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NOTE
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Survey of bovine foamy virus infection among cattle in Japan and comparison with bovine leukemia virus infection

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ABSTRACT. The prevalence of bovine foamy virus (BFV) infections in cattle on farms in the Kanto region of Japan was determined using agar gel immunodiffusion (AGID) test and polymerase chain reaction (PCR). Six out of 20 farms contained BFV-positive cattle. Furthermore, 16.7% (91/545) of all cattle tested positive for BFV. This suggested that BFV-infected cattle are widely prevalent in Japan. Positive results for BFV infection were consistent between AGID and PCR tests. Additionally, we tested for bovine leukemia virus (BLV) infections at nine farms, primarily those containing BFV-infected cows. At each farm, the infection rate of BFV was lower than that of BLV. Further, cattle that were PCR-positive but antibody-negative, indicating immune tolerance to BFV, were not detected.

KEY WORDS: bovine foamy virus, bovine leukemia virus, cattle, epidemiology, retrovirus

Cattle are infected by retroviruses from three genera: *deltaretrovirus*, *lentivirus*, and *bovispumavirus* [1, 14]. Bovine leukemia virus (BLV) is a *deltaretrovirus*, the pathogenesis and epidemiology of which has been well characterized [11, 20]. However, as with other retroviral infections among cattle, such as those caused by bovine foamy virus (BFV) and bovine immunodeficiency virus (BIV), the primary mode of transmission is not yet known, and relatively few epidemiological reports have been published on BFV and BIV [8, 15, 26]. BFV, which was first isolated from lymphosarcomatous cattle [25], is a typical spumavirus that resembles the primate foamy viruses and feline foamy virus in its morphology and molecular structure [21]. However, it has not been clearly linked to any disease yet [10, 13, 19, 23]. BFV infection is persistent and its seroprevalence in cattle ranges from 7% to 85%, with most farms having a seroprevalence of greater than 30% [2, 4, 5, 7–9, 15, 28]. Based on antibody surveys conducted in some farms, the infection rates of BFV were found to be higher than or almost equal to those of BLV [1, 5, 7]. Recently, BFV was isolated from the peripheral blood leukocytes (PBLs) of a cow in a farm in the Kanto region of Japan [6], prompting a BFV antibody survey in the farm. Interestingly, the infection rate of BFV among the cattle was lower than that of BLV [6]. Therefore, we investigated the prevalence of BFV among cattle in a wider area and compared it with the prevalence of BLV in Japan.

The virus neutralization (VN) test is an ideal antibody assay for viral diseases. However, BFV produces very few cell-free viruses in the infected cells [3]; thus, it is difficult to produce a viral solution with high infectivity, rendering the VN test impractical in this case. Thereby, agar gel immunodiffusion (AGID) test and polymerase chain reaction (PCR) were used to investigate BFV infection in the present study. Blood samples were collected from cattle in 20 farms from 2 prefectures (Kanagawa and Ibaraki) in the Kanto district of Japan. In the Kanagawa prefecture, blood samples were collected from all cattle in 4 farms, followed by assays for detection of BFV and BLV infections. In Ibaraki prefecture, blood samples were collected from cattle in 16 farms. In farms with less than 50 cattle, 10 cattle were selected randomly, and in farms with more than 50 cattle, 30 cattle were selected randomly for the serological survey of BFV. If BFV-positive cattle were detected in a farm, blood was collected from all the cattle to investigate BFV and BLV infections. Plain tubes were used for serum collection and Ethylenediaminetetraacetic acid (EDTA)-containing tubes were used to obtain the PBLs. Erythrocytes were lysed by mixing blood with 2 volumes of 0.83% NH_4Cl solution containing 0.01% EDTA. The PBLs were then separated from blood by centrifugation at 200 × g for 10 min followed by three washes with phosphate buffered saline (PBS). PBL concentration was adjusted to 1×10^7/m*l* and used for PCR.

Fetal bovine muscle (FBM) cells and Madin–Darby bovine kidney (MDBK) cells, infected with BFV No. 43 strain, were used

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as antigens for the AGID test [6]. Briefly, BFV-infected FBM, which were used for BFV isolation and caused clear cytopathic effect (CPE), were mixed with MDBK and cultivated until CPE appeared in more than 30% of the cells. FBM cells were cultivated in Eagle's MEM containing 10% fetal bovine serum (FBS), 100 μg/m*l* of streptomycin and 100 U/m*l* of penicillin at 37°C. MDBK cells and the mixed (FBM+MDBK) cells were cultivated in Eagle's MEM containing 5% FBS, 0.3% tryptose phosphate broth, 100 μg/m*l* of streptomycin and 100 U/m*l* of penicillin at 37°C. The infected cells were detached from the culture bottle with a rubber policeman, transferred to a centrifuge bottle, and washed 3 times with PBS by centrifugation at 1,200 × g for 10 min. The centrifuged cell pellet was suspended in a small volume of PBS containing 1.0% Triton X-100 (approximately 1/100th volume of the original cell suspension culture fluid), sonicated, and used as the antigen for AGID tests. BLV-infected fetal lamb kidney cell line (FLK-BLV) was used for antigen preparation for the BLV AGID test [27]. The cells were cultivated in Eagle's MEM containing 5% FBS, 100 μg/m*l* of streptomycin, and 100 U/m*l* of penicillin at 37°C. Culture fluid of FLK-BLV was concentrated using ammonium sulfate and used as an antigen, as described previously [12].

The AGID tests were performed with minor modifications of the methods reported by Malmquist *et al.* and Kono *et al.* [12, 16]. The wells were 5 mm in diameter, and 6 circumferential wells were placed at a distance of 3 mm from the central well. The central well was filled with the antigen and the other wells were filled with positive control antisera and undiluted serum samples. Positive control antiserum, which yielded a dense precipitation line, was selected from bovine serum samples, and its specificity was confirmed using immunized rabbit serum [6]. The gel diffusion plate was allowed to stand at room temperature (20–27°C) for 2 days to observe for precipitation lines. A sample was considered positive when a precipitation line was formed and it joined in continuation with the positive line of the control, formed between the antigen well and the control antiserum well. If a precipitation line was not formed, but the control line curved slightly towards the inside of the test serum well, the sample was considered weakly positive and classified as antibody-positive serum.

BFV DNA was detected by nested PCR. PBLs, re-suspended in approximately 1.0 m*l* PBS, were transferred to 1.5 m*l* microcentrifuge tubes and centrifuged at 2,000 × g for 5 min. DNA was extracted directly from the cell pellet, composed of approximately 1 × 10^6 cells, using the DNeasy® Blood & Tissue Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions.

A region of the BFV *env* gene was amplified by nested PCR, as described by Materniak *et al.* (after modification) [17, 22]. The primer sequences of the first PCR were BFV-P1 (5′-TGGACTCTAGTAGTCTCACC-3′) and BFV-P2 (5′-CTTAGAAAGCGTGGTAATGGC-3′), resulting in a 1,248-bp product. For the second PCR, 1 μ*l* of the first PCR product was re-amplified using primers, BFV-P3 (5′-TGTCATTAGAGGACTTCAGG-3′) and BFV-P4 (5′-TTGATTGTCCTGCTATCTGG-3′), producing a 915-bp product. The cycling conditions of the two consecutive PCRs were as follows: 1 cycle of 95°C for 2 min; 35 cycles of denaturation (95°C, 30 sec), annealing (55°C, 30 sec) and extension (72°C, 80 sec); and final extension (72°C, 7 min). PCR was performed using the GoTaq Green Master Mix (Promega, Madison, WI, USA). DNA was amplified using a PCR Thermal Cycler Dice TP600 (Takara-bio, Kusatsu, Japan). The PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

In the AGID test, BFV-positive cattle were detected in 5 out of 16 farms in the Ibaraki prefecture and in 1 out of 4 farms in the Kanagawa prefecture. BFV was detected at different rates in each positive farm, ranging from 10.9% to 79.7%. In total, 91 out of 545 (16.7%) cattle were positive for BFV (Table 1). All cattle from the 5 farms in Ibaraki prefecture, where positive cattle were detected, and the 4 farms in Kanagawa prefecture, were serologically tested for BFV and BLV. BLV-positive cattle were detected on all farms, and the number of BLV-infected cattle was higher than the number of BFV-infected cattle in all the farms investigated. PCR, which was used to detect the BFV gene in PBL, validated the results of the antibody tests. BFV and BLV infections among the cattle were not correlated, and both

Table 1. Prevalence of bovine foamy virus and bovine leukemia virus infections in 20 farms in Kanto district of Japan

Prefecture	Farm	No. of sera tested	BFV[a]		BLV[b]
			AGID[c]	PCR[d]	AGID
Ibaraki	A	69	55	55	61
	B	27	6	6	18
	C	14	3	3	10
	D	35	13	13	31
	E	57	7	7	34
	F	10	0	0	ND[e]
	G	10	0	0	ND
	H	30	0	0	ND
	I	10	0	0	ND
	J	30	0	0	ND
	K	10	0	0	ND
	L	30	0	0	ND
	M	10	0	0	ND
	N	30	0	0	ND
	O	10	0	0	ND
	P	10	0	0	ND
Kanagawa	Q	64	7	7	17
	R	27	0	0	11
	S	20	0	0	6
	T	42	0	0	15

a) BFV; bovine foamy virus, b) BLV; bovine leukemia virus, c) AGID; agar gel immunodiffusion, d) PCR; polymerase chain reaction, e) ND; not done.

Table 2. Summary of bovine foamy virus and bovine leukemia virus infection among cattle

		BLV[a]		
		+	−	Total
BFV[b]	+	78	13	91
	−	125	139	264
	Total	203	152	355

a) BLV; bovine leukemia virus, b) BFV; bovine foamy virus.

infections spread without affecting each other (Table 2).

BFV appears to be prevalent among cattle in the Kanto district of Japan, although the rate of infection differs in each farm. Serological surveys for BFV infections have been performed in several countries. BFV seropositive cattle comprise approximately 40% of the cattle in Canada [7], 34% to 40% of the cattle in Great Britain [2, 28], and approximately 40% of the cattle in Australia [8, 9]. These surveys were performed in limited areas in each country, and the results may not be representative. However, the present results show a lower infection rate in Japan compared to the other countries.

There are possible reasons for the existence of areas with high BFV infection prevalence. PCR-positive but antibody-negative cattle are considered immune-tolerant cattle and have been postulated to be a source of BFV infection via nasal discharge and saliva [10]. Such cattle were not detected on any farm in the present study. Interestingly, one farm in the Ibaraki Prefecture showed a high infection rate of BFV in this study, suggesting that immune-tolerant cattle may have existed there in the past. However, this farm is a common breeding farm and cattle are frequently replaced.

It has been reported that the BFV antibody-positive cattle have a high percentage of cell-free viruses in their saliva and that they are a source of horizontal infection [18]. This study was performed by detecting syncytium formation using canine thymus cells (Cf2Th) as target cells. However, both detection of the BFV gene and a proliferation assay of BFV in Cf2Th were not performed in this study. On the contrary, there are also reports that deny horizontal spread of BFV from antibody-positive cattle [2, 15, 26]. The release of BFV from infected cells has been reported to be less [3]. Therefore, it is necessary to reconsider whether the saliva of antibody-positive cattle is a source of horizontal transmission.

Other natural modes of BFV transmission that have been postulated include perinatal modes of transmission via colostrum or milk from dam to fetus [10, 15, 22, 26]. This could lead to the infection of calves, uninfected at birth, but infected upon being kept together with infected adults [8]. In the acute infection stage, before the antibody is produced, shedding of BFV could occur via saliva and infect others, possibly through sneezing or licking [8]. However, these modes of infection do not seem to cause strong dissemination of BFV.

The present study suggests that BFV-infected cattle are likely to be widespread in Japan, and the rate of BFV infection in each farm is lower than that of BLV. However, in some countries, BFV infection in cattle may occur at a higher or equivalent rate as BLV infection [1, 5, 7].

Real-time PCR was used for the detection and quantification of BFV-DNA in PBLs, milk samples and saliva [18]. The number of viral DNA copies was highest in PBLs and varied from 240 to 7,490 per 0.5 μg genomic DNA (48 to 149.8/10 *n*g DNA). Whereas, BLV copy numbers in the blood of BLV-infected cattle (BLV antibody positive, but clinically healthy) ranged from 0 to 2,600 per 10 *n*g DNA (the median was 110). Ten point eight % of the BLV-infected cattle had more than 1,000 copies per 10 *n*g DNA [24]. Therefore, the horizontal transmission of BFV via contact infection and blood-sucking insects is less likely to occur, compared to BLV. Therefore, the transmission ability of BFV from infected cattle is weaker than that of BLV, if immune-tolerant cattle did not exist [15].

In conclusion, this study provides basic information about the epidemiology of BFV infection in the dairy cattle of Japan. Our results can be used for future studies that investigate the transmission and dissemination of BFV infection.

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