FULL PAPER *Virology*

Direct polymerase chain reaction from blood and tissue samples for rapid diagnosis of bovine leukemia virus infection

Asami NISHIMORI[1)], Satoru KONNAI[1)], Ryoyo IKEBUCHI[1)], Tomohiro OKAGAWA[1)], Ayako NAKAHARA[1)], Shiro MURATA[1)] and Kazuhiko OHASHI[1)]*

[1)]*Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku Kita 18- jo Nishi 9-chome, Sapporo, Hokkaido 060–0818, Japan*

(Received 5 October 2015/Accepted 17 January 2016/Published online in J-STAGE 25 February 2016)

ABSTRACT. Bovine leukemia virus (BLV) infection induces bovine leukemia in cattle and causes significant financial harm to farmers and farm management. There is no effective therapy or vaccine; thus, the diagnosis and elimination of BLV-infected cattle are the most effective method to eradicate the infection. Clinical veterinarians need a simpler and more rapid method of diagnosing infection, because both nested polymerase chain reaction (PCR) and real-time PCR are labor intensive, time-consuming, and require specialized molecular biology techniques and expensive equipment. In this study, we describe a novel PCR method for amplifying the BLV provirus from whole blood, thus eliminating the need for DNA extraction. Although the sensitivity of PCR directly from whole blood (PCR-DB) samples as measured in bovine blood containing BLV-infected cell lines was lower than that of nested PCR, the PCR-DB technique showed high specificity and reproducibility. Among 225 clinical samples, 49 samples were positive by nested PCR, and 37 samples were positive by PCR-DB. There were no false positive samples; thus, PCR-DB sensitivity and specificity were 75.51% and 100%, respectively. However, the provirus loads of the samples detected by nested PCR and not PCR-DB were quite low. Moreover, PCR-DB also stably amplified the BLV provirus from tumor tissue samples. PCR-DB method exhibited good reproducibility and excellent specificity and is suitable for screening of thousands of cattle, thus serving as a viable alternative to nested PCR and real-time PCR.

KEY WORDS: bovine leukemia virus (BLV), PCR directly from whole blood, provirus

doi: 10.1292/jvms.15-0577; *J. Vet. Med. Sci.* 78(5): 791–796, 2016

Bovine leukemia virus (BLV) is an oncogenic retrovirus which can infect cattle and sheep *in vivo*. Approximately 30–40% of BLV-infected cattle show persistent lymphocytosis, among which 2%–3% will have bovine leukemia. In Japan, a law dictates that cattle with bovine leukemia must be killed. Thus, BLV infection exerts a severe negative influence on farm management [14].

There is no effective therapeutic strategy or vaccine against BLV infection available. Currently, the only countermeasure against the possible development of bovine leukemia is early detection and eradication of BLV-infected cattle before transmission. In Denmark, a country-wide government initiative of detecting and culling all BLV-infected cattle resulted in the elimination of BLV [8]. However, in many countries, including Japan, the precise rate of infection is not known, because a nationwide survey has not been conducted.

Screening for BLV-infected cattle is critical to freeing a farm or region of BLV infection. However, most BLV-infected cattle do not show clinical symptoms, and field veterinarians cannot detect them through routine examination practices [7]. Thus, molecular biology techniques are needed to detect the virus. The major methods for identifying an infected carrier are agar gel immunodiffusion (AGID) and enzyme-linked immunosorbent assay (ELISA), which detect antibodies in serum or milk that are specific to a BLV antigen, and nested polymerase chain reaction (PCR) and real-time PCR, which amplify the BLV provirus from genomic DNA. In Sweden, which is free of BLV, an ELISA test is applied to milk samples in an ongoing surveillance program [6]. However, AGID and ELISA tests exhibit poor sensitivity and specificity compared to PCR test results [11, 15], and testing calf serum for the anti-BLV antibody is not appropriate because of the presence of maternal antibodies. Thus, nested PCR and real-time PCR are now regarded as the most sensitive methods for detecting the BLV provirus and active infection.

Although these PCR methods are suitable for diagnosing BLV infection, PCR contamination resulting from modest contamination of DNA templates is frequent. In particular, nested PCR is labor intensive when thousands of cattle are being screened, since it requires many experimental steps, such as DNA purification, first round PCR, secondary PCR and electrophoresis. To reduce the experimental steps and the risk of sample cross contamination, we developed methods for amplifying the BLV provirus from whole blood (PCR directly from whole blood; PCR-DB). This method facilitates the diagnosis of BLV infection without special techniques and is more suitable than nested PCR for first screening of thousands of cattle.

*CORRESPONDENCE TO: OHASHI, K., Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Kita-ku Kita 18- jo Nishi 9-chome, Sapporo, Hokkaido 060–0818, Japan. e-mail: okazu@vetmed.hokudai.ac.jp

MATERIALS AND METHODS

Blood samples: Peripheral blood samples were obtained from several farmers and veterinarians for diagnosis of BLV infection at the Hokkaido University veterinary teaching hospital. Informed consent was obtained from each owner, and approval for all procedures was obtained from the Institutional Animal Care and Use Committee of Hokkaido University. DNA was purified from 500 μl blood samples using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI, U.S.A.) according to the manufacturer's protocol and finally suspended in 50 μl buffer.

Cells: KU-1 cells infected with BLV [13] were maintained at 37°C in Roswell Park Memorial Institute (RPMI)-1640 medium (Sigma-Aldrich, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum (Cell Culture Technologies, Gravesano, Switzerland) and penicillin/streptomycin/L-glutamine (Life Technologies, Carlsbad, CA, U.S.A.). Blood samples not infected with BLV that contained various numbers of KU-1 cells (10^2–10^7 cells/1,000 μl of blood; 10^{-1}–10^4 cells/μl) were used as PCR-DB templates, and DNA purified from those blood samples was used as nested PCR templates.

PCR-DB: Amplification of BLV provirus from whole blood used KOD FX Neo (Toyobo, Osaka, Japan), which is superior for amplification from crude samples, and a specific primer pair (PV2-F 5′-ACT TTC AGA CCC CCT TGA CTG ACA-3′ and PV2-R 5′-AAA CCT CTG CCC TGG TGA TTA AGG-3′). This primer pair was designed by Primer3 to amplify BLV provirus (GeneBank accession number: both K02120 and AF033818), and the intron region of the provirus (3308−3580 in K02120) was amplified by the primer pair. Briefly, each 30 μl reaction mixture contained 0.4 mM dNTPs, 0.5 μM of primers, 1 U of KOD FX Neo and 1 μl of whole blood (10-, 50- or 100-fold dilutions in double distilled water; Fig. 2). Amplifications were performed under the following conditions: one lysis cycle at 94°C for 2 min and then 45 cycles of template denaturation at 94°C for 15 sec followed by annealing and extension at 68°C for 50 sec. All experiments were carried out in duplicate or sextuplicate. The *β-globin* gene was amplified as an internal control using the following primer pairs: 5′-TGC TGA CTG CTG AGG AGA AGG CTG-3′ and 5′-GTC CTC ACA CGC CCA GGT GCA TTT C-3′.

Nested PCR: To amplify the long terminal repeat (LTR) in the BLV provirus, nested PCR was performed using rTaq (Takara-bio, Otsu, Japan) as previously described [9]. Briefly, the BLV LTR was amplified using primer pairs, BLV-LTR1 and BLV-LTR533, for the first PCR reaction, and 1.5 μl of DNA from whole blood or BLV-uninfected blood with KU-1 cells were used as templates. And then, 1.5 μl of the first PCR products were reamplified using BLV-LTR256 and BLV-LTR453 for the second PCR reaction. The *β-globin* gene was amplified as an internal control.

Real-time PCR: To confirm the provirus loads of BLV-infected cattle diagnosed by nested PCR, we used a real-time PCR system (LightCycler 480 system II; Roche Diagnostics, Mannheim, Germany), SYBR Premix Dimer-Eraser (Takara-bio), and the primers PV2-F and PV2-R for BLV and 5′-ACA CAA CTG TGT TCA CTA GC-3′ and 5′-CAA CTT CAT CCA CGT TCA CC-3′ for *β-globin*. Amplification of DNA samples from whole blood was performed as follows: one cycle at 95°C for 30 sec, followed by a 3-step PCR procedure consisting of 5 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C for 45 cycles. To obtain a standard curve, serial dilutions of the standard plasmid containing from 10^7 to 10^1 copies were prepared and stored at −20°C until use.

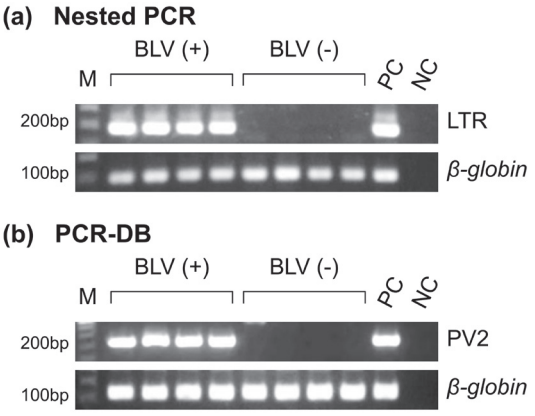

Fig. 1. Amplification of the BLV provirus from whole blood. BLV provirus amplification was performed by (a) nested PCR and (b) PCR-DB. Blood samples were collected from cattle with (n=4) and without (n=4) diagnosed BLV infections. DNA samples purified from individual blood samples were used as templates. The *β-globin* gene was amplified as an internal control. For both of nested PCR and PCR-DB, DNA samples purified from BLV-positive cattle were used as a positive control (PC), and double distilled water was used as a negative control (NC).

Tumor samples: To diagnose bovine leukemia by using PCR-DB, tumor cells from three cattle with clinically diagnosed lymphoma were collected: case No. 1 (Holstein-Friesian, 4 months old, enzootic bovine leukosis), case No. 2 (Japanese black, 5 years old, enzootic bovine leukosis) and case No. 3 (Holstein-Friesian, 2 years old, thymic lymphosarcoma). The tumor cells were stained with an antibody specific to B cell markers and analyzed by flow cytometry as described previously [10]. In brief, double staining was performed using anti-bovine IgM (IL-A30; AbD Serotec, Oxford, U.K.) pre-labeled with Zenon Alexa Fluor 488 (Life Technologies) and the following antibodies: anti-WC4 (CC55; CD19-like; AbD Serotec); anti-B-B7 (GB25A; CD21-like; VMRD, Pullman, WA, U.S.A.); and anti-bovine CD5 (CACT105A; VMRD). Alexa Fluor 647-conjugated anti-mouse IgG (Life Technologies) was used for bound antibody detection (anti-WC4, anti-B-B7 and anti-CD5). Tumor cells were incubated with anti-WC4, anti-B-B7 and anti-CD5 as the first antibody, anti-mouse IgG as the second antibody and anti-IgM as the third antibody. BLV infection was diagnosed from blood and tumor cells by nested PCR as described above. Approximately 1–2 mm^3 of tumor tissue suspended in 1 ml of phosphate buffered saline was used as a template for PCR-DB.

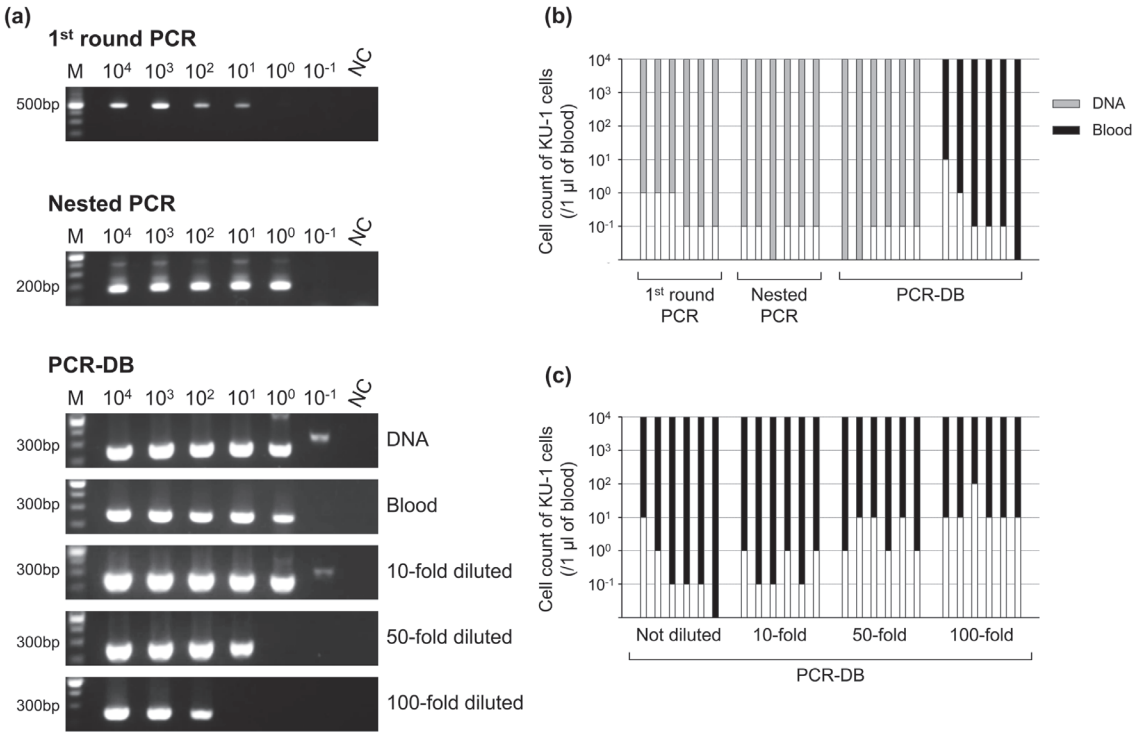

Fig. 2. Comparison of nested PCR and PCR-DB sensitivity. To assess PCR method sensitivity, DNA samples purified from BLV-uninfected blood containing KU-1 cells (10^{-1}–10^4 cells/μl) were used as nested PCR templates, and the blood samples were used as PCR-DB templates (1-, 10-, 50- or 100-fold diluted in double distilled water). (a) Representative images of electrophoresis of amplicons generated using each PCR condition. The numbers along the top indicate KU-1 cell counts per 1 μl blood. (b, c) The results from detectable samples using each PCR condition are presented. White bars indicate KU-1 cell counts which were undetectable by electrophoresis, while the gray (DNA) and black (blood) bars indicate the templates used for each PCR condition. All amplification procedures were performed in sextuplicate.

RESULTS

PCR-DB amplification of BLV provirus from whole blood: First of all, we tried to use BLV-LTR 256 and BLV-LTR 453 for PCR-DB amplification of BLV provirus. But, the Tm value of this primer pair was not suitable for the polymerase we used for PCR-DB, resulted in appearance of many extra bands (data not shown). Because of that, we designed an optimal primer pair for the polymerase of PCR-DB following the manufacturer's protocol. The PV2 primer pair was determined from seven ones based on their ability to show the most sensitive and reproducible results (data not shown). To confirm the amplification of BLV provirus from whole blood, PCR-DB was performed using blood samples for which BLV infection had been previously diagnosed by nested PCR. We found single bands approximately 272 bp using the PV2 primer pair, with the results from PCR-DB amplification completely consistent with those from nested PCR reactions (Fig. 1). The amplification of *β-globin* was observed as bands of approximately 100 bp in all samples, although a second band of approximately 400 bp was also produced in PCR-DB using the *β-globin* primer pair (data not shown).

PCR-DB results are reproducible with sample dilution: To compare the sensitivity of nested PCR and PCR-DB methods, BLV provirus was amplified from KU-1 cells mixed in blood from healthy cattle (Fig. 2a). In sextuplicate reactions, the provirus was detected by nested PCR even from samples containing 0.1 cells or 1 cell per 1 μl of blood (average level of undetectable cell numbers: 0.083 cells/μl; Fig. 2b). PCR-DB amplification using DNA samples as templates was also performed to confirm the effects of its primer pair and polymerase on assay sensitivity. Results showed a similar sensitivity to that of nested PCR (0.067 cells/μl; Fig. 2b). However, the results of PCR-DB used undiluted blood samples indicated less sensitivity and reproducibility. On the other hand, PCR-DB performed under variable conditions, with blood samples diluted 10-, 50- and 100-fold with double distilled water, showed improved reproducibility, even though the sensitivity was lower than for undiluted samples (0.55, 5.5 and 25 cells/μl, respectively; Fig. 2c).

PCR-DB diagnosis of most of BLV-infected cattle that showed detectable provirus loads: In total, 225 bovine blood samples were tested by nested PCR and PCR-DB. Whole blood was diluted 50-fold with double distilled water for PCR-DB, representing the middle condition tested in Fig. 2c. Using both methods, 37 samples were positive, and 176 samples were negative, with no samples that were positive

Table 1. Amplification of BLV provirus in clinical blood samples

PCR Result	Nested PCR Positive	Nested PCR Negative	Total
PCR-DB Positive	37	0	37
PCR-DB Negative	12	176	188
Total	49	176	225

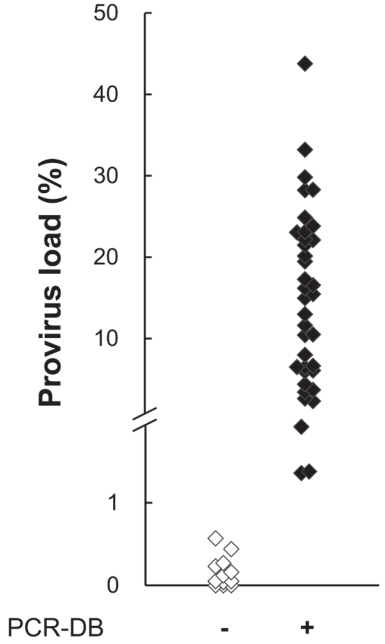

Fig. 3. Genomic DNA provirus loads for clinical samples that were positive by nested PCR. DNA samples from BLV-infected cattle diagnosed by nested PCR were evaluated to determine provirus loads using real-time PCR (n=49). The Y-axis indicates the rates of BLV genome copies in 100 cells as determined by *β-globin* amplicon copy numbers. The dot colors depict the PCR-DB results (white: negative; black: positive). Each amplification procedure was performed in duplicate.

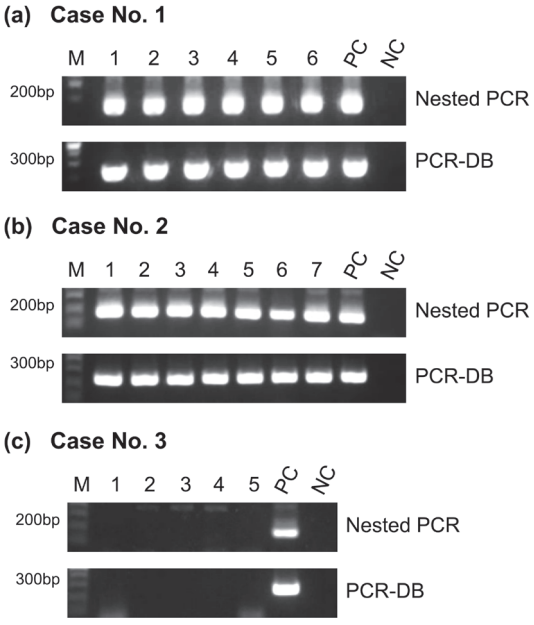

Fig. 4. Direct detection of the BLV provirus in tumor samples. Amplification of the BLV provirus in blood and tumor samples isolated from three cattle with bovine leukemia was performed using PCR-DB, with 50-fold diluted blood and tumor suspensions used as templates. Each amplification procedure was performed in duplicate. LN: lymph node. (a) Case No. 1 (Holstein-Friesian, 4 months old): 1, blood; 2, thymus; 3, spleen; 4, gastric LN; 5, mesenteric LN; and 6, inguinal LN. (b) Case No. 2 (Japanese black, 5 years old): 1, blood; 2, spleen; 3, heart; 4, superficial cervical LN; 5, mesenteric LN; 6, mediastinal LN; and 7, renal LN. (c) Case No. 3 (Holstein-Friesian, 2 years old): 1, blood; 2, cervical thymus; 3, thoracic thymus; 4, superficial cervical LN; and 5, bronchial LN.

by PCR-DB and negative by nested PCR, thus indicating a PCR-DB specificity of 100% (Table 1). The provirus in 12 samples was detected only using nested PCR and not with PCR-DB, indicating a PCR-DB sensitivity of 75.51%. Because we considered that those false-negative results are responsible for poor infection levels, provirus loads of clinical samples that were positive by nested PCR were measured by real-time PCR. The results showed that the provirus loads of these 12 samples were quite low, suggesting that the animals were asymptomatic carriers at the aleukemic stage (Fig. 3).

PCR-DB directly detected BLV provirus in tumor samples: To investigate whether PCR-DB can be used to diagnose BLV infection from tumor tissues, the provirus in tumor samples was amplified using PCR-DB. In the three sample cases, two out of three cases were BLV-positive tumor tissues, and all PCR-DB results were entirely consistent with those of nested PCR (Fig. 4). Flow cytometry analysis showed that case No. 1, a BLV-positive tumor, expressed several B-cell markers, such as IgM, WC4 (CD19-like) and B-B7 (CD21-like) on the tumor cell membrane, and on the other hand, case No. 3, which was BLV-negative, did not show B-cell phenotypes (Table 2). These results strongly supported the clinical diagnosis, cases No.1 and No.2 as enzootic bovine leukosis and case No. 3 as thymic lymphosarcoma of sporadic bovine leukosis.

DISCUSSION

In this study, we developed novel diagnosis method named PCR-DB for amplifying the BLV provirus directly from whole blood. Our method showed high specificity and reproducibility with diluted blood samples, while undiluted ones resulted in less reproducibly probably because of endogenous PCR inhibitors contained in the blood samples and

Table 2. Phenotyping and diagnosis of tumor samples from cattle

Case No.	BLV infection	Lymphocytes ($10^2/\mu l$)	Cell surface markers				Tumor cell types	Diagnosis
			Lymphocytes	B cells				
			CD5	WC4	B-B7	IgM		
1	+	3,881	+	+/−	+	+	B cells	Enzootic bovine leukosis
2	+	N.T.		N.T.			Unknown	Enzootic bovine leukosis
3	-	23	+	-	-	-	T cells	Thymic lymphosarcoma

blood viscosity, which made it difficult to measure the sample volume accurately. Although the sensitivity of PCR-DB was lower than that of nested PCR, all clinical samples detected only using nested PCR and not with PCR-DB showed slight provirus loads, suggesting that those were from carriers at early stage. So, we believe that our study demonstrates the utility of PCR-DB for rapid diagnosis of BLV infection.

In previous studies, some researchers have reported the amplification of DNA directly from whole blood using PCR methods for diagnosing bacterial or viral infections, including *Mycoplasma haemofelis* [16], *Bartonella quintana* [12] and hepatitis B virus [4]. Moreover, in another report, PCR-DB was used for mutation screening of G_{M1} gangliosidosis in dogs, demonstrating that this technique can also be a good method for hereditary disease screening and not only for detecting infectious diseases [17]. These reports suggest that PCR-DB has the potential to be applied in multiple clinical situations as a novel, rapid and viable method of diagnosis.

One of the primary methods used to identify BLV-infected cattle is a serological test (AGID, ELISA, etc.) that detects antibodies against BLV antigen in serum and milk. Those tests are appropriate for screening thousands of cattle, because of easy sample preparation compared with PCR tests which require DNA extraction and availability of a rapid and cost-effective diagnosis kit. However, in some cases, these serological tests show several problems, including poor sensitivity compared with PCR testing, false positive samples, detection of maternal antibodies and inability to perform a diagnosis using tissue or semen samples [3, 5, 11, 15]. Although Sweden achieved eradication of BLV-infected cattle and elimination of BLV using only an ELISA test [6], PCR testing would enable a more definitive surveillance program to eradicate BLV infection.

There are many advantages of using PCR-DB to detect the BLV provirus: i) PCR-DB specificity as calculated by diagnosing clinical samples was 100%; ii) the technique does not require a special and expensive thermal cycler like real-time PCR; iii) it is not labor intensive or time-consuming and is cost-effective; iv) because it is a rapid and straightforward procedure, there is less possibility of contamination; and v) less than 10 μl of blood is enough to run the PCR-DB assay, even in duplicate. The biggest challenge with PCR-DB is the existence of PCR inhibitors in whole blood, which contains IgG, hemoglobin and lactoferrin [1, 2]. Our results demonstrate that endogenous PCR inhibitors do not impact the reproducibility of the PCR reaction by sample dilution. Thus, PCR-DB is a suitable method for use by clinical veterinarians to perform BLV diagnosis in a typical veterinarian's office.

Nested PCR using purified genomic DNA is likely the best method for detecting BLV infection, because provirus in the blood from several cattle showing slight provirus loads was not detected by PCR-DB. Although the amplification of low-copy provirus may be difficult using PCR-DB, the sensitivity of PCR-DB can be improved by simply increasing the template blood volume. As demonstrated in Fig. 2c, PCR-DB using 10-fold diluted blood samples showed higher sensitivity than other dilutions and adequate reproducibility. Therefore, we consider PCR-DB to be appropriate in first screening diagnosis in an individual farm or region to systematically eradicate BLV-infected cattle. This technique is preferred, because the use of nested PCR for diagnosis in a large number of cattle is both labor intensive and time-consuming. PCR-DB represents the best practical way for eliminating BLV infection through periodic screening and isolation or culling of all infected animals at intervals of several months or a few years.

PCR-DB amplification of BLV provirus was also evaluated using tumor tissues. Our preliminary data showed that the cell number in the PCR reaction buffer influenced the stability of the results and that excessive cell numbers inhibited the PCR reaction (data not shown). Thus, suspending tumor cells in phosphate buffered saline or other suitable solutions are important in order to adjust the templates to the appropriate PCR conditions. In tumors, PCR-DB may be a better method for diagnosing BLV infection than nested PCR or other methods, because the most rapid and definitive method for diagnosing BLV infection in cattle with lymphoma is required by clinical veterinarians and because serological tests are not suitable for tissue diagnosis. Incidentally, calf lymphoma (case No.1) was diagnosed with enzootic bovine leukosis which is caused by BLV infection, because the lymphoma was constructed with clonal $CD5^+$ IgM^+ B cells highly expressing viral protein gp51 (data not shown).

BLV infection is difficult to eliminate due to its latency. However, Denmark did so by peripheral blood cell counts without serological tests [8]. In our opinion, other countries can eliminate the infection more definitively than Denmark using PCR-DB. Countries with a much higher prevalence can work to eradicate all infected cattle through PCR-DB screening and other strategies, such as isolating BLV-positive cattle and reducing the incidence of bovine leukemia. We conclude that this PCR-DB assay is a highly simplified, cost-effective and rapid method (results obtained in less than 3 hr) that serves as a new alternative way to diagnosis BLV infection without DNA purification.

ACKNOWLEDGMENTS. This work was supported by the Science and Technology Research Promotion Program for Agriculture, Forestry, Fisheries and Food Industry, and Japan Society for the Promotion of Science (JSPS).

REFERENCES

1. Al-Soud, W. A., Jönsson, L. J. and Rådström, P. 2000. Identification and characterization of immunoglobulin G in blood as a major inhibitor of diagnostic PCR. *J. Clin. Microbiol.* **38**: 345–350. [Medline]
2. Al-Soud, W. A. and Rådström, P. 2001. Purification and characterization of PCR-inhibitory components in blood cells. *J. Clin. Microbiol.* **39**: 485–493. [Medline] [CrossRef]
3. Asadpour, R. and Jafari, R. 2012. Detection of bovine leukosis provirus in blood and semen samples of bulls. *Comp. Clin. Pathol.* **21**: 187–191. [CrossRef]
4. Cheng, Z. J., Hu, L. H., Fu, W. R. and Li, Y. R. 2007. Rapid quantification of hepatitis B virus DNA by direct real-time PCR from serum without DNA extraction. *J. Med. Microbiol.* **56**: 766–771. [Medline] [CrossRef]
5. Choi, K. Y., Monke, D. and Stott, J. L. 2002. Absence of bovine leukosis virus in semen of seropositive bulls. *J. Vet. Diagn. Invest.* **14**: 403–406. [Medline] [CrossRef]
6. Emanuelson, U., Scherling, K. and Pettersson, H. 1992. Relationships between herd bovine leukemia virus infection status and reproduction, disease incidence, and productivity in Swedish dairy herds. *Prev. Vet. Med.* **12**: 121–131. [CrossRef]
7. Florins, A., Gillet, N., Asquith, B., Boxus, M., Burteau, C., Twizere, J. C., Urbain, P., Vandermeers, F., Debacq, C., Sanchez-Alcaraz, M. T., Schwartz-Cornil, I., Kerkhofs, P., Jean, G., Théwis, A., Hay, J., Mortreux, F., Wattel, E., Reichert, M., Burny, A., Kettmann, R., Bangham, C. and Willems, L. 2007. Cell dynamics and immune response to BLV infection: a unifying model. *Front. Biosci.* **12**: 1520–1531. [Medline] [CrossRef]
8. Gillet, N., Florins, A., Boxus, M., Burteau, C., Nigro, A., Vandermeers, F., Balon, H., Bouzar, A. B., Defoiche, J., Burny, A., Reichert, M., Kettmann, R. and Willems, L. 2007. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology* **4**: 18. [Medline] [CrossRef]
9. Ikebuchi, R., Konnai, S., Shirai, T., Sunden, Y., Murata, S., Onuma, M. and Ohashi, K. 2011. Increase of cells expressing PD-L1 in bovine leukemia virus infection and enhancement of anti-viral immune responses *in vitro* via PD-L1 blockade. *Vet. Res.* **42**: 103. [Medline] [CrossRef]
10. Ikebuchi, R., Konnai, S., Okagawa, T., Yokoyama, K., Nakajima, C., Suzuki, Y., Murata, S. and Ohashi, K. 2013. Blockade of bovine PD-1 increases T cell function and inhibits bovine leukemia virus expression in B cells *in vitro*. *Vet. Res.* **44**: 59. [Medline] [CrossRef]
11. Kohara, J., Konnai, S. and Onuma, M. 2006. Experimental transmission of Bovine leukemia virus in cattle via rectal palpation. *Jpn. J. Vet. Res.* **54**: 25–30. [Medline]
12. Morton, B. A. and de Paiva Diniz, P. P. V. 2013. Detection of *Bartonella quintana* DNA in the presence of human and feline whole blood by single-tube PCR without DNA extraction. *Microbiology Discovery* **1**: 7. [CrossRef]
13. Onuma, M., Koyama, H., Aida, Y., Okada, K., Ogawa, Y., Kirisawa, R. and Kawakami, Y. 1986. Establishment of B-cell lines from tumor of enzootic bovine leukosis. *Leuk. Res.* **10**: 689–695. [Medline] [CrossRef]
14. Ott, S. L., Johnson, R. and Wells, S. J. 2003. Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms. *Prev. Vet. Med.* **61**: 249–262. [Medline] [CrossRef]
15. Trono, K. G., Pérez-Filgueira, D. M., Duffy, S., Borca, M. V. and Carrillo, C. 2001. Seroprevalence of bovine leukemia virus in dairy cattle in Argentina: comparison of sensitivity and specificity of different detection methods. *Vet. Microbiol.* **83**: 235–248. [Medline] [CrossRef]
16. Watanabe, M., Hisasue, M., Souma, T., Ohshiro, S., Yamada, T. and Tsuchiya, R. 2008. Molecular detection of *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' Infection in cats by direct PCR using whole blood without DNA extraction. *J. Vet. Med. Sci.* **70**: 1095–1099. [Medline] [CrossRef]
17. Yamato, O., Jo, E. O., Shoda, T., Yamasaki, M. and Maede, Y. 2004. Rapid and simple mutation screening of $G_{(M1)}$ gangliosidosis in Shiba dogs by direct amplification of deoxyribonucleic acid from various forms of canine whole-blood specimens. *J. Vet. Diagn. Invest.* **16**: 469–472. [Medline] [CrossRef]