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Latency and Persistence of Bovine Herpesvirus Type 4, Strain B11-41, in Bovine Nervous Tissues

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ABSTRACT. Three cattle were experimentally infected with bovine herpesvirus type 4 (BoHV-4), strain B11-41, isolated from the spinal cord of a cow, and monitored for clinical symptoms. None of them showed any clinical signs except increases of leukocyte numbers in two of them, and the body temperature remained normal throughout the experiment. Antibody titers against BoHV-4 continuously increased for one month and were maintained at a high level for more than 1 year by enzyme-linked immunosorbent assay (ELISA). The virus was isolated only from serum and peripheral blood leukocytes (PBL) of one cow in the early stage of infection, but the viral genome was detected in PBL continuously by PCR. When they were euthanized, the viral genome was detected in the lymph nodes and nervous tissues such as medulla, spinal cord, and trigeminal ganglion. These results indicate that cattle are infected with the virus latently and persistently, and the latency site would be in the tissues of the central nervous system as well as lymphoid tissues. When a seroepidemiological survey was performed on antibodies to BoHV-4 among cattle in Japan by ELISA, the rate of antibody-positive cattle was 8.9% and they were found irregularly on certain farms.

KEY WORDS: BoHV-4, bovine herpes virus, cattle, nervous tissue, persistent infection.

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Bovine herpesvirus type 4 (BoHV-4) has been isolated worldwide from cattle with several clinical conditions, such as respiratory tract diseases, skin lesions, reproductive tract disease, abortion, mammilitis, encephalitis and enteric infections [13, 17, 28]. BoHV-4 is a member of the subfamily Gammaherpesvirinae on the basis of its genomic structure [25], and has been divided into two groups by restriction endonuclease analysis [5, 29]. One is the Movar 33/63-like (Movar-like) group and the other is the DN 599-like group [9, 12, 14, 24, 27]. Movar-like viruses were mainly isolated in Europe and DN 599-like viruses in North America. The main targets for viral replication are the lymphoid organs, the upper respiratory tract, and urogenital and alimentary tracts [13, 17]. Like other herpesviruses, BoHV-4 is capable of producing a persistent infection and the lymphoid organs and mononuclear blood cells are sites of BoHV-4 latency [3, 7, 10, 23]. On the other hand, as the tissue distribution of BoHV-4 was investigated in cattle and rabbits experimentally infected with Movar-like viruses, the virus was detected in many organs, including nervous tissues [6, 20, 22]. Therefore, the nervous system was also suspected to persistently harbor Movar-like viruses. However, since gammaherpesviruses usually persist latently in lymphocytes, and since BoHV-4 has been isolated from a wide variety of clinical conditions in cattle, it is not evident that persistent infection in the nervous system is a common biological property of all BoHV-4 strains. Recently strain B11-41 of BoHV-4, which belongs to the DN 599-like group, was isolated from the spinal cord of a cow with astasia in Japan [21, 30]. Our aims in the present study were to investigate the pathogenicity and tissue tropism of the virus in cattle. Seroprevalence of BoHV-4 infection was also investigated among cattle in Japan.

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MATERIALS AND METHODS

Viruses: Strain B11-41 of BoHV-4, isolated from the spinal cord of a cow with astasia, was used in this study [21, 30]. The Los Angeles strain of bovine herpesvirus type 1 (BoHV-1) [18], preserved in the National Institute of Animal Health, Tsukuba, Japan, was used as a control to determine the specificity of the enzyme-linked immunosorbent assay (ELISA).

Cell culture: Madin-Darby bovine kidney (MDBK) cells were used for virus propagation. The cells were cultivated in Eagle's minimal essential medium (Eagle MEM) containing 5% fetal bovine serum, 0.3% triptose phosphate broth, 100 μg/m*l* of streptomycin and 100 U/m*l* of penicillin.

Experimental infection of the cattle: Three cattle (Nos. 1, 2 and 3) were used. They were 2 years old and seronegative for BoHV-4 as determined by ELISA. All cows were inoculated intranasally and intravenously with 10 m*l* of culture fluid (total 20 m*l*) of BoHV-4 strain B11-41, which had an infectivity of 10^4 $TCID_{50}$/0.1 m*l*. They were kept in isolated pens (3.4 m × 3 m) that were air conditioned with filtrated air. Body temperature was measured twice daily over the experimental period. Cows 1 and 2 were euthanatized at 42 days after virus inoculation. Cow 3 was euthanatized at 479 days after virus inoculation.

Table 1. Isolation and detection of BoHV-4 from PBL and serum

		Days after virus infection														
		0	1	2	3	4	5	6	7	10	11	14	21	28	35	42
Cow 1																
Virus isolation from	PBL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Serum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Detection of viral DNA in PBL		–	–	–	+	–	ND	ND	+	–	ND	+	–	–	+	+
Cow 2																
Virus isolation from	PBL	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	Serum	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Detection of viral DNA in PBL		–	–	–	–	–	ND	ND	+	–	ND	–	–	+	–	+
Cow 3																
Virus isolation from	PBL	–	–	–	–	–	+	–	–	ND	ND	ND	ND	ND	ND	ND
	Serum	–	–	–	+	+	+	+	–	ND	ND	ND	ND	ND	ND	ND

ND: Not done.

Table 2. Detection of BoHV-4 by virus isolation and nested-PCR from various tissue samples

Cow No.	1		2		3	
	Virus Isolation	Nested PCR	Virus Isolation	Nested PCR	Virus Isolation	Nested PCR
Heart	–	+	–	+	–	+
Lung	–	+	–	–	NT	NT
Liver	–	–	–	+	–	–
Spleen	–	–	–	–	–	+
Kidney	–	+	–	+	–	+
Superficial cervical lymph nodes	–	+	–	+	–	+
Mediastinal lymph nodes	NT	NT	–	+	–	+
Mesenteric lymph nodes	–	+	–	–	–	+
Brain	–	+	–	–	–	+
Medulla oblongata	–	+	–	+	–	+
Breast spinal cord	NT	NT	–	+	–	+
Sternum spinal cord	–	+	NT	NT	NT	NT
Lumbar spinal cord	–	+	–	+	–	+
Trigeminal nerve	–	+	–	+	–	+
Sciatic nerve	–	–	–	+	–	+
Popliteal lymph nodes	–	+	NT	NT	–	–
Axillary nerve	–	+	NT	NT	NT	NT

NT: Not tested.

Blood and tissue samples: After virus inoculation, serum and blood samples were collected periodically from these cows as shown in Table 1. Basically, hematological examinations were carried out at least once a week using ethylenediaminetetraacetic acid (EDTA) treated blood taken from the jugular vein. The total leukocyte and erythrocyte counts, hemoglobin value and hematocrit value were determined using a Coulter counter (S-Plus JR: Coulter Electronic, Hialeh, FL, U.S.A.). Leukocytes were collected from EDTA-treated blood after lysis of erythrocytes by mixing two volumes of 0.83% NH_4Cl solution and 3 washes in phosphate-buffered saline solution (PBS). Tissue samples of various organs were collected from all cattle after they were euthanatized. These samples were submitted for histological investigation, detection of the viral genome by nested PCR and virus isolation by inoculation onto MDBK cell culture. The list of tissue samples collected from each animal is shown in Table 2.

Histopathology: Tissue samples were processed by routine paraffin embedding methods. Sections were cut and stained with hematoxylin and eosin for routine morphological studies.

Virus isolation: Peripheral blood leukocytes (PBL) were frozen and thawed three times and were centrifuged at 1,000 g for 20 min. Then the supernatant was inoculated onto confluent cultures of MDBK cells. Various tissue samples were cut into pieces, suspended in 9 volumes of Eagle MEM, homogenized and centrifuged at 1,000 g for 10 min. The supernatant was inoculated onto confluent cultures of MDBK cells. Cells were cultivated in tissue culture plates

in a CO_2 incubator at 37°C for at least 12 days. The samples showing cytopathic effects were considered positive. Fluids of negative cultures were subpassaged into new cultivated cells a further two times.

Extraction of DNA from tissue samples: About 0.5 g of each tissue sample was taken and cut into pieces. These pieces were transferred to centrifuge tubes filled with 400 μl of lysis buffer containing 30 mM NaCl, 10 mM Tris-HCl (pH 7.4), 20 mM EDTA, 1% SDS, and 0.2 mg/m*l* Proteinase K. After vortexing, the samples were incubated at 37°C overnight with rotation. DNA was extracted from these pretreated tissue samples using a commercially available test kit (SepaGene, Sankoujunyaku Co., Tokyo, Japan), dissolved in a small volume of TE buffer (10mM Tris-HCl, pH 8.0 and 1mM EDTA) and the DNA concentration was measured.

Nested PCR for BoHV-4: For nested PCR study, we followed the method described by Egyed*et al.* [10]. In the first PCR, 1 μg of DNA from each sample was added to the PCR reaction mixture (50 μl of total volume) containing 0.2 μM of each primer, 200 μM of dNTP, 10mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$ and 1 U of AmpliTaq Gold DNA polymerase (Perkin Elmer, CT, U.S.A.). The sequences of the first PCR primers were 5'-GTTGGGCGTCCTGTATGGTAGC-3' and 5'-ATGTATGCCCAAAACTTATAATATGACCAG-3', and 567 bp product was predicted. DNA was amplified with a DNA Thermal Cycler, PE9700 (Perkin Elmer) by 1 cycle of 95°C for 9 min, 5 cycles of denaturation (94°C, 45 sec), annealing (56°C, 60 sec) and extention (72°C, 90 sec) and 25 cycles of denaturation (94°C, 45 sec), annealing (51°C, 60 sec) and extention (72°C, 90 sec). A final extension time of 7 min at 72°C was included at the end of the last cycle. Nested PCR was carried out using 5 μl of the first PCR product in the same conditions. The sequences of the second PCR primers were 5'-TTGATAGTGCGTTGTTGGGATGTGGT-3' and 5'-CACTGCCCGGTGGGAAATAGCA-3', and amplified a 260 bp product. The PCR products were electrophoresed in 1.5% agarose gels and stained with ethidium bromide. PBL samples collected from cattle during preinfection were used as negative controls. The specificity of PCR products was confirmed by restriction fragment length polymorphism (RFLP) analysis. The nucleotide sequence data obtained from the GenBank EMBL data bank (accession number AB035515) allowed us to search for restriction enzyme recognition sites specific to BoHV-4. The nested PCR products were digested with *Hind* III (81 and 179 bp), *Kpn* I (28 and 232 bp) and *Xba* I (105 and 155 bp), respectively.

Preparation of Antigen for ELISA: For detection of BoHV-4-specific antibodies, we chose the method of ELISA. BoHV-4 B11-41 strain-infected cells were scraped from the culture flask with a rubber policeman when CPE appeared all over the cells (approximately 5 to 7 days after inoculation), frozen and then thawed 3 times and centrifuged at 1,000 g for 20 min at 4°C. The supernatant was further centrifuged at 44,000 g for 2 hr at 4°C. Then the pellet was suspended in a small volume of PBS, pH 7.4, disrupted by sonication , and stored at –80°C until used as the viral antigen for ELISA. A negative antigen control was prepared in the same manner using noninfected-cells. The viral antigen was diluted with 15 mM carbonate-35 mM bicarbonate buffer (pH 9.6), and the optimal antigen concentration for the assay was determined on the basis of the optimal density (OD) values of positive and negative control sera.

Serologic analysis: ELISA microplates were coated with 50 μl of the antigen per well, sealed and incubated at 4°C overnight. After washing the plates three times with PBS containing 0.02% Tween 20 (PBS-T), 100 μl of blocking solution, which consisted of PBS (pH 7.2) containing 0.15% Tween 20, 2% Block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) and 0.1% NaN_3, was added to each well. The plates were sealed, incubated at 37°C for 1 hr and washed with PBS-T as described above. Serum samples were diluted to a final concentration of 1:100 in a diluent that consisted of PBS (pH 7.2) containing 0.15% Tween 20, 2% Block Ace, and 0.1% NaN_3. Fifty microliters of diluted serum was added to each well, which was then sealed, and incubated at 37°C for 1 hr. After washing the plates with PBS-T, 50 μl of a 1:2,000 dilution of peroxidase-conjugated mouse anti-bovine IgG (Cappel Research Reagents, ICN Biomedicals, Inc., Irvine CA, U.S.A.) was added. After incubation at 37°C for 1 hr, the plates were washed again and 100 μl of the enzyme substrate solution containing 50 mM citric acid, 0.01% hydrogen peroxidase and 2.2.azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) was added, followed by incubation at 37°C for 30 min. The plates were read at 414 nm with an Immuno Mini ELISA reader (Immuno Mini NJ-2300, InterMed, Tokyo, Japan). The ELISA value of each sample was determined by a calibration procedure that used positive and negative control serum samples on each plate as shown below.

ELISA value = (sample OD value – negative control OD value)/positive control OD value – negative control OD value)

Serum samples for surveillance: A total of 1,819 bovine serum samples were obtained in 27 prefectures in Japan in 1997 and 1998. In each prefecture, more than 50 serum samples were collected. In principle, 5–10 farms distributed in different areas were selected in each prefecture, and 5–10 serum samples were collected from clinically healthy cattle at random in each farm. A serum sample, with an indirect-fluorescent antibody (IFA) titer of more than 1:1,280 to BoHV-4 strain B11-41, was used as a positive control. A serum sample with no antibody titer to either strain BoHV-1 (less than 1:1 by virus neutralization test) or BoHV-4 (less than 1:10 by IFA test), was used as a negative control.

RESULTS

Clinical observations and pathological findings in the experimentally infected cattle: All the experimentally infected cattle were monitored daily for signs of disease. None of them showed any clinical signs, and the body tem-

Table 3. The specificity of the BoHV-4 ELISA

Samples	BoHV-4 ELISA OD value	VN antibody[a)] titer to BoHV-1	IFA antibody[b)] titer to BoHV-4	ELISA[c)] value
BoHV-1 positive serum				
1	0.09	4	<10	0.01
2	0.10	8	<10	0.03
3	0.12	16	<10	0.06
4	0.30	64	<10	0.32
5	0.32	≥128	<10	0.35
6	0.36	≥128	<10	0.41
7	0.53	≥128	<10	0.65
BoHV-4-positive serum				
8	0.73	<1	80	0.94
9	0.79	<1	320	1.03
10	0.76	<1	640	0.99
11	0.79	<1	640	1.03
12	0.72	<1	1280	0.93
BoHV-4-positive control serum	0.77	<1	≥1280	1.00
BoHV-1- and BoHV-4-negative control serum	0.08	<1	<10	0.00

a) Virus neutralizing antibody titer.
b) Immunofluorescene antibody titer.
c) ELISA value=(sample OD value–negative control OD value)/(positive control OD value–negative control OD value).

peratures of the cattle remained normal throughout the experiment. In two cattle, the number of blood leukocytes was increased temporarily. The number of blood leukocytes increased from 7,500/μl at pre-inoculation time to 11,000/μl at 3 days after infection in cow 1, and from 8,000/μl to 10,900/μl in cow 2. In all the experimentally infected cattle, no distinct microscopic lesion was found except for shrinkage of Peyer's patches.

Distribution of BoHV-4 in tissues: The virus was isolated from serum and PBL of cow 3 in the early stage of infection, but other attempts at virus isolation, from blood samples in the later stage of infection and tissue samples when cattle were killed, were all negative (Table 1). On the other hand, the viral DNA was frequently detected by nested PCR from PBL during the experimental period, and from several tissues examined (Tables 1 and 2). Nested PCR products were all confirmed their specificity by RFLP analysis. Positive reactions were observed mainly in the tissues of the nervous system and lymphoids. However, viral DNA was not detected in Peyer's patches, which were the only sites with distinct microscopic lesions.

The specificity of the BoHV-4 ELISA: Because antigenic cross-reactivity between BoHV-1 and BoHV-4 has been reported by Mohanty *et al.* [19], the specificity of the BoHV-4 in the ELISA was preliminarily assessed. Bovine sera, which had antibody titers to BoHV-1 (1:4, 1:64 and more than 1:160 by virus neutralization tests) but did not have antibody titers to BoHV-4 (less than 1:10 by IFA test), showed ELISA values of less than 0.7 with the BoHV-4 antigen. Whereas serum samples, which had IFA titers to BoHV-4 of more than 1:80 showed ELISA values of more than 0.9 with BoHV-4 antigen. Therefore serum samples from the field were designated antibody positive to BoHV-4 if their ELISA values were more than 0.8 in this experiment (Table 3).

ELISA titers of experimentally infected cows: The BoHV-4 antibody titer continuously increased during 1 month or more in the three experimentally infected cows (Fig. 1). The ELISA values reached to 0.57 at 28 days after infection in cow 1, 0.70 at 42 days in cow 2, and 0.99 at 35 days in cow 3. The level was maintained at a high value for more than 1 year in the one cow that was observed for a long period.

Seroprevalence in Japan: A total of 1,819 serum samples were tested by ELISA for seroprevalence of BoHV-4 antibodies in cattle in Japan. The data in Table 4 show that 162 (8.9%) sera were positive, and seropositive cattle were found in most of the prefectures tested. The seropositive rate in each prefecture differed, ranging from 0% to 46%. There was a tendency for positive cattle to be distributed irregularly and found on certain farms at high rates.

DISCUSSION

BoHV-4 has been isolated from a wide variety of clinical conditions in cattle, and the biological properties, including tissue tropism, of each strain might be different. Though a high antigenic relationship is shown between the BoHV-4 strains, many differences are observed between DN599 and Movar subtypes by restriction analysis [4], like equine herpesvirus 1 and 4 [1]. Therefore, it was uncertain that the bio-

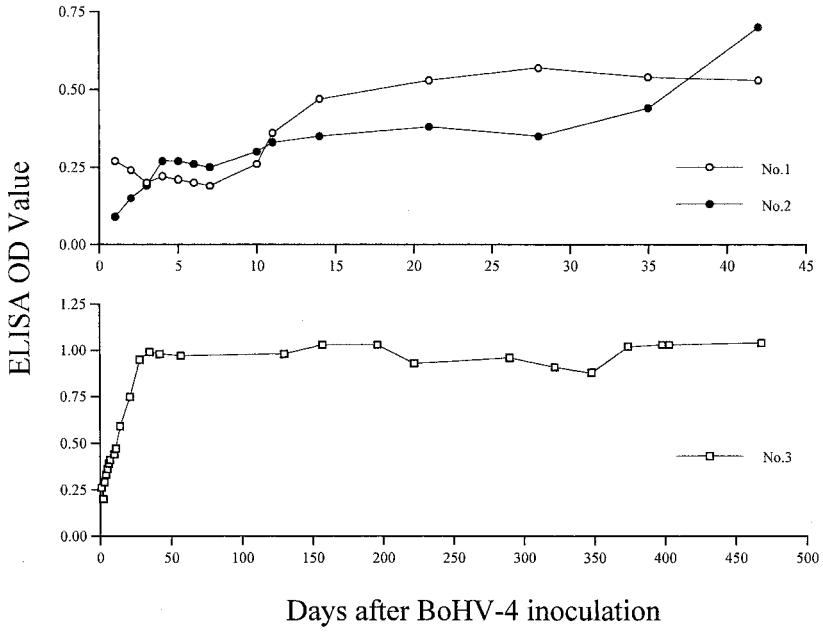

Fig. 1. Antibody responses of three cows experimentally infected with BHV-4, strain B11. Antibody responses to BoHV-4 were examined by ELISA.

logical properties observed in one subtype were common to the other. Recently, strain B11–41, a virus classified into the DN599-like group of BoHV-4, was isolated from the spinal cord of a cow with astasia [30]. The present study was conducted to obtain additional information on latency and persistence of BoHV-4 in cattle.

The BoHV-4 antibody titer was maintained at a high level for more than 1 year by ELISA, but was not detected by virus neutralization test. This implies infective virus is not eliminated effectively by humoral immunity, and activate the immune system of the host animal continuously or intermittently. As a result, latent and persistent infection of BoHV-4 was established and viral DNA was detected from PBL of experimentally infected animals for a long period after infection.

Most alphaherpesviruses avoid immune elimination by remaining within the cells of the nervous system as DNA in ganglion cells during the intervals between disease episodes. On the other hand, betaherpesviruses and gammaherpesviruses avoid immune elimination by persisting in lymphocytes. We attempted to survey viral DNA in various tissues from experimentally infected cows to see where BoHV-4 was harbored. Since viral DNA of BoHV-4 was detected in the nervous tissues as well as in lymphoid tissues by nested PCR, it was strongly indicated that BoHV-4 was harbored in protected sites of these tissues. Boerner *et al.* reported that the latently BoHV-4 infected cell types in cattle are most likely to be B lymphocytes [3]. Therefore, it could not be completely denied that the positive PCR reactions of nervous tissue were induced by contaminating lymphocytes. However, nested PCR was negative in the spleen and liver which contain many lymphocytes, so the suspicion of PBL contamination in the nervous tissues could be eliminated.

The *in vivo* distribution of BoHV-4 had been previously examined using experimentally infected cattle and the results were different in each experiment testing for the presence of BoHV-4 in the nervous system. Egyed *et al.* reported that the nervous system and the muscles were free of viral DNA of BoHV-4 [10]. They used tissues from calves in the early stage of infection with strain Movar 33/63 isolated from calves with respiratory disease. The presence of BoHV-4 was confirmed by virus isolation in their experiments. Later, they detected BoHV-4 genome from nervous tissues of experimentally infected calves by nested PCR [11]. Castrucci *et al.* also reported that the virus was recovered from nerve tissues of calves experimentally infected with Movar-like strains in the early stage of infection and after dexamethasone injection in the later stage of infection [6]. The different results of these studies might have been due to the differences of virus strain and infection stage of the cattle when investigated, and further procedure to detect virus. If sensitive procedure such as nested PCR had been used for detection of virus genome in the experiments, BoHV-4 would be detected frequently in nervous system. Some BoHV-4 strains have been isolated from bovine nervous tissues even in U.S.A. [15, 16]. These strains seem to be DN599 subtype geographically. However, their subtype was not identified genetically. As far as we know, the isolates from nervous system and identified genetically as DN599 subtype has not been reported.

In present study, we used tissues from cows in a later

stage of infection with strain B11-41, which was isolated from the spinal cord of a cow and belongs to the DN 599-like group, and BoHV-4 DNA was detected in nervous tissues. Therefore, the latency and persistence style of strain B11–41 was found in the tissues of the nervous system as well as lymphoid tissues. We insist that persistent infection in nervous tissues is a common biological property of BoHV-4 group, though BoHV-4 is a gammaherpesvirus.

BoHV-4 has been isolated from cattle with respiratory symptoms [26]. In this experiment, however, no cows showed any clinical sign such as bronchitis, coughing, diarrhea or the elevation of body temperature. Thus, the virulence of strain B11–41 against cows is considered very weak and other immunosuppressive factors might be necessary for clinical symptoms to appear. All experimentally infected cattle showed the shrinkage of Peyer's patches. However, viral DNA was not detected in these tissues, and the shrinkage of Peyer's patches in these cattle appeared not to have relation to the BoHV-4 infection.

Since infective viruses were not isolated from the blood except in the early stage of infection and since nested PCR was required to detect viral DNA from blood and tissues in later stages of infection, the infective dose of the virus was rather low in the persistently infected cattle when they were in normal condition without clinical symptoms. However, the result of a seroepidemiological survey for BoHV-4 by ELISA showed that there were many antibody-positive cattle all over Japan. Since a high cut-off ELISA value was used because of cross-reaction between BoHV-1 and BoHV-4, the rate of positive cattle might be higher than the present result (Table 4). There was also a tendency for positive cattle to be irregularly distributed and found on certain farms at high rates. Thus, some cattle might work as a reservoir in the field. Since BoHV-4 was isolated from nasal swab samples [2], infective BoHV-4 was present in nasal epithelial cells, nasal exudates, or leukocytes of the respiratory mucosa. The epithelial cells of the upper respiratory tract are considered to be the main targets and replication sites of BoHV-4 [2]. In a recent study, infective BoHV-4 was detected in milk, and transmission of BoHV-4 from the dam to the calf was indicated [8]. BoHV-4 that exists in lymphocytes might be transmitted through milk.

The reason for the wide prevalence of BoHV-4 among cattle in Japan might be due to the management system of dairy farms. Cattle about eight months old are often carried to breeding farms from various regions and then moved to the milking farm after half a year. Additionally, the transportation of cattle between milking farms is also increasing. BoHV-4 existing persistently in these animals could be activated when they are subjected to stress and immunosuppression is induced. Such conditions could be caused by long-distance transportation, parturition and sudden changes of atmospheric temperature in the field.

Table 4. Distribution of BoHV-4 antibody-positive cattle in various prefectures in Japan

Perfecture	Number of sera tested	Number of positive sera	Positive rate (%)
Hokkaido	330	11	3.3
Aomori	50	13	26.0
Iwate	50	23	46.0
Yamagata	50	2	4.0
Ibaraki	50	2	4.0
Chiba	110	3	2.7
Tochigi	50	5	10.0
Gunma	59	7	11.9
Niigata	50	5	10.0
Nagano	50	1	2.0
Gifu	50	2	4.0
Mie	50	0	0.0
Shiga	50	7	14.0
Wakayama	50	1	2.0
Okayama	55	0	0.0
Tottori	60	26	43.3
Hiroshima	61	14	23.0
Ehime	77	8	10.4
Kochi	64	5	7.8
Fukuoka	53	9	17.0
Oita	50	2	4.0
Saga	50	0	0.0
Nagasaki	50	0	0.0
Kumamoto	50	3	6.0
Miyazaki	50	2	4.0
Kagoshima	50	2	4.0
Okinawa	100	9	9.0
Total	1819	162	8.9

Sera with ELISA values of 0.8 or higher were considered positive.

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