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Methods Article

METHODS ARTICLE

Development of a LAMP assay using a portable device for the real-time detection of cotton leaf curl disease in field conditions

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Abstract

Cotton production is seriously affected by the prevalent cotton leaf curl disease (CLCuD) that originated from Nigeria (Africa) to various parts of Asia including Pakistan, India, China and Philippines. Due to CLCuD, Pakistan suffers heavy losses approximately 2 billion USD per annum. Numerous reports showed that CLCuD is associated with multiple species of begomoviruses, alphasatellites and a single species of betasatellite, that is 'Cotton leaf curl Multan betasatellite' (CLCuMuB). The most prevalent form of CLCuD is the combination of 'Cotton leaf curl Kokhran virus'-Burewala strain (CLCuKoV-Bur) and CLCuMuB. Thus, the availability of an in-field assay for the timely detection of CLCuD is important for the control and management of the disease. In this study, a robust method using the loop-mediated isothermal amplification (LAMP) assay was developed for the detection of CLCuD. Multiple sets of six primers were designed based on the conserved regions of CLCuKoV-Bur and CLCuMuB-βC1 genes. The results showed that the primer set targeting the CLCuMuB-βC1 gene performed best when the LAMP assay was performed at 58°C using 100 ng of total plant tissue DNA as a template in a 25 µl reaction volume. The limit of detection for the assay was as low as 22 copies of total purified DNA template per reaction. This assay was further adapted to perform as a colorimetric and real-time LAMP assay which proved to be advantageously applied for the rapid and early point-of-care detection of CLCuD in the field. Application of the assay could help to prevent the huge economic losses caused by the disease and contribute to the socio-economic development of underdeveloped countries.

Keywords: loop-mediated isothermal amplification (LAMP); cotton leaf curl disease (CLCuD); cotton leaf curl Kokhran virus; cotton leaf curl Multan betasatellite; βC1 gene

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Introduction

Cotton contributes 1.5% to Pakistan's economy but its production has been drastically reduced in the past few years by the cotton leaf curl disease (CLCuD) complex. CLCuD in the Indian sub-continent is caused by multiple begomoviruses of the family Geminiviridae (members with single-stranded DNA genomes) in association with a single beta-satellite, that is 'Cotton leaf curl Multan betasatellite' (CLCuMuB). Multiple species of alphasatellites have also been reported associated with CLCuD complex [1–3]. In the CLCuD complex, the betasatellite is essentially required for pathogenicity [4]. However, evidence suggested that *Gossypium darwinii* symptomless alphasatellites and *Gossypium mustelinium* symptomless alphasatellites are involved in overcoming the RNA interference (RNAi)-mediated host defense [5]. Multiple alphasatellites have been found to be associated with CLCuD that includes cotton leaf curl Egypt alphasatellite, cotton leaf curl Gezira alphasatellite, cotton leaf curl Lucknow alphasatellite, etc. [6].

Genomic studies revealed that betasatellites are half the length of geminiviruses (almost 1350 bp) divided into three regions with only one open-reading frame encoding βC1 gene without which begomovirus (also called helper virus; HV) itself cannot cause disease. Like other satellites, the betasatellite of the begomoviruses is dependent on the HV for replication, encapsidation, movement and transmission [1–7].

In Pakistan, CLCuD was first observed in 1967 on a few cotton plants, which were later reported to reach infection levels of up to 80% in the field [8]. In 1991–1993, CLCuD appeared as an epidemic and cotton yields were reduced by 75% and Pakistan suffered a loss of 5 billion US dollars [1]. The disease epidemic was controlled by the introduction of resistant varieties. However, during 2001, a resistant-breaking recombinant strain, that is the 'Cotton leaf curl Khokhran virus'-Burewala strain (CLCuKoV-Bur)/'Cotton leaf curl Multan betasatellite' (CLCuMuB), was found to infect all the resistant cultivars [9, 10]. CLCuMuB is found to be a recombinant, with most of the molecules originating from CLCuMuB but with some sequences from 'Tomato leaf curl betasatellite' [11]. Infected plants showed various disease symptoms, including curling of leaf margins, stunted plant growth, yellowing as well as thickened veins which resulted in enations on the underside of newly grown young leaves giving them a deep downward cup conformation [1].

CLCuD detection methods include DNA amplification of the conserved genomic region/s by polymerase chain reaction (PCR) [12, 13] using universal primers for DNA A [14, 15] as well as for CLCuMuB [15, 16], membrane blot [17], enzyme linked immunosorbent assay (ELISA) [17–19], flow cytometry [20, 21] and real-time quantitative PCR [22]. However, these techniques are often time-consuming, labourious, requiring instrumentation and proper laboratory infrastructure along with a certain level of the skills of scientists or technicians [23]. These techniques are not suitable for in-field diagnosis and, therefore, a simple, user-friendly platform needs to be developed for this purpose.

The loop-mediated isothermal amplification (LAMP) assay involves the amplification of nucleic acid at a single temperature [23–25]. An isothermal DNA polymerase is the prime requirement of the LAMP assay because of its strand displacement property and work at a single temperature in the range of 55–65°C [26]. This assay requires six primers to perform the exponential amplification [27]. Betaine was used to denature secondary structures [28] while $MgSO_4$ enhances the amplification efficiency [29]. Due to its isothermal property, LAMP has the potential to be conducted in the field as a point of care diagnosis of plant pathogens.

Previously, LAMP has been reported for 'Tomato leaf curl Bangalore virus' [30], 'Squash leaf curl virus' [31], 'Tomato yellow leaf curl virus' [32], 'Tomato leaf curl Sinaloa virus' [23] and 'Tomato leaf curl New Delhi virus-[potato]' [33]. However, to the best of our knowledge, no LAMP assay has been established for cotton infecting viruses or CLCuD. In this report, a protocol is described for the detection of CLCuD in field-infected samples. This protocol is based on the detection of the CLCuMuB-βC1 gene which is the pathogenicity determinant of CLCuD and essentially present in all cases of the disease [3, 4]. Optimizations were performed for the various components: DNA polymerase, template concentration as well as reaction temperature. The LAMP product was visualized by gel electrophoresis followed by colorimetric and real-time LAMP assay to make the system user friendly and in-field diagnosis of disease.

Materials and methods

Lamp reaction assays

To develop LAMP-reaction assay for in-field detection of CLCuD infection, three reaction sets were designed including LAMP optimization for CLCuMuB-βC1 gene by gel electrophoresis, colorimetric detection and finally real-time detection of CLCuD using a portable device.

Primers design

Primer sets for LAMP assay were designed on reference sequence of accession number AM084379 retrieved from an online available database of National Center for Biotechnology Information (NCBI) using an online available software, that is PrimerExplorer V5 software (http://primerexplorer.jp/lamp4.0.0/index.html) which generates all six primers (Table 1). Later, these primer sequences were modified manually and synthesized commercially.

Reaction components

For LAMP assay, *Bsm* DNA polymerase (ThermoFisher, USA) was used. For a 25-µl reaction volume, 8 units of *Bsm* DNA polymerase were added along with a total of six primers including pair of outer, inner and loop primers at concentration of 0.2, 1.6 and 0.8 µM, respectively. Betaine (GeneLink, USA) at a final concentration of 0.5 M was added in each reaction while 6 mM $MgSO_4$ was added in the reaction. In addition to this, 1.4 mM dNTPs (ThermoFisher) were also added. The LAMP reaction was first carried out using DNA plasmid containing the cloned CLCuMuB-βC1 gene and infected-cotton plant DNA samples.

Table 1: LAMP primers sequence for the CLCuMuB-βC1 gene: outer primers (F3 and B3), internal primers (FIP and BIP) and loop primers (LF and LB)

Outer primers	βc1-1F3	5′-CTGTGAACTATATCTTCTATCTCG-3′
	βc1-1B3	5′-ATCTTCATTCACATGAGGATAC-3′
Internal primers	βc1-1FIP	5′-GCTTTGAAGGGAGCATCATAG CttttCTCTTCTATCTTTGCCCCG-3′
	βc1-1BIP	5′-CGTTGAAGTCGAATGGAACGTGt tttACAAAGTCACCATCGCTAA-3′
Loop primers	βc1-1LF	5′-GAATTTCCTATTCGCATACAA-3′
	βc1-1LB	5′-TACGTGTACTGGACGATC-3′

Table 2: Details of field-infected cotton samples; sample code, location name and longitudinal and latitudinal coordinates on map

Srial number	Sample label	Symptoms	Location name	Coordinates
1	37	SG, LC, LE, VD, VT	CCRI, Multan	30 00.621, 71 57.752
2	52	SG, LC, VT	PCCC, Multan	30 16.252,
3	53	SG, LC, LE, VT		71 37.241
4	54	SG, LC, VT		
5	61	SG, LC, VD, VT,	Kabirwala, Khanewal	30 19.556,
6	62	SG, VD, VT		71 51.497
7	64	SG, LC, LE, VD, VT		
8	116	VT, VD	Sahiwal	30 33.598, 72 46.745
9	119	SG, LC, VT	Chichawatni, Sahiwal	30 30.261, 72 43.901
10	123	LC, VT	Chak 561, Vehari	30 05.747,
11	125	LC, LE, VT		72 29.779
12	126	SG, LC, LE, VD, VT	Chak 425, Vehari	30 13.249,
13	127	SG, LC, LE, VD, VT		72 42.249
14	130	SG, LC, LE, VD, VT		
15	143	SG, LC, VT	Vehari Tehsil	30 01.473, 72 22.007
16	163	SG, LC, LE	Chak 6 Lot Joyanwali, Vehari	30 01.405,
17	166	LC, VT, VD		72 22.154
18	167	LC, VT		
19	186	SG, LC, LE, VD, VT	Vehari	30 02.828, 72 10.174
20	192	SG, LC, VT	Kamalia, Toba Tek Singh	30 35.70, 72 40.876
21	194	SG, LC, LE, VT	NIAB, Faisalabad	31 39.9108, 73 03.3155
22	206	VT, VD	NIBGE, Faisalabad	31 39.8200,
23	207	LC, VT, VD		73 02.568

*SG, stunted growth; LC, leaf curling; LE, leaf enation; VT, vein thickening; VD, vein darkening.

Infected sample collection

Infected cotton samples were collected from the field. Origins and symptoms of each sample are given in Table 2 while district wise locations are shown in Fig. 1. The genomic DNA from cotton leaves was extracted using CTAB method [34]. The concentration of extracted DNA was assessed by NanoDrop (Thermo). Initially, the assay was tested for three samples. Later, the developed assay was studied on large data set of 20 infected cotton plants.

Reaction temperature optimization

All six primers used in LAMP reaction have different melting temperatures which decide the annealing in normal PCR. However, in LAMP, a single temperature was optimized first by using different temperatures to amplify CLCuMuB. The incubation temperatures tested were 55°C, 58°C, 60°C, 62°C and 65°C using thermal cycler on 'incubation mode' to obtain isothermal temperature conditions. Later, the DNA extracts from field samples were subjected to optimized temperature conditions along with plasmid DNA containing CLCuMuB-βC1 gene as a positive control while a non-specific DNA template and water were used as negative controls.

Template concentration optimization

Various template concentrations ranging between 50 and 150 ng/µl were tested in the LAMP optimizations using plasmid DNA containing the CLCuMuB-βC1 gene.

Optimization of colorimetric detection of LAMP

For the colorimetric LAMP assay, a master mix (New England Biolab, UK) was used at a final concentration of 1× (compared with supplied concentration) in each reaction volume. LAMP (outer, inner and loop) primers were added at concentrations of 0.2, 1.6 and 0.8 µM, respectively, and 100 ng/µl of template plasmid DNA containing the CLCuMuB-βC1 gene was used as a positive control. In addition, the same set of three extracted DNA samples from virus-infected cotton plants grown in-field, along with two negative controls containing water and non-specific DNA, were then placed at a temperature 58°C in a preheated dry heat block for 60 min for visual monitoring of the LAMP reaction.

Optimization of real-time detection of LAMP using a portable device

Real-time detection of CLCuD was performed using an isothermal master mix (OptiGene, Horsham, UK) containing all components of LAMP reaction while reaction readouts were taken at ESE Quant tube scanner (Qiagen, The Netherlands). LAMP isothermal master mix was obtained in the form of dried reagents containing a novel DNA polymerase along with double-stranded (ds) DNA binding dye and its respective resuspension buffer. All these components were resuspended in the supplied buffer and directly used in the reaction mixture. A reaction of 25 µl volume was prepared containing 1× master mix, outer, inner and loop primers at concentrations of 0.2, 1.6 and 0.8 µM, respectively, while 100 ng/µl of template DNA was added. Similar to the reaction mentioned previously, three DNA extracts from field samples were tested to detect the virus load while optimization of real-time LAMP was carried out for CLCuMuB-βC1 gene cloned in a TA plasmid, which was used as a positive control. In addition, two separate negative controls were set up containing water and non-specific DNA, respectively.

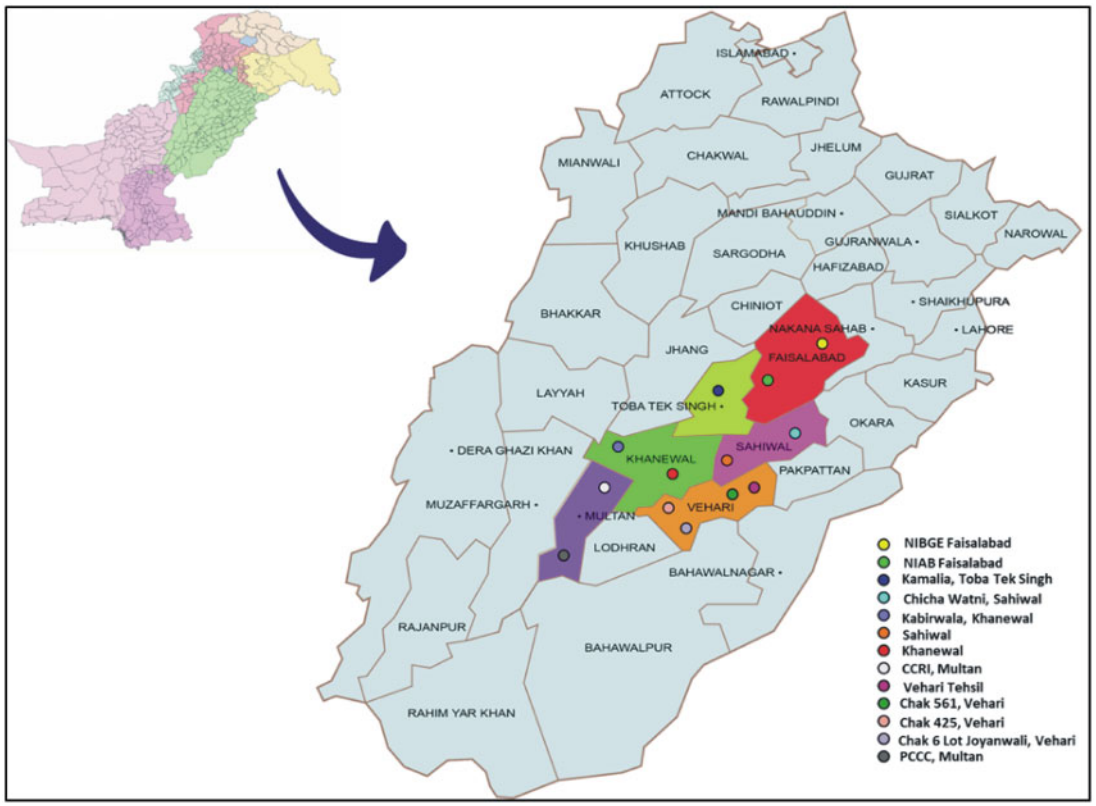

FIGURE 1: map of study districts in Punjab Province, Pakistan.

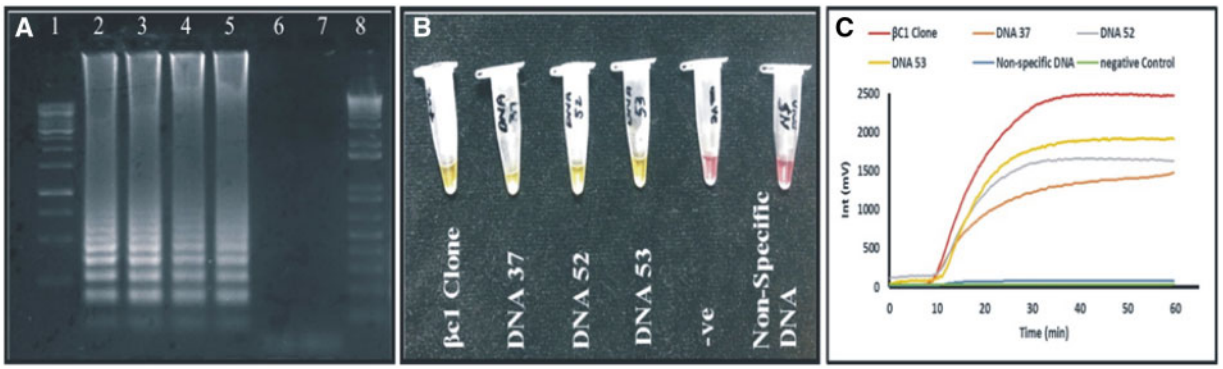

FIGURE 2: LAMP assay optimization, colorimetric and real-time LAMP assay for βC1 gene. (**A**) Molecular optimization of LAMP assay; Lane 1: 1 kb DNA ladder, Lane 2: amplification from βC1 gene cloned in plasmid DNA. Lane 3–5: amplification from field-infected cotton DNA samples, Lanes 6 and 7: negative controls, Lane 8: 1 kb PLUS DNA ladder. (**B**) Colorimetric detection of LAMP. (**C**) Real-time amplification of LAMP products.

Analytical sensitivity of LAMP assay

To measure the detection limits of the LAMP assay, a total of 12 reactions were prepared as 10-fold serial dilutions from equivalent to 2.2×10^{10} copies of virus DNA used as initial template concentration. The reaction products were assessed using 1% agarose gel electrophoresis under UV light after staining with ethidium bromide. Images were taken with a Gel Doc EZ imager (Bio-Rad). Image J software was used to measure the fluorescence intensity of the products. Fluorescence intensity for each reaction was recorded in triplicate. The mean value was then calculated and plotted against the respective concentration to determine the limit of detection (LOD).

Results

Lamp optimization for CLCuMuB-βC1 gene

Above discussed-parameters, including reaction temperature and template concentration, were optimized. After preliminary optimizations, the primer set designed for the CLCuMuB-βC1 gene amplified to generate the distinct ladder-like banding pattern, characteristic of the LAMP assay, at 58°C when 100 ng/µl DNA was used. Afterwards, optimized parameters were tested for amplification of three infected field samples and observed similar banding pattern (Fig. 2A). This ladder-like pattern as shown in Fig. 2A is ascribed as amplification by *Bsm* DNA

polymerase due to six different primers at various locations in an amplicon.

Colorimetric detection of LAMP

The one-step colorimetric detection of LAMP assay for CLCuMuB was performed using a master mix containing phenol red dye for the indication of reaction. A similar reaction configuration was designed for plasmid DNA containing the βC1 gene as positive control with DNA samples of infected cotton plants along with negative controls of water and non-specific DNA. Positive reactions, after amplification by *Bst* DNA polymerase, show a color change from violet pink to golden yellow (Fig. 2B). This color change is attributed to change in pH as reaction mixture contains phenol red dye, which is a pH indicator.

Real-time detection of LAMP using a portable device

Another experiment for real-time detection of CLCuD was carried out in which a real-time response was recorded during the progression of the LAMP assay. These real-time datasets are possible due to the inclusion of fluorescent dye present in the supplied reaction mixture. Response signals (datapoints) were plotted against time as shown in Fig. 2C. The observed figure illustrates the expected sigmoidal curve for each positive sample, including plasmid DNA and infected cotton-plant samples from the field. No significant amplification was observed in both negative controls.

LOD of LAMP assay

Limits of the detection for the assay were measured via both gel electrophoresis and colorimetric method using various concentrations of template DNA starting from 2.2×10^{10} (100 ng/µl), with 10-fold serial dilution. The ensuing gel pattern is shown in Fig. 3A, with fluorescence intensity calculated using image J software. Fluorescence was measured in triplicate and the mean value was plotted against the log of template concentration (Fig. 3B). The amplification product concentration from different initial DNA concentrations, after LAMP assay, was found to vary as added concentration of template was serially diluted. The most concentrated sample, with 2.2×10^{10} (100 ng/µl) DNA template, shows bright amplification and a subsequent decline was observed for serially diluted amplification products. Figure 3B shows a graph of fluorescence intensity plotted against log of concentrations. Limits of detection were then calculated to be as low as 22 copies of βC1 gene in purified DNA template per reaction. In Fig. 3C, a similar amplification pattern can be observed for colorimetric LAMP assay of same serially diluted template concentration. A bright amplification (color change from violet pink to bright yellow as described in the 'Colorimetric detection of LAMP' section) was observed in reaction having 2.2×10^{10} DNA copies. A very little color change can be observed in reaction with 2.2×10 virus DNA but this change is more inclined towards violet pink therefore LOD for colorimetric LAMP was 22 copies of satellite in purified DNA template per reaction.

Assay evaluation on infected field samples

For further validation of obtained results, initial optimizations of LAMP assay for CLCuD were then extended to 20 more DNA samples taken from infected cotton plants grown in field along with five different non-specific DNA as negative control. Non-specific DNA includes non-infected cotton plant, potato, *Nicotiana benthamiana*, *Nicotiana tabacum* and tomato. DNA samples of cotton plants infected with CLCuD showed a range of symptoms (mild to severe) and from diverse locations of Punjab Pakistan. All DNA samples were first confirmed for the presence of CLCuMuB-βC1gene by conventional PCR as shown in Fig. 4. Same DNA samples were used for LAMP assay and then subject

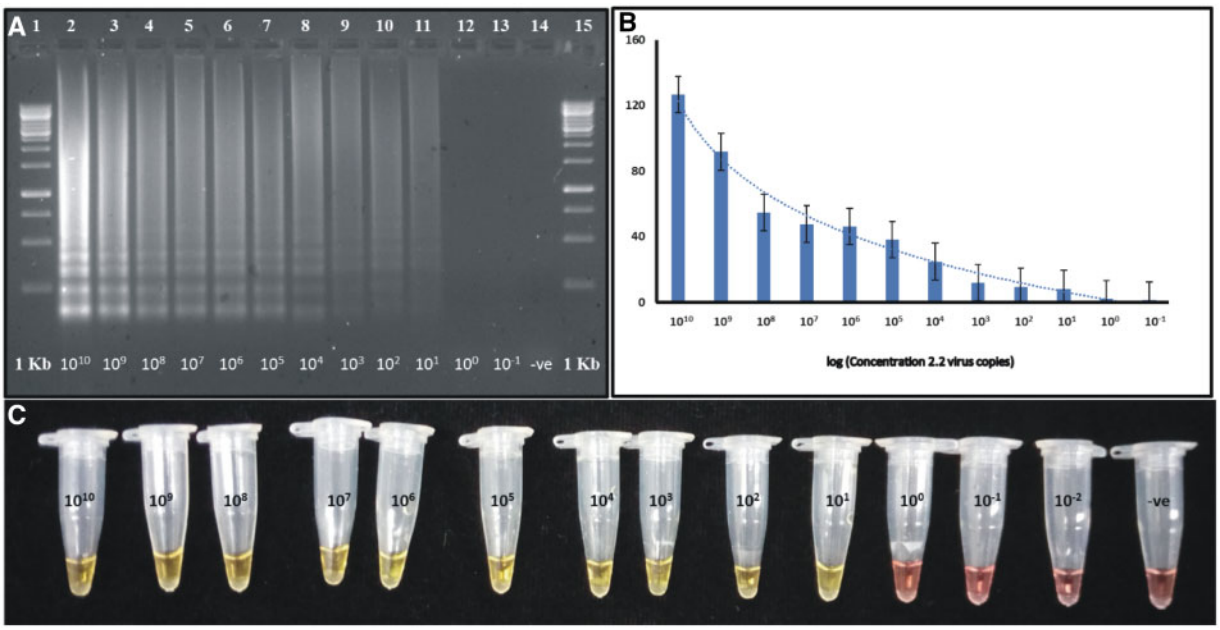

FIGURE 3: Determination of LOD for optimized LAMP assay. **(A)** Agarose gel electrophoresis of the products from the LAMP assays of the serially diluted template DNA; Lane: 1 kb DNA ladder, Lanes 2–13: products from the 10-fold serial dilutions of template, 2.2×10^{10} virus DNA copies (100 ng/µl) to 2.2×10^{-1} virus copies (1×10^{-9} ng/µl), respectively. Lane 14: negative control, Lane 15: 1 kb DNA ladder. **(B)** A plot of the fluorescence intensity against the products of the assays containing different concentrations of plasmid template containing the βC1 gene as indicated in Figure 3A. **(C)** Colorimetric LAMP assay products from the 10-fold serial dilutions of template, 100 ng/µl (2.21×10^{10} virus DNA copies) to 1×10^{-10} ng/µ; (2.21×10^{-2} virus copies), respectively.

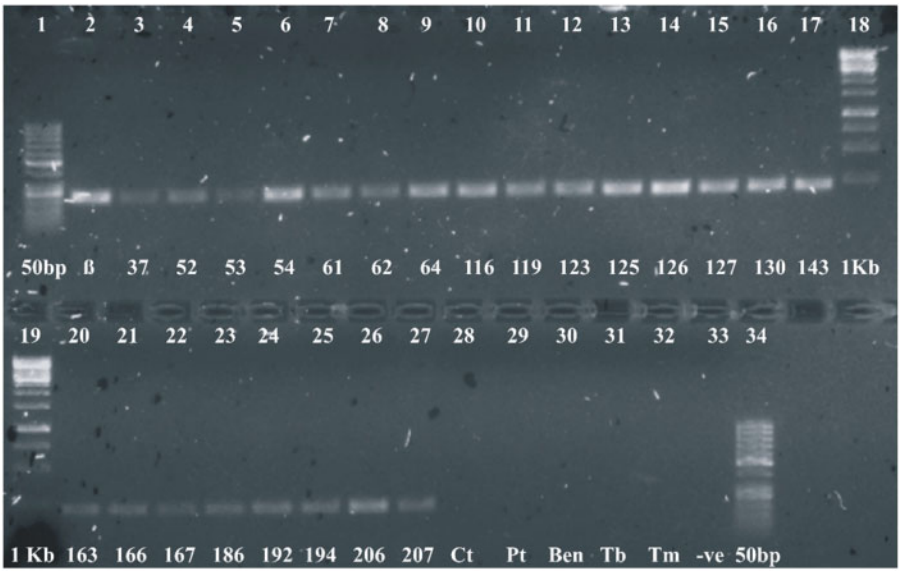

FIGURE 4: detection of the CLCuMuB-βC1 gene in field-infected cotton samples using PCR. Lane 1: 50 bp DNA ladder, Lane 2: amplification from CLCuMuB-βC1 gene cloned in plasmid DNA, Lanes 3–17: an amplicon of 213 bp from field-infected DNA samples, Lanes 18 and 19: 1 kb DNA ladder, Lanes 20–27: amplification 213 bp from infected DNA samples, Lanes 28–32: negative controls of nonspecific DNA from non-infected cotton (Ct), Potato (Pt), Benthamiana (Ben), Tobacco (Tb) and Tomato (Tm) plants, Lane 33: negative control without any DNA sample, Lane 34: 50 bp DNA ladder.

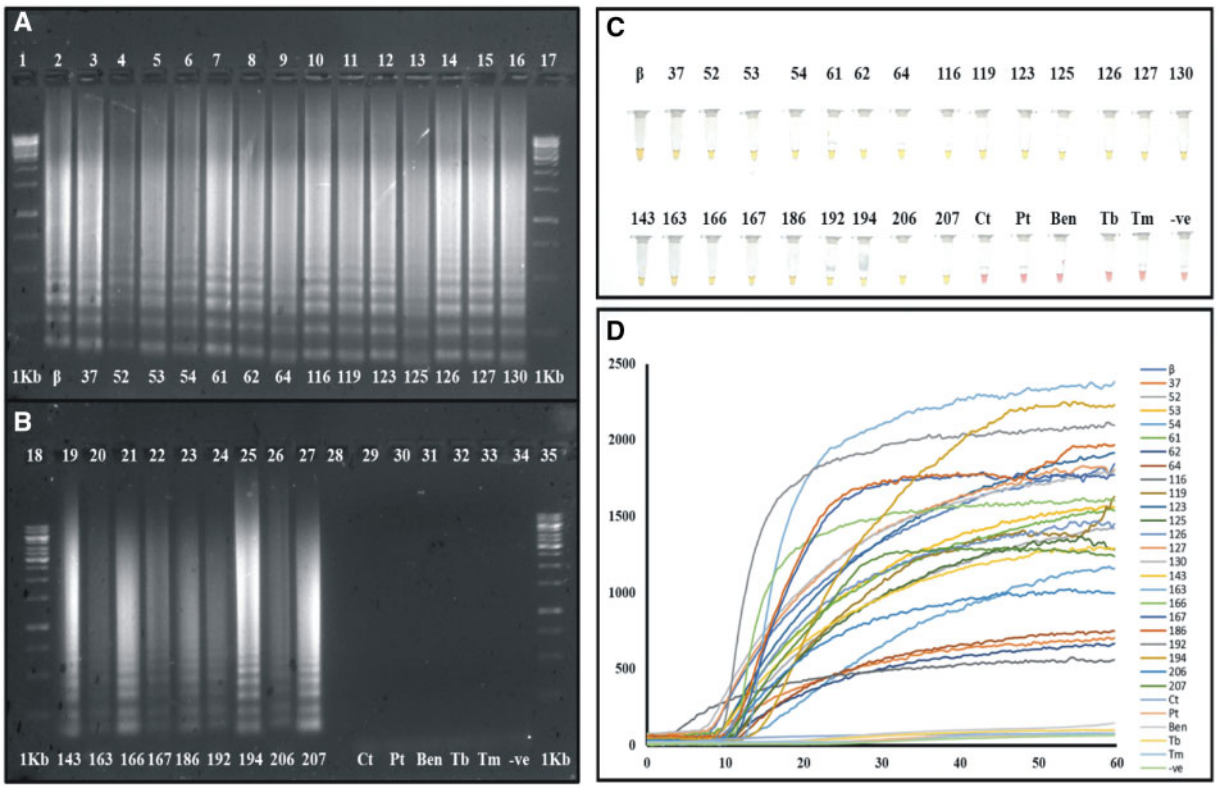

FIGURE 5: LAMP assays on infected cotton DNA collected from diverse locations of Punjab. (**A**) and (**B**) Agarose gel analysis of LAMP assay; Lane 1: 1 kb DNA ladder, lane 2: CLCuMuB-βC1 gene as positive control. Lanes 3–16: LAMP amplification from DNA of infected field plants, Lanes 17–18: 1 kb DNA ladder, Lanes 19–27: LAMP amplification of DNA collected from infected field plants, Lanes 29–33: negative controls of non-specific DNA from non-infected cotton (Ct), potato (Pt), benthamiana (Ben), tobacco (Tb) and tomato (Tm) plants, Lane 34: negative control with water, Lane 35: 1 kb DNA ladder. (**C**) Colorimetric detection of LAMP. (**D**) Detection through real-time amplification of LAMP products.

to colorimetric and real-time LAMP detection using reaction mixtures having fluorescent dyes, that is phenol red and FAM, respectively. In all LAMP assays, an optimized concentration of 100 ng/µl per reaction was maintained. Figure 5A–D shows respective resultant amplification in terms of DNA bands, color change due to change in pH as well as real-time amplification response.

Discussion

The ability to perform rapid infected-plant screening on-site can reduce huge economic losses, especially in agriculture-based economies [23]. In this study, we proposed a LAMP assay that detects CLCuD on-site. The primers used in this study amplify LAMP products at 58°C within 1 h. Following the reported optimized concentrations of given primers, it was found that LAMP products can efficiently be amplified by the use of 0.8 μM of loop primers in the final reaction setup. In contrast, a few studies reported that the addition of loop primers may result in non-specific amplification [35]. However, we have not observed non-specific amplification and our results are in accordance with previous reports in which LAMP has been found to be accelerated by the addition of loop primers thus enhancing sensitivity [27, 36–38]. Other primers that are outer and internal primers were used in their standard concentration of 0.2 and 1.6 μM, respectively.

To make LAMP assay more efficient for on-site diagnostics, it is important to develop a method that requires minimal instrumentation. For this, a one-step colorimetric test has been designed in which LAMP amplification gives a distinct color change. Previously, a colorimetric test has been optimized for tomato yellow leaf curl virus detection using different dyes including ethidium bromide, SYBR green, hydroxy naphthol blue and magnesium pyrophosphate as the LAMP-reaction indicator [32]. Other studies have also reported color-based testing for mosaic and streak viruses [25, 39, 40]. In this study, a colorimetric test has been reported for the detection of an economically important plant virus disease complex by using a reaction mixture which contains phenol red dye as an amplification indicator utilizing its color change capability in different pH environments. In the case of color-based detection using LAMP, as the reaction proceeds, DNA is amplified by the addition of dNTPs. Each dNTP incorporation in the newly synthesized DNA strand results in the release of a proton which lowers pH of the reaction, thus, indicated by color change. Our results are in agreement with prior reports that show similar sort of color change upon isothermal amplification [41–43].

After the optimization of LAMP for CLCuD at 58°C and with colorimetric analysis, another reaction of real-time LAMP was designed in which amplification of cloned virus and infected plant DNA was observed in real time through fluorescence monitoring. Resultant signals were recorded in data sets which then generated sigmoidal plots for signal against time (Figs 2C and 5D). These sigmoidal curves indicate spatio-temporal amplification of respective template DNA by DNA polymerase. This real-time in-field amplification is in line with previous reports where they have optimized the detection of other plant viruses [23, 44, 45].

LOD for the designed assay was also examined by setting up LAMP for a serially diluted template which was initially taken as 2.2×10^{10} (100 ng/μl). The calculated LOD of this assay for diagnosis of CLCuD was found to be 22 copies of betasatellite in purified DNA template (both by gel electrophoresis and colorimetric LAMP methods) which was better than the previously reported detection limits [36, 38]. Therefore, this assay is comparably sensitive to qualitative PCR, while requiring only a single incubation temperature, less time and similar reaction costs. In order to assess the in-field suitability of the assay, it has also been tested on infected plant samples taken from the field, which results in LAMP amplicons that can be distinguished from those of negative control reactions with water or plant with no disease symptoms [23]. The predictive value for both positive and negative field samples was found to be similar to the cloned DNA; therefore, this assay could possibly be extended to the detection of other economically and medically important viruses. Because of its low detection limits, speed and ease of use under field conditions, LAMP may provide a useful assay for the on-site diagnosis of CLCuD.

Conclusions

In summary, we have described a LAMP assay for the detection of CLCuD in which 100 ng/μl template concentration and 58°C temperature have been found to be optimal. The LOD was found to be as low as 22 copies of viral DNA. In addition to being colorimetric and real-time, LAMP was also optimized for portability of the system. This method can be linked to other sensor platforms like quartz crystal microbalance or electrochemical setup and its applications can be extended to other economically important pathogens.

Data Data availibilty

CComplete data sets generated and analysed for this article are shown in the manuscript

Author's contribution

A.R. performed all experimental work. W.R.A., M.A. and N.A. assisted in sample collection, primer designing and in optimizing real-time assay. S.Z.B. and I.A. conceived the study and supervised the work. A.R. prepared the first draft of the manuscript which was edited by W.S.K., S.M., S.Z.B. and I.A. The final manuscript was read and approved by all authors.

Conflict of interest statement. None declared.

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