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RAPID AND SENSITIVE DETECTION OF XANTHOMONAS *AXONOPODIS* PV. *PUNICAE* INFECTING POMEGRANATE USING LOOP - MEDIATED ISOTHERMAL AMPLIFICATION

T. R. USHARANI*, S. MEENAKSHI, C. SUNISHA, H. D. SOWMYA, SHIVSHANKAR G KUMBDALE, S. POOVARASAN AND S. MOHANDAS
Indian Institute of Horticultural Research, Hessaraghatta, Bangalore - 560 089, INDIA
e-mail: usharanitr@iihr.res.in

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***Corresponding author**

ABSTRACT
In the present study, Loop – mediated isothermal amplification (LAMP) assay was used as a novel tool for diagnosis of *Xanthomonas axonopodis* pv. *punicae* (Xap), a gram negative host specific bacterium causing bacterial blight of pomegranate (*Punica granatum*). LAMP assay based on the amplification of the *Gyrase*â (gyrâ) gene showed the detection limit of 1fg/µl of purified Xap DNA when compared with conventional PCR showing amplification only upto 1pg/µl concentration of DNA. The time limit of detection was found to be 45 and 60 min of incubation. The sensitivity of this LAMP assay was determined to be 10 copies per reaction mixture as against single copy detection using real time PCR.The LAMP assay was able to detect Xap from crude extract of the infected samples collected from different locations without isolating the pathogen or purified genomic DNA. Pathogenic extract from infected leaf was visualized as increase in turbidity as well as color change from violet to sky blue by pre addition of HNB. LAMP assay showed a great potential for monitoring the disease incidence and it could prove to be a powerful supplemental tool for current diagnostic methods.

INTRODUCTION

Bacterial blight of pomegranate caused by Xap has severely hampered pomegranate cultivation in all major pomegranate-growing states of India including Maharashtra, Karnataka, and Andhra Pradesh causing huge losses to pomegranate growers and also affecting the export. Bacterial blight of pomegranate affects leaves, twigs, and fruits which serve as potential sources of primary inoculum. Early diagnosis for Xap is essential to prevent the primary source of inoculum from being transmitted to newer locations. PCR based detection of Xap using gyrâ gene is being used to detect the pathogen (Kalyan *et al*., 2012). LAMP assay is a method to detect specific nucleic acid sequences and has the potential to overcome many of the limitations of PCR based methods (Tsai *et al*., 2009). Positive reactions of LAMP assay could be visually detected by colorimetric HNB dye (Goto *et al*., 2009), intercalating fluorescent dye (Tomlinson *et al*., 2007), magnesium pyrophosphate accumulation (Mori *et al*., 2001), cationic polymer precipitation (Mori *et al*., 2006), labelled primers using flow devices (Tomlinson *et al*., 2010), and gel electrophoresis where the amplified product exhibits a ladder like fashion. LAMP method has been successfully used for detection of many plant pathogenic bacteria, fungi, virus and nematodes (Tomlinson *et al*., 2010, Fukuta *et al*., 2003, Kikuchi *et al*., 2009, Kubota *et al*., 2008) as well as human and animal pathogens (Dukes *et al*., 2006, Iwamoto *et al*., 2003). As LAMP assay is being found as a novel tool for disease detection with high specificity, efficiency and rapidity in short duration under isothermal condition which eliminates the need for thermal cycling equipment (Notomi *et al*., 2000), the present study was taken up to identify the gyrâ gene sequence to efficiently detect Xap infection and to compare its effectiveness with that of conventional and real-time PCR. The paper deals with the findings of the study.

MATERIALS AND METHODS

Sample collection and DNA extraction

Naturally infected pomegranate plant parts like leaf, flower and stem were collected from farmers field in Chitradurga dist, Karnataka, India. Xap was isolated and purified by inoculating the infected portion of pomegranate on nutrient glucose medium at 28p C (Raghuwanshi *et al.,* 2013; Sujatha and Saigopal, 2010). The identification of Xap was confirmed by gyrB gene specific primer (Mondal and Kumar, 2011). Xap liquid culture (optical density at 600 nm = 1, approximately $1X10^9$ CFU/ml) was ten fold serially diluted (***10^{-1} to 10^{-9}***) and plated on agar medium for viable count while an aliquot of 10 *i*L was used for the PCR and LAMP assay using crude lysate method (Edward *et al.*,1991).

Bacterial genomic DNA was extracted from 72 hr grown culture by following cetyltrimethyl ammonium bromide (CTAB) method (Rogers and Bendich 1994). The concentration and quality of the purified DNA were determined using an ND-

1000 Spectrophotometer (Nanodrop Technologies, USA) for subsequent PCR and LAMP reactions. The crude DNA extracted from the bacterial blight infected leaf was used for LAMP assay.

Optimization of LAMP assay

The LAMP primer sets (Table 1, Fig. 1) were designed from the 198 nucleotides of C-terminal region in the gyrâ gene of the published sequence of Xap strain NCPPB466 (GenBank accession no. EU285148.1) using the software (Primer Explorer V4, Eiken Co., Ltd., Japan). To initiate the reaction the F3, B3 were used and further acceleration was achieved with the additional loop primers (Floop, Bloop). The LAMP reaction mixture with a total volume of 25 ìl contained: 1 × Thermopol reaction buffer, 0.8 M Betaine, 4mM of dNTP, 7mM of MgSO4, 8 units of Bst DNA polymerase (New England Biolabs, Massachusetts, USA), FIP and BIP at 40 pmol each, F3 and B3 at 10 pmol each, Loop primers (forward and backward) at 20 pmol each, 25ng of template DNA and double distilled water to make up the volume. LAMP was performed at 65°C in a water bath for 1 hour followed by incubating at 85^{0}C for 5 mins to stop the reaction, and samples were visualised on 1.5% agarose gel electrophoresis. LAMP reactions were also stained with HNB dye, a colorimetric reagent at 120μM / reaction (TaKaRa Biotechnology Co., Ltd, Dalian, China) as a visual indicator for positive reaction by a color change from violet to sky blue. The assay was performed at different time intervals (5, 15, 30, 45 and 60 min) with Xap genomic DNA diluted at 10 fold increments (10^{-1} to 10^{-5}) to determine the time limit of reaction. The LAMP amplified bands were cut and sequenced (data not shown). The sequence results were used to perform BLAST search to confirm the identity.

LAMP sensitivity assay

The sensitivity of real-time PCR assay was compared with LAMP reaction using known concentration of serially diluted recombinant plasmid pTZgyrâ (copy number of 1000, 500,100, 10, 1). Real-time PCR was performed with the detection of the gyrâ gene of Xap on an ABI PRISM 7500 thermocycler. The copy number was calculated as described (Wang *et al*., 2008). Briefly, the concentration of pTZgyrâ (X) was determined by spectrophotometry and converted to number of molecules using the following formula: (concentration in nanograms × 6.023 × 10^{23}) / (bp size of plasmid + insert × 10^9 × 650). The sensitivity of the assay was then determined using the 10-fold serial dilutions of pTZgyrâ. 25μl of reaction was set up which includes SYBR green I Rio mix 8μl, 1μM of both primers, 25ng/ul of template DNA and water to make up the volume. The thermal cycling conditions included three segments; and started with a pre-incubation at 95°C for 10 min (segment 1), followed by 40 cycles of amplification at 95°C for 10 s and 55°C for 30 s (segment 2), and finally the melting curve analysis at 95°C for 10 s, 55°C for 30 s and 95°Cfor 30 s (segment 3). All the reactions were performed in triplicates using plasmids (pTZgyrâ), reactions without addition of DNA served as negative control. Crude DNA isolated from symptomatic field samples of pomegranate collected from different locations were tested for the infection of Xap using LAMP assay.

RESULTS

Sample selection and DNA extraction

The yellow colour pin head colonies were isolated from infected pomegranate leaf on nutrient agar medium. The template DNA from the isolate was PCR analysed with Gyrase B specific primers and the amplified product of 490bp was confirmed as Xap by sequencing (Eurofins). The initial concentration of the DNA was adjusted to 100ng/μl and serially diluted in the range of 10^{-1} to 10^{-9} for PCR and LAMP assay.

Optimization of LAMP assay

The optimum reaction conditions were established for fast and efficient LAMP assay. The LAMP assay performed at different time detected Xap at 45 and 60 min of incubation. However the optimal amplification of the gyrâ gene fragment was 65°C for 60 min, as shown by agarose gel electrophoresis and the amplified products exhibited a typical ladder-like pattern. No products were observed in negative control without template DNA (Fig 2). The sensitivity of LAMP was compared with that of conventional PCR, and results showed that the detection limit of LAMP assay was 1fg/μl (10^{-9} dilution) of purified Xap DNA when compared with conventional PCR showing amplification only upto 10^{-6} dilution (1pg/μl concentration) of DNA (Fig 3).

Xap liquid culture exhibited similar sensitivity levels where as bacteria at 10^{-9} dilution was efficiently detected using LAMP which was limited to 10^{-6} dilution with PCR (Fig. 4) confirming LAM P to be more sensitive and rapid than PCR. Pathogenic extract from infected leaf were visualized as increase in turbidity as well as color change from violet to sky blue by pre addition of HNB showing positive result (Fig 5). LAMP assays carried out using healthy host plant gave no amplification as evidenced by gel electrophoresis. A BLAST search performed using LAMP product showed homology with gyrase gene sequence of Xap (Acc No. EU285148).

Sensitivity of LAMP assay

Table 1: Location and Sequence of LAMP primer sets targeting Xap gyrâ gene (198 bp)

Primer name	Amplicon size (5'- 3')	Sequence
F3	296 – 311	GCCTGCCACGTTGGTG
B3	454 – 471	TGATCGGGTGGCTCTTGT
FIP	F1C+F2	CTTTCGAAGGCCCCCATCGG-ATCCGCCGCCACATG
BIP	B1C+B2	CTGCGTGAGGTGTCGCTGG-TGCCGCGGACGATCTG
F2	314 – 329	CCGCCGCCACATG
F1c	354 – 373	CTTTCGAAGGCCCCCATCGG
B2	438 – 453	TGCCGCGGACGATCTG
B1c	390 – 408	CTGCGTGAGGTGTCGCTGG

F3 F2 F LOOP (RC)
GCCTGCCACGTTGGTGGCATCCGCCGCCACATGGCGAAGAGTTCACCCAGGTGCT
F1C (RC) B1C
GCCGATGGGGGCCTTCGAAAGCGGCGAGCTGCGCCCGCTGCGTGAGGTGTCGCTGG
B LOOP B2 (RC) B3 (RC)
CCCTGCACGACCTGGTGCGCGAAGGCGCCCAGATCGTCCGCGGCAACAAGAGCCAC

CCGATCA

Figure 1: Schematic representation of binding pattern of LAMP primers for amplification.

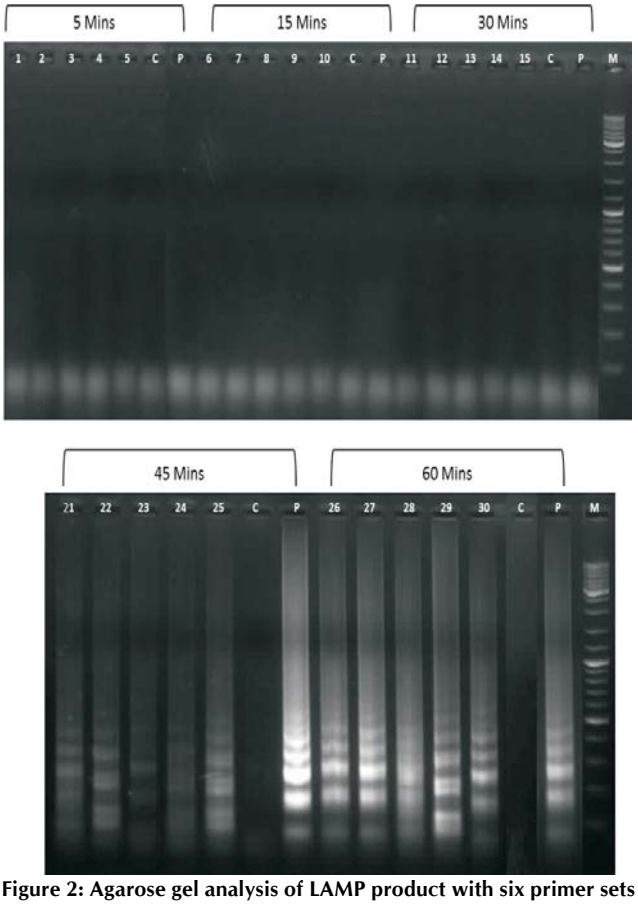

Figure 2: Agarose gel analysis of LAMP product with six primer sets at different time interval (5 to 60 min). Lane 1 - 5, 6-10, 11-15, 16-20, 21-25, 26-30 represents Xap DNA (initial concentration of 100 ng/μl) dilution ranging from 10^{-1} to 10^{-5} and C -Negative control, P – Positive control plasmid pTZgyrâ

The detection limit of Real time PCR was found to be as low as single copy/μl. A linear relation between the cycle threshold and copy number was observed and the standard curve (Fig 6a) generated a slope of -3.3669 with a reaction efficiency of 98.16%. The correlation coefficient of standard (R^2) was above 0.994. The amplification curve (Fig. 6b) analysis showed a unique peak at 91.7p C which indicates that only the target DNA was amplified without the interference of non-specific amplification or primer dimer. All copy numbers (1000, 500, 100, 10, 1) of recombinant plasmid were amplified in realtime PCR while LAMP detection levels were upto 10 copies of plasmid DNA (Fig. 7). The sensitivity of LAMP in detecting Xap was greater than conventional PCR but not as sensitive as real-time PCR. Crude DNA extract of symptomatic pomegranate field samples collected from different locations gave positive reaction (Fig. 8) suggesting that LAMP amplifications are successfully carried with crude DNA extract without need for purification. Amplification of Xap was successfully achieved from Xap DNA, cultured cells and extract of infected plant hosts, thus confirming the robustness of LAMP in disease diagnosis.

DISCUSSION

Bacterial blight of pomegranate is a deadly disease of pomegranate infecting almost all plant parts. The early detection of disease helps to minimize damage to the crop and prevents further spread. PCR based detection of Xap currently followed may not be applicable for field screening due to the requirement of non portable equipments. In the present work, LAMP based technology has been applied for quick detection of Xap without the requirement of PCR machine. The gene gyrâ has proved to be effective in detection of Xap as it has been described by previous workers (Kalyan *et al.,* 2012). LAMP required much lesser time for detection of Xap showing a typical ladder-like pattern on agarose gel electrophoresis unlike PCR based application routinely followed Utilisation of LAMP assay has resulted in favourable response in detecting number of plant and animal pathogens as well (Hodgetts *et al.*, 2015; Jeong *et al.,* 2015). The LAMP assay showed higher sensitivity compared to PCR in terms of detecting the number of colonies or purified DNA. Tsai *et al.* (2013) demonstrated the detection limit of the LAMP assay to be approximately 300 CFU using crude bacterial lysates, 100-fold more sensitive than PCR. Rigano *et al.* (2010) demonstrated the sensitivity of CBC-LAMP as 5 CFU in cultured samples and 10fg in pure Xcc DNA. In our studies the LAMP assay was 1000- fold more sensitive than PCR. Detection limit of purified Xap DNA by LAMP assay was 1fg/μl (10^{-9} dilution) when compared with conventional PCR which showed amplification only upto 10^{-6} dilution (1pg/μl

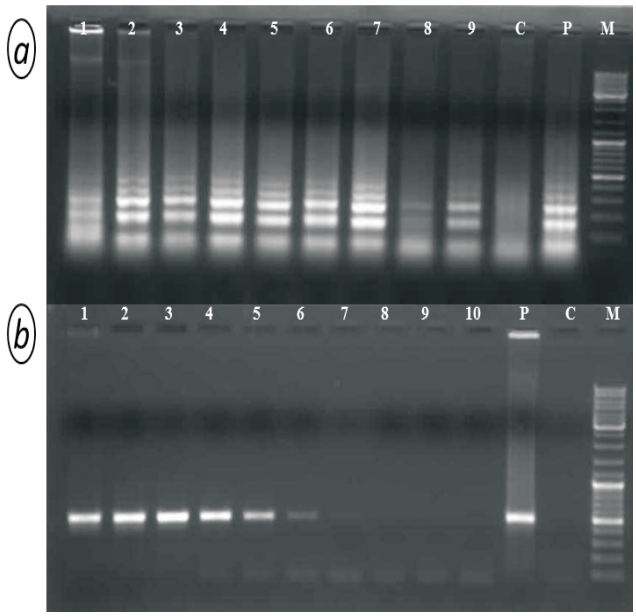

Figure 3: Sensitivity of LAMP and conventional PCR using serially diluted Xap DNA (initial concentration of 100 ng/μl) from 10^{-1} to 10^{-9}. (a) Lane 1 to 9 LAMP detection product on agarose gel stained with EB, P - Positive control plasmid p T Zgyr â, C- Negative control and M - Generuler mix 1 Kb ladder, (b) Lane 1 to 10 conventional PCR, P - Positive control plasmid pTZgyrâ, C - Negative control and M - Generuler mix 1 Kb ladder

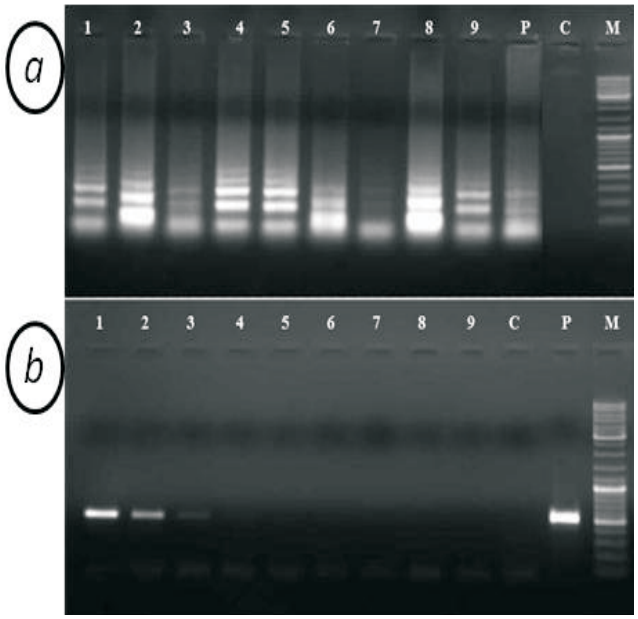

Figure 4: Sensitivity of LAMP and conventional PCR using serially diluted Xap culture (OD $_{600\ nm}$ = 1, approximately 10^9 CFU/ml) from 10^{-1} to 10^{-9}. (a) Lane 1 to 9 LAMP detection product on agarose gel stained with EB and M - Generuler mix 1 Kb ladder, (b) Lane 1 to 9 conventional PCR, P- positive control plasmid pTZgyrâ, C - Negative control and M - Generuler mix 1 Kb ladder

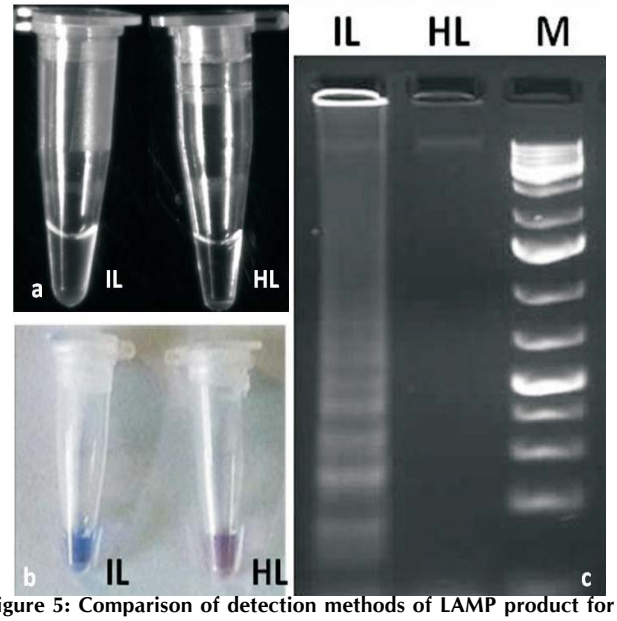

Figure 5: Comparison of detection methods of LAMP product for Xap infected and healthy leaf sample (a)Visual assessment based on formation of white precipitate in infected leaf sample (IL), Absence of precipitation in healthy leaf sample (HL) (b) Visualization of colour change from violet (HL-healthy leaf) to blue (IL: Infected leaf sample) using HNB (c) LAMP product detected by agarose gel electrophoresis stained by EB- IL: Infected leaf sample, HL–Healthy leaf sample and M - Generuler mix 1 Kb ladder

concentration) of DNA. The visual assessment of LAMP product was confirmed by formation of white precipitate as a result of accumulation of Magnesium pyrophosphate similar assesment was used for the detection of *Vibrio* sps by Yamazaki *et al*. (2010). The results obtained by using a metal-ion indicator, hydroxynaphthol blue (HNB) for low-cost detection were consistent with those obtained by gel electrophoresis. Ghosh *et al*. (2015) have successfully employed similar dye for detection of wilt pathogen of chickpea. However other dyes like SYBR Green (Wang and Turechek, 2016), ethidium bromide, Pico Green (Tomlinson *et al.,* 2007) are also reported to be useful for detection of LAMP product. Besides visual indicator, the use of such dyes to the reaction mixture eliminates the contamination risk.

Comparison of real time assay with LAMP showed real time assay to be more sensitive than LAMP in the present study. The results were in consistent among LAMP assays designed to detect bacterial pathogens from infected plants (Harper *et al*., 2010 Kubota *et al*., 2008; Okuda *et al*., 2005). However the sensitivity of both assays were proven to be similar with the DNA purified from a *Candidatus* Liberibacter asiaticus positive plant (Rigano *et al.,* 2014)

The crude DNA extraction method using Edward *et al*. (1991) from bacterial spots collected from leaves shortened the sample preparation protocol and need of equipments. The amplification of crude DNA extracted from infected samples using LAMP showed that the procedure tolerates certain inhibitors in crude DNA extract as evidenced in earlier reports (Kaneko *et al*., 2007, Francois *et al*., 2011). Hence the detection speed and the capability to perform field trial inspection aids plant protection measures for a quality production.

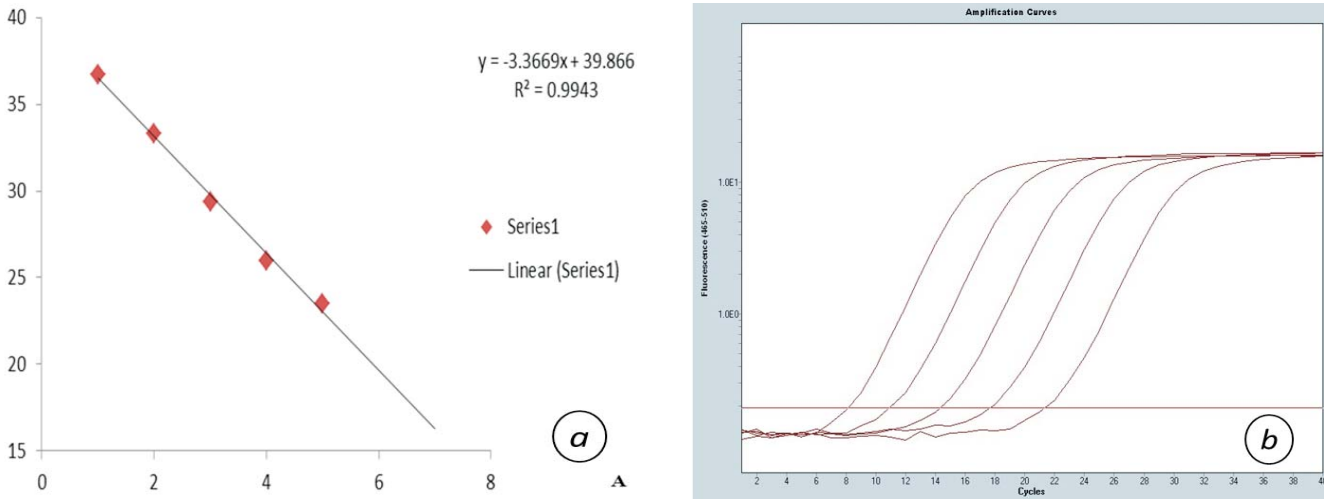

Figure 6: Sensitivity of real-time PCR assay. (a) The standard curve, (b) amplification curve generated using known concentration of recombinant plasmid containing the serially diluted gyrâ gene (copy number of 1000, 500,100, 10, 1)

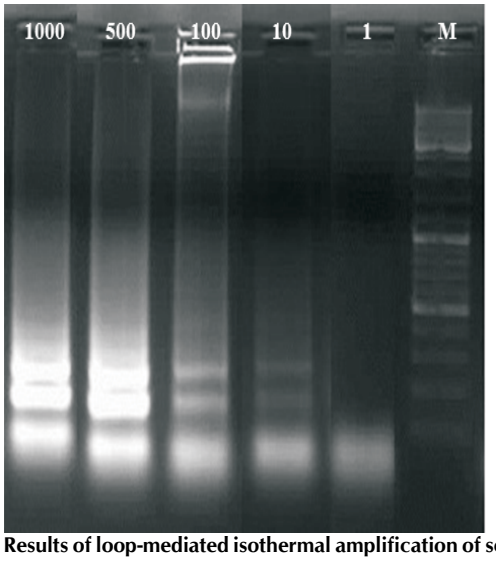

Figure 7: Results of loop-mediated isothermal amplification of serially diluted TAcloning vector containing gyrâ gene (copy numbers of 1000, 500,100, 10, 1) after 60 min of incubation at 65°C, M – Generuler mix 1 Kb ladder.

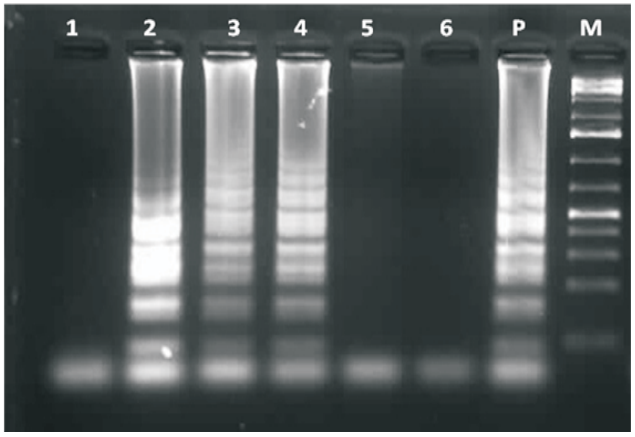

Figure 8: Diagnosis of field samples from different locations for LAMP amplification on agarose gel. Lane 1,5 & 6-no amplification due to absence of Xap infection. Lane 2, 3and 4-show amplification due to the presence of Xap infection, P represents positive control plasmid pTZgyrâ and M - Generuler mix 1 Kb ladder

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