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Immunocapture Loop Mediated Isothermal Amplification for Rapid Detection of Tomato Yellow Leaf curl Virus (TYLCV) without DNA Extraction

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Abstract

To diminish the time required for some diagnostic assays including polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP; due to mainly DNA extraction step) and also DAS-ELISA into a minimum level, an innovative immunocapture LAMP (IC–LAMP) and immunocapture PCR (IC-PCR) protocol on the basis of Tomato Yellow Leaf curl Virus (TYLCV) genome were used and optimized. Even though DAS-ELISA, IC-PCR and IC–LAMP assays could successfully detect positive infected plant samples, considering the time, safety, sensitivity, cost, no need of DNA extraction and simplicity the last one was overall superior. The hydroxynaphthol blue could produce long stable colour change and brightness in a close tube-based approach to prevent cross-contamination risk. Altogether, as IC–LAMP is sensitive, cost effective, fairly user friendly and also can generate more accurate results than previous diagnostic procedures, we accordingly propose this assay as a highly reliable alternative viral recognition system regarding TYLCV recognition and probably other viral-based diseases.

Keywords: DAS-ELISA assay; IC–LAMP assay; IC-PCR assay; Tomato yellow leaf curl virus

Introduction

The Tomato yellow leaf curl virus (TYLCV) belongs to the *Begomovirus* genus within the *Geminiviridae* family. Begomoviruses are exclusively transmitted by *Bemisia tabaci* (Gennadius) (Homoptera: Aleyrodidae) [1,2]. Tomato yellow leaf curl diseases (TYLCD) are associated to a complex of viral species, including Tomato yellow leaf curl virus (TYLCV), Tomato yellow leaf curl Mali virus (TYLCMLV), Tomato yellow leaf curl Sardinia virus (TYLCSV), Tomato yellow leaf curl Malaga virus (TYLCMalV), and Tomato yellow leaf curl Axarquia virus (TYLCAxV) and all including rather similar symptoms on tomato (*L. esculentum*) plants. Hence, TYLCD-associated virus isolates belonging to two or more different species, and sometimes recombinants, have been found in the same country [3-8]. This disease was first identified in Israel in 1930 and has since the 1960s become the most important tomato viral disease in different countries [9-14]. In Iran, TYLCV was first reported in 1996 from central and southern provinces of Iran (Kerman, Hormozgn, Khuzestan, Bushehr, and Sistan- Baluchestan) [9,13]. The *Lycopersicon esculentum* is the primary host of TYLCV [15]. Symptoms induced by TYLC viruses consist of foliar curling and yellowing, reduced leaflet area, plant stunting and reduced fruit size and yield [16,17]. The virus was isolated in 1988 and its genome DNA sequenced in 1991 [18]. TYLCV is unusual in that it has a monopartite genome, composed of a single-stranded virion DNA (2787 nt) [19]. The genome consists of six open reading frames (ORFs) that are organized bidirectionally; two of these ORFs (V1 and V2) are in the virion sense orientation, and four (C1-C4) in the complementary orientation. Between the two transcription units resides an intergenic region (IR) of about 300 nucleotides [20-23]. As a result, fast, costeffective, reliable and sensitive indexing techniques are requisite tools to determine the virus status, particularly during early stages of viral infection, double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA), in this context, being considered as one of the first detection approaches [24-26]. Some alternative approaches were gradually developed including TAS-ELISA (Triple Antibody Sandwich ELISA) [4,27] and molecular methods including PCR [28], dot blot hybridization [3], tissue blotting immuno-binding assay (TBIA) [24], Hybridization with probe [12] and LAMP [29,30], all of which were unfortunately time consuming and require expensive or carcinogenic materials to visualize DNA amplification [31]. Meanwhile, extraction of DNA is another exhausting task, accomplished commonly under various protocols, all of which are typically accompanied by some drawbacks. Among various isothermal amplification systems developed over the recent years, the most frequently applied approach seems to be LAMP, implemented first by [32]. Due to its enormous rate of amplification paired with a very high specificity, sensitivity, rapidity and low artifact susceptibility, the method together with its modifications have been strongly recommended for detection of a great number of strains of bacteria as well as viruses worldwide [33,34]. Briefly, each reaction is carried out with four oligonucleotide primer sets which recognize six distinct regions on the target DNA in conjunction with two loop primers to accelerate the reaction [35]. LAMP assay, alternately, can also amplify nucleic acid 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, although a large number of studies have been accomplished using LAMP or reverse transcription LAMP (RT-LAMP) [36-39]. Notably, despite a few number of studies about immunocapture RT-LAMP (IC–RT-LAMP) and immunocapture LAMP (IC–LAMP) [40,41] because the technique has not been yet introduced for detection of TYLCV,

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an attempt was accordingly made to optimize a new protocol of it to save time, particularly remove DNA extraction. As the second purpose, since the existence of LAMP-positive amplicons has been proved to be confirmed by adding hydroxyl naphthol blue prior to the reaction, allowing observation with the naked eye [42-49]. In the study here, IC-LAMP was employed to detection of TYLCV.

Materials and Methods

Virus samples

Survey studies were conducted in three provinces (Dezfoul, Zanjan and Mashhad) where tomatoes are commonly grown in Iran. A total of 180 healthy and infected leaf samples with foliar curling and yellowing, reduced leaflet area, plant stunting symptoms which were infected naturally with TYLCV in the field were collected in summer of 2010 and 2011, respectively, from 42 farms in 20 major tomato-growing areas of 3 provinces and kept at -80°C until use. The samples were screened for the presence of TYLCV using serological and molecular techniques.

Double antibody sandwich enzyme linked immune sorbent assay (DAS-ELISA)

Double antibody sandwich ELISA (DAS-ELISA) was carried out as described by Clark and Adams [48] with some minor modifications. 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 infected tomato 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 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 A405nm.

IC–PCR assay

One set of primers (F and B) was used on the basis of the SF gene (GenBank accession number: AB014347) of the DNA sequences of TYLCV for the amplification of the DNA genomic component. Primer sequences were designed from the nucleotide sequence of the TYLCV genome [29] (Table 1). The protocol, to generate IC–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 TYLCV 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 9 positive tomato 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 DNA template in IC–PCR reactions.

Section 2 in this part, IC–PCR was carried out in a Bio-Rad thermocycler. The amplification was performed in a 25 µl volume containing 200 mM of each dNTPs, 0.2 mM of each primers (F and B), 2 mM $MgCl_2$, 10 Mm Tris-HCl (pH 8.3), 50 mM KCl and 2.5 units of Taq DNA polymerase (Cinagen Co, Cat. No TA7505C). The PCR reactions were performed in a Thermal Cycler (iCycler, BIO RAD, CA, USA) with 40 cycles of denaturing for 20 s at 94°C, annealing for 40 s at 55°C and DNA extension for 20 s at 72°C, followed by a single 7 min extension step. The products were lastly analyzed by gel electrophoresis in which 5 µl of the IC–PCR products (187 bp) were loaded on a 1.5% agarose gel and visualized by staining with ethidium bromide.

IC–LAMP assay

In order to perform IC–LAMP, on the basis of the SF gene, four specific primers, including outer primers (F3 and B3) and inner primers (FIP and BIP) were used (Table 1) [29]. Even though the principles of the first section of IC–LAMP assay exactly followed the IC–PCR with no DNA 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:

Section 1 just the same as the section 1 of IC–PCR procedure (see above).

Section 2 each reaction was performed in a total volume of 25 µl: 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 each dNTP, 0.2 µM each of primer F3 and B3, 0.8 µM each of primer FIP and BIP, and 8 U of Bst DNA polymerase (New England Biolabs Inc.). Tubes were then incubated at 60°C for 60 min in water bath. An agarose gel electrophoresis system (optional; 1.5%) under UV illumination could be also employed to visualize positive reactions: 5 µl of each IC–LAMP amplicon is loaded on a 1.5% agarose gel. In order to visual detection, 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. 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.

Sensitivity of the LAMP assay

Both quality and quantity of DNA template may have a dramatic influence on the results of each PCR method. In order to determine limit of the IC-LAMP assay, total nucleic acids were extracted from

Primer	Type	Position on gene	Length	Sequence(5′-3′)
F	Forward	701-720	20 mer	GTCTTATGAGCAACGGGATG
B	Backward	867-887	21 mer	GAACATGACCTGATTAGTGTG
F3	Forward outer	387-405	19 mer	TGCAGTCCGTTGAGGAAAC
B3	Backward outer	598-617	19 mer	CCTGTACGTCCATGATCGTC
FIP	Forward inner	453-473 and 413-431	40 mer	AGTCACGGGCCCTTACAA-CAGCCCAATACATTGGGCCACG
BIP	Backward inner	515-534 and 564-581	37 mer	TCGAAGGTTCGCCGAAGGCGA-CAATGGGGACAGCAGC

Table 1: Oligonucleotide primers used for IC-LAMP and IC-PCR.

both TYLCV-infected and healthy tomato cultivars using a modified procedure of Dellaporta heat extraction method [41]. An eight dilution series (2×10^1 to 2×10^8 CFU/ml) of DNA were prepared in water; 2 µl of each dilution was used for LAMP and PCR reactions. Furthermore, Hydroxynaphthol blue (HNB) dye was added to LAMP products and positive reactions were directly detected by visual inspection. Similarly, the detection limit of the LAMP and PCR was approved by electrophoresis on 1.5% agarose gel.

Results

DAS-ELISA results revealed that 38 out of 180 (21.1%) symptomatic tomato samples obtained from different regions of 3 provinces of 7 farms were infected with TYLCV. All positive samples were utilized lastly for further analyses.

As regards IC–PCR, the amplification occurred via both backward and forward primers to generate ultimate products. The method, overall, could successfully identify positive samples with no attempt to DNA extraction. As expected, a fragment with the size band of 187 bp was detected when the IC–PCR products were run on 1.5% agarose gel and stained with ethidium bromide (Figure 1a). The same as IC–PCR, our new IC–LAMP protocol could successfully identify positive samples, interestingly with no use of DNA isolation in a water bath. 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). IC–LAMP amplicons were able to be detected with the naked eye by adding visual dye (HNB) followed by colour changing in the solutions (Figure 1c). Our results, interestingly, indicated that LAMP can produce reliable

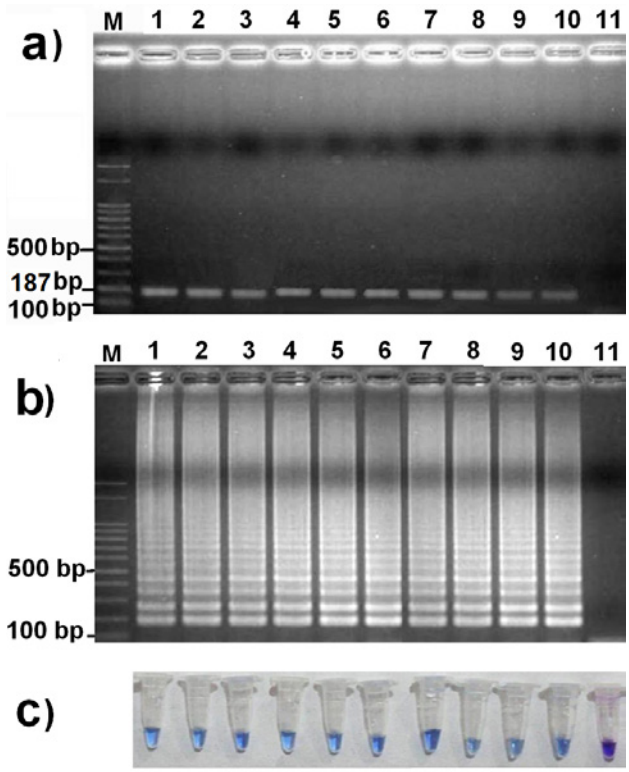

Figure 1: Gel electrophoresis pattern on 1.5 % agarose gel (a) IC-PCR assay; (b) IC-LAMP assay; (c) visual detection by HNB. Left to right: lane M, DNA size marker (100 bp; Fermentas); lanes and tubes 1-7, positive samples of 7 farms; lane and tube 8, negative control.

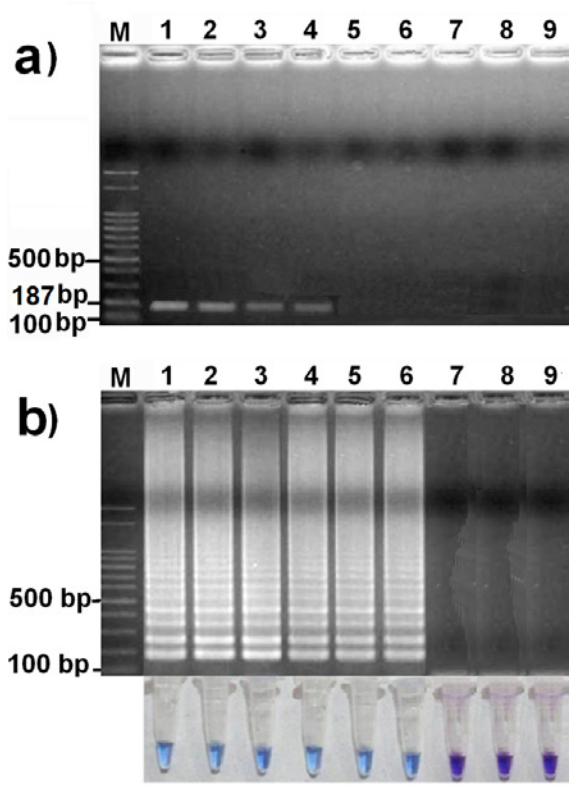

Figure 2: Comparative analysis of the sensitivity of LAMP and PCR, using an eight dilution series of DNA as template (a) Electrophoresis analysis of PCR; (b) Electrophoresis analysis and visual detection (HNB) of LAMP. Left to right: Lane M, DNA size marker (100 bp; Fermentas); Lanes 1-8, 2×10^8, 2×10^7, 2×10^6, 2×10^5, 2×10^4, 2×10^3, 2×10^2 and 2×10^1 CFU/ml, respectively; Lane 9 negative control (water).

products even under lower DNA concentrations (2×10^3 CFU/ml or more), whilst PCR, requires higher level of DNA (at least 2×10^5 CFU/ml) (Figures 2a and 2b).

Discussion

In this study, as a result, three detection methods including DAS-ELISA, IC-PCR and IC-LAMP were assessed to explore positive and negative aspects of each one, followed by introducing the best one regarding TYLCV detection. Even though all three techniques had enough potential to make differentiation and detect infected plant samples accurately, IC–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 IC–LAMP and IC–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), IC–LAMP overall requires just 60 min to accomplish (as the least demanding detection method), while regarding IC–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 [27,50]. More surprisingly, in IC–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 IC–PCR assays, all of which are undoubtedly costly. On the contrary, IC–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 [38-40]. Likewise, exclusive of the primer designing process which is somehow complicated and sensitive, other phases are simply applicable. It is noticeable that in all DNA extraction-based methods, DNA extraction is an unavoidable step, needing different protocol(s) followed by optimization (mostly is a time-consuming process) to acquire purified DNA stock [51], whereas IC–LAMP can be easily performed with no attempt for DNA 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 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 [42-44]. In the current study, therefore, IC–LAMP amplified products were confirmed by adding HNB prior to the reaction along with forming diverse colour pattern depending upon the chemical characteristics of the applied chemical substances as dye.

According to our results, despite the precise detection of positive IC–LAMP products using HNB dye, some was significantly superior when the time of stability, cost and the safety were taken into consideration. To abbreviate the contamination hazard and also increase colour stability, as a result, additional metal indicator (HNB) known as close-tube IC–LAMP detection were lastly used. Interestingly, HNB dye-based assays were accompanied by several remarkable advantages compared with other colorimetric-based methods [46-48] in that of which are mixed prior to amplification, a need to open the assayed samples to add the dye is thereby omitted, and the risk of cross-contamination will be excluded drastically [31,47,48]. Meanwhile, the visual inspection of IC–LAMP products by means of HNB dye was seen as advantageous as there was no need for electrophoresis and subsequent staining with carcinogenic ethidium bromide [45]. Lastly, the colour brightness and stability of the both HNB in the solutions with positive/negative reactions were remained constant after 2 weeks of exposure to ambient light [49]. For example, at the study of Goto et al. [31], HNB was reported as the best visual system, while the brightness of SYBR green fluorescence and calcein fluorescence was significantly weaker than that of HNB. It is noticeable that since the colour presented by HNB was light blue for positive results and dark blue for negative results, which cannot be discriminated precisely [51], so such based detection methods involve a little more attention to provide accurate decision.

In summary, a novel IC–LAMP assay for rapid and easy detection of TYLCV was developed here, its potential compared with DAS-ELISA and IC–PCR assays. The method, on the whole, had the following advantages over the two mentioned procedures and also the methods including LAMP and PCR: (1) fascinatingly, no need of DNA 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 dye. 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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