Development of a Loop-Mediated Isothermal Amplification Assay for Detection of Tomato Leaf Curl New Delhi Virus in Ridge Gourd [Luffa acutangula (L) Roxb.]

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A B S T R A C T

Ridge gourd yellow mosaic disease (RgYMD) caused by the strain of Tomato leaf curl New Delhi virus (ToLCNDV) is an emerging disease in India. Early detection of ToLCNDV in ridge gourd has great significance in the management of the disease. An innovative loop-mediated isothermal amplification (LAMP) assay was standardized for rapid detection of ToLCNDV. Assay was carried out using the set of six primers (F3, B3, FIP, BIP, LF and BF) specific to the coat protein (CP) gene of the virus. Reaction time, temperature, primers concentration and DNA dilutions were optimized for detection of ToLCNDV. The reaction was optimized in a single tube at 63 °C for 45 min and terminated by keeping it at 80 °C for 10 min. The sensitivity of LAMP based detection assay was more than 100 times as that of conventional PCR. The LAMP primers specifically targeted to the CP gene of ToLCNDV resulted in typical water fall like bands by the agarose gel electrophoresis. The amplified LAMP products were also identified by visualization with dyes. No amplification was observed in DNA from tissues of healthy plants and water control either by LAMP or PCR assays. The LAMP assay was validated by testing field samples of RgYMD collected from various ridge gourd growing areas in southern India. The results indicated that the LAMP assay standardized will be useful for monitoring and detection of ToLCNDV associated with RgYMD. The assay has many advantages over PCR with respect to cost, accuracy, sensitivity and specificity.

Keywords
Begomovirus; Coat protein gene; LAMP assay; Ridge gourd; Yellow mosaic disease.

Introduction

Ridge gourd [Luffa acutangula (L.) Roxb.] is one of the important vegetables in the daily food chain of southern and eastern parts of India. The crop is known to be affected by several plant diseases such as downy mildew (Pseudoperonospora cubensis), powdery
mildew (*Sphaerotheca fulginea*), Pythium rot (*Pythium butleri*), collar rot (*Rhizoctonia solani*) (Gopalakrishnan, 2007) and RgYMD (Patil et al., 2017) and thus limiting the crop yield.

The RgYMD is more prevalent in southern India with the disease incidence up to 100 per cent (Patil et al., 2017). The disease is associated with the strain of ToLCNDV in India and transmitted by whitefly *Bemisia tabaci* (Gennadius). The RgYMD affected plants display mosaic with light chlorotic areas on the leaf lamina, mottling, reduction in leaf size, crinkling, blistering of leaves, leaf distortion, finally leading to downward and upward curling and stunting of plant (Patil et al., 2017). ToLCNDV belongs to genus Begomovirus under family Geminiviridae. Members of Geminiviridae have small circular single stranded DNA (ssDNA) with distinctive twinned isometric particles of 20 nm x 30 nm size. The family Geminiviridae consists of nine genera viz., *Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus* and *Turncurtovirus* (Zerbini et al., 2017). The begomovirus has either monopartite genome having ss DNA component of about 2.7 kb designated as DNA A or bipartite genome having two similar size components designated as DNA A and DNA B (Lazarowitz and Lazdins 1991; Rybicki 1994). The DNA A component has five open reading frames (ORFs) encoding all the factors required for virus replication, overcoming host defenses, insect transmission and control of gene expression. The DNA B component has two ORFs encoding factors required for inter- and intra-cellular movement in host plants (Rojas et al., 2005).

Several serological (DAS-ELISA, DAC-ELISA, TAS-ELISA, tissue blotting immune binding assay etc) and molecular (PCR, RCA-PCR, qPCR, microarray, dot blot hybridization etc.) (Kushwaha et al., 2010) diagnostic methods for begomoviruses detection have been developed. Although these methods are sensitive and specific, they are either complicated or expensive and need equipped laboratories, technical experts and more time (De la Pena and Flores 2002). To address these limitations, Notomi et al., (2000) developed an alternative isothermal molecular detection technique *i.e.* LAMP. The LAMP assay can specifically amplify DNA sequences by using a set of six primers, which recognize six distinct regions on the target DNA. In the LAMP reaction, nucleic acids amplify rapidly in a standard heat block or water bath under isothermal conditions with a temperature range of 60 °C – 65 °C (Mori et al., 2001; Nagamine et al., 2001; Ihira et al., 2004; Soliman and El-Matbouli 2005) and does not require a thermocycler (Notomi et al., 2000; Tomlinson et al., 2010). It relies on strand displacement DNA synthesis performed by *Bst* DNA polymerase (Notomi et al., 2000; Nagamine et al., 2002). Unlike other nucleic acid amplification techniques, the LAMP assay has advantages of higher simplicity with good amplification efficiency (Parida et al., 2008). The LAMP can be monitored in different ways; gel electrophoresis to view LAMP amplicons; through visual inspection by inspecting turbidity (Mori et al., 2001), intercalating dyes (Maeda et al., 2005) or colorimetric indicators (Iwamoto et al., 2003; Goto et al., 2009). This assay was found to be a highly versatile diagnostic tool and has been used in the detection of a wide range of pathogens (Parida et al., 2008).

The LAMP assay was utilized for detection plant pathogenic DNA viruses such as banana bunchy top virus (BBTV) (Peng et al., 2012), squash leaf curl virus (SLCV) (Kuan et al., 2010), tomato yellow leaf curl virus (TYLCV) (Fukuta et al., 2003; Almasi et al., 2013), ToLCNDV in potato (Jeevalatha et al., 2017) and tomato leaf curl Bangalore virus
(ToLCBaV) in tomato (Arutselvan et al., 2017). With this backdrop, we attempted to standardize LAMP assay for rapid and efficient detection of ToLCNDV associated with RgYMD in the present study.

**Materials and Methods**

**Virus samples**

The ToLCNDV associated with RgYMD characterized from the leaf sample collected from Sanganakeri village, Gokak (taluk), Belgaum (district), Karnataka, India (Patil et al., 2017) was used for standardization of LAMP and PCR assays. For the validation of LAMP assay, RgYMD affected ridge gourd leaf samples were collected from the different locations in southern India (Patil et al., 2017).

**DNA isolation**

The total DNA was extracted from leaves of healthy ridge gourd and RgYMD affected ridge gourd by following method developed by Lodhi et al., (1994) and Maruthi et al., (2002). One hundred milligrams of fresh ridge gourd leaf tissue samples were ground with 1000 µl of pre-warmed (60 °C) DNA extraction buffer (2 per cent CTAB (w/v); 1.4 M NaCl; 20 mM EDTA of pH 8.0; 100 mM Tris-HCl of pH 8.0; 10 per cent polyvinyl pyrrolidone and 0.2 per cent mercaptoethanol (added in situ just before DNA extraction)) using sterile mortar and pestle. The extracted crude sap was transferred into a fresh 1.5 ml micro centrifuge tube and incubated for 30 min at 60 °C in a water bath with occasional mixing. The supernatant (750 µl) without disturbing the debris was transferred into a fresh 1.5 ml micro centrifuge tube and mixed with an equal amount (750 µl) of chloroform: isoamyl alcohol (24:1) by vortexing. The samples were then centrifuged at 13000 rpm for 10 min using micro centrifuge. The aqueous supernatant was collected into a fresh 1.5 ml micro centrifuge tube. The DNA was precipitated by mixing with 300 µl of chilled isopropanol by inversion and incubated at -20 °C at least for one hour. The tubes were centrifuged at 13000 rpm for 10 min. The resulted pellet was washed with 70 per cent ethanol, dried in a vacuum drier for 10 min and re-suspended with 50 µl of T10E1 buffer (10 mM Tris-HCl of pH 8.0 and 0.1 mM EDTA of pH 8.0). The quality and quantity of DNA were assessed at 260 nm and 280 nm using UV spectrophotometer. Stored at -20 °C for further use.

**LAMP primers design**

The specific LAMP primers were designed based on sequence of CP gene of ToLCNDV associated with RgYMD (Patil et al., 2017) by using the Primer Explorer V5 software (http://primerexplorer.jp/e) program. The primers sequences and their respective binding sites are shown in the Fig 1. All the primers were selected based on the criteria described by Notomi et al., (2000) and their specificity was confirmed with BLAST search (Standard nt BLAST available at http://blast.ncbi.nlm.nih.gov/blast.cgi). The primers were synthesized at GCC Biotech (India) PVT Ltd, Parganas, West Bengal, India.

**PCR assay for amplification of coat protein gene of ToLCNDV**

The PCR amplification of CP gene of ToLCNDV was carried out by using the set of CP gene primers (Sohrab et al., 2010) which were used to detect ToLCNDV. PCR reaction was carried out in a thermal cycler (Eppendorf, Cambridge, UK) with the following conditions; hot start at 92 °C for 2 min, followed by 35 cycles of denaturation at 90 °C for 1 min, annealing at 40 °C for 30 seconds and extension at 72 °C for 2 min followed by final extension at 72 °C for 10 min. A total of 25 µl PCR reaction mixture contained 12.5 µl of 2x Fast PCR mix.
(Thermoscientific Pvt Ltd), 2 µl of each forward and reverse primer (10µM), 2 µl of genomic DNA (100 ng/µl) and 6.5 µl of nuclease free water. The PCR was also carried out with two LAMP primers i. e. F3 and B3 as forward and reverse primers, respectively.

**LAMP-based detection**

The LAMP assay was performed in a 0.5 ml PCR tubes by keeping them in a water bath. The 25 µl LAMP reaction mixture contains 1.0 µl (10 µM) of F3, B3, 2.0 µl of (10µM) FIP, BIP, LF and LB primers, 1.5 µl of 10 mM dNTPs, 1.0 µl of 5 M betaine, 6.5 µl of H$_2$O, 2.5 µl of 1x ThermoPol Reaction buffer (20 mM Tris-HCl, 10 mM (NH$_4$)$_2$SO$_4$, 10 mM KCl, 2 mM MgSO$_4$, 0.1% Triton X-100, pH 8.8 @ 25 °C), 0.5 µl of 100 mM MgSO$_4$ and 1.0 µl of 8U Bst DNA Polymerase (New England Biologicals, USA).

The reaction mixture was incubated at 63 °C for 45 min followed by 80 °C for 10 min to terminate the reaction. The DNA from the healthy sample and distilled water were included as negative controls.

The LAMP reaction products were analyzed on two per cent agarose gel with 65 volts for 3 h and then visualized using ethidium bromide stain under alpha imager gel documentation system. DNA ladder set (100 bp, G-Biosciences, USA) was included as a sized molecular marker.

Naked eye inspection by turbidity and nucleic acid staining was done with ethidium bromide, hydroxynapthol blue, VeriPCR and thiazole orange. Details of the dyes used in the present study were given in the Table 1.

**Results and Discussion**

**LAMP primers**

Based on the information of ToLCNDV genome sequence associated with RgYMD, CP gene was selected for LAMP primer designing. The LAMP primer sets (F3, B3, FIP, BIP, LF and LB primers) were designed using the 771 nucleotides (nt) of CP gene of ToLCNDV (Fig. 1).

The Tm value, GC content, primer position and primer length (nt) were given in the Supplementary Table 2. The nt sequence of the LAMP amplicon and the LAMP primers were BLAST searched against the NCBI database to ensure their specificity. All the sequences were found to have 100 percent nt sequence identity to the corresponding to the CP gene of several ToLCNDV isolates.

**Optimization of LAMP**

Using DNAs extracted from RgYMD affected leaves, the LAMP reactions were carried out at different temperatures and products were detected at 57, 59, 61, 63 and 65 °C (Fig. 2). Lower amplification was found when the reactions were performed at 57 °C for 45 min. No difference was found when the reactions were performed at 57 °C, 59 °C, 61°C, 63 °C and 65 °C and no amplification was observed at 67 and 69 °C.

In order to find out optimum reaction time, amplified products were detected at 15 to 90 min. The optimum reaction times for the LAMP reactions were 30, 45 and 60 min (Fig. 3). Amplified products were observed as early as 15 min. The positive reactions of LAMP were obtained using outer and inner primer ratios ranging from 1:1 to 1:8. A more distinct and optimum patterns were seen when outer and inner primer ratios were from 1:1 to 1:4 (Fig. 4).

All subsequent experiments were conducted for 45 min reaction time, at reaction temperature of 63 °C with 1:2 outer and inner primer ratios to ensure optimum sensitivity.
Comparison of sensitivity between LAMP and PCR assay

The comparative sensitivities of LAMP and PCR assays were detected by using serial dilutions of total DNA extracted from RgYMD affected ridge gourd leaf tissue. Dilutions were made using double distilled water and range from $10^0$ (100 ng/µl) to $10^{-10}$. Results showed that the virus was detected by both LAMP (Fig. 5a) and PCR up to DNA dilution of $10^{-6}$ (Fig. 5b). However, LAMP assay (Fig. 5a) was able to detect the virus up to the DNA dilution of $10^{-10}$ but the PCR failed to detect beyond the dilution of $10^{-6}$ (Fig. 5b).

Specificity of LAMP detection

The results of optimized LAMP assay analyzed by agarose gel electrophoresis revealed the successful amplification of CP gene of ToLCNDV from the DNA extracted from RgYMD affected plant leaf tissue (Fig. 6A). No amplification was observed from the DNA of healthy sample and water control. The LAMP amplified products were also tested by visual inspection with ethidium bromide, hydroxynapthol blue, VeriPCR and thiazole orange, which also showed positive results (Fig. 6B). This was further confirmed with PCR assay (Fig. 6C).

Validation of LAMP assay against the RgYMD affected field samples of ridge gourd

The standardized LAMP assay was validated against the twenty leaf samples of ridge gourd showing RgYMD and suspected to be infected with ToLCNDV collected from different locations of southern India (Table 3). All the samples showed positive amplification in the LAMP assay. This was further confirmed by PCR assay. Representative agarose gel showing amplification of ToLCNDV in field samples through PCR and LAMP assays were shown in the Fig 7A and Fig 7B respectively.

Ridge gourd is one of the important vegetable crops grown across the world. It is affected by many diseases, of which RgYMD caused by the strain of ToLCNDV is wide spread in southern India (Patil et al., 2017). The ToLCNDV was initially identified on solanaceous crops in India (Padidam et al., 1995) and later reported in 43 diverse plant species belong to different families including Caricaceae, Cucurbitaceae, Euphorbiaceae, Solanaceae, Malvaceae and Fabaceae in Pakistan, India, Bangladesh, Iran, Sri Lanka, Malaysia, Taiwan, Thailand, Indonesia, Tunisia, Philippines, Spain Mediterranean basin and Italy (Fortes et al., 2016; Morines et al., 2017; Zaidi et al., 2017). The PCR, non-radioactive nucleic acids spot hybridization, squash and tissue-print PCR, immunocapture PCR, real time PCR and RCA-PCR were used for detection of ToLCNDV (Tiwari et al., 2010; Sridhar et al., 2016; Venkatasalam et al., 2011; Jeevalatha et al., 2013; 2014; 2016). Most of them were time consuming and required sophisticated equipment. Thus we developed LAMP assay for specific detection of ToLCNDV associated with ridge gourd.

The LAMP primers were designed based on CP gene sequence of ToLCNDV associated with RgYMD (Patil et al., 2017). Reaction time, temperature, primers concentration and DNA dilution were optimised for quick, sensitive and specific detection of ToLCNDV- (Ridge gourd). The results of temperature optimization showed detectable amplification at 57 to 65 °C. Which is same with the most of the studies indicated earlier (Bhat et al., 2013; Banerge et al., 2016; Jeevalatha et al., 2017). Low and higher temperature resulted in lower or no amplification, it might be due to inactivation of enzyme or reaction instability caused by too high or low temperature (Jeong et al., 2015).
Table 1: Details of dyes used in LAMP assay

| Sl. No. | Name of the dye          | Company                                      | Stock Conc. | Volume per reaction (µl) | Need to UV ray | Use time              | Positive reaction       |
|--------|--------------------------|----------------------------------------------|-------------|--------------------------|----------------|-----------------------|-------------------------|
| 1      | VeriPCR                  | Chromus Biotech PVT Ltd, Bengaluru, India    | 1x          | 5.0                      | Yes            | After amplification   | Green Fluorescent       |
| 2      | Ethidium bromide         | Sisco Research Laboratories Pvt. Ltd, Mumbai, India | 10 mg/ml    | 1.0                      | Yes            | After amplification   | Red Fluorescent         |
| 3      | Hydroxy naphthol blue    | Sigma Aldrich, USA                           | 20 mM       | 0.2                      | No             | Before amplification  | Sky blue                |
| 4      | Thiazole orange          | Sigma Aldrich, USA                           | 1 mM        | 1.0                      | Yes            | After amplification   | Light orange            |

Table 2: Information of the primers used for loop mediated isothermal amplification of ToLCNDV associated with Ridge gourd yellow mosaic disease

| Name of the primer | Type        | Sequence (5’-3’)                                                                 | Primer position | Primer length (nt) | GC (%) | Tm      |
|--------------------|-------------|--------------------------------------------------------------------------------|-----------------|--------------------|--------|---------|
| F3                 | Forward outer | TCGAAGCGACCAGCAGAT                                                             | 4-21            | 18                 | 56.0   | 60.33   |
| B3                 | Backward outer | CTTACACGGGCCTTCACAG                                                            | 201-219         | 19                 | 58.0   | 59.66   |
| FIP (F1c+F2)       | Forward inner | CGTGTTCATAGGGGCTGTCG-CATTTCAACTCCCGCATCGA                                      | (69-89)+(27-46) | 41                 | 62-50  | 64.66-60.14 |
| BIP (B1c+B2)       | Backward inner | CAAAAGCAAGGCGCTGGA CGAA-TGGGACGTCGACTTTCT                                      | (116-137)+(178-195) | 40                 | 55-56  | 65.64-59.9 |
| LF                 | Loop forward  | TTGAGCCGTCGGCGTACCT                                                           | 47-65           | 19                 | 63.0   | 65.56   |
| LB                 | Loop backward | CAGGGCCCATGAACAAGAAA ACC                                                        | 138-158         | 21                 | 52.0   | 61.4    |
Table 3 Detection of ToLCNDV infection in field collected ridge gourd samples using optimized LAMP or conventional PCR assays

| Sample No. | Collection site                                          | Detection of ToLCNDV through LAMP assay | Detection of ToLCNDV through PCR assay |
|------------|----------------------------------------------------------|----------------------------------------|---------------------------------------|
|            | **Karnataka state**                                     |                                        |                                       |
| 1          | Chabbhi, Bagalkot taluk, Bagalkot district               | +                                      | +                                    |
| 2          | Lakhamapur, Badami taluk, Bagalkot                      | +                                      | +                                    |
| 3          | Bidari, Jamakhandi taluk, Bagalkot                       | +                                      | +                                    |
| 4          | Sanganakeri, Gokak taluk, Belagavi                       | +                                      | +                                    |
| 5          | Badachi, Athani taluk, Belagavi                          | +                                      | +                                    |
| 6          | Gorbal, Savadhati taluk, Belagavi                        | +                                      | +                                    |
| 7          | United Genetics Company, Hesaraghatta, Bengaluru urban district | +                                      | +                                    |
| 8          | Janakhirama, Devanahalli, Bengaluru rural district       | +                                      | +                                    |
| 9          | Muddebihal, Muddebihal taluk, Bijapur district           | +                                      | +                                    |
| 10         | Atharga, Indi taluk, Bijapur district                    | +                                      | +                                    |
| 11         | Gondivarihalli, Chintamani taluk, Chikkaballapur district | +                                      | +                                    |
| 12         | Suravanne, Honnali taluk, Davanagere district            |                                        |                                       |
| 13         | Jalihal, Ron taluk, Gadag district                       | +                                      | +                                    |
| 14         | Bhatrahalli, Kolar taluk, Kolar district                 | +                                      | +                                    |
| 15         | Hallikere, Maddur taluk, Mandya district                 | +                                      | +                                    |
| 16         | Sindhuvalli, Nanjangudu taluk, Mysore district           | +                                      | +                                    |
| 17         | Handralu, Madugiri taluk, Tumukur district               | +                                      | +                                    |
|            | **Andhra Pradesh state**                                 |                                        |                                       |
| 18         | Poragi, Hindupur taluk, Ananthapur district              | +                                      | +                                    |
| 19         | Vempalle, Madanpalli taluk, Chittur district             | +                                      | +                                    |
|            | **Tamil Nadu state**                                     |                                        |                                       |
| 20         | Bagalur, Hosur taluk, Krishnagiri district               | +                                      | +                                    |
Fig. 1 Schematic representation of position and sequence of primers within the nucleotide sequence of coat protein gene of ToLCNDV associated with ridge gourd yellow mosaic disease used for LAMP assay. Arrows and box indicate the position of the target sequences.

Fig. 2 Effect of temperature on LAMP reaction. Lane M: 100 bp DNA marker; lanes indicate temperature of 57, 59, 61, 63, 65, 67 and 69 °C.
Fig. 3 LAMP amplification results using ToLCNDV infected DNA obtained at different reaction times and its visualization on 2% agarose gel. Lane M: 100 bp DNA marker; Lane W-Water control; H- Healthy ridge gourd; lanes indicate LAMP reaction time of 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min.

Fig. 4 Effect of outer and inner primers concentration on LAMP amplification. Lane M: 100 bp DNA marker; lanes 1 & 2: 1:1; lanes 3 & 4: 1:2; lanes 4 & 6: 1.4;lanes 7 & 8: 1:8
**Fig. 5** Sensitivity analysis of optimized LAMP assay (A) for detection of ToLCNDV and was compared with PCR assay (B) on agarose gel electrophoresis. Lane M: 100 bp DNA marker; Lane W: Water control; H: Healthy; lanes 1 to 11 are serial dilutions of DNA from $10^0$ to $10^{-10}$.

**Fig. 6** LAMP assay for detection of ToLCNDV in ridge gourd. Visualization: A) After 2% agarose gel electrophoresis, B) examining with dyes a) Ethidium bromide, b) VeriPCR, c) Hydroxy napthol bule and d) Thiazole orange. C) Detection of ToLCNDV in ridge gourd through PCR. Lane M: 100 bp DNA marker, Lane W: water control, H: healthy ridge gourd, Lanes 1 -4: RgYMD affected ridge gourd samples.
Fig. 7 Representative agarose gel showing amplification of ToLCNDV in ToLCNDV infected ridge gourd samples collected from ridge gourd in different locations in southern India through LAMP assay (A) and PCR assay (B). Lane M: 100 bp DNA marker, Lane W: Water control; H: healthy ridge gourd, RgI: Positive control; Lanes 1-11; RgYMD affected ridge gourd samples from different locations in southern India (Table 3).

In the present study, three optimum reaction times (30, 45 and 60 min.) were showed good amplification. However several reports revealed that 60 min as optimum reaction time for detection of plant pathogens (Kuan et al., 2010; Almasi et al., 2013; Arutselvan et al., 2017; Jeevalatha et al., 2017). The LAMP assay has indicated high specificity in detection of plant viruses (Fan et al., 2013; Almasi et al., 2013; Arutselvan et al., 2017; Jeevalatha et al., 2017) which was evident in the present study showing 100 times more sensitivity than traditional PCR assay. Traditionally gel electrophoresis followed by ultraviolet imaging are used to check amplification of LAMP products, addition of dyes allows formation of the products to be easily observed with the naked eye (Iwamoto et al., 2003; Goto et al., 2009; Almasi et al., 2013; Wang et al., 2013). The visual inspection to save the time required for detection by avoiding gel electrophoresis was carried out using nucleic acid stains. Higher sensitivity was observed in detection of LAMP products by colour change method using hydroxy napthol blue, veriPCR, ethidium bromide and thioazloe orange. The colour differences were observed between
healthy, water control and ToLCNDV infected LAMP products indicating the possibility of using them in quick detection of plant pathogens.

In the present study, we report optimization of LAMP assay for specific detection of ToLCNDV in ridge gourd. The standardized assay could successfully detect ToLCNDV in RgYMD affected samples collected from different locations in southern India. This is the first report of optimization of LAMP assay for detection of ToLCNDV associated with RgYMD.

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