Journal of Virological Methods 315 (2023) 114706

Contents lists available at ScienceDirect

Journal of Virological Methods

journal homepage: www.elsevier.com/locate/jviromet

Development of droplet digital PCR for quantification of bovine leukemia virus proviral load using unpurified genomic DNA

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ARTICLE INFO

Keywords:
Bovine leukemia virus
Diagnostic test
Droplet digital PCR
Enzootic bovine leukosis
Quantification
Unpurified genomic DNA

ABSTRACT

Bovine leukemia virus (BLV) is the causative agent of a B-cell tumor called enzootic bovine leukosis. Preventing BLV spreading is required to reduce economic loss related to BLV infection of livestock. To quantify proviral load (PVL) more easily and rapidly, we developed a quantification system of PVL using droplet digital PCR (ddPCR). This method uses a multiplex TaqMan assay of the BLV provirus and housekeeping gene RPP30 for the quantification of BLV in BLV-infected cells. Furthermore, we combined ddPCR with DNA purification-free sample preparation (unpurified genomic DNA). The percentage of BLV-infected cells based on unpurified genomic DNA was highly correlated with that based on purified genomic DNA (correlation coefficient: 0.906). Thus, this new technique is a suitable method to quantify PVL of BLV-infected cattle in a large sample number.

1. Introduction

Bovine leukemia virus (BLV) belongs to the *Deltaretrovirus* genus of the *Retroviridae* family, which is closely related to human T cell lymphotropic virus 1 (Aida et al., 2013). BLV mainly infects B lymphocytes and integrates its genome into the host chromosome during viral replication, where the BLV genome is integrated as a provirus. Although most infected cattle remain asymptomatic, approximately 30% progress to persistent lymphocytosis and less than 10% develop malignant B-cell lymphoma called enzootic bovine leukosis (Aida et al., 2013; Burny et al., 1988).

The transmissibility of the virus and disease progression of infected animals are associated with the proviral load (PVL) (Juliarena et al., 2007; Kobayashi et al., 2020; Kuczewski et al., 2021). Previous studies reported that cattle with a high proviral load (HPL) were a major infectious source in a herd (Ruggiero et al., 2019; Kuczewski et al., 2021). In contrast, cattle with a low proviral load (LPL) had low or no infectivity to other cattle (Juliarena et al., 2016; Mekata et al., 2015). Based on these studies, the separation of infected cattle from uninfected cattle (Maresca et al., 2015) and the prioritization of isolating cattle with HPL are critical approaches to prevent BLV from spreading throughout a herd (Ruggiero et al., 2019). Therefore, updating BLV PVL diagnostics to improve the simplicity and time-benefit is important to prevent and control BLV.

Despite the high sensitivity and specificity of qPCR, it uses relative quantification based on a standard curve. Thus, new laboratory techniques are needed to obtain accurate results. Recently, digital PCR has emerged as third generation PCR. Droplet digital PCR (ddPCR) uses a TaqMan or intercalate assay whereby the reaction is portioned into droplets and a readout of the fluorescence magnitude of each droplet is obtained (Hindson et al., 2011). Small amounts of DNA/RNA are distributed into the partition and the PCR reaction is performed independently by partition This enables the precise quantification of ultra-diluted samples without the need for a standard curve. The objective of this study was to develop a quantification system of BLV PVL using a multiplex digital PCR assay based on the co-quantification of BLV provirus and housekeeping gene RPP30. Furthermore, we combined this assay with a DNA purification-free method (unpurified

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https://doi.org/10.1016/j.jviromet.2023.114706
Received 14 November 2022; Received in revised form 23 February 2023; Accepted 24 February 2023
Available online 26 February 2023

genomic DNA) for sample preparation to provide time and cost benefits for users.

2. Materials and methods

2.1. Ethics

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and that the appropriate ethical review was received from the Animal Ethics Committee of the Faculty of Agriculture at the University of Miyazaki (University of Miyazaki, 1–1 Gakuen-Kibanadai-Nishi, Miyazaki 889–2192, Japan) (number 2018–03–29-Z20).

2.2. Samples for the validation of digital PCR for purified DNA

From 2019–2021, peripheral blood samples were collected in EDTA tubes from 111 Japanese black cattle from 41 farms in Miyazaki Prefecture, Japan. Blood samples were centrifuged at 1000g for 5 min to isolate plasma. We determined the presence of anti-gp51 antibody using a commercial ELISA kit (Nippon Gene Co., Ltd. Tokyo, Japan) according to the manufacturer's instructions. Whole blood samples were stored at − 20 °C until genomic DNA extraction with high purity using MagDEA Dx SV reagent (Precision System Science, Co., Ltd., Chiba Japan) with an automated nucleic acid extraction system magLEAD 12gC, according to the manufacturer's instructions. The extracted DNA was stored at − 20 °C until analysis.

The percentage of BLV-infected cells was determined using a commercial qPCR kit (RC202A Takara Bio Inc., Shiga, Japan), targeting the BLV pol gene and housekeeping gene RPPH1. We used the laboratory-optimized protocol for this kit. The reaction mixture contained 7.5 μl of probe qPCR Mix-UNG, 3 μl of Primer/probe Mix (BLV), 0.1 μl of Rox reference Dye, 2.4 μl of H_2O, and 2 μl of DNA sample up to 15 μl of final reaction volume. The amplification profile was 95 °C for 30 s, 45 cycles of 95 °C for 5 s, and 60 °C for 30 s qPCR was performed using the QuantStudio™ 3 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, USA). The percentage of BLV-infected cells was calculated using the following equation.

$$\text{The percentage of BLV} - \text{infected cells} = \frac{\text{BLV copy number}}{\text{RPPH1copy number} \times 0.5} \times 100 \quad (1)$$

We considered blood samples that were ELISA-positive and qPCR-positive as BLV-infected, and ELISA-negative and qPCR-negative as BLV-uninfected. Samples that were ELISA-positive and qPCR-negative, and ELISA-negative and qPCR-positive were excluded from this study. Using the above criteria, 57 samples were determined to be BLV-infected and 53 samples were determined to be BLV-uninfected.

2.3. Assay design for the quantification of BLV PVL using ddPCR

BLV-specific primers and FAM-labeled probes targeting the env and pol genes were designed according to the sequence of the pvAN003 strain (Acc. No. AP018024.1). Housekeeping RPP30-specific primers and a HEX-labeled probe were designed according to the sequence of bovine chromosome 26 (Acc. No. NC037353.1) (Table 1). The components of the reaction mix were 10 μl of 2 × ddPCR Supermix (Bio-Rad Laboratories Inc., Hercules, USA), 475 nM of env primers, 450 nM of pol primers, 125 nM of env and pol probes in each, 950 nM of RPP30 primers, 250 nM of RPP30 probe mix, < 20 ng of purified DNA or 2 μl of unpurified genomic DNA (see *Materials and methods 2.5*), and H_2O up to 20 μl of the final volume. The amplification profile was 95 °C for 10 min, 40 cycles of 94 °C for 30 s and 51 °C for 1 min, 98 °C for 10 min, and 8 °C until the amplification was completed. In the data analysis, QuantaSoft software version 1.3.2.0 (Bio-Rad Laboratories Inc.) was used to quantify the number of droplets. We set the threshold of the fluorescence of the magnitude of BLV and RPP30 positive droplets to 3000 and 2500, respectively. We calculated the percentage of BLV-infected cells using the following equations including the "Copy Number Variation (CNV) 2″ function of the software.

$$\text{CNV2} = \frac{\text{The number of BLV positive droplet}}{\text{The number of RPP30positive droplet} \times 0.5} \quad (2)$$

$$\text{The percentage of BLV infected cells} = \text{CNV2} \times 100 \quad (3)$$

2.4. Validation of ddPCR for purified DNA

First, we determined a consensus between ddPCR-positive or ddPCR-negative vs BLV-infected or uninfected samples as described in the Materials and methods 2.3. Second, a consensus on the measurement of the percentage of BLV-infected cells by ddPCR with qPCR was determined using 110 samples from BLV-infected cattle with a variable copy number. Finally, we determined the limit of detection (LoD) of the ddPCR based on purified DNA. Whole blood samples in which the percentage of BLV-infected cells was 12% (quantified using qPCR, see *Materials and methods 2.2*) were serially diluted 10-fold with whole blood from uninfected cattle. DNA was extracted by magLEAD 12gC (n = 3). The concentration of each purified DNA sample was determined by a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific Inc.), and each sample was diluted to 10 ng/μl by PCR-grade water. We performed ddPCR for 20 ng of sample input. The LoD of ddPCR was compared with that of qPCR using 20 ng sample input.

2.5. Samples for the validation of digital PCR using unpurified genomic DNA

We collected 70 peripheral blood samples from Japanese black cattle (52 heads), Holstein cattle (16 heads), and Jersey cattle (2 heads) from 38 farms in Miyazaki Prefecture, Japan. We determined the BLV-infectious status of these samples according to the criteria described in Materials and methods 2.2 by ELISA and qPCR using purified DNA extracted using magLEAD 12gC. Forty samples were determined to be infected and 30 samples were determined to be uninfected. Whole blood samples were stored at 4 °C until further experiments based on

Table 1
Sequence of primers and probes for ddPCR.

Target	Primer/probe	Sequence (5′ – 3′)	Acc. No. of reference	Position
BLV	env-F	CCGAAATATTAGTATATAACAA	AP018024.1	5496–5517
	env-R	ACCCAGAAGATTTGG	AP018024.1	5560–5574
	env-P	FAM-ACCATCTCCAGCTCTG-lowa Black	AP018024.1	5519–5534
	pol-F	CCTCAAGGCTTCATTA	AP018024.1	2703–2718
	pol-R	GACACCAGAAGAGAC	AP018024.1	2786–2800
	pol-P	FAM-AACGAGCACTACAGGAA-lowa Black	AP018024.1	2737–2753
RPP30	F	TGGGCTTTGAACTTC	NC037353.1	12,514,468–12,514,482
	R	GCGTTGGAAATTGTG	NC037353.1	12,514,523–12,514,537
	P	HEX-AGACTCTACGATGAGAAGG-lowa Black	NC037353.1	12,514,502–12,514,520

unpurified genomic DNA were performed within 2 weeks. To lyse the cells and nucleolus membranes, and release DNA, 2 μl of blood sample was mixed with 100 μl of 0.1% sodium dodecyl sulfate (SDS) solution. The mixture was heated at 95 °C for 10 min.

The bovine B-cell lymphoma line BLSC-KU17 (KU-17) was purchased from the RIKEN BioResource research center (Koyama et al., 1992; Yamanaka et al., 2022). KU-17, established from the lymphoma cells of BLV-infected cattle with enzootic bovine leukosis, contains a single copy of an intact BLV provirus per cell. KU-17 was cultured at 37 °C with RPMI 1640 medium (Thermo Fisher Scientific Inc.) supplemented with 10% fetal bovine serum (HyClone Laboratories Inc., South Logan, USA), 100 IU/mL penicillin, 100 μg/mL streptomycin (Thermo Fisher Scientific Inc.), 2 mM L-glutamine (Thermo Fisher Scientific Inc.), and 50 μM 2-mercaptoethanol (Thermo Fisher Scientific Inc.).

2.6. Validation of ddPCR using unpurified genomic DNA

First, we determined a consensus between ddPCR-detected or undetected using unpurified genomic DNA samples vs BLV-infected or uninfected samples as described in Materials and methods 2.5. Second, a correlation between the percentage of BLV-infected cells and PVL using 70 BLV-infected cattle were determined. Finally, we determined the LoD of ddPCR using unpurified genomic DNA. KU-17 cells were adjusted to 5×10^6 cells/mL and serially diluted 5-fold with whole blood from uninfected cattle to prepare six different conditions: 100,000, 20,000, 4000, 800, 160, and 32 cells/mL. Then, 2 μl of sample was added as a template into a PCR mixture.

2.7. Validation of ddPCR for amplicon-containing plasmid DNA

In order to convert the quantitative performance of ddPCR uniformly according to viral copies, we used amplicon-containing plasmid DNA (Eurofins Genomics, Tokyo, Japan). The pEX-A2J2 vector plasmid DNAs containing the sequence of env (Acc no. AP018024.1), pol (Acc no. AP018024.1) (Table 1). The copy number of plasmid DNA that determined LOD was calculated using the following formula:

DNA concentration (copy number) = (6.02 × 1023 copies/mol × -plasmid concentration [ng/μl] × 10 −9)/ (DNA length in nucleotides × 660 g/mol).

Solution containing artificially synthesized DNA was diluted 5-fold with DW to prepare five different conditions: 100 copies, 20copies, 4 copies, 0.8 copies and 0.016copies. Then, 2 μl of sample was added as a template into a PCR mixture.

2.8. Statistical analysis

Samples were judged to be positive when a signal exceeded the cut-off value regardless of the percentage of infected cells in the assay. The kappa value was calculated for the agreement of the results (positive or negative) between two different methods: ddPCR using purified DNA vs qPCR, and ddPCR using unpurified genomic DNA vs purified DNA. Spearman's coefficient was used to calculate the correlation between the measurement of the percentage of BLV-infected cells using two different methods: ddPCR using purified DNA vs qPCR, and ddPCR using unpurified genomic DNA vs purified DNA. All statistical analyses were performed using R software (version 4.0.2, https://www.r-project.org/).

3. Results

3.1. Performance of ddPCR using purified DNA

ddPCR using purified DNA correctly detected PVL in all infected cattle (n = 53) and no PVL in all uninfected cattle (n = 57) from 110 samples. Thus, the kappa coefficient of this assay against combined ELISA-qPCR was 1. A result of ddPCR assay displayed as 2D plot is shown in Fig. 1. We found a high correlation (Spearman's coefficient $r = 0.994$, $P < 0.01$) between the measurement of the percentage of BLV-infected cells by ddPCR using purified DNA and qPCR (Fig. 2). In this analysis, we confirmed that a sample with the lowest PVL by qPCR (0.249% of BLV-infected cells) was positive by ddPCR. The results of the LoD analysis showed this assay could detect BLV provirus more than 0.012% of BLV-infected cells (Table 2). Results of triplicate tests of this sample were 0.09%, 0.03%, and 0.08% BLV-infected cells by ddPCR.

3.2. Performance of ddPCR using unpurified genomic DNA

ddPCR using unpurified genomic DNA correctly detected PVL in all infected cattle (n = 40) and no PVL in all uninfected cattle (n = 30) from 70 samples. Thus, the kappa coefficient of this assay against combined ELISA-qPCR was 1. We found a high correlation (Spearman's coefficient $r = 0.906$, $P < 0.01$) between the measurement of the percentage of BLV-infected cells using ddPCR with unpurified genomic DNA and that using purified DNA (Fig. 3). In this analysis, we confirmed that a sample with the lowest PVL (0.06% of the cells were BLV positive) was detected by ddPCR using unpurified genomic DNA. The results of the LoD analysis showed this assay could detect BLV provirus from whole blood containing 800 cells/mL with a single copy of PVL per infected cell (Table 3), indicating 1.6 infected cells in a 2 μl of sample input in a reaction mixture. Results of triplicate tests of this sample were 0.3%, 0.7%, and 1.6% BLV-infected cells by ddPCR. We also found a high correlation (Spearman's coefficient $r = 0.94$, $P < 0.01$) between the percentage of BLV-infected cells using ddPCR with unpurified genomic DNA and qPCR with purified one (Fig. S1).

3.3. Performance of ddPCR for amplicon-containing plasmid DNA

In this experiment, we confirmed the amplicon-containing plasmid DNA template with the minimum detection limited by ddPCR was 4 copies per reaction (Table 4).

4. Discussion

In this study, we developed a quantitative method for determining the BLV PVL using ddPCR by detecting the BLV provirus with the housekeeping gene RPP30. Furthermore, we combined this assay with unpurified genomic DNA-based sample preparation. This protocol does not require the purification of DNA from blood so that the operating time for template preparation is reduced. In addition to its ease of use and time benefits, we demonstrated the high accuracy of this protocol for BLV testing including BLV testing to identify provirus-positive cattle and BLV quantitation to measure the PVL. Thus, this method is a simple and reliable tool for the high-throughput diagnosis of BLV infection.

Our updated ddPCR method for BLV has three advantages compared with previous techniques (De Brun et al., 2022). First, our assay is a duplex ddPCR that targets the BLV and housekeeping RPP30 genes in a single well. This system enables the quantification of BLV PVL by calculating the percentage of BLV-infected cells without sample quantity adjustment and standard curve. Second, our assay enables us to quantify BLV PVL using unpurified genomic DNA template so that reduced the time, cost, and manpower necessary for genomic DNA purification. Third, our assay used two kinds of primers (env and pol) to avoid failed PCR reaction by point mutation in env or pol gene. These points are distinct from the previous techniques (De Brun et al., 2022) and novelty. Therefore, our system might be suitable for the high-throughput diagnosis of BLV PVL.

ddPCR using unpurified genomic DNA was sufficient to identify provirus-positive cattle and measure the PVL. When our method was compared with the DNA purification method using magLEAD 12gC, the time required for DNA extraction was reduced by more than one third, and the extraction cost was reduced by about 98%. Therefore, this method is suitable for large-scale screening testing to identify spreader

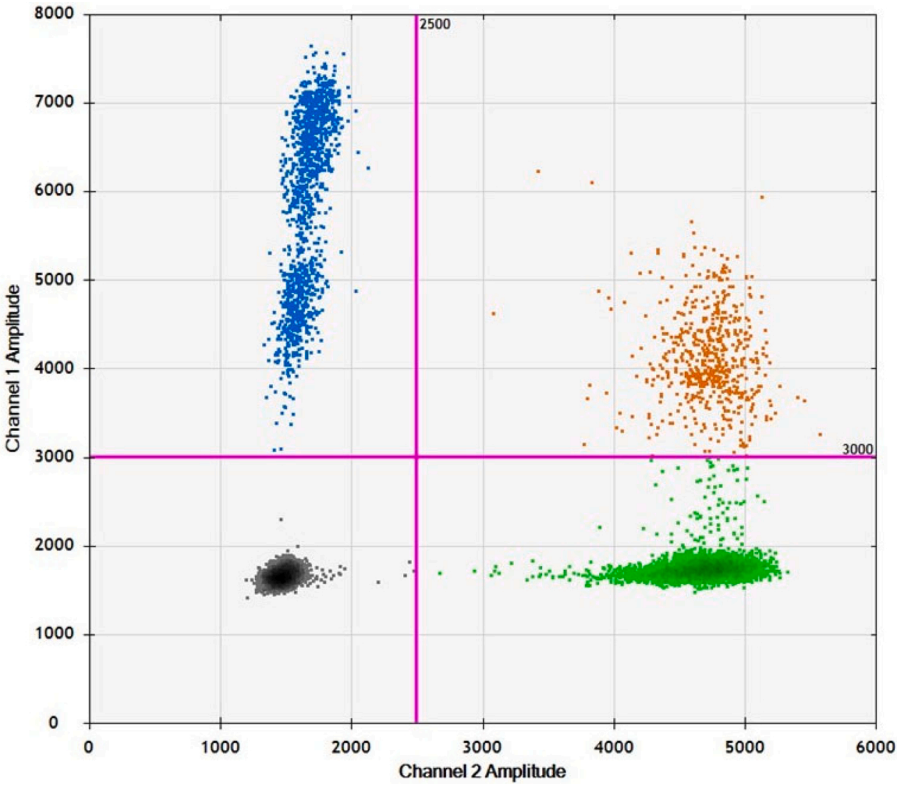

Fig. 1. 2D plot display of ddPCR assay, The x-axis (Channel 2 Amplitude) indicates the amplitude of HEX fluorescence, corresponding to the housekeeping gene RPP30, whereas the y-axis (Channel 1 Amplitude) indicates the amplitude of FAM fluorescence, corresponding to BLV (env and pol). For each plot, droplets in the lower left quadrant are negative for both targets (black), droplets in the upper left quadrant are positive for BLV only (blue), droplets in the lower right quadrant are positive for RPP30 only (green), and droplets in the upper right quadrant are positive for BLV and RPP30 (orange).

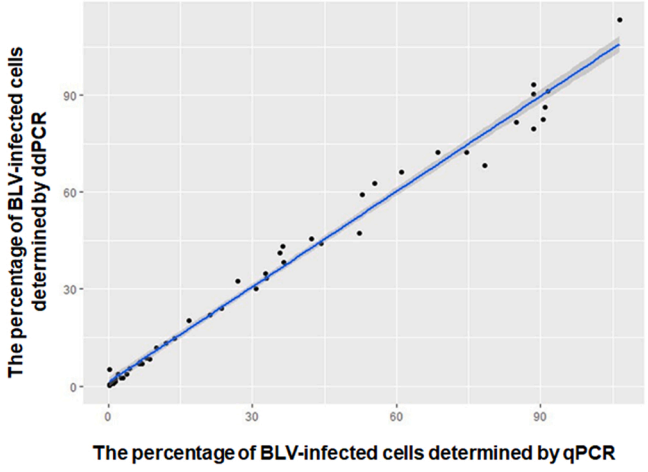

Fig. 2. Correlation of the percentage of BLV-infected cells between qPCR and ddPCR results, The x-axis indicates the percentage of BLV-infected cells determined by qPCR and the y-axis indicates the percentage of BLV-infected cells determined by ddPCR using purified DNA. Each dot indicates a single sample. The blue line and shadow indicate the linear model and 95% confidence interval, respectively.

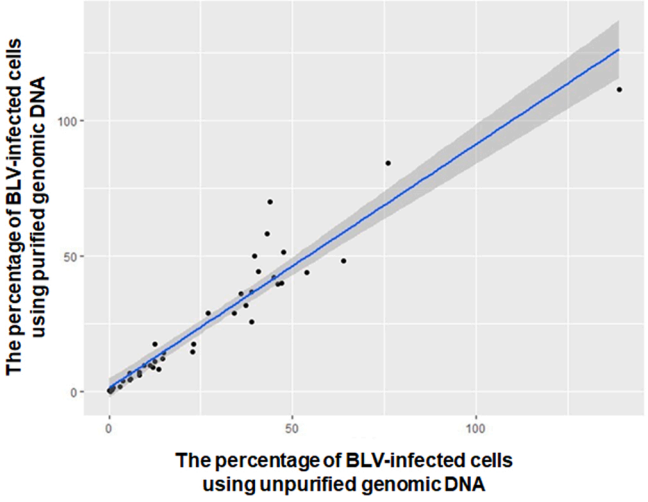

Fig. 3. Correlation of the percentage of BLV-infected cells between unpurified genome DNA and purified DNA results, The x-axis indicates the percentage of BLV-infected cells determined by ddPCR using unpurified genomic DNA and the y-axis indicates the percentage of BLV-infected cells determined by ddPCR using purified genomic DNA. Each dot indicates a single sample. The blue line and shadow indicate the linear model and 95% confidence interval, respectively.

Table 2
LoD of ddPCR using purified DNA.

The percentage of BLV infected cells (%)	12	1.2	0.12	0.012	0.0012	0.00012
The No. of positive wells in ddPCR	3/3	3/3	3/3	3/3	1/3	0/3

Table 3
LoD of ddPCR using unpurified genomic DNA.

Number of BLV Infected cells (cells/mL)	100,000	20,000	4000	800	160	32
The No. of positive wells in ddPCR	3/3	3/3	3/3	3/3	0/3	0/3

Table 4
LoD of ddPCR using amplicon-containing plasmid.

Concentration of BLV targeting DNA (Copies/reaction)	100	20	4	0.8	0.16	0.032
The No. of positive wells in ddPCR	3/3	3/3	3/3	2/3	0/3	0/3

cattle to prevent BLV spread.

BLV-infected cattle having extremely low PVL cannot be detected by even this assay. A relevant example of this is cattle with the BLV-resistant bovine MHC-DRB3 haplotype (*BoLA-DRB3 *009:02*). Previous research indicated such BLV resistance genome-carrying cattle have no or extremely low PVL after BLV infection (Hayashi et al., 2017), which cannot be detected by genetic testing. Another limitation is the possibility of overestimating the BLV PVL when the BLV env and pol in the same provirus are enveloped in different droplets. As shown in Fig. 2, the percentage of infected cells we calculated was similar to that when using a commercial qPCR kit. Because of the low impact of the DNA cleavage effect on the accuracy of the results, we consider it negligible, and avoiding false negatives related to single-target detection in BLV should be prioritized. Although the LoD analysis with suspension cells showed this assay could detect 1.6 infected cells in a 2 μl of sample input in a reaction mixture, the condition of the experiment was a very clean method. Therefore, this result is not equal to what could be detected using blood sample.

In conclusion, we updated the BLV ddPCR method for the quantification of the percentage of BLV provirus with high accuracy and combined this PCR using unpurified genomic DNA preparation using SDS. This method using unpurified genomic DNA might be more practical for the identification of BLV-infected cattle with minimized cost, time, and manpower compared with purification method. Thus, this study accelerates the risk-based control of BLV infection by measuring PVL levels.

Funding

This work was supported by KAKENHI Grant Number JP20K06413 funded by JSPS, and "Regulatory research projects for food safety, animal health and plant protection (JPJ008617. 21453644)" funded by the Ministry of Agriculture, Forestry and Fisheries of Japan, JST Adaptable and Seamless Technology transfer Program through Target-driven R&D (A-STEP) Grant Number JPMJTM22EQ.

CRediT authorship contribution statement

X.W. performed Data curation, and Formal analysis and Writing – original draft. **S.S.** contributed to Conceptualization, Methodology, and Project administration. **X.W. and T.I.,** performed the experiments. **S.S. and K.N.** contributed to Writing – review & editing. **Y. M., J.N., S.M., and H.E.D.** contributed to the commentary and revision of the manuscript.

Declaration of Competing Interest

The authors have no conflicts of interest to declare. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We thank Toshie Iwanaga, Rika Nohara, and Mire Serita for assistance with data collection. We thank Chika Ryu and Yuichi Ushitani for the coordination of sampling. We thank J. Ludovic Croxford, PhD, from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jviromet.2023.114706.

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