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Broad detection and quick differentiation of bovine viral diarrhea viruses 1 and 2 by a reverse transcription loop-mediated isothermal amplification test

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ABSTRACT. For broad detection of *pestivirus A* (*bovine viral diarrhea virus 1*: BVDV1) and *pestivirus B* (BVDV2) by a reverse transcription loop-mediated isothermal amplification (RT-LAMP) test, the P25 primer set was designed using nucleotide sequences of 5′-UTR region of 1454 BVDVs. The base coverage of each primer against diverse BVDVs were more than 99% in each base position. The one step LAMP test with the P25 primer set could detect both BVDV1 (TK) and BVDV2 (KZ), but did not amplify 5 other bovine viruses. Detection limit of the LAMP test was 10^3 copies of synthesized DNAs, and 10^{-3} and 10^{-4} dilutions of viral RNAs of TK and KZ strains, respectively, whereas that with current Aebischer's primer set was 10^{-2} dilution and negative of these RNAs, respectively. All of the 63 viral RNA samples of persistently infected (PI) cattle, consisting of the 1a (12), 1b (31), 1c (11), and 2a (9) subgenotypes, were broadly detected with the P25, while only 65% of them were positive with Aebischer's primer set. The validation study showed that the RT-LAMP test with the P25 had 100% sensitivity and 100% specificity against that with updated Vilcek's PCR primers. Also, by using the P26 primer set which contained 3 species-specific primers, all 63 RNA samples were clearly distinguished from BVDV1 or BVDV2 by the typing RT-LAMP test. These results indicate that the one step RT-LAMP test using P25 or P26 primer sets would be useful for broad detection and rapid differentiation of BVDV1 and BVDV2.

KEY WORDS: bovine viral, broad detection, diarrhea, lamp, typing

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The genus *Pestivirus* in the family *Flaviviridae* is a single-stranded positive-sense RNA virus. Of the 11 pestivirus species (*A* to *K*), *Pestivirus A* (*Bovine viral diarrhea virus 1*: BVDV1), and *pestivirus B* (*Bovine viral diarrhea virus 2*: BVDV2) are important pathogens of cattle, causing the transient/chronic diarrhea and mucosal disease [7, 12, 13]. The infection of a susceptible pregnant cow may cause abortion, difficulty of breeding, and birth of persistently infected (PI) cattle. The PI cattle appear healthy, but continuously excrete the viruses into the farm environment, and moreover, cause mucosal disease accompanied with high mortality when non-cytopathogenic virus become cytopathogenic one. These economic losses caused by BVDV infection can be reduced by identification and elimination of the PI cattle from herds, in combination with vaccine inoculation to cows before pregnancy.

The genomes of BVDV1 and BVDV2 are genetically diverse [14]. The BVDV1 is divided into at least 21 subgenotypes (1a–1u), and the BVDV2 is divided into 4 subgenotypes (2a–2d). The genetic variation of BVDVs hinders the development of a reliable molecular diagnostic test with broad coverage of diverse BVDVs.

Currently, reverse transcriptase (RT)-polymerase chain reaction (PCR) and real-time RT-PCR have been used to identify PI cattle [12]. These tests are specific to BVDVs and sensitive enough to detect PI cattle, but must be carried out by well-trained

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technicians. In contrast, the loop-mediated isothermal amplification (LAMP) test is superior to PCR tests in terms of rapidity, simplicity, and easy applicability to many laboratories. However, sequence variations of BVDVs hinder the design of LAMP primers with broad detection spectra. Current LAMP tests have narrow detection spectrum against diverse BVDVs, and can detect some subpopulations of BVDV1 and limited subpopulations of BVDV2 [1, 2, 15]. If a LAMP test has broad detection spectrum, it can be used to detect PI cattle in many developing countries.

Design of the LAMP primers with broad detection spectra for diverse BVDVs is not a straightforward task. A LAMP test usually requires eight conserved primer sites in a small target region. The 5′-untranslated region (UTR) (approximately 385 bp) is well-conserved in the BVDV genomes, but even in this region, there are considerable variations in the sequence. This makes it difficult to design primers with a broad detection spectrum. A new method to design primers with a broad detection spectrum is needed.

Recently, we have shown that exhaustive detection of diverse hemagglutinin (HA) genes of H5 subtype avian influenza viruses was possible by using subpopulation primers in the RT-LAMP test [3]. The subpopulation primers can be designed on conserved regions as consensus sequences of each subpopulation of HA genes. This subpopulation primer approach is theoretically applicable to primers of the BVD-LAMP test.

In this study, we have designed subpopulation primers (P25 primer set) with wide-detection spectrum against both BVDV1 and BVDV2 for an one-step real-time RT-LAMP test. The RT-LAMP test with P25 primer set could detect all of the 63 BVDV field RNA samples. Also, the P26 primer set was designed to distinguish BVDV1 from BVDV2 by using 3 of the 6 P25 primers as species-specific primers. By using the P26 primer set in the RT-LAMP test, all 63 BVDV field RNA samples were clearly classified into BVDV1 or BVDV2. This is the first report of the universal RT-LAMP test for broad detection and quick differentiation of BVDV1 and BVDV2.

MATERIALS AND METHODS

Primer database

Nucleotide sequence data of the 5′-UTR region of the BVDV1 (1235 genes) and BVDV2 (283 genes) were retrieved from GenBank (https://www.ncbi.nlm.nih.gov/pubmed/nucleotide). Length and positions of the sequenced regions of BVDVs varied considerably. These sequences were aligned using Genetyx software (Genetyx, Tokyo, Japan), and transferred to a spreadsheet to assign one base to each cell. This primer database is used for counting numbers of A, G, T, and C bases at each base position and to design of subpopulation primers [3].

Design of LAMP primers

Subpopulation primers with broadly reactivity to diverse genes of BVDV1 and BVDV2 were designed based on our primer guideline [3, 10]. Six primers (F3, B3, FL, BL, FIP, and BIP) were assigned on the eight conserved regions of the 5′-UTRs of BVDV, and the resulting primer set was named P25 primer set (Table 1).

Viruses, synthesized DNA templates, and field RNA samples

Viral culture fluids of BVDV1 Tokachi strain (TK Ct value 24.2), BVDV2 KZ-91 strain (KZ Ct value 19.3), bovine ephemeral fever virus (BEFV, Ct value 13.7), bovine respiratory syncytial virus KY5 strain (BRSV, Ct value 8.9), bovine coronavirus (Kakegawa strain) (BCV, Ct value 13.7), bovine adenovirus (serotype 7) (BAV, Ct value 11.2). These viruses were kindly supplied by Dr. H. Sentsui (Department of Veterinary Medicine, Nihon University). The infectious cDNA clone of bovine leukemia virus (AJ003 strain) (BLV, Ct value 23) isolated in our laboratory were used. The primer sets used to determine Ct values was those published previously [5, 6, 8].

Additionally, the 5′-UTR (400 bases long) of 5 pestivirus DNA templates, including BVDV1 (NADL strain, M31182), BVDV2 (GBK E- strain, AB894424), classical swine fever virus (CSFV) (*pestivirus C*) (HuN23/2013 strain, KP233071), and Border disease virus (BDV) (*pestivirus D*) (Aveyron strain, KF918753) were synthesized by Eurofins Genomics (Tokyo, Japan), and concentrations of these gene fragments were adjusted to 10^6 copies/µl with Easy dilution (Takara Bio Inc., Kusatsu, Japan). These synthesized DNA templates were used for specificity test and sensitivity test.

A total of 63 BVDV-positive and 120 BVDV-negative field RNA samples were obtained from Livestock Hygiene Service Centers of Chiba and Tochigi Prefectures of Japan. These viral RNA samples were stored at the Livestock Hygiene Service Centers, and used to determine the detection spectrum and the validation of the RT-LAMP test. Sixteen Chiba RNA samples were derived from serum, buffy coat, and liver tissues of PI cattle or bulk milk samples, whereas 47 Tochigi RNA samples were prepared from the sera of PI cattle [4].

Viral genome preparation

Eleven viral RNA genomes were extracted from bovine serum or cell culture fluids using the High Pure Viral RNA kit (Roche) according to the manufacturer's instructions (Table 1). Genomes of DNA viruses (bovine adenovirus, infectious bovine rhinotracheitis virus and bovine leukosis virus) were purified by a phenol-chloroform extraction method. These genome samples were stored at −80°C before use.

cDNA synthesis

Viral RNA samples were reverse-transcribed to cDNA using the PrimeScript RT reagent kit (Takara, Bio Inc., RR037) according to the manufacture's instruction. Briefly, 2 µl of 5× PrimeScript Buffer (for real-time), 0.5 µl of PrimeScript RT enzyme mixture, 0.5 µl of random primer (6-mer), 4 µl of RNase free H_2O, and 3 µl of viral RNA were mixed. The mixture was incubated at 37°C for 15 min followed by 85°C for 5 sec. The cDNA was used in the PCR or LAMP test.

Table 1. Primers of one-step real-time loop mediated amplification test for the common detection and differentiation of bovine viral diarrhea viruses 1 and 2

Primer site	Target genes	ID number	Position*[1]	Sequences*[2]	Primer sets		
					P25	P26	
					Common	V1	V2
F3	V1, V2	P2843	110–130	CATRCCYTYAGTAGGACKAGC	○		
	V1	2872	142–161	GGGKAGCRRCAGTGGYGAGTT		○	
	V2	2873	142–161	GACTAGCGGTAGCAGTGAGTT			○
B3	V1, V2	P2853	427–404	aaaagttcatttgwrawcaaytcc	○	○	○
FL	V1, V2	P2695	212–193	gtyraaccaytgacgact	○	○	○
BL	V1	P2847	361–378	CCACTGYWYYGCTACTRA	○	○	
	V2	P2848	361–378	CTGCTAYYYCGCTAGTAW	○		○
FIP	V1, V2	P2844	257–246, 165–184	cctcgtccacrtTGGATGGCYKAABCCCTGAG	○	○	○
BIP	V1	P2850	341–358, 402–384	TGATAGGRYGCTGCAGAGtgtgcmatktacagcagag	○	○	
	V2	P2851	341–358, 402–384	TGATAGGGYGTWGCAGAGtgtgcmatktacagcagag	○		○

*[1] Primer position is shown as the nucleotide number of serotype 1 Nose E-strain (AB558134) or serotype 2 KZ-91 (LC006970) strains. *[2] Captal letters indicate one sense, while lower case letters with underline means antisense.

Preparation of primer mixture

The P25 primer set with broad coverage for BVDV1 and BVDV2 consisted of 1 forward inner primer (FIP), 2 backward inner primer (BIP), 1 FL (forward loop), 2 BL (backward loop), 1 F3, and 1 B3 primers. Concentrations these primers were adjusted to 40, 40, 20, 20, 5, and 5 pmol/µl, respectively.

RT-LAMP

The RT-LAMP reactions were carried out in a 5 µl reaction volume containing 3 µl of isothermal master mix (IMM) (OptiGene Ltd., West Sussex, UK), 0.05 µl (1 unit) of avian myeloblastosis virus (AMV) reverse transcriptase (Nippon Gene, Tokyo, Japan), 0.4 µl of primer mix, 0.25 µl of random primer (6-mer), 0.3 µl of RNase free H_2O, and 1 µl of viral RNA. After mixing by tapping twice, the amplification was performed by using the Genie II machine (OptiGene Ltd.). The reaction was initially incubated at 60°C for 40 min, followed by an annealing cycle 98°C for 1 min. The Aebischer's primer set was used as a current primer set for a RT-LAMP test, and the incubation protocol was the same to the above.

Comparison of sensitivity and specificity

The detection limit of the RT-LAMP test with the P25 primer set was determined using 10-fold serial dilutions of viral RNAs and synthesized DNAs of TK and KZ strains with Easy Dilution (Takara Bio Inc.). The detection limit of the RT-LAMP test was compared with that of the RT-LAMP test with the Aebischer's primer set, and with that of the real-time RT-PCR (SYBR) using Vilcek original primer set [11], which is currently used at the Livestock Hygiene Service Centers in Japan.

Real-time RT-PCR (SYBR)

The SYBR green-based real-time PCR with the original Vilcek's primer set (p324F and p326R) [11] was carried out with the 7500 Real Time PCR System (Table 1). The PCR reaction mixture contained 5 µl of (2×) SYBR premix (Takara Bio Inc., No. RR420), 1 µl each of forward and reverse primers (20 pmol/µl) [11], 2 µl of RNase free H_20, and 1 µl of cDNA sample. The RT-PCR was performed with the following protocol: 95°C × 30 sec and then 35 cycles of 95°C for 30 sec, 50°C for 20 sec, and 72°C for 20 sec, followed by a dissociation cycle (95°C for 15 sec, 60°C for 60 sec, and 95°C for 15 sec). Also, the original Vilcek primers were updated based on the nucleotide sequence information of the field RNA samples, and used for the detection and sequencing of the 63 field RNA samples.

Sequencing and differentiation of pestivirus species

The 5′-UTR region of the 63 field BVDV RNA samples were sequenced to determine the species and subgenotypes. Briefly, PCR products of the 5′-UTR region which covered entire primer sites of LAMP and PCR tests were amplified by the RT-PCR with forward primer P2733 (5′-CTYWGCGAAGGCCGAARAG-3′) or P2882 (5′-CCCYYAGCGAMGGCCGAAM-3′) and reverse primer P2734 (5′-RGGTTTTTGTTTRTATGTT-3′). The products were purified with High Pure PCR Product Purification Kit (Roche Diagnostics Corp., Indianapolis, IN, USA), and sequenced with the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific K.K., Tokyo, Japan). After pretreatment with BigDye X Terminator purification kit, the products were analyzed with ABI PRISM 3130 Genetic Analyzer (Thermo Fisher Scientific).

Validation of the RT-LAMP test using field RNA samples

Sensitivity and specificity of the RT-LAMP test was validated against the Vilcek's RT-PCR test using those of the RT-PCR test with updated Vilcek's primers (P2745: 5′-ATRCCMTKAGTAGGACKAGC-3′, P2746: 5′-WCAAYTCCATRTGCCATGTAC-3′).

For this validation study, a total of 183 field RNA samples which were composed of 63 BVDV (+) (plus 1 BVDV (+) sample) and 120 BVDV (−) field RNA samples were used.

Differentiation between BVDV1 and BVDV2 by RT-LAMP

The 63 LAMP (+) field RNA samples (Table 5) were used to differentiate BVDV1 from BVDV2 by the RT-LAMP test with two P26 primer sets (P26V1, P26V2). Concentration of the BL and typing F3 primers were increased to 40 and 15 pmol/µl, respectively, for quick amplification of genes.

In silico analysis of gene amplification for primer evaluation.

The gene coverage of each LAMP primer as well as those of the original Vilcek's PCR primers was validated by using 1518 5′-UTR gene sequences (BVDV1, 1235: BVDV2, 273). Numbers of mismatched bases in each gene against each primer were counted. According to our previous experiments [3, 9], genes with 0–2 mismatched bases per primer were determined amplifiable, whereas those with more than 3 mismatches were not.

RESULTS

Construction of BVDV primer database

The BVDV primer database was constructed with aligned nucleotide sequences of 5′-UTR region of the 1180 BVDV1 and 273 BVDV2. Target region (332 bp) of the primer database corresponded to positions 106 to 437 of Nose E strain (BVDV1) (AB558134) which consisted of 5′-UTR (282 bp, positions 106–387) and a part of Npro protease (51 bp, positions 388–438). The sequenced positions and length varied considerably among genes, and therefore numbers of registered genes in the central part (positions 142 to 379) were more than 1,000 genes, while those in both terminal ends were approximately 200 genes. In addition, two regions (215–243, 283–319) were not used for primer sites since there were addition/deletion of bases in some strains. Therefore, 3 central regions of 5′-UTR which did not contain addition or deletion of bases (base positions 142–214, 244–282, and 320–379) were selected as suitable primer sites.

Design of LAMP primers

Several combinations of our primers and mixed combinations of ours and Aebisher's primers were tested for amplification of RNAs of BVDV1 TK and BVDV2 KZ strains by the RT-LAMP test. As a result, the P25 primer set which could amplify both genes quickly was obtained (Table 1). The TK and KZ RNAs (10-fold dilution) were amplified in 20 min and 14 min, respectively, while these RNAs were detected in 28 min and negative with Aebishcer's primer set. The P25 primer set consists of the three original primers designed in this study (B3, BL, and BIP) and the three primers designed on the similar positions of Aebisher primers (F3, FL, and FIP) (Table 1). Five primer sites (F2, FL, F1, B1, and BL) were located on the middle region (base positions 139–377) which contained large numbers of genes, whereas 3 primer sites (F3, B2, and B3) were located at both ends of the primer database (base positions 109–129 and 383–426) which were composed of small numbers of genes.

Specificity of P25 primers

The specificity of the P25 primer set in one step RT-LAMP test was tested using BVDV RNAs and 5 other virus genomes (DNA and RNA samples). As a result, only two BVDV RNAs, TK (BVDV1) and KZ (BVDV2) strains, were detected after 18 and 17 min incubation, respectively. However, DEFV, BRSV, BCV, BAV, and BLV were not detected. These results suggest that the P25 primer set is specific to BVDV1 (*pestivirus A*) and BVDV2 (*pestivirus B*) species.

Specificity of P25 primer set was further tested by using synthesized 5′-UTR DNA templates. In this experiment, 100-fold higher concentrations of temperate (10^8 copies/µl) of the following pestiviruses were used to investigate the cross-reactivity: BVDV1 NADL strain (*pestivirus A*, M31182), BVDV2 GBK E- strain (*pestivirus B*, AB894424), Hobi-like virus D32/00 strain (*pestivirus H*, AB871953), CSFV HuN23/2013 strain (*pestivirus C*, KP233071), and BDV Aveyron strain (*pestivirus D*, KF918753). As a result, BVDV1 and BVDV2 genes were detected quickly (24 min and 20 min, respectively), but Hobi-like virus and BDV were detected lately (29 min and 33 min, respectively), and CSFV was not detected. These pestivirus specificity test indicates that the RT-LAMP test with the P25 primer set can detect BVDV1 and BVDV2 strains rapidly, but pestiviruses H and D were weakly reactive.

Sensitivity of the P25 primers

Detection limit of the RT-LAMP test with P25 was determined using 10-fold serial dilutions of viral RNAs. As a result, TK RNA (BVDV1) was positive up to 10^{-3} dilutions by the RT-LAMP, while it was positive up to 10^{-2} dilutions with the Aebischer's primer set. Also, the KZ-91 RNA (BVDV2) was detectable up to a dilution of 10^{-4} and 10^{-1} by the RT-LAMP test with P25 and Aebischer primer sets, respectively. These results show that the P25 primer set is more sensitive than the Aebischer primer set to detect BVDV1 and BVDV2 genes.

Statistical validation with field RNA samples

Sensitivity and specificity of the RT-LAMP test with P25 primer set was validated statistically using the RT-PCR test with the updated Vilcek's primers which was the Japanese standard BVDV test. In this experiment, the 63 BVDV (+) and 120 BVDV (−) field RNA samples were used. As a result, all of the 63 BVDV (+) field RNA samples were detected by the RT-LAMP test with the P25 primer set (Table 2), and all of the 120 BVDV (−) RNA samples were negative. Therefore, the sensitivity of the RT-LAMP test is 100%

Table 2. Comparison of three detection tests for detecting bovine viral diarrhea viruses from field samples

Name of sample	Origin	Subgenotype type	RT-LAMP Detection time (min) with P25		RT-LAMP Detection time (min) with Aebischer		rt RT-PCR Vilcek (35 cycles)
CB2	Buffy coat	1b	+	17	+	21	+
CB3	Serum	2a	+	25	+	32	-
CB4	Liver	1b	+	17	+	22	+
CB7	Serum	2a	+	23	-	-	+
CB8	Serum	1c	+	22	+	26	+
CB9	Serum	1b	+	20	+	26	+
CB10	Serum	1c	+	19	+	25	+
CB11	Serum	1b	+	19	+	25	+
CB13	Buffy coat	2a	+	21	-	-	+
CB14	Buffy coat	1b	+	19	-	-	+
CB15	Buffy coat	1b	+	21	+	24	+
CB16	Buffy coat	1b	+	20	+	28	+
CB17	Buffy coat	1b	+	25	+	30	+
CB18	Buffy coat	1b	+	19	+	25	+
CB19	Serum	1b	+	21	+	24	+
CB21	Bulk milk	1b	+	26	-	-	+
TG1	Serum	1b	+	22	+	29	+
TG2	Serum	1b	+	20	+	25	+
TG3	Serum	1b	+	21	+	27	+
TG4	Serum	1b	+	24	+	26	-
TG5	Serum	1b	+	23	+	28	+
TG6	Serum	1c	+	26	-	-	+
TG7	Serum	1a	+	23	+	23	+
TG8	Serum	1a	+	26	+	28	+
TG9	Serum	1a	+	26	+	27	+
TG10	Serum	1a	+	26	+	27	+
TG11	Serum	1c	+	27	+	32	+
TG12	Serum	1c	+	27	+	30	+
TG13	Serum	1c	+	27	+	32	+
TG14	Serum	1a	+	27	-	-	+
TG15	Serum	1b	+	29	-	-	+
TG16	Serum	1a	+	26	-	-	+
TG17	Serum	1a	+	28	-	-	+
TG18	Serum	1b	+	24	+	26	+
TG19	Serum	1a	+	24	+	30	+
TG20	Serum	1b	+	21	+	29	+
TG21	Serum	2a	+	26	-	-	+
TG22	Serum	1b	+	22	+	27	+
TG23	Serum	1b	+	26	+	29	+
TG24	Serum	2a	+	32	-	-	+
TG25	Serum	2a	+	35	-	-	+
TG26	Serum	1c	+	29	+	31	+
TG27	Serum	2a	+	26	-	-	+
TG28	Serum	1b	+	28	-	-	+
TG29	Serum	1a	+	38	-	-	+
TG30	Serum	2a	+	35	-	-	+
TG31	Serum	1b	+	30	-	-	+
TG32	Serum	2a	+	30	-	-	+
TG33	Serum	1c	+	25	+	27	+
TG34	Serum	1b	+	25	+	26	+
TG35	Serum	1b	+	24	-	-	+
TG36	Serum	1b	+	26	-	-	+
TG37	Serum	1c	+	27	+	32	+
TG38	Serum	1b	+	23	+	28	+
TG39	Serum	1c	+	31	+	37	+
TG40	Serum	1c	+	31	+	37	+
TG41	Serum	1b	+	20	+	24	+
TG42	Serum	1a	+	28	-	-	+
TG43	Serum	1a	+	31	+	31	+
TG44	Serum	1a	+	38	+	32	+
TG45	Serum	1b	+	27	+	34	+
TG46	Serum	1b	+	40	-	-	-
TG47	Serum	1b	+	30	+	39	-
Total			63 (100%)		41 (65%)		59 (94%)

RT-LAMP, reverse transcription loop-mediated isothermal amplification.

and the specificity is 100%, and 54 of the 63 (86%) samples were detected within 30 min by the RT-LAMP test. In contrast, only 41 (63%) BVDV (+) field RNA samples were positive with the Aebischer primer set, and only 30 (48%) of the samples were detected within 30 min.

As shown in Table 3, all of the 1a (12), 1b (31), 1c (11), and 2a (9) subgenotypes were detected with the P25 primer set. On the other hand, 7 of the subgenotype 1a (7/12, 58%), 23 of the subgenotype 1b (23/31, 74%), 10 of the subgenotypes 1c (10/11, 91%), and 1 of the subgenotypes 2a (1/9, 11%) were detected with the Aebisher's primer set, respectively (41/63, 65%). Also, 12 (100%), 28 (90%), 11 (100%), and 8 (89%) of these subgenotypes, respectively, were detected by the RT-PCR with the original Vilcek primers (59/63, 94%).

These results indicate that the P25 primer set has broad detection spectrum against diverse BVDV1 and BVDV2.

Table 3. Genotyping of bovine viral diarrhea virus RNAs by one-step real-time loop-mediated amplification test with P26 primer sets

Genes tested	Detection time (min) with		
	P25	P26V1	P26V2
BVDV1 TK	18	15	21
BVDV2 KZ	24	38	21
CSFV	-	-	-
BDV	34	31	35

Synthesized DNA templetes ($10^{\wedge 6}$ copies/tube) of CSFV and BDV were used. BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus; BDV, boader disease virus.

Quick differentiation of BVDVs by the RT-LAMP test

The P25 primer set, which detects BVDV1 and BVDV2 in common, consisted of four common primers (F3, B3, FL, FIP) and two species-specific primers (BL, BIP) (mixture of V1 and V2 primers). If some of the four common primers could be replaced with species-specific primers, it was possible that BVDV1 and BVDV2 could be distinguished by RT-LAMP test. Trying to design species-specific primers of these common primer sites, two species-specific primers of F3 (P2872 for BVDV1 and P2873 for BVDV2) could be obtained at 32 bases downstream of the original F3 primer site. The species-specific F3 primers, named P26V1 for BVDV1 and P26V2 for BVDV2, were designed by substituting 2–5 bases of the P2843 with species-specific bases. Differentiation of two species was not possible by positive/negative, but they could be distinguished by the difference in detection time. Also, increased concentration of the F3 and BL primers as triple and double amounts, respectively, was effective for quick amplification of a homologous gene. For example, TK (BVDV1) RNA became positive at 15 min and 21 min with the P26V1 and P26V2 primer sets, respectively, and KZ (BVDV2) RNA was positive at 38 and 21 min with the P26V1 and P26V2 primer sets, respectively (Table 2). However, the CSFV DNA template was negative with any of the P26 primer sets, but the BDV DNA template was detected lately after 30 min incubation with the P26 primer sets. These results suggested that quick differentiation of BVDV1 and BVDV2 might be possible by using the P26 primer sets.

Verification of the quick differentiation test using field RNA samples

Applicability of the quick differentiation test was evaluated using the 63 field BVDV (+) RNA samples consisted of the 1a, 1b, 1c and 2a subgenotypes. As a result, all of the 63 BVDV (+) field RNA samples were correctly differentiated by the RT-LAMP test with P26 primer sets (Table 4). These results demonstrate that the one step real-time RT-PCR test with the P26 primer sets is useful for quick differentiation of BVDV1 and BVDV2.

In silico evaluation of gene coverage of P25 and P26 primers

The *in-silico* evaluation of the 10 LAMP primers and two original Vilcek PCR primers for gene coverage was performed using 1518 BVDV genes. The number of genes retrieved from the GenBank varies considerably depending on the primer region. Only genes containing the entire sequences in each primer site were used in this analysis, and genes with less than 2 mismatched bases per primer were determined amplifiable. The results were summarized in Table 5. All of the 10 LAMP primers had more than 99% coverage of the BVDV1 and BVDV2. On the other hand, the original Vilcek's forward and reverse primers had 88.6% and 100% coverage, respectively, although these primers were located at 5′ or 3′ ends of our primer database (114 genes for P324F and 146 genes for P326R). This *in silico* validation study suggests that all 10 LAMP primers designed in this study have quite high gene coverage against diverse BVDV genes.

DISCUSSION

The LAMP test would be a quick screening test for the detection of PI cattle since the test has high sensitivity, quickness, and easy-to-handle. However, the narrow detection spectrum of the current LAMP tests hinders its application [1, 2]. Gene coverage of the Fan's LAMP test was estimated as 95% and 70.6% for BVDV1 and BVDV2 [2], and Fan's F3 primer site was partially located out of our database, and Fan's B3 primer site was completely out of our database. Also, gene coverage of the Aebischer's primers was estimated as 100% (7/7) and 25% (1/4) of BVDV1 and BVDV 2, respectively [1], and this study showed that the coverage was 74% (40/54) and 10% (1/10), respectively. By using the BVDV primer database, the common P25 primer set could be designed. The evaluation analysis with the field RNA samples and *in silico* validation study suggests that the RT-LAMP test with P25 primer set has high gene coverage against diverse BVDV1 and BVDV2. Thus, the one step, real-time RT-LAMP test with P25 primer set would be useful for rapid detection of PI cattle. It is necessary to use it carefully for diagnosis since the P25 primer set weakly interacted with *Pestivirus D* and *Pestivirus H*.

After identification of PI cattle, next step is to identify the species of BVDV, BVDV1 or BVDV2 for selection of the vaccine. However, a rapid typing RT-LAMP test of BVDVs has not been developed. Based on the difference in the detection time between

Table 4. Genotyping of bovine viral diarrhea viruses directly from field RNA samples by the one-step real-time loop-mediated amplification test

Name of sample	Origin	Genotype by sequencing	PCR*[1]	LAMP		
			Ct value	Detection time (min) with P26V1	Detection time (min) with P26V2	Genotype
TK		1	29	21	27	1
KZ		2	24	26	16	2
CB2	Buffy coat	1	24	19	32	1
CB3	Serum	1	25	27	-	1
CB4	Liver	1	18	19	36	1
CB7	Serum	2	26	-	27	2
CB8	Serum	1	25	23	37	1
CB9	Serum	1	27	21	39	1
CB10	Serum	1	25	23	39	1
CB11	Serum	1	25	23	37	1
CB13	Buffy coat	2	25	-	23	2
CB14	Buffy coat	1	24	22	35	1
CB15	Buffy coat	1	25	18	32	1
CB16	Buffy coat	1	21	20	30	1
CB17	Buffy coat	1	23	24	40	1
CB18	Buffy coat	1	23	18	-	1
CB19	Serum	1	26	20	32	1
CB21	Bulk milk	1	30	23	-	1
TG1	Serum	1	28	20	29	1
TG2	Serum	1	27	20	26	1
TG3	Serum	1	27	20	27	1
TG4	Serum	1	27	20	28	1
TG5	Serum	1	29	23	32	1
TG6	Serum	1	29	27	35	1
TG7	Serum	1	29	19	26	1
TG8	Serum	1	28	20	26	1
TG9	Serum	1	29	20	28	1
TG10	Serum	1	29	20	26	1
TG11	Serum	1	28	24	29	1
TG12	Serum	1	27	24	28	1
TG13	Serum	1	28	22	28	1
TG14	Serum	1	28	18	-	1
TG15	Serum	1	27	17	23	1
TG16	Serum	1	29	18	25	1
TG17	Serum	1	29	24	35	1
TG18	Serum	1	27	19	28	1
TG19	Serum	1	28	22	32	1
TG20	Serum	1	28	18	29	1
TG21	Serum	2	28	38	23	2
TG22	Serum	1	27	18	27	1
TG23	Serum	1	27	19	29	1
TG24	Serum	2	29	-	24	2
TG25	Serum	2	27	38	22	2
TG26	Serum	1	28	23	32	1
TG27	Serum	2	28	40	21	2
TG28	Serum	1	26	20	27	1
TG29	Serum	1	29	26	-	1
TG30	Serum	2	26	40	23	2
TG31	Serum	1	29	22	33	1
TG32	Serum	2	31	-	22	2
TG33	Serum	1	26	21	29	1
TG34	Serum	1	26	21	29	1
TG35	Serum	1	25	20	29	1
TG36	Serum	1	27	20	28	1
TG37	Serum	1	26	22	30	1
TG38	Serum	1	27	19	28	1
TG39	Serum	1	28	26	-	1
TG40	Serum	1	29	26	34	1
TG41	Serum	1	23	16	23	1
TG42	Serum	1	28	20	26	1
TG43	Serum	1	29	22	28	1
TG44	Serum	1	27	21	-	1
TG45	Serum	1	28	21	28	1
TG46	Serum	1	28	28	39	1
TG47	Serum	1	29	25	-	1

*[1] The updated Vicek's primers were used for the PCR test and for sequencing the PCR products. LAMP, loop-mediated isothermal amplification.

Table 5. *In silico* validation of the P25 and P26 primers for amplification of bovine viral diarrhea viruses

Primer site	By the one-step real-time loop-mediated amplification test			B3	FL	BL		FIP	BIP		RT-PCR Vilcek*2	
Primer ID	P2843	P2872	P2873	P2853	P2895	P2847	P2848	P2844	P2850	P2851	P324F	P326R
Target	V1 V2	V1	V2	V1 V2	V1 V2	V1	V2	V1 V2	V1	V2	V1 V2	V1 V2
No. of genes detected	114	945	43	175	1,378	1,046	198	1,209	62	92	101	146
No. of genes not detected	0	5	0	0	1	5	1	7	0	1	13	0
Detection rate*1	100%	99.5%	100%	100%	99.9%	99.4%	99.5%	99.4%	100%	99.0%	88.6%	100%

*1 The detection rate was calculated as follows: no. of genes detected/ (no. of genes detected + no. of genes not detected). *2 No genes of bovine viral diarrhea virus 2 (BVDV2) were available for Vilcek's reverse primer.

homologous and heterologous genes, all of the 63 BVDV (+) field RNA samples could be differentiated within 40 min. This result indicate that the typing RT-LAMP test would be useful for quick differentiation between BVDV1 and BVDV2.

The spreadsheet-based primer database was indispensable to design and evaluate primers with broad gene coverage. All of the 10 primers developed in this study (P25 and P26 primer sets) have more than 99% gene coverage against over 1,000 BVDV genes retrieved from the GenBank, and these primers are sensitive enough to detect PI cattle and to classify BVDV species as shown in Tables 2 and 4. Until now, it was considered that species-specific primers could not be designed in the highly conserved 5′-UTR region. However, we found that BVDV can be differentiated by substituting three of the six common primers with species-specific primers which contained two to five species-specific bases. This finding suggests that common detection and differentiation of diverse viruses by LAMP or PCR methods may be possible by combination of primers designed on a single conserved region.

Another BVDV-specific problem to develop genetic test methods is that the 5′-UTR sequences of BVDVs registered in GenBank are fragmented, and the sequenced positions varies considerably among virus strains. In this study, numbers of genes at both ends (F3, B2 and B3 primer sites or Vilcek primers) are very small. It is recommended that sequences of the entire 5′-UTR region should be registered in the GenBank, not a part of the region. Two primers may be useful to amplify and sequence the entire 5′-UTR region of BVDVs: P2733 forward primer (5′-cccYYagcgaMggccgaaM-3′) and P2734 reverse primer (5′-KggtttYtgtttRtatgtt-3′). In order to develop a reliable diagnostic test for easily mutating RNA viruses, it is necessary to register the nucleotide sequences of the common region in the GenBank.

In conclusion, the LAMP tests for the broad detection and quick differentiation of BVDV1 and BVDV2 were established based on the primer database. The field sample test showed that the tests were useful for identification of PI cattle and quick differentiation of BVDVs.

CONFLICT OF INTEREST. The authors have no conflicts of interests.

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