LAMP PCR for Detection of Rice Tungro Virus

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Abstract. Routine monitoring of tungro virus on rice field in several regions in Indonesia is necessary as an important part of disease control strategy. Therefore, a fast, accurate, and simple method to detect tungro virus is required. A research was conducted to develop loop-mediated isothermal amplification polymerase chain reaction (LAMP PCR) method as a detection approach for tungro virus. Rice leaf samples infected by tungro virus from Garut (West Java), Sidrap (South Sulawesi), and Pesisir Selatan (West Sumatera) were collected and used for this experiment. Conventional PCR was conducted as comparison to LAMP PCR. Specific DNA fragments of tungro virus (Rice tungro bacilliform virus/RTBV) was successfully amplified by conventional PCR as well as LAMP PCR. Sensitivity of LAMP PCR was higher than those of conventional PCR. Better result for detection of RTBV using LAMP PCR was achieved with sample incubation condition at 63°C for 60 min and termination reaction at 80°C for 10 min. The result of LAMP PCR can be visualized obviously using HNB dye without UV light. LAMP PCR should be recommended for routine rice tungro virus detection method due to its simplicity with accurate and high sensitivity result.

Keywords: conventional PCR, fast detection, LAMP PCR, Rice tungro bacilliform virus

1. Introduction
At the beginning of viral research, virus detection and identification was based solely on the development of symptoms on infected plants or biological estimates. However, the use of symptoms for diagnosis is less precise because the same symptoms can be caused by different viruses and non-pathogenic disorders, such as nutrient deficiency, excess water, dryness, or insect damage can also induce the same symptoms as those caused by viral infections. Another fact that, in the field, rice plants may be infected by some other viruses. This causes the diagnosis of rice disease based on symptom observation rather difficult [1,2].

Detection of the rice virus, including the tungro virus, developed rapidly as serology began to be introduced. Serology method is a specific and sensitive detection method for detecting virus in low numbers [3]. In addition, the workmanship is relatively simple. Enzyme-linked immunosorbent assay (ELISA) is one of the most commonly used serological detection examples for detecting tungro virus [4,5]. However, tungro virus detection with this method is rather difficult because of the limited availability of antiserum. The antiserum for this virus is not produced commercially as any other virus.
Along with the development of tungro disease that increasingly penetrated into new areas, the monitoring of tungro virus that infects rice routinely in various regions in Indonesia needs to be done as part of disease control strategy. To facilitate monitoring, fast, accurate, and simple detection techniques is necessary. Accordingly, a simple and fast detection technique is required with high accuracy, does not require expensive equipment, and is efficiently used in large quantities of samples. This detection technique should also be used alone directly by farmers, so that decision-making related disease control can be as early as possible.

Loop-mediated isothermal amplification (LAMP) is a rapid and simple detection technique [6,7], particularly in areas with limited tools [8,9]. LAMP is an easily prepared method of amplifying specified nucleic acids that can amplify nucleic acids under isothermal conditions at 60-65°C [6,10]. The research was conducted to develop the method of loop-mediated isothermal amplification polymerase chain reaction (LAMP-PCR) as a method of tungro virus detection.

2. Materials and methods

2.1. Collecting rice leaf sample infected by virus tungro

Rice leaf samples were collected from West Java (Subang, Garut and Bogor District), South Sulawesi (Sidrap), West Sulawesi (Polman), East Sulawesi (South Minahasa), Central Sulawesi (Parigi Moutong), West Nusa Tenggara (Lombok), Bali (Badung), West Papua and West Sumatera (South Pesisir).

2.2. Extraction of samples

Total DNA extraction from fresh rice leaf sample was done by cetyl trimethyl ammonium bromide (CTAB) method [11].

2.3. Detection sample using PCR conventional and LAMP PCR

Detection using PCR Conventional. A pair of primer, DAF (5’-GGAATTCCGGCCCTCAAAAA CCTAGAAG-3’)/DAR (5’GGGGGTACCCCCCTCCGATTTCCCATGTATG-3’), were used to amplified DNA target from all samples. RTBV coat protein gene is the amplification target of the primers, which size 1400 bp [12]. The amplification reaction was made with a total volume of 25 µl, comprising 8.5 µl ddH2O, 12.5 µl DreamTaq Green PCR Master mix (Thermo ScientificTM, US), 1 µl primary DAR 10 µM, 1 µl primary DAF 10 µM, and 2 µl DNA. The amplification process consisted of 5 minutes of initial denaturation at 94°C, followed by 34 amplification cycles including 1 minute denaturation at 94°C, annealing for 1 minute at 62.2°C, synthesis for 2 minutes at 72°C, then added synthesis step End for 10 minutes at 72°C. The amplification results were visualized with electrophoresis on 1% agarose gel (TBE) with ethidium bromide staining (0.5 µg / ml) for ± 15 min.

Detection using LAMP PCR. The LAMP method uses four primary pairs to detect RTBV and RTBV [13] (table 1). LAMP primers for detecting RTBV and RTSV are designed based on conserved region, ie, respectively on ORF3 and ORF1.

LAMP was carried out using a LoopAmp DNA amplification kit (Eiken Chemicals Co. Ltd, Tokyo, Japan), in a 25 µl reaction mixture with 40 mM Tris buffer (pH 8.8), 20 mM KCl, 16 mM MgSO4, 20 mM (NH4)2SO4, 0.2% Tween 20, 1.6 M Betaine, 2.8 mM dNTP.

The addition of the dye is carried out before the reaction heating (incubation). The nuclease free water is used as a negative control to be included in each test. The reaction composition is presented in table 2. Beside that, lamp sensitivity testing is also performed, by testing several series of dilutions, ranging from 10⁻¹ to 10⁻⁷.

Temperature optimization for LAMP amplification visualization was performed at 60, 63, and 65°C for 60 minutes. In this study temperature optimization is not carried out, because it
uses only four primers without primary LOOP (B-Loop and F-Loop), where it is known that the primary Loop use to accelerate the reaction in the incubation process. Subsequently, the reaction was stopped by heating using a water bath at 80 °C for 10 minutes.

### Table 1. LAMP primer to detect RTBV (Le et al. 2000 [14])

| Primer | Sequen (5’-3’) |
|--------|----------------|
| F3     | 5’-ACTCTTTGTATAGACTACCAGAAG-3’ |
| B3     | 5’-GGATTTTTCGTTTCTTTATAATCTCC-3’ |
| FIP    | 5’-GCTATTTCATTCTGCTTCATAGGGGAAAGGTAGTAAAGCGGA-3’ |
| BIP    | 5’-CATGGATGAGGCAAAATGCATTAAGATCTACAGAATGCTAAGGATG-3’ |

The LAMP visualization is done in three ways, namely 1) the color change reaction with fluorescent detection reagent (FDR) (Eiken Chemicals Co., Japan) that can be observed by the naked eye or using UV rays; 2) discoloration with hydroxy naphthol blue (HNB) (Dojindo Laboratories, Japan) that can be observed with the naked eye, and 3) gel electrophoresis analysis. The color change reaction is done by adding 1 µl each of FDR or HNB. Observation of staining using FDR with the naked eye will show a change of color from a clear orange to transparent green if positive and if the negative sample remains orange. Observations under UV lamps (wavelength 240 ~ 260 nm or 350 ~ 370) will distinguish positive samples from visible light green from fluorescent colored negative samples. When using HNB dyes, a positive sample will turn blue, while the negative sample will remain purple. A total of 3 µl of LAMP detection results in electrophoresis on 1.2% agarose gel. Positive samples will show DNA bands of several sizes composed downward, whereas negative samples show no DNA bands [6].

### Table 2. Reagent composition of LAMP detection

| Component                      | Stock Concentration | Work Concentration | Volume Needed Per Reaction (µL) |
|--------------------------------|---------------------|--------------------|---------------------------------|
| 2x Reaction Mix                | -                   | -                  | 12.5                            |
| Primer FIP                     | 40 mM               | 1.6 mM             | 1                               |
| Primer BIP                     | 40 mM               | 1.6 mM             | 1                               |
| Primer F3                      | 10 mM               | 0.2 mM             | 0.125                           |
| Primer B3                      | 10 mM               | 0.2 mM             | 0.125                           |
| FDR or HNB                     | -                   | -                  | 1                               |
| Bst DNA Polymerase             | 8 U/µL              | 8 U/µL             | 1                               |
| sterile water                  | -                   | -                  | 8.5                             |
| DNA Template                   | -                   | -                  | 2                               |
| Reaction total volume          | -                   | -                  | 25                              |

### 3. Results

#### 3.1. Detection of tungro virus using conventional PCR.

The presence of RTBV and RTSV in fresh samples was successfully detected by conventional PCR. Fresh sampling results using PCR showed that all positive rice samples were infected with RTBV. This is evident from the amplification results showing the same DNA fragment size of all isolates of about 1400 pb for RTBV (figure 1).
3.2. Detection of tungro virus using LAMP PCR.

The LAMP PCR method was tested to detect RTBV using fresh rice samples from Sidrap, Pesisir Selatan and Garut areas. All three samples gave positive reactions based on visualization of PCR LAMP results using agarose gel, FDR dye, and HNB dye. In the visualization results with the agarose gel a unique pattern of DNA bands, which resembles the DNA marker band pattern, in a positive test sample infected with RTBV (figure 2).

Optimization of LAMP PCR method is done by testing some incubation temperature and DNA dilution rate. Testing of some incubation temperatures showed that RTBV DNA amplification was successfully amplified at 60°C, 63°C, and 65°C (figure 2). However, the DNA bands generated at 63°C incubation temperature appear brighter and are more clearly separated than incubation at other temperatures, particularly in Sidrap isolate samples. These results prove that PCR LAMP can amplify DNA in isothermal conditions.

The positive reactions to the visualization results with FDR dyes observed with the naked eye are characterized by the discoloration of clear young orange to light green, whereas in the negative reaction the color remains unchanged, i.e remains clear young orange (figure 3a).
Positive reactions to visualization results with FDR dyes observed with UV light are characterized by discoloration from light green to light green as fluorescent because of flavorescens, whereas in negative color reactions does not change, i.e remain light green (figure 3b). Color change also occurs in positive reactions to visualization results with HNB dye, i.e from purple to light blue blues (figure 3c).

**Figure 3.** Comparison of visualization of LAMP PCR detection results with different dyes. (A) FDR is observed with the naked eye, negative samples are light orange clear (left) and positive samples turn yellowish (right), (b) FDR is observed with UV light, negative samples are light green (left) and samples positives change color to light green (right), (c) HNB is observed with the naked eye, negative samples are purplish blue (left) and positive samples change color to light blue (right).

In addition to temperature optimization, there is also optimization of DNA dilution levels to see the level of LAMP sensitivity. RTBV DNA dilution is performed up to $10^{-7}$. The result of the test shows that LAMP can still amplify sample DNA until $10^{-6}$ dilution, it can be seen from the color change that happened. RTBV DNA samples without dilution give the blue color younger than the other dilution series. The higher DNA concentration, the younger the resulting color (figure 4).

**Figure 4.** The sensitivity of LAMP at some level of DNA dilution, the more concentrated the DNA concentration, the younger the resulting color. (1) and (2) no dilution, (3) dilution $10^{-1}$, (4) dilution $10^{-2}$, (5) dilution $10^{-3}$, (6) dilution $10^{-4}$, (7) dilution $10^{-5}$, (8) dilution $10^{-6}$.

The LAMP PCR method has a higher sensitivity in detecting RTBV than conventional PCR methods (figure 5). As the test material is a sample of origin Sidrap with extraction DNA concentration of about 439.2 ng/µl. The conventional PCR method is only capable of detecting RTBV to $10^{-5}$ dilution or equivalent to 100 pg/µl, whereas the LAMP PCR method is able to detect up to $10^{-6}$ dilution or equivalent to 1 fg/µl (table 3). Garut and Pesisir Selatan isolates was also tested for the sensitivity of LAMP. The initial concentration of extracted DNA from both samples was 374.6 ng/µl and 386.5 ng/µl, respectively, lower than the initial concentration of extracted DNA from samples from Sidrap origin. The RTBV detection limit using both samples is 10-4 or equivalent 10 pg/µl and 10-5 or equivalent to 100 pg/µl, respectively with
conventional PCR and PCR LAMP. Thus, detection sensitivity using conventional PCR method and PCR LAMP is determined by the initial concentration of sample DNA.

Figure 5. Comparison of sensitivity of LAMP (top) and conventional PCR (bottom) detection techniques with the same dilution series using a Sidrap isolate sample confirmed by gel electrophoresis analysis. (-) negative control, (M) Marker, (1) dilution $10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$.

4. Discussion
Detection of the presence of *tungro virus* in samples collected from the field was successful. The detection results using PCR method showed that samples obtained during the positive survey were infected with *tungro virus*. Similarly, LAMP detection using fresh samples shows the same results as conventional PCR detection. All three samples detected with LAMP were also positively infected by RTBV.

Based on the optimization that has been done, it is seen that to obtain a good detection result then the sample is incubated at 63 °C for 60 minutes and termination at 80 °C for 10 minutes. This is consistent with the results of Le *et al.* [13] who successfully detected RTBV in rice plants in quick time, accurate results, and high levels of sensitivity and selectivity. As for visualization of incubation results, it is advisable to use HNB dyes because the color changes that occur can be observed with the naked eye, does not require UV light. Parida *et al.* [7] suggests that reading these LAMP results does not require expensive tools, since the use of dyes such as SYBR green, HNB, FDR, and Calcein facilitate the interpretation of detection results with the naked eye. Analysis with agarose gel was performed only to confirm the visualization results obtained.

The presence of RTBV in the sample of rice plants can be known in a short time (less than two hours) with more accurate results than conventional PCR, but with simple equipment. According to Parida *et al.* [7], the advantages of LAMP comparing to PCR detection are easier and faster procedures where amplification can be completed in less than an hour, the entire incubation process is reagent in just one tube. Mori *et al.* [10] adds another advantage of LAMP that is its ability to amplify specific DNA sequences under isothermal conditions.
LAMP sensitivity is higher than conventional PCR. This is evident from the comparison of conventional LAMP and PCR detection with several series dilutions of DNA sample concentrations. LAMP can still detect DNA samples at concentrations of 10-5. This is supported by Le et al. [13] that detected rice dwarf virus (RDV), rice stripe virus (RSV) and rice transitory yellowing virus (RTYV) with RT-LAMP using samples with up to 10-5 dilutions. Notomi et al. [6] adds that the sensitivity of LAMP is also high because it can still amplify DNA with low concentrations.

Table 3. Comparison of LAMP and PCR sensitivity with five levels of DNA dilution

| RTBV Isolate | Parameter | 10^0 | 10^-1 | 10^-2 | 10^-3 | 10^-4 | 10^-5 | 10^-6*** |
|--------------|----------|------|-------|-------|-------|-------|-------|----------|
| Garut        | DNA concentration | 374.6 | 37.46 | 3.746 | 0.38 | 0.038 | 0.004 | 0.0004   |
|              | LAMP     | +    | +     | +     | +    | +     | -     |           |
|              | PCR      | +    | +     | +     | +    | +     | -     |           |
| Sidrap      | DNA concentration | 439.2 | 43.92 | 4.39  | 0.439 | 0.044 | 0.004 | 0.0004   |
|              | LAMP     | +    | +     | +     | +    | +     | +     |           |
|              | PCR      | +    | +     | +     | +    | +     | -     |           |
| Pesisir     | DNA concentration | 386.5 | 38.65 | 3.87  | 0.387 | 0.039 | 0.004 | 0.0004   |
| Selatan     | DNA concentration | 374.6 | 37.46 | 3.746 | 0.38 | 0.038 | 0.004 | 0.0004   |

*10^0 = 1 nanogram/µl, **10^-3 = 1 picogram/µl, ***10^-6 = 1 femtogram/µl
+ = infected by RTBV, - = uninfected by RTBV

5. Conclusion

Based on the sensitivity and accuracy, LAMP can be used as an alternative to detect rice tungro virus. LAMP detection will give better results if the test sample was incubated at 63 ° C for 60 minutes and terminated at 80 ° C for 10 minutes. To facilitate the application in the field it is advisable to use HNB dyes because the color changes that occur can be observed with the naked eye, does not require UV light. The sensitivity of LAMP is still higher than that of conventional PCR.

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