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Detection of Coat Protein Gene of the *Potato Leafroll Virus* by Reverse Transcription Loop-Mediated Isothermal Amplification

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Abstract

Loop-mediated isothermal amplification assay amplifies DNA/RNA with high specificity and sensitivity. In this study, we describe an optimized reverse transcription- LAMP assay for detection of *Potato Leafroll Virus*. Firstly, DAS-ELISA assay was performed to detect of the virus in a collection containing 40 suspicious samples. Lastly, two samples were detected as the positive samples. Then, the positive samples were verified by RT-PCR and RT-LAMP methods. Furthermore, the results demonstrated that the RT-LAMP assay was 40 times sensitive and 4 time faster compared to RT-PCR. RT-LAMP assay was accomplished in the water bath either frees from any thermal cycler machine or sophisticated laboratories facility. Moreover, in RT-LAMP reaction the positive samples were detected through turbidity which produced by magnesium pyrophosphate. Interestingly, the application of $CaCl_2$ instead of $MgSO_4$ which create calcium pyrophosphate in reaction could significantly increase both stability and concentration of turbidity. Consequently, it could be an interesting alternative to MgSO4. Overall, the newly developed RT-LAMP assay can be a sensitive, specific and low-cost method for early detection of *Potato Leafroll Virus* and also other viral plant pathogens.

Keywords: *Potato Leafroll virus*; Coat protein gene; RT-LAMP; RT-PCR

Introduction

Potato (*Solanum tuberosum*) is the fourth important food resources in the daily food chain which was affected by several pathogenic agents including viruses, resulting a significant diminution in yield production worldwide. Despite the presence of a broad spectrum of diverse deleterious viruses, the most common and economic ones are *Potato virus A*, *Potato virus Y* (PVA, PVY, both from genus *Potyviruses*), *Potato leafroll virus* (PLRV, genus *Polerovirus*), *Potato virus X* (PVX, genus *Potexvirus*) and *Potato virus S* (PVS, genus *Carlavirus*) [1-4]. Viruses of the family Luteoviridae belongs to ssRNA plant viruses, divided into three genera: *Enamovirus, Luteovirus* and *Polerovirus* [5]. Members of this family infect a broad range of mono- and dicotyledonous plants that they exclusively replicate in the phloem tissue [6]. PLRV infects potato plants, causing economic losses on the yield [7,8]. PLRV causes characteristic rolling of leaves, chlorosis and stunting of infected plants [9]. In a number of cultivars, PLRV also causes a dark discoloration of the tubers so-called "net necrosis" that refers to a selective death and damage of cells in the phloem tissue of tubers [10, 11]. Additionally, PLRV can be transmitted in a persistent manner by a limited number of aphid species [12]. Also, the two main vector aphids are the green peach aphid (Myzus persicae) and the potato aphid (*Macrosiphum euphorbiae*) [2,9]. PLRV has isometric capsids containing a single-stranded (ss) messenger-sense RNA (5.9 kb) molecule with several open reading frames (ORFs) linking to a small protein (VPg) [13,14]. Vegetative part of potato (the tubers) is used as planting materials in most countries; a tuber-borne PLRV infection can indefinitely propagate. Furthermore, one of the most important measures in favor of diseases management in potato fields is to use of the certified virus-free tuber as 'seed' for planting [15].

PLRV control measures are time-consuming and expensive. Thus, development of rapid and reliable diagnostic method(s) to detect virus-free tubers can be effective to control of pathogen. Typically, in the recent decades the molecular techniques have been applied for discrimination of viruses and other plant pathogens. In this regards, several molecular methods have been developed for detection of the PLRV, including nucleic acid sequence-based amplification (NASBA) [16], Northern blotting [7], immunecapture RT-PCR (IC-RT-PCR) [3], reverse transcription polymerase chain reaction (RT-PCR) [16-27], Multiplex AmpliDet RNA [18], and real-time RT-PCR [1,19]. Although those methods are sensitive and specific, they are either complicated or expensive. Alternatively, they need equipped laboratories and educated experts [8]. To address these limitations, isothermal-based detection methods such as loop-mediated isothermal amplification (LAMP) were developed. Some different form of this technique including RT-LAMP and IC-RT-LAMP were universally used [2,3,20,21]. Briefly, the LAMP assay can specifically amplified DNA sequences by using a set of 4 primers, which recognize 6 distinct regions on the target DNA. Additionally, a pair of Loop primers (i.e., LF and LB) can accelerate LAMP reaction as well as reduce the time. In the LAMP reaction, nucleic acids amplify under isothermal conditions in a range of 60°C -65°C. Hence, it allows using of simple and cost effective equipments [23,20,21]. Up to now, numerous studies in both plants and animals have been reported to recognize pathogens by LAMP technique. For instance, RT-LAMP method were utilized for detection of *Plasmodium falciparum* gametocytes [9], *Fusarium graminearum* [23], *Peste des petits ruminants virus* (PPRV) [24], *Potato virus Y* (PVY) [10], *Japanese yam mosaic potyvirus* (JYMV) [22], *Rabies virus* (RABV) [19], *Macrobrachium rosenbergii noda virus* (MrNV) [25], *Cymbidium*

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mosaic virus (CymMV) [26] and *Potato Leafroll Virus* (PLRV) [2,3]. As described earlier, some different visualizing systems are employed for verification of LAMP products. Among these, utilization of $MgSO_4$ which produces magnesium pyrophosphate (white precipitate) in positive sample considered a simple, cost-effective and safe verification approach [27]. Unfortunately, the turbidity is unstable and after a few seconds vanishes. To improve such shortcomings, the application of $MgCl_2$ instead of $MgSO_4$ was suggested but, no significant achievement was observed [10].

The aim of this study, were to develop and optimize a sensitive and specific RT-LAMP assay for detection of PLRV based on a new visualizing system by using $CaCl_2$ instead of $MgSO_4$ in order to increase both stability and concentration of turbidity.

Materials and Methods

Viruses samples and DAS-ELISA assay

Forty suspicious potato leaf samples which showed symptoms of infection with PLRV were collected from some potato fields (Zanjan, Iran). The presence of the virus in the whole collection was first confirmed via DAS-ELISA assay by polyclonal PLRV specific antibodies (IgG: AS-110612, RAM AP: AS-110621, Bioreba AG). DAS-ELISA was carried out according to previous protocols with some minor modifications, firstly, using a commercial PLRV IgG and the alkaline phosphatase-conjugated PLRV IgG [28]. Polystyrene microtiter plates were coated for 3 h at 34°C, with 200 µl per well of IgG coating, in 50 mM carbonate buffer (pH 9.6). Then, the plates were incubated for 1 h at 34°C with PBS (10 mM phosphate buffer pH 7.2, 0.8% NaCl and 0.02% KCl). Afterwards, the plates were washed three times, with washing buffer (0.8% NaCl, pH 7.2 and 0.05% Tween 20). The infection free (control) and PLRV-infected potato leaf samples were ground in 10 volumes (w/v) of PBS buffer (pH 7.2), containing 0.2% polyvinyl pyrolidone (PVP) and 2% of egg albumin (Sigma A5253).The infected preparations were serially diluted (five-fold dilution) at the same buffer. Next, aliquots of 195 µl of prepared samples were added to each well, followed by incubation at 4°C overnight. Plates were then washed three times by washing buffer and incubated for 4 h at 37°C, with 190 µl per well of alkaline phosphatase-conjugated PLRV IgG diluted in sample buffer and washed again. Finally, it incubated for 90 min, with p-nitrophenylphosphate (1 mg/ml), in 10% diethanolamine pH 9.8.

RNA extraction

RNA was purified from PLRV-infected potato leaves according to former protocol [29]. 2 ml of extraction buffer (21.7 g $K_2HPO_4.3H_2O$, 1.4 g KH_2PO_4, pH 7.4, 100 g sucrose, 1.5 g BSA, 20 g PVP, and 5.3 g ascorbic acid) was transferred into a mortar containing 0.2 g of leave samples. Next, two successive centrifugations were performed, 10 min (1100 g) and 20 min (16800 g) at 4°C, respectively. Then, the resultant precipitate was mixed with 0.2 ml TE buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8, 0.1% mercaptoethanol) and 10% Sodium Dodecyl Sulphate (SDS). Afterwards, 80 µl of 5 M acetate potassium was added and the solution incubated for 10 min at 60°C and for overnight at 4°C, respectively. Then, the tubes were centrifuged at 4°C for 15 min (16800 g). The aqueous phase was harvested and 30 µl acetate sodium 3 M was added followed by isopropanol. After incubation at –20°C for 2 h, the last centrifugation was performed at 4°C for 20 min (16800 g). The resultant pellet was washed with 70% ethanol, dried under a vacuum, and dissolved in a total volume of 15 µl of double distilled water.

cDNA preparation and RT-PCR reaction

Extracted RNA (5 µl) was incubated at 75°C for 3 min and chilled on ice for 3 min. Then, 20 pmol backward primer, 50 mM Tris-HCL, pH 8.3, 10 mM dithiothreithol (DTT), 2.5 mM $MgCl_2$, 10 mM of each dNTP (dATP, dTTP, dCTP and dGTP), 5 U of RNasin Ribonuclease Inhibitor (Fermentas Co, Cat. No EO0381), and 100 U of *Moloney murine leukemia virus* (M-MLV) reverse transcriptase (Fermentas Co, Cat. No EP0351) were added to RNA. Afterwards, mixtures were incubated at 45°C for 45 min. lastly; the obtained cDNA was used as template for PCR or two-step RT-LAMP. RT-PCR was performed by using specific primers which were designed based on CP gene [29]. Performing, on a Thermal Cycler (iCycler, BIO RAD, CA, USA) in a 25 µl volume containing 1X PCR buffer (10 mM Tris-HCL, pH 8.3, 50 mM KCl), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pmol of each forward and backward primers, 0.625 U of *Taq* DNA polymerase (Cinagen Co, Cat. No TA7505C) and 2 µl cDNA. Subsequently, mastermix were amplified at 94°C for 3 min, for 35 cycles followed by for 1 min at 94°C, 1 min at 54°C and 1 min at 72°C. A final extension was accomplished for 10 min at 72°C. Finally, amplified products (5 µl) were loaded on a 1.5% agarose gel containing ethidium bromide, eventually visualized under UV illuminator.

Developing of primers for LAMP

The complete genome sequence of PLRV is available at Gen bank (accession no. U73777.1). The specific LAMP primers were designed based on conserved sequence (ORF3) of CP gene by using the Primer Explorer V4 software (http://primerexplorer.jp/e/) and primers including *Outer primers* (F3 and B3) and *Inner primers* (FIP and BIP). Moreover, In order to accelerate as well as to reduce the time of LAMP reaction, an additional *Loop primers* (LF and LB) were developed (Table 1). In addition, figure 1 shows the position of the RT-LAMP primers on ORF3.

Two-step RT-LAMP

Two-step RT-LAMP was carried out by using obtained cDNA which was previously described. cDNA (3 µl at 10 ng/µl) was incubated at 95°C for 5 min and chilled on ice and it was served as a template in

Primer	Type	Position on gene	Length of primer	Length of product	Sequence(5'-3')
Forward	Forward outer	85-104	20 nt	336 bp	CGCGCTAACAGAGTTCAGCC
Backward	Backward outer	420-401	20 nt		GCAATGGGGGTCCAACTCAT
F3	Forward outer	61-78	18 nt		AGAAGGCAATCCCTTCGC
B3	Backward outer	245-264	20 nt		GGTGAAACTTCCTTGGGTGT
FIP	Forward inner	139-159 and 79-96	43 nt	163 bp	GCCTCCTCTTCTGCGTCTTCGTTTTAGGCGCGCTAACAGAGTT
BIP	Backward inner	161-182 and 223-241	45 nt		ATCGCCGCTCAAGAAGAACTGGTTTTCCACGAGGTTGTCCTTTGT
LF	Loop forward outer	97-118	22 nt		CCGTGACCATAACCACTGGCTG
LB	Loop backward outer	191-210	20 nt		GAGGACGAGGCTCAAGCGAG

Table 1: Oligonucleotide primers used for RT-LAMP and RT-PCR of coat protein gene of PLRV. RT-PCR primers (forward and backward) were designed by Oligo7 software that amplifies a 336 bp fragment. RT-LAMP primers (F3, B3, FIP, BIP, LF and LB) were designed by PrimerExplorer V.3 that produced many fragments with different sizes from which the smallest fragment was 163 bp in size.

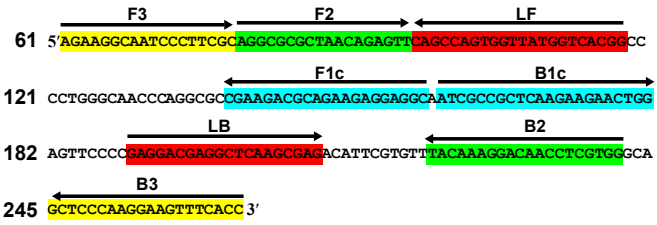

Figure 1: Position of the Oligonucleotide primers used for RT-LAMP on coat protein gene of PLRV.

LAMP reaction. The LAMP mixture containing 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 0.1% Triton X-100, 2 mM Betaine (Sigma-Aldrich, Oakville, Ontario, Canada), 1 mM $MgSO_4$, 10 mM of each dNTP, 0.2 µM each of F3 and B3, 0.8 µM each of FIP and BIP, 0.6 µM each of LF and LB, and 8 U of Bst DNA polymerase (New England Biolabs Inc.). The mixture was incubated at 62°C for 1 h, followed by 5 min at 80°C to terminate of reaction in a water bath. Finally, LAMP products (5 µl) were visualized by naked eye and also analyzed by electrophoresis as described earlier.

One-step RT-LAMP

This reaction was performed in 25 µl volume by using RNA as a template. The reaction mixture contained 5 µl of RNA, 100 U of M-MLV reverse transcriptase, RNasin Ribonuclease Inhibitor (5 U) and 10 mM DTT. The RNA was incubated at 75°C for 3 min and chilled on ice. Then, the denatured RNA solution was added to the RT-LAMP mixture. Thereafter, the solution was incubated at 45°C for 45 min and then 62°C for 1 h, followed by 5 min at 80°C in a water bath. Finally, the products were analyzed by gel electrophoresis.

Real-time RT-LAMP

In this study, real-time RT-LAMP assay was performed for precise detection of PLRV by using fluorescence which was derived from SYBR® Premix Ex Taq™ II that it can be bind to the dsDNA. Consequently, it was measured by real-time thermal cycler (Roto Gene CR6000). Fluorescence-intercalation dye (SYBR® Premix Ex Taq™ II) based real-time RT-LAMP (FRt-RT-LAMP) was carried out with and without LF and LB primers. 3 µl cDNA was considered as a template. Furthermore, 0.8 µM SYBR® Premix Ex Taq™ II (Perfect Real TIME, TAKARA Bio Co, LTD, RR081A) was added to 22 µl of LAMP mixture. The mixture was placed on a real time thermal cycler at 62°C for 1 h.

Optimization of $CaCl_2$

In the LAMP reaction when DNA polymerizes, producing pyrophosphate ions and interaction between pyrophosphate and magnesium lead to produce magnesium pyrophosphate (white precipitate) which can be observed and detectable as turbidity in positive samples. In contrast, negative control remains transparent without any turbidity [27]. Use of $MgCl_2$ or $MgSO_4$ in LAMP reaction in order to create turbidity has some drawbacks such as, low stability and concentration. In this research, we used $CaCl_2$ to address these disadvantages. Thus, different dilutions of $CaCl_2$ (0.01 mM to 0.5 mM) were examined to improve both stability and concentration of turbidity in LAMP reaction.

Results

DAS-ELISA and RT-PCR reaction

DAS-ELISA assay could successfully detect two (5%) positive sample among 40 natural samples. Remarkably, changes in the color of the infected samples in wells were much clear, so that there was no need for ELISA Reader (Figure 2a). The positive samples were nominated as PLRV10 and PLRV16 and ultimately, they used for further analyses. Additionally, the positive samples were detected by RT-PCR assay and a predictable fragment (336 bp) was observed on agarose gel (Figure 2b).

Optimization of LAMP reaction

The effects of temperatures and times as well as the concentrations of dNTP, cDNA, primers, *Bst* DNA polymerase, Betaine and $MgSO_4$ were examined. Temperatures range was considered between 45 -75°C. The results showed that the amplification occurred at 55°C, 60°C, 63°C and 65°C (Figure 3a). Moreover, the minimum time for completion of reaction was 30 min (Figure 3b). To test the effects of dNTPs concentration on RT-LAMP reaction, final concentration of 0.2 to 10 mM was prepared. The results showed that at 5 mM and 10 mM, ladder-like DNA fragments were clearly observed (Figure 4a).

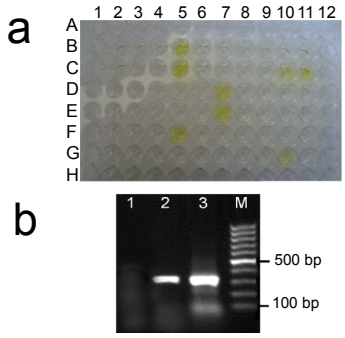

Figure 2: a) the results of DAS-ELISA assay for PLRV. The positive control (5F and 10C), PLRV10 (5B, 5C and 11C) and PLRV16 (7D, 7E and 10G) are shown in green. b) Electrophoresis of the RT-PCR product on 1.5% agarose gel visualized by Ethidium Bromide staining under UV illumination. The RT-PCR reaction was carried out using PLRV specific primers and amplified a 336 bp fragment. Lanes: 1, water control; 2 PLRV10; 3 PLRV16; M, 100 bp ladder.

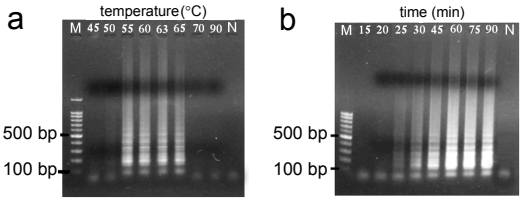

Figure 3: Optimization of RT-LAMP reaction for detecting of PLRV: a) Effects of temperature; The optimized temperature was 65°C; b) Effects of the reaction time ; the optimized reaction time was 60 min. Lanes: M, 100 bp ladder; N, water control.

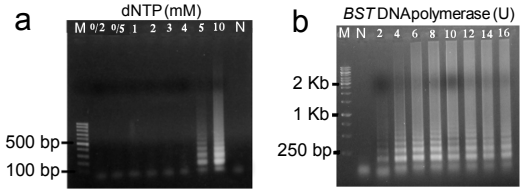

Figure 4: Optimization of RT-LAMP reaction for detecting of PLRV: a) Effects of dNTPs concentration; 10 mM was selected as the optimized concentration; b) Effects of Bst DNA polymerase concentrations; 8U of enzyme was selected as optimized concentration. Lanes: M, 100 bp ladder; N, water control.

Different concentrations (2 U to 16 U) of Bst DNA polymerase were used to select the minimum concentrations with the good performance. With low concentration of the enzyme (2U), poor amplification of DNA was observed, but with increasing of the enzyme concentration to 4 U or 8 U, the amplification considerably improved (Figure 4b). In order to optimize the concentration of primers the outer primers (B3 and F3), inner primers (FIP and BIP), and loop primers (LF and LB) were used with 5 to 50 pmol. The results demonstrated that 10, 20 and 20 pmol were selected for outer, inner, and loop primers, respectively (Figure 5a). A Betaine concentration from 0.2 mM to 250 mM was examined. When the concentration of Betaine increased from 0.2 to 100 mM, the intensity of the amplified products increased but, no visible products were detected when the concentration increased to 250 mM. Different concentrations (0.2 to 250 mM) of $MgSO_4$ were inspected and results showed that at the 0.2 to 1 mM of $MgSO_4$ concentrations amplification were observed while at 5 to 250 mM there were no visible products (Figure 5b). Due to drawbacks of $MgCl_2$ (low stability and concentration). In this study, $CaCl_2$ was employed to achieve significant improvement in both stability and concentration of turbidity. The results showed that at 10 mM $CaCl_2$ concentration the best quality was observed. Moreover, it is noticeable that, even though the 20 mM created positive results, the resolution of the bands were a little low than 10 mM (Figure 6).

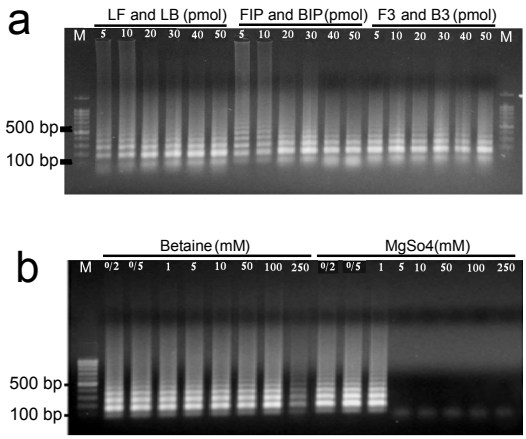

Figure 5: Optimization of RT-LAMP reaction for detecting of PLRV: a) Effects of primer concentrations; 5 pmol concentrations for F3 and B3 primers, and 20 pmol for LF, LB, FIP and BIP were selected as optimized concentration of primers; b) Effects of Betaine and $MgSO_4$ concentrations; 100 mM concentration for Betaine and 1mM concentration for $MgSO_4$ were selected as optimized concentrations. Lanes: M, 100 bp ladder.

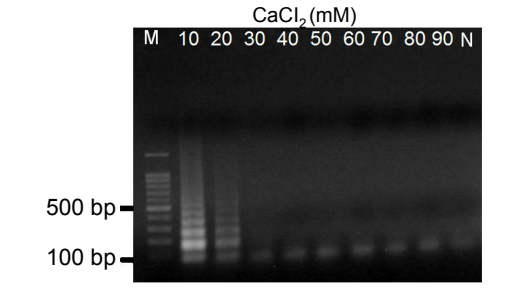

Figure 6: Effects of $CaCl_2$ and cDNA concentrations. 0.01 mM $CaCl_2$ concentrations were selected as optimized concentrations. Lanes: M, 100 bp ladder; N, water control.

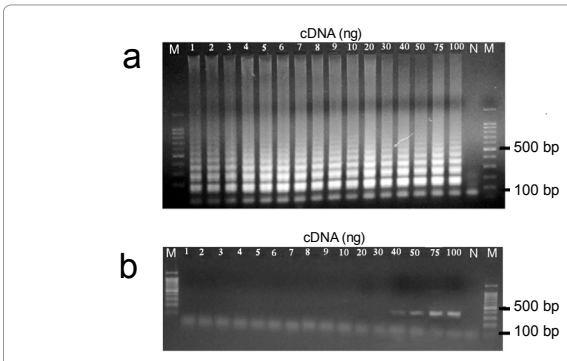

Figure 7: The results of sensitivity for PLRV: a) Amplification was observed in 1 ng/µl concentration of cDNA for RT-LAMP; b) Amplification was observed in 40 ng/µl concentration of cDNA for RT-PCR respectively. Lanes: M, 100 bp ladder; N, water control.

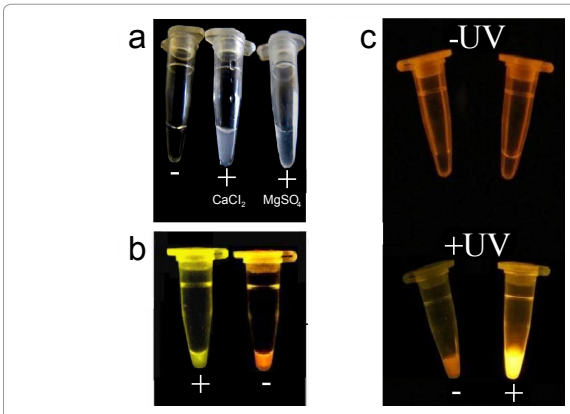

Figure 8: Visual detection of positive RT-LAMP reaction: a) Visible precipitate due to the formation of magnesium pyrophosphate and Calcium pyrophosphate with naked-eye; b) Using SYBR Green dyes under UV illumination; c) Using Ethidium Bromide under UV illumination.

Sensitivity of the RT-LAMP assay

To determine the sensitivity of RT-LAMP reaction different concentrations of cDNA (1 ng/µl up to 100 ng) were tested. The results indicated that even with 1 ng/µl of cDNA amplification perfectly worked while RT-PCR reaction was completed with a minimum of 40 ng/µl of cDNA. The all products (per each 5 µl) were analyzed on 1.5% agarose gel (Figures 7a and b).

Visual detection of RT-LAMP products and Real-time RT-LAMP

Positive reactions were accompanied with cloudy phase due to the formation of magnesium pyrophosphate and calcium pyrophosphate (Figure 8a). In addition, 2 µl SYBR® Premix Ex Taq™ II (Perfect Real TIME, TAKARA Bio Co, LTD, RR081A) was added to 25 µl RT-LAMP product under UV illumination (302 nm); the positive reaction appeared in green color (Figure 8b). Also, positive reaction was visualized by using Ethidium bromide under UV illumination, adding 0.5 µl of the diluted ethidium bromide turned colour of the positive sample in to ocherish yellow (Figure 8c).

Fluorescence-intercalation dye (SYBR® Premix Ex TaqTM II)-based real time RT-LAMP (FRt-RT-LAMP) was carried out with LF and LB and without those primers. The results demonstrated that in the presence of the loop primers the amplification began at 15 min while under the absence of loop primers, amplification started at 44 min

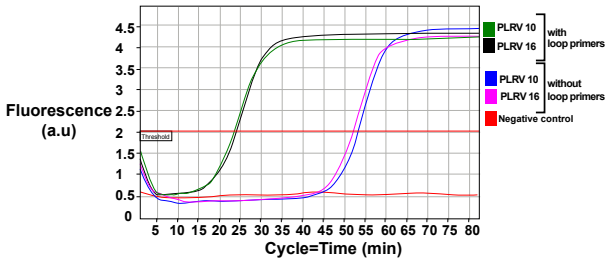

Figure 9: Fluorescence-intercalation dye (SYBR® Premix Ex Taq™ II)-based real time RT-LAMP (FRt-RT-LAMP), with and without using loop primers. The amplification started by 15 min using the loop primers and by 44 min without loop primers.

(Figure 9). Therefore, using loop primers would speed up the reaction in real- time RT-LAMP assays.

Discussion

Potato is a vulnerable host for abundant viral infectious agents. In crops in which true seeds are used for propagation, most of the time, flower works as a powerful barrier against transmission of viruses to the seeds of the next generation. While in potato fields, the true seeds are rarely used and the tubers are mainly used for propagating. Therefore, for viral pathogens is quite straightforward way to infect host plants to the new-formed tubers. Since there is no effective viricide to control of the viruses in potato, using virus free seed potatoes is an effective approach for management of potato viral disease. In Iranian seed potato market, along with other part of the world, demands for certified potato seed tubers are increasing [2-3,30]. In order to produce infection free potato seed tubers, using rapid, cost-effective and sensitive methods for detection of important potato viruses, including PLRV, in different stages of infection process would provide a great step forward. Various experimental methods have already been utilized for detecting of PLRV as well as other important viral agents in potato from which DNA/RNA-based methods are now considered among most important detection methods. The RT-LAMP assay is one of those nucleic acid-based methods that were introduced by beginning of 21st century. This method was first applied for detecting of human and animal pathogens. It is not for a long time that this technique is being used in plant pathology. However, the number of plant pathogens that have been detected with this technique in the last few years is remarkably increased. For several reasons we encourage potato pathologists and potato seed inspectors to use of this method for detecting of PLRV.

It is safe and easy method to work with

The amplification products of RT-LAMP reaction, which are stem-loop DNA structure with several inverted repeats and cauliflower-like structure with multiple loops, can be monitored visually and therefore using of toxic staining materials can be avoided [22]. This would simplify the detection procedure and would result in saving of significant time which is needed for separating of the amplified products on the gel and the analyzing of the data which is commonly used in other PCR–based methods.

It is a technician friendly method

The isothermal conditions required by RT-LAMP could be met easily using a water bath or a temperature block, whereas the rapid and accurate temperature change required by other nucleic acid-based methods including RT-PCR.

It is a rapid and a highly specific method

The higher specificity and sensitivity of this method has already been compared with other nucleic acid-based methods such as RT-PCR [8,31]. The results of the present study showed that the LAMP reaction can be completed within 30-40 min with about 1 ng/µl of cDNA, while RT-PCR needed at the least 3 hours with 40 ng/µl of cDNA to complete a reaction. One may conclude that RT-LAMP works faster (4-fold) with higher sensitivity (40-fold) for detecting of PLRV compared with RT-PCR. These results parallel with previous studies, suggesting that RT-LAMP can be 10 to 100-fold more sensitive than RT-PCR [24,32-34]. Overall, our results showed that some ingredients of RT-LAMP reaction, such as $MgSO_4$, Betaine, primers, dNTPs, and DNA polymerase concentrations, as well as temperature and time period of the reaction play important roles in efficiency of the assay. At the end of the reaction, the infected samples can be easily visualized with naked eye. However, the turbidity of the positive samples is not stable for a longer time and it should be judged soon after taking out of the samples from the water bath or from the thermal cycler. As a solution, adding SYBR Green and Ethidium Bromide to the tubes will provide enough time to monitor changes in the color of the tubes and to detect the positive samples under UV illumination as stressed by other workers [25,33]. However, using of toxic staining materials and UV irradiation would not be compatible with the main feature of this technique which is "safety". It seems that using of some other alternative chemicals to stabilize the turbidity of positive samples for a longer time would increase the efficiency of this method. Interestingly, our results showed that the application of $CaCl_2$ can accompany with more reliable outputs than $MgSO_4$ when both stability and concentration of the turbidity are taken into account. Consequently, $CaCl_2$ can be proposed as the best alternative options for $MgSO_4$ to enhance the stability and also to avoid applying toxic materials.

In conclusion, the RT-LAMP method was successfully developed for rapid and sensitive detection of PLRV. Furthermore, it was approximately 40 times more sensitive compared to RT-PCR. The RT-LAMP assay did not require costly or sophisticated equipment and also use of $CaCl_2$ instead of $MgSO_4$ create significant turbidity which may be make RT-LAMP methodology safe, cost-effective and portable diagnostic tool for assessment of distribution of pathogen in the field. Additionally, this technique can be applied for routine screening of pathogens in large samples in epidemiological and pathological programs. Undoubtedly, RT-LAMP characteristics turn it in to a suitable alternative to ELISA and RT-PCR methods in order to accelerate decision-making processes in potato disease-management.

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