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# The Comparison of RT-LAMP, RT-PCR and Dot-Blot Hybridization for Detection of Jembrana Disease Virus

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**Abstract:** Jembrana disease virus is a viral pathogen that causes Jembrana disease in Bali cattle (*Bos javanicus*) with high mortality rate. Infection of Jembrana Disease Virus (JDV) on Bali cattle have caused substantial economic losses for farmers in Indonesia and Australia. In order to control the spread, development of a sensitive detection method is important. In this study, we used three different detection methods based on genomic approach, i.e., reverse transcriptase-polymerase chain reaction (RT-PCR), reverse transcriptase-loop mediated isothermal amplification (RT-LAMP) and dot-blot hybridization to detect JDV. Utilization of pGEX-TM, a recombinant plasmid containing *env-tm* gene as a positive control showed that RT-LAMP is the most sensitive method compares the two others. It could detect template concentration as low as $10^{-6}$ ng $\mu L^{-1}$ or equivalent to $1.52\times10^{2}$ plasmid copy number, 100 and 10000 more sensitive than RT-PCR and dot-blot hybridization, respectively.

**Keywords:** Jembrana Disease, RT-PCR, RT-LAMP, dot-blot hybridization, Comparative Analysis

## Introduction

Jembrana Disease Virus (JDV) is a lentivirus associated with an acute disease syndrome on Bali cattle *(Bos javanicus)* in Indonesia and Australia (Kusumawati *et al*., 2014a). After short latent period, infected cattle show clinical signs of fever, lymphadenopathy and lymphopenia, at which stage high viral titres of $10^{8}$ infectious units/mL are found in the plasma (Kusumawati *et al*., 2014b; Soeharsono *et al*., 1990; Soesanto *et al*., 1990). In fatal infection, death is attributed to multisystem involvement. In non-fatal infection, regression of lesions commences about 5 weeks post-infection and the recovered cattle is resistant to further development of clinical disease (Dharma *et al*., 1991). A recovered cattle will develop a delayed immune respone to the same or different isolates of JDV but still in state of viraemia with no recurrence for at least 2 years after infection (Kusumawati *et al*., 2014b). A carrier cattle is a potential source of infection. The etiology of the disease and the mode of transmission in nature are as yet unknown but the disease can be readily transmitted to susceptible cattle by the exposure of blood, spleen, or lymph node material from infected cattle (Soeharsono *et al*., 1990; Kusumawati *et al*., 2014b). The disease have been spread to several islands in Indonesia such as Sumatra and Kalimantan and it is believed occurred via the distribution of carrier cattle (Kusumawati *et al*., 2014b).

In order to control the spread of JDV infection, development of a sensitive detection method is important. Furthermore, the method used has to give accurate result because it is known there is a possibility to cross react with another bovine lentivirus i.e., Bovine Immunodeficiency Virus (BIV) since BIV was genetically and antigenically the most closely related to JDV although the respective associated disease is quite different (Kusumawati *et al*., 2014a). In this study, we describe the comparison of genomic based-method of reverse transcriptase-polymerase chain reaction (RT-PCR), reverse transcriptase-loop mediated isothermal amplification (RT-LAMP) and dot-blot hybridization to detect JDV genomic material. Those methods are commonly used in detecting for many infectious diseases (Kusumawati *et al*., 2014c; Parida *et al*., 2008). RT-PCR and RT-LAMP work by amplifying the genomic material until sufficient amount of amplicons can be detected by electrophoresis (Notomi *et al*., 2000; Parida *et al*., 2008), while dot-blot hybridization works by using spesific probe which hybridize to certain genomic material

(Kusumawati *et al*., 2014c). The aim of this study is to compare the sensitivity among all of the three methods.

## Materials and Methods

### *Sample and Material Preparation*

Whole blood samples were obtained from carrier cattle on several livestocks in South Kalimantan, Indonesia. Each sample was collected in 5 mL EDTA K2/K3 blood collection tube and storaged in 4°C until it was used. In this study, we used pGEX-TM, a recombinant plasmid containing JDV *env-tm* gene for positive control. All used glasswares were treated with 0.1% Diethyl Pyrocarbonate in water overnight (12 h) at 37°C and then autoclaved or heated to 100°C for 15 min.

### *RNA Viral and Positive Control Sample Preparation*

RNA viral was extracted with High Pure Viral Nucleid Acid Kit (Roche®), following the manufacturer's instructions. pGEX-TM was obtained from previous study (Kusumawati *et al*., 2010), propagated in Luria Bertani liquid medium and extracted with GenJet Plasmid Miniprep Kit (Fermentas), following the manufacturer's instructions. RNA and pGEX-TM concentration and quality were also measured by spectrophotometric analysis at 260 and 280 nm. Each RNA sample from whole blood extraction and pGEX-TM was prepared in 10 ng $\mu L^{-1}$ concentration. They were then diluted with double-distilled $H_2O$ RNAse free in a series of 10-fold dilution and used as a template for PCR and LAMP detection.

### *Detection by Polymerase Chain Reaction (PCR)*

DNA synthesis from each RNA sample was performed in a 25 µL reaction volume containing 6.75 µL of RNA and 5 µM of B3 primer. RNA mixture was warmed 70°C for 10 min (min) and cooled in ice water for 5 min. A mixture containing 10 mM of dNTP mix (Bioron, Germany), 7.5U of AMV reverse transcriptase (Promega), 1x of AMV reaction buffer, 4U of Protector RNAse Inhibitor (Roche, Germany) and nuclease free water was added to RNA mixture. The mixture was incubated at 37°C for 60 min and terminated at 95°C for 2 min using waterbath (Haake L).

The detection by PCR assay was performed using a DreamTaq™Green PCR Master Mix 2X in a 25 µL reaction volume. The mixture consist of 2 µL each of sample (cDNA or pGEX-TM), 12.5 µL of PCR master mix, 5 µM each of F3 and B3 primer and nuclease free water. The amplification program was performed as follows: Initial denaturation at 95°C for 5 min, followed by 30 cycles at 95°C for 45 (sec), 58°C for 30 sec; 72°C for 45 sec and then a terminal extention step of 72°C for 10 min. 5 µL of PCR products then were electrophoresed on 1.8% agarose gel to verify the presence of the expected 211 bp band target.

### *Detection by Loop-Mediated Isothermal Amplification (LAMP)*

LAMP reaction mixture consist of 1,8 µM each of FIP and BIP primer, 0.2 µM each of F3 and B3 primer, 1.2 mM of dNTP mix, 0.6 M betaine, 10 mM MgSO4, 8 U of Bst DNA polymerase, 1x of Bst DNA polymerase reaction buffer and 1 µL each of sample (pGEX-TM or RNA). For RNA sample, 0.125U of AMV reverse transcriptase and 4U of Protector RNAse Inhibitor were added on the LAMP reaction mixture. Both LAMP and RT-LAMP was performed in a 25 µL reaction volume, incubated at 60°C for 1 (h) and terminated at 80°C for 4 min.

### *Probe Preparation of Dot-Blot Hybridization*

Linearized pGEX-TM was used as a template for probe synthesizing with PCR DIG Labeling Mix (Roche®). According to the manufacturer's instructions, reaction was performed in 50 µL containing 5 ng pGEX-TM, PCR DIG labelling mix (200 µM dNTP, digoxigenine-11-dUTP) (1 uL), 1-5 U Taq DNA polymerase and 50 pmol each of F3 and B3 primer. PCR program was performed as "PCR detection method".

Quantification of probe was performed using DIG Quantification Teststrips (Roche). Probes were diluted to obtain 1 ng $\mu L^{-1}$ final concentration. 1 uL of diluted probe were blotted into membrane DIG Quantification Teststrips (Roche), dried for 2 min, blocked into blocking solution for 2 min, dipped into antibody solution for 3 min and blocked again into blocking solution for 1 min. The strips then was washed with washing buffer for 1 min, dipped into detection buffer for 1 min and finally incubated in color-substrate solution (NBT/BCIP) for 5-30 min. The color signal produced then compared to control quantification on the kit (Roche).

### *Detection by Dot-Blot Hybridization*

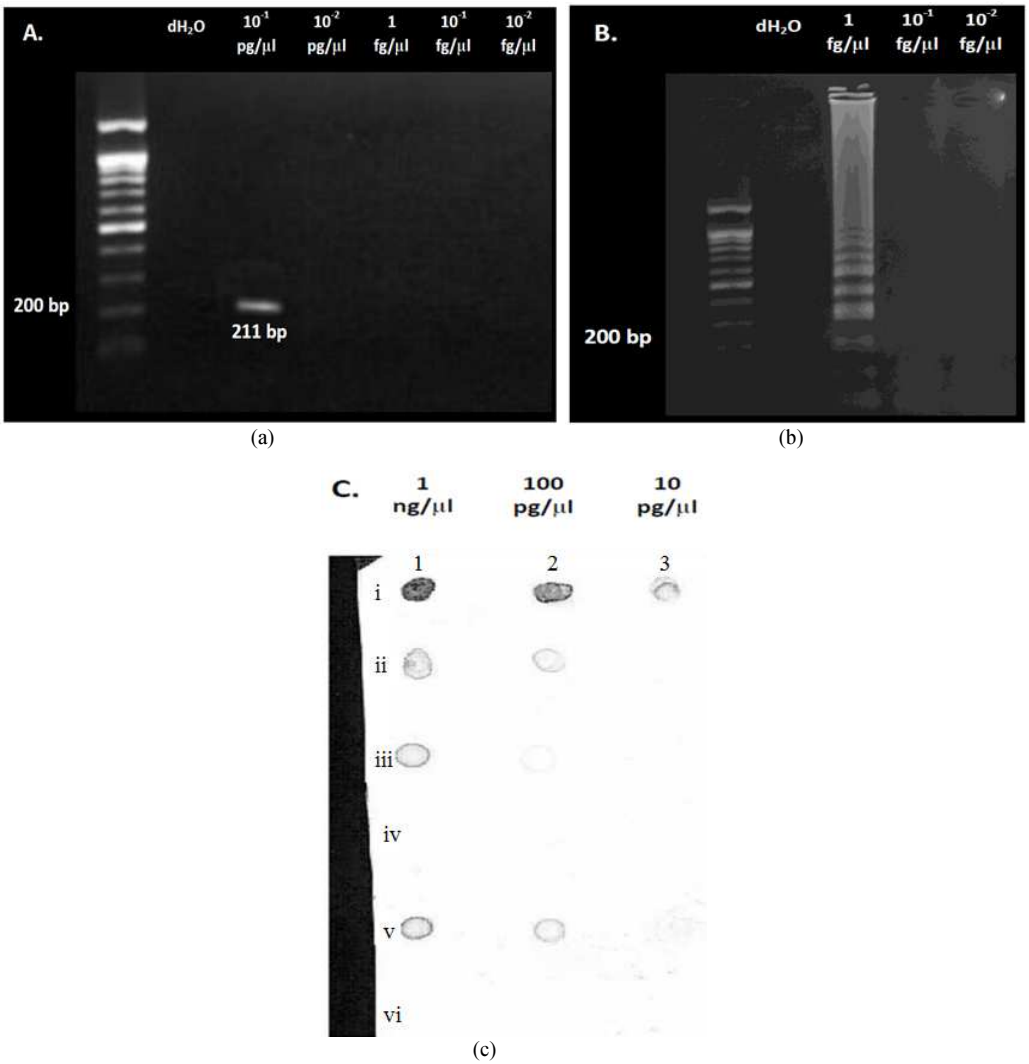
RNA was diluted into 10 ng $\mu L^{-1}$, 1 ng $\mu L^{-1}$, 100 pg $\mu L^{-1}$, 10 pg $\mu L^{-1}$. Each of sample was denaturated in MOPS buffer, 50% formamide and 2 M formaldehyde at 65°C for 5 min and then placed into ice tube. 1 µL of denatured sample was immobilized on nylon Hybond-N membrane (Amersham Pharmacia). Membrane was dried at 50°C for 1 h then crosslinked by UV crosslinker for 5 min.

Prehybridization was carried out at 42°C for 3-4 h in prehybridization medium (buffer 50% formamide, 2 X SSC, 50 mM sodium phosphate, 2% blocking reagent, 0,1% sodium sarkosyl, 7% SDS), followed hybridization by added 25 ng $mL^{-1}$ of DIG labeled probe which had been denaturated at 100°C for 10 min in prehybridization medium. Hybridization was carried out at 45°C overnight. After hybridization, membrane was washed twice with 2X SSC and 0,1% SDS at room temperature for 15 min and 0,5 X SSC and 0,1% SDS, at 68°C for 15 min. Hybridization was detected with alkaline phosphatase-conjugated and anti-DIGoxigenin antibody according to the manufacturer's instruction by washed the

hybridization membrane with washing buffer I (100 mM maleic acid, 150 mM NaCl pH 7.5, 0.320) for 1-3 min, blocked into blocking reagent (100 mM maleic acid, 150 mM NaCl, pH 7,5, 1% blocking reagent) for 1 h, washed twice with washing buffer I for 15 min and wash once with washing buffer II (100 mM Tris-HCl, pH 9,5, 100 mM NaCl, 50 mM $MgCl_2$) for 15 min. The process was continued by incubation of the membrane into 2 mL buffer II containing 7 µL Nitro Blue Tetrazolium (NBT) dan 7 µL solution 5-Bromo-4-Chloro-3-indolylphosphate (BCIP) (Sigma) in dark room for 2 h to overnight.

## Results

The sensitivity of RT-PCR and LAMP assay were evaluated by using serial 10-fold dilutions of pGEX-TM as a positive control template. In range from $10^{-1}$ pg µL$^{-1}$ to $10^{-2}$ fg µL$^{-}$1, PCR products were analysed using electrophoresis on agarose gel showed the presence of the expected 211 bp band target up to concentration $10^{-1}$ pg µL$^{-1}$ or equivalent to $1.52\times10^4$ plasmid copy number (Fig. 1a). RT-LAMP products were also analysed using electrophoresis on agarose gel (DNA ladder like pattern result), ranging from 1 fg uL$^{-1}$ to $10^{-2}$ fg uL$^{-1}$. As shown in Fig. 1b, RT-LAMP was able to detect *env-tm* gene till dillution which contained 1 fg uL$^{-1}$ of template or equivalent to $1.52\times10^2$ plasmid copy number. The sensitivity of dot-blot hybridization was evaluated on different concentration of RNA sample, i.e., 1 ng uL$^{-1}$, 100 pg uL$^{-1}$ and 10 pg uL$^{-1}$. From five samples (Fig. 1c), probe could detect up to 10 pg µL$^{-1}$ for positive control sample (pGEX-TM) and up to concentration 100 pg uL$^{-1}$ for RNA sample.




(a) (b)

(c)

Fig. 1. Comparison of sensitivity of three molecular methods. Electrophoresis on 1.8% agarose gel of RT-PCR (a) and RT-LAMP (b) reaction products and probe hybridization on DIG quantification membrane of dot-blot hybridization (c). Serial 10-fold dilutions of pGEX-TM and RNA from whole blood sample, ranging from $10^{-1}$ pg µL$^{-1}$ to $10^{-2}$ fg µL$^{-}$1 (RT-PCR), from 1 fg uL$^{-1}$ to $10^{-2}$ fg uL$^{-1}$ (RT-LAMP) and different concentration of five (RNA) samples, i.e., 1 ng uL$^{-1}$, 100 pg uL$^{-1}$ and 10 pg uL$^{-1}$ (dot-blot hybridization)

## Discussion

The aim of this study is to compare the sensitivity among RT-PCR, RT-LAMP and dot-blot hybridization. Infection of Jembrana disease virus on Bali cattle have caused substantial economic losses for farmers in Indonesia and Australia (Kusumawati *et al*., 2014a). JDV has spread to almost west region in Indonesia (Soeharsono *et al*., 1995; Burkala *et al*., 1999) and has high prevalence which is more than 50% (Kusumawati *et al*., 2014b). Therefore it is very important to identify the presence of the virus as early as possible to prevent further disease transmission among the Bali cattle or outbreak to other areas. Immunodiagnostic method 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 (Kusumawati *et al*., 2014b). Furthermore, antibody-based diagnostic methods do not enable to distinguish JDV-from BIV-infection as the two bovine lentiviruses are antigenically very closely related (Kusumawati *et al*., 2014a). 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 (Kusumawati *et al*., 2014a). During the acute phase, high titre of infectious JDV viral particles is found in plasma. Viruses are also abundantly present in secreted fluids, i.e., milk and saliva (Kusumawati *et al*., 2014b). In this case, detection method based on genomic approach is an appropriate method because of its ability to detect JDV in acute phase of the disease. In this study, we described three kind of molecular methods, i.e., RT-PCR, RT-LAMP and dot-blot Hybridization and compared them to each other in term of their sensitivity.

In this study, the comparison of both RT- LAMP and PCR result assumed that RT-LAMP is 100 times more sensitive than PCR assay (comparison of sensitivity of RT-LAMP and PCR are, 1 fg $uL^{-1}$ and $10^{-1}$ pg $\mu L^{-1}$, respectively). The application of both method on RNA sample give the same results as the positive control sample. Quantification of labeling probe showed that probe could detected up to concentration of 3 pg $uL^{-1}$ (data not shown). Based on the dot comparison between probe and the standard of the Roche®, it was obtained that probe have a high labeling efficiency. This information then used to determine the amount of probe in hybridization process. In other case, the comparison of both RT-LAMP and dot-blot hybridization shows that RT-LAMP is 10000 times more sensitive than dot-blot hybridization assay (comparison of sensitivity of RT-AMP and dot-blot hybridization are, 1 fg $uL^{-1}$ and 10 pg $\mu L^{-1}$, respectively). Theoretically, the sensitivity of LAMP is due to the methodology is based on the high strand displacement activity of the polymerase used, allowing amplification reactions to produce more amount of DNA (Notomi *et al*., 2000). LAMP method is also known to be has high specificity due to the utilization of 2 or 3 primer pairs in the process (Notomi *et al*., 2000). LAMP is also a rapid analysis method which is due to there is no time losses during the process because of temperature changing as in PCR (Parida *et al*., 2008). LAMP is easy to perform and does not require expensive equipments or high technical skill. It is simple procedure on isotermal temperature, which can carried out on a simple waterbath, make this method is appropriate to be applied in field condition which has minimal facility, replaces PCR as the common method for detection purpose. Dot-blot hybridization generate specific results because of utilization of specific probes but this method is time consuming and very laborious (Kusumawati *et al*., 2014c).

## Conclusion

Infection of JDV on Bali cattle have caused substantial economic losses for farmers in Indonesia and Australia. In order to control the spread, development of a sensitive detection method is important. In this study, we used three different detection methods based on genomic/molecular approach, i.e., RT-PCR, RT-LAMP and dot-blot hybridization to detect JDV genomic material. Utilization of pGEX-TM, a recombinant plasmid containing *env-tm* gene as a positive control showed that RT-LAMP is the most sensitive method compares the two others. It could detect template concentration as low as $10^{-6}$ ng $\mu L^{-1}$ or equivalent to $1.52\times10^{2}$ plasmid copy number, 100 and 10000 more sensitive than RT-PCR and dot-blot hybridization, respectively.

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## Author's Contribution

**Asmarani Kusumawati:** Participated in all experiments, aquisition of data, analyzed all the data and contributed to the writing of the manuscript, provided technical guidance, coordinated the writing of the manuscript, designed the research plan and organized the study.

**Tenri Ashari Wanahari:** Participated in all experiments, aquisition of data, analyzed all the data and contributed to the writing of the manuscript.

**Issabelina Dwades Tampubolon:** Participated in all experiments, aquisition of data, analyzed all the data and contributed to the writing of the manuscript.

**Basofi Ashari Mappakaya:** Participated in interpretation of data and writing of the manuscript.

## Ethics

This study was approved by Research Ethics Commitee, Faculty of Veterinary Medicine, Gadjah Mada University, Indonesia.

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