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Citation	Japanese Journal of Veterinary Research, 56(1), 3-8
Issue Date	2008-05
DOI	https://doi.org/10.14943/jjvr.56.1.3
Doc URL	https://hdl.handle.net/2115/33848
Type	departmental bulletin paper
File Information	56-1_p3-8.pdf



Availability of oral swab sample for the detection of bovine viral diarrhea virus (BVDV) gene from the cattle persistently infected with BVDV

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Received for publication, April 8, 2008; accepted, May 8, 2008

Abstract

Bovine nasal and oral discharges were used as samples for bovine viral diarrhea virus (BVDV) gene detection. Viral genes in serum (S), nasal discharge (N) and oral discharge (O) were quantified with real-time polymerase chain reaction using SYBR Green by the relative quantification method, and findings were compared among samples. Although the quantity of the BVDV gene in S was greater than those in N and O, all samples were available to identify persistently infected (PI) cattle with BVDV by reverse transcription polymerase chain reaction (RT-PCR). The swab samples were able to be stored for a few days at 4°C with a little decrease of amplification signal in RT-PCR. Oral swab sampling was easier than nasal swab sampling, and was also less uncomfortable for the cattle than other sampling methods without pain or unnecessary retention. This sampling method can be performed without any special technique and equipment. Therefore, the oral swab sampling method is useful for screening to detect BVDV PI cattle by RT-PCR.

Key Words: bovine viral diarrhea virus, BVDV, gene detection, swab sampling

Bovine viral diarrhea virus (BVDV) is distributed all over the world¹⁵⁾. There are many BVDV-infected cattle that do not show the typical clinical manifestations of diarrhea and mucosal diseases. These cattle including those persistently infected (PI) with BVDV are a latent source of infection of BVDV in the herd, which could cause serious eco-

nomic damage to the farmer^{4,9)}. Detection and elimination of BVDV from the herd are important to prevent its spread^{11, 12)}. In the dairy herd, bulk tank milk (BTM) is an available sample for the detection of BVDV. To identify PI cattle in BTM test-positive herd, serum or leukocytes are usually collected from individual cattle on the farm. In the

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present study, more convenient and easier methods of collecting samples to detect BVDV by reverse transcription polymerase chain reaction (RT-PCR) from individual cattle were evaluated.

Nasal and oral discharges were used as samples for BVDV detection. They were collected by swab and the swabs were stored in 2 ml of saline. Then 140 μ l of these samples were used for the extraction of RNA. RNA was extracted using QIAamp Viral RNA Mini Kit (Qiagen Inc., Tokyo, Japan) according to the manufacturer's instructions. Synthesis of cDNA was carried out using Moloney murine leukemia virus reverse transcriptase (MMLV-RT, Invitrogen Inc., Tokyo, Japan) and a random hexamer (Promega Inc., Tokyo, Japan)¹⁴. Specific primers¹³ for 5' UTR of BVDV1 and BVDV 2 were used for the detection by RT-PCR. The cycling conditions were 94°C for 1 min, 35 cycles (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec) and a final extension step (72°C for 2.5 min). The amplification was confirmed by 2% agarose gel electrophoresis after RT-PCR. The viral genes in serum (S), nasal discharge (N) and oral discharge (O) were quantified with the real-time PCR method using SYBR Green (Applied Biosystems Japan Inc., Tokyo, Japan) by the relative quantification method (qPCR), and findings were compared among samples. The conditions for real-time PCR were 50°C for 2 min, 50 cycles (denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 60 sec) and a dissociation step for the confirmation of specific amplification. The primers were the same as those for RT-PCR and the reaction mixture was prepared according to the manufacturer's instructions for SYBR Green. The number of copies of BVDV gene per μ l of sample was estimated from the 10 times serial dilutions of 10 ng of amplified products corresponded to 3.67×10^{10} copies. The standard curve showed a highly significant ($R^2 = 0.999$) negative correlation between the threshold cycle (Ct) value and gene copies/ μ l (Fig. 1). The number of gene copies was calculated using the molecular weight of the amplified product (242 bp) and Avogadro's number.

By RT-PCR, specific amplification bands of BVDV gene were recognized using S, N and O as examination samples. It has been reported that the sensitivities of virus isolation (VI) and RT-PCR for detecting BVDV were almost the same or RT-PCR was high sensitivity^{5,6,8}. Moreover, nasal, ocular and tracheal swabs and sera showed the same results for the detection of BVDV by VI and RT-PCR⁵. In the present study, although the quantity of the BVDV gene in S was greater than those in N and O by real-time PCR, the appearances of the bands on agarose gel electrophoresis were almost the same among the three samples (Fig. 2).

By qPCR, the quantities of viral gene from 13 PI cattle were estimated. The relative quantities of viral gene calculated by Ct differed individual PI cattle between 10^5 to 10^7 copies/ μ l of serum. Thus the results of qPCR of swabs were evaluated as relative value against that of serum sample. As shown in Table 1, in 6 of 13 PI cattle, oral swabs included more virus gene than serum. This was thought depend on the sampling manner in the field such as manipulation of the cotton stick (vigorously or mild in oral or nasal cavity), volume of saline, and so on, because the samples were collected from individual BVDV-PI cattle by different operators (veterinarian or farmer). In general, serum did not include the cells, however, swab samples sometimes included some cells by vigorous manipulation of the cotton stick. In all cases, the amplification bands of RT-PCR appeared almost the same on gel electrophoresis, therefore, swab samples could be used for the detection of PI-cattle by RT-PCR. This indicated that RT-PCR was satisfiable method and swab was enough as the examination sample. All samples could be stored for a few days at 4°C with a little decrease of amplification signal on RT-PCR. However, freezing and thawing of the swab sample markedly decreased amplification signal. These could be easily recognized on RT-PCR as shown in Fig. 3. The decreasing of signal by freezing might resulted from the freezing damage by saline as stock solution of cotton stick. The causes could not identify in the present study.

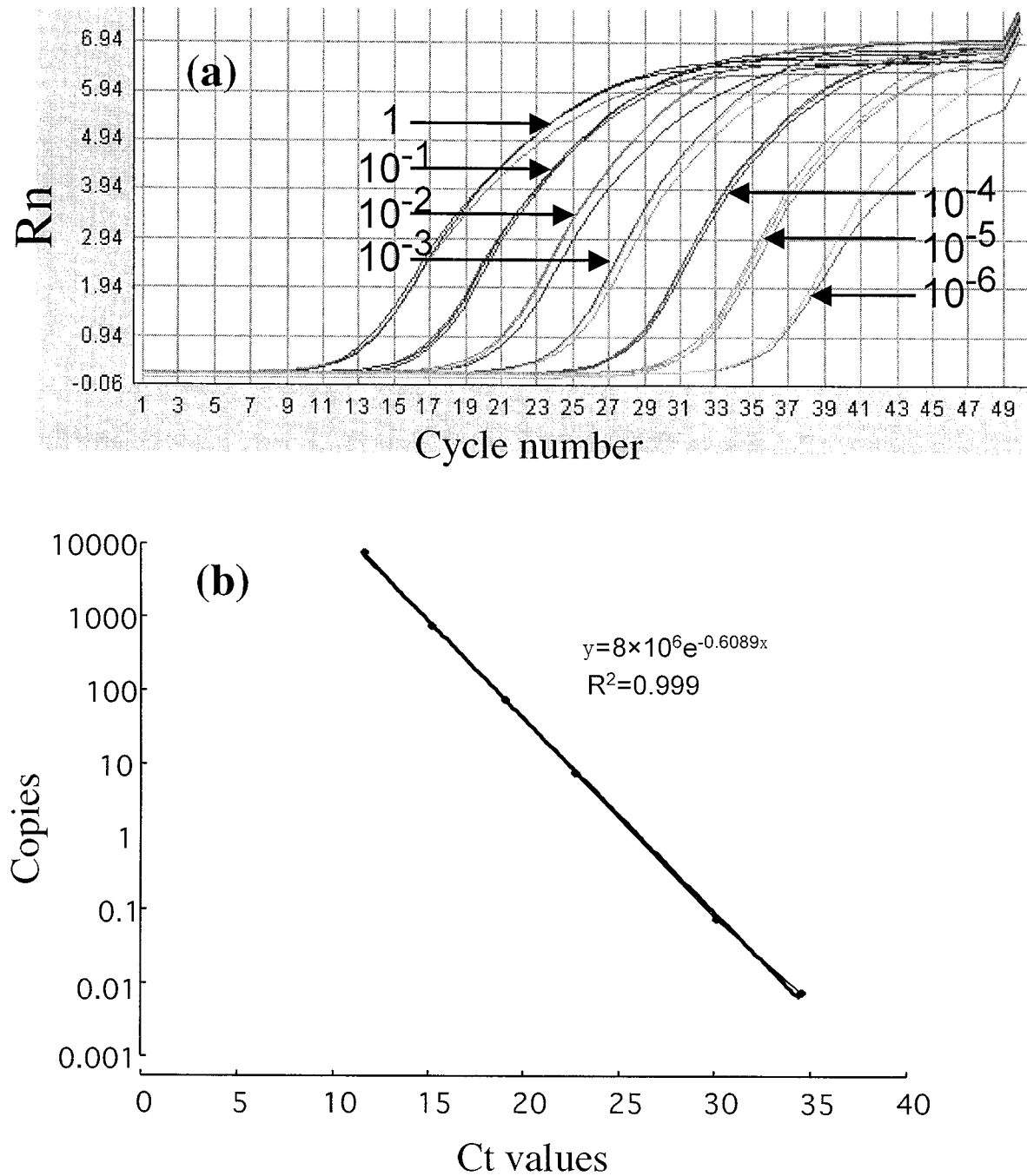


Fig. 1. Correlation between the BVDV gene copies and the threshold cycle (Ct).

(a) The number of copies of the BVDV gene per μ l of sample was estimated from the 10 times serial dilutions (1 to 10^{-6}) of 10 ng of amplified products. The curves of each dilution were indicated from the results of triplicate reactions. (b) The standard curve showed a highly significant ($R^2=0.999$) negative correlation between the Ct value and the number of gene copies.

By the use of oral swab sampling, 1,488 newborn beef calves were examined by RT-PCR in 2

farms for a year. 2 PI calves were detected. Acute infection could not be identified from swab sam-

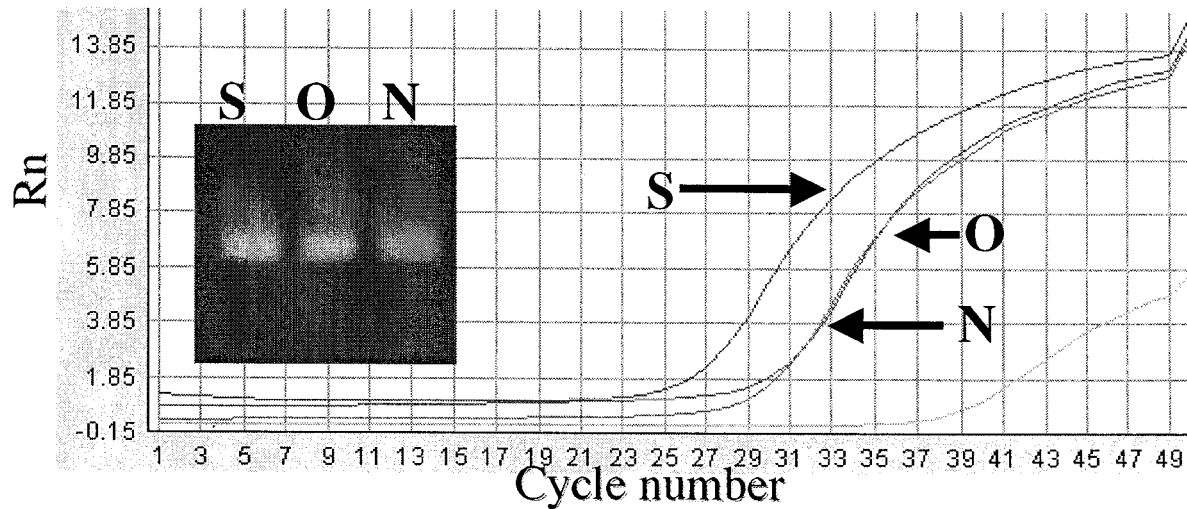


Fig. 2. Amplification signal of real-time PCR of viral gene in each sample

S, serum; O, oral swab sample; N, nasal swab sample. Appearance of 2% agarose gel electrophoresis is almost same each other.

Table 1. Relative quantities of gene copies in the each sample from BVDV-PI cattle

Cattle No.	Breed ¹⁾	Age (Months)	Genotype	Serum ²⁾	Oral ²⁾	Nasal ²⁾
K492	F1	1	1b	1	0.03	0.65
K512	Hol	37	1b	1	0.34	0.42
76	Hol	1	1b	1	17.15	4.66
2	Hol	1	1a	1	17.68	NT
408	JB	1	1c	1	0.04	1.84
409	JB	1	1c	1	0.57	1.09
AK2025	JB	6	1b	1	0.05	NT
602	F1	1	1b	1	10.63	NT
631	Hol	1	1b	1	1.26	NT
IZ	JB	6	2	1	0.16	1.57
45	Hol	45	1a	1	0.11	0.11
52	JB	1	1b	1	6.95	6.37
600	Hol	40	1a	1	1.08	5.38

¹⁾Hol, Holstein; JB, Japanese Black; F1, Mix

²⁾Relative quantity is indicated as relative value (Rv) calculated by following equation.

$Rv = \frac{\text{the number of copies of BVDV gene in the sample}}{\text{the number of copies of BVDV in serum}}$

The number of copies computed by Fig. 1-b). NT: not tested

ples in the present study. Also Antonis et al.¹⁾ reported that acute infection of BVDV could not isolated from swab sample but peripheral leukocyte in experimentally infected calf with BVDV.

BVDV distributes throughout the body of PI cattle^{2,10)}. PI cattle spread the virus in the herd through nasal discharge, saliva, urine, feces, blood,

milk, and so on. Serum or leukocytes are usually used as a sample for BVDV detection. In North America, an ear notch skin sample is widely utilized for the detection of BVDV by immunohistopathological examination or enzyme linked immunosorbent assay^{3,7)}. A scrap of ear lobe obtained during the attachment of ear tag is usually used as

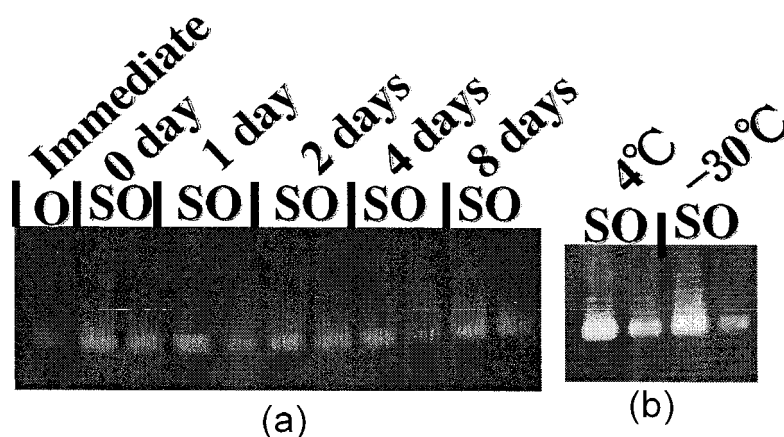


Fig. 3. Appearances of RT-PCR amplified products of serum and oral swab samples from a PI cattle in different store conditions. S, serum sample; O, oral swab sample. (a) Different storage period between 0 to 8 days at 4°C. Immediate, manipulation of RNA extraction started within few minutes after swab sampling. 0 day, stored 4 hrs. (b) Each sample stored at 4°C or -30°C for a day.

a sample. In Japan, ear tags are attached by the pierce method, without a skin piece being extruded during attachment. Therefore, the ear-notch method is not widely used for surveillance of PI cattle in Japan.

Oral swab sampling was easier than nasal swab sampling, and was also less uncomfortable for the cattle than the other sampling methods such as blood collection and ear notching, without pain causing or unnecessary retention. This sampling method can be performed without any special technique and equipment. Therefore, the oral swab sampling method is useful for the screening to detect BVDV.

We are grateful to the veterinary practitioners of Hokkaido Agricultural Mutual Aid Associations for their cooperation. This work was supported by a grant-in-aid from the Ministry of Education, Science, Sports and Culture, Japan, and the Ministry of Agriculture, Forestry and Fisheries of Japan.

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