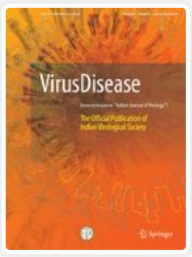

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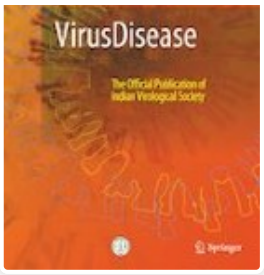

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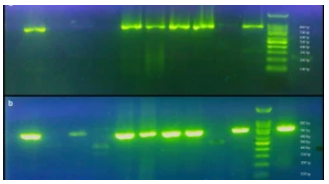

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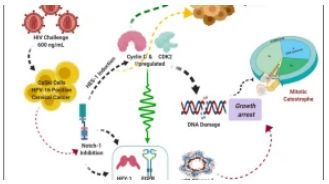

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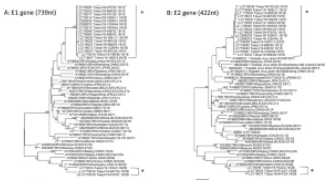

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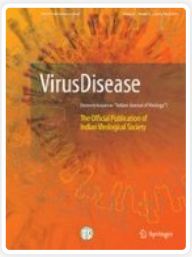

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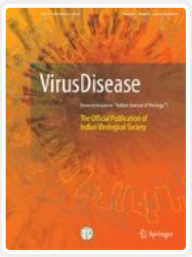

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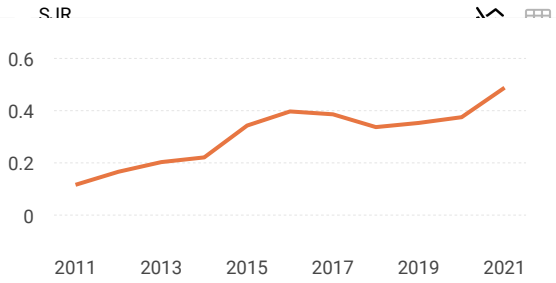

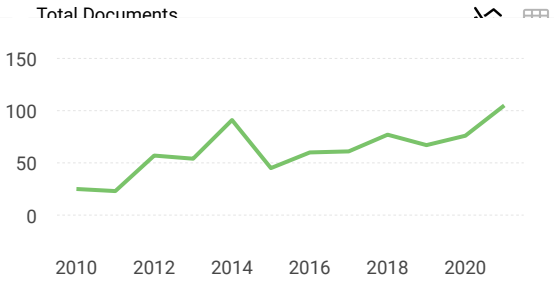

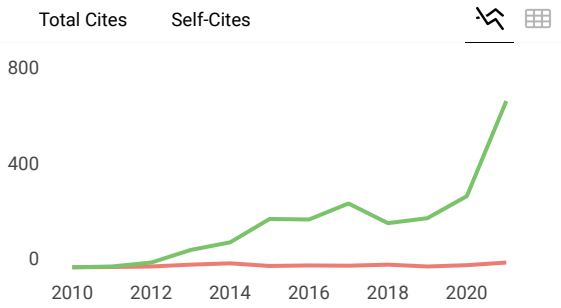

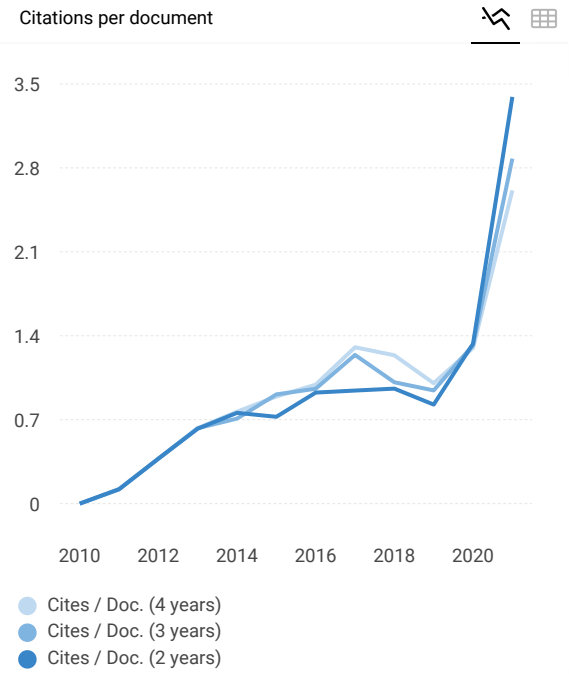

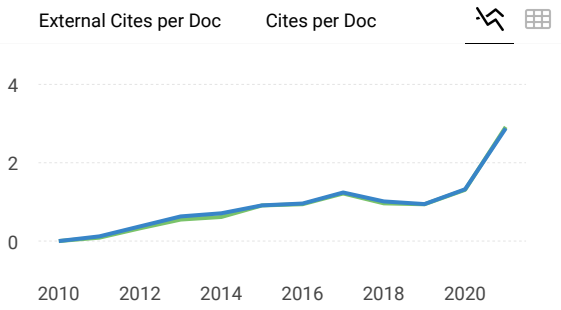

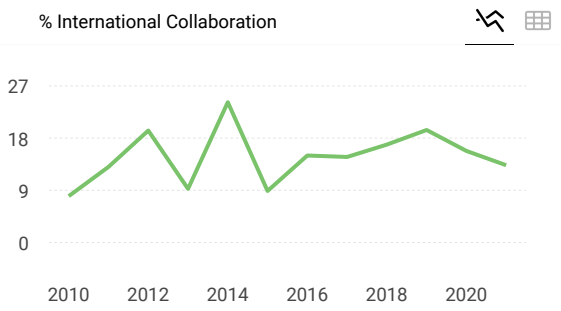

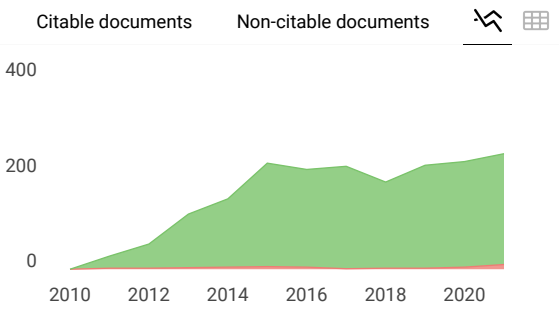

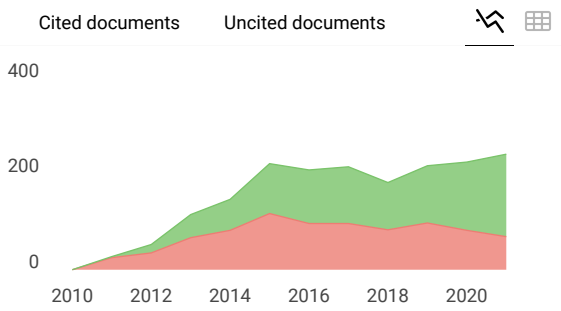

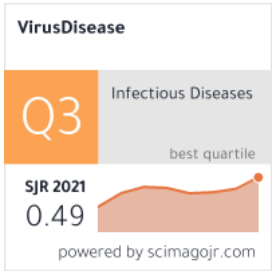

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Use of reverse transcription loop-mediated isothermal amplification combined with lateral flow dipstick for an easy and rapid detection of Jembrana disease virus

Asmarani Kusumawati, Issabellina Dwades Tampubolon, Narendra Yoga Hendarta, Siti Isrina Oktavia Salasia, Tenri Ashari Wanahari, Basofi Ashari Mappakaya & Sri Hartati

VirusDisease **26**, 189–195 (2015)

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Jembrana disease virus (JDV) is a viral pathogen that causes Jembrana disease in Bali cattle (*Bos javanicus*) with high mortality rate. An easy and rapid diagnostic method is essential for further control this disease. We used a reverse transcription loop-mediated isothermal amplification (RT-LAMP) combined with lateral flow dipstick (LFD), based on conserved *tm* subunit of Jembrana disease virus *env* gene. The RT-LAMP conditions were optimized by varying the concentration of $MgSO_4$, betaine, dNTP, and temperature as well as the time and duration of reaction. The primers sensitivity for JDV was confirmed. The method was able to detect *env-tm*

gene dilution which contained 2×10^{-15} g of template. Comparatively, the sensitivity of RT-LAMP/LFD was 100-fold more sensitive than reverse transcription-polymerase chain reaction. The primers specificity for JDV was also confirmed using positive and negative controls. This work also showed that virus detection could be done not only on total RNA extracted from blood but various organs could also be analyzed for the presence of JDV using RT-LAMP/LFD method. The whole process, including the LAMP reaction and the LFD hybridization step only lasts approximately 75 min. Results of analysis can be easily observed with naked eyes without addition of any chemical or further analysis. The combination of RT-LAMP with LFD makes the method a more suitable diagnostic tool in conditions where sophisticated and expensive equipments are not available for field investigations on Jembrana disease in Bali cattle.

References

1. Budiarso IT, Hardjosworo S. Jembrana disease in Bali cattle. Aust Vet J. 1976;52:97.

2. Chadwick BJ, Coelen RJ, Sammels LM, Kertayadnya G, Wilcox GE. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. J Gen Virol. 1995;76:189–92.

3. Chadwick BJ, Coelen RJ, Wilcox GE, Sammels LM, Kertayadnya G. Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. J Gen Virol. 1995;76:1637–50.

4. Chadwick BJ, Desport M, Brownlie J, Wilcox GE, Dharma DM. Detection of Jembrana disease virus in spleen, lymph nodes, bone marrow and other tissues by in situ hybridization of paraffin-embedded sections. J Gen Virol. 1998;79:101–6.

5. Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1997.

6. Desport M, Stewart ME, Sheridan CA, Ditcham WG, Setiyaningsih S, Tenaya WM, Hartaningsih N, Wilcox GE. Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and nonpathogenic bovine lentiviruses. J Virol Methods. 2005;124:135–42.

7. Dharma DM, Ladds PW, Wilcox GE, Campbell RS. Immunopathology of experimental Jembrana disease in Bali cattle. Vet Immunol Immunopathol. 1994;44:31–44.

8. Ding WC, Chen J, Shi YH, Lu XJ, Li MY. Rapid and sensitive detection of infectious spleen and kidney necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Arch Virol. 2010;155:385–9.

9. Hartaningsih N, Wilcox GE, Dharma DM, Soetrisno M. Distribution of Jembrana disease in cattle in Indonesia. Vet Microbiol. 1993;38:23–9.

10. Hartaningsih N, Wilcox GE, Kertayadnya G, Astawa M. Antibody response to Jembrana disease virus in Bali cattle. Vet Microbiol. 1994;39:15–23.

11. Kertayadnya G, Wilcox GE, Soeharsono S, Hartaningsih N, Coelen RJ, Cook RD, Collins E, Brownlie J. Characteristics of a retrovirus associated with Jembrana disease in Bali cattle. J Gen Virol. 1993;74:1765–78.

12. Kiatpathomchai W, Jaroenram W, Arunruta N, Jitrapakdee S, Flegel TW. Shrimp taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J Virol Methods. 2008;153:214–7.

13. Kusumawati A, Pratiwi R, Astuti P, Hamid PH. Characterization of envelope transmembrane gene of Jembrana disease virus Tabanan 1995 isolate. Indo J Biotechnol. 2010;15:15–9.

14. Lu M, Zheng L, Mitchell K, Kapil S, Wood C, Minocha H. Unique epitope of bovine immunodeficiency virus gag protein spans the cleavage site between p16(MA) and p2L. Clin Diagn Lab Immunol. 2002;9:1277–81.

15. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun. 2001;289:150–4.

16. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification reaction of DNA. Nucleic Acids Res. 2000;28:e63.

17. Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-time reverse loop mediated isothermal amplification for rapid detection of West Nile virus. J Clin Microbiol. 2004;42:257–63.

18. Soeharsono S, Hartaningsih N, Soetrisno M, Kertayadnya G, Wilcox GE. Studies of experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. J Comp Pathol. 1990;103:49–59.

19. Soeharsono S, Wilcox GE, Dharma DM, Hartaningsih N, Kertayadnya G, Budiantoro A. Species differences in the reaction of cattle to

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20. Soesanto M, Soeharsono S, Budiantono A, Sulistyana K, Tenaya M, Wilcox GE. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. J Comp Pathol. 1990;103:61–71.

21. Wilcox GE, Chadwick BJ, Kertayadnya G. Recent advances in the understanding of Jembrana disease. Vet Microbiol. 1995;46:249–55.

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Asmarani Kusumawati,✉ Issabellina Dwades Tampubolon, Narendra Yoga Hendarta, Siti Isrina Oktavia Salasia, Tenri Ashari Wanahari, Basofi Ashari Mappakaya, and Sri Hartati

Abstract

Jembrana disease virus (JDV) is a viral pathogen that causes Jembrana disease in Bali cattle (*Bos javanicus*) with high mortality rate. An easy and rapid diagnostic method is essential for further control this disease. We used a reverse transcription loop-mediated isothermal amplification (RT-LAMP) combined with lateral flow dipstick (LFD), based on conserved *tm* subunit of Jembrana disease virus *env* gene. The RT-LAMP conditions were optimized by varying the concentration of $MgSO_4$, betaine, dNTP, and temperature as well as the time and duration of reaction. The primers sensitivity for JDV was confirmed. The method was able to detect *env-tm* gene dilution which contained 2×10^{-15} g of template. Comparatively, the sensitivity of RT-LAMP/LFD was 100-fold more sensitive than reverse transcription-polymerase chain reaction. The primers specificity for JDV was also confirmed using positive and negative controls. This work also showed that virus detection could be done not only on total RNA extracted from blood but various organs could also be analyzed for the presence of JDV using RT-LAMP/LFD method. The whole process, including the LAMP reaction and the LFD hybridization step only lasts approximately 75 min. Results of analysis can be easily observed with naked eyes without addition of any chemical or further analysis. The combination of RT-LAMP with LFD makes the method a more suitable diagnostic tool in conditions where sophisticated and expensive equipments are not available for field investigations on Jembrana disease in Bali cattle.

Keywords: Bovine disease, Env gene, Jembrana disease virus, Lateral flow immunoassay, Loop mediated isothermal amplification

Introduction

Jembrana disease was recognized for the first time in 1964 as an acute and infectious disease of Bali cattle (*Bos javanicus*) in the Jembrana district of Bali island in Indonesia [1]. The disease was endemic through-out parts of Indonesia, including Java, Sumatra [9], and also Australia [4]. Jembrana disease virus (JDV), belongs to the lentivirus family of retrovirus [2, 11]. Genetically and antigenically, the most closely related to JDV is bovine immunodeficiency virus (BIV) although the respective associated disease is quite different [2]. Jembrana disease constitutes the main concern in Bali cattle industry due to the high mortality of the infected cattle. In experimentally infected Bali cattle, the mortality rate was about 17 % [20]. This was consistent

with data obtained from field observations [4]. JDV infection causes an acute febrile illness in infected Bali cattle, with a short incubation time and duration, and a high mortality rate, attributed to multisystem involvement [21]. This is in contrast to the chronic and progressive diseases over a long incubation periods typically associated with most lentiviral infections [5]. During the febrile stage of the disease, a high titre of viral particles is found in the plasma fraction of the blood [18, 20]. Viruses are also detected in secreted fluids, i.e. saliva, milk, nasal discharge. The sampling of biological materials that can be used for virus detection in routine health controls, is so made easier. Various organs are also infected, i.e. spleen, lymph nodes, lungs, bone marrow, liver and kidney [4].

Due to the disastrous effects of Jembrana disease on Bali cattle industry and also for lack of efficient medical treatments, it is essential that JDV infection can be diagnosed as early as possible to prevent the disease spread. Among the different diagnostic tools developed up to now, in situ hybridization is certainly the most cumbersome to perform and therefore cannot be routinely used. A simpler dot-blot hybridization can instead be used as it is unexpensive and several samples can be simultaneously processed. Nevertheless, in situ hybridization has allowed to determine the JDV distribution in tissues and cells. Due to the high virus titre in blood during the acute phase, viral genome amplification methodology, i.e. quantitiave-reverse transcription-polymerase chain reaction (q-RT-PCR), is ideal detection tool among the up to date developed diagnostic tools. It allows to accurately quantify the viral particles and to monitor the disease progression. However methods such as q-RT-PCR is not always applicable as it requires a high technical skill and expensive equipments. Though less sensitive, standard RT-PCR can instead be used for routine health controls. Other molecular diagnostic methods may be developed such as the more recently developed “loop mediated isothermal amplification” or LAMP [15–17]. LAMP methodology is now currently used for the detection of various pathogens [8, 12, 17]. The method is based on the high strand displacement activity of the polymerase used, allowing amplification reactions to be carried out at a single temperature [16, 17]. It allows the amplification of DNA (LAMP) as well as RNA (RT-LAMP). LAMP exhibits a high sensitivity and specificity due to the use of six primers which recognize eight distinct regions of the target [16, 17]. It is a rapid analysis method which is easy to perform and does not require expensive equipments or high technical skill. As the reaction occurs at a unique temperature, a simple water bath, a heating block or any heating device can be used. Its combination with an additional hybridization step using lateral flow dipstick (LFD) method renders the method even easier to perform as both the amplification and the hybridization steps do not require sophisticated and expensive equipments and results can be observed with naked eyes by the appearance or absence of grey line on LFD sticks [8, 12]. This makes the method ideal for in field routine health controls.

This report describes the applicability of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) combined with LFD (RT-LAMP/LFD), based on *tm* subunit of JDV *env* gene (*env-tm* gene), as a diagnostic tool of Jembrana disease.

Materials and methods

Samples and RNA extraction

50–100 mg of various organs were taken from healthy and experimentally JDV-infected cattle (obtained from BPPV [Center of Veterinary Investigations], Denpasar, Bali, Indonesia) and blood from suspected cattle was collected from Barambai region, Kalimantan (Borneo), Indonesia. Organs were first homogenized in PBS (phosphate buffer saline, pH 7.2) and total RNAs were then extracted using High Pure Viral Nucleid Acid Kit (Roche®). Total RNAs from 200 ul blood were directly extracted using the same extraction kit. Total RNAs were then eluted in 50 µl sterile distilled water. 1 µl of this RNA solution contained so the RNA content of 1–2 mg of organs or 4 µl of blood.

LAMP primers and probe

The primers used for LAMP and the probe for LFD were designed from *env-tm* gene, using the program "Primer Explorer" (LAMP primer designing support software program: http://primerexplorer.jp/e/), from the published JDV genome sequence (Acc. no. U21603; version U21603.1 GI: 733067; www.ncbi.nlm.nih.gov/nucleotide/) [2, 3] and synthetized by "1st Base Custom Oligos" (Singapore).

LAMP/LFD and one step RT-LAMP/LFD

Due to the use of *Bsm* Polymerase, instead of *Bst* polymerase, optimal conditions of LAMP reactions were beforehand defined by varying the concentration of $MgSO_4$, betaine, dNTP, and temperature as well as the time and duration of reaction, using 20 ng of *tm-env* gene cloned in pGEX (pGEX-*tm*) as template. The defined optimal conditions were as follows: reactions in 25 µl of reaction buffer, containing 1.6 µM primers FIP and BIP each, 0.2 µM F3 and B3 each, 1 µM dNTPs mix, 1 M betaine, 10 mM $MgSO_4$, 8 u *Bsm* DNA polymerase (Fermentas, Life Science Fermentas, Life Science), and variable amounts of template, at 61 °C for 60 min. Reactions were ended by increasing the temperature to 80 °C for 4 min. When RNA template was used 5 unit reverse transcriptase and 40 unit protector RNAse inhibitor were added in the reaction mixture. The reverse transcription and LAMP reactions occurred in the same reaction tube. One half of the amplification products was analyzed by electrophoresis on a 1.8 % agarose gel and the other half was used for hybridization, using LFD method, to determine further whether the LAMP amplified products actually corresponded with the target. LFD hybridization was performed by incubating LAMP products with 30 pmol of 6-Fam-labeled probe at 61 °C for 10 min, in a final volume of 20 µl. 10 µl of the hybridization products were then added to 100 µl of PBS. LFD sticks (Milenia Biotec, Germany) were then dipped into the mixture of probe-LAMP products for 5 min at room temperature. Analysis was considered positive if two lines appeared on the stick, one corresponding to the control (CL) and one to the positive hybridization of the LAMP products with the probe/test line (TL).

PCR

PCR was carried out in 25 µl reaction buffer, with various templates and using LAMP outer primers B3 and F3 (0.5 µM each), specific to JDV *env-tm* gene and 1× DreamTaq™Green PCR Master Mix (Fermentas, Life Science Fermentas, Life Science) with the following condition: prior denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at

56 °C for 30 s, elongation at 72 °C for 45 s then additional incubation at 72 °C for 10 min and finally samples were kept at 4 °C till analysis. Half of the reaction products were analyzed by electrophoresis on a 1.8 % agarose gel.

Results

LAMP primers and probe

For the detection of JDV, the designed LAMP primers and the probe were specific to the subunit *tm* of *env* gene (*env-tm*). Their nucleotide sequence, orientation as well as their localization within the gene are shown in Fig. 1. The inner primers FIP and BIP are composed of 2 oligonucleotides with opposite orientations, linked by two T residues (Fig. 1). FIP consists of F1 (in anti-sense orientation), linked at its 3′-end by 2 T residues to the 5′-end of F2 (in sense orientation). BIP is made up of B1 (in sense orientation), linked at its 3′-end to the 5′-end of B2 (in anti-sense orientation) by 2 T residues. The probe is included within the LAMP-amplified fragment.

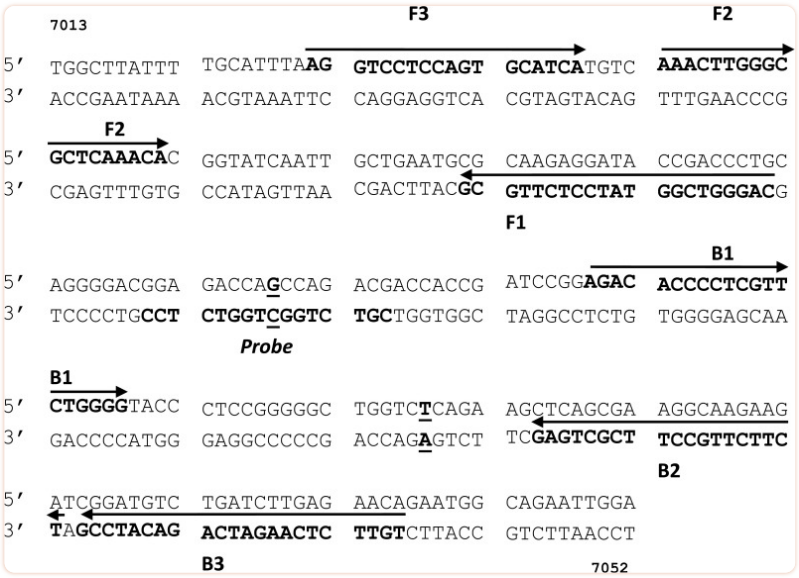

Fig. 1

Nucleotide sequence, localization and orientation of RT-LAMP primers and probe on the cDNA of *env-tm* JDV genome. *Env-tm*, localized between nucleotides 6463 and 7542 within the JDV genome, is a subgene of *env* gene which spans from nucleotide 5197 to 7542. The nucleotide sequence shown in fugure 1 spans from nucleotide 7013 to 7252 of the JDV genome. The primers and the probe are in bold. F1 (in anti-sense orientation) is linked at its 3′-end to the 5′-end of F2 (in sense orientation) by 2 T residues to form the inner primer FIP while B1 (in sense orientation) is linked at its 3′-end to the 5′-end of B2 (in anti-sense orientation) by 2 T residues to form the inner primer BIP. FIP was biotin-labeled at its 5′-end and the probe was labeled with 6-Carboxyfluorescein (6-Fam) at its 5′-end

Optimal conditions of LAMP

Optimal conditions of LAMP reactions were defined by varying the concentration of $MgSO_4$, betaine and dNTP, the temperature as well as the time and duration of reaction, using 20 ng of *tm-env* gene cloned in pGEX (pGEX-*tm*) as template. The optimal conditions, described in Materials and Methods, were consequently used for all LAMP or RT-LAMP reactions.

The sensitivity and specificity of LAMP/LFD

The sensitivity of LAMP/LFD was evaluated by using serial 10-fold dilutions of pGEX-*tm*, ranging from 2 ng (dilution 1) to 0.002 fg (dilution 10). It was compared to that obtained for LAMP alone and for PCR using primers F3 and B3. As shown in Fig. 2, LAMP, PCR and LAMP/LFD allowed to detect the gene target till respectively dilutions 8, 5 and 7. These dilutions corresponded respectively to the initial amounts of 0.2, 200 and 2 fg of pGEX-*tm* in 25 µl of LAMP or PCR reaction. PCR allowed to see a clearly observable positive band till dilution 5. By attentively examining the electrophoretic analysis, it seems however that a faint band is still observable at dilutions 6 and 7 in PCR results. As shown in Fig. 2c, LAMP/LFD was able to detect *env-tm* gene till dilution 7 which contained 2 fg of template. Considering the length of pGEX-*tm* (ca. 6000 bp), its molecular mass and the Avogadro constant, 2 fg of pGEX-*tm* corresponds to ca. 170 molecules of the gene target. As only half of LAMP reactions was analyzed further by LFD, the detection threshold of LAMP/LFD was therefore ca. 85 molecules of the gene target in the experimental conditions we used. Comparatively, the sensitivity of LAMP alone was 10-fold higher (Fig. 2a) but LAMP/LFD was 100-fold more sensitive than PCR (Fig. 2b). LAMP alone allowed the detection of as few as ca. 8 molecules of the target. The detection threshold of LAMP/LFD was however more than sufficient when the method was applied to sampled organs or blood.

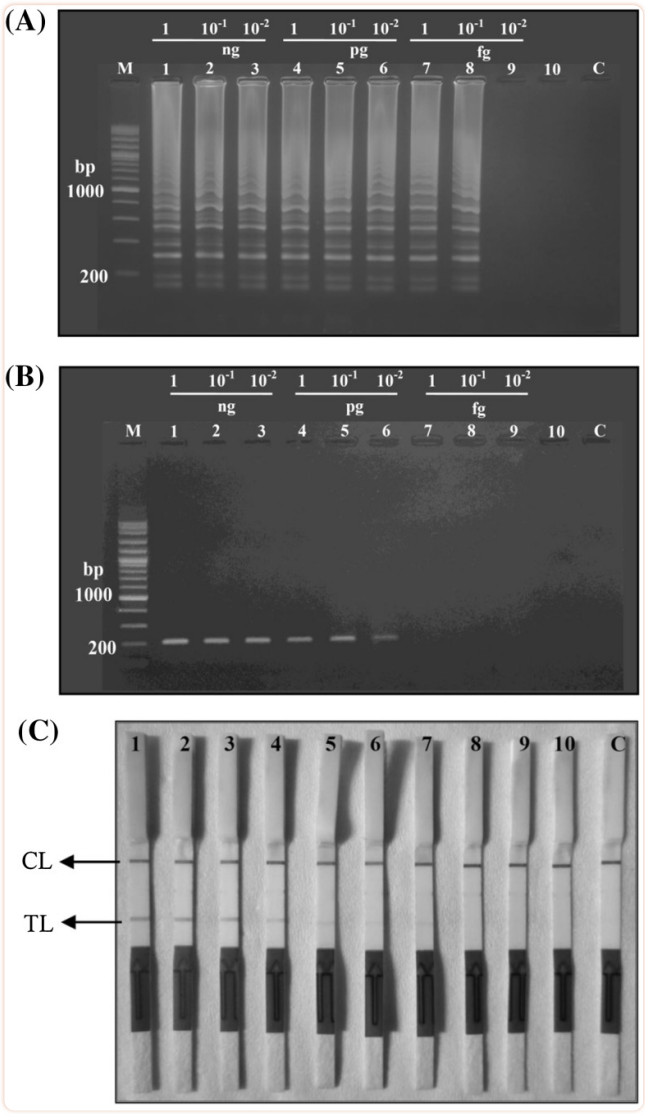

Fig. 2

Comparative sensitivity of LAMP, PCR and LAMP/LFD. Serial 10-fold dilutions (1–10) of pGEX-*tm*, ranging from 2 ng to 0.002 fg of DNA. Electrophoresis on a 1.8 % agarose gel of half of LAMP (**a**) and PCR reaction products (**b**) and further analysis by LFD of the other half of LAMP products (**c**)

The specificity of LAMP primers for JDV was tested against positive and negative controls. As positive control, we used *env-tm* gene cloned in pGEX (pGEX-*tm*) which has been identified by sequencing (Fig. 3; lane 1), and total RNA extracted from blood and organs of JDV-infected Bali cattle (Fig. 4), as templates. As negative control, total RNA extracted from blood of JDV non-infected Bali cattle and Ongole cattle (a crossbreed between a male *Bos indicus* and a female Javanese cattle) (Fig. 3; lane 2 and lane 3). As shown in Fig. 3, sole pGEX-*tm* gave a positive response, following analysis of LAMP products by electrophoresis on agarose gel (Fig. 3a). LFD analysis, using the probe specific to part of LAMP amplified fragment, confirmed LAMP amplification and the amplicon identity (Fig. 3b).

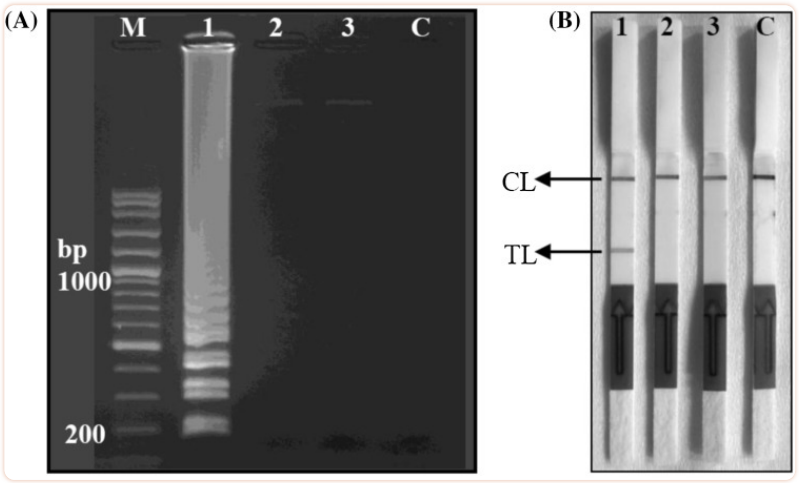

Fig. 3

Specificity of RT-LAMP and RT-LAMP/LFD. Analysis by electrophoresis on a 1.8 % agarose gel of LAMP or RT-LAMP products (**a**) and further analysis by LFD (**b**). LAMP reactions gave the characteristic ladder-pattern of bands. *Lane 1* pGEX-*tm*, *lane 2* healthy Bali cattle, *lane 3* PO cattle, *M* DNA marker, *C* water, *CL* control line, *TL* test line

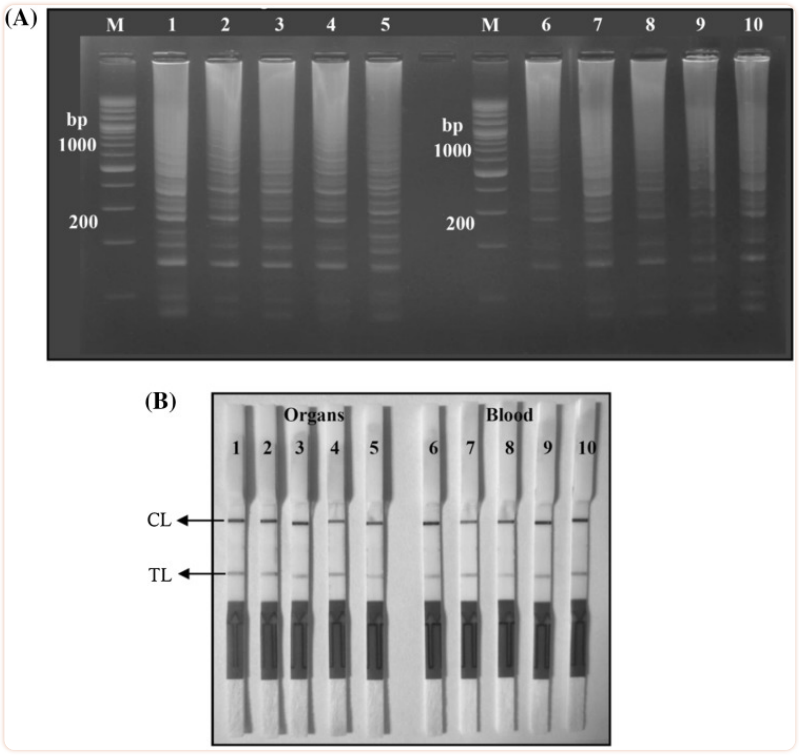

Fig. 4

Application of LAMP/LFD to organs and blood of JDV-infected Bali cattle. One step RT-LAMP on total RNA extracted from organs (1–5) or from blood (6–10) and analysis by electrophoresis on a 1.8 % agarose gel (**a**) and further by LFD (**b**). *Lanes 1, 2* spleen, *lane 3* hearth, *lane 4* lung, *lane 5* tongue, *lanes 6–10* blood, *M* DNA markers, *CL* control line, *TL* test line

Application of LAMP/LFD to sampled organs and blood

The application of the method was carried out by one-step RT-LAMP on total RNA extracted from different organs of experimentally JDV-infected Bali cattle and from selected blood samples of Bali cattle which were suspected to suffer from Jembrana disease. Sample selection was done by RT-PCR and only positive samples (data not shown) were used. As shown in Fig. 4a, b both in analysis by electrophoresis on agarose gel and further hybridization with 6-FAM-labeled *env-tm*-specific probe, all the sampled organs and blood gave a positive response. It turned out therefore that RT-LAMP as well as RT-LAMP/LFD can be used for the detection of JDV present in organs or blood. In this experiment, RT-LAMP was performed on total RNA corresponding to the RNA content of 2–4 mg of organs or 8 µl of blood. As only half of the RT-LAMP products was used in hybridization using LFD method, 1–2 mg of organs or 4 µl of blood were largely sufficient for JDV detection. Only a small amount of initial materials was thus required for analysis by RT-LAMP/LFD.

Discussion

This paper describes the applicability of RT-LAMP and RT-LAMP/LFD for the detection of the pathogenic agent of Jembrana disease. Due to the high mortality rate of infected cattle and the consequent economic losses, it is essential that JDV infection can be diagnosed as early as possible to limit the disease spread. Immunodiagnosis based on host humoral response can not be used in early stages of the disease as like the other lentiviruses, JDV infection induces a delayed humoral response and JDV-specific antibodies are not produced in most infected cattle untill 11 weeks post infection [7, 10]. Furthermore, antibody-based diagnostic methods do not enable to distinguish JDV- from BIV-infection as the two bovine lentiviruses are antigenically very closely related [6, 10]. Distinguishing BIV-infection was only made feasible by using a BIV-specific monoclonal antibody that only recognizes the unique BIV GAG epitope, which is not shared by JDV [14]. During the acute phase, high titre of infectious JDV viral particles is found in plasma [18, 20]. Viruses are also abundantly present in secreted fluids, namely milk and saliva. This make viral antigen identification by molecular method is ideal detection tool in order to detect viral infection as early as possible during the course of the disease.

The RT-LAMP/LFD we used proved to be fast, easy to perform, specific and highly sensitive (Figs. 2, 3). It does not require sophisticated and expensive devices for both the amplification and hybridization steps. Using cloned *env-tm* subunit gene, RT-LAMP/LFD allowed the detection of as few as 80 molecules of the gene target. Positive results can be easily and immediately observed by the appearance of grey lines on the LFD stick. This avoids the analysis step by electrophoresis on agarose gel and eliminates the need to handle carcinogenic ethidium bromide.

The RT-LAMP/LFD we used proved to be 100-fold more sensitive than RT-PCR (Fig. 2). Besides, the additional hybridization process by LFD method also enables to confirm the amplicon identity. The whole process, including the LAMP reaction and the hybridization step only lasts ca. 75 min. The sensitivity of RT-LAMP/LFD is more than sufficient as for its application to the analysis of sampled organs or blood, only 1–2 mg of organs and 4 ul of whole blood were needed and less initial materials can still be used. This work also showed that virus detection could be done not only on total RNA extracted from blood but various organs could also be analyzed for the presence of JDV.

The specificity of RT-LAMP/LFD method was established using positive and negative samples. As positive samples, we used *env-tm* gene cloned in pGEX (pGEX-*tm*) and total RNA extracted from blood and various organs of JDV-infected Bali cattle. As negative samples, we used total RNA extracted from blood JDV non-infected Bali cattle and Ongole cattle. It is worthwhile mentioning that JDV infections of Ongole cattle only induce mild clinical signs and the infectious agents persist for less than 9 months [18, 19]. Therefore the diagnosed animal may be healthy, or too few viruses are found in the sampled blood, or they have been rapidly eliminated. For checking the cross reactivity of the primers with the related virus (e.g. BIV), a meticulous search of possible sequence homology between the nucleotide sequence of *env-tm* region (LAMP-amplified region) with any of the nucleotide sequences published in GenBank, including the entire genome of BIV (including *env* region of BIV) has conducted. It did not reveal significant degrees of homology with any of the nucleotide sequences published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). More importantly, no sequence homology is found in *env-tm* subunit gene of the genome of BIV which is genetically the most related to JDV (data not shown). Reasonably, this will avoid cross reactions and allow to distinguish JDV- from BIV-infection. Besides, the additional step "hybridization-LFD" was also reasonably play an essential control role as it ensures that the JDV *env-tm* fragment (LAMP-amplified fragment) is actually the chosen part of JDV gene target. Furthermore, the choice of *env-tm* subunit gene as target was determined by the fact that this part of JDV genome is highly conserved among the strains or isolates so far studied [13]. Sequence comparison of *env-tm* gene of 8 Indonesian JDV isolates, using bioinformatic analyst (BLASTN), showed a very high degree of conservation among the 8 JDV isolates studied [13], rendering so this part of the genome an ideal target for amplification of all JDV strains or isolates. Only one variation, at position 666, was found within the oligonucleotide probe and a mismatch at this position will not prevent hybridization to take place. Another variation, at position 726, is not comprised in any of the designed LAMP primers (Fig. 1).

LAMP primers proved so to be specific to *env-tm* subunit gene of JDV genome. LFD analysis, using the probe specific to part of LAMP amplified fragment, confirmed further the specificity of LAMP amplification and the amplicon identity (Fig. 3b). The amplification of JDV *gag*-ca gene also resulted in negative response (data not shown). The combination of RT-LAMP with LFD makes the method a more suitable diagnostic tool in conditions where sophisticated and expensive equipments are not available for field investigations on Jembrana disease in Bali cattle. Thus RT-LAMP and RT-LAMP/LFD both can be potentially used for routine monitoring of JDV infections.

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References

1. Budiarso IT, Hardjosworo S. Jembrana disease in Bali cattle. *Aust Vet J.* 1976;52:97. doi: 10.1111/j.1751-0813.1976.tb13867.x. [PubMed] [CrossRef] [Google Scholar]

2. Chadwick BJ, Coelen RJ, Sammels LM, Kertayadnya G, Wilcox GE. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. *J Gen Virol.* 1995;76:189–192. doi: 10.1099/0022-1317-76-1-189. [PubMed] [CrossRef] [Google Scholar]

3. Chadwick BJ, Coelen RJ, Wilcox GE, Sammels LM, Kertayadnya G. Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. *J Gen Virol.* 1995;76:1637–1650. doi: 10.1099/0022-1317-76-7-1637. [PubMed] [CrossRef] [Google Scholar]

4. Chadwick BJ, Desport M, Brownlie J, Wilcox GE, Dharma DM. Detection of Jembrana disease virus in spleen, lymph nodes, bone marrow and other tissues by in situ hybridization of paraffin-embedded sections. *J Gen Virol.* 1998;79:101–106. doi: 10.1099/0022-1317-79-1-101. [PubMed] [CrossRef] [Google Scholar]

5. Coffin JM, Hughes SH, Varmus HE, editors. *Retroviruses.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1997. [Google Scholar]

6. Desport M, Stewart ME, Sheridan CA, Ditcham WG, Setiyaningsih S, Tenaya WM, Hartaningsih N, Wilcox GE. Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and nonpathogenic bovine lentiviruses. *J Virol Methods.* 2005;124:135–142. doi: 10.1016/j.jviromet.2004.11.015. [PubMed] [CrossRef] [Google Scholar]

7. Dharma DM, Ladds PW, Wilcox GE, Campbell RS. Immunopathology of experimental Jembrana disease in Bali cattle. *Vet Immunol Immunopathol.* 1994;44:31–44. doi: 10.1016/0165-2427(94)90167-8. [PubMed] [CrossRef] [Google Scholar]

8. Ding WC, Chen J, Shi YH, Lu XJ, Li MY. Rapid and sensitive detection of infectious spleen and kidney necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. *Arch Virol.* 2010;155:385–389. doi: 10.1007/s00705-010-0593-4. [PubMed] [CrossRef] [Google Scholar]

9. Hartaningsih N, Wilcox GE, Dharma DM, Soetrisno M. Distribution of Jembrana disease in cattle in Indonesia. *Vet Microbiol.* 1993;38:23–29. doi: 10.1016/0378-1135(93)90072-F. [PubMed] [CrossRef] [Google Scholar]

10. Hartaningsih N, Wilcox GE, Kertayadnya G, Astawa M. Antibody response to Jembrana disease virus in Bali cattle. *Vet Microbiol.* 1994;39:15–23. doi: 10.1016/0378-1135(94)90082-5. [PubMed] [CrossRef] [Google Scholar]

11. Kertayadnya G, Wilcox GE, Soeharsono S, Hartaningsih N, Coelen RJ, Cook RD, Collins E, Brownlie J. Characteristics of a retrovirus associated with Jembrana disease in Bali cattle. *J Gen Virol.* 1993;74:1765–1778. doi: 10.1099/0022-1317-74-9-1765. [PubMed] [CrossRef] [Google Scholar]

12. Kiatpathomchai W, Jaroenram W, Arunruta N, Jitrapakdee S, Flegel TW. Shrimp taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. *J Virol Methods.* 2008;153:214–217. doi: 10.1016/j.jviromet.2008.06.025. [PubMed] [CrossRef] [Google Scholar]

13. Kusumawati A, Pratiwi R, Astuti P, Hamid PH. Characterization of envelope transmembrane gene of Jembrana disease virus Tabanan 1995 isolate. *Indo J Biotechnol.* 2010;15:15–19. [Google Scholar]

14. Lu M, Zheng L, Mitchell K, Kapil S, Wood C, Minocha H. Unique epitope of bovine immunodeficiency virus gag protein spans the cleavage site between p16(MA) and p2L. *Clin Diagn Lab Immunol.* 2002;9:1277–1281. [PMC free article] [PubMed] [Google Scholar]

15. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. *Biochem Biophys Res Commun.* 2001;289:150–154. doi: 10.1006/bbrc.2001.5921. [PubMed] [CrossRef] [Google Scholar]

16. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification reaction of DNA. *Nucleic Acids Res.* 2000;28:e63. doi: 10.1093/nar/28.12.e63. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

17. Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-time reverse loop mediated isothermal amplification for rapid detection of West Nile virus. *J Clin Microbiol.* 2004;42:257–263. doi: 10.1128/JCM.42.1.257-263.2004. [PMC free article] [PubMed] [CrossRef] [Google Scholar]

18. Soeharsono S, Hartaningsih N, Soetrisno M, Kertayadnya G, Wilcox GE. Studies of experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. *J Comp Pathol.* 1990;103:49–59. doi: 10.1016/S0021-9975(08)80134-X. [PubMed] [CrossRef] [Google Scholar]

19. Soeharsono S, Wilcox GE, Dharma DM, Hartaningsih N, Kertayadnya G, Budiantoro A. Species differences in the reaction of cattle to Jembrana disease virus infection. *J Comp Pathol.* 1995;112:391–402. doi: 10.1016/S0021-9975(05)80020-9. [PubMed] [CrossRef] [Google Scholar]

20. Soesanto M, Soeharsono S, Budiantono A, Sulistyana K, Tenaya M, Wilcox GE. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. *J Comp Pathol.* 1990;103:61–71. doi: 10.1016/S0021-9975(08)80135-1. [PubMed] [CrossRef] [Google Scholar]

21. Wilcox GE, Chadwick BJ, Kertayadnya G. Recent advances in the understanding of Jembrana disease. *Vet Microbiol.* 1995;46:249–255. doi: 10.1016/0378-1135(95)00089-S. [PubMed] [CrossRef] [Google Scholar]

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ORIGINAL ARTICLE

Use of reverse transcription loop-mediated isothermal amplification combined with lateral flow dipstick for an easy and rapid detection of Jembrana disease virus

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Abstract Jembrana disease virus (JDV) is a viral pathogen that causes Jembrana disease in Bali cattle (*Bos javanicus*) with high mortality rate. An easy and rapid diagnostic method is essential for further control this disease. We used a reverse transcription loop-mediated isothermal amplification (RT-LAMP) combined with lateral flow dipstick (LFD), based on conserved *tm* subunit of Jembrana disease virus *env* gene. The RT-LAMP conditions were optimized by varying the concentration of $MgSO_4$, betaine, dNTP, and temperature as well as the time and duration of reaction. The primers sensitivity for JDV was confirmed. The method was able to detect *env-tm* gene dilution which contained 2×10^{-15} g of template. Comparatively, the sensitivity of RT-LAMP/LFD was 100-fold more sensitive than reverse transcription-polymerase chain reaction. The primers specificity for JDV was also confirmed using positive and negative controls. This work also showed that virus detection could be done not only on total RNA extracted from blood but various organs could also be analyzed for the presence of JDV using RT-LAMP/LFD method. The whole process, including the LAMP reaction and the LFD hybridization step only lasts approximately 75 min. Results of analysis can be easily observed with naked eyes without addition of any chemical or further analysis. The combination of RT-LAMP with LFD makes the method a more suitable diagnostic tool in conditions where sophisticated and expensive equipments are not available for field investigations on Jembrana disease in Bali cattle.

Keywords Bovine disease · Env gene · Jembrana disease virus · Lateral flow immunoassay · Loop mediated isothermal amplification

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Introduction

Jembrana disease was recognized for the first time in 1964 as an acute and infectious disease of Bali cattle (*Bos javanicus*) in the Jembrana district of Bali island in Indonesia [1]. The disease was endemic through-out parts of Indonesia, including Java, Sumatra [9], and also Australia [4]. Jembrana disease virus (JDV), belongs to the lentivirus family of retrovirus [2, 11]. Genetically and antigenically, the most closely related to JDV is bovine immunodeficiency virus (BIV) although the respective associated disease is quite different [2]. Jembrana disease constitutes the main concern in Bali cattle industry due to the high mortality of the infected cattle. In experimentally infected Bali cattle, the mortality rate was about 17 % [20]. This was consistent with data obtained from field observations [4]. JDV infection causes an acute febrile illness in infected Bali cattle, with a short incubation time and

duration, and a high mortality rate, attributed to multisystem involvement [21]. This is in contrast to the chronic and progressive diseases over a long incubation periods typically associated with most lentiviral infections [5]. During the febrile stage of the disease, a high titre of viral particles is found in the plasma fraction of the blood [18, 20]. Viruses are also detected in secreted fluids, i.e. saliva, milk, nasal discharge. The sampling of biological materials that can be used for virus detection in routine health controls, is so made easier. Various organs are also infected, i.e. spleen, lymph nodes, lungs, bone marrow, liver and kidney [4].

Due to the disastrous effects of Jembrana disease on Bali cattle industry and also for lack of efficient medical treatments, it is essential that JDV infection can be diagnosed as early as possible to prevent the disease spread. Among the different diagnostic tools developed up to now, in situ hybridization is certainly the most cumbersome to perform and therefore cannot be routinely used. A simpler dot-blot hybridization can instead be used as it is unexpensive and several samples can be simultaneously processed. Nevertheless, in situ hybridization has allowed to determine the JDV distribution in tissues and cells. Due to the high virus titre in blood during the acute phase, viral genome amplification methodology, i.e. quantitiave-reverse transcription-polymerase chain reaction (q-RT-PCR), is ideal detection tool among the up to date developed diagnostic tools. It allows to accurately quantify the viral particles and to monitor the disease progression. However methods such as q-RT-PCR is not always applicable as it requires a high technical skill and expensive equipments. Though less sensitive, standard RT-PCR can instead be used for routine health controls. Other molecular diagnostic methods may be developed such as the more recently developed "loop mediated isothermal amplification" or LAMP [15–17]. LAMP methodology is now currently used for the detection of various pathogens [8, 12, 17]. The method is based on the high strand displacement activity of the polymerase used, allowing amplification reactions to be carried out at a single temperature [16, 17]. It allows the amplification of DNA (LAMP) as well as RNA (RT-LAMP). LAMP exhibits a high sensitivity and specificity due to the use of six primers which recognize eight distinct regions of the target [16, 17]. It is a rapid analysis method which is easy to perform and does not require expensive equipments or high technical skill. As the reaction occurs at a unique temperature, a simple water bath, a heating block or any heating device can be used. Its combination with an additional hybridization step using lateral flow dipstick (LFD) method renders the method even easier to perform as both the amplification and the hybridization steps do not require sophisticated and expensive equipments and results can be observed with naked eyes by the appearance or absence of grey line on LFD sticks [8, 12]. This makes the method ideal for in field routine health controls.

This report describes the applicability of a reverse transcription loop-mediated isothermal amplification (RT-LAMP) combined with LFD (RT-LAMP/LFD), based on *tm* subunit of JDV *env* gene (*env-tm* gene), as a diagnostic tool of Jembrana disease.

Materials and methods

Samples and RNA extraction

50–100 mg of various organs were taken from healthy and experimentally JDV-infected cattle (obtained from BPPV [Center of Veterinary Investigations], Denpasar, Bali, Indonesia) and blood from suspected cattle was collected from Barambai region, Kalimantan (Borneo), Indonesia. Organs were first homogenized in PBS (phosphate buffer saline, pH 7.2) and total RNAs were then extracted using High Pure Viral Nucleid Acid Kit (Roche®). Total RNAs from 200 ul blood were directly extracted using the same extraction kit. Total RNAs were then eluted in 50 µl sterile distilled water. 1 µl of this RNA solution contained so the RNA content of 1–2 mg of organs or 4 µl of blood.

LAMP primers and probe

The primers used for LAMP and the probe for LFD were designed from *env-tm* gene, using the program "Primer Explorer" (LAMP primer designing support software program: http://primerexplorer.jp/e/), from the published JDV genome sequence (Acc. no. U21603; version U21603.1 GI: 733067; www.ncbi.nlm.nih.gov/nucleotide/) [2, 3] and synthetized by "1st Base Custom Oligos" (Singapore).

LAMP/LFD and one step RT-LAMP/LFD

Due to the use of *Bsm* Polymerase, instead of *Bst* polymerase, optimal conditions of LAMP reactions were beforehand defined by varying the concentration of $MgSO_4$, betaine, dNTP, and temperature as well as the time and duration of reaction, using 20 ng of *tm-env* gene cloned in pGEX (pGEX-*tm*) as template. The defined optimal conditions were as follows: reactions in 25 µl of reaction buffer, containing 1.6 µM primers FIP and BIP each, 0.2 µM F3 and B3 each, 1 µM dNTPs mix, 1 M betaine, 10 mM $MgSO_4$, 8 u *Bsm* DNA polymerase (Fermentas, Life Science Fermentas, Life Science), and variable amounts of template, at 61 °C for 60 min. Reactions were ended by increasing the temperature to 80 °C for 4 min. When RNA template was used 5 unit reverse transcriptase and 40 unit protector RNAse inhibitor were added in the reaction mixture. The reverse transcription and LAMP reactions occurred in the same reaction tube. One half of

the amplification products was analyzed by electrophoresis on a 1.8 % agarose gel and the other half was used for hybridization, using LFD method, to determine further whether the LAMP amplified products actually corresponded with the target. LFD hybridization was performed by incubating LAMP products with 30 pmol of 6-Fam-labeled probe at 61 °C for 10 min, in a final volume of 20 µl. 10 µl of the hybridization products were then added to 100 µl of PBS. LFD sticks (Milenia Biotec, Germany) were then dipped into the mixture of probe-LAMP products for 5 min at room temperature. Analysis was considered positive if two lines appeared on the stick, one corresponding to the control (CL) and one to the positive hybridization of the LAMP products with the probe/test line (TL).

PCR

PCR was carried out in 25 µl reaction buffer, with various templates and using LAMP outer primers B3 and F3 (0.5 µM each), specific to JDV *env-tm* gene and 1× DreamTaq^{TM}Green PCR Master Mix (Fermentas, Life Science Fermentas, Life Science) with the following condition: prior denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 45 s, annealing at 56 °C for 30 s, elongation at 72 °C for 45 s then additional incubation at 72 °C for 10 min and finally samples were kept at 4 °C till analysis. Half of the reaction products were analyzed by electrophoresis on a 1.8 % agarose gel.

Results

LAMP primers and probe

For the detection of JDV, the designed LAMP primers and the probe were specific to the subunit *tm* of *env* gene (*env-tm*). Their nucleotide sequence, orientation as well as their localization within the gene are shown in Fig. 1. The inner primers FIP and BIP are composed of 2 oligonucleotides with opposite orientations, linked by two T residues (Fig. 1). FIP consists of F1 (in anti-sense orientation),

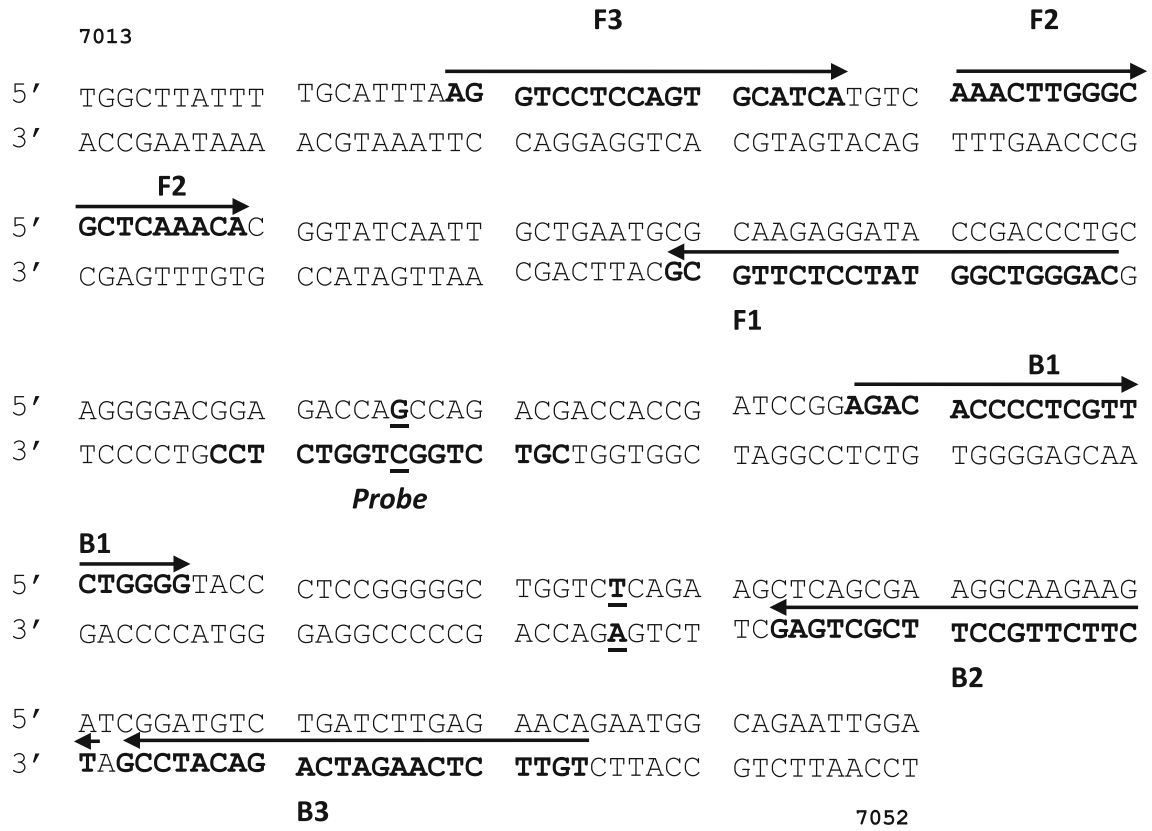

Fig. 1 Nucleotide sequence, localization and orientation of RT-LAMP primers and probe on the cDNA of *env-tm* JDV genome. *Env-tm*, localized between nucleotides 6463 and 7542 within the JDV genome, is a subgene of *env* gene which spans from nucleotide 5197 to 7542. The nucleotide sequence shown in fugure 1 spans from nucleotide 7013 to 7252 of the JDV genome. The primers and the probe are in bold. F1 (in anti-sense orientation) is linked at its 3′-end to the 5′-end of F2 (in sense orientation) by 2 T residues to form the inner primer FIP while B1 (in sense orientation) is linked at its 3′-end to the 5′-end of B2 (in anti-sense orientation) by 2 T residues to form the inner primer BIP. FIP was biotin-labeled at its 5′-end and the probe was labeled with 6-Carboxyfluorescein (6-Fam) at its 5′-end

linked at its 3′-end by 2 T residues to the 5′-end of F2 (in sense orientation). BIP is made up of B1 (in sense orientation), linked at its 3′-end to the 5′-end of B2 (in anti-sense orientation) by 2 T residues. The probe is included within the LAMP-amplified fragment.

Optimal conditions of LAMP

Optimal conditions of LAMP reactions were defined by varying the concentration of $MgSO_4$, betaine and dNTP, the temperature as well as the time and duration of reaction, using 20 ng of *tm-env* gene cloned in pGEX (pGEX-*tm*) as template. The optimal conditions, described in Materials and Methods, were consequently used for all LAMP or RT-LAMP reactions.

The sensitivity and specificity of LAMP/LFD

The sensitivity of LAMP/LFD was evaluated by using serial 10-fold dilutions of pGEX-*tm*, ranging from 2 ng (dilution 1) to 0.002 fg (dilution 10). It was compared to that obtained for LAMP alone and for PCR using primers F3 and B3. As shown in Fig. 2, LAMP, PCR and LAMP/LFD allowed to detect the gene target till respectively dilutions 8, 5 and 7. These dilutions corresponded respectively to the initial amounts of 0.2, 200 and 2 fg of pGEX-*tm* in 25 µl of LAMP or PCR reaction. PCR allowed to see a clearly observable positive band till dilution 5. By attentively examining the electrophoretic analysis, it seems however that a faint band is still observable at dilutions 6 and 7 in PCR results. As shown in Fig. 2c, LAMP/LFD was able to detect *env-tm* gene till dilution 7 which contained 2 fg of template. Considering the length of pGEX-*tm* (ca. 6000 bp), its molecular mass and the Avogadro constant, 2 fg of pGEX-*tm* corresponds to ca. 170 molecules of the gene target. As only half of LAMP reactions was analyzed further by LFD, the detection threshold of LAMP/LFD was therefore ca. 85 molecules of the gene target in the experimental conditions we used. Comparatively, the sensitivity of LAMP alone was 10-fold higher (Fig. 2a) but LAMP/LFD was 100-fold more sensitive than PCR (Fig. 2b). LAMP alone allowed the detection of as few as ca. 8 molecules of the target. The detection threshold of LAMP/LFD was however more than sufficient when the method was applied to sampled organs or blood.

The specificity of LAMP primers for JDV was tested against positive and negative controls. As positive control, we used *env-tm* gene cloned in pGEX (pGEX-*tm*) which has been identified by sequencing (Fig. 3; lane 1), and total RNA extracted from blood and organs of JDV-infected Bali cattle (Fig. 4), as templates. As negative control, total RNA extracted from blood of JDV non-infected Bali cattle and Ongole cattle (a crossbreed between a male *Bos indicus* and a female Javanese cattle) (Fig. 3; lane 2 and lane 3). As shown in Fig. 3, sole pGEX-*tm* gave a positive response, following analysis of LAMP products by electrophoresis on agarose gel (Fig. 3a). LFD analysis, using the probe specific to part of LAMP amplified fragment, confirmed LAMP amplification and the amplicon identity (Fig. 3b).

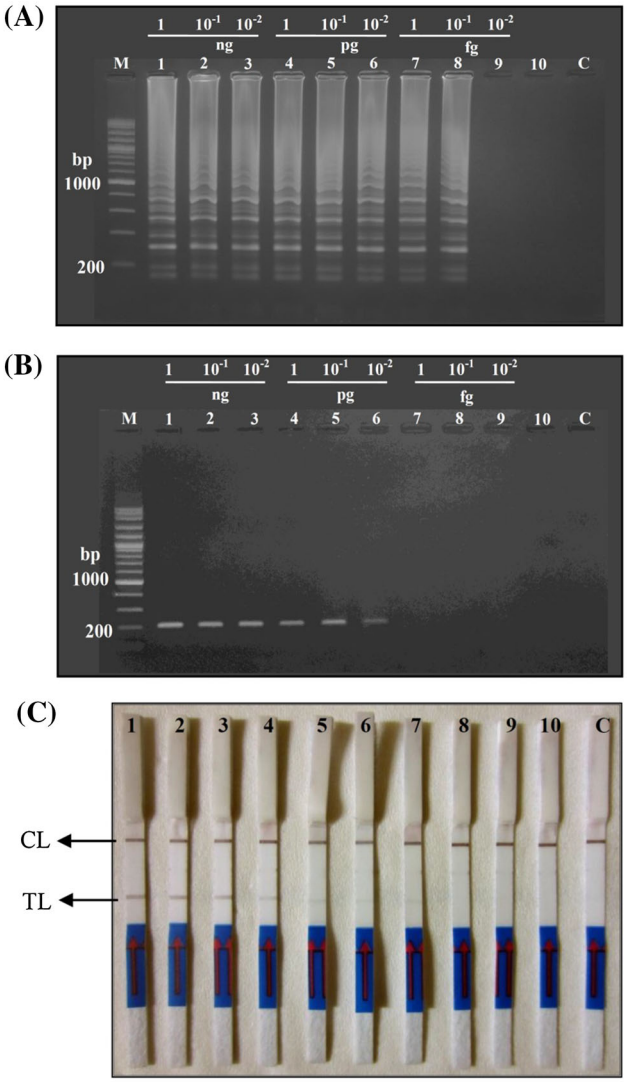

Fig. 2 Comparative sensitivity of LAMP, PCR and LAMP/LFD. Serial 10-fold dilutions (1–10) of pGEX-*tm*, ranging from 2 ng to 0.002 fg of DNA. Electrophoresis on a 1.8 % agarose gel of half of LAMP (**a**) and PCR reaction products (**b**) and further analysis by LFD of the other half of LAMP products (**c**)

Application of LAMP/LFD to sampled organs and blood

The application of the method was carried out by one-step RT-LAMP on total RNA extracted from different organs of experimentally JDV-infected Bali cattle and from selected

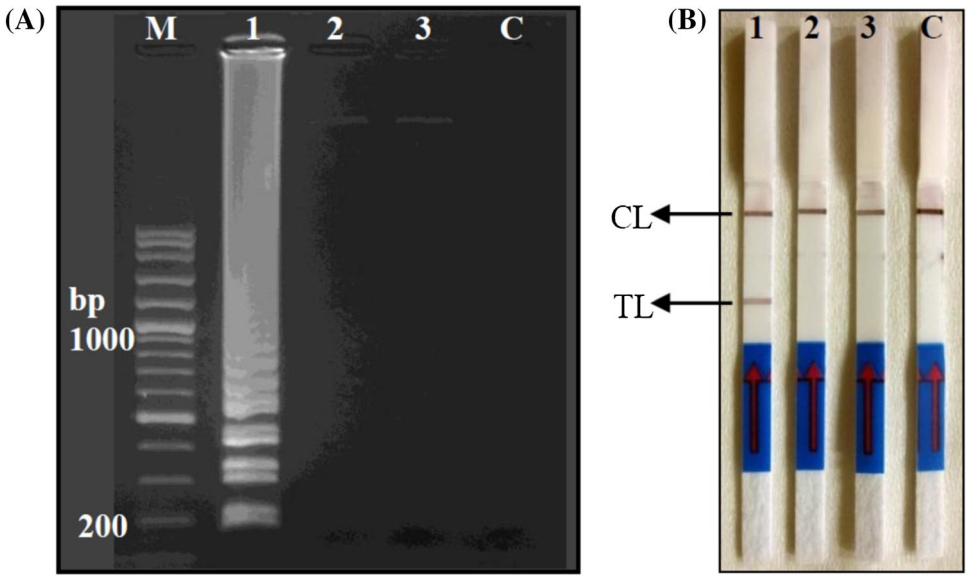

Fig. 3 Specificity of RT-LAMP and RT-LAMP/LFD. Analysis by electrophoresis on a 1.8 % agarose gel of LAMP or RT-LAMP products (**a**) and further analysis by LFD (**b**). LAMP reactions gave the characteristic ladder-pattern of bands. *Lane 1* pGEX-*tm*, *lane 2* healthy Bali cattle, *lane 3* PO cattle, *M* DNA marker, *C* water, *CL* control line, *TL* test line

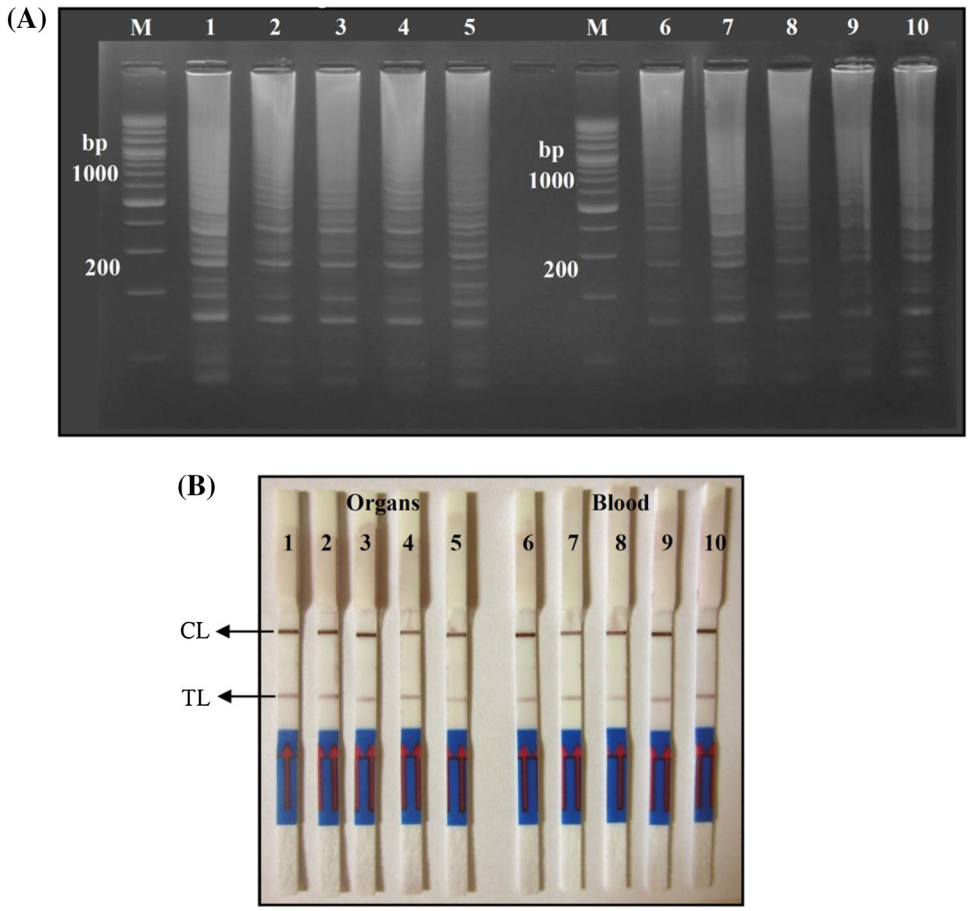

Fig. 4 Application of LAMP/LFD to organs and blood of JDV-infected Bali cattle. One step RT-LAMP on total RNA extracted from organs (1–5) or from blood (6–10) and analysis by electrophoresis on a 1.8 % agarose gel (**a**) and further by LFD (**b**). *Lanes 1, 2* spleen, *lane 3* hearth, *lane 4* lung, *lane 5* tongue, *lanes 6–10* blood, *M* DNA markers, *CL* control line, *TL* test line

blood samples of Bali cattle which were suspected to suffer from Jembrana disease. Sample selection was done by RT-PCR and only positive samples (data not shown) were used. As shown in Fig. 4a, b both in analysis by electrophoresis on agarose gel and further hybridization with 6-FAM-labeled *env-tm*-specific probe, all the sampled organs and blood gave a positive response. It turned out therefore that RT-LAMP as well as RT-LAMP/LFD can be

used for the detection of JDV present in organs or blood. In this experiment, RT-LAMP was performed on total RNA corresponding to the RNA content of 2–4 mg of organs or 8 µl of blood. As only half of the RT-LAMP products was used in hybridization using LFD method, 1–2 mg of organs or 4 µl of blood were largely sufficient for JDV detection. Only a small amount of initial materials was thus required for analysis by RT-LAMP/LFD.

Discussion

This paper describes the applicability of RT-LAMP and RT-LAMP/LFD for the detection of the pathogenic agent of Jembrana disease. Due to the high mortality rate of infected cattle and the consequent economic losses, it is essential that JDV infection can be diagnosed as early as possible to limit the disease spread. Immunodiagnosis based on host humoral response can not be used in early stages of the disease as like the other lentiviruses, JDV infection induces a delayed humoral response and JDV-specific antibodies are not produced in most infected cattle untill 11 weeks post infection [7, 10]. Furthermore, antibody-based diagnostic methods do not enable to distinguish JDV- from BIV-infection as the two bovine lentiviruses are antigenically very closely related [6, 10]. Distinguishing BIV-infection was only made feasible by using a BIV-specific monoclonal antibody that only recognizes the unique BIV GAG epitope, which is not shared by JDV [14]. During the acute phase, high titre of infectious JDV viral particles is found in plasma [18, 20]. Viruses are also abundantly present in secreted fluids, namely milk and saliva. This make viral antigen identification by molecular method is ideal detection tool in order to detect viral infection as early as possible during the course of the disease.

The RT-LAMP/LFD we used proved to be fast, easy to perform, specific and highly sensitive (Figs. 2, 3). It does not require sophisticated and expensive devices for both the amplification and hybridization steps. Using cloned *env-tm* subunit gene, RT-LAMP/LFD allowed the detection of as few as 80 molecules of the gene target. Positive results can be easily and immediately observed by the appearance of grey lines on the LFD stick. This avoids the analysis step by electrophoresis on agarose gel and eliminates the need to handle carcinogenic ethidium bromide.

The RT-LAMP/LFD we used proved to be 100-fold more sensitive than RT-PCR (Fig. 2). Besides, the additional hybridization process by LFD method also enables to confirm the amplicon identity. The whole process, including the LAMP reaction and the hybridization step only lasts ca. 75 min. The sensitivity of RT-LAMP/LFD is more than sufficient as for its application to the analysis of sampled organs or blood, only 1–2 mg of organs and 4 ul of whole blood were needed and less initial materials can still be used. This work also showed that virus detection could be done not only on total RNA extracted from blood but various organs could also be analyzed for the presence of JDV.

The specificity of RT-LAMP/LFD method was established using positive and negative samples. As positive samples, we used *env-tm* gene cloned in pGEX (pGEX-*tm*) and total RNA extracted from blood and various organs of JDV-infected Bali cattle. As negative samples, we used total RNA extracted from blood JDV non-infected Bali cattle and Ongole cattle. It is worthwhile mentioning that JDV infections of Ongole cattle only induce mild clinical signs and the infectious agents persist for less than 9 months [18, 19]. Therefore the diagnosed animal may be healthy, or too few viruses are found in the sampled blood, or they have been rapidly eliminated. For checking the cross reactivity of the primers with the related virus (e.g. BIV), a meticulous search of possible sequence homology between the nucleotide sequence of *env-tm* region (LAMP-amplified region) with any of the nucleotide sequences published in GenBank, including the entire genome of BIV (including *env* region of BIV) has conducted. It did not reveal significant degrees of homology with any of the nucleotide sequences published in GenBank (http://www.ncbi.nlm.nih.gov/genbank/). More importantly, no sequence homology is found in *env-tm* subunit gene of the genome of BIV which is genetically the most related to JDV (data not shown). Reasonably, this will avoid cross reactions and allow to distinguish JDV- from BIV-infection. Besides, the additional step "hybridization-LFD" was also reasonably play an essential control role as it ensures that the JDV *env-tm* fragment (LAMP-amplified fragment) is actually the chosen part of JDV gene target. Furthermore, the choice of *env-tm* subunit gene as target was determined by the fact that this part of JDV genome is highly conserved among the strains or isolates so far studied [13]. Sequence comparison of *env-tm* gene of 8 Indonesian JDV isolates, using bioinformatic analyst (BLASTN), showed a very high degree of conservation among the 8 JDV isolates studied [13], rendering so this part of the genome an ideal target for amplification of all JDV strains or isolates. Only one variation, at position 666, was found within the oligonucleotide probe and a mismatch at this position will not prevent hybridization to take place. Another variation, at position 726, is not comprised in any of the designed LAMP primers (Fig. 1).

LAMP primers proved so to be specific to *env-tm* subunit gene of JDV genome. LFD analysis, using the probe specific to part of LAMP amplified fragment, confirmed further the specificity of LAMP amplification and the amplicon identity (Fig. 3b). The amplification of JDV *gag-*

ca gene also resulted in negative response (data not shown). The combination of RT-LAMP with LFD makes the method a more suitable diagnostic tool in conditions where sophisticated and expensive equipments are not available for field investigations on Jembrana disease in Bali cattle. Thus RT-LAMP and RT-LAMP/LFD both can be potentially used for routine monitoring of JDV infections.

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References

1. Budiarso IT, Hardjosworo S. Jembrana disease in Bali cattle. Aust Vet J. 1976;52:97.
2. Chadwick BJ, Coelen RJ, Sammels LM, Kertayadnya G, Wilcox GE. Genomic sequence analysis identifies Jembrana disease virus as a new bovine lentivirus. J Gen Virol. 1995;76:189–92.
3. Chadwick BJ, Coelen RJ, Wilcox GE, Sammels LM, Kertayadnya G. Nucleotide sequence analysis of Jembrana disease virus: a bovine lentivirus associated with an acute disease syndrome. J Gen Virol. 1995;76:1637–50.
4. Chadwick BJ, Desport M, Brownlie J, Wilcox GE, Dharma DM. Detection of Jembrana disease virus in spleen, lymph nodes, bone marrow and other tissues by in situ hybridization of paraffin-embedded sections. J Gen Virol. 1998;79:101–6.
5. Coffin JM, Hughes SH, Varmus HE, editors. Retroviruses. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1997.
6. Desport M, Stewart ME, Sheridan CA, Ditcham WG, Setiyaningsih S, Tenaya WM, Hartaningsih N, Wilcox GE. Recombinant Jembrana disease virus gag proteins identify several different antigenic domains but do not facilitate serological differentiation of JDV and nonpathogenic bovine lentiviruses. J Virol Methods. 2005;124:135–42.
7. Dharma DM, Ladds PW, Wilcox GE, Campbell RS. Immunopathology of experimental Jembrana disease in Bali cattle. Vet Immunol Immunopathol. 1994;44:31–44.
8. Ding WC, Chen J, Shi YH, Lu XJ, Li MY. Rapid and sensitive detection of infectious spleen and kidney necrosis virus by loop-mediated isothermal amplification combined with a lateral flow dipstick. Arch Virol. 2010;155:385–9.
9. Hartaningsih N, Wilcox GE, Dharma DM, Soetrisno M. Distribution of Jembrana disease in cattle in Indonesia. Vet Microbiol. 1993;38:23–9.
10. Hartaningsih N, Wilcox GE, Kertayadnya G, Astawa M. Antibody response to Jembrana disease virus in Bali cattle. Vet Microbiol. 1994;39:15–23.
11. Kertayadnya G, Wilcox GE, Soeharsono S, Hartaningsih N, Coelen RJ, Cook RD, Collins E, Brownlie J. Characteristics of a retrovirus associated with Jembrana disease in Bali cattle. J Gen Virol. 1993;74:1765–78.
12. Kiatpathomchai W, Jaroenram W, Arunruta N, Jitrapakdee S, Flegel TW. Shrimp taura syndrome virus detection by reverse transcription loop-mediated isothermal amplification combined with a lateral flow dipstick. J Virol Methods. 2008;153:214–7.
13. Kusumawati A, Pratiwi R, Astuti P, Hamid PH. Characterization of envelope transmembrane gene of Jembrana disease virus Tabanan 1995 isolate. Indo J Biotechnol. 2010;15:15–9.
14. Lu M, Zheng L, Mitchell K, Kapil S, Wood C, Minocha H. Unique epitope of bovine immunodeficiency virus gag protein spans the cleavage site between p16(MA) and p2L. Clin Diagn Lab Immunol. 2002;9:1277–81.
15. Mori Y, Nagamine K, Tomita N, Notomi T. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochem Biophys Res Commun. 2001;289:150–4.
16. Notomi T, Okayama H, Masubuchi H, Yonekawa T, Watanabe K, Amino N, Hase T. Loop-mediated isothermal amplification reaction of DNA. Nucleic Acids Res. 2000;28:e63.
17. Parida M, Posadas G, Inoue S, Hasebe F, Morita K. Real-time reverse loop mediated isothermal amplification for rapid detection of West Nile virus. J Clin Microbiol. 2004;42:257–63.
18. Soeharsono S, Hartaningsih N, Soetrisno M, Kertayadnya G, Wilcox GE. Studies of experimental Jembrana disease in Bali cattle. I. Transmission and persistence of the infectious agent in ruminants and pigs, and resistance of recovered cattle to re-infection. J Comp Pathol. 1990;103:49–59.
19. Soeharsono S, Wilcox GE, Dharma DM, Hartaningsih N, Kertayadnya G, Budiantoro A. Species differences in the reaction of cattle to Jembrana disease virus infection. J Comp Pathol. 1995;112:391–402.
20. Soesanto M, Soeharsono S, Budiantono A, Sulistyana K, Tenaya M, Wilcox GE. Studies on experimental Jembrana disease in Bali cattle. II. Clinical signs and haematological changes. J Comp Pathol. 1990;103:61–71.
21. Wilcox GE, Chadwick BJ, Kertayadnya G. Recent advances in the understanding of Jembrana disease. Vet Microbiol. 1995;46:249–55.