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NOTE

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Development of a fluorescent loop-mediated isothermal amplification assay for rapid and simple diagnosis of bovine leukemia virus infection

Konomu NAGAO[1)], Ryohei MAKINO[1)], Francis Victor APEGO[1)], Hirohisa MEKATA[2,3)] and Wataru YAMAZAKI[1,2)]*

[1)]Department of Veterinary Science, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki, Miyazaki 889-2192, Japan
[2)]Center for Animal Disease Control, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki, Miyazaki 889-2192, Japan
[3)]Organization for Promotion of Tenure Track, University of Miyazaki, 1-1 Gakuen Kibanadai-nishi, Miyazaki, Miyazaki 889-2192, Japan

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ABSTRACT. Bovine leukemia virus (BLV) causes enzootic bovine leukosis (EBL), a condition that threatens the sustainability of the livestock industry. A fluorescent loop-mediated isothermal amplification (fLAMP) assay targeting BLV *env* sequences was developed and used to evaluate 100 bovine blood samples. Compared with a conventional real-time PCR (rPCR) assay, the fLAMP assay achieved 87.3% (62/71) sensitivity and 100% (29/29) specificity. The rPCR assay took 65 min, while the fLAMP assay took 8 min to 30 min from the beginning of DNA amplification to final judgement with a comparable limit of detection. The fLAMP is a potential tool for the rapid and simple diagnosis of BLV infection to supplement ELISA testing and can be used by local laboratories and slaughterhouses without special equipment.

KEY WORDS: BLV, bovine leukemia virus, enzootic bovine leucosis, fLAMP, fluorescent loop-mediated isothermal amplification

Bovine leukemia virus (BLV), a member of the Retroviridae family and the *Deltaretrovirus* genus, causes enzootic bovine leukosis (EBL). Although BLV-infections are mainly asymptomatic, within several years approximately 30% and 0.1–10% of BLV-infected cattle develop persistent lymphocytosis and fatal lymphosarcoma, respectively [19, 23]. In the European Union (EU), BLV infections have been eradicated, and several nations are officially recognized as free from BLV infection [13, 23]. In contrast, increases in BLV infection and EBL have occurred in Japan [9–11]. In Japan, cattle diagnosed with lymphosarcoma, which are disapproved for meat consumption by legal regulations, cause vast economic losses to the Japanese livestock industry. Accurate and reliable diagnosis of BLV infection is therefore crucial to accelerate efforts to implement an effective eradication program.

Serological tests that detect persistent anti-BLV antibodies, such as agar-gel immunodiffusion and enzyme linked immunosorbent assays (ELISAs) are routinely used worldwide for the effective screening of BLV-infected cattle [9, 11, 16]. However, these tests are insufficient to correctly diagnose the early stages of infection [5, 9, 16]. Therefore, supplemental use of a rapid and sensitive assay for the detection of BLV-genomic DNA is required to reinforce accurate diagnosis.

Although nested PCR assays detect BLV-genomic DNA with high sensitivity [1, 14, 23], they require time-consuming and tedious electrophoretic analysis and are plagued by laboratory contamination. Although real-time PCR assays are more rapid and easier to perform compared with nested PCR assays and achieve equivalent analytical sensitivity (limit of detection, LOD), the assays require expensive equipment [3, 16]. Further, detection of at least two distinct genomic targets is desirable to prevent including false-negatives [20].

Loop-mediated isothermal amplification (LAMP) is a promising candidate diagnostic tool for the rapid and sensitive detection of BLV genomic DNA. The LAMP assay involves isothermal amplification at 60–67°C within 60 min and one-step detection of a target gene using simple analysis of turbidity (turbidimetric LAMP or tLAMP) [5, 14, 15, 24]. Therefore, the assay is faster and easier to perform than nested PCR and real-time PCR assays with equivalent analytical sensitivity [5, 12, 14, 15, 24]. tLAMP assays afford rapid diagnosis of viral diseases [15, 24], including BLV detection through targeting the long terminal repeat (LTR)

*Correspondence to: Yamazaki, W.: yamazakiw@cc.miyazaki-u.ac.jp

Table 1. Primers used for fluorescent LAMP and real-time PCR assays for the detection of BLV proviral sequences

Assay primer	Sequences (5′–3′)	Sequence of the FLK strain (Genbank accession no. LC164083)	Reference
Fluorescent LAMP			This study
BLV-2_FIP	GGCCARTAGATCTTAGGATAGCCA-TTTT-ACCTTTCTGTGCCAAGTCTCC	5,100–5,077-(TTTT)-5,032–5,052	
BLV-2_BIP	GAGCCAGGGCCATGGTC-TTTT-CCCCACATAAGGGCATCG	5,127–5,143-(TTTT)-5,179–5,162	
BLV-2_F3	CCATTGACCAAATACTAGAGGC	4,998–5,019	
BLV-2_B3	CAGTCGAACCGATCHGC	5,180–5,196	
BLV-2_LF	TTACAGAGTCCAAGGTGTATC	5,075–5,055	
BLV-2_LB	CACATATGATTGCGAGCC	5,143–5,160	
Real-time PCR			Rola-Łuszczak *et al*, 2013 [16]; OIE, 2018 [23]
MRBLVL	CCTCAATTCCCTTTAAACTA	2,326–2,345	
MRBLVR	GTACCGGGAAGACTGGATTA	2,445–2,426	
MRBLV probe	6FAM-GAACGCCTCCAGGCCCTTCA-BHQ1	2,346–2,365	

[5, 14]. Fluorescent (fLAMP) reagents are commercially available, and this real-time amplification approach allows extremely rapid and accurate diagnosis through the use of an improved chain replacement enzyme and annealing analysis compared with tLAMP [4, 6, 17].

Here we used 100 bovine blood samples obtained from farms in the Kagoshima, Miyazaki and Oita prefectures in Japan to develop an fLAMP assay that we compared with a published real-time PCR assay.

From March to June 2017, blood samples of 20 Brown-Swiss, 85 Holstein and 304 Japanese Black cattle were collected from five farms in Kagoshima, Miyazaki and Oita prefectures, Japan. An evacuated tube containing EDTA was used for blood collection, all samples were centrifuged at 1,500 × g for 5 min, and the plasma was used for the anti-BLV antibody-ELISA (BLV ELISA test; JNC Co., Ltd., Tokyo, Japan) according to manufacturer's instructions, including with previously described modifications [8, 9].

Genomic DNA extraction was performed according to our previous study [9]. Briefly, among 409 blood samples, 80 seropositive and 20 seronegative samples were randomly chosen. The buffy coat was collected from the centrifuged EDTA-treated blood sample described above, and genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Fitchburg, WI, U.S.A.) in accordance with the manufacturer's instructions. Using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, U.S.A.), the DNA concentration was measured and adjusted to 20 *ng*/*µl* using distilled water. The genomic DNA of fetal lamb kidney cells infected with BLV (FLK-BLV) [21] was extracted in the same manner described above for the comparative determination of the LOD. Genomic DNAs were used immediately, otherwise they were stored at −20 or −80°C.

We used Primer Explorer V5 software (Fujitsu System Solutions Ltd., Tokyo, Japan) to design a new primer set based on BLV *env* sequences. We used Clustal omega for multiple alignment of 180 BLV complete *env* sequences (https://www.ebi.ac.uk/Tools/msa/clustalo/), as well as Jalview to identify conserved *env* nucleotide sequences in an alignment of 401 BLV partial *env* gene sequences [22]. Details of the primers are shown in Table 1. The predicted specificities of the six primers were determined using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The fLAMP assay was performed using a Genie III (OptiGene, Horsham, U.K.). Amplification was performed at 67°C for 30 min, followed by inactivation of enzymatic activity at 98°C for 2 min, and cooling to 80°C for annealing analysis with ramping at 0.05°C/sec. The 25-*µl* fLAMP reaction comprised 15 *µl* of an isothermal master mix (ISO-002, Optigene), 0.4 *µl* each of FIP and BIP primers (100 pmol/*µl*), 0.2 *µl* each of LF and LB primers (100 pmol/*µl*), 0.05 *µl* each of F3 and B3 primers (100 pmol/*µl*), 3.7 *µl* of nuclease-free water, and 5 *µl* of the DNA template. All LAMP primers were produced using column-grade purification methods by Hokkaido System Science (Sapporo, Japan). When the fluorescence intensity reached 20,000 within a 30-min amplification, and the annealing temperature (Ta) value ranged between 89.0 and 92.0°C, the results were interpreted as positive. Time of positivity (Tp) was automatically calculated by the Genie III.

rPCR was performed using a LightCycler 96 (Roche Molecular Systems, Pleasanton, CA, U.S.A.) to detect *pol* sequences according to Rola-Łuszczak, *et al.* [16] and the OIE terrestrial manual [23]. Reactions were modified using shorter times for denaturation and annealing according to the instructions provided with the Cycleave PCR reaction mixture (TaKaRa Bio Inc., Otsu, Japan). Briefly, 20-*µl* rPCR reactions comprised 10 *µl* of 2x Cycleave PCR reaction mixture (TaKaRa Bio), 0.08 *µl* each of primers (100 pmol/*µl*, Hokkaido System Science, 0.04 *µl* of probe (100 pmol/*µl*, Hokkaido System Science), 4.8 *µl* of nuclease-free water, and 5 *µl* of the DNA template. The cycling conditions were as follows: one cycle at 95°C for 10 sec, 50 cycles each at 94°C for 5 sec, and 60°C for 30 sec. Each amplification was performed in duplicate, and an average C_T (threshold cycle) value was automatically calculated. Details of the primers and probes are shown in Table 1.

Based on the sequence of LC164083, a partial sequence of *pol* for rPCR encompassing primer target sequences was synthesized

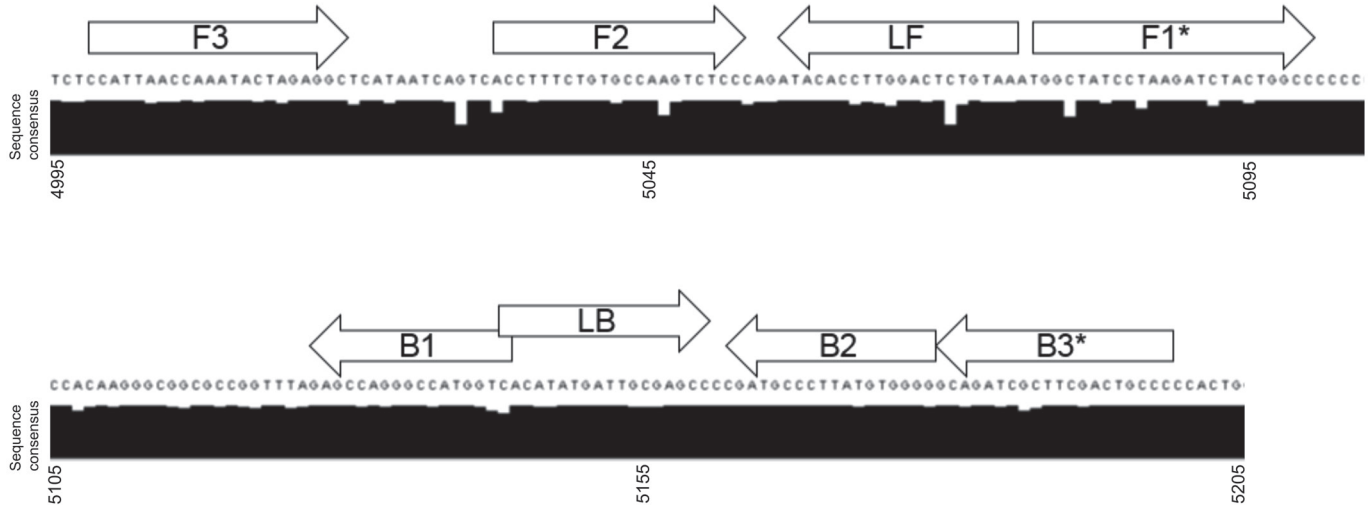

Fig. 1. Location of each primer used in the fLAMP assay. Numbers correspond to the sequence of the BLV FLK strain (LC164083). *LAMP primers containing mixed degenerate sequences.

and cloned into the pEX-K4J1 vector by Eurofins Genomics, Co., Ltd (Tokyo, Japan). The amplified sequence was used to calculate the proviral loads in clinical samples as well as to prepare serial 10-fold serial dilutions of the DNA templates for LOD determination. Further, the tLAMP assay targeting the LTR region was performed to compare the LOD according to a published method [5]. Two DNA templates were used for further comparisons of different genotypes of the FLK strain (genotype 1) and a BLV-genomic DNA obtained from a naturally BLV-infected cow in Miyazaki (JA366, genotype 3). Ten-fold serial dilutions in distilled water were prepared, and the fLAMP, tLAMP and rPCR assays were performed as described above. When a sample was positive or negative in duplicate analyses, the result was interpreted as a positive in all three assays.

We retrieved 401 BLV complete and partial *env* sequences from GenBank to design a BLV-specific LAMP primer set. The locations and nucleotide variations of the LAMP primers representing 180 complete *env* sequences are shown in Fig. 1. Although LAMP primers were derived from the conserved region of *env*, to maintain amplification speed and accuracy, mixed bases were incorporated into the FIP and B3 primers because of the variability of the target sequences that introduce mismatches at the crucial regions between target sequences and primers (Table 1 and Fig. 1).

In a preliminary test, we attempted to design a LAMP primer set using well-conserved *pol* sequences. The *pol* gene was not amplified by any LAMP primer set that we designed using Primer Explorer V5 software. This may be explained by the the low GC content of *pol*. Therefore, we chose the conserved sequence in *env* to design the specific LAMP primer set. To determine primer specificities, each of the six primers representing eight distinct regions of *env* were analysed using BLAST to query GenBank. There were no matches between the first six bases of the eight primer-binding regions with non-BLV sequences. (Table 1). Further, 20 samples did not yield C_T values using the BLV-specific real-time PCR (rPCR) assay, and their values were significantly below the threshold of baseline absorbance in the anti-BLV antibody-ELISA. These samples were all BLV-negative in the fLAMP assay. Further, false-positive signals were not detected (Fig. 2, Table 2).

We used 100 bovine clinical blood samples, comprising 80 ELISA-positive and 20 ELISA-negative samples, to evaluate the performance of the BLV specific fLAMP assay. The results were compared with those of the rPCR assay [16, 23]. As shown in Table 2 and Fig. 2, among the 80 ELISA-positive samples, 62 and 71 were positive and 18 and 9 were negative in the fLAMP and rPCR assays, respectively. The 20 ELISA-negative samples were negative in both assays. Compared with the ELISA results, the fLAMP assay achieved 77.5% (62/80) sensitivity and 100% (20/20) specificity and the rPCR assay showed 88.8% (71/80) sensitivity and 100% (20/20) specificity. Compared with a conventional real-time PCR (rPCR) assay, the fLAMP assay achieved 87.3% (62/71) sensitivity and 100% (29/29) specificity. The rPCR assay required 65 min from the beginning of reaction to the final extension step. In contrast, the fLAMP assay yielded 62 positive results between 7 min, 15 sec and 28 min (mean Tp 10 min 8 sec ± SD 3 min 23 sec). Among the 13-samples that were low proviral loads (2–39 copies per 100 *n*g DNA), corresponding to weak rPCR positives (C_T range, 35.37–39.00), the fLAMP assay generated four true-positives and nine false-negatives (Table 2, Fig. 2, and Table S1). The fLAMP assay did not detect BLV sequences in 29 samples comprising nine rPCR-negative/ELISA-positive and 20 rPCR- and ELISA-negative samples (Table 2).

A comparison between fLAMP and rPCR data sets is shown in Fig. 2. The rPCR assay yielded 71 positives with 2–25,883 proviral loads (copies per 100 *n*g DNA), corresponding to C_T values ranging from 24.94 to 39.00 (mean C_T, 29.74 ± SD 3.86). In contrast to the ELISA results, the remaining nine samples were negative in the rPCR assay. A 10^{-5}-dilution of the BLV reference strain FLK (genotype 1) returned a "weak" C_T value (39.32) in one of two duplicate samples (Table 3). The LODs of the fLAMP assay were comparable, or 1-log less sensitive, compared with those of the rPCR and tLAMP assays (Table 3). The difference in the LOD values may explain why nine samples were fLAMP-negative but rPCR-positive.

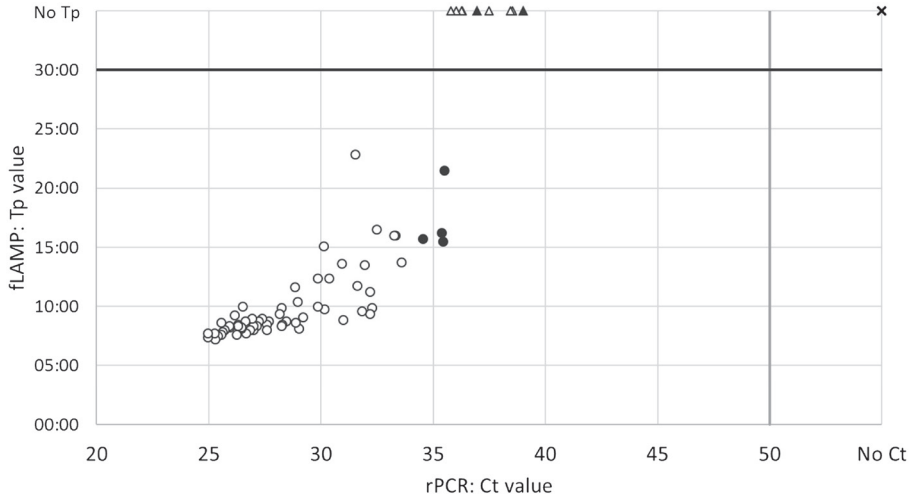

Fig. 2. Comparison of real-time fluorescent LAMP and real-time PCR assays for the detection of BLV proviral sequences from 100 clinical bovine blood samples. Circles show the mean C_T and Tp values of duplicate samples ○: C_T and Tp values positive in duplicate (*n*=58); ●: duplicate positive C_T but one Tp value negative and one Tp value positive (*n*=4); Δ: duplicate positive C_T but duplicate negative Tp values (*n*=7); ▲: One C_T value negative and one Ct value positive, but duplicate negative Tp values (*n*=2); ×: C_T and Tp values negative in duplicate (*n*=29).

Table 2. Diagnostic performance of the fluorescent LAMP assay compared with a real-time PCR assay

	n	fLAMP results
ELISA positive/rPCR positive	71	62/71
ELISA positive/rPCR negative	9	0/9
ELISA negative/rPCR negative	20	0/20

Table 3. Limit of detection of the fluorescent LAMP, turbidimetric LAMP and real-time PCR assays

Strain	Assay	Target	Dilutions of DNA template							
			10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
FLK	fLAMP (Tp)	*env*	7:23	8:30	10:08	24:15[a)]	No. Tp	No. Tp	No. Tp	No. Tp
	tLAMP (Tp)	LTR	15:12	16:09	17:36	21:45	No. Tp	No. Tp	No. Tp	No. Tp
	rPCR (C_T)	*pol*	25.73	28.69	31.82	35.31	39.32[a)]	No. C_T	No. C_T	No. C_T
	rPCR (copy number)		5.0×10^4	5.6×10^3	5.5×10^2	4.1×10^1	2.1×10^0	N/A	N/A	N/A
JA366	fLAMP (Tp)	*env*	16:53	23:30[a)]	No. Tp	No. Tp	No. Tp	No. Tp	ND	ND
	tLAMP (Tp)	LTR	ND	22:12	33:36[a)]	No. Tp	No. Tp	No. Tp	ND	ND
	rPCR (C_T)	*pol*	ND	33.56	37.69	No. C_T	No. C_T	No. C_T	ND	ND
	rPCR (copy number)		N/A	1.5×10^2	8.3×10^0	N/A	N/A	N/A	N/A	N/A

C_T, Threshold cycle number; LTR, Long terminal repeat region; N/A, Not applicable; ND, Not done; Tp, Time of positivity. a) In duplicate analysis, one was positive and one was negative in the reaction.

The fLAMP assay was remarkably rapid. Two reports describe the development of a LAMP assay for BLV based on the LTR region [5] and its application to routine surveys [14]. However, these studies used a tLAMP assay, requiring amplification times (22–60 min) [5, 14] that are significantly longer compared with those reported here (8–30 min). In our present study, the tLAMP and fLAMP assays took 16–34 min and 8–25 min to amplify target DNAs, respectively (Table 3). Further, the tLAMP assay is unable to confirm the specificity of the amplified product as judged by annealing temperatures [4, 6, 17]. For this purpose, the tLAMP assay requires an open reaction tube for electrophoresis, which may be susceptible to contamination.

A diagnostic system based on single genomic targets risks a false-negative diagnosis [20]. For this reason, the development of a fluorescent-based assay using a distinct target gene with high specificity should be useful as a safeguard. Therefore, the fLAMP provides a potential tool for the rapid and simple diagnosis of BLV infection to supplement ELISA testing and can be used by local laboratories and slaughterhouses without special equipment.

The worldwide spread of BLV emphasizes the requirement for early diagnosis and control of disease to minimize economic

losses, as well as to ensure animal welfare. The fLAMP assay was applied to rapid and simple diagnosis of human and veterinary infectious diseases [4, 6, 17]. Specific amplification using the LAMP assay occurs at a constant temperature, minimizing reliance on expensive equipment [2, 24]. Consequently, these assays may facilitate the development of an inexpensive test. For example, the fLAMP takes 8–30 min from the beginning of amplification and <10 min for enzyme inactivation and annealing to final judgment.

The application of the LAMP assay for rapid screening of clinical samples would save time and costs, enabling detection of BLV-positive cases during the early phase of infection because of its lower or equivalent LODs compared with those of conventional PCR and nested PCR assays [5, 14]. Further, the fLAMP assay can be used at farms, slaughterhouses, and wholesale markets in combination with direct DNA detection techniques of clinical samples [4, 17], which would enhance the utility of fLAMP when performed using a portable real-time detector such as the Genie III [4, 6].

As ideal quantitative tools, digital LAMP assays have been developed for clinically important infectious diseases [7, 18]. In the future, application of the BLV-specific LAMP primers described here to the LAMP assay should facilitate more precise and rapid quantification of BLV as a countermeasure in countries with endemic BLV infections. The determination of the precise BLV proviral loads of individual cattle within a herd and the isolation of cattle with high BLV proviral loads is essential to develop an effective strategy to control the transmission of BLV [8–11]. In conclusion, the fLAMP assay serves as a simple and rapid tool that has the potential for effectively controlling of BLV infection.

CONFLICT OF INTEREST. We declare that there are no conflicts regarding the subject matter of the manuscript.

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