NOTE

Virology

Simple and rapid method for routine screening of bovine leukemia virus by loop-mediated isothermal amplification assay

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ABSTRACT. Various techniques for screening and detection of bovine leukemia virus (BLV) were compared to ascertain a rapid and simple technique for routine examination. The performance of real-time PCR, nested PCR and loop-mediated isothermal amplification (LAMP) assays was compared using DNA extracted from whole blood instead of white blood cells (WBCs) of 23 cattle. Real-time PCR, LAMP and nested PCR detected 18, 16 and 11 BLV-positive cattle, respectively. These results suggest that LAMP using DNA from whole blood could enable rapid examination, as isolation of WBCs and electrophoresis is time-consuming and could be useful as a simple and rapid method for routine screening of BLV.

KEY WORDS: blood DNA, bovine leukemia virus, cattle, loop-mediated isothermal amplification, screening

Bovine leukemia virus (BLV) is the etiological agent of enzootic bovine leukosis [7]. BLV infects circulating B cells and induces malignant leukosis in cattle [16]. The majority of cattle infected with BLV are sub-clinical carriers of the virus, and about 30% of BLV-infected cattle develop persistent lymphocytosis without clinical signs [7].

BLV infection has been reported globally and is prevalent in Japan [2, 11]. According to a recent nationwide survey in Japan, BLV is spreading widely among dairy cattle, where BLV seroprevalence from 2009 to 2011 (40.9%) [11] was approximately 10-fold higher than that reported in 1980 (3.7%) [1]. It is thought that sub-clinical carriers of BLV become the source of infection and further spread of the disease. BLV-infected herds without clinical signs show decreased milk production and increased risk of suffering from an infectious disease [3]. Thus, if cattle are infected with BLV, selection and restrictions are imposed on the commerce; the economic loss due to BLV infection is thus immense. Therefore, it is important to diagnose BLV infection accurately in order to eradicate it.

There are various techniques for diagnosis of BLV infection. The syncytia induction assay and the polymerase chain reaction (PCR) have been used for detection of BLV antigen and BLV DNA, and indirect hemagglutination (IHA), enzyme-linked immunosorbent assay (ELISA), radioimmunoassay, indirect fluorescent antibody test and syncytium inhibition test have been used for detection of antibody against BLV, respectively [1, 6, 7, 9]. These serological tests and removal of seropositive cattle from farms will enable the reduction in the number of BLV-infected cattle significantly, but these tests are not adequate to identify cattle during the early stages of infection when the antibody is not yet induced [5]. Since BLV infection is chronic and persistent, it is important to detect BLV DNA as well as antibody. Proviral BLV DNA is found in isolated white blood cells (WBCs) [16], and it can be detected by PCR [4, 12]. However, single PCR does not have sufficient sensitivity, and thus, it is essential to use nested PCR [4, 12]. Nested PCR is time-consuming, and there is a high risk of DNA cross-contamination from the first PCR. Although real-time PCR is much more specific and sensitive than nested PCR, it is an expensive technique and requires special facility and equipment.

Therefore, in this study, to shorten time for a routine examination and reduce laborious efforts, a simple and rapid screening procedure was evaluated for detection of BLV infection by using DNA extracted from whole blood instead of WBCs and loop-mediated isothermal amplification (LAMP) as a DNA amplification method.

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Blood was collected from 23 Holstein cattle in Ishikawa prefecture in 2015. Four to five milliliters of blood were treated with ethylenediaminetetraacetic acid (EDTA), and WBC was isolated by hemolysis with 0.83% ammonium chloride followed by washing twice with phosphate buffered saline (PBS). DNA was extracted both from EDTA-treated whole blood (200 μl) and WBCs by using a QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instructions, and the extracted DNA was used as blood DNA and WBC DNA, respectively. Plasma samples were also obtained from the same cattle.

After measurement of DNA concentration of WBC DNA by a spectrophotometer UV-1700 (SHIMADZU, Kyoto, Japan), DNA concentration was adjusted to 20 $ng/\mu l$ and 5 μl was used for real-time PCR detecting BLV Tax gene using a commercially available Cycleave PCR BLV detection kit (TaKaRa Bio, Otsu, Japan) according to the manufacturer's instructions. The BLV copy numbers per 10 ng were determined.

Primers to amplify the envelope or pX region of BLV were used for nested PCR, according to the protocols by Fechner *et al*. [4] and Murakami *et al*. [12]. PCR was carried out in a total reaction volume of 25 μl containing 0.625 U of TAKARA Ex Taq (TaKaRa Bio), 0.4 $\mu mol/l$ of forward and reverse primers, and 2 μl of extracted blood DNA or WBC DNA. PCR products were electrophoresed on 2% agarose gel in Tris acetate EDTA (TAE) buffer, stained with ethidium bromide and visualized under a UV illuminator.

LAMP assay was carried out using primers specific to the LTR region of BLV DNA as described previously [8] with the Loopamp DNA amplification kit (Eiken Chemical, Tochigi, Japan). The 25 μl reaction mixture contained 40 pmol/25 μl of each inner primer (FIP, BIP), 5 pmol/25 μl of each outer primer (F3, B3), 20 pmol/25 μl of each loop primer (FLP, BLP), 1× reaction mixture, 8 U of *Bst* DNA polymerase and 5 μl of extracted blood DNA or WBC DNA from each sample. In the LAMP reaction mixture, 1 μl of Fluorescent Detection Reagent (Eiken Chemical) was added according to the manufacturer's instructions to facilitate visual detection. The mixtures were incubated at 63°C for 1 hr and then heated at 80°C for 5 min to terminate the reaction in a Loopamp EXIA real-time turbidimeter (Teramecs, Kyoto, Japan). The amplification of DNA was also confirmed by the detection of fluorescence visualized in a UV illuminator.

An anti-BLV antibody ELISA kit (JNC Inc., Tokyo, Japan) was used to detect anti-BLV antibodies according to the manufacturer's instructions.

First, real-time PCR was carried out for detection of BLV DNA and measurement of BLV copy number using DNA extracted from the WBCs of 23 cattle. Five samples were negative, and 18 were positive for BLV DNA. The copy numbers of positive samples detected by real-time PCR were in the range of 0.3–360 copies/10 ng DNA. The same number of positive results was obtained by nested PCR. Among these 18 positive samples by real-time PCR, BLV DNA was not amplified from one sample in the LAMP assay (Fig. 1). The copy number of this sample was 0.3 copies/10 ng DNA. Whereas, five negative samples detected by real-time PCR were also detected to be negative by both nested PCR and LAMP assays.

Further, to shorten time and reduce efforts for isolation of WBCs from whole blood, the sensitivity of nested PCR and LAMP assays was evaluated using blood DNA. The number of positive samples detected by real-time PCR of blood DNA was the same as that detected by real-time PCR of WBC DNA, i.e., 18 blood DNA samples were positive for BLV DNA (data not shown). Among these 18 positive samples, 16 samples were found to be positive, but 2 samples were found to be negative by the LAMP assay (Fig. 1). In contrast, 11 of 18 samples were found to be positive, but seven samples were found to be negative by the nested PCR (Fig. 1). BLV copy numbers of the seven negative samples were detected by nested PCR, and they ranged widely from 0.3 to 215 copies/10 ng DNA, whereas those of the two negative samples detected by LAMP assay were less than 1 copy/10 ng DNA (0.3 and 0.4 copies/10 ng DNA) (Table 1). These results were confirmed by repeated experiments. As for negative results by nested PCR, but positive results by real-time PCR, we think a possibility that different numbers of WBCs in 200 μl of whole blood in each sample caused this discrepancy. In the negative samples by nested PCR, but positive by real-time PCR, the number of WBCs was significantly lower than the positive samples ($P<0.1$), resulting in lower DNA concentration (data not shown). The LAMP assay has a characteristic feature of being more sensitive than PCR [8, 13]. In this study, LAMP assay was more sensitive than nested PCR in blood DNA. Moreover, real-time PCR, nested PCR and LAMP assays can be performed within 1.5, 4 and 1 hr, respectively. These results revealed that LAMP assay using blood DNA could be more suitable for simple and rapid screening of BLV than nested PCR.

ELISA measured antibody against BLV in 23 cattle. The results showed 18 out of the 23 samples to be positive for antibody, which was almost consistent with the number of positive results of BLV DNA detected by real-time PCR (Table 1). Among these 18 ELISA antibody-positive samples, one sample was found to be negative for BLV DNA by the real-time PCR, nested PCR and LAMP assays. This sample might be positive for BLV antibody due to the presence of maternal antibody as the sample was obtained from 1-month-old cattle. Conversely, one out of the five BLV antibody-negative samples was found to be positive for BLV DNA by real-time PCR, nested PCR and LAMP assays using WBC DNA. However, three weeks later, this sample became positive for the antibody, suggesting that real-time PCR, nested PCR and LAMP assays can diagnose cattle in the early stage of BLV infection where antibody was not yet induced; this is in accordance with a previous report [8]. To prevent missing out of cattle in the early stage of BLV infection, both genetic screening and serological examinations should be carried out.

In conclusion, our results suggest that LAMP assay using blood DNA could be more suitable for simple and rapid screening of BLV. Real-time PCR is the most sensitive method as compared to other methods used in this study. It can detect BLV infection even in the early stages when anti-BLV antibody is not yet induced and can measure the viral copy numbers [15]. However, it is unsuitable for screening of BLV, because the analysis cost per sample is high. Nested PCR is cheaper than real-time PCR, but it requires longer time and has a high risk of DNA cross-contamination, causing misdiagnosis. The time required for LAMP assay is shorter than that required for nested PCR, and it could reduce a contamination risk because it does not require a downstream
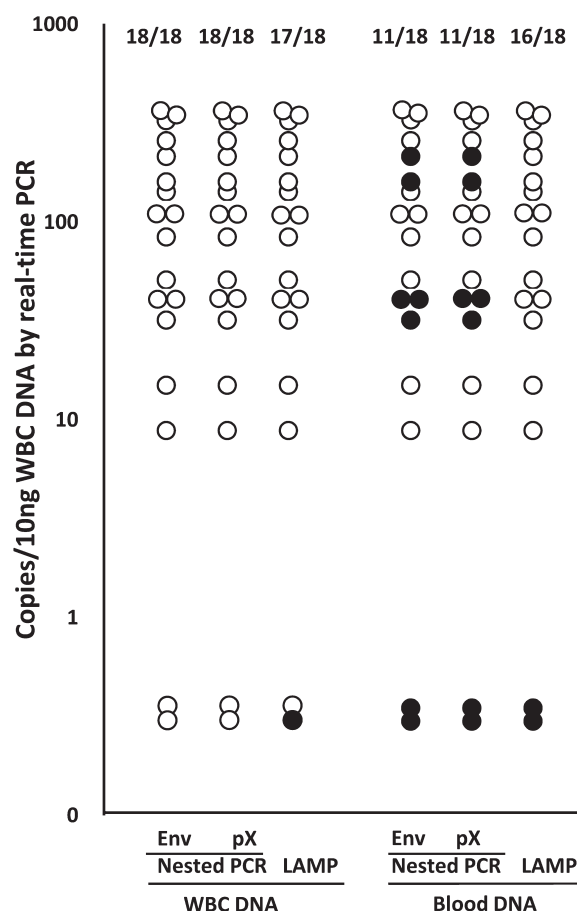
Fig. 1. Comparison of detectability of BLV among real-time PCR, nested PCR and LAMP assays using WBC DNA or blood DNA. ○, positive by nested PCR or LAMP assay. ●, negative by nested PCR or LAMP assay. The numbers of the top indicate the numbers of positive samples/18 positive samples by real-time PCR using WBC DNA.

analysis like electrophoresis and it can be detected visually, leading to a rapid analysis. BLV-infected cattle carrying high viral copy numbers could be a high risk factor in farms as a source of spreading the infection [10, 14]. LAMP assay could not detect the samples having less than 1 copy/10 *n*g DNA, and the sensitivity would be enough for screening because the risk of spreading BLV infection from these cattle is presumably lower than that from cattle having high copy numbers. LAMP assay can be performed only with a water bath or a heating block for maintaining isothermal conditions [13], which are available in facilities where special machines and laboratory equipment, such as a thermal cycler, are unavailable. Therefore, combination LAMP assay with blood DNA could be suitable for the inspection of many samples, including the routine screening of BLV.

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Table 1. Relation of age and quantitative proviral load to the qualitative results of nested PCR and LAMP assay, and anti-BLV antibody

Cattle No.	Age (Month)	WBC DNA Real-time PCR (Copies/10 *ng* DNA)	Blood DNA Nested PCR Envelope	pX	LAMP	Antibody
1	1	–	–	–	–	+
2	27	–	–	–	–	–
3	4	–	–	–	–	–
4	1	–	–	–	–	–
5	7	–	–	–	–	–
6	68	0.3	–	–	–	+
7	53	0.4	–	–	–	+
8	46	9	+	+	+	+
9	5	15	+	+	+	+
10	54	32	–	–	+	+
11	53	40	–	–	+	+
12	52	40	–	–	+	–
13	89	51	+	+	+	+
14	8	84	+	+	+	+
15	32	111	+	+	+	+
16	82	114	+	+	+	+
17	36	143	+	+	+	+
18	27	160	–	–	+	+
19	44	215	–	–	+	+
20	32	258	+	+	+	+
21	52	346	+	+	+	+
22	57	350	+	+	+	+
23	34	360	+	+	+	+

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