NOTE *Virology*

# Prevalence of Borna Disease Virus Antibodies in Healthy Japanese Black Cattle in Kyushu

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ABSTRACT. Epidemiological studies have demonstrated that asymptomatic infection of Borna disease virus (BDV) is found in various species of animals in Japan. Recent reports have also revealed that neurological diseases caused by this virus could exist in horses, cattle, a dog, and cats in this country. In this study, we investigated seroprevalence of BDV antibodies in Japanese black cows reared in Kyushu, the southernmost main island of Japan, using ELISA and Western-immunoblotting. Of 101 serum samples, 11 (10.9%) and 21(20.7%) sera were identified as having antibodies to the BDV N and P antigens, respectively. Among the positive sera, three cows (2.9%) were seropositive for both of the antigens. Furthermore, interestingly, only female cows showed antibodies to P, whereas N antibodies were detected in male and female cows with a comparative ratio. Together with previous studies, our results indicate that BDV might be widely spread in cattle raised in Japan. Furthermore, this is the first report to show that beef cattle, Japanese black cattle, have antibodies against a possible zoonotic pathogen, BDV.

KEY WORDS: Borna disease virus, epidemiology, Japanese black cattle.

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Borna disease (BD) is a severe, frequently fatal, neurological disease characterized by a progressive, nonpurulent meningoencephalitis in horses and sheep [19, 27, 29]. Although BD was originally described only in the endemic areas of southern Germany and Switzerland, BD horses have recently been found outside these endemic countries, including in Japan [2, 28]. Current efforts have also revealed that BD or Borna-like disease occurs in other species of animals, such as goats, cattle, cats, dogs, and ostriches [1, 23, 24, 26], from various geographic regions [1, 23, 26], indicating that this emerging disease is a matter of great importance in the veterinary field. The causative agent, Borna disease virus (BDV), is a negative-strand RNA virus that belongs to the *Mononegavirales* family. Epidemiological studies have demonstrated that asymptomatic natural infection of BDV is found worldwide in a wide variety of vertebrate species and have suggested that the host range of this virus probably includes all warm-blooded animals [18, 27]. Furthermore, mounting evidence indicates that BDV infection occurs in humans and that it may be related to neuropsychiatric diseases [5, 13, 17]. These observations give rise to the possible risk of BDV as a zoonotic pathogen.

In recent years, seroepidemiological and molecular epidemiological studies of BDV infection have been reported in Japan. These studies indicate that BDV antibodies and/or BDV-specific RNAs are present in apparently healthy animals from various species [9, 10]. Furthermore, it has been reported that BDV infection is demonstrated at high rates in horses having locomotor abnormalities with unknown etiology in Hokkaido [11]. Although almost all infected animals seem to be asymptomatic, interestingly, BD or Borna-like disease has been identified in horses, cattle, a dog, and cats in Japan [23, 24, 28]. Two cases of BD were found in horses and one in cattle in an area of Hokkaido, and Borna-like diseases were demonstrated in a dog and in cats in Kyushu and Honshu, respectively. These observations might be cause for alarm concerning the expansion of BDV infection in animals in Japan, although the mode of transmission for this virus is not well understood.

In Kyushu, the southernmost main island of Japan, BDV infection and transmission were well documented in the Toimisaki herd of Japanese feral horses [14]. Inoue *et al.* revealed that 26.9% of Misaki feral horses are seropositive for BDV after four years of surveillance using an electrochemiluminescence immunoassay. This observation suggested a relatively high prevalence of BDV infection in Kyushu. Despite the prosperity of stock raising in Kyushu, seroprevalence of BDV in the domestic animals of this area has not yet been investigated. In addition, epidemiological survey of BDV infection in beef cattle has never been performed.

The present study was undertaken to examine seroprevalence of BDV in healthy Japanese black cattle reared in Kyusyu. A total of 101 apparently healthy cattle were sampled from various regions of Kyushu in the winter season of 2002 (between November 2002 and January 2003). The mean age of the animals was 8.78 ± 3.17 years (4–16 years old). None of the animals showed neurological abnormalities at time of sampling. Sera were isolated from the EDTA-treated blood by centrifugation. All males and females examined were capable of breeding.

Initially, we performed enzyme-linked immunosorbent assay (ELISA) using soluble recombinant BDV phosphoprotein (P) expressed in *Escherichia coli*. BDV P is a major viral antigen that could be involved in viral replication as a

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polymerase cofactor and is commonly used as the target of epidemiological studies [10, 21]. Microtiter plates were coated with 100 $\mu l$ of PBS containing 1 $\mu$g of anti-P monoclonal antibody for 14 hr at 4°C. The plates were subsequently blocked with 0.5% skimmed milk in phosphated-buffered saline (PBS) for 3 hr at 37°C. After washing three times with 0.05% tween-20 in PBS (PBST), the wells were incubated with 0.02 $\mu$g of the recombinant antigen for 1 hr at 37°C. The microtiter plates were then washed three times and incubated with 100 $\mu l$ of a 1:100 dilution of the serum samples for 1 hr at 37°C. After washing 5 times with PBST, 50 $\mu l$ of 1:2,000 diluted HRP-conjugated anti-bovine IgG (Jackson ImmunoResearch Laboratories Inc.) was added and incubated for 1 hr at 37°C. After washing, 100 $\mu l$ of 0.4 mg/m*l* O-phenylenediamine in citrate-phosphate buffer (pH 5.4) with 0.012% hydrogen peroxide was added. The reaction was then stopped with 100 $\mu l$ of 3N $H_2SO_4$. The absorbance was measured at 492 nm. As negative controls, we assigned two bovine sera, which were judged as seronegative for both BDV N and P antigens by Western blotting and which induced a low absorbance in ELISA testing. In addition, 100 $\mu l$ of 1:500 diluted rabbit anti-P polyclonal antibody was used as a positive control. The fold values of the absorbance for the specimen to BDV-seronegative control sera are plotted in Fig. 1A. Interestingly, the female sera exhibited significantly higher absorbance compared with the male samples (male, 1.47 ± 0.42; female, 3.24 ± 2.72, $P<0.01$) (Fig. 1A). When the cut-off value was positioned at three fold of the mean absorbance for the negative samples, only females were seropositive to BDV P antigen (21 sera, 20.7%; Table 1) (sex differences, Chi-square test: $X^2=14.6838$, $P<0.01$). We performed Western blotting to confirm the reactivity of the ELISA. The recombinant BDV P fusion protein was separated by 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. The membrane strips were incubated with a 50-fold dilution of the serum samples at 37°C for 1 hr, and then the reacted antibodies were visualized using a Konika immunostain HRP-1000 kit. As a positive control, a rabbit polyclonal antibody to BDV P was used. To avoid non-specific reaction, we performed an absorption test as follows. Twenty $\mu$g of soluble recombinant P was added to each sample and incubated at 37°C for 1 hr. After centrifugation, the sera were reacted to membrane strips. Figure 1B shows representative results of the Western blot analysis. The assay indicated that serum containing a high absorbance (#39) by ELISA develops a strong intensity of reacted bands on the membrane, whereas sera with low absorbance induced none or weak bands for this assay (Fig. 1B and Table 1). As was the case in other studies [12, 20], the reactivity of the sera to P antigen did not necessarily correlate between the ELISA and Western blotting assays in this study. The major difference between the two methods may be caused by the form of the antigen. In Western blotting, the antigen is denatured into a linear structure and fixed on the membrane, whereas the ELISA reaction is performed in the liquid phase with the conformational structure of the antigen preserved.

Previous studies demonstrated that seroprevalence between BDV N and P antibodies is not necessarily correlated in many animals, including humans [7, 11, 22, 30]. Therefore, we next tried to detect the antibody to N antigen in the bovine sera. We selected Western blot analysis for

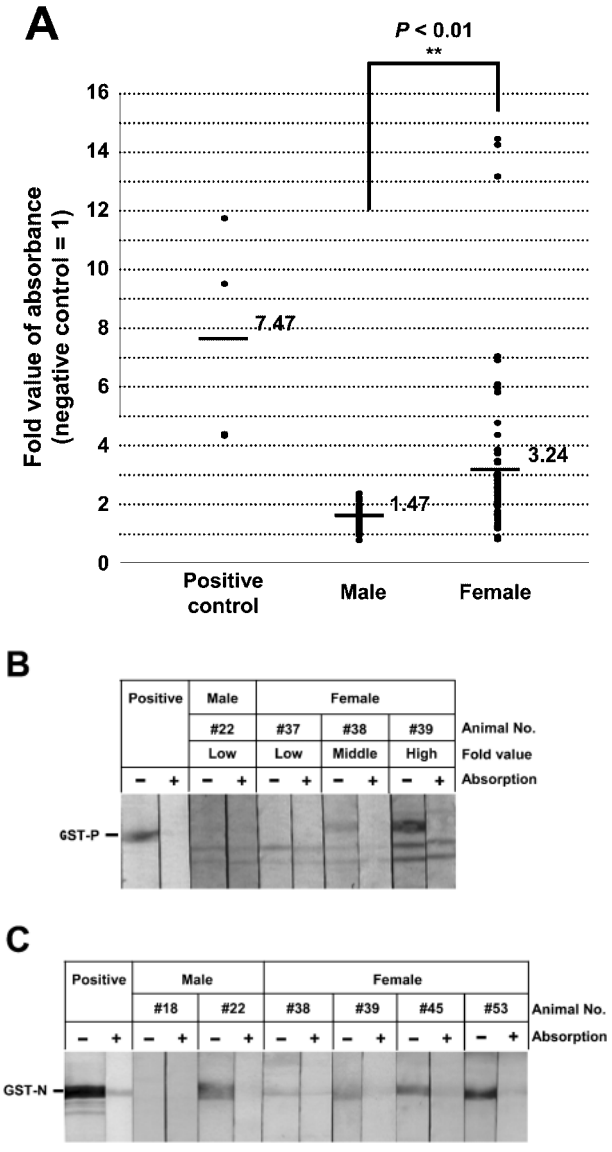


Fig. 1. Detection of anti-BDV antibodies in sera from Japanese black cattle. (A) Detection of anti-BDV P antibody by ELISA. The values plotted are the fold values of the absorbance for sample sera against the negative control. The numbers at the plots indicate the mean of the fold value for each group. BDV-infected rat sera were used as positive controls. (B) and (C) Representative positive and negative results of Western blotting to the BDV P (B) and N (C) antigens. The results of absorption test are also shown. Positive, BDV-infected rat serum. The fold value represents the fold absorbance to the negative control. Low, < 3 fold; Middle, 3–4 fold; High, > 7 fold.

Table 1. Prevalence of anti-BDV antibodies in Japanese black cows

| Animal # | Sex[a)] | Ages (Years) | BDV antibodies | |
|---|---|---|---|---|
| | | | N[b)] | P[c)] |
| 7 | M | 12 | + | – |
| 16 | M | 6 | + | – |
| 22 | M | 6 | + | – |
| 30 | M | 5 | + | – |
| 38 | F | 9 | – | + |
| 39 | F | 9 | + | +++ |
| 40 | F | 9 | – | + |
| 41 | F | 9 | – | ++ |
| 44 | F | 9 | + | – |
| 45 | F | 9 | + | + |
| 47 | F | 9 | – | +++ |
| 48 | F | 9 | – | ++ |
| 49 | F | 9 | + | – |
| 50 | F | 13 | – | + |
| 53 | F | 5 | + | +++ |
| 54 | F | 5 | – | + |
| 56 | F | 5 | – | + |
| 58 | F | 4 | – | + |
| 66 | F | 10 | – | + |
| 67 | F | 16 | + | – |
| 68 | F | 10 | – | ++ |
| 69 | F | 6 | – | + |
| 70 | F | 8 | – | +++ |
| 72 | F | 7 | – | + |
| 74 | F | 17 | – | + |
| 79 | F | 8 | – | ++ |
| 83 | F | 8 | – | ++ |
| 88 | F | 6 | – | + |
| 96 | F | 7 | + | – |

a) M: male, F: female
b) Seroprevalence of N antibodies was detected by Western blotting.
c) Seroprevalence of P antibodies was detected by ELISA and Western blotting. The fold value of the ELISA absorbance against negative control is indicated. +, 3–4 fold; 5–7 fold; +++, > 7 fold

detection of N antibody because ELISA using recombinant N antigen shows only low specificity to the antibody under our conditions (data not shown). The absorption test indicated that four (11.1%) and seven (10.7%) sera showed a positive-reaction to BDV N antigen in the male and female cattle, respectively (Fig. 1C and Table 1), demonstrating a discrepancy between N and P antibodies in the bovine sera. In the prevalence of N antibody, we found no sex and age differences for the animals (Chi-square test: $X^2$=0.032 [sex]; seropositive animal: 8.45 ± 3.29, seronegative animal: 8.82 ± 3.17 [age]). The Western blot analysis indicated that three (2.9%) female cows, #39, #45, and #53, had both of the antibodies to BDV N and P (Table 1).

In cattle, neurological diseases or neuropathology resembling classical BD have been found in endemic and nonendemic areas in Europe [3, 4]. A recent study has demonstrated a case of Borna-like disease in a Holstein cow in Hokkaido, where several species are seropositive for BDV even in apparently healthy animals [23]. Although Japan is not an endemic country for BD, recent findings reveal that BDV infection and neurological diseases induced by the virus may be commonly found in domestic animals living in Japan. This suggests that a systematic survey of BDV infection is important to understanding the expansion of this virus, as well as its potential as a zoonotic pathogen. The prevalence of BDV in animals, including livestock, however, is not clear in Japan, except in Hokkaido. Furthermore, there are no reports regarding seroprevalence of BDV in Japanese black cows, which are dominantly reared in the east part of Japan, especially in Kyushu.

In this study, we demonstrated BDV-seropositivity in healthy Japanese black cows raised in Kyusyu. We found that 10.9% and 20.7% of the sera showed positive reactions to BDV N and P antigens, respectively. Previous data indicated that the BDV P antibody was found in 20.3% of Holstein cows by Western blot analysis in Hokkaido [10]. Although BDV's natural reservoir(s) or vector(s) has not yet been identified, our data, together with previous studies [10, 14], suggests that a follow-up study with area isolated cattle could be important for exploring the natural reservoir or vector of this virus.

Hokkaido is Japan's northernmost island, and its main livestock are dairy cattle and horses, many of which are originally imported from abroad, including BD epidemic countries. On the other hand, Kyushu is Japan's southernmost main island, and beef cows and pigs are dominantly reared in this area. Furthermore, it has been demonstrated that Japanese black cattle originated from native bovine species in Japan. Considering these differences, it is likely that the origin of BDV in Japanese black cattle in Kyushu might be different from that in Holstein cows raised in Hokkaido. Supporting this hypothesis is a recent study that BDV isolates from the same geographical area exhibited a clearly higher degree of identity to each other than to BDV isolates from other regions, independent of host species and year of isolation [16]. Virological characterization as well as nucleotide sequence analysis of bovine BDV from different areas could be helpful in discovering the origin of BDV in cattle in Japan. In addition, Hagiwara *et al.* reported that no BDV antibodies were detected in serum samples from 20 cattle in Xinjiang, China [8]. In contrast, BDV RNAs were detected in brain samples by RT-PCR technique from 9.7% of cattle in France [6]. Although only a few studies have been reported concerning BDV prevalence in cattle in other countries at present, these observations suggest that BDV prevalence in cattle might be dependent on the geographic region.

As reported in previous studies [7, 11, 22, 30], we found a discrepancy in the prevalence between BDV N and P antibodies in bovine samples. In our investigation, only three sera were positive to both of the antigens. Although it is unclear why the humoral immune responses between BDV antigens do not correlate in a host, some reports have suggested possibilities regarding this discrepancy in BDV-infected animals. Johansson *et al.* have demonstrated that cats experimentally infected with a laboratory strain devel-

oped a higher response to N antigen, whereas a cat-derived wild strain of BDV induced a strong reactivity against P antigen in experimentally infected cats [15]. In addition, Ouchi *et al.* reported that cats with neurological symptoms tend to show a higher prevalence of antibodies to P than to X (p10) antigen [25]. We have also demonstrated that BDV P is dominantly expressed in the persistently infected stage of the virus in the brains of experimentally infected gerbils [31]. These observations suggest that the humoral immune response to BDV is most likely dependent on the strain of the virus, as well as the stage (e.g., acute or persistent) of the infection, in the host. More interestingly, we revealed that the P antibody has only developed in the female cattle. This observation indicated the possibility that the immune reaction to the virus may also be dependent on the host environments in which the virus replicates.

In conclusion, we demonstrated anti-BDV antibodies in sera from healthy Japanese black cows in Kyusyu, Japan. Together with previous studies, this result suggests that BDV might be widespread in Japanese cattle, as an asymptomatic infection. Further studies are needed of more accurate incidences of BDV in Japanese domestic animals and also for evaluation of the risk of BDV infection.

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