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ORIGINAL ARTICLE

Development of a direct blood-based PCR system to detect BLV provirus using CoCoMo primers

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Abstract Bovine leukemia virus (BLV), the etiologic
agent of enzootic bovine leucosis, has caused pandemic
outbreaks worldwide. Because transcription of the BLV is
quickly blocked after infection, detecting integrated pro-
virus at host genome is an important method of identifying
whether an animal is infected. The aim of the present study
was to develop a novel direct blood-based PCR system to
detect the BLV provirus with high specificity and at low
cost. The assay was based on the BLV-CoCoMo degen-
erate primers, which amplify all known BLV strains. Cattle
blood samples (n = 182) were collected from the same
BLV-positive farm and subjected to BLV-CoCoMo-direct-
PCR to detect the BLV provirus. The proviral load was
then estimated. This novel PCR method showed 100 %
specificity. The BLV-CoCoMo-direct-PCR can be used in
a variety of laboratory situations because it does not require
expensive equipment/reagents, DNA purification, or a
second round of PCR. Therefore, the method is extremely
cost-effective and the risk of a false-positive result due to
DNA contamination is very low.

Introduction

Bovine leukemia virus (BLV), which belongs to the
Retroviridae family within the genus *Deltaretrovirus*, is the
etiologic agent of enzootic bovine leucosis (EBL), a
disease characterized by an extended course that often
involves persistent lymphocytosis and culminates in B cell
lymphosarcoma [1]. BLV pandemics have occurred
worldwide (World Animal Health Information Database
Interface; http://www.oie.int/wahis_2/public/wahid.php/
Wahidhome/Home). In Japan, 99 head of cattle were
diagnosed as bovine leukosis in 1998. The number of
Bovine leukosis cattle were increasing every year, and
2,415 cattle were diagnosed as bovine leukosis in 2014
(Ministry of Agriculture, Forestry and Fisheries of Japan,
http://www.maff.go.jp/j/syouan/douei/kansi_densen/kansi_
densen.html). Moreover, a recent study found that 40.9 %
of dairy cows and 28.7 % of beef cattle in Japan were
infected [8].

As with other *Deltaretroviruses* such as human T-cell
leukemia virus type 1 (HTLV-1), transcription of the BLV
is quickly blocked after infection [5, 16]. Therefore,
because viral mRNA and viral antigens are difficult to
detect in infected cattle, they are not suitable targets for
assays designed to detect BLV infection. By contrast,
integrated reverse transcribed viral DNA (provirus) is a
good target for direct detection of BLV because the
proviral genes remains integrated in cellular genomes and
can be amplified by polymerase chain reaction (PCR). The
copy number of the BLV provirus is usually very low
compared with that of host genes because of the cell
population were the mixture of infected and non-infected
cells; therefore, most PCR systems designed to detect BLV
used a nested design [15, 18]. These nested assays are
extremely sensitive, but there is a high risk of obtaining
false-positive results due to DNA contamination.

Antibody-based assays such as enzyme-linked
immunosorbent assays (ELISAs), agar-gel immuno-diffu-
sion tests (AGIDs), and passive hemagglutination assays
(PHAs) were widely used to identify BLV-infected cattle

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[4] because most infected animals develop BLV-specific antibodies. AGIDs and PHAs are cost-effective ways of determining whether an animal is BLV-positive. However, because the sensitivity and specificity of these two methods are quite low, new diagnostic systems are required [4]. ELISA-based detection systems show quite high sensitivity and specificity for identifying BLV-positive cattle [4]; however, cattle can be identified as negative even if they have a provirus [11]. Also, these three antibody-based detection methods cannot be used to test calves less than 6 months old due to the presence of maternal antibodies, which may trigger a false-positive result [12].

Recently, we developed BLV-CoCoMo-qPCR-2 assay to measure the BLV proviral load with extremely high sensitivity. The assay is based on degenerate CoCoMo primers (constructed using the CoCoMo algorithm), which amplify both known and novel BLV variants [3, 4, 7, 11, 13, 14, 17, 19]. This assay enabled us to demonstrate that the proviral load correlates not only with BLV infection capacity (as assessed by syncytium formation), but also with BLV disease progression [3]. Recently, we used the BLV-CoCoMo-qPCR-2 method to detect the BLV provirus in nasal secretions and saliva samples obtained from cattle with BLV provirus-positive blood test results [19].

In addition, Jimba et al. [4] compared four BLV detection systems (ELISA, PHA, AGID, and nested PCR) and concluded that the antibody titer does not correlate with infection status. Although the quantitative PCR system is one of the best for amplifying proviruses with high sensitivity and a low risk of contamination, the method requires expensive real-time PCR machines and reagents and tricky sample preparation protocols. Here, we developed a novel blood-based PCR system that amplifies the target region of DNA without the need for DNA isolation and purification. Developed PCR system, namely BLV-CoCoMo-direct-PCR, were compared with real-time PCR system as BLV proviral DNA detection method, and national test certificated ELISA system as BLV antibody-based method for estimating the specificity and sensitivity. The assay can detect BLV provirus with high specificity and at low cost, enabling identification of BLV-infected cattle without the need for real-time PCR systems.

Materials and methods

Animals

Blood samples (collected in ethylenediaminetetraacetic acid (EDTA)) were obtained from 182 cattle on a BLV-positive farm (Table 1) and used for both direct PCR and isolation/purification of genomic DNA. At the same time, blood samples were collected without anti-coagulant and the serum was separated from the red blood cells. White blood cell and lymphocyte counts were obtained for each sample using a cell counter (Table 1).

All experiments were conducted in accordance with the Guidelines for Laboratory Animal Welfare and Animal Experiment Control set out by the NARO Institute of Livestock and Grassland Science (permit numbers: NILGS-13053101 and NILGS-14112050).

Diagnosis of BLV infection using the BLV-ELISA system

Anti-Env gp51 antibodies were detected using an anti-BLV antibody ELISA Kit (JNC Inc., Tokyo, Japan), according to the manufacturer's instructions.

Measurement of the BLV proviral load using BLV-CoCoMo-qPCR-2

Genomic DNA was isolated from EDTA-treated blood samples using the Wizard Genomic DNA Purification Kit (Promega Corporation, Tokyo, Japan) and used for quantitative PCR. The BLV-CoCoMo-qPCR-2 assay was used to determine the presence of BLV and to measure the proviral load, as described previously [3, 17]. A 179 bp sequence of the BLV long terminal repeat (LTR) was amplified using the degenerate primer pair, CoCoMo-FRW and CoCoMo-REV, and a 15 bp 6-carboxyfluorescein (FAM)-labeled MGB probe. *BoLA-DRA* (the internal control) was amplified using the primer pair DRA-F and DRA-R and a FAM-labeled DRA MGB probe, as previously described [17]. Proviral load were estimated as the copy number existing in 10^5 of white blood cells.

Comparison of three DNA polymerases for use in the direct blood-based PCR assay

The CoCoMo-FRW and CoCoMo-REV primers (RIKEN genesis, Tokyo, Japan), which are specific for the BLV-LTR region, were used to detect BLV provirus in blood samples.

We tested three different DNA polymerases (KOD FX neo (Toyobo, Tokyo, Japan), MightyAmp DNA polymerase ver. 2 (TaKaRa, Tokyo, Japan), and Phusion Blood2 DNA polymerase (Life Technologies, Tokyo, Japan)) for suitability in the assay. Briefly, 1 µl of blood was added to 19 µl of PCR buffer (provided by each company) containing 0.4 units of Taq polymerase and 2 µl of CoCoMo primers (RIKEN genesis, Tokyo, Japan). The thermal cycling profile was as follows: initial denaturation for 10 min at 95 °C, followed by 60 cycles of 15 s at 95 °C and 1 min at 60 °C. The PCR products were treated with

Table 1 Comparison of different BLV detection methods using samples from 186 cattle

Cattle ID	No.[a]	ELISA[b]	PVL[c]	Direct PCR
1-36	36	-	0	-
37-38	2	+	0	-
39	1	-	1	-
40-44	5	-	2	-
45-46	2	-	3	-
47	1	+	3	-
48-55	8	-	4	-
56	1	+	4	-
57-59	3	-	5	-
60-61	2	+	5	-
62-64	3	-	6	-
65-66	2	+	6	-
67-68	2	-	7	-
69	1	-	7	+
70-73	4	-	9	-
74-75	2	-	10	-
76	1	+	10	-
77	1	-	11	-
78	1	-	13	-
79	1	-	14	-
80	1	+	14	-
81	1	+	23	-
82	1	+	24	-
83	1	+	28	-
84	1	+	34	-
85	1	+	35	-
86	1	+	39	-
87	1	+	40	-
88-89	2	+	43	-
90	1	+	50	-
91	1	+	51	-
92	1	-	51	-
93	1	+	52	-
94	1	-	59	-
95	1	+	67	-
96	1	+	71	-
97	1	-	76	-
98	1	+	85	-
99	1	+	90	-
100	1	+	109	-
101	1	+	133	-
102	1	+	145	+
103	1	+	299	+
104	1	+	328	+
105	1	+	525	+
106	1	+	589	+
107	1	+	1,142	+

Table 1 continued

Cattle ID	No.[a]	ELISA[b]	PVL[c]	Direct PCR
108	1	+	1,335	+
109	1	+	1,354	+
110	1	+	1,478	+
111	1	+	1,528	+
112	1	+	1,620	+
113	1	+	1,733	+
114	1	+	2,272	+
115	1	+	2,310	+
116	1	+	2,716	+
117	1	+	2,731	+
118	1	+	2,783	+
119	1	+	3,161	+
120	1	+	3,267	+
121	1	+	3,386	+
122	1	+	3,576	+
123	1	+	3,690	+
124	1	+	4,045	+

[a] Number of cattle which corresponding to each categories

[b] ELISA was performed using an anti-BLV-ELISA kit according to the manufacturers' instructions (JNC Inc., Tokyo, Japan). +, positive for anti-BLV antibodies; −, negative for anti-BLV antibodies

[c] The proviral load (expressed as the copy number per 10^5 cells) was evaluated by BLV-CoCoMo-qPCR, as previously described (Jimba et al., 2010). The copy number of the bovine leukocyte antigen *BoLA-DRA* gene was used to evaluate cell numbers in the blood

Hae III and analyzed by agarose gel electrophoresis to confirm that the product was a BLV-LTR amplicon.

Results

Selection of an optimal DNA polymerase for the BLV-CoCoMo-direct-PCR

To select the optimal Taq polymerase for the direct PCR using CoCoMo primers, three systems, provided by TaKaRa co. (Might Amp ver.2.), Toyobo co. (KOD Fx neo), and Thermo fisher co. (Phusion Blood2 DNA polymerase), were tested (Fig. 1, panel A). The host gene *BoLA-DRA* was used as a positive control. All three enzymes successfully amplified the 151 bp product derived from *BoLA-DRA* gene. However, only KOD FX neo Taq polymerase amplified the 179 bp product derived from the BLV-LTR gene when using whole blood as a template. Therefore, we chose KOD Fx neo Taq polymerase for the BLV-CoCoMo-direct-PCR (Fig. 1, panel A).

Selection of optimal DNA polymerase for developping Direct CoCoMo PCR

(A)

(B)

(C)
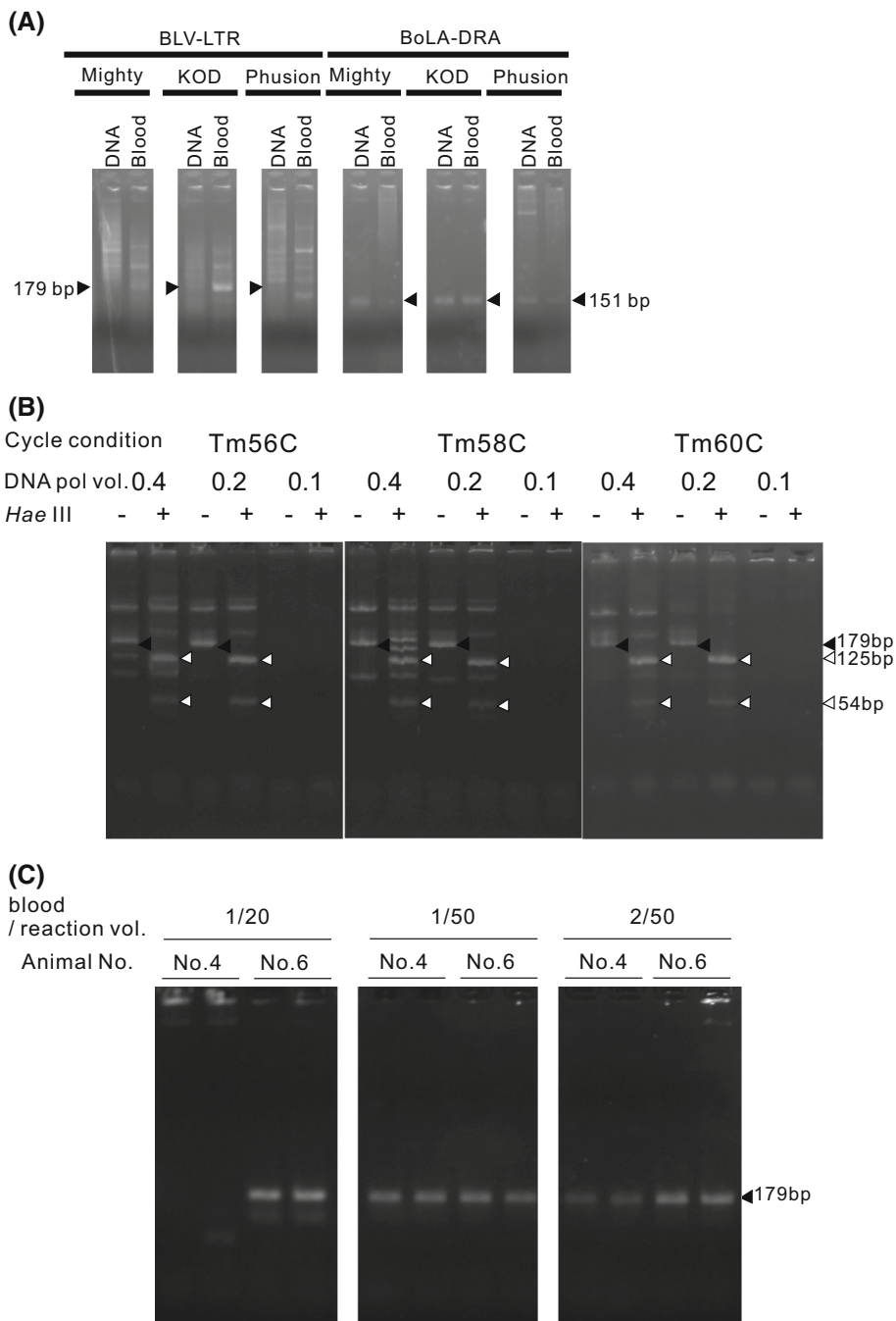
Next, we optimized the PCR reaction to amplify the BLV-LTR gene using CoCoMo primers and KOD Fx neo Taq polymerase. We found that a DNA polymerase concentration of 0.2 μl per 20 μl of PCR reaction and a Tm60C cycling profile yielded the best results (Fig. 1, panel B). Finally, we used two kinds of BLV-positive cattle blood for the direct PCR (Fig. 1, panel C). For blood sample number four, different volumes of PCR solution and different amounts of blood yielded different results; therefore, for samples 4 and 6, we added 1 μl of blood to 50 μl of PCR reaction solution to obtain a signal of sufficient strength to determine BLV positivity.

Thus, the following protocol was used when performing the BLV-CoCoMo-direct-PCR: PCR reactions were

◀**Fig. 1** Selection of the optimal DNA polymerase for the CoCoMo-direct-PCR. (**A**) Amplification of the BLV-LTR using CoCoMo primers, and of the *BoLA-DRA* gene using gene-specific primers. Purified DNA and whole blood were used as templates. The three Taq polymerases were Mighty amp ver.2, KOD plus, and Phusion Blood2. (**B**) Optimization of the PCR conditions used to amplify the BLV-LTR sequence using the KOD plus DNA polymerase kit (Toyobo). Three different polymerase volumes and three cycling conditions were tested. The amplicon (179 bp) was digested with *Hae* III and the products (125 bp and 54 bp) were detected on agarose gels. Three cycle conditions (Tm56C, Tm58C and Tm60C) which use different annealing temperature were compared. Tm56C; initial denaturation for 2 min at 96 °C, followed by 5 cycles of 10 s at 96 °C, 10 s at 66 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 64 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 62 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 60 °C, 5 cycles of 10 s at 96 °C, 10 s at 58 °C, and 30 cycles of 10 s at 96 °C, 10 s at 56 °C. Tm58C; initial denaturation for 2 min at 96 °C, followed by 5 cycles of 10 s at 96 °C, 10 s at 66 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 64 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 62 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 60 °C, and 35 cycles of 10 s at 96 °C, 10 s at 58 °C. Tm60C; initial denaturation for 2 min at 96 °C, followed by 5 cycles of 10 s at 96 °C, 10 s at 66 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 64 °C, and 30 s at 68 °C, 5 cycles of 10 s at 96 °C, 10 s at 62 °C, and 30 s at 68 °C, and 40 cycles of 10 s at 96 °C, 10 s at 60 °C. (**C**) The effects of different blood samples and reaction volumes were tested using samples from BLV-positive cattle (no. 4 and no. 6)

performed using 1 μl of cattle blood and 5 μl of CoCoMo primer mix in 50 μl of reaction mixture containing 44 μl of PCR buffer, 400 μM dNTPs, and 0.5 unit KOD FX neo Taq polymerase. The thermal cycling profile was as follows: initial denaturation for 2 min at 96 °C, followed by 5 cycles of 10 s at 96 °C, 10 s at 66 °C, and 30 s at 68 °C; 5 cycles of 10 s at 96 °C, 10 s at 64 °C, and 30 s at 68 °C; 5 cycles of 10 s at 96 °C, 10 s at 62 °C, and 30 s at 68 °C; and 50 cycles of 10 s at 96 °C and 10 s at 60 °C. Five microliters of the reaction mixture was then loaded onto a 3 % agarose/tris-acetate-EDTA gel, which was then stained with ethidium bromide. DNA bands were visualized under UV light.

Validation of the BLV-CoCoMo-Direct-PCR using samples from 182 cattle

To determine the specificity and sensitivity of the BLV-CoCoMo-direct-PCR, we collected 182 blood samples from cattle on the same BLV-positive farm (Table 1), detected the BLV provirus using BLV-CoCoMo-direct-PCR, and estimated the proviral load using BLV-CoCoMo-qPCR. All 38 samples that tested negative in the BLV-CoCoMo-qPCR-2 were also negative in the BLV-CoCoMo-direct-PCR, meaning that the specificity of the BLV-CoCoMo-direct-PCR was 100 % (Table 2). Overall, BLV-CoCoMo-qPCR-2 detected BLV provirus in 144/182

samples, whereas BLV-CoCoMo-direct-PCR detected BLV provirus in only 82 samples (sensitivity = 57 %) (Table 2). Notably, all 62 cattle that tested negative in the BLV-CoCoMo-direct-PCR assay had a proviral load less than 133 copies/10^5 cells (as determined by BLV-CoCoMo-qPCR-2) (Table 1), suggesting that the BLV-CoCoMo-direct-PCR could not detect BLV-positive cattle with a low proviral load. However, one cow (No.69) with a proviral load of 7 copies/10^5 cells did test positive in the BLV-CoCoMo-direct-PCR (Table 1).

Comparison of BLV-ELISA and BLV-CoCoMo-direct PCR

Historically, BLV-positive cattle were identified by AGID, PHA, or ELISA. In Japan, the BLV-ELISA (JNC, Inc.) is the most reliable and sensitive kit for identifying BLV-positive cattle. Therefore, we tested all 186 samples by BLV-CoCoMo-direct-PCR and by BLV-ELISA (Table 3). Based on the ELISA results, the BLV-CoCoMo-direct-PCR showed 98.6 % specificity and 74.3 % sensitivity. Two animals (nos. 37 and 38) that tested negative in the BLV-CoCoMo-direct-PCR and BLV-CoCoMo-qPCR-2 assays tested positive in the ELISA (Table 1). In addition, 26 animals that tested negative in the BLV-CoCoMo-direct-PCR, but positive in the BLV-CoCoMo-qPCR-2 assay, tested positive in the ELISA (Table 1). The proviral load in these cattle was quite low (<133 copies/10^5 cells) when compared with the mean proviral load in BLV-positive cattle (6,372 copies/10^5 cells).

Discussion

This is the first report of a direct blood-based PCR that can detect the BLV provirus. The BLV-CoCoMo-direct-PCR showed high specificity for the provirus, making it suitable for use in a wide variety of scenarios due to the lack of need for real-time PCR assays, DNA purification, or nested reactions. Thus, the method is very cost-effective and the risk of a false-positive result due to DNA contamination is low. Additionally, BLV-CoCoMo-direct-PCR showed extremely high sensitivity (92 %) for detecting BLV positive cattle with over 100 copies / 10^5 cells of proviral load. Panei et al. [13] showed that the cattle with <100 copies / 10^5 cells of proviral load were difficult to detect the pathogenesis by syncytium assay and also their CD5^{+} B cell population were completely normal. Additionally, Yuan et al., [19] showed that BLV proviruses were detected in nasal and saliva of BLV-infected cattle when the proviral load is over 14,000 copies / 10^5 cells. These results clearly showed that the cattle infected with low proviral load have low risk to spread BLV to other animal and our developed BLV-CoCoMo-direct-PCR can identify these

Table 2 Comparison of results from the BLV-CoCoMo-Direct PCR and BLV-CoCoMo-qPCR-2 assays

		CoCoMo-qPCR-2		Total
		Positive	Negative	
CoCoMo-Direct PCR	Positive	82	0	82
	Negative	62	38	100
Total		144	38	182
		Sensitivity (%)	Specificity (%)	
		(95 % CI)	(95 % CI)	
		56.9	100	
		(48.4–65.2)	(90.7–100.0)	

CI, confidence interval

Table 3 Comparison of results from the BLV-CoCoMo-Direct PCR and BLV-ELISA

		ELISA		Total
		Positive	Negative	
CoCoMo-Direct PCR	Positive	81	1	82
	Negative	28	72	100
Total		109	73	182
		Sensitivity (%)	Specificity (%)	
		(95 % CI)	(95 % CI)	
		74.3	98.6	
		(65.1–82.2)	(92.6–100.0)	

CI, confidence interval

cattle. Moreover, our result showed 84.9 % of BLV positive cattle in Japan had more than 100 copies / 10^5 cells of proviral load (data not shown) [11]. In contrary, our samples in this study showed only 17.4 % of BLV positive cattle with proviral load more than 100 copies / 10^5 cells. The sensitivity of BLV-CoCoMo-direct-PCR may be increase if we use the sample from other farms with high proviral load.

In present time, nine major BLV sub-groups have been reported worldwide [20], and 1,306 BLV sequences have been uploaded to the GenBank database. The drawback of PCR-based detection systems is that a mismatching between PCR primers and BLV sequences results in failure to detect the BLV provirus. The CoCoMo algorithm, the method used to design degenerate primer sets that amplify all available sequences within a target region, was developed to solve this problem. Recently, this algorithm was used to design the CoCoMo primer set used to measure the BLV proviral load [3]. The BLV-CoCoMo-direct-PCR method described herein is based on CoCoMo primers that amplify almost all known and novel BLV strains, meaning that the assay can be used worldwide.

Although the BLV-CoCoMo-direct-PCR method showed quite high specificity, the sensitivity was low compared with that of ELISA. The discrepancy between the results obtained with PCR-based provirus detection systems and antibody-based detection systems has been reported previously [4, 11, 19]. These reports show that some cattle with a low proviral load do not produce anti-BLV antibodies; therefore, although some cattle test positive by ELISA, no DNA-integrated provirus is detected by PCR. Here, we also identified two cattle (no. 1185 and no. 804) that had anti-BLV antibodies, but no detectable BLV provirus in the BLV-CoCoMo-qPCR2 and BLV-CoCoMo-direct PCR assays. On the other hand, 37 provirus-positive cattle did not have anti-BLV antibodies. This result clearly shows that antibody- and PCR-based assays can generate different results for the same sample.

In Japan, there are three standard serological tests for detecting BLV-positive cattle: PHAs, AGIDs, and ELISAs. Jimba et al. [4] used 370 clinical samples to compare the sensitivity of BLV-CoCoMo-qPCR with that of these three serological techniques. The results led us to conclude there are three problems with these methods: (1) some cattle can be negative for anti-BLV antibodies according to AGID and PHA, but have a high proviral load, due to low sensitivity of AGID and PHA tests; (2) some cattle can be positive according to serological tests, but still have a low proviral load; indeed, these tests could not discriminate cattle with a moderate proviral load from cattle with a high proviral load; and (3) anti-BLV antibodies could be raised in dam exposed to BLV antigens and these antibodies can then be transferred from mother to calf; the latter may then test positive for antibodies but is not infected with BLV. The BLV-CoCoMo-direct-PCR showed 100 % specificity

when compared with BLV-CoCoMo-qPCR2. The presence of provirus, as determined by BLV-CoCoMo-direct-PCR, clearly identifies animals infected with BLV. Therefore, we conclude that BLV-CoCoMo-direct-PCR can be used for the definitive diagnosis of BLV-infected cattle.

Although many EU countries are officially free from BLV, others countries such as Japan [8], the United States [9], Canada [10], and Argentina [2] are not. In these countries, it is hard to segregate all infected cattle due to difficulties in management. As suggested by several researchers [6, 11], the proviral load is an important factor that determines transmission of BLV from infected cattle to non-infected cattle. Indeed, we detected BLV provirus in nasal secretions and saliva in host animals with a proviral load greater than $14,000/10^5$ cells, as determined by BLV-CoCoMo-qPCR [19]. Taken together, these results suggest that BLV-infected cattle with a low proviral load are less of a transmission risk than those with a high proviral load. Therefore, detection of infected cattle with a high proviral load is important to prevent the transmission of BLV from high proviral load cattle to uninfected cattle. The BLV-CoCoMo-direct-PCR system identified cattle with a high proviral load cost-effectively and with a low risk of cross-contamination. Therefore, the BLV-CoCoMo-direct-PCR system is eminently suited to identifying BLV-infected cattle on farms with high levels of contamination.

In Conclusion, BLV-CoCoMo-direct-PCR has quite high specificity for the BLV provirus in infected cattle and can be used under a wide variety of circumstances because it does not require expensive equipment and reagents or multiple sample preparation steps. Thus, the method is extremely cost-effective and carries a low risk of providing false-positive results due to DNA contamination.

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