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ORIGINAL ARTICLE

Development and evaluation of loop-mediated isothermal amplification assay for rapid and sensitive detection of potato cyst nematode, *Globodera pallida* from soil

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Abstract

Potato cyst nematodes, *Globodera pallida* and *G. rostochiensis*, are economically important and difficult to manage pests of the potato crop. The cyst of both the species looks similar and it is difficult to differentiate once it turns brown upon maturity. Early detection of the PCN at the species level is crucial to avoid its further spread and for adopting the appropriate management strategies. Therefore, in the present study, highly specific and sensitive loop-mediated isothermal amplification (LAMP) assay was developed to amplify mitochondrial-Sequence Characterized Amplified Region (SCAR) sequence of potato cyst nematode, *G. pallida*. The LAMP assay was completed within a shorter incubation period of 60 min at 60 °C followed by the reaction termination at 80 °C for 5 min. The developed LAMP assay exhibited high specificity for *G. pallida* and did not detect any other species including its sibling species, *G. rostochiensis.* In sensitivity tests, the assay detected *G. pallida* at 1000 times less DNA concentration (10 fg/µl) as compared to conventional PCR (10 pg/µl). In addition to this, the developed LAMP assay was tested for the detection of *G. pallida* directly from the soil samples, and even a single cyst mixed with soil was successfully detected by the developed assay. Moreover, the utility of low-cost instruments like hot water bath was also demonstrated for the detection of *G. pallida* from the soil. The developed LAMP is a rapid, highly specific, sensitive, and cost-effective technique for the species-specific detection of *G. pallida*. The developed assay will facilitate the rapid detection of *G. pallida* at quarantine stations as well as from the fields which will help to stop its further spread in new areas and also to devise effective management strategies for sustainable potato production.

Keywords Detection · *Globodera pallida* · LAMP · Management · Mitochondrial-SCAR · Potato

Introduction

Potato (*Solanum tuberosum* L.) is the third most important food crop of the world after rice and wheat, considered as a food for nutritional security of the growing world population (Mishra et al. 2020). The increasing demand for potatoes can be attained by enhancing the productivity potential as well as by reducing the yield losses caused by several biotic and abiotic stresses. Among the biotic stresses, potato cyst nematodes (PCN) are the major problems of potato cultivation worldwide. It comprises two important species (*Globodera rostochiensis* and *G. pallida*), can cause serious yield reductions (Oerke et al. 1994), and severely affect the movement of potatoes around the globe including India. In India, PCN was first reported from Udhagamandalam, The Nilgiri, Tamil Nadu (Jones 1961), whereas recently due to the presence of PCN in North India, the domestic quarantine was implemented in 35 locations from Himachal Pradesh, Jammu & Kashmir, and Uttarakhand hills (Chandel et al. 2020).

Among the PCN species, *G. pallida* was alone reported to cause up to 80% yield reductions in potatoes (Talavera

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et al. 1998). This necessitates the appropriate management of the species. The perfect identification of the species is a prerequisite for suggesting the efficient management strategies. Traditionally, the identification of plant parasitic nematode species is relying upon morphology and morphometric observations which are complex, laborious, time-consuming, and need taxonomic expertise (Oliveira et al. 2011). In particular, due to the microscopic size and presence of sibling species, it is very difficult to differentiate different stages including cysts of *G. pallida* from *G. rostochiensis* (Stone et al. 1973; Kaushal et al. 2007). Therefore, the use of polymerase chain reaction (PCR) became the most favored method to identify PCN species that produce specific and accurate results. Among the PCR-based techniques, use of the internal transcribed spacer (ITS) region of rDNA and species-specific markers became popular for the identification of PCN species (Mulholland et al 1996; Bairwa et al. 2017; Niragire et al. 2019; Hajjaji et al. 2021). But, these techniques require expensive and sophisticated laboratory facilities, such as thermo-cycler, imaging system, sequencer, etc., and, also it cannot be applied directly under the field conditions. This provides an opportunity for utilization of the novel technique like loop-mediated isothermal amplification (LAMP) for rapid amplification of the targeted region of DNA (Notomi et al. 2000; Tomita et al. 2008). This technique is superior to PCR in terms of its simplicity, rapidity, specificity, sensitivity, and cost-effectiveness. Above all, only a simple heating block or hot water bath is needed that could maintain a constant temperature for amplification of the target gene. Because of these advantages, LAMP has been used commercially in a variety of pathogens detection kits including nematodes (Mori and Notomi 2009; Ahuja and Somvanshi 2020).

Accordingly, Kikuchi et al. (2009) were the first to demonstrate the application of the LAMP technique in plant parasitic nematodes by detecting an Aphelenchid pinewood nematode, *Bursaphelenchus xylophilus* which is responsible for a devastating "pine wilt" disease and is of quarantine importance in many countries. Other than *B. xylophilus*, LAMP assay was developed for few other Aphelenchid nematodes, such as *Aphelenchoides fragariae* (Bennison et al. 2015), *B. cocophilus* (Ide et al. 2017), *A. besseyi* (Yang and Yu 2019), and *A. ritzemabosi* (Wang et al. 2019), whereas among the Tylenchid nematodes, the LAMP has been developed to detect several economically important nematode species, such as *Meloidogyne incognita* (Niu et al. 2011), *M. enterolobii* (Niu et al. 2012), *Radopholus similis* (Peng et al. 2012), *M. mali* (Zhou et al. 2017), *H. avenae*, *H. filipjevi* (Peng et al. 2015; Wei et al. 2016), *M. hapla* (Peng et al. 2017), *Tylenchulus semipenetrans* (Song et al. 2017), *M. chitwoodi, M. fallax* (Zhang and Gleason 2019), *M. partityla* (Waliullah et al. 2020), *Anguina wevelli* (Yu et al. 2018), and *M. graminicola* (He et al. 2020). In addition to this, recently, the LAMP assays have been developed for the detection of *G. rostochiensis (*Ahuja et al. 2021) and *G. pallida* (Camacho et al. 2021) using ITS region. However, in the present study, an attempt has been made to develop a species-specific LAMP assay for the detection of *G. pallida* using a mitochondrial-Sequence Characterized Amplified Region (SCAR). The mitochondrial-SCAR sequence of *G. pallida* was selected based on the higher specificity exhibited by the gene when sequence similarity was confirmed using NCBI database. In addition to this, quick and specific detection of *G. pallida* directly from the soil is the need of an hour for the development of management strategy against this species. In view of this, efforts were also made to extend the utility of the developed LAMP protocol directly from the soil samples for on-field detection of *G. pallida*.

Materials and methods

Potato cyst nematodes samples

The pure populations of *G. pallida* and *G. rostochiensis* were maintained on susceptible potato cultivar Kufri Jyoti by inoculating single cyst of each species separately under glasshouse conditions at ICAR-Central Potato Research Institute, Regional Station, Kufri, Himachal Pradesh. At 55–60 days, the color of the developing globose females was observed for the morphological confirmation. The female cysts were collected and used for DNA extraction. The species confirmation at the molecular level was done using species-specific primers (Mulholland et al 1996).

Genomic DNA extraction and PCR amplification of the mitochondrial-*SCAR sequence of G. pallida.*

The genomic DNA of *G. pallida* and *G. rostochiensis* was extracted from a single female cyst nematode following the CTAB method with few modifications (Bairwa et al. 2019). The quality and the quantity of the purified DNA sample were determined using the NanoDrop 2000/2000c spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA).

A nucleotide sequence of mitochondrial-SCAR specific to *G. pallida* retrieved from GeneBank (Accession numbers DQ631912) was used for designing the primers. Primers were designed using FastPCR software. PCR reaction was carried out with 20 µl reaction mixture containing 2.0 µl genomic DNA (50 ng/µl), 1.0 µl of each primer mix containing 10 pmol of each primer (Forward primer: 5′-ACA GGGGCTGGTGCTTTA-3′ Backward primer: 5′-GCAAAA TTTGGGGCGGGA-3′), 1.0 µl Red Taq DNA polymerase (8 U) (Genei), 2.0 µl Taq buffer A, 1.0 µl 2.5 mM dNTP mix and 12 µl double distilled water (DDH_2O). The amplification was carried out using a Veriti 96-Well Fast Thermal Cycler (Applied Biosystems™, Thermo Fisher Scientific

Inc.), which was preheated at 95 °C for 1 min followed by 35 cycles of 95 °C for 20 s, 60 °C for 20 s, and 72 °C for 45 s and then a final elongation at 72 °C for 10 min. The PCR products were resolved on 2% agarose gel stained with ethidium bromide for visualization under the gel documentation unit. Amplified product was purified using a Qiagen Gel Extraction Kit, and DNA fragment was sequenced using the BigDye Direct terminator cycle sequencing kit (Applied Biosystems, UK). An automated DNA sequencer (Genetic Analyzer 3500, Applied Biosystems) was used to perform the sequencing analysis. Traces were aligned and visualized using the Sequence Scanner Software version 2.0 for Windows (Applied Biosystems 2012). The obtained nucleotide sequences were subjected to NCBI-BLAST to confirm its identity before utilizing it for designing *G. pallida* specific LAMP primers.

LAMP primer design for specific detection of G. pallida

Primers were designed using the Primer Explorer V4 software program (http://primerexplorer.jp/e/) to detect *G. pallida*. A total of four primers were designed and designated as *G. pallida*-Forward outer primer (*Gp*-F3), *G. pallida*-Backward outer primer (*Gp*-B3), *G. pallida*-Forward inner primer (*Gp*-FIP: F1c-F2), and *G. pallida*-Backward inner primer (*Gp*-BIP: B1c-B2).

Optimization of LAMP reaction and conditions

The LAMP reaction was performed in a 25 µl reaction mixture containing 2 µl template DNA (50 ng/µl), 1 µl each *Gp*-FIP and *Gp*- BIP primers (30 pmol), 0.5 µl each of F3 and B3 primers (10 pmol), 1.0 µl of *Bst* DNA polymerase (8000 U/ml, New England Biolabs), 2.5 µl of 10X isothermal amplification buffer 3.5 µl of 10 mM dNTP mix, 1.5 µl of 100 mM MgSO4, and 11.5 µl of double distilled water. For optimizing temperature and amplification time, the reaction was performed at a temperature ranging from 54 °C to 64 °C at five different time points (15, 30, 40, 50, and 60 min). Finally, the reactions were ended at 80 °C for 5 min. The reactions were visualized on agarose gel (2%) stained with ethidium bromide and also visually examined using 2 µl SYBR Gold nucleic acid (Invitrogen) (1:10 diluted) in 8 µl LAMP product.

Sensitivity and specificity of LAMP assay

The sensitivity of the LAMP assay was determined by ten-fold serial dilutions of genomic DNA isolated from a single cyst of *G. pallida* with an initial concentration of 10 ng/µl. The DNA was serially diluted for eight times to reach the DNA concentration of 1 femtogram (fg)/µl (10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl, 100 fg/µl, 10 fg/µl, and 1 fg/µl). 2.0 µl of DNA template from each dilution was used for LAMP and PCR reactions. The LAMP and PCR reactions were set as described above. The sensitivity test was repeated thrice for confirmation of the obtained results.

To check the cross-reactivity or specificity of the LAMP assay, DNA of other plant parasitic nematodes was obtained from the Department of Nematology, Tamil Nadu Agricultural University, Coimbatore (*M. incognita*, *M. javanica*, and *Heterodera cajani*) and ICAR-Central Potato Research Institute, Regional Station, Muthorai, Udhagamandalam, Tamil Nadu (*G. pallida* and *Cactodera estonica*), whereas the population of *H. avenae* used in the present study was collected from the soil samples from wheat growing areas of Kangra, Himachal Pradesh (Latitude: 32.1087, Longitude: 76.2777, Altitude: 733 m above mean sea level), followed by its multiplication on a susceptible wheat cultivar HD 2894 (Mhatre et al. 2017) and DNA extraction as described above.

Optimization of LAMP assay directly from soil

To check the utility of standardized LAMP assay at field conditions, we have checked the performance of LAMP assay for the detection of *G. pallida* directly from the soil samples. This has been accomplished by two methods i.e., by artificial inoculating the soil with the cysts of *G. pallida* and using naturally infested soils from different areas of the country. Accordingly, for the first assay the soil samples (250 mg) were artificially inoculated with 1, 3, 5, and 10 numbers of *G. pallida* cysts. DNA template from 250 mg soil samples was isolated using a NucleoSpin soil kit (Macherey–Nagel, GmbH & Co. KG, Germany) following the manufacture's protocol. The temperature required for LAMP reaction was maintained using a water bath (60 °C) and the products were analyzed as described earlier. In another study, the soil samples were collected from PCN infested area of Himachal Pradesh (Kufri, Fagu, and Jubbal) and The Nilgiris (Muthorai, Appokodu, and Porthyhada), Tamil Nadu, India to check the efficacy of the developed LAMP assay. The coordinate details of these places are given in Supplementary table 1. For validation, DNA isolated from a single cyst of *G. pallida* was used as a standard positive control. DNA from healthy soil was used as no-template water control and double distilled water as a negative control. The reactions were repeated thrice to confirm the results.

Results

Molecular confirmation of PCN species

After 55–60 days of potato planting, the protruding females were observed on the roots of the PCN susceptible potato

cultivar Kufri Jyoti. A single female cyst nematode of each species was used for DNA extraction to confirm the identity at the molecular level using species-specific PCR primers. The *G. pallida* yielded an amplicon of 391 bp whereas *G. rostochiensis* yielded 238 bp (Fig. 1). These populations of PCN were used in the present work for optimization of the LAMP assay.

Details of primers for LAMP assay

The designed PCR primers of the mitochondrial-SCAR of *G. pallida* yielded an amplicon of 187 bp (Supplementary Fig. 1) and the sequence was deposited into the GeneBank database (Accession no. MZ766254). The sequence revealed 99.14% nucleotide similarity only with the reference sequence of *G. pallida* (Accession numbers DQ631912). A set of four *LAMP* primers were designed for species-specific detection of *G. pallida.* The details of these primers viz., *Gp*-F3/B3 and *Gp*-FIP (F1c-F2)/BIP (B1c-B2) are given in Table 1 and the position is shown in Fig. 2. Forward and backward outer primers were designated as *Gp*-F3 and *Gp*-B3, respectively, whereas, forward and backward inner primers as *Gp*-FIP and *Gp*-BIP, respectively.

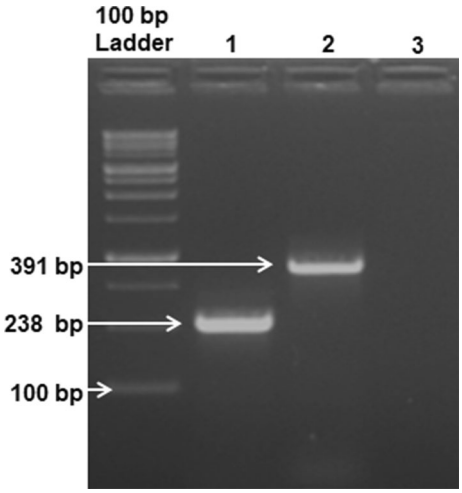

Fig. 1 Molecular confirmation of PCN species using species-specific PCR primers, Lane 1, agarose gel showing species-specific PCR amplification of 238 bp sized amplicon for *Globodera rostochiensis*; Lane 2, an amplicon of 391 bp for *G. pallida*; Lane 3, no-template water control

Standardization of LAMP conditions

Standardization of LAMP assay for determining reaction time and incubation temperature was done at five different time points (15 min, 30 min, 40 min, 50 min, and 60 min) and six different temperatures (54 °C, 56 °C, 58 °C, 60 °C, 62 °C, and 64 °C). The reaction was successful at 40, 50, and 60 min run times with prominent amplification at 6 min, however no clear detectable signals were obtained at 15 and 30 min run time (Fig. 3A). Additionally, the reaction was successful in almost all the tested temperatures, and the maximum yield of product in terms of the sharp amplicon was noted with 60 °C and 60 min run time (Fig. 3). Therefore, the present study standardized the conditions for species-specific detection of *G. pallida* using LAMP primers are an incubation period of 60 °C for 60 min followed by the termination of the reaction at 80 °C for 5 min. In the case of a positive LAMP reaction, a characteristic ladder-like pattern was resolved on agarose gel, whereas, no amplification was observed in no-template water control. The positive samples resulted in a visual change of color from orange to green after the addition of SYBR Gold nucleic acid dye in normal day-light; however, no change in color was noticed in the no-template water

Table 1 Oligonucleotide primers for PCR and LAMP assay used in the present study

S. No	Primer name	Sequence (5′–3′)	Length (bp)	Reference
1	Species-specific primers for *Globodera pallida* and *G. rostochiensis*			
	Gp ITS-1	GGTGACTCGACGATTGCTGT	20	Mulholland et al., (1996)
	Gr ITS-1	TGTTGTACGTGCCGTACCTT	20	
	Universal 5.8S rDNA PCN primer	GCAGAAGGCTAGCGATCTTC	20	
2	LAMP primers specific to *G. pallida*			
	Gp-F3	ACAGGGGCTGGTGCTTTA	18	Designed in this study
	Gp-B3	GCAAAATTTGGGGCGGGA	18	
	Gp-FIP	CCGTAGGCGCATACTGTG GATC-ATCCTACCTCTACTG ACCCG	42	
	Gp-BIP	AACTCGGCAGTTTGGTGT TCGA-AATTCGATACCGCGC TCG	40	

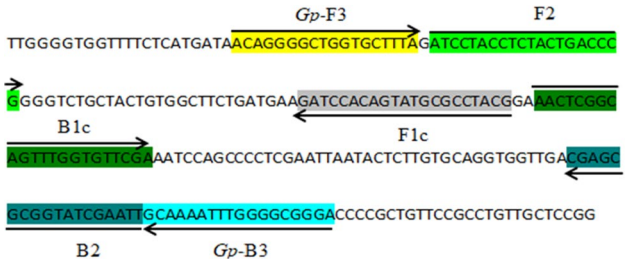

Fig. 2 The position of respective LAMP primers on mitochondrial-Sequence Characterized Amplified Region, *Gp*-F3 (forward outer primer); *Gp*-FIP (forward inner primer, a conjugated primer of F2 and a complementary sequence i.e., F1c); *Gp*-B3 (Backward outer primer), *Gp*-BIP (Backward inner primer, a conjugated primer of B2 and a complementary sequence i.e., B1c)

control. Under ultraviolet light, strong fluorescence was observed for positive reactions, while the fluorescence was absent from no-template water control (Fig. 3). Additionally, mitochondrial- cytochrome c oxidase subunit I (mt-COI) sequences of fourteen other plant parasitic nematodes were retrieved from NCBI database and the multiple sequence alignment was done using BioEdit software with *G. pallida* mitochondrial-SCAR sequence. The analysis revealed that the selected sequence and primers are highly specific with *G. pallida* (Supplementary Fig. 2). Our study demonstrated the utility of LAMP assay for species-specific detection of *G. pallida.*

Determination of sensitivity of LAMP assay

The sensitivity of the LAMP assay was tested on a tenfold serial dilution of crude genomic DNA preparation from a single cyst of *G. pallida*. The LAMP could detect DNA up to 10 femtograms (fg)/µl genomic DNA, whereas, conventional PCR could detect DNA only up to 10 picograms (pg)/µl genomic DNA (Fig. 4). The positive reactions produced the green-fluorescent color when added with SYBR Gold nucleic acid dye and also exhibited a ladder-shaped pattern when resolved on the gel. The water control and negative reaction did not exhibit any fluorescence and remain orange in color. The present study demonstrated a 1000 fold sensitivity of LAMP over conventional PCR assay (Fig. 4).

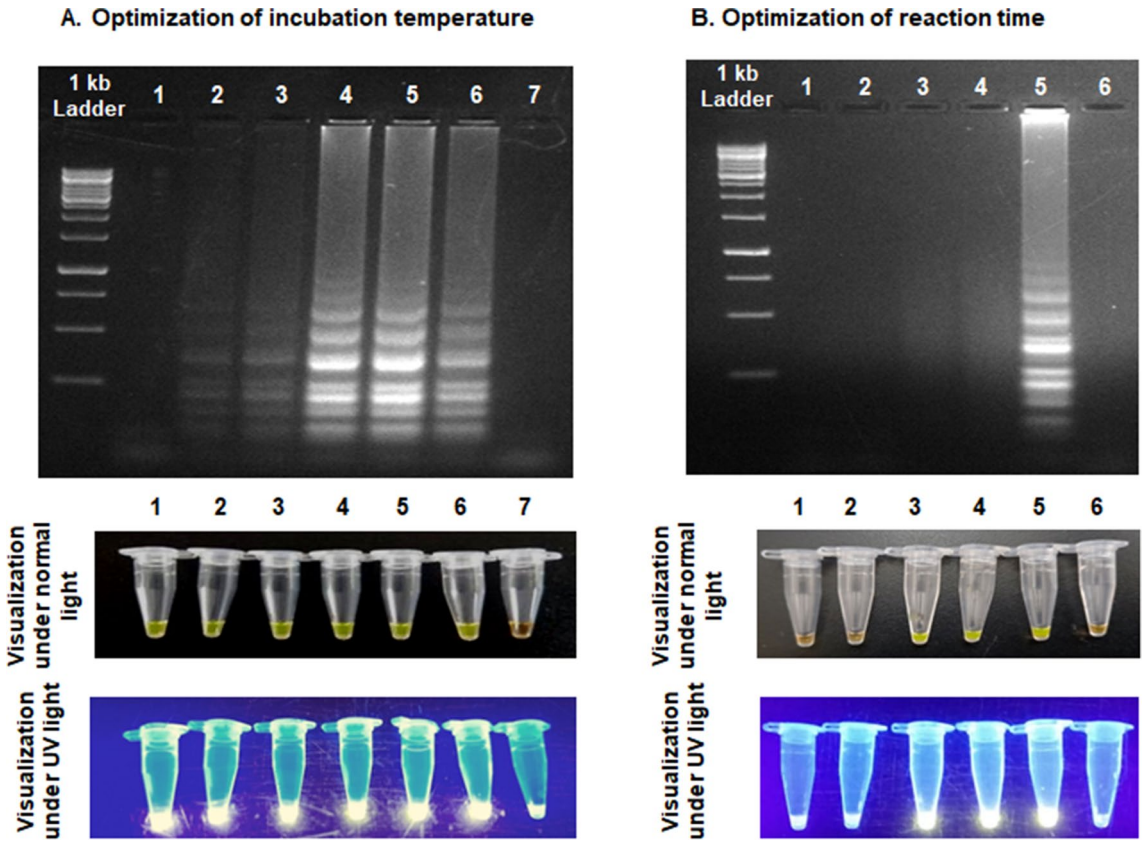

Fig. 3 Standardization of LAMP conditions. **A**. Optimization of incubation temperature at six different temperatures- Lane 1, 54 °C; Lane 2, 56 °C; Lane 3, 58 °C; Lane 4, 60 °C; Lane 5, 62 °C; Lane 6, 64 °C; Lane 7, No template water control. **B**. Optimization of reaction time at five time points-Lane 1, 15 min; Lane 2, 30 min; Lane 3, 40 min; Lane 4, 40 min; Lane 5, 60 min; Lane 6, No template water control. The bottom panel shows the visualization of amplified products under normal and UV lights, respectively

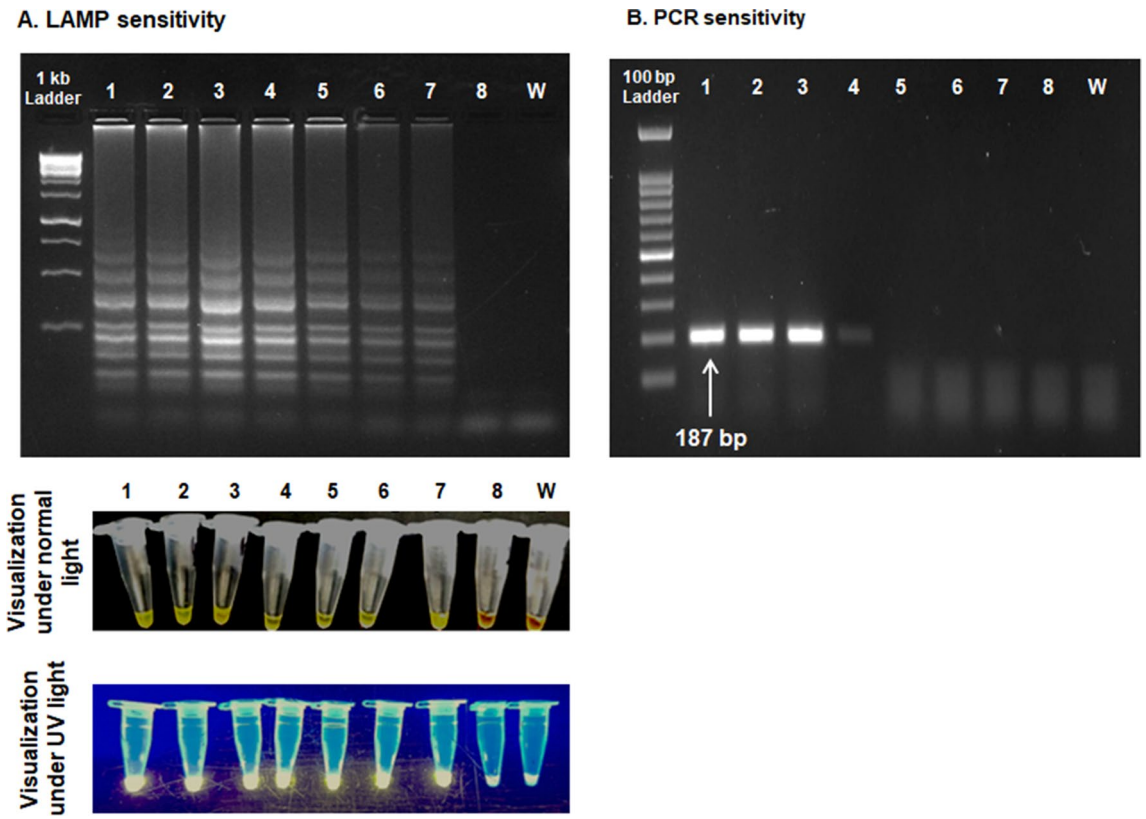

Fig. 4 Determination and comparison of sensitivity of LAMP assay and PCR assay. **A** Determination of the sensitivity of standardized LAMP assay, Lane 1, 10 ng/µl; Lane 2, 1 ng/µl; Lane 3, 100 pg/µl; Lane 4, 10 pg/µl; Lane 5, 1 pg/µl; Lane 6, 100 fg/µl; Lane 7, 10 fg/µl; Lane 8, 1 fg/µl; Lane 9, No template water control. The bottom panel shows the visualization of amplified products under normal and UV lights, respectively. **B** Determination of the sensitivity of the PCR assay, Lane 1, 10 ng/µl; Lane 2, 1 ng/µl; Lane 3, 100 pg/µl; Lane 4, 10 pg/µl; Lane 5, 1 pg/µl; Lane 6, 100 fg/µl; Lane 7, 10 fg/µl; Lane 8, 1 fg/µl; Lane 9, No template water control

Determination of specificity of LAMP assay

In the specificity assay, the genomic DNA of different species of plant parasitic nematodes, such as *G. rostochiensis*, *M. incognita*, *M. javanica*, *H. avenae*, *H. cajani*, and *C. estonica*, was used to determine the specificity of standardized LAMP assay. The reaction was conducted at an incubation of 60 °C for 60 min thereafter final termination at 80 °C for 5 min. The LAMP assay positively amplified only the DNA of *G. pallida* and not the DNA of other tested nematodes. The visualization of LAMP product under normal day conditions, UV light, and gel electrophoresis perfectly differentiated positive and negative reactions (Fig. 5). This assay concluded that the developed LAMP assay was specific to *G. pallida.*

Detection of G. pallida using LAMP assay directly from the soil

In addition to the development, optimization, sensitivity, and specificity of LAMP assays, we have also studied the utility of the assay for the detection of *G. pallida* directly from soil samples. The studies were conducted for the detection of *G. pallida* from artificially inoculated soil (1, 3, 5, and 10 cysts per 250 mg soil) and naturally infested soils (6 samples from two parts of India). After incubation at 60 °C for 60 min thereafter the final termination at 80 °C for 5 min, the LAMP reaction showed positive amplification for all the inoculated and naturally infested soil samples. The visualization of LAMP product under normal day light, UV light, and agarose gel differentiated the positive reactions in all the inoculated and infested soil samples and negative reactions of healthy soil sample and no-template water control (Fig. 6). In the current experiment, a hot water bath was used as a source of maintaining the reaction temperature and the reaction was successful using the same.

Discussion

PCNs are the highly specialized pests of potato crop with the host range restricted to very few species of the *Solanaceae* family (Mhatre et al. 2022). The morphological marker for identification of *Globodera* spp. females is the

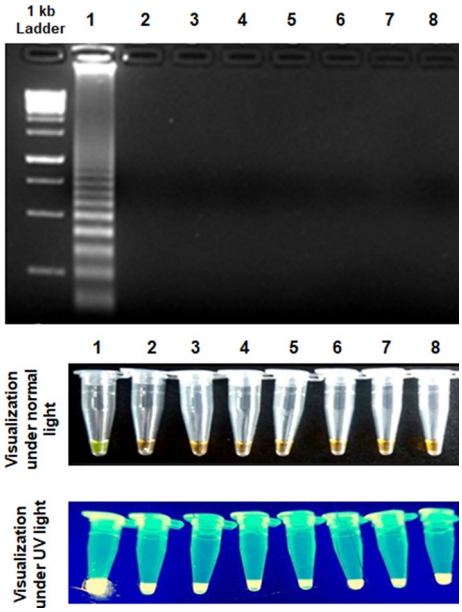

Fig. 5 Determination of LAMP specificity with different nematode species, such as, Lane 1, *Globodera pallida*; Lane 2, *G. rostochiensis*; Lane 3, *Meloidogyne incognita*; Lane 4, *M. javanica*; Lane 5, *Heterodera avenae*; Lane 6, *H. cajani*; Lane 7, *Cactodera estonica*; Lane 8, No template water control. The bottom panel shows the visualization of amplified products under normal and UV lights, respectively

color of developing female that can be seen only when the female is alive (during crop season). After completion of life cycle the female dies and form a resistant protective cover called cyst which is black to brown in color for all the *Globodera* spp. Other than this, most of the people are still relying on the traditional morphology of cyst which is laborious, time-consuming, and require expertise. However, PCR-based identification is elaborate and require sophisticated and costly laboratory equipment like thermal cycler (Bulman and Marshall 1997; Thiery and Mugniery 1996). Despite all the quarantine measures, PCN has crossed the international boundaries specifically through the soil carried by the potato seed material. Once established in the field, it is almost impossible to eradicate the PCN; therefore precise and timely detection of the species at an early stage either in the field or at quarantine centers is a prerequisite for its efficient management (Camacho et al. 2020). Therefore in the present study, an attempt was made to develop a species-specific LAMP assay for the detection of *G. pallida* using crude genomic DNA and infested soil samples. The results of the present study have been validated with the sibling species i.e., *G. rostochiensis,* which confirmed the specificity of the developed protocol for species-specific detection of *G. pallida*. The LAMP protocol worked well without loop primers hence only two pairs of oligonucleotide primers along with specific incubation time and period is enough for the assay to work efficiently. Our results are consistent with the LAMP protocols developed for *Tylenchulus semipenetrans* (Lin et al. 2016), *M. hapla* (Peng et al. 2017), *Anguina wevelli* (Yu et al. 2018), *G. rostochiensis* (Ahuja et al. 2021), and *G. pallida* (Camacho et al. 2021), where the LAMP assay was optimized without loop primers.

In the present study, it was also found that the developed LAMP assay is 1000 times more sensitive than the conventional PCR, as the produced the positive results to the least genomic DNA concentration of 10 fg/µl whereas, the conventional PCR has detected *G. pallida* up to 100 pg/µl. These results of the higher sensitivity of LAMP assay were consistent with earlier studies where the sensitive LAMP assays were developed for *M. incognita* (Niu et al. 2012), *R. similis* (Peng et al. 2012), *M. chitwoodi,* and *M. fallax* (Zhang and Gleason 2019) than PCR. Several other researchers also demonstrated the higher sensitivity of LAMP over conventional PCR (Notomi et al. 2000; Njiru et al. 2010; McKenna et al. 2011).

In the present study, the developed LAMP assay was found highly specific to *G. pallida* and no false reaction was observed even with closely related sibling species, *G. rostochiensis*. The specificity of LAMP primers was also reported by Zhang and Gleason (2019) for the specific detection of *M. chitwoodi* and *M. fallax* and suggested that the closely related species should be tested to avoid false reactions. Recently, Ahuja et al. (2021) reported the specificity of the developed PCN-LAMP protocol for specific detection of *G. rostochiensis* but the reactivity of the primers has not been checked with the DNA of *G. pallida* due to the unavailability of the nematode. However in the present study the specificity of the LAMP assay was evaluated considering the closely related sibling species, *G. rostochiensis*, and other related cyst nematodes species like *H. avenae*, *H. cajani*, and *C. estonica.*

The interesting part of the study was the successful demonstration of the developed LAMP assay for species-specific detection of *G. pallida* directly from soil samples. This enables the rapid detection of the species with high precision. The present study demonstrated that the developed LAMP assay can detect even a single cyst of *G. pallida* from the soil. Moreover, a hot water bath was used for this study to check the utility of low-cost instruments for species-specific detection of the species using the developed LAMP protocol. The results are interesting and will be useful for rapid and species-specific detection of *G. pallida* at quarantine centers where the presence of the cysts can be checked easily as it is being carried by the infested soil along with potato seed tubers.

Very recently, Camacho et al. (2021) also developed a LAMP protocol specific to *G. pallida* but the protocol is completely different than the one which is developed in the present study. Moreover, it's always better to have more than one protocol to detect a pathogen as it was developed for

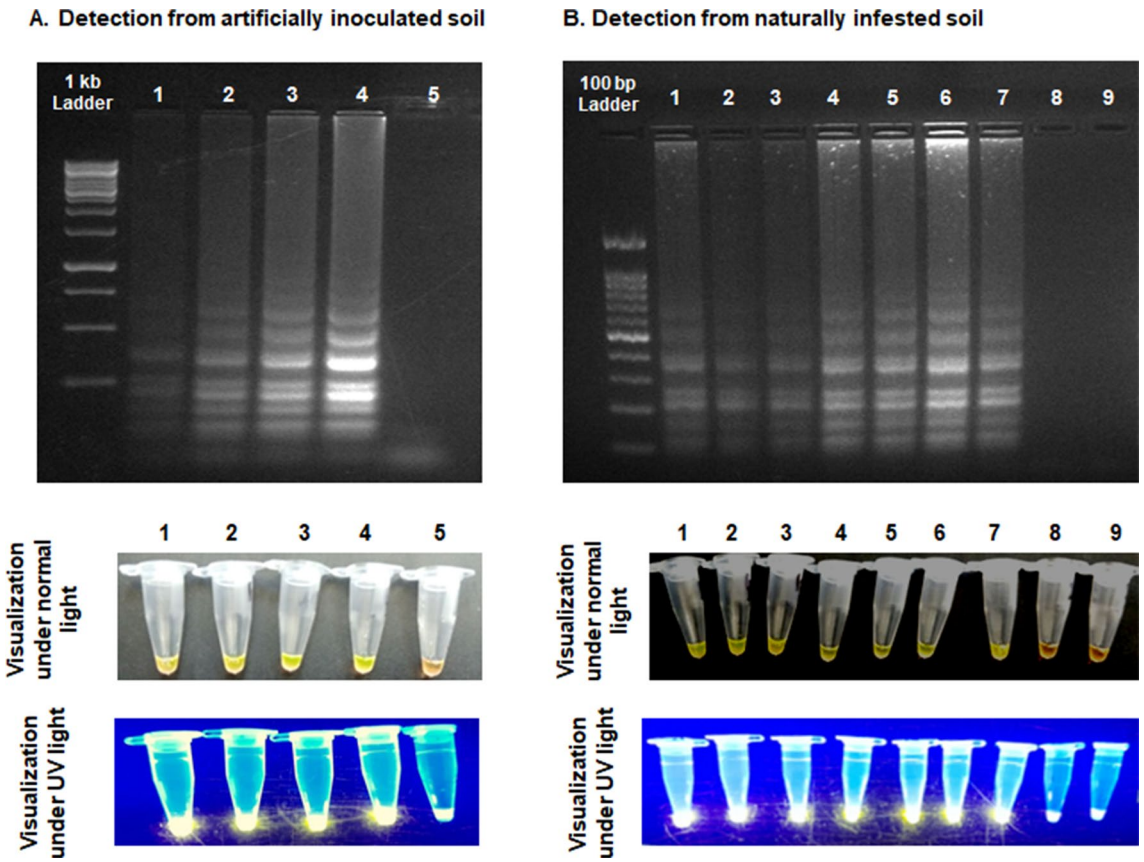

Fig. 6 Detection of *Globodera pallida* directly from soil using LAMP assay. **A**. Detection of *G. pallida* from artificially inoculated soil with, Lane 1, Single cyst; Lane 2, Three cysts; Lane 3, Five cysts; Lane 4, ten cysts; Lane 5, No template water control. **B**. Detection of *G. pallida* from naturally infested soil samples from, Lane 1, Muthorai; Lane 2, Appokodu; Lane 3, Porthyhada; Lane 4, Kufri; Lane 5, Fagu; Lane 6, Jubbal; Lane 7, positive control-*G. pallida*; Lane 8, Healthy soil without *G. pallida*; Lane 9, No template water control. The bottom panel shows the visualization of amplified products under normal and UV lights, respectively

B. xyllophillus, a quarantine important pine wilt nematode (Kikuchi et al. 2009; Kang et al. 2015; Meng et al. 2018). The LAMP assay developed in the present study showed higher sensitivity i.e., 10 fg/µl verses 5 pg/ul of the one developed by Camacho et al. (2021). Additionally the developed LAMP assay has demonstrated the utility for species-specific detection of *G. pallida* directly from the soil samples. This will facilitate the rapid, specific and more efficient detection of *G. pallida* at quarantine stations as well as from the field.

In conclusion, the primers developed for the LAMP assay specifically detected *G. pallida* in a short time with higher sensitivity as compared to conventional PCR. The reaction can be completed within 60 °C for 60 min thereafter final termination at 80 °C for 5 min for obtaining specific results. Our study proposes that the assay developed targeting mitochondrial-SCAR sequence combined with SYBR Gold nucleic acid dye was found to be highly reliable to detect *G. pallida* directly from the infested soil samples in the field conditions for advising proper management protocols. In addition to this, to stop the further spread of *G. pallida* in new localities the developed protocol can be adopted at quarantine stations of different countries to check the presence of *G. pallida* from the infested soil samples carried away by the seed potatoes.

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Declarations

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

Ethical statement This paper is not been considered in this or any other form for publication elsewhere. No ethics have been violated in compiling this article.

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