The Journal of Veterinary Medical Science

FULL PAPER

Virology

Screening of persistently infected cattle with bovine viral diarrhea virus on dairy farms by using milk tanker and bulk tank milk samples for viral RNA and viral-specific antibody detection

Masataka AKAGAMI[1)]*, Mariko TAKAYASU[1)], Shoko OOYA[1)], Yuki KASHIMA[1)], Satoko TSUZUKU[1)], Yoshiko OOTANI[1)], Yoshinao OUCHI[1)] and Yoko HAYAMA[2)]

[1)]Kenhoku Livestock Hygiene Service Center, Ibaraki Prefecture, 966-1 Nakagachi, Mito, Ibaraki 310-0002, Japan
[2)]Viral Disease and Epidemiology Research Division, National Institute of Animal Health, National Agriculture and Food Research Organization, 3-1-5 Kannondai, Tsukuba, Ibaraki 305-0856, Japan

ABSTRACT. The objective of this study was to provide a screening scheme of persistently infected (PI) cattle on dairy herds by combining reverse-transcription polymerase chain reaction (RT-PCR) to detect bovine viral diarrhea virus (BVDV) in milk tanker samples and commercial enzyme-linked immunosorbent assay to detect BVDV antibodies in bulk tank milk. We conducted a pilot survey and regional survey targeting all dairy farms in Ibaraki Prefecture by using milk tanker and bulk tank milk samples to screen PI cattle. Farms with positive samples underwent a follow-up test to identify PI cattle. In the pilot study, all virus-positive samples in bulk tank milk were included in the positive milk tanker samples. The RT-PCR assay successfully detected BVDV at dilutions of 1:1,600 by using two PI cows' milk. In the regional survey, 5 of 79 milk tanker samples were virus-positive. The virus was detected in three PI lactating cows and one PI calf on three farms. Antibody screening using bulk tank milk samples revealed 15 of 363 samples were positive, and 12 of 348 farms were BVDV antibody-positive. Follow-up tests on one farm identified three PI calves. Thus, eight PI cattle on five farms were identified in this study. In conclusion, combining BVDV detection using milk tanker samples and antibody detection using bulk tank milk is a feasible and economical method to efficiently screen PI cattle and confirm the PI-free status among dairy herds.

KEY WORDS: antibody enzyme-linked immunosorbent assay, bovine viral diarrhea virus, milk tanker, persistently infected cattle, reverse-transcription polymerase chain reaction

J. Vet. Med. Sci.
82(5): 607–614, 2020
doi: 10.1292/jvms.19-0634

Received: 26 November 2019
Accepted: 3 March 2020
Advanced Epub:
13 March 2020

Bovine viral diarrhea (BVD) is a chronic infectious disease in cattle caused by infection with the BVD virus (BVDV) [10]. It results in significant economic losses to the cattle industry worldwide [9, 14, 33]. Clinical signs are frequently fever approximately 6–9 days postinfection, inappetence, and mucosal lesions; however, most cases of BVDV infection are subclinical in susceptible nonpregnant cows [23]. If clinical signs are severe, acute BVD infection may be followed by reproductive disorders immediately or shortly after seroconversion because of the general condition of the cow [15]. Moreover, transplacental infection can cause fetal death and abortion, significant fetal abnormalities [6, 7], or the birth of persistently infected (PI) calves [3, 30]. BVDV can be transmitted to the fetus in pregnant cattle during days 40–120 of gestation, and thereby induces fetal immune tolerance to BVDV and results in the delivery of PI calves [3, 5]. The PI calves continuously excrete large amounts of virus throughout their lives, show few symptoms, and are a continual source of infection in a herd [13, 18, 24]. Furthermore, they are at risk of developing fatal mucosal disease [4, 5].

Infection with BVDV has severely damaged the cattle industry throughout Japan. Based on the results of the latest survey on PI animals on dairy farms in Japan, the prevalence of farms with PI animals is calculated as 7.6% (95% confidence interval [CI], 3.1–16.4%), and the prevalence of cattle tested as PI animals was 0.12% (95% CI, 0.05–0.25%) [17]. In addition, based on the investigation of BVDV epidemics from 2006 to 2014 in Hokkaido, where most dairy cattle are reared in Japan, BVDV-1b and BVDV-2a viruses were the predominant BVDV subgenotypes [1].

The reverse-transcription polymerase chain reaction (RT-PCR) technique has been used to detect BVDV-infected cattle among

*Correspondence to: Akagami, M.: m.akagami@pref.ibaraki.lg.jp

dairy herds. This testing technique for bulk tank milk is useful as a screening test to detect any PI cattle among lactating cows [19]. It is a cost-effective diagnostic method. The virus has been detected by RT-PCR when milk from a PI animal was diluted to 1:600 with milk from a BVDV-free herd [28].

In Nordic countries such as Denmark, Sweden, Norway, and Finland where BVDV vaccines are not used in BVDV control programs, antibody detection in bulk tank milk by using indirect or blocking enzyme-linked immunosorbent assays (ELISAs) has been used as a diagnostic tool for monitoring the BVDV infection-free status [15, 25]. Houe *et al.* [15] revealed that PI animals were found only among young stock, and that PCR analysis of bulk tank milk was therefore unsuitable to test a herd for the presence of PI animals. Furthermore, they stated that, because of the high sensitivity and low specificity of bulk tank milk antibody testing, this method identifies nearly all true-positive herds, but tends to produce a certain number of false-positive herds [15]. Thus, for a BVDV control program, combining several available diagnostic tests that can detect the virus itself or detect viral-specific antibodies is important to improve the accuracy of detecting PI cattle.

In consideration of the more effective and rapid assessment of BVDV status in dairy herds, we focused on screening for BVDV in milk tanker samples. The milk tanker samples, which include commingled milk collected from several dairy farms, are often used to detect antibiotics to prevent them from getting mixed into the plant [31]. For pathogen screening, milk tanker samples are used to detect *Listeria* spp. [29]. Milk tanker samples save the labor involved in sampling bulk tank milk or serums on each dairy farm. Therefore, the milk tanker samples have the potential to screen BVDV from cattle on several dairy farms simultaneously. However, whether milk tanker samples are sufficiently sensitive to identify PI animals among dairy herds has not been fully studied. Our objective was to provide a screening scheme of PI cattle in dairy herds by combining RT-PCR to detect BVDV in milk tanker samples and commercial ELISA to detect BVDV antibodies in bulk tank milk samples. By using these methods, we conducted a pilot survey and regional survey that targeted all dairy farms in Ibaraki Prefecture in eastern Japan, and we investigated the feasibility of using milk tanker samples and bulk tank milk samples for efficiently detecting PI cattle.

MATERIALS AND METHODS

Study area

In this study, all dairy farms in Ibaraki Prefecture were targeted for screening PI cattle by using milk tanker samples and bulk tank milk samples. In this area, milk tankers make multifarm pick-ups in which milk is collected from several farms and commingled before transport and unloading to a plant. On average, a milk tanker travels to four or five dairy farms to collect milk. The capacity of a milk tanker is 4.2–6.7 t for a mid-sized vehicle and 10.5−13.5 t for a large vehicle. The raw milk collected by milk tankers is transported to two cooling stations (CSs) in Ibaraki Prefecture. These CSs cover the raw milk distribution from nearly all dairy farms in this area.

Screening scheme

The screening scheme for identifying PI cattle on dairy herds is shown in Fig. 1. This screening scheme consists of two streams. The first stream is BVDV gene detection in milk tanker samples to screen PI cattle in a milk cow herd. The second stream was the screening of PI cattle, including calves and dry cows, by using BVDV antibody detection in bulk tank milk.

To establish this scheme, the possibility of false-negative results due to dilution in milk tanker samples was investigated. To address this, BVDV gene detection was conducted on milk tanker samples and their corresponding bulk tank milk samples in the pilot survey. Furthermore, to verify gene detection in milk tanker samples, we investigated the detection limits of BVDV genes diluted in milk from PI cattle. For antibody detection, we collected individual milk and serum samples of cattle from farms with PI cattle, and then investigated whether BVDV antibodies in milk could be detected with available ELISA kits.

For farms in which the bulk tank milk tested positive, we conducted a follow-up test of all cattle and attempted to identify PI cattle. The BVDV antigen-positive cattle were re-tested after three weeks. Cattle that were antigen-positive on both tests were diagnosed as PI cattle. For the follow-up test, all calves that were born up to 10 months after the removal of the last PI animal were tested for BVDV antigen by ELISA. Epidemiological information on identified PI cattle was collected to evaluate the validity of this scheme.

Pilot survey and regional survey

In this study, the local veterinary officers collected milk tanker samples at the CSs, which included milk collected from several dairy farms. At the time of collecting the milk, the milk tanker driver took bulk tank milk samples from every bulk tank. Milk samples of approximately 15 m*l* were collected from the milk tanker and the bulk tank.

First, to assess the feasibility of milk tanker samples, a pilot survey was initially conducted. Fifty milk tanker samples and 220 bulk tank milk samples on each farm were collected at a CS on Sept. 18, 2016. Second, a regional survey was conducted on nearly all 348 dairy farms in Ibaraki Prefecture. In this survey, 79 milk tanker samples and 363 bulk tank milk samples from each dairy farm were collected at two CSs on May 30, 2017 and on July 19, 2017. If virus-positive or antibody-positive samples were detected, follow-up tests were conducted on each farm, based on the screening scheme (Fig. 1).

Milk was collected in 15-m*l* plastic tubes, and centrifuged at 3,000 rpm for 10 min at 4°C. Milk somatic cells from the bottom of the tubes (i.e., cell pellets) were then washed with phosphate-buffered saline (PBS) and re-pelleted. All cell pellets were used for ribonucleic acid (RNA) extraction.

For antibody ELISA, skimmed milk was collected from below the fat layer after the centrifugation of whole milk at 3,000 rpm

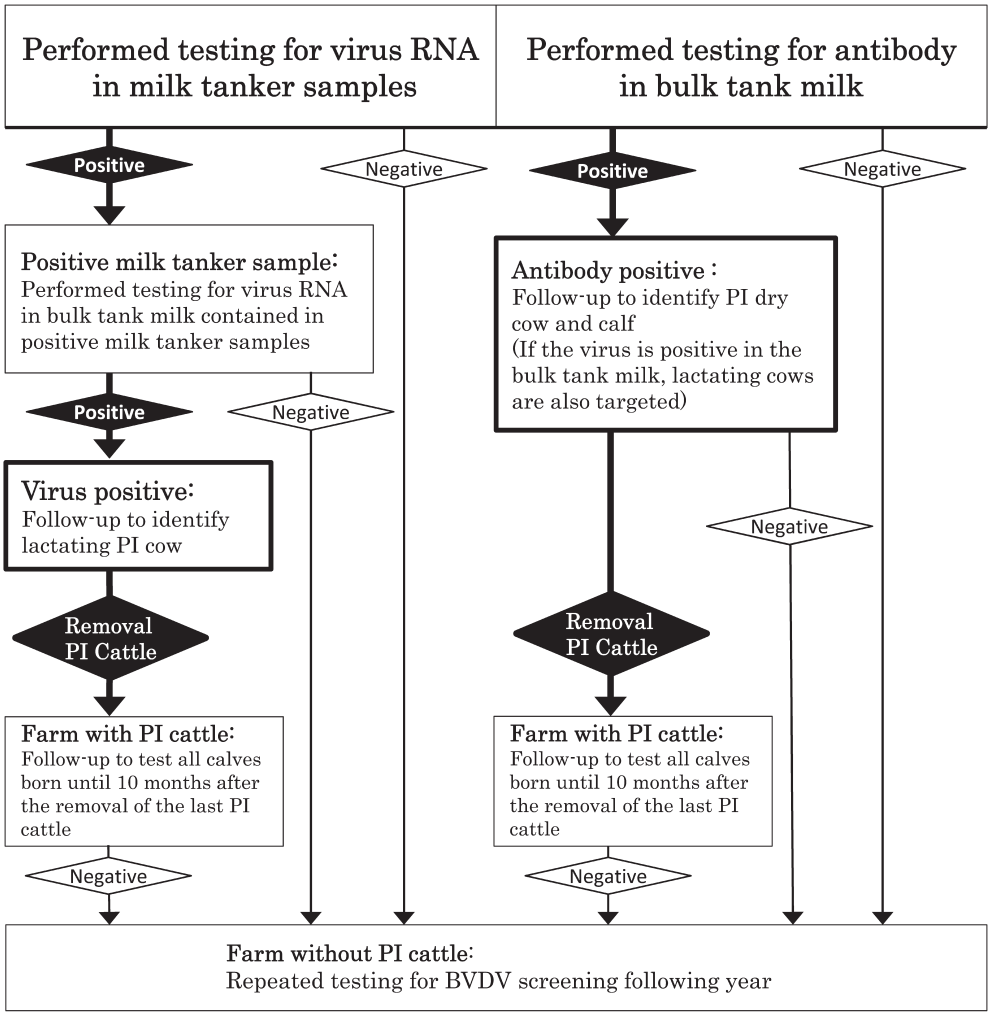

Fig. 1. Systematic bovine viral diarrhea virus (BVDV) screening scheme to identify individual persistently infected (PI) cattle in infected herds, and continuous monitoring to confirm BVDV-free status.

for 10 min at 4°C.

To examine the sensitive limits of virus detection from the milk of PI cows, two milk samples were collected from cows that tested positive on both tests and were thus defined as PI cattle.

Two samples of milk from a known PI cow were diluted from 1:10 to 1:1,600 with PBS. Pelleted cells recovered from the milk were processed, based on the method described previously.

Virus detection in tank milk by using RT-PCR assay

Viral RNA was extracted from the pelleted cells recovered from milk by using fully automated instrumentation with an in-tip nucleic acid extractor (magLEAD 12gC; Precision System Science Co., Chiba, Japan). The RT-PCR assay was conducted as follows. Published primer sequences (primer pair 324 and 326) were used [34]. The 324–326 primer pair is widely used to detect BVDV including new genotypes in a recent report [12] and to monitor BVDV infections in dairy herds using bulk tank milk [21]. The solutions of the PCR reactions each contained 25 μl of RNase-free water, 10 μl of 5 × PCR buffer, 2 μl of dNTP mix, 2 μl of Enzyme Mix, 20 pmol of each primer and 8 μl of viral RNA; this made a total volume of 50 μl in the One Step RT-PCR Kit (QIAGEN, Hilden, Germany). Each reverse transcriptase reaction was performed at 50°C for 30 min. The reactions were then initially incubated at 95°C for 15 min, and cycled 35 times, as follows: 94°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. The amplification products were visualized on 1.5% agarose gels stained with GelRed (Biotium, Hayward, CA, USA).

For the follow-up test, all calves born until 10 months after the removal of the last PI cattle were screened for viral antigen using a BVDV ELISA test kit (IDEXX BVDV ag ELISA; IDEXX Laboratories, Tokyo, Japan), according to the protocol provided by the manufacturer.

Antibody detection in bulk tank milk by using BVDV antibody ELISA

A commercial BVDV antibody ELISA kit (VDPro BVDV AB ELISA; Median Diagnostics Inc., Chuncheon, Korea) was

used to detect antibodies in all bulk tank milk samples, individual milk samples, and sera samples. This blocking ELISA was designed to detect the presence of anti-E2 protein-specific antibody for BVDV. Based on the test procedure, enzyme-conjugated immunosorbent reaction was performed in skimmed milk. The optical density (OD) was thereafter measured by absorbance at 450 nm. The results were judged by the sample-to-negative (S/N) ratio for each sample, as follows: S/N=the OD of the sample/the OD of the negative control. Samples with S/N ratios ≤0.7 were positive. Data handling and statistical analysis were carried out using Microsoft Excel 2010.

RESULTS

Virus detection in the milk tanker samples

The results of the pilot survey are shown in Table 1. Three of 50 milk tanker samples were positive for the virus, based on RT-PCR. Among these positive samples, two positive samples were detected in the samples from milk tankers with a maximum capacity of 4.2–6.7 t, and one sample was detected in the samples from milk tankers with a maximum capacity of 10.5–13.5 t.

Three of 220 bulk tank milk samples were positive. These bulk tank milk samples corresponded to the farms where the milk tanker samples were collected. All farms with virus-positive bulk tank milk were included in virus-positive milk tanker samples. No false-negative results occurred (i.e., negative in the milk tanker sample and positive in the bulk tank milk sample). In this pilot study, one PI lactating cow was identified on one farm (Farm No. 1).

The results of regional survey, based on the scheme, are presented in Table 2. Five of 79 milk tanker samples were positive, and three of 18 bulk tank milk samples that were included in the milk tanker samples were positive. In the regional survey, three PI lactating cows were identified on three farms (Farm Nos. 2–4; Table 3).

When testing the detection limit of BVDV gene diluted in the milk tanker samples, RT-PCR assay successfully detected BVDV at a dilution of 1:1,600 by using the milk collected from two PI cows on Farms Nos. 1 and 2 (Fig. 2).

Antibody detection in bulk tank milk

Table 4 shows the results of BVDV antibody detection in bulk tank milk samples from nearly all dairy farms in Ibaraki Prefecture. Fifteen of 363 bulk tank milk samples were positive for BVDV antibody ELISA. Based on the results of these positive

Table 1. Detection of bovine viral diarrhea virus (BVDV) in milk tanker samples and bulk milk samples on dairy farms, based on reverse-transcription polymerase chain reaction (RT-PCR) assay in the pilot study

Maximum capacity of the milk-collecting vehicle	Milk tanker sample			Bulk tank milk		
	n	Positive	Negative	n	Positive	Negative
4.2–6.7 t	37	2	35	191	2	189
10.5–13.5 t	13	1	12	29	1	28
Total	50	3	47	220	3	217

Table 2. Results of viral ribonucleic acid (RNA) detection using milk tanker samples in the regional survey

CS	Testing for viral RNA in pooled milk from milk tanker samples			
	Pooled milk samples (no.)	Samples positive for virus (no.)	Number of farms	Farms positive for virus (no.)
No. 1	24	2	8	2
No. 2	55	3	10	1
Total	79	5	18	3

CS No. 1 =Kensei cooler station, CS No. 2 =Keno cooler station. CS=cooling station.

Table 3. Numbers of persistently infected (PI) cattle to identify and remove from infected herds in the regional survey

Factor detected	No. of positive farms	No. of farms with PI cattle	No. of PI cattle		
			Total	Cows	Calves
Viral RNA	3	3	4	3	1
Antibody	10	1	3	0	3
Total	13	4	7	3	4

RNA=ribonucleic acid.

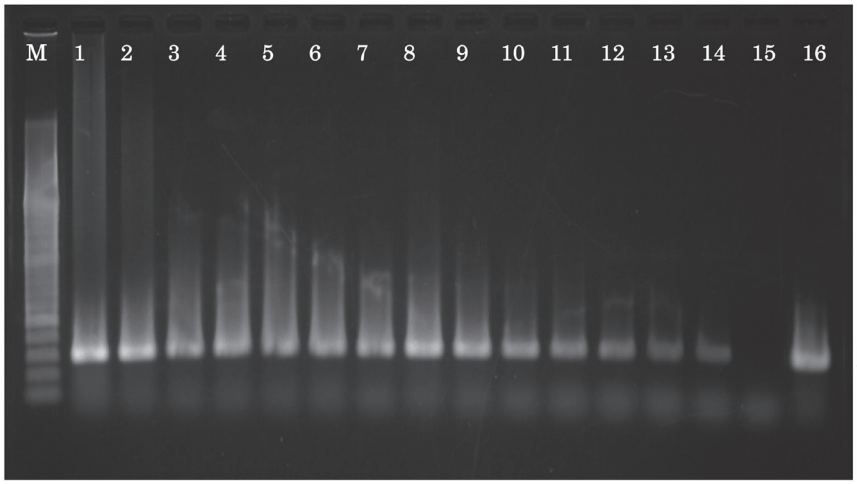

Fig. 2. Agarose gel electrophoresis of the amplification products of bovine viral diarrhea virus (BVDV) ribonucleic acid (RNA). Line M: pUC Mix Marker 8, (Fermentas, Vilnius, Lithuania); Lines 1–7: the milk of a persistently infected (PI) cow from Farm No. 1; Line 8–14: the milk of a PI cow from Farm No. 2; Line 15: the negative control (i.e., commercial fetal bovine serum); Line 16: the BVDV positive control (i.e., BVDV-1a nose strain). As for the dilution ratio, lane 1 and 8 is 1:1, lane 2 and 9 is 1:10, lane 3 and 10 is 1:100, lane 4 and 11 is 1:200, lane 5 and 12 is 1:400, lane 6 and 13 is 1:800, and lane 7 and 14 is 1:1,600. bp=base-pair.

Table 4. Results of viral-specific antibody detection in bulk tank milk

CS	Testing for antibodies in bulk tank milk			
	No. of bulk milk samples	Positive samples (no.)	No. of farms	Positive farms [a)]
No. 1	103	4	102	4 (1)
No. 2	260	11	246	8 (1)
Total	363	15	348	12 (2)

a) The number within the parentheses indicate the number of viral RNA-positive farms among antibody positive farms. CS=cooling station, RNA=ribonucleic acid.

bulk tank milk samples, 12 of 348 farms were classified as BVDV antibody-positive, including two farms in which viral RNA was already detected in the survey by using milk tanker samples. In the follow-up test, three PI calves were identified on one farm (Farm No. 5; Table 3).

Figure 3 shows the results of BVDV antibody detection by using ELISA on the sera and individual milk on two farms with PI cattle: Farm No. 1, which kept 120 adult cows, and Farm No. 5, which kept 48 adult cows. On Farm No. 1, a PI cow was kept between October 25, 2013 and October 24, 2016, and 120 (100%) sera samples and 26 (22%) milk samples of 120 samples, respectively, were positive after the PI cow was culled. In re-testing after two months, among 19 milk samples collected from antibody-positive lactating cows, eight samples were positive. On Farm No. 5, a PI cow was kept between February 11 and October 28, 2017, and 45 (95%) sera samples and 18 (38%) milk samples of 48 samples were positive after the PI calves were culled. Two months later, 29 of 51 milk samples were positive.

Detection of PI cattle

In the pilot survey and the regional survey, a total of eight PI cattle on five farms were detected (Table 5). In the pilot study, after the detection of virus-positive milk tanker samples, one lactating cow was identified as PI cattle on Farm No. 1. In the regional survey, after the detection of virus-positive milk tanker samples, three lactating PI cows were identified on Farm Nos. 2–4.

The PI cattle raised on Farm No. 2 was detected in the regional survey, but not in the pilot survey. Because this cattle was introduced to the farm on Sept. 13, 2016 in a pregnant state before giving birth, it was not detected in the pilot survey on Sept. 18, 2016. On Farm No. 4, a PI calf was detected in the follow-up test. In addition, in the regional survey using bulk tank milk, one farm (Farm No. 5) among 12 antibody-positive farms was identified as having PI cattle, and three PI calves were detected. The two PI calves raised on Farm No. 5 were born on February 11, 2017 and February 12, 2017. The two PI calves were born from dams that were pregnant with PI fetuses that returned from summer pasturing in Hokkaido.

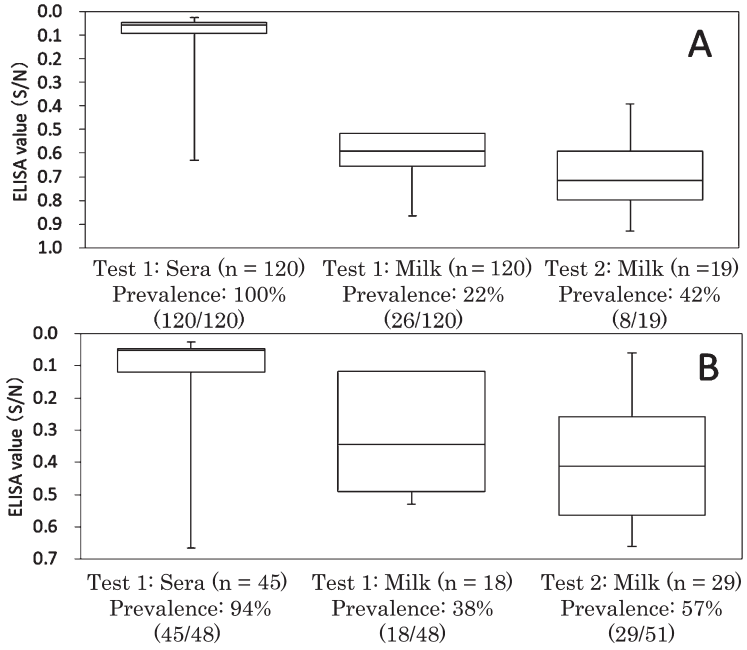

Fig. 3. Boxplot of enzyme-linked immunosorbent assay (ELISA) values (S/N) for sera and individual milk immediately after persistently infected (PI) cattle removal (Test 1), and the ELISA values (S/N) for individual milk 2 months after Test 1 (Test 2) on the farms with detected PI cattle (Panel "A" is Farm No. 1 and panel "B" is Farm No. 5). S/N=the optical density of the sample / the optical density of the negative control.

Table 5. Epidemiologic information of culled persistently infected (PI) cattle and result of testing for bulk tank milk

Farm no.	Period sampling	Type of screening [a)]	Viral RNA [b)] in MTS	Antibody in BTM	No. of PI cattle	Stage of PI cattle	Date of introduction
1	9/18/2016	Pilot-MTS/BTM	+ (6.5t)	+	1	Lactating cow	10/25/2013
	7/19/2017	Regional-MTS/BTM	-	-	0	-	-
2	9/18/2016	Pilot-BTM	-	+	0	-	-
	7/19/2017	Regional-MTS/BTM	+ (11.5t)	+	1	Lactating cow	9/13/2016
3	5/30/2017	Regional-MTS	+ (4.2t)	-	1	Lactating cow	8/24/2013
4	5/30/2017	Regional-MTS/BTM	+ (3.2t)	+	1	Lactating cow	12/15/2015
					1	Calf	11/17/2016
5	7/19/2017	Regional-BTM	-	+	3	Calves	2/11/2017 2/12/2017 9/6/2017

a) Pilot: pilot survey, regional: regional survey, MTS: milk tanker sample, BTM: bulk tank milk. b) ( ): Maximum capacity of milk collecting vehicle detected viral RNA. RNA=ribonucleic acid.

DISCUSSION

This study aimed to demonstrate the feasibility of using milk tanker samples and bulk tank milk samples to efficiently screen PI cattle in dairy herds. When using RT-PCR assay, BVDV was detected in milk samples from milk tankers with a maximum capacity exceeding 10 t, if the tanker contained milk from a lactating PI cow. Although the milk tanker samples were mixed with a large amount of milk collected from several farms, no false-negative results were observed in this study. Moreover, RT-PCR assay successfully detected BVDV at dilutions of 1:1,600 when using PI cows' milk. To verify that the use of PBS as a diluent did not affect the detection sensitivity, the authors compared it with samples diluted with bulk tank milk from a BVDV-free herd, and confirmed no difference between these dilute solutions (data not shown). These results suggested that RT-PCR assay of a milk tanker sample has the potential to detect BVDV in a total volume of 16 t of pooled milk containing at least 10 kg/day of the milk of an individual PI cow. The detection of BVDV in milk tanker samples is an efficient and economical diagnostic tool because the number of PCR runs when using milk tanker samples is approximately one-fourth the number when using bulk tank milk samples from all dairy farms in a prefecture. However, a disadvantage of testing only for the virus in milk samples is that it does not fully screen for the presence of BVDV because PI animals are likely to be among the young stock rather than among the lactating herd [15]. In addition, all cows were not represented in a milk sample. If a PI cow was dry or her milk was not entered into the tank at

the time of sampling, then it could not be detected.

The 324–326 primer pair was used to screen for BVDV in the milk samples in this study. This primer pair has been shown to be reliable, and is widely used to detect BVDV, including new genotypes identified in a recent report [12]. A previous study reported that the UTR1-UTR2 primer pair has a higher detection sensitivity than 324–326 primer pair for detecting the BVDV gene in bulk tank milk [28]. However, that study detected BVDV in acutely infected cattle, rather than PI cattle, among the parts of farms with BVDV-positive in bulk tank milk. This may explain the difference in the detection sensitivity of these primer pairs. Although the primer pair used in our study may be inappropriate to detect the viral gene excreted by acutely infected cattle, these cattle can be followed up by antibody testing of bulk tank milk. Further, no acute infection cases were observed during the study period. Thus, we believe that the primer pair used in the present study did not affect the detection accuracy in PI cows.

The commercial BVDV antibody ELISA kit used in this study sufficiently detected BVDV antibodies in individual milk samples collected from two farms with PI cattle. Immediately after removing the last PI cattle, the overall prevalence, based on individual milk samples, was 22% and 38% in the lactating herd on each farm, respectively. In immunologically naïve herds, the presence of a single antibody-positive animal (especially in an animal carrying a PI fetus) could make the bulk tank milk test result positive, even in herds with up to 250–500 cows [22]. Therefore, bulk tank milk antibody testing is useful for identifying herds with PI cattle. A more important finding is that antibody detection in bulk tank milk is suitable as a herd test for the presence of PI calves and PI cows, and can compensate for the disadvantage of RT-PCR assay in bulk tank milk. However, among 12 farms in which antibody was detected in bulk tank milk, only one farm kept PI calves and four farms had kept PI cattle in the past, whereas seven of 12 farms did not keep PI cattle. Many false-positive results in bulk tank milk predictions of the presence of PI animals are attributable to the high prevalence of seropositive animals in herds from which PI animals had recently been removed [15]. A repeated bulk tank milk test a few months later would solve this problem because antibody-free primiparous cows replace herd populations and the antibody titer of individual milk would decrease, which occurred in cows kept in Farm Nos. 1 and 5.

In nonvaccinated herds, bulk tank milk samples can be used to test for BVDV-specific antibodies because vaccination may interfere with bulk tank milk testing. In Ibaraki Prefecture, BVDV vaccines (including live and inactivated vaccine) are not used for cows on most of the dairy farms, except for several large-sized farms. Therefore, bulk tank milk testing is a useful method for continuous monitoring to confirm BVDV infection-free status. However, the areas where vaccination has been used for systematic BVDV control require another approach to testing of herds because the results for bulk tank milk samples are not necessarily indicative of a herd's BVDV infection status. One example of antibody detection is that the testing of milk samples for antibodies to NS3 is suitable for differentiating between animals vaccinated against BVDV and animals infected with a field virus [20].

To compensate for the disadvantages of each diagnostic technique for detecting BVDV, various herd-level diagnostic tools such as antibody detection in bulk tank milk and BVDV detection in milk tanker samples need to be combined appropriately to obtain effective strategies at low cost. We demonstrated that the BVDV screening scheme shown in Fig. 1 is useful with the aim of efficiently identifying and removing PI animals. As a result of the regional survey, which is based on this screening scheme, seven head of PI cattle, which consisted of three cows and four calves on four farms, were identified and culled. The remaining 336 farms did not have BVDVs and antibodies detected in the bulk tank milk. The movement of cattle such as for animal trade and summer pasturing are particular epidemiological risk factors associated with the introduction of PI cattle, which are the source of infection for dairy herds [11, 14, 27]. For regional BVDV control, implementing a screening scheme for all dairy farms to determine the BVDV status is important.

In the regional survey, approximately 4% of the dairy farms in Ibaraki Prefecture had anti-BVDV antibodies in bulk tank milk. To some extent, the BVDV prevalence was relatively low, compared to the national level prevalence of 7.6% (95%CI, 3.1–16.4%) [17]. According to Lindberg's review [23] on BVDV infections and its control, the annual incidence risk at the herd level varies between countries and regions, and has consequently been estimated to range from 8% to 48% under endemic conditions. In areas with a systematic control of the infection, reports indicate decreasing risk and levels of approximately 2–3% after 4–5 years of implementation [2, 32]. In actuality, the estimated prevalence of infected herds with detected antibodies in bulk tank milk is less than 1% in Sweden [16], Finland [26] and Denmark [8], based on national programs for the control and eradication of BVDV.

The regional survey used in the study revealed the current prevalence of PI cattle. However, a continuous screening test is required to ascertain the effect of control measures against BVDV. To identify and cull PI animals introduced during an early stage and to prevent a new PI calf outbreak on farms in Ibaraki Prefecture, maintaining a low prevalence of PI cattle in this region is essential by repeating this screening scheme with as short an interval as possible. In conclusion, combining antibody testing of bulk tank milk to confirm the infection-free status and screening tests on milk tanker samples to classify and identify infected herds is feasible and economical.

ACKNOWLEDGMENTS. This study was supported by Ibaraki Prefectural Federation of Dairy Cooperative Associations; Keno CS, Kensei CS, and Kensei Livestock Hygiene Service Center in Ibaraki Prefecture; and funded by the TOKUDEN Project from the Ministry of Education, and Science and Technology (Tokyo, Japan).

REFERENCES

1. Abe, Y., Tamura, T., Torii, S., Wakamori, S., Nagai, M., Mitsuhashi, K., Mine, J., Fujimoto, Y., Nagashima, N., Yoshino, F., Sugita, Y., Nomura, T., Okamatsu, M., Kida, H. and Sakoda, Y. 2016. Genetic and antigenic characterization of bovine viral diarrhea viruses isolated from cattle in Hokkaido, Japan. *J. Vet. Med. Sci.* **78**: 61–70. [Medline] [CrossRef]

2. Alban, L., Stryhn, H., Kjeldsen, A. M., Ersbøll, A. K., Skjøth, F., Christensen, J., Bitsch, V., Chriél, M. and Strøger, U. 2001. Estimating transfer of bovine virus-diarrhoea virus in Danish cattle by use of register data. *Prev. Vet. Med.* **52**: 133–146. [Medline] [CrossRef]
3. Baker, J. C. 1987. Bovine viral diarrhea virus: a review. *J. Am. Vet. Med. Assoc.* **190**: 1449–1458. [Medline]
4. Bolin, S. R. 1995. The pathogenesis of mucosal disease. *Vet. Clin. North Am. Food Anim. Pract.* **11**: 489–500. [Medline] [CrossRef]
5. Brownlie, J., Clarke, M. C. and Howard, C. J. 1984. Experimental production of fatal mucosal disease in cattle. *Vet. Rec.* **114**: 535–536. [Medline] [CrossRef]
6. Carlsson, U., Fredriksson, G., Alenius, S. and Kindahl, H. 1989. Bovine virus diarrhoea virus, a cause of early pregnancy failure in the cow. *Zentralbl. Veterinarmed. A* **36**: 15–23. [Medline] [CrossRef]
7. Done, J. T., Terlecki, S., Richardson, C., Harkness, J. W., Sands, J. J., Patterson, D. S., Sweasey, D., Shaw, I. G., Winkler, C. E. and Duffell, S. J. 1980. Bovine virus diarrhoea-mucosal disease virus: pathogenicity for the fetal calf following maternal infection. *Vet. Rec.* **106**: 473–479. [Medline] [CrossRef]
8. Foddai, A., Enøe, C., Stockmarr, A., Krogh, K. and Uttenthal, Å. 2015. Challenges for bovine viral diarrhoea virus antibody detection in bulk milk by antibody enzyme-linked immunosorbent assays due to changes in milk production levels. *Acta Vet. Scand.* **57**: 32. [Medline] [CrossRef]
9. Fourichon, C., Beaudeau, F., Bareille, N. and Seegers, H. 2005. Quantification of economic losses consecutive to infection of a dairy herd with bovine viral diarrhoea virus. *Prev. Vet. Med.* **72**: 177–181, discussion 215–219. [Medline] [CrossRef]
10. Gillespie, J. H., Baker, J. A. and McENTEE, K. 1960. A cytopathogenic strain of virus diarrhea virus. *Cornell Vet.* **50**: 73–79. [Medline]
11. Graham, D. A., Clegg, T. A., Lynch, M. and More, S. J. 2013. Herd-level factors associated with the presence of bovine viral diarrhoea virus in herds participating in the voluntary phase of the Irish national eradication programme. *Prev. Vet. Med.* **112**: 99–108. [Medline] [CrossRef]
12. Han, D. G., Ryu, J. H., Park, J. and Choi, K. S. 2018. Identification of a new bovine viral diarrhea virus subtype in the Republic of Korea. *BMC Vet. Res.* **14**: 233. [Medline] [CrossRef]
13. Houe, H. 1995. Epidemiology of bovine viral diarrhea virus. *Vet. Clin. North Am. Food Anim. Pract.* **11**: 521–547. [Medline] [CrossRef]
14. Houe, H. 1999. Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. *Vet. Microbiol.* **64**: 89–107. [Medline] [CrossRef]
15. Houe, H., Lindberg, A. and Moennig, V. 2006. Test strategies in bovine viral diarrhea virus control and eradication campaigns in Europe. *J. Vet. Diagn. Invest.* **18**: 427–436. [Medline] [CrossRef]
16. Hult, L. and Lindberg, A. 2005. Experiences from BVDV control in Sweden. *Prev. Vet. Med.* **72**: 143–148, discussion 215–219. [Medline] [CrossRef]
17. Kameyama, K., Konishi, M., Tsutsui, T. and Yamamoto, T. 2016. Survey for detecting persistently infected cattle with bovine viral diarrhea in Japan. *J. Vet. Med. Sci.* **78**: 1329–1331. [Medline] [CrossRef]
18. Kirkland, P. D., Richards, S. G., Rothwell, J. T. and Stanley, D. F. 1991. Replication of bovine viral diarrhoea virus in the bovine reproductive tract and excretion of virus in semen during acute and chronic infections. *Vet. Rec.* **128**: 587–590. [Medline] [CrossRef]
19. Kozasa, T., Tajima, M., Yasutomi, I., Sano, K., Ohashi, K. and Onuma, M. 2005. Relationship of bovine viral diarrhea virus persistent infection to incidence of diseases on dairy farms based on bulk tank milk test by RT-PCR. *Vet. Microbiol.* **106**: 41–47. [Medline] [CrossRef]
20. Kuijk, H., Franken, P., Mars, M. H., bij de Weg, W. and Makoschey, B. 2008. Monitoring of BVDV in a vaccinated herd by testing milk for antibodies to NS3 protein. *Vet. Rec.* **163**: 482–484. [Medline] [CrossRef]
21. Kuta, A., Polak, M. P., Larska, M. and Żmudziński, J. F. 2013. Monitoring of bovine viral diarrhoea virus (BVDV) infection in Polish dairy herds using bulk tank milk samples. *Bull. Vet. Inst. Pulawy* **57**: 149–156. [CrossRef]
22. Lindberg, A. L. and Alenius, S. 1999. Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. *Vet. Microbiol.* **64**: 197–222. [Medline] [CrossRef]
23. Lindberg, A. L. E. 2003. Bovine viral diarrhoea virus infections and its control. A review. *Vet. Q.* **25**: 1–16. [Medline] [CrossRef]
24. Meyling, A. and Jensen, A. M. 1988. Transmission of bovine virus diarrhoea virus (BVDV) by artificial insemination (AI) with semen from a persistently-infected bull. *Vet. Microbiol.* **17**: 97–105. [Medline] [CrossRef]
25. Niskanen, R., Emanuelson, U., Sundberg, J., Larsson, B. and Alenius, S. 1995. Effects of infection with bovine virus diarrhoea virus on health and reproductive performance in 213 dairy herds in one county in Sweden. *Prev. Vet. Med.* **23**: 229–237. [CrossRef]
26. Nuotio, L., Juvonen, M., Neuvonen, E., Sihvonen, L. and Husu-Kallio, J. 1999. Prevalence and geographic distribution of bovine viral diarrhoea (BVD) infection in Finland 1993–1997. *Vet. Microbiol.* **64**: 231–235. [Medline] [CrossRef]
27. Presi, P., Struchen, R., Knight-Jones, T., Scholl, S. and Heim, D. 2011. Bovine viral diarrhea (BVD) eradication in Switzerland—experiences of the first two years. *Prev. Vet. Med.* **99**: 112–121. [Medline] [CrossRef]
28. Renshaw, R. W., Ray, R. and Dubovi, E. J. 2000. Comparison of virus isolation and reverse transcription polymerase chain reaction assay for detection of bovine viral diarrhea virus in bulk milk tank samples. *J. Vet. Diagn. Invest.* **12**: 184–186. [Medline] [CrossRef]
29. Rodriguez, J. L., Gava, P., Medina, M. and Nuñez, M. 1994. Incidence of *Listeria monocytogenes* and other *Listeria* spp. in ewes' raw milk. *J. Food Prot.* **57**: 571–575. [Medline] [CrossRef]
30. Ross, C. E., Dubovi, E. J. and Donis, R. O. 1986. Herd problem of abortions and malformed calves attributed to bovine viral diarrhea. *J. Am. Vet. Med. Assoc.* **188**: 618–619. [Medline]
31. Sternesjö, Å. and Johnsson, G. A. 1998. A novel rapid enzyme immunoassay (Fluorophos BetaScreen) for detection of *β*-lactam residues in ex-farm raw milk. *J. Food Prot.* **61**: 808–811. [CrossRef]
32. Valle, P. S., Wayne Martin, S. and Skjerve, E. 1999. A hierarchical trend model for bovine virus diarrhoea virus (BVDV) sero-conversion in Norwegian dairy herds from 1993 through 1997. *Prev. Vet. Med.* **47**: 39–52. [Medline] [CrossRef]
33. Valle, P. S., Skjerve, E., Martin, S. W., Larssen, R. B., Østerås, O. and Nyberg, O. 2005. Ten years of bovine virus diarrhoea virus (BVDV) control in Norway: a cost-benefit analysis. *Prev. Vet. Med.* **72**: 189–207, discussion 215–219. [Medline] [CrossRef]
34. Vilcek, S., Herring, A. J., Herring, J. A., Nettleton, P. F., Lowings, J. P. and Paton, D. J. 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. *Arch. Virol.* **136**: 309–323. [Medline] [CrossRef]