*Int.J.Curr.Microbiol.App.Sci* (2019) *8*(9): 2282-2295

EXCELLENT PUBLISHERS

*International Journal of Current Microbiology and Applied Sciences*
*ISSN: 2319-7706* **Volume 8 Number 09 (2019)**
Journal homepage: http://www.ijcmas.com

**Original Research Article** https://doi.org/10.20546/ijcmas.2019.809.264

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

**Priya Naganur[1, 2], U. Premchand[1], K. S. Shankarappa[1*], R. K. Mesta[2], C. Manjunatha[3] and C. V. Patil[1]**

[1]*Department of Plant Pathology, College of Horticulture, Bengaluru-560 065, University of Horticultural Sciences, Bagalkot, Karnataka, India*
[2]*Department of Plant Pathology, College of Horticulture, Bagalkot - 587101, University of Horticultural Sciences, Bagalkot, Karnataka, India*
[3]*ICAR- Indian Agricultural Research Institute, Regional Station, Wellington - 643 231, Tamil Nadu, India*

**Corresponding author*

## ABSTRACT

**Keywords**

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

**Article Info**

***Accepted:***
22 August 2019
***Available Online:***
10 September 2019

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.

## 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 (*Pseudoperonspora 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 isotheral 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 pyrolidone 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 $T_{10}E_1$ 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 $H_2O$, 2.5 µl of 1x ThermoPol Reaction buffer (20 mM Tris-HCl, 10 mM $(NH_4)SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8 @ 25 °C), 0.5 µl of 100 mM MgSO4 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 | CGTGTTCCATAGGGGCTGTCG-CATTTCAACTCCCGCATCGA | (69-89)+(27-46) | 41 | 62-50 | 64.66-60.14 |
| **BIP (B1c+ B2)** | Backward inner | CAAAAGCAAGGGCCTGGACGAA-TGGCACGTCTGGACTTCT | (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 | CAGGCCCATGAACAGAAAACC | 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 | 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

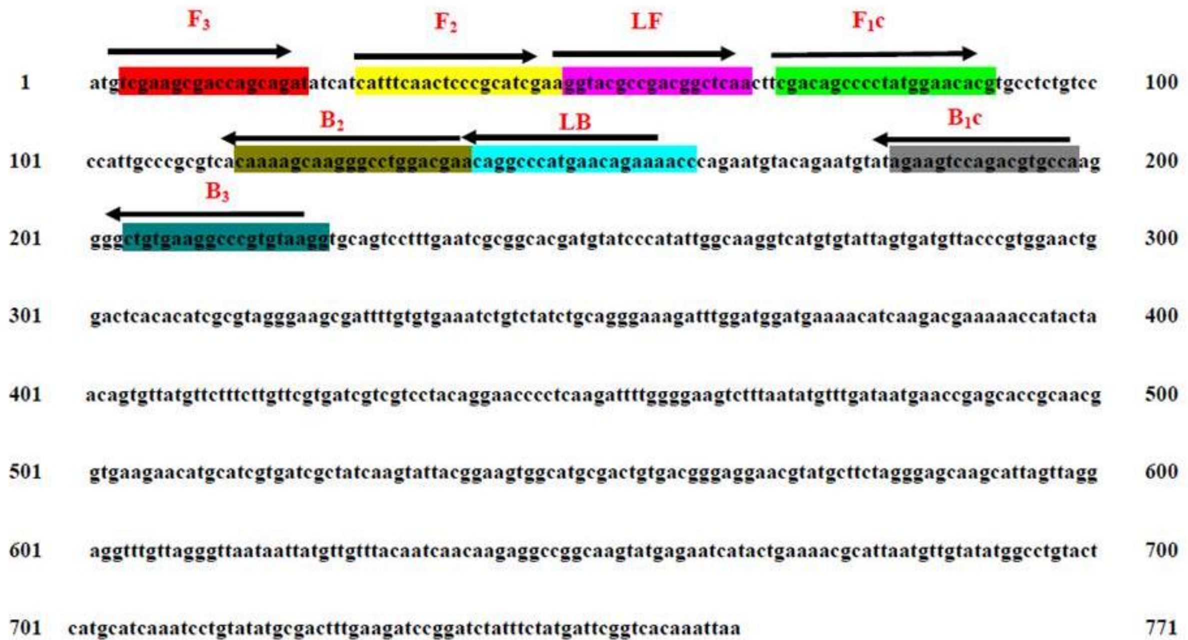



**Fig. 2** Effect of temperature on LAMP reaction. Lane M: 100 bp DNA marker; lanes indicate temperature of 57, 59, 61, 63, 65, 67 and 69 °C.

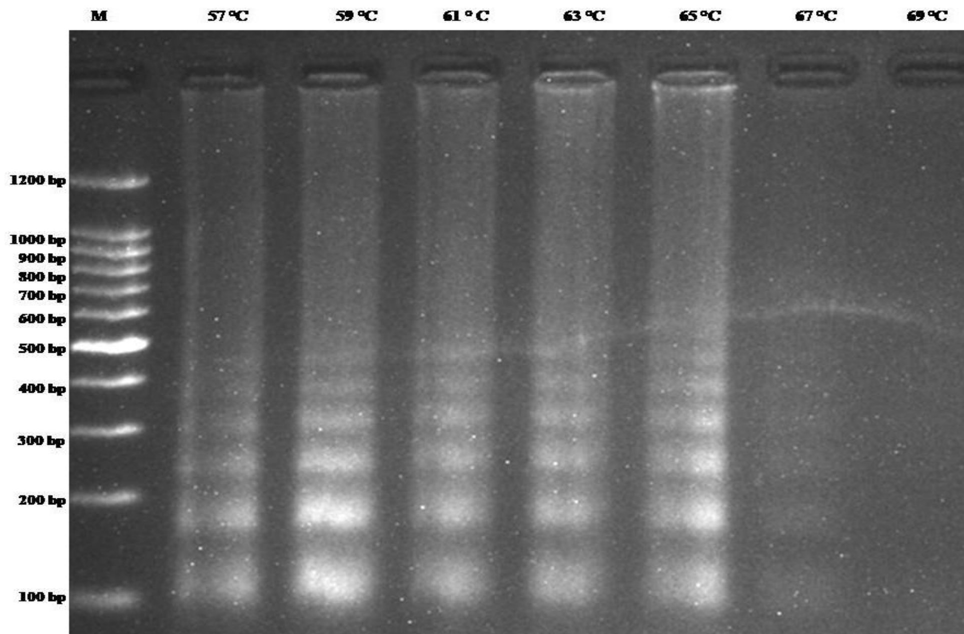


**Fig. 3** LAMP amplification results using ToLCNDV infected DNA obtained at different reaction times and its visualization on 2% agarose gel. Lane M: 100 bp DNA marker; Lane W-Water control; H- Healthy ridge gourd; lanes indicate LAMP reaction time of 5, 10, 15, 20, 25, 30, 45, 60, 75 and 90 min.

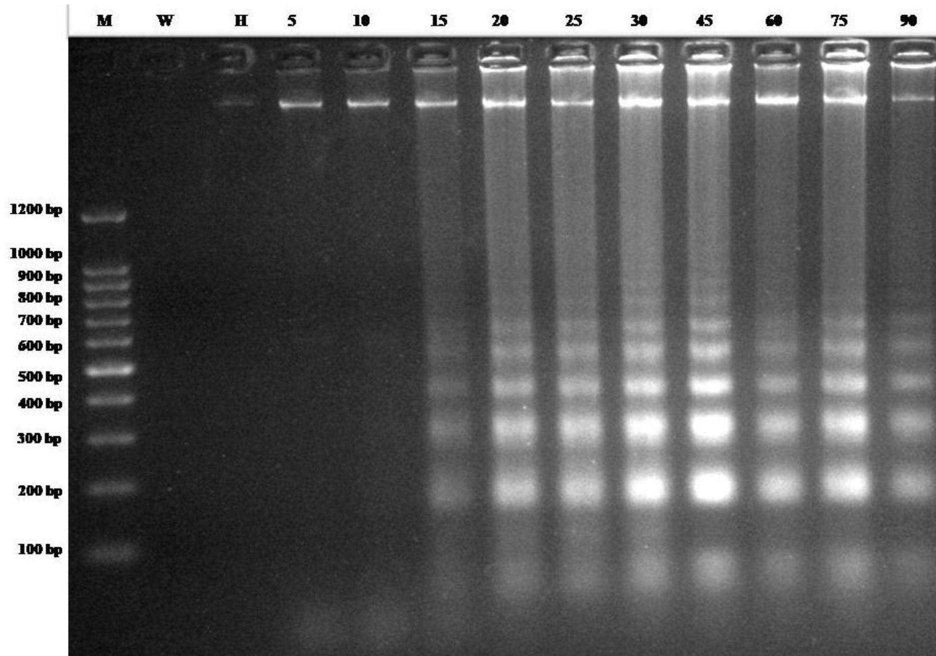



**Fig. 4** Effect of outer and inner primers concentration on LAMP amplification. Lane M: 100 bp DNA marker; lanes 1 & 2: 1:1; lanes 3 & 4: 1:2; lanes 4 & 6: 1.4;lanes 7 & 8: 1:8

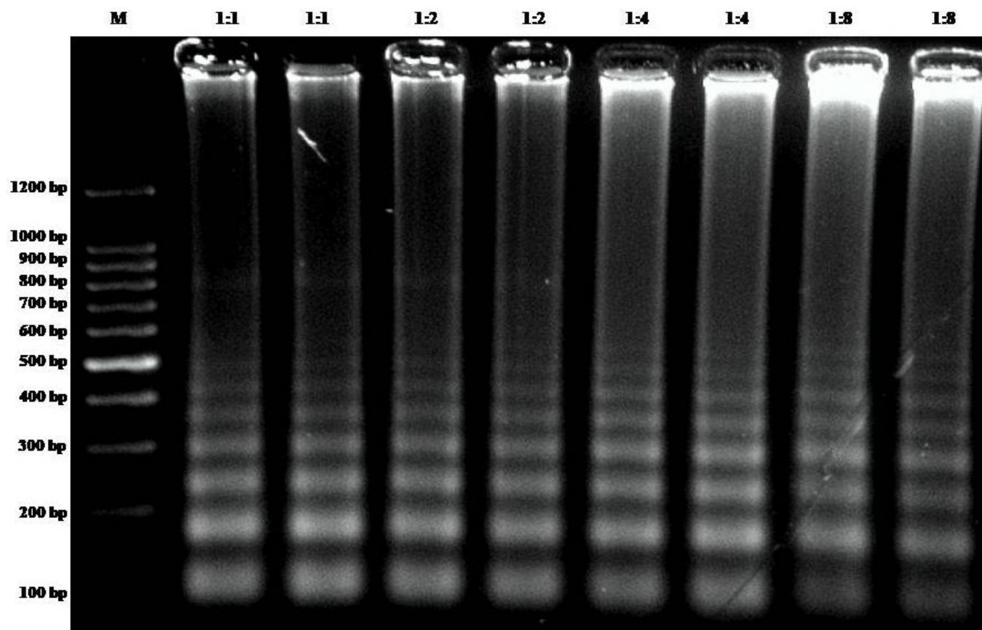


**Fig. 5** Sensitivity analysis of optimized LAMP assay (A) for detection of ToLCNDV and was compared with PCR assay (B) on agarose gel electrophoresis. Lane M: 100 bp DNA marker; Lane W- Water control; H- Healthy; lanes 1 to 11 are serial dilutions of DNA from $10^0$ to $10^{-10}$

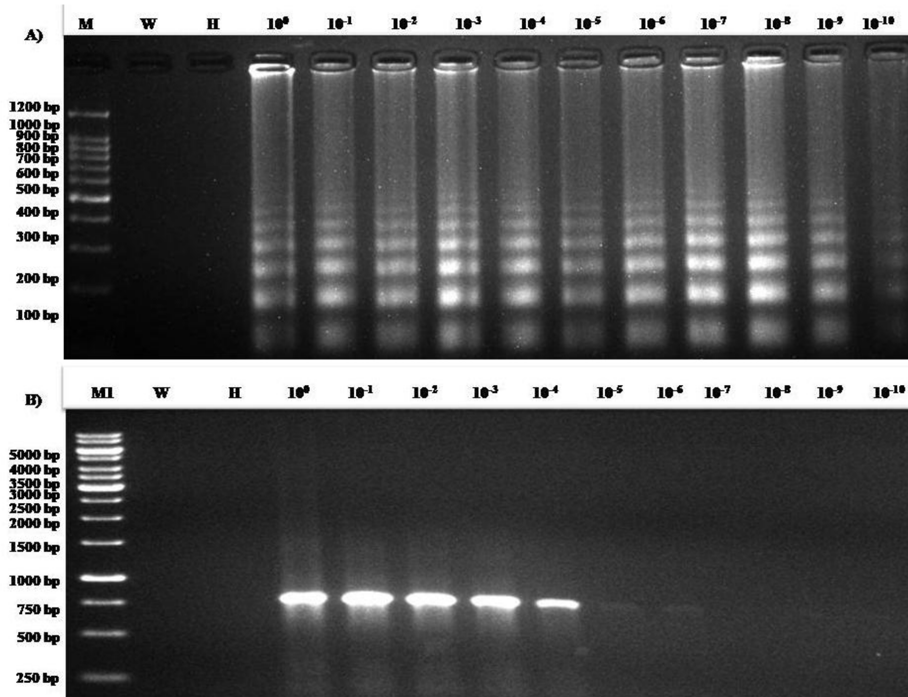



**Fig. 6** LAMP assay for detection of ToLCNDV in ridge gourd. Visualization: A) After 2% agarose gel electrophoresis, B) examining with dyes a) Ethidium bromide, b) VeriPCR, c) Hydroxy napthol bule and d) Thiazole orange. C) Detection of ToLCNDV in ridge gourd through PCR. Lane M: 100 bp DNA marker, Lane W: water control, H: healthy ridge gourd, Lanes 1 -4; RgYMD affected ridge gourd samples

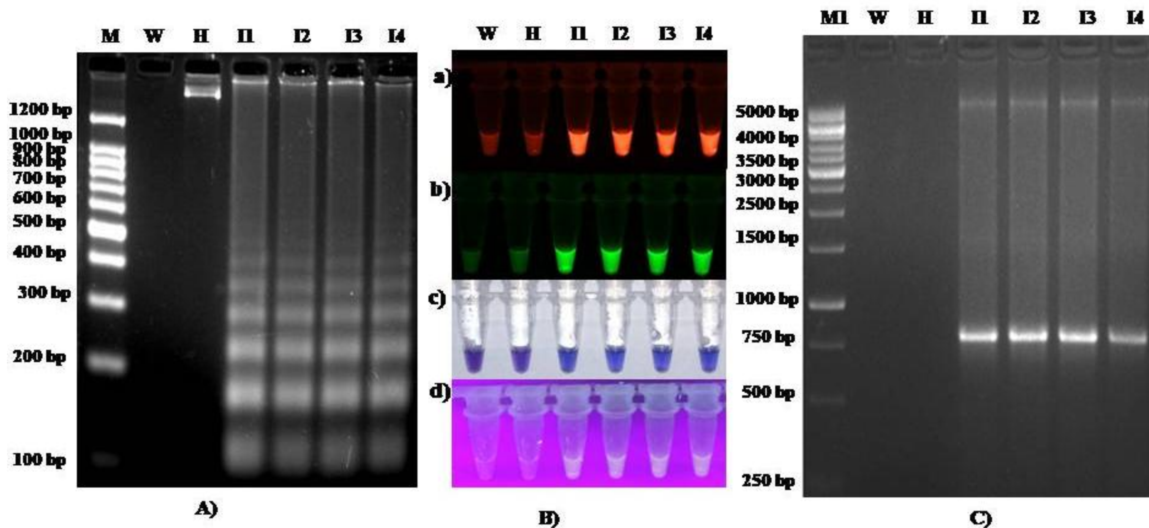
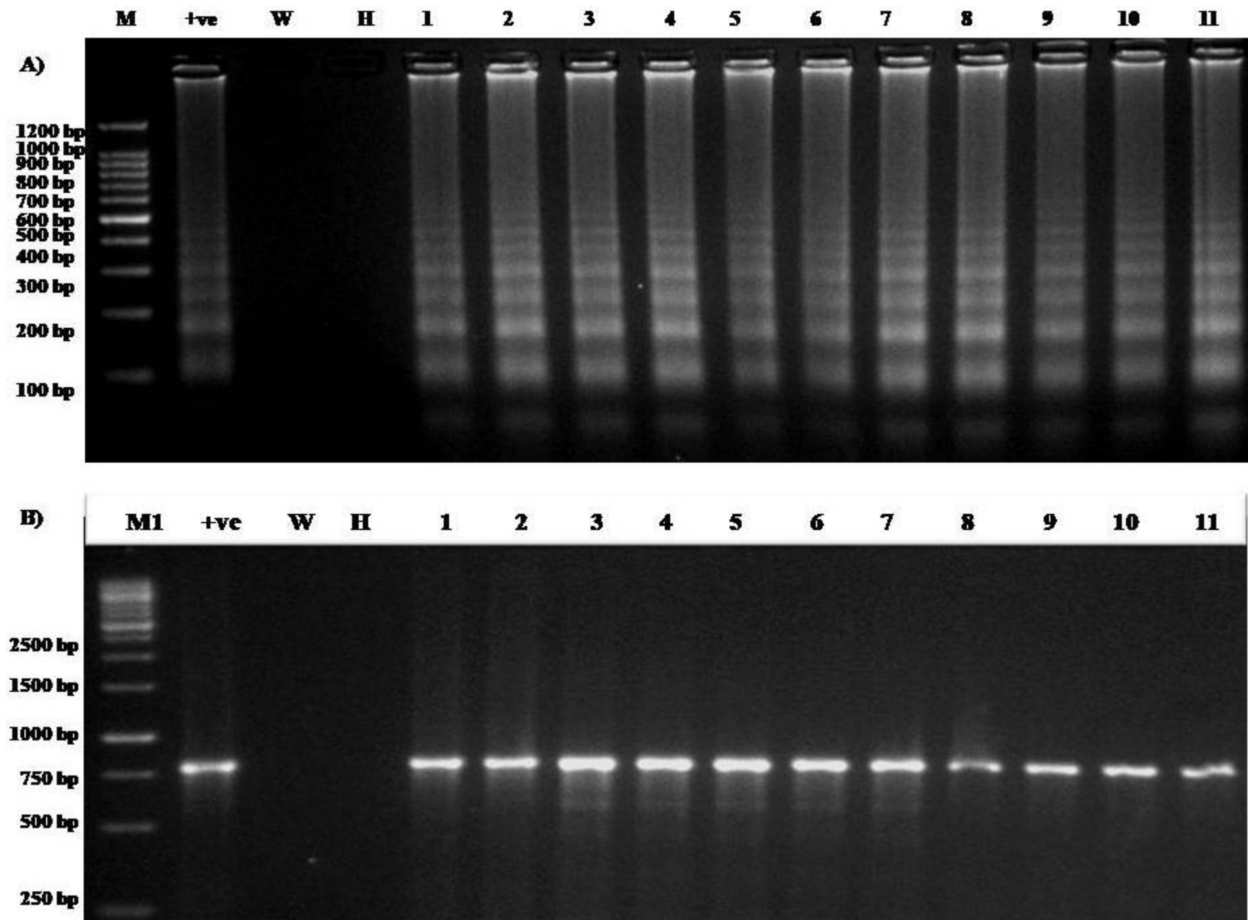


**Fig.7** Representative agarose gel showing amplification of ToLCNDV in ToLCNDV infected ridge gourd samples collected from ridge gourd in different locations in southern India through LAMP assay (A) and PCR assay (B). Lane M: 100 bp DNA marker, Lane W: Water control; H: healthy ridge gourd, RgI; Positive 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.

## Acknowledgements

This work supported by Science and Engineering Research Board (SERB), New Delhi, India. We are thankful to Inspire-DST, New Delhi for granting fellowship to Priya Naganur. We are thankful to Dr. Umesha, K., Dr. B. Fakruddin and Dr. V. Devappa for providing laboratory facilities and Dr. Basavaraj for technical help.

## References

Almasi, M.A, Dehabadi, S.H, Eftekhari, Z., 2013. Immunocapture loop mediated isothermal amplification for rapid detection of tomato yellow leaf curl virus (TYLCV) without DNA extraction. J Plant Pathol Microb, 4: 185.

Arutselvan, R., Krishnareddy, M., Maheshkumar, T. 2017. Rapid detection of tomato leaf curl Bengaluru virus through loop mediated isothermal amplification assay. Virus disease, 28:146-155.

Banerjee, A., Roy, S., Sharma, S.K., Dutta, S.K., Chandra, S., Ngachan, S.V., 2016. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for rapid diagnosis of chilli veinal mottle virus. Arch Virol, 161:1957–1961.

Bhat, A.I., Siljo, A., Deeshma, K.P., Siljo, A., Deeshma, K.P. 2013. Rapid detection of piper yellow mottle virus and cucumber mosaic virus infecting black pepper (*Piper nigrum*) by loop-mediated isothermal amplification (LAMP). J Virol Methods, 193: 190–196.

De la Pena, M., Flores, R., 2002. Chrysanthemum chlorotic mottle viroid RNA: dissection of the pathogenicity determinant and comparative fitness of symptomatic and non-symptomatic variants. J Mol Biol, 321: 411–421.

Fan, X., Dong, Y.F., Zhang, Z.P., Ren, F., Hu, G.J., Zhu, H.J. 2013. RT-LAMP assay for detection of grapevine rupestris stem pitting associated virus. Acta Phytopathol, 43:286–293.

Fortes, I.M., Sanchez-Campos, S., Fiallo-Olive, E., Diaz-Pendon, A.J., Navas-Castillo, J., Moriones, E. 2016. A novel strain of tomato leaf curl New Delhi virus has spread to the mediterranean basin. Viruses, 8: 307.

Fukuta, S., Kato, S., Yoshida, K., Mizukami, Y., Ishida, A., Ueda, J., Kanbe, M., Ishimoto, Y. 2003. Detection of tomato yellow leaf curl virus by loop-mediated isothermal amplification reaction. J Virological Methods, 112:35-40.

Gopalakrishnan, T.R., 2007. Vegetable Crops. In: Peter, K. V. (ed.), Horticulture Science Series, vol 4, New India Publishing Agency, New Delhi, India, 343 pp.

Goto, M., Honda, E., Ogura, A., Nomoto, A., Hanaki, K., 2009. Colorimetric detection of loop-mediated isothermal amplification reaction by using hydroxyl naphthol blue. BioTechniques, 46:167–172.

Ihira, M., Yoshikawa, T., Enomoto, Y., Akimoto, S., Ohashi, M., Suga, S., Nishimura, N., Ozaki, T., Nishiyama, Y., Notomi, T., Ohta, Y., Asano, Y., 2004. Rapid diagnosis of human herpesvirus 6 infection by a novel DNA amplification method, loop-mediated isothermal amplification. J Clin Microbiol, 42:140–145.

Iwamoto, T., Sonobe, T., Hayashi, K., 2003. Loop-mediated isothermal amplification for direct detection of *Mycobacterium tuberculosis* complex, *M. avium,* and *M. intracellulare* in sputum samples. J CliniMicrobiol, 41: 2616-2622.

Jeevalatha, A., Kaundal, P., Kumar, A., Guleria, A., Sundaresha, S., Pant, R.P., Sridhar, J., Venkateswarlu, V., Singh, B.P., 2016, SYBR green based duplex RT-qPCR detection of a begomovirus, tomato leaf curl New Delhi virus-[Potato] along with potato virus X and potato leaf roll virus in potato. Potato J., 43: 125–137.

Jeevalatha, A., Kaundal, P., Kumar, R., Raigond, B., Kumar, R., Sharma, S., Chakrabarti, K.S., 2017. Optimized loop-mediated isothermal amplification assay for tomato leaf curl New Delhi virus-[Potato] detection in potato leaves and tubers. European J Plant Pathol, 149: 1-9.

Jeevalatha, A., Kaundal, P., Venkatasalam, E.P., Chakrabarti, S.K., Singh, B.P., 2013. Uniplex and duplex PCR detection of geminivirus associated with potato apical leaf curl disease in India. J Virol Methods, 193: 62–67.

Jeevalatha, A., Singh, B.P., Kaundal, P., Kumar, R., Raigond, B., 2014. RCA-PCR: a robust technique for the detection of tomato leaf curl New Delhi virus-[Potato] at ultra-low virus titre. Potato J, 41: 76–80.

Jeong, J., Cho, S.Y., Lee, W.H., Lee, K.J., Ju, H.J., 2015. Development of a rapid detection method for potato virus X by reverse transcription loop-mediated isothermal amplification. The Plant Pathol J, 31: 219–225.

Kuan, C.P., Wu, M.T., Lu, Y.L., Huang, H.C., 2010. Rapid detection of squash leaf curl virus by loop-mediated isothermal Amplification. J Virol Methods, 169: 61–65.

Kushwaha, N., Achuit, K., Singh, K.A., Chattopadhyay, B., Chakraborty, S., 2010, Recent advances in geminivirus detection and future perspectives. The J Plant Prot Sci, 2: 1-18.

Lazarowitz, S.G., Lazdins, I.B., 1991. Infectivity and complete nucleotide sequence of the cloned genomic components of a bipartite Squash leaf curl virus with a broad host range phenotype. Virol, 180:58–69.

Lodhi, M.A., Ye, G.N., Weeden, N.F., Reisch, B., 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. Pl Mol Biol Report, 2:6-13.

Maeda, H., Kokeguchi, S., Fujimoto, C., Tanimoto, I., Yoshizumi, W., Nishimura, F., Takashiba, S., 2005. Detection of periodontal pathogen *Porphyromonas gingivalis* by loop-mediated isothermal amplification method. FEMS Immunol and Medical Microbiol, 43: 233–239.

Maruthi, M.N., Colvin, J., Seal, S.E., Gibson, G., Cooper, J., 2002. Co adaptation between cassava mosaic geminiviruses and their local vector populations. Virus Res, 86:71–85.

Mori, Y., Nagamine, K., Tomita, N., Notomi, T., 2001. Detection of loop-mediated isothermal amplification reaction by turbidity derived from magnesium pyrophosphate formation. Biochemical and Biophysical Research Communications, 289: 150-154.

Moriones, E., Praveen, S., Chakraborty, S., 2017, Tomato leaf curl New Delhi virus: An emerging virus complex threatening vegetable and fiber crops, Viruses, 9: 264.

Nagamine, K., Hase, T., Notomi, T., 2002. Accelerated reaction by loop-mediated isothermal amplification using loop primers. Mol Cell Probes, 16: 223-229.

Nagamine, K., Watanabe, K., Ohtsuka, K., Hase, T., Notomi, T., 2001. Loop-mediated isothermal amplification reaction using a no denatured template. Clinical Chemistry, 47: 1742–1743.

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., Hase, T., 2000. Loop-mediated isothermal amplification of DNA. Nucleic Acids Res, 28:63.

Padidam, M., Sawyer, S., Fauquet, C.M., 1999, Possible emergence of new geminiviruses by frequent recombination. Virol, 265:218–25.

Parida, M., Sannarangaiah, S., Dash, P., Rao, P., Morita, K., 2008. Loop-mediated isothermal amplification (LAMP): a new generation of innovative gene amplification technique; perspectives in clinical diagnosis of infectious diseases. Reviews in Medical Virology, 18: 407-421.

Patil, C.V., Ramdas, S.V., Premchand, U., Shankarappa, K.S. 2017. Survey, symptomatology, transmission, host range and characterization of begomovirus associated with yellow mosaic disease of ridge gourd in southern India. Virus disease, 28:146–155.

Peng. J., Shi, M., Xia, Z., Huang, J., Fan, Z., 2012, Detection of cucumber mosaic virus isolates from banana by one-step reverse transcription loop-mediated isothermal amplification. Arch Virol, 157: 2213–2217.

Rojas, M.R., Hagen, C., Lucas, W.J., Gibertson, R.L., 2005. Exploiting chinks in the plant's armor: evolution and emergence of geminiviruses. Ann Rev Phytopathol, 43:361–94.

Rybicki, E.P., 1994. A phylogenetic and evolutionary justification for three genera of Geminiviridae. Arch Virol, 39:49–77.

Sohrab, S.S., Mandal, B., Ali, A., Varma, A., 2010. Chlorotic curly stunt: a severe begomovirus disease of bottle gourd in northern India. Indian J Virol, 21:56–63.

Soliman, H., El-Matbouli, M., 2006. Reverse transcription loop-mediated isothermal amplification (RT-LAMP) for rapid detection of viral hemorrhagic septicaemia virus (VHS). Vet Microbiol, 114:205–213.

Sridhar, J., Venkateswarlu, V., Jeevalatha, A., Malik, K., Bhatnagar, A., Singh, B.P., 2016, Squash and tissue print protocols for quick detection of tomato leaf curl New Delhi virus-potato in fresh and ethanol preserved single whitefly. Potato J, 43: 62-69.

Tiwari, A.K., Snehi, S.K., Singh, R., Raj, S.K., Rao, G.P., Sharma, P.K., 2012. Molecular identification and genetic diversity among six begomovirus isolates affecting cultivation of cucurbitaceous crops in Uttar Pradesh. India. Arch Phytopathol Plant Prot, 45:62–72.

Tomlinson, J.A., Dickinson, M.J., Boonham, N., 2010. Detection of *Botrytis cinerea* by loop-mediated isothermal amplification. Lett Appl Microbiol, 51:650–657.

Venkatasalam, E.P., Singh, S., Sivalingam, P.N., Malathi, V.G., Garg, I.D., 2011. Polymerase chain reaction and nucleic acid spot hybridization detection of begomovirus (es) associated with apical leaf curl disease of potato. Arch Phytopathol Plant Prot, 44: 987-992.

Wang, Z., Gu, Q., Sun, H., Li, H., Suna, B., Lianga, X., Yuana, Y., Liua, R., Shi, Y., 2014. One-step reverse transcription loop mediated isothermal amplification assay for sensitive and rapid detection of cucurbit chlorotic yellows virus. J Virol Methods, 195: 63–66.

Zaidi., SSEA, Martin, D.P., Amin, I., Farooq, M., Mansoor, S., 2017. Tomato leaf curl New Delhi virus: a widespread bipartite begomovirus in the territory of monopartite begomoviruses. Molecular Plant Pathol, 18: 901-911.

Zerbini, F.M., Briddon, R.W., Idris, A., Martin, D.P., Moriones, E., Navas-Castillo, J., Rivera-Bustamante, R., Roumagnac, P., Varsani, A., 2017. ICTV report consortium ICTV virus taxonomy profiles: geminiviridae. J Gen Virol, 98:131–13.

**How to cite this article:**

Priya Naganur, U. Premchand, K. S. Shankarappa, R. K. Mesta, C. Manjunatha and Patil, C. V. 2019. Development of a Loop-Mediated Isothermal Amplification Assay for Detection of Tomato Leaf Curl New Delhi Virus in Ridge Gourd [*Luffa acutangula* (L) Roxb.]. *Int.J.Curr.Microbiol.App.Sci.* 8(09): 2282-2295. doi: https://doi.org/10.20546/ijcmas.2019.809.264