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One-step Colorimetric Immunocapture Reverse Transcription Loop-Mediated Isothermal Amplification for Detection of *Potato virus Y* without RNA Extraction

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Abstract

To diminish the time required for some diagnostic assays including reverse transcription PCR (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP) (due to mainly RNA extraction step), and also DAS-ELISA into a minimum level, an innovative one-step colorimetric IC-RT-LAMP and one-step IC-RT-PCR protocol on the basis of *Potato virus Y* (PVY) genome were used and optimized. In this regard, all four one-step colorimetric IC–RT-LAMP primers (i.e. F3, B3, FIP and BIP) together with one-step IC-RT-PCR primers (F and B) were selected on the basis of coat protein gene (CP) of PVY genome. Even though DAS-ELISA, one-step IC-RT-PCR and one-step colorimetric IC–RT-LAMP assays could successfully detect positive infected plant samples, considering the time, safety, sensitivity, cost and simplicity, the last one was overall superior. Meanwhile, among six different visual dyes to accurately detect one-step IC–RT-LAMP products, Hydroxynaphthol blue, GeneFinder™ and SYBR Green I could produce long stable colour change and brightness in a close tube-based approach to prevent cross-contamination risk, concluded eventually as the best ones. Altogether, as one-step IC–RT-LAMP is sensitive, cost effective, fairly user friendly, and also can generate more accurate results than previous diagnostic procedures; we accordingly propose this colorimetric assay as a highly reliable alternative viral recognition system regarding PVY recognition and probably other viral-based diseases.

Keywords: Colorimetric assay; DAS-ELISA assay; IC-RT-LAMP assay; IC-RT-PCR assay; *Potato virus Y*

Introduction

Potato (*Solanum tuberosum L.*), as the world's most important non-grain food crop [1], historically originated in the Andean mountains of South America about 8000 years ago, and gradually has undergone an immense spread around the world. It is mainly cultivated for its underground storage called tubers, and is central to global food security as well. Iran, with the yield of 5240000 tones, is assigned as the world's number 12 *Potato* producer and the third largest in Asia, after China and India, respectively, as the world's first and third largest potato-producing nations, with the output value of nearly 72 and 26 million tones) (http://www.potato2008.org/en/world/asia.html). *Potato* plants, like other crops, are exposed frequently to plenty of infectious *virus*es, economically the most important of which are *Potato leafroll virus* (PLRV, genus *Polerovirus*), *Potato virus Y* (PVY, genus *Potyvirus*), *Potato virus* X (PVX, genus *Potexvirus*) and *Potato virus* S (PVS, genus *Carlavirus*) [2]. Infections arisen from PVY and PLRV entail commonly substantial yield losses, but those caused by single infections of PVX or PVS are rarely significant, particularly regarding the last one [3]. PVY, one species of the genus *Potyvirus* is a phloem-limited *virus* that infects potato, leading to a significant reduction in *Potato* annual yield, as well as tuber quality worldwide [4].

As a result, fast, cost effective, reliable and sensitive indexing techniques are requisite tools to determine the *virus* status of *Potato* seeds, particularly during early stages of viral infection, double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), in this context, being considered as one of the first detection approaches [5]. This method, however, is expensive, time-consuming and generally cannot be carried out on dormant tubers [6]. Some alternative approaches were gradually developed including bioassay [7], DAS-ELISA [5] and polymerase chain reaction (PCR) [8], all of which were unfortunately time-consuming and require expensive or carcinogenic materials to visualize DNA amplification [9]. Meanwhile, extraction of RNA is another exhausting task, accomplished commonly under various protocols, all of which are typically accompanied by some drawbacks. At the time of extraction, for instance, the *virus*es may be captured on a filter, present in a pellet or suspended in solution. RNA molecule is also sensitive to autolysis, and prolonged incubation may cause degradation and decreased yields. The presence of RNase is another significant problem, as well. Neither ethylene-diamine-tetra-acetic acid nor ethylene glycol tetra-acetic acid, both as the most popular detergents, inactivates RNase, so other RNase-inhibiting agents may be required if one plans to extract RNA-containing *virus*es by simple thermal destabilization; the other RNase inhibitors like RNasin [10] and ribonucleoside–vanadyl complex [11], as well as other commercially available proprietary reagents (e.g. RNAsecure, Ambion; RNaseOUT, Invitrogen), or even novel isolation approaches should be employed, all of which are generally costly, time-consuming and necessitate more precise extraction protocol(s) [12]. Among various isothermal amplification systems developed over the recent years, the most frequently applied approach seems to be loop-mediated isothermal amplification (LAMP), implemented first by Notomi et al. [13]. Due to its enormous rate of amplification paired with a very high specificity, sensitivity, rapidity and low artifact susceptibility

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[14], the method together with its modifications have been strongly recommended for detection of a great number of strains of bacteria, as well as *virus*es worldwide [15]. Briefly, each reaction is carried out with four oligonucleotide primer sets, which recognize six distinct regions on the target DNA [16]. RT-LAMP assay, alternately, can also amplify nucleic acid (RNA) under isothermal condition in the range of 60 to 65°C, all turbidity and fluorescent-based detections, as well as agarose gel electrophoresis system are applied to visualize suspicious samples [17], although a large number of studies have been accomplished using LAMP or RT-LAMP, including *Potato virus Y* [4], *Potato leafroll virus* [18], *Japanese yam mosaic Potyvirus* [19], *Tomato Yellow Leaf Curl virus* [20], *Rabies virus* [21], *Macrobrachium rosenbergii nodavirus* [22] and *Cymbidium mosaic virus* [23], all of which basically require more precise extraction protocol(s) about generating higher concentrations of DNA or RNA, followed by the least amounts of probable contaminations, which act as inhibitors in an amplification process. Notably, despite a few number of studies about immunocapture reverse transcription loop-mediated isothermal amplification (IC–RT-LAMP) [17,24], because the technique has not been yet introduced for detection of PVY, an attempt was accordingly made to optimize a new protocol of it to save time and remove RNA extraction. As the second purpose, since the existence of one-step IC-RT-LAMP positive amplicons has been proved to be confirmed by adding a number of fluorescent dsDNA intercalating dye, including ethidum bromide [17,25], SYBR Green [26] and propidium iodide [27], after the reaction is completed or metal indicators such as calcein [28], Gene-Finder™ [29,30], hydroxyl naphthol blue [31-33], and magnesium pyrophosphate [13,15,17,18,25], prior to the reaction, allowing observation with the naked eye, here, six different visualization systems were consequently employed to assess the colour stability, as well as safety feature of each one in a viral detection procedure of PVY.

Materials and Methods

Plant samples

A total of 95 fresh *Potato* leaf samples infected dubiously with PVY on the basis of plant symptoms containing yellowing and erect leaves, as well as plant stunting were collected from ten separate *Potato* fields in Provinces Zanjan, Hamadan, Kordestan and East Azarbaijan of Iran.

DAS-ELISA assay

DAS-ELISA was carried out as described by Clark and Adams [5] with some minor modifications, using a commercially available PVY IgG and the alkaline phosphatase-conjugated PVY IgG. 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. The plates were then incubated for 1 h at 34°C with PBS (10 mM phosphate buffer, pH 7.2, 0.8% NaCl and 0.02% KCl). After that, the plates were washed three times using washing buffer (0.8% NaCl, pH 7.2 and 0.05% Tween 20). The infection-free (control) and PVY-infected *Potato* leaf samples were ground in ten volumes (w/v) of PBS buffer pH 7.2, containing 0.2% polyvinyl pyrrolidone and 2% of egg albumin (Sigma A5253). The infected preparations were serially diluted (fivefold dilution) at the same buffer. Aliquots of 195 µl of prepared samples were added to each well, and the plates were incubated overnight at 4°C. Plates were then washed three times with washing buffer, incubated for 4 h at 37°C, with 190 µl per well of alkaline phosphatase-conjugated PVY IgG diluted in sample buffer, washed again, and incubated lastly for 90 min, with p-nitrophenylphosphate (1 mg/ml), in 10% diethanolamine, pH 9.8. Data were expressed and recorded using Multiskan at A_{405} nm.

One-step IC–RT-PCR

The one-step IC–RT-PCR was developed using a PVY-specific primer designed on the basis of *virus* coat protein (CP) gene (Table 1) [4]. The protocol, to generate one-step IC–RT-PCR products, was divided into two successive sections as below:

Section 1: The same as DAS-ELISA method, here, PCR tubes were first coated with PVY specific IgG diluted in coating buffer and incubated for 4 h in 37°C. Tubes, in the following, were washed with washing buffer (see "DAS-ELISA Assay" section). The extractions of positive *Potato* samples (i.e. previously detected by DAS-ELISA assay as positive control) and a free *virus* plant sample (as negative control) were added to IgG-coated tubes and kept overnight at 4°C. Tubes, the next day, these were washed using washing buffer, dried and employed as RNA template in one-step IC–RT-PCR reactions.

Section 2: In this part, the reaction was carried out in a Bio-Rad thermocycler. The amplification was performed in a 40 µl volume containing 20 mol of each backward and forward primers (F and B), 10 mM dithiothreithol (DTT), 2.5 mM $MgCl_2$, 10 mM of each dNTP, 5 U of RNasin Ribonuclease Inhibitor (Fermentas Co, cat. no. EO0381), 1.25 U of Avian myeloblastosis *virus* (AMV) reverse transcriptase (Fermentas Co, cat. no. EP0641), 1×PCR buffer (10 mM Tris–HCL, pH 8.3, 50 mM KCl) and 0.625 U of Taq DNA polymerase (Cinagen Co, cat. no.TA7505C). First, strand of cDNA was amplified at 60°C for 60 min from RNA extracted by immunocapture method using the backward primer. One set of primer (Table 1), that is backward and forward primers, was used for the PCR amplification of the cDNA. Amplification was performed with the following PCR profile: 94°C for 3 min; 35 cycles of 1 min at 94°C, 1 min at 54°C, 1 min at 72°C and 10 min at 72°C for final extension. The products were lastly analyzed by gel electrophoresis, in which 5 µl of the products (480 bp) was loaded on a 1.5% agarose gel and visualized by staining with ethidium bromide.

One-step colorimetric IC–RT-LAMP

In order to perform one-step IC–RT-LAMP, on the basis of CP gene of the PVY genome, four specific primers, including outer primers (F3 and B3) and inner primers (FIP and BIP) were used (Table 1) [4]. Even though the principles of the first section of IC–RT-LAMP assay exactly followed the IC–RT-PCR with no RNA extraction step, in the second part, a different methodology was employed, leading to a significant reduction in the time as well as the cost. The details are as follows:

Primer	Type	Position on gene	Length	Sequence(5'-3')
F	Forward	8721-8740	20 mer	ACGTCCAAAATGAGAATGCC
B	Backward	9181-9200	20 mer	TGGTGTTCGTGATGTGACCT
F3	Forward outer	8870-8890	21 mer	ATACGACATAGGAGAAACTGA
B3	Backward outer	9059-9078	20 mer	ACGCTTCTGCAACATCTGAG
FIP	Forward inner	8931-8951 and 8900-8920	42 mer	GTTTGGCGAGGTTCCATTTTC-TGTGATGAATGGGCTTATGGT
BIP	Backward inner	9004-9024 and 9036-9056	42 mer	TGAAACCAATCGTTGAGAATG-ATGTGCCATGATTTGCCTAAG

Table 1: Oligonucleotide primers used for one-step colorimetric IC-RT-LAMP and one-step IC-RT-PCR for detection CP gene of PVY.

Section 1: Just the same as the section 1 of IC–RT-PCR procedure (see above).

Section 2: Each reaction was performed in a total volume of 50 µl: 10 mM DTT, 5 U of RNase Ribonuclease Inhibitor (Fermentas Co., cat. no. EO0381), 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, ON, Canada), 1 mM $MgSO_4$, 10 mM each dNTP, 0.2 µM each of F3 and B3, 0.8 µM each of primer FIP and BIP, 1.25 U of AMV reverse transcriptase (Fermentas Co., cat. no.EP0641), and 8 U of Bst DNA polymerase (New England Biolabs Inc.). Tubes were then incubated at 60°C for 90 min in water bath. It is noticeable that the first 45 min is allocated only to synthesize cDNA, while in the second round, LAMP amplicons are amplified. An agarose gel electrophoresis system (optional; 1.5%) under UV illumination could be also employed to visualize positive reactions: 5 µl of each product is loaded on a 1.5% agarose gel. The details of colorimetric assay are described below:

Magnesium pyrophosphate: Like other metal indicators, magnesium pyrophosphate must be added before reaction. At the end of the amplification process, positive reactions were accompanied by a visible darker phase in the tubes in consequence of the formation of magnesium pyrophosphate [13,34], which can be easily visualized with the naked eye. It is noticeable that the turbidity of the positive samples is stable, but just for a short time, which should be consequently judged soon after taking out of the samples either from the water bath or the thermal cycler.

SYBR® Premix Ex TaqTM II: To conquer time-dependent instability of magnesium pyrophosphate-based detection method, an alternative visual system using SYBR® Premix Ex TaqTM II was employed [20,25,35]. Hence, 2 µl SYBR® Premix Ex TaqTM II (Perfect Real Time, Takara Bio Co., Ltd., RR081A) was added into each completely finished one-step IC–RT-LAMP reaction containing 25 µl of products; all positive reactions were effectively identified. Under UV illumination (302 nm), a green colour pattern is an identical characteristic of all positive reactions as the same was monitored in this study.

Hydroxynaphthol blue (HNB) dye: In this protocol, 1 µl of the hydroxynaphthol blue dye (3 mM, Lemongreen, Shanghai, China) is mixed prior to amplification; all positive reactions can be easily identified using the naked eye, interestingly with no probable cross contaminations, which usually arise from opened tubes after amplification [20,31-33]. In this context, a sky blue colour pattern implies the existence of the reference *virus*, whereas a violet colour change is observed when the control(s) are taken into consideration.

GeneFinder™: An obvious Green fluorescence pattern was observed to confirm positive one-step IC–RT-LAMP products through visual observation with the naked eye when 1 µl of GeneFinder™, diluted to 1:10 with 6×loading buffer (TaKara, Dalian, China), was added to each reaction as described previously [29,30]. Remarkably, concerning negative reaction, the original orange colour could be observed.

SYBR Green I: 1 µl of SYBR Green I (Invitrogen, Sydney, Australia) diluted to 1:10 with 6×loading buffer was separately added to each reaction, as described previously [35]. Remarkably, concerning negative reaction, the original orange colour could be observed.

Ethidium bromide: About 0.5 µg ethidium bromide/ml (Sigma) was added to each tube [18,25,34]. Under a UV transilluminator, positive products will be consequently marked if a detectable yellow colour pattern is observed. As a result, the intensity of the fluorescent emissions moves up in positive tubes, while the reverse is true regarding negative tubes with no amplified fragments.

Results

On the whole, 5 out of 95 leaf samples suspicious of having infection with PVY (5.3%) showed positive responses against DAS-ELISA assay. All five samples, subsequent to nomination as PVY 16, PVY 28, PVY 45, PVY 78 and PVY 81, were utilized lastly for further analyses. As regards one-step IC–RT-PCR, following provide RNA template and cDNA (see "Materials and Methods" section for details), the amplification occurred via both backward and forward primers to generate ultimate products. The method, overall, could successfully identify five aforementioned positive samples with no attempt to RNA extraction. As expected, a fragment with the size band of 480 bp was detected when the one-step IC–RT-PCR products were run on 1.5% agarose gel and stained with ethidium bromide (Figure 1a). The same as one-step IC–RT-PCR, our new one-step IC–RT-LAMP protocol could successfully identify five positive samples, interestingly with no use of RNA isolation in a water bath. One-step IC–RT-LAMP amplicons were finally electrophoresed on a 1.5% agrose gel (as an optional system), and a large number of fragments (a ladder-like pattern) were eventually visualized (Figure 1b). One-step IC–RT-LAMP amplicons were able to be detected with the naked eye by adding different visual dyes, followed by colour changing in the solutions. In this regard, all used visual components could successfully make a clear distinction between positive infected samples and negative ones (Figure 2a-2f).

Discussion

Potato is a versatile, carbohydrate-rich food highly popular worldwide, prepared and served in a variety of ways. In Iran, potato, after wheat and rice, plays a fundamental role in a daily food chain that is extremely used around the country. Unfortunately, during the last few years, PVY activity commercially has led to a noteworthy diminution in the total yield of potato. The same, as some viral diseases,

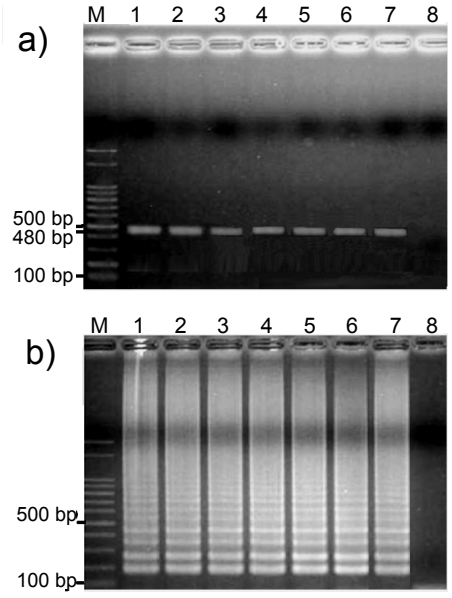

Figure 1: Gel electrophoresis pattern on 1.5% agarose gel (a) one-step IC-PCR assay; (b) one-step IC-LAMP assay. Left to right: lane M, DNA size marker (100 bp; Fermentas); lanes 1-7, PVY 16, PVY 28, PVY 45, PVY 78 and PVY 81, respectively; lane 8, negative sample.

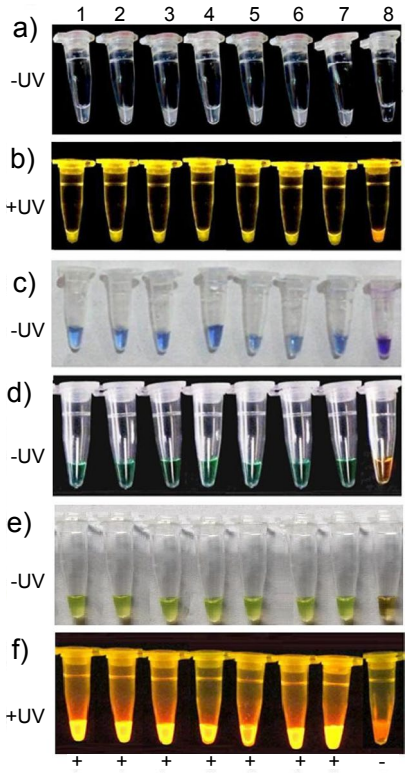

Figure 2: Detection of positive one-step colorimetric IC-RT-LAMP reaction using six visualizing methods (different dyes) (a) magnesium pyrophosphate-based method; (b) SYBR® Premix Ex TaqTM II-based method; (c) hydroxynaphthol blue (HNB)-based method; (d) GeneFinder™ based method; (e) SYBR Green I-based method; (f) ethidium bromide-based method. Left to right: tubes 1-7, PVY 16, PVY 28, PVY 45, PVY 78 and PVY 81, respectively; tube 8 negative sample.

even though presumptive diagnosis of PVY can be relatively simple when typical symptomatology is evident, symptoms in plants are not always specific, and can be confused with those caused by other biotic or abiotic agents. On the other hand, detection of deleterious *virus*es in symptomless plant material for preventive control is a compulsory task but can be extremely difficult, since low populations with uneven distribution of the pathogen can occur; developing fast, easy, highly sensitive, cost-effective and reliable diagnostic protocols to make an accurate discrimination is accordingly required [4,17,18,30,33].

In this study, as a result, three detection methods including DAS-ELISA, one-step IC–RT-PCR and One-step colorimetric IC–RT-LAMP were assessed to explore positive and negative aspects of each one, followed by introducing the best one regarding PVY detection. Even though all three techniques had enough potential to make differentiation and detect infected plant samples accurately, one-step colorimetric IC–RT-LAMP proved to be much more useful as some factors including time, safety, simplicity, cost and being user friendly are taken into account:

Time DAS-ELISA, as compared with one-step colorimetric IC–RT-LAMP and one-step IC–RT-PCR commonly needs a long time to identify positive infected samples (two or few additional days). In reality, with the exception of section one which takes equal time (see "Material and Methods" section), one-step colorimetric IC–RT-LAMP overall requires just 90 min to accomplish (as the least demanding detection method), while regarding one-step IC–RT-PCR and DAS-ELISA, 3 h and at least 1 day should be served, respectively. This, in turn, would simplify the detection procedure and result in saving of significant time needing for separating of the amplified products on the gel and the analyzing of the data, which are commonly used in the other PCR-based methods.

Safety regarding a number of detection methods, application of gel electrophoresis systems has emerged as a routine approach with enough potential to observe related amplicons. Just the same, such visual methods not only involve some expensive instruments, but also during a period of time, exposure to the UV ray (because it is harmful to the eyes, even watching for a short period would irritate eyes and cause symptoms similar to conjunctivitis), as well as ethidium bromide could accompany a number of serious negative effects on researchers who use these methods [17,25,30,33,35]. More surprisingly, in one-step IC–RT-LAMP and other LAMP variants, amplified products can be easily visualized by means of different in-tube colour indicators with no essential requirement of additional staining systems; thus, toxic staining materials would be significantly avoided.

Simplicity, Cost and User Friendly Equipped labs with some molecular instruments, as well as trained personnel are prerequisites to perform DAS-ELISA and one-step IC–RT-PCR assays, all of which are undoubtedly costly. On the contrary, one-step colorimetric IC–RT-LAMP can be easily accomplished just in a water bath or temperature block, with no need of thermocyler and gel electrophoresis, as the same results were recorded by [17,18,25,33]. Likewise, exclusive of the primer designing process, which is somehow complicated and sensitive, other phases are simply applicable. It is noticeable that in all RT-based methods, RNA extraction is an unavoidable step, needing different protocol(s), followed by optimization (mostly is a time-consuming process) to acquire purified RNA stock [12,36], whereas one-step IC–RT-LAMP can be easily performed with no attempt for RNA isolation. This method would lastly simplify the detection procedure, and would result in saving of significant time which is needed for separation of the amplified products on the gel. On the other hand, the presence of LAMP and RT-LAMP-positive amplicons proved to be confirmed by adding a number of fluorescent or metal dyes to the reaction tubes, allowing observation with the naked eye [18,30,33]. In the current study, therefore, one-step IC–RT-LAMP-amplified products were confirmed by adding all aforementioned visual systems (see "Materials and Methods" section), either prior to or after the reaction, along with forming diverse colour patterns depending upon the chemical characteristics of the applied chemical substances as dye.

In summary, a novel one-step colorimetric IC–RT-LAMP assay for rapid and easy detection of PVY was developed here, its potential compared with DAS-ELISA and one-step IC–RT-PCR assays. The method, on the whole, had the following advantages over the two mentioned procedures, and also the methods including RT-LAMP and RT-PCR: (1) fascinatingly, no need of RNA extraction (2) no requirement of expensive and sophisticated tools for amplification and detection; (3) no post-amplification treatment of the amplicons; and (4) a flexible and easy detection approach, that is visually detected by naked eyes using diverse visual dyes. On the other hand, among different visual systems, HNB, SYBR Green I and GeneFinder™ were proved to be more powerful, since tubes do not have to be opened after amplification consequent to accompany by no cross-contamination, judging positive reactions can be visualized using the naked eye, no need of post amplification treatment of the amplicons, and finally the brightness and stability produced by these two dyes were significantly

stronger than the others. Due to such remarkable features, the application of this colorimetric assay using visual observation systems, particularly, HNB, SYBR Green I and GeneFinder™ seems to be more effective as a new viral diagnostic method for epidemiological studies of PVY, particularly in less well equipped laboratories, and might be helpful in clarifying *virus*–vector interaction. As the last point of view, the current diagnostic approach can be suitable not only for laboratory research, but also regarding field diagnoses of many infectious diseases worldwide.

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