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Real-Time loop-mediated isothermal amplification assay for rapid detection of Banana bunchy top virus in North-east India

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Abstract

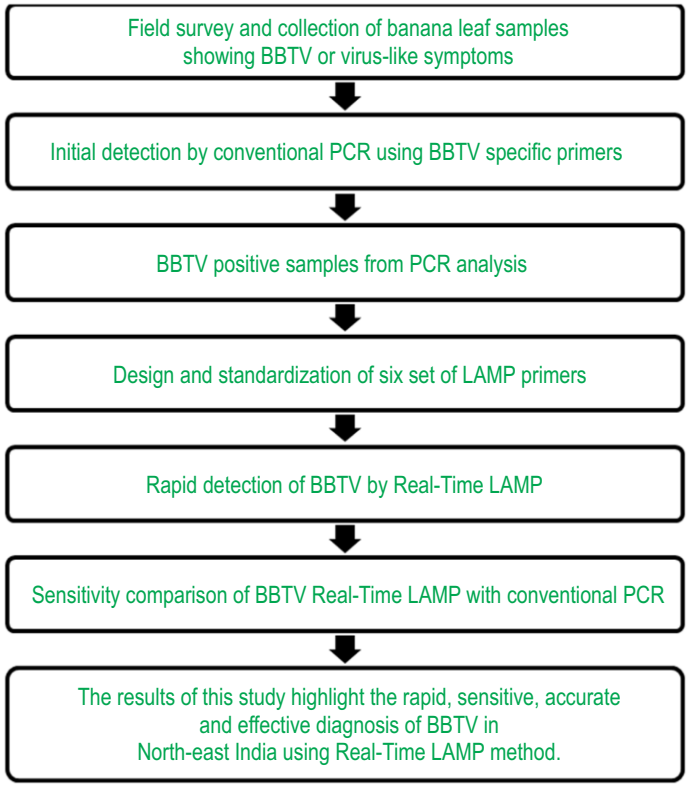
Aim: The study aims to standardize a Real-Time LAMP assay for effective, highly sensitive, and rapid detection of BBTV in North-east India.

Methodology: Forty samples of banana showing BBTV like symptoms were collected from Assam, India and subjected to conventional PCR for confirmation. Six sets of BBTV LAMP primers were designed and the PCR positive samples were subjected to Real-Time LAMP assay for detection of BBTV. Finally, a sensitivity test of BBTV LAMP assay and comparison of BBTV LAMP assay with conventional PCR was done using seven 10-fold dilutions of total genomic DNA of leaf samples with the highest dilution starting from 100 ng μl^{-1}.

Results: Initially a total of twenty six out of forty banana samples were tested positive for BBTV with conventional PCR method. The Real-Time LAMP assay for BBTV detection resulted in typical sigmoidal amplification curves with the peak values ranging between 8.00 to 12.15 min and annealing derivatives ranging between 83.3°C to 84.3°C in the tested samples. Sensitivity testing and comparison of BBTV Real-Time LAMP assay with conventional PCR revealed that the BBTV LAMP assay could efficiently detect up to 0.0001ng μl^{-1} of total DNA against 0.01ng μl^{-1} in conventional PCR.

Interpretation: The findings highlight rapid, sensitive, accurate and effective diagnosis of BBTV using Real-Time LAMP method. This method can be preferred over conventional diagnostic techniques like PCR or ELISA for rapid large scale detection of BBTV in banana plants in North-east India.

Key words: Banana, *Banana bunchy top virus*, Rapid detection, Real-Time LAMP assay



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Introduction

Banana (*Musa* spp.), is a widely consumed fruit globally. In India, banana and plantains are viewed as the fourth most important fruit crop with annual production of 30 million tons (FAOSTAT, 2018) and grown mostly by the small farmers with less holding of land (Dash and Rai, 2016; Kaur *et al.,* 2018). *Banana bunchy* top disease (BBTD) caused by Banana bunchy top virus (BBTV) belongs to genus *Babuvirus*, family *Nanoviridae* is considered as a major threat to global banana and plantain cultivation (Vetten *et al.,* 2012). The virus is non-enveloped and contains six components of circular ssDNA (DNA1-6) each approximately 1 kb in size. Since it's first report from Fiji in 1889 (Magee, 1927), the disease has been recorded in 33 countries from Africa, Asia, Australia, and the South Pacific Islands (Dale, 1987, Diekmann and Putter, 1996, Ferreira *et al.,* 1997; Amin *et al.,* 2008). Due to high destructive potential of disease, BBTV is listed as one of the world's 100 worst invasive diseases, and the International Plant Protection Convention has include it as a pathogen to be subjected to rigorous quarantine measures (IPPC, 2010; Kumar *et al.,* 2011). In India, BBTD is considered as one of the most important viral disease of banana.

Selvarajan and Balasubramanian (2014) reported BBTD outbreaks in Kodur, Andhra Pradesh and Jalgaon, Maharashtra, India during 2007-2010 resulted in production loss of worth 50 million US dollars per annum. The North-east India comprising eight states is a bio-diversity hot spot and possess diverse germplasm of banana including both wild and cultivated. BBTD is widely spread in the entire North-east India and possess a grave risk to banana cultivation in the area. Banana plants affected by BBTV show vein clearing, foliar chlorosis, and a typical bunchy top-like appearance with stunted growth and distorted bunches. It is transmitted by banana aphid (*Pentalonia nigronervosa*) in a persistent, circulative, and non-propagative manner (Kumar *et al.,* 2015). It has been reported that early infection of this virus can lead up to 100% of crop loss (Dale, 1987) and so far, no reports of resistance have been found in *Musa* spp. to BBTV. The management of BBTD is solely dependent on the use of virus-free propagating materials, using tissue culture-based techniques, rouging and destruction of infected plants, insect vector management, and implementation of quarantine barriers. Therefore, the development of rapid and efficient detection is the need of the hour for effective management of BBTD (Peng *et al.,* 2012).

Some of the previously described molecular diagnostic protocols for routine detection of BBTV are Enzyme-Linked Immunosorbent Assay (ELISA) (Wu and Su, 1990; Thomas and Dietzgen, 1991), Double Antibody Sandwich ELISA (DAS-ELISA) (Wanitchakorn *et al.,* 2000), Polymerase Chain Reaction (PCR) (Manikckam *et al.,* 2002; Su *et al.,* 2003) and high- throughput real-time PCR methods (Chen and Hu, 2013). Although ELISA and PCR are the most common method used in diagnostics, these methods are cumbersome and time consuming. Moreover, ELISA although convenient for processing a large number of samples, it is comparatively less sensitive than PCR (Su *et al.,* 2003). Conventional PCR for BBTV generally requires high quality DNA that is free off polyphenols, polysaccharides, and endogenous nucleases. It is difficult to achieve as banana leaves contain a high quantity of polysaccharides, polyphenols, and secondary metabolites like alkaloids, flavonoids, phenols, and terpenes (Das *et al.,* 2009; Shankar *et al.,* 2011). The quantification of BBTV through real-time PCR using SYBR green has been reported (Fu *et al.,* 2009; Watanabe and Bressan, 2013). In recent years, sensitive quantification assays using TaqMan probes in real-time PCR (TaqMan PCR) have been developed and used successfully to detect BBTV (Chen and Hu, 2013).

Loop-mediated isothermal amplification (LAMP) is a molecular detection technique developed in the year 2000 (Notomi *et al.,* 2000). During LAMP, the reaction can be performed in a single tube maintaining an isothermal condition at 60-65 °C for less than 30 min in a LAMP machine (OptiGene, UK) and the amplification is measured on real-time basis. For the LAMP reaction, four sets of specially designed primers are required which recognizes six distinct sequences on the target gene. Moreover, the addition of loop primers is known to accelerate this LAMP reaction (Notomi *et al.,* 2000; Nagamine *et al.,* 2002). Compared to PCR, it is less affected by inhibitors and hence, widely used now days for detecting of plant pathogens (Kaneko *et al.,* 2007). Peng *et al.* (2012) reported that the detection limit of LAMP assay was approximately 1 pg μl^{-1} DNA and is about 100-fold more sensitive than PCR. Hence, LAMP can be considered as an efficient alternative to conventional PCR. The objective of the present study is rapid detection of BBTV from North East India using Real Time LAMP method. The study also aims to evaluate the sensitivity of Real Time LAMP over conventional PCR so as to use LAMP as an effective alternative to conventional detection methods like ELISA or PCR for the rapid and highly sensitive detection of BBTV in banana.

Materials and Methods

Virus source and sample collection: Banana fields in the Jorhat district of Assam, India were surveyed for BBTV virus infection during the growing season of 2019 and 2020. A total of 40 leaf samples of banana plants showing BBTV or virus-like symptoms were collected from four different fields. The collected samples were brought to the laboratory of Plant Virology, Department of Plant Pathology, Assam Agricultural University and stored at - 80°C for around a month until the time of detection.

Genomic DNA extraction and conventional PCR: The total genomic DNA was extracted from the collected leaf samples of banana plants by using a modified CTAB protocol (Cullings, 1992). The genomic DNA was also extracted from a mass propagated virus-free plantlet of Grand Naine cultivar (AAA genome) of banana which was taken as a healthy control for further experiments. Total DNA was quantified using a NanoDrop spectrophotometer (Eppendorf Biospectrophotometer) and the concentration of extracted DNA was adjusted to 100ng μl^{-1} in

nuclease-free water and stored at -20°C before testing. The forty test samples along with one healthy sample were subjected to a conventional PCR for confirmation using a set of standard primer that amplifies a region of the coat protein in DNA3 of BBTV genome (Kakati and Nath, 2018; Baldodiya *et al*., 2019). PCR primers used were: BBTV-DNA3 forward primer (5' ATCAAGAAGAGGCGGGTTGG 3') and BBTV-DNA3 reverse primer (5' GGATTTCTTCGGA TACCTAGCCAT 3'). PCR was carried out in a 10 µl reaction mixture containing 5 µl of EmeraldAmp GT 2x PCR Master Mix, 0.5 µl each of forward and reverse primer (10 pmol concentrations), 0.5 µl of 50ng of total genomic DNA, and 3.5 µl of distil sterile water in Agilent Technologies Sure Cycler 8800. The samples were initially denatured at 94 °C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing for 30 sec at 53°C and synthesis at 72°C for 1 min, and final extension at 72°C for 5 minutes. The PCR products were subjected to gel electrophoresis using 1.5% agarose stained with ethidium bromide. The electrophoresis was carried out and visualized under Bio-Rad Gel Doc TM EZ Imager.

Primer design for LAMP: The coat protein coding sequences of ten BBTV isolates were obtained from GenBank and multiple alignment analysis was performed using Clustal Omega EMBL-EBI software (Chojnacki *et al.,* 2017). The Gene Bank accession numbers of the sequences used in the alignment were; JN250599, MG825490, MF688998, KF246091, EU190967, EU190966, EU190965, EU190968, AF246122, and DQ996466. Based on the alignment, six sets of primers targeting a conserved region of coat protein sequence were designed with the help of primer design software, Primer Explorer V5 (Eiken Co., Ltd., Japan:http://primerexplorer.jp). LAMP assay was performed using the following primers shown in Table 1, which includes forward and reverse outer primers (F3_CP and B3_CP), forward and reverse inner primers (FIP_CP and BIP_CP), and forward and reverse loop primers (loop F_CP and loop B_CP).

Reaction mixtures and optimal condition for LAMP assays: Real-Time LAMP was carried out on a Genei II instrument (OptiGene, UK) in 25 µl reaction volume consisting of 5µl of primer mix containing 20 pmol each of inner primers FIP and BIP, 5 pmol each of outer primers F3 and B3, 10 pmol each of loop primers FLP and BLP; 15 µl of isothermal master mix ISO 0001 (OptiGene, UK) which included *Geobacillus* species DNA polymerase, thermostable inorganic pyrophosphatase, optimized buffer (containing $MgCl_2$, deoxynucleotide triphosphates, and double-stranded DNA dye) and 5 µl of extracted DNA with a concentration of 100ng μl^{-1} as a template. LAMP assay was optimized at 65°C for 30 min on a real-time fluorometer Genie II (Optigene, UK). The Genie II device allows creating an annealing curve for confirmation of amplification specificity by additional heating and cooling step from 98°C to 80°C (0.05°C/s) for 6 min to allow re-annealing of amplified product.

Comparison of LAMP with conventional PCR: In order to determine the sensitivity of Real-Time LAMP assay for BBTV and to compare the results with conventional PCR, seven 10-fold dilutions were made of total genomic DNA with the highest dilution starting from 100ng μl^{-1}. DNA from a healthy Grand Naine plant was used as a negative control. Both Real-Time LAMP assay and a conventional PCR were carried out using these dilutions and the results were compared.

Results and Discussion

Preliminary testing of forty samples by conventional PCR showed that twenty-six were infected with BBTV. A consistent band of 1075 bp was obtained with the BBTV primer (DNA-3) in all the positive samples whereas no band was observed during agarose gel electrophoresis in healthy Grand Naine plantlet which was taken as negative control (Fig. 3 a). DNA extracted from twenty-six BBTV positive samples and one healthy Grand Naine plantlet as negative control were used for BBTV Real-Time LAMP assay using primers designed under this study.

All the BBTV positive samples showed amplification during Real-Time LAMP assay whereas no amplification was observed in the case of healthy sample. It was observed that the peak amplification time typically ranged between 8.00 to 12.15 min and the annealing derivatives ranged between 83.3°C to 84.3°C in all the tested samples. The loop-mediated isothermal amplification (LAMP) assay, a nucleic acid-based detection assay, can rapidly amplify the target DNA with high specificity under isothermal conditions (Notomi *et al.,* 2000). The LAMP assay takes advantage of high strand displacement activity of

Table 1: Primers for BBTV LAMP assays used under this study

Primer name	Primer type	Primer sequence (5' – 3')	Length (bp)	Position on CP gene (bp)
F3_CP	Forward outer	CTACTGATAAAACATTACCCAGAT	24	146-169
B3_CP	Reverse outer	TC CCACTACATACCAGTT	19	329-347
FIP_CP (F1c + F2)	Forward inner	CTCTTGATCATAGCCCAATGAAGTATGGAAAA TGTTTATGCTTCTTGT	48	F1c: 224 - 248 F2: 178-200
BIP_CP(B1c +B2)	Reverse inner	TCTTGGGAAATCAACCAGCCGTAACCAGATG GCTATGTTCAG	42	B1c: 250-270 B2: 308-328
Loop F_CP	Forward loop	CGGGCTTCACCTTGCAC	17	72-88
Loop B_CP	Reverse loop	TCTGGAAGCCCCAGGTTTAT	20	149-168

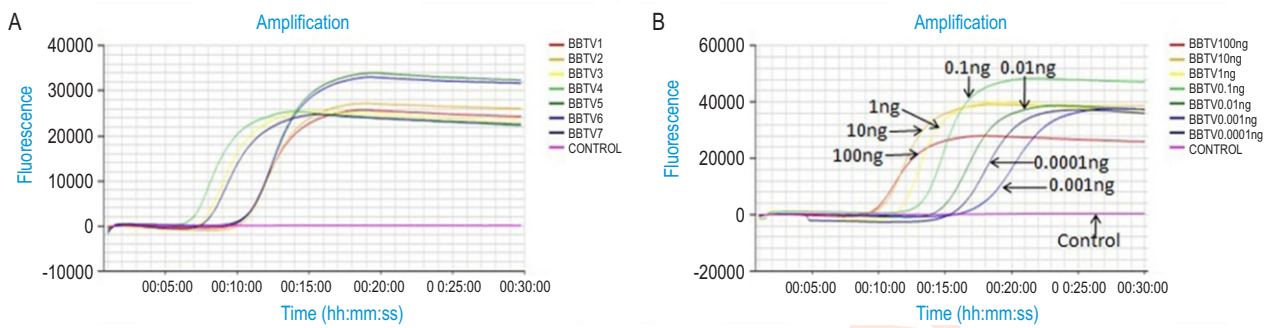

Fig. 1: Loop-mediated isothermal amplification curves showing BBTV detection on Real-Time basis. (a) Amplification of BBTV infected samples along with negative control, (b) BBTV amplification of seven 10-fold dilutions of total genomic DNA starting from 100ng to 0.0001ng along with negative control.

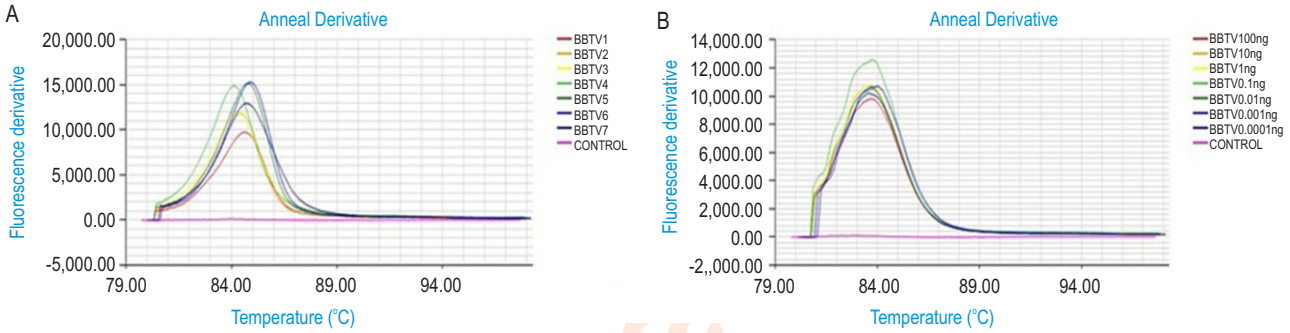

Fig. 2: Annealing derivatives and dissociation curves of Real Time LAMP product showing BBTV detection (a) Annealing derivatives and dissociation curves of BBTV infected samples along with negative control, (b) Annealing derivatives and dissociation curves of seven 10-fold dilutions starting from 100ng to 0.0001ng along with negative control.

certain polymerases allowing for isothermal amplification of nucleic acids. A LAMP assay, therefore, allows for rapid detection of infectious diseases without using complex equipment which enables it to be used on-site (Fukuta *et al.,* 2003; Hansen *et al.,* 2016). Selvarajan *et al.* (2015) developed a simple, rapid as well as solvent-free nucleic acid extraction protocol for detecting of BBTV by LAMP and well conventional PCR methods. Moreover, as principle, LAMP does not require a thermal cycler. In LAMP, the reactions can be performed using a heating block or a water bath under isothermal conditions (Peng *et al.,* 2012). However in the present study, the use of OptiGene, UK kit helped in maintaining stability in the preparation of reaction mixture for LAMP assay.

The use of a real-time fluorometer Genie II (Optigene, UK) device allowed easy monitoring of amplification in real-time using fluorescence. Another advantage of LAMP over other detection methods is that in LAMP, the risk of cross-contamination can be kept to a minimal level using closed-tube detection system (Peng *et al.,* 2012). During sensitivity testing of Real-Time LAMP assay of BBTV and comparing it with conventional PCR, it was observed that the BBTV LAMP assay could efficiently detect up to 0.0001ng μl^{-1} of DNA. However, the amplification time was found to increase progressively with reducing quantity of sample. Whereas in the case of conventional PCR, no bands were observed beyond 10-5 dilution, that is 0.01ng μl^{-1} of DNA as shown in Fig. 3 (b). Therefore, from these results, it can be concluded that the Real-Time LAMP assay for BBTV was at least a hundred times sensitive than conventional PCR. Typical amplification curve of BBTV LAMP assay for the tested samples along with negative control is shown in Fig. 1 (a) and the amplification curves of seven 10-fold dilutions of total genomic DNA during LAMP assay is shown in Fig. 1 (b).

The annealing derivatives and dissociation curves of LAMP products of the tested samples and negative control as well as the seven 10-fold dilutions are depicted in Fig 2 (a and b). Similar work on BBTV LAMP was done by Peng *et al.* (2012) where they reported a detection limit of 1pg μl^{-1} plasmid DNA through LAMP assay which was lower than 100pg μl^{-1} plasmid DNA for BBTV detected through PCR assay. This proves the sensitivity of LAMP over PCR. In a recent study, Galvez *et al.* (2020), reported the limit of detection for BBTV LAMP protocol was 10pg μl^{-1} whereas it was 100pg μl^{-1} for its corresponding PCR assay indicating higher levels of sensitivities of BBTV LAMP assays compared to the corresponding PCR assays for detecting of the virus. As LAMP is a sensitive method, hence using LAMP,

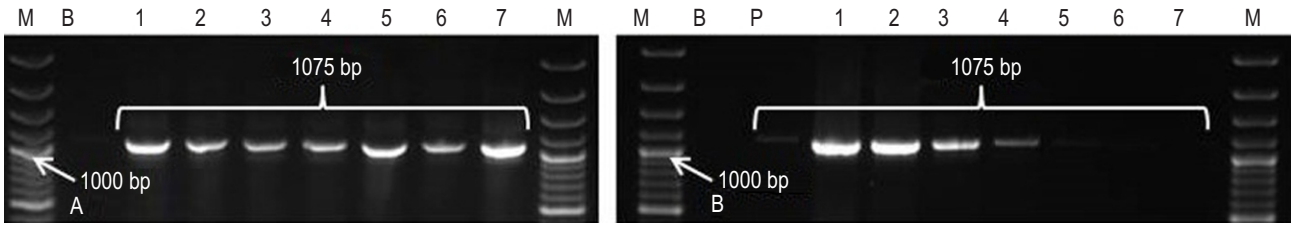

Fig. 3: Agarose gel electrophoresis photographs showing BBTV detection using PCR assay (a) Amplification of 7BBTV infected representative samples along with negative control. Lane M: 100 bp DNA ladder (Takara); Lane B: Negative control or blank; Lane 1-7: Seven BBTV infected samples (b) BBTV amplification of seven 10-fold dilutions starting from 100ng along with negative control. Lane M: 100 bp DNA ladder (Takara); Lane B: Negative control or blank; Lane P: Positive control; Lane 1-7: Seven 10-fold serial dilutions of total genomic DNA at 100, 10, 1, 0.1 0.01. 0.001 and 0.0001 ng/µl.

mild infections can also be detected effectively which might give false negative during detection using conventional PCR methods. Apart from BBTV, the LAMP technique is also being widely used globally for rapid and sensitive detection of several plant viruses like *Odontoglossum Ring Spot Virus* (ORSV) (Tsai *et al.,* 2016), *Banana Bract Mosaic* Virus (BBrMV) (Koh *et al.,* 2020), *Potato Leaf Roll Virus* (PLRV) (Almasi *et al.,* 2013; Ju, 2011), *Potato Virus* Y (PVY) (Tredar *et al.,* 2018) *etc.* In this study, the Real-Time LAMP method was standardized for detecting BBTV in North-east India as it gives us cumulative advantages over conventional diagnostic methods like ELISA or PCR. Apart from being highly sensitive, this method has proven to be both time as well as cost effective. Moreover, the closed tube detection system of LAMP is highly suitable for general laboratories as it minimizes the risks of cross contamination.

In conclusion, the results of the present study suggest that the Real-Time LAMP method can be a preferred option for rapid and sensitive detection of BBTV during disease monitoring as well as during large-scale propagation of virus-free plantlets by tissue culture techniques.

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Add-on Information

Authors' contribution: D. Kaushik: Conceptualization, design, material preparation, data collection as a part of PhD research; **M.H. Halabi:** Standardization and design of LAMP assay; **P. Barua:** Conceptualization, manuscript preparation and proofreading; **P.D. Nath:** Major advisor and project PI to Dipshika Kaushik, fund acquisition.

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