            | 5           | 5.6        | 79          | 3           | 11          | 2           |
| Gunma      | 73            | 8           | 11         | 73          | 8           | –           | –           |
| Chiba      | 5             | 0           | 0          | –           | –           | 5           | –           |
| Kanagawa   | 37            | 1           | 2.7        | 37          | 1           | –           | –           |
| Yamashita  | 109           | 12          | 11         | 109         | 12          | –           | –           |
| Nagano     | 139           | 24          | 17.3       | 123         | 23          | 16          | 1           |
| Gifu       | 81            | 3           | 3.7        | 81          | 3           | –           | –           |
| Shizuoka   | 120           | 6           | 5          | 117         | 6           | 3           | 0           |
| Iwate      | 78            | 0           | 0          | 78          | 0           | –           | –           |
| Shiga      | 111           | 4           | 3.6        | 111         | 4           | –           | –           |
| Kyoto      | 72            | 0           | 0          | 72          | 0           | –           | –           |
| Hyogo      | 123           | 4           | 3.3        | 122         | 4           | 1           | 0           |
| Wakayama   | 64            | 0           | 0          | 64          | 0           | –           | –           |
| Tottori    | 40            | 0           | 0          | 40          | 0           | –           | –           |
| Shimane    | 87            | 1           | 1.1        | 87          | 1           | –           | –           |
| Hiroshima  | 41            | 0           | 0          | 36          | 0           | 5           | 0           |
| Yamaguchi  | 24            | 0           | 0          | 24          | 0           | –           | –           |
| Tokushima  | 5             | 0           | 0          | –           | –           | 5           | 0           |
| Ehime      | 28            | 0           | 0          | 28          | 0           | –           | –           |
| Kochi      | 25            | 0           | 0          | 25          | 0           | –           | –           |
| Fukuoka    | 28            | 0           | 0          | 28          | 0           | –           | –           |
| Oita       | 36            | 0           | 0          | 33          | 0           | 3           | 0           |
| Miyazaki   | 34            | 1           | 2.9        | 24          | 0           | 10          | 1           |
| Kagoshima  | 32            | 1           | 3.1        | 28          | 1           | 4           | 0           |
| Kumamoto   | 4             | 0           | 0          | –           | –           | 4           | 0           |

Total 1,747 116 6.6 1,609 87 138 29

<sup>1)</sup>: Zamoto-Niikura et al. Emerg Infect Dis. 2014. (Ref.1).
- : not done.
Fig. 1. Sensitivity of the direct PCR. (A) Direct PCR approach using various volumes of hamster’s RBCs infected with *B. microti* (infection rate, 5.7%) as template. M, marker. (B) Comparison of general (left) and direct PCR (right) methods. For direct PCR, 0.4 µl of RBCs was used in a 20 µl mixture for the 1st PCR. DNA extracted from 0.4 µl of RBCs was used for general PCR. Nested PCRs were performed in the same way.

Fig. 2. (Color online) Infection of *B. divergens* Asia lineage in sika deer in Japan. (A) Infection rate by prefecture. Since small number (below 5) of samples were examined in Aomori, Fukushima, Chiba, Tokushima and Kumamoto prefectures, these prefectures are colored as white (not done) (Table 1). Prefectures, in which infection rates are above 10%, are shown as Bold. (B left) Spots where deer infected with *B. divergens* were hunted. Note that 18 positives in Hokkaido were not included (see text). The map was generated using map tool provided by Geospatial Information Authority of Japan (http://maps.gsi.go.jp/#5/36.104611/140.084556/). (B right) Presumed distribution of *I. persulcatus* (gray).
sika deer in Honshu were mainly distributed along the mountainous areas including the Ryohaku, Hida, Kiso, Akashi, Kanto, and Echigo mountains in central part of Honshu, and Kitakami Mountains in northern part of Honshu. In addition, positive sika deer were sporadically detected in the eastern and southern mountainous areas. We did not observe any significant differences between the PCR-positive and PCR-negative deer with regard to weight, age, and sex (data not shown).

The Asia lineage was specifically carried by *I. persulcatus* tick in Hokkaido and all the sympatric ticks, such as *I. ovatus* and *Hemaphysalis* spp., were negative for the pathogen upon examination by the lineage-specific PCR (2). Although the tick survey was only conducted in Hokkaido, we speculated that *I. persulcatus* transmitted the Asia lineage in the eastern mountainous areas of the Honshu mainland, where *I. persulcatus* is the prevalent tick species. Strikingly, the present study revealed that *B. divergens* Asia lineage in sika deer (Fig. 2B left) was mainly detected in the areas which were the habitats of *I. persulcatus* (Fig. 2B right). Thus, our results strongly suggest that in east Japan, the lifecycle of the *B. divergens* Asia lineage exclusively included sika deer and *I. persulcatus* as the reservoir and vector, respectively. Further detailed studies are needed to define the vectorial capacity of *I. persulcatus*. We were unable to conduct thorough investigations in the northeastern areas of Honshu such as the Yamagata, Akita, Aomori, and Fukushima Prefectures. Additional studies are needed to further improve our understanding of the geographical distribution of the lineage.

PCR-positive sika deer were also found in the southern as well as western Japan including Kagoshima, Miyazaki, Shimane, and Hyogo prefectures. The presence of *I. persulcatus* has been recorded in the southern mountains of Kyushu at over 1,000 m elevation (4,5). Since *B. divergens* survives through three stages (egg, larva to nymph, and nymph to adult) (6,7), we speculated that it might be possible that the small number of *I. persulcatus* was enough for maintaining the *B. divergens* population in nature. Furthermore, the overabundant sika deer serve both as the reservoir and a blood meal for the female ticks. The Asia lineage might be spreading by the resulting larvae infected with this lineage. Extensive field survey of the ticks in the west and south would elucidate whether the tick played an important role as the vector.

Patients infected with *B. divergens* in Europe (or *B. divergens* EU lineage), as well as the emerging *B. divergens*-like parasites in the United States (or *B. divergens* US lineage), were either splenectomized and/or immunologically suppressed (8,9). However, recent serological studies in European countries indicated that asymptomatic infection occurred at a low level (0.1–2.1%) in a healthy person (10–13). Moreover, *B. divergens*-like infection occurred through transfusion in Arkansas, United States (14). Such transfusion-related infection cases raised concerns about blood safety in areas where *B. divergens* and *B. divergens*-like pathogens were distributed. In conclusion, our study revealed that sika deer infected with the *B. divergens* Asia lineage were widely distributed across eastern Japan. Thus, there is an urgent need to investigate infections in humans and to further assess the risk of acquiring the parasite through blood transfusions.

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**Conflict of interest** None to declare.

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