Development and evaluation of a point‐of‐care test with a combination of EZ‐Fast DNA extraction and real‐time PCR and LAMP detection: evaluation using blood samples containing the bovine leukaemia DNA

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Article type : Original Article

Development and evaluation of a point-of-care test with a combination of EZ-Fast DNA extraction and real-time PCR and LAMP detection: evaluation using blood samples containing the bovine leukemia DNA

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/LAM.13376

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Running headline: Highly sensitive POCT from blood

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Significance and impact of the study

We developed and evaluated a point-of-care test (POCT) with a combination of new rapid DNA extraction method and real-time PCR or LAMP detection suitable for field settings that does not require advanced laboratory equipment and expensive DNA extraction kits. The developed POCT achieves crude DNA extraction within 10 min at extremely low cost, and high diagnostic performance under combination use of real-time PCR or LAMP analyses. This economical, easy, rapid, highly sensitive and specific POCT promises to provide an important new tool for routine veterinary medicine as well as zoonotic diseases in a field setting.

Abstract

Along with progress in globalization of society, the spread of infectious diseases has accelerated worldwide. The deployment of highly sensitive genetic tests is essential for early diagnosis and early containment of potential outbreaks and epidemics, as well as routine surveillance, however tedious and expensive nucleic acid extraction steps represent a major drawback. Here we developed a simple and rapid DNA extraction method, named as an EZ-Fast kit, applicable to the field setting. The kit does not require

advanced laboratory equipment or expensive DNA extraction kits and achieves crude DNA extraction within 10 min at extremely low cost and can easily be performed in field settings. When combined with real-time PCR and LAMP analyses, the performance of the POCT, using 183 bovine blood samples, was similar to that of the existing DNA extraction method: 92.5% (135/146) (real-time PCR) and 93.7% (133/142) (LAMP) diagnostic sensitivities, and 100% diagnostic specificities. The developed POCT provides a powerful tool to facilitate on-site diagnosis in a field setting.

Keywords: EZ-Fast; Field setting; LAMP; Point-of-care test; POCT; Real-time PCR

Introduction

With progress in globalization, the risk of spreading animal infectious diseases has accelerated worldwide (OIE, 2018b, 2018c; Yamazaki et al, 2019). Further, transboundary animal diseases cause serious global economic damage to livestock and meat industries worldwide (OIE, 2018b, 2018c; Yamazaki *et al*. 2013, 2019). It is therefore important to minimize the spread of pathogens through correct and rapid diagnosis of diseases of humans and animals (Howson et al, 2018). This in turn highlights the necessity for on-site diagnosis in field settings (Almassian, *et al*. 2013; Armson *et al*. 2017; Howson *et al*. 2018; Madi *et al*., 2012).

As a representative POCT (Point-of Care Test), the immunochromatographic assay (ICA) or a lateral flow device (LFD) is widely used for rapid screening of transboundary animal diseases such as avian influenza and foot-and-mouth disease on farms (OIE, 2018b, 2018c). Although POCT such as ICA or LFD is useful for rapid diagnosis of infected animals, for a high number of pathogens, its lack of sensitivity is a major drawback. For example, antigen detection of avian influenza using POCT requires $\geq 10^{4.7}$–$10^{5.7}$ EID_{50} (50% egg-infective dose) of virus per ml, potentially leading to false-negative diagnoses of

recently-infected animals with a low number of pathogens (OIE, 2018b).

Deployment of POCT based on genetic tests such as real-time reverse transcription polymerase chain reaction (RT-PCR) or reverse transcription loop-mediated isothermal amplification (RT-LAMP) for on-site diagnosis in field settings is an attractive option to achieve early diagnosis because of their high diagnostic performance (Almassian, *et al*. 2013; Kurosaki *et al*. 2016; Howson *et al*. 2018). The nucleic acid extraction step requires expensive commercial extraction kits, however, and is therefore a major disadvantage. Therefore, a simple dilution technique with nuclease-free water was applied to successfully detect target pathogens (Howson *et al*. 2018; Waters *et al*. 2014). However, this simple technique decreases limit of detection(LOD) in the range of a 10^1–10^3-fold reduction compared with conventional laboratory nucleic acid extraction methods (Howson *et al*. 2018). Several studies have investigated POCTs with rapid inbuilt extraction steps (Armson et al, 2017; Semper et al, 2016; Madi et al, 2012), however, so far these have been limited by being low throughput.

More sophisticated approaches that minimize false-negative results will enable simple and

sensitive diagnosis. Sodium dodecyl benzenesulfonate (SDBS) is an attractive candidate to develop a simple, rapid, and cost-effective nucleic acid extraction method used in combination with real-time PCR and real-time LAMP amplification (Sekikawa, 2017; Yamazaki *et al*. 2019). SDBS is an analog of sodium dodecyl sulfate (SDS), which provides equivalent performance for DNA extraction from *cryptosporidium* cells with lower inhibitory effects than SDS (Sekikawa, 2017).

Bovine leukemia virus (BLV) has been spread throughout Japan. POCT is required at farms to quickly distinguish between BLV-positive and -negative cattle (Hayashi *et al*. 2017), as well as, at cattle auction markets. The development here should be also applicable to transboundary animal diseases with high needs for POCT. For this purpose, we developed and evaluated a simple and cost-effective POCT through the combination of rapid DNA extraction using SDBS, named as an EZ-Fast kit, and real-time PCR or real-time LAMP amplifications that maintains diagnostic accuracy. Here, we used 183 bovine blood samples containing BLV and evaluated diagnostic performance of the EZ-Fast kit compared with a conventional automated DNA extraction method.

Results and Discussion

The results of the conventional and the EZ-Fast extraction methods were highly consistent. Diagnostic sensitivities and specificities were 92.5% (135/146) and 100% (37/37) for real-time PCR or 93.7% (133/142) and 100% (41/41) for real-time LAMP (Tables 1 and 2). In contrast to the conventional automatic DNA extraction platform, which requires 26 min to complete and costs >$3.50 (USD) for DNA extraction reagents, the EZ-Fast kit required 10 min for crude DNA extraction and costs <$0.10 (USD) (Figure S1). When the LAMP assay was performed with extracted DNA by the EZ-Fast kit, the results of endpoint judgement by the unaided eye and the fluorescent real-time LAMP detection by Genie III were constantly matched with each other, despite the presence of residual blood pigment. A representative result is shown in Figure S1.

Using both the real time PCR and LAMP nucleic amplification methods, the LOD using the EZ-Fast method was lower (>10-fold) than when using the conventional extraction method (Table 3). The real-time PCR showed positive until 10^3-fold dilution using DNAs from both conventional and the EZ-Fast extraction. However, at 10^4-fold dilution, the conventional method yielded one positive and one negative in duplicate analyses, although negative in

the EZ-Fast extraction. Compared with the real-time PCR assay, the real-time LAMP assay yielded five false-negative (Table 1) and one false-positive samples using the conventional method and thirteen false negatives and no false positives samples using the EZ-Fast kit (Tables 1 and 2), which is likely explained by the difference between the LODs of the real-time PCR vs LAMP assays (Table 3).

The average difference in C_T value between the two extraction methods was 2.65. The EZ-Fast kit dilutes blood 1:1 with SDBS, whereas the conventional method inputs 200ul of bovine blood and elutes the DNA in 50ul of nuclease-free water. In DNA extraction using the EZ-Fast kit, it was possible to collect 50ul of the supernatant. However, to avoid the risk of contamination of the supernatant with blood debris, therefore, 20ul of the supernatant was simply collected as a safeguard. Although real-time PCR using the EZ-Fast kit reproducibly amplified target DNA, individual samples may be affected by different inhibitory effects of blood-derived substances (Schrader *et al*. 2012; Wiemels *et al*. 1999; Wilson, 1997). Therefore, a qualitative interpretation should be employed, regardless of C_T values, suggesting that the developed system is unsuitable for quantifying copy number.

Among 183 bovine blood samples, the real-time PCR data for one sample (no. 167) were discrepant, between the two extraction methods (C_T 30.63, automatic extraction and C_T 22.48, EZ-Fast extraction) (Table 4 and Table S1). Considering the possibility of mixing samples, we repeated both DNA extraction methods in duplicate using the blood sample stored at –80 °C. The results were comparable to those of the initial analyses: C_T 30.37 (conventional automatic extraction) and C_T 24.59 (EZ-Fast extraction). We therefore prepared 10-fold serial dilutions of both automatically extracted and crude DNAs using distilled water, and evaluated LODs using the real-time PCR and LAMP assays performed in duplicate (Table 4). In both assays, crude DNA obtained using the EZ-Fast kit achieved 4-5 log10 greater sensitivity compared with the conventional automatic extraction method (Table 4). Although we are unable to explain the reason for this discrepancy, we determined that more target DNA was contained in the EZ-Fast-extracted crude supernatant than in DNA automatically extracted from whole blood.

Compared with SDS, SDBS performs equally well for extracting DNA from *cryptosporidium* cells with reduced inhibitory effects on real-time PCR reactions when the

SDS in the samples is neutralized with 5% Tween 20 (Sekikawa, 2017). Here we were surprised to find that simple heating at 95 °C for 5 min followed by 5-min centrifugation using a portable tabletop centrifuge operated at full speed (1,260–2,840 *g*) was sufficient to obtain crude DNA suitable for real-time PCR and LAMP amplifications, despite significant contamination with blood components. Further, tedious neutralization using a Tween 20 solution was not required for reproducible DNA amplification with both amplification techniques.

EBL caused by BLV infection has been widespread throughout Japan (Hayashi *et al*. 2017; Komiyama *et al*. 2009). POCT needs are therefore high, as example, BLV-positive and -negative cattle are bred separately to prevent BLV-infection in a farm (Hayashi *et al*.2017). Additionally, in some cattle auction markets in Japan, BLV-negative certified cattle by testing win higher prices, which have brought about economic benefits to farmers (Yamazaki, personal communication). Despite of these situations, the collected blood samples need to be brought to the laboratory, due to the lack of a highly sensitive POCT. Eliminating this time lag is a clear benefit to farmers. Furthermore, the developed POCT could be easily applied to on-site diagnosis in a field setting for detecting

pathogens that require detection of DNA in blood, including serum and plasma, such as African swine fever and protozoan diseases.

However, the developed POCT has some limitations. The POCT is qualitative, and therefore unable to perform quantitative analysis of the viral load. Further evaluation is also essential for various RNA viruses, and in clinical samples from different sources such as swab, milk and probang, possibly containing inhibitors. In combination use of EZ-fast kit and LAMP, judgement by the unaided eye through the change of the CFI is possible but not probable due to the influence of the residue of the blood pigment. Therefore, as the safeguard, use of the portable LAMP device such as a Genie III should be prioritised for real-time monitoring rather than use of simple heat-block amplification for end point analysis. Also, use of a portable PCR machine is essential for the evaluation of feasibility of POCT with the combination of EZ-Fast and real time PCR detection with clinical samples in a field setting. Additionally, in ~~In~~ a field setting, concerns regarding the biosecurity of zoonotic pathogens such as anthrax and brucellosis.

In conclusion, EZ-Fast using SDBS is a rapid, simple and cost-effective DNA extraction kit from bovine whole blood. Combination use of real-time PCR or LAMP detection by portable devices could expand potentiality of the POCT for veterinary infectious diseases as well as zoonotic diseases in a field setting.

Materials and Methods

The EZ-Fast kit for on-site diagnosis was developed by combining a portable heat-block and a dry-cell battery-powered tabletop centrifuge, which achieves simple DNA extraction using SDBS. Unpurified DNA was subsequently amplified using a real-time PCR and a portable battery-powered LAMP device (Figure S1). To evaluate the performance of this kit, we compared results with those of the gold standard (conventional method) real-time PCR and LAMP analyses of DNA using an automatic nucleic acid extractor.

Bovine blood samples.

From March 2017 to January 2020, we collected whole blood samples from 183 Japanese Black cattle (*Bos tourus*) from seven cattle farms in Kagoshima, Miyazaki, and Oita prefectures, in the southernmost part of mainland Japan, which is one of the areas with

the highest number of cattle. Using evacuated blood collection tubes containing EDTA, whole blood samples were collected from all breeding cattle and heifers in each farm, kept cool, and delivered to our laboratory where they were stored at 4 °C. DNA was immediately extracted from the bovine blood samples and used for real-time PCR and LAMP analyses ,the remaining samples were stored at –80 °C.

DNA extraction using the EZ-Fast kit.

Each crude DNA sample was prepared as follows: 0.5 ml of bovine blood and 0.5 ml of 1% SDBS aqueous solution (Tokyo Chemical Industry Co., Tokyo, Japan) was mixed in a 1.5-ml microcentrifuge tube by pipetting 5–10 times. The sample tube was placed in a portable heat block (Mini heating dry bath incubator, MD-MINI, Major Science, Co. Ltd., Saratoga, CA, USA), incubated at 95 °C for 5 min, and centrifuged in a dry-cell battery-powered tabletop centrifuge (Puchimaru 8, Wakenbtech, Co., Ltd., Kyoto, Japan) for 5 min at full speed (1,260–2,840 *g*) at room temperature. As the safeguard to avoid contamination by blood debris, a 20-µl sample of the supernatant was transferred to a new 0.2-ml microcentrifuge tube for real-time PCR and LAMP analyses. Special attention was paid to retrieving the very top layer of the supernatant to avoid contamination with solid

blood debris that may contain DNA polymerase inhibitors. The protocol for DNA amplification steps is shown in Figure S1.

DNA extraction using the automated magnetic bead-based extraction system.

For conventional real-time PCR and LAMP analyses, DNA was automatically extracted from the bovine blood samples using an automated magnetic bead-based extraction system (magLEAD 6gC, Precision System Science Co., Matsudo, Japan) with a reagent cartridge magDEA Dx SV (Precision System Science).The bovine blood sample input for extraction was 200ul and the DNA was eluted in 50ul of nuclease-free water.

Real-time PCR.

Using a LightCycler 480 System II (Roche Molecular Systems, Pleasanton, CA, USA) or a QuantStudio 3 (Thermo Fisher Scientific, Inc., Waltham, MA, USA), real-time PCR was performed according to the World Organization for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2018a) based on *pol* sequences reported by Rola-Łuszczak, and colleagues (2013). Reactions were slightly modified using shorter times for denaturation and annealing according to the instructions provided with

the probe qPCR mix (TaKaRa Bio Inc., Otsu, Japan). Briefly, 20 µl of rPCR reaction mixtures comprising 10 µl of 2x probe qPCR mix (TaKaRa Bio), 0.08 µl each of forward and backward primers (100 pmol/µl; Hokkaido System Science, Sapporo, Japan), 0.04 µl of probe (100 pmol/µl; Hokkaido System Science), 7.8 µl of nuclease-free water (TaKaRa Bio), and 2 µl of the DNA template. The cycling conditions were as follows: one cycle at 95 °C for 20 s, 45 cycles each at 95 °C for 5 s, and 60 °C for 30 s. The threshold cycle (C_T) value used as the cut-off was 40.95 according to the OIE Manual (2018a).

Real-time LAMP (Loop-mediated isothermal amplification).

Real-time LAMP was performed using a Genie III (OptiGene, Horsham, UK) according to the LTR sequences reported by Komiyama, *et al*. (2009), originally based on turbidimetric amplification at 63 °C for 60 min, with a slight modification as follows: amplification was performed at 63 °C for 30 min, followed by inactivation at 98 °C for 2 min, and cooling to 80 °C for annealing, with ramping at 0.05 °C/s. The 20-µl LAMP reaction comprised 10 µl of a Loopamp DNA amplification kit (Eiken Chemical, Co., Ltd., Tokyo, Japan) or an in-house LAMP reaction mixture with the identical composition (Yashiki *et al*. 2019); 0.32 µl each of FIP and BIP primers (100 pmol/µl); 0.16 µl each of LF and LB primers (100

pmol/µl); 0.04 µl each of F3 and B3 primers (100 pmol/µl); 0.8 µl of *Bst* polymerase 2.0; 0.8 µl of CFI (Colori- Fluorescent Indicator); 5.36 µl of nuclease-free water; and 2 µl of the DNA template. CFI was added to accelerate the amplification rate and enable analysis of fluorescence, comprising 967 µl of distilled water, 30 µl of 100 mmol/µl hydroxynaphthol blue (MP Biomedicals, Aurora, OH, USA), and 3 µl of Gel Green (x10 000; Biotium, Hayward, CA, USA) (Hayashida *et al*. 2015; Yashiki *et al*. 2019). Primers were produced by Hokkaido System Science (Sapporo, Japan) (column-grade purification). As the threshold definition, the real-time LAMP results were interpreted as positive when the fluorescence intensity reached 10,000 within 30 min of amplification, and the annealing temperature (Ta) ranged between 81.5 °C and 84.5 °C. Time-of-positivity (Tp) was automatically calculated using the Genie III setting. Details of the primers and probes are shown in S-Table 2 (Rola-Łuszczak *et al*. 2013; OIE, 2018a; Komiyama *et al*. 2009). Also, endpoint judgement by the unaided eye was supplementary performed. When the color of the reagent remained purple or dark purple, the result was interpreted as negative. On the other hand, the changes to sky blue (extraction by automated magnetic bead-based extraction system) or green (extracted by EZ-Fast kit) were defined as positive.

LOD (Limit of detection)

The LOD was determined using a BLV DNA-positive blood sample, no. 12 (S-Table 1). Ten-fold serial dilutions of the BLV DNA positive blood sample (no. 12) was prepared into a pool of BLV-DNA-negative samples (nos. 1, 15, 18, 22, 23, 24, 25 and 26), and the real-time PCR and LAMP assays were performed as described above. When a sample showed one positive and one negative in duplicate analyses, the result was interpreted as a positive in both assays.

Acknowledgments

The authors thank Mari Yamamoto for technical assistance. This research was supported by JSPS KAKENHI, Grant Number JP18K05535, JSPS Bilateral Open Partnership Joint Research Projects and AMED under Grant Number JP19jk0210007.

Conflict of Interest Statement

The concept of EZ-Fast is pending for a patent in Japan (Japanese Patent Application no. 2020-98951). The authors declare that they have no other conflict of interest.

All authors contributed to the study conception and design. YY, UTS and KN carried out DNA extraction, real-time LAMP and PCR analyses. HM performed material preparation. WY organized the study. YY and WY wrote the manuscript, and all authors read and approved the final manuscript.

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Table 1. Diagnostic performance of real-time PCR and LAMP using automatically or EZ-Fast-extracted DNAs.

	n	Real-time PCR with EZ-Fast-extracted DNAs	LAMP with automatically extracted DNAs	LAMP with EZ-Fast-extracted DNAs
Real-time PCR-positive with automatically extracted DNAs	146	135/146 (Sen, 92.5%)	141/146 (Sen, 96.6%)	133/146 (Sen, 91.0%)
Real-time PCR-negative with automatically extracted DNAs	37	37/37 (Spe, 100%)	36/37 (Spe, 97.3%)	37/37 (Spe, 100%)

Sen, Diagnostic sensitivity. Spe, Diagnostic specificity.

Table 2. Diagnostic performance of LAMP using automatically and EZ-Fast-extracted DNAs.

	n	LAMP with EZ-Fast-extracted DNAs
LAMP-positive with automatically extracted DNAs	142	133/142 (Sen, 93.7%)
LAMP-negative with automatically extracted DNAs	41	41/41 (Spe, 100%)

Sen, Diagnostic sensitivity. Spe, Diagnostic specificity.

Table 3. Limit of detection of real-time PCR and LAMP assays.

Assays	Dilutions of pooled BLV-positive blood samples					
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Real-time PCR (C_T)						
Automatically extracted DNAs	29.79	32.82	36.78	39.48 †	No. C_T	No. C_T
EZ-Fast-extracted DNAs	33.92	36.54	39.46	No. C_T	No. C_T	No. C_T
LAMP (Tp)						
Automatically extracted DNAs	13:30	14:00	19:07	20:00*	No. Tp	No. Tp
EZ-Fast-extracted DNAs	14:07	16:23	24:45 †	No. Tp	No. Tp	No. Tp

No. C_T, no threshold cycle values detected using real-time PCR. No. Tp, time-of-positivity values undetectable using LAMP.

*, In duplicate analyses, one sample was positive, and one was negative using the LAMP reaction.

Table 4. Limit of detection of a sample-with discrepant results using automatically and EZ-Fast-extracted DNAs (No. 167).

Assays	Dilutions								
	10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}
Real-time PCR (C_T)									
Automatically extracted DNAs	30.37	34.96	37.95	No. C_T	No. C_T	No. C_T	No. C_T	No. C_T	No. C_T
EZ-Fast-extracted DNAs	24.59	26.18	28.12	30.40	33.01	35.90	38.45	No. C_T	No. C_T
LAMP (Tp)									
Automatically extracted DNAs	13:00	17:52	No. Tp	No. Tp	No. Tp	No. Tp	No. Tp	No. Tp	No. Tp
EZ-Fast-extracted DNAs	14:00	15:15	10:00	10:37	12:15	16:00	23:45*	No. Tp	No. Tp

No. C_T, threshold cycle values undetectable using real-time PCR.

No. Tp, time-of-positivity values undetectable using LAMP.

*, In a duplicate analysis, one sample was positive and one was negative using the real-time PCR or LAMP reaction.

Figure S1. Concept of the development of a POCT using a combination of simple DNA extraction with EZ-Fast and a portable battery-powered LAMP device, potentially applicable to a portable real-time PCR device.