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Development of reverse transcription loop mediated isothermal amplification assay for visual detection of Grapevine fleck virus (GFkV)

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Summary

Grapevine fleck virus (GFkV) produces a ubiquitous disease, latent in grapevine causing simple or complex infections with other more dangerous viruses. The aim of the present study is to detect GFkV through application of reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay; its efficiency has been contrasted with other procedures such as Double antibody sandwich ELISA (DAS-ELISA) and RT-polymerase chain reaction (PCR). The coat protein (CP) gene of the virus is basically used for designing the primers. Using hydroxynaphthol blue (HNB) Dye, RT-LAMP was placed in a water bath after the optimization was done. In order to detect GFkV easily and rapidly, a new immunocapture (IC)-RT-LAMP assay was developed as well; it was further compared with other assays. The results show RT-LAMP is an advantageous method because it is highly sensitive, quite cheap, user-friendly, and safe; in addition, it is performed quickly by visual detection and does not require RNA extraction (in IC-RT-LAMP).

K e y w o r d s : DAS-ELISA; Grapevine fleck virus (GFkV); immunocapture assay; RT-LAMP assay; RT-PCR assay.

Introduction

One of the diseases targeting grapevine is Grapevine fleck; it is a graft-transmissible disease caused by Grapevine fleck virus (GFkV), which is a phloem-limited and non-mechanically transmissible virus (Boscia *et al*. 1995). This virus has isometric particles ca. 30 nm in diameter and belongs to the genus *Maculavirus* in the family of *Tymoviridae* (Boscia *et al*. 1991). Its genome is a positive-sense consisting of single stranded RNA with a genome size of 7,564 nucleotides (nt) (Naidu and Mekuria 2010). Latent infections in *Vitis vinifera* L. cultivars are brought about by GFkV. However, this virus causes specific foliar symptoms in the indicator host *Vitis rupestris* L. (Sabanadzovic *et al.* 2000). Leaf vein clearing, chlorotic ringspots, oak leaf patterns, and/or distortion of leaf blades are the symptoms caused by GFkV infections (Naidu and Mekuria 2010).

Recently, isothermal amplification methods providing simple and cost-effective molecular tests in low resource settings have been developed with an increasing demand (Notomi *et al*. 2000). LAMP is one of these methods, which has been most widely adopted today. Hydroxy naphthol blue (HNB) is a metal indicator for calcium and a colorimetric reagent for alkaline earth metal ions which is used for colorimetric assay of the LAMP reaction (Goto *et al*. 2009). In the pH range between 12 and 13, the solution of the indicator is reddish pink in the presence of calcium ions and deep blue in the presence of excess (Ethylenedinitrilo) tetraacetate (Ito and Ueno 1970). The purpose of this study was to develop a rapid, sensitive, specific, and easy method to detect GFkV using a visualized detection system using the HNB dye.

Material and Methods

P l a n t s a m p l e s : 12 grapevine fields of three Iranian provinces including East and West Azerbaijan and Ardebil were the target area for the surveys (38 leaf samples) in 2016.

D A S - E L I S A a s s a y : In order to detect the infection in the samples, Polyclonal GFkV-specific antibody (IgG: Art.No. 120612, Conjugate: Art. No. 120622, Positive control: Art. No. 120653, negative control: Art. No. 180043, Bioreba AG, Switzerland) was applied first. As Clark and Adams (1977) specified, Double antibody sandwich ELISA (DAS-ELISA) was performed.

R N A e x t r a c t i o n a n d p r i m e r d e s i g n : As it was specified by Rowhani and Stace-Smith (1979) before, 100 mg of leaf tissue was used to isolate Total RNA. The Primer Explorer V.4 (http://primerexplorer.jp/elamp3.0.0/index.html) and Oligo7 software were utilized to design RT-LAMP and RT-PCR specific primers based on the coat protein (CP) (GenBank: GU372374.1), respectively. The positions of the designed primers on the sequence are displayed in Figure A. Using the BLASTN algorithm, the primers were put to test.

R T - P C R a s s a y : First, incubation was performed on the extracted RNA for 3 min at 75 °C; it was then chilled for 3 min on ice. Using a 25 µL volume tube, 50 mM Tris-HCL (pH 8.3), 20 pmol B primer, 10 mM dithiothreitol

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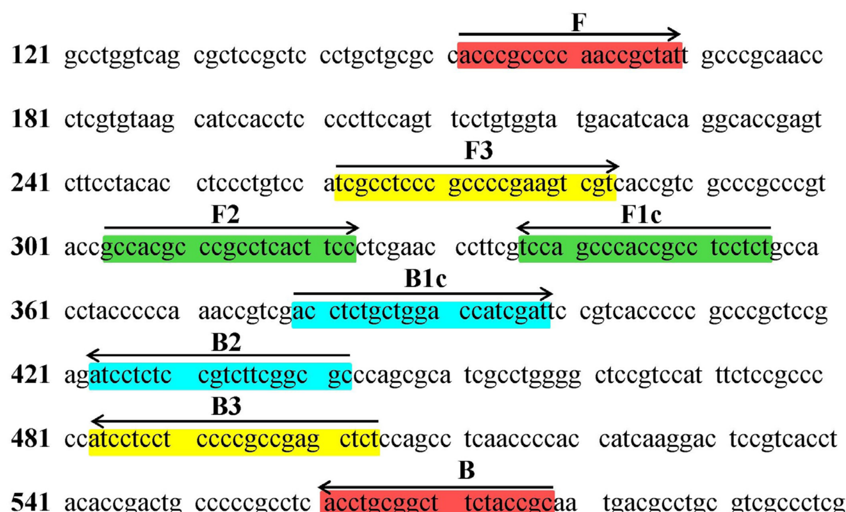

Figure A: Oligonucleotide primers.

(DTT), 10 mM of each dNTP, 2.5 mM $MgCl_2$, 5 U of RNasin Ribonuclease Inhibitor (Fermentas Co, Cat. No EO0381) and 1.25 U of *AMV* reverse transcriptase (Fermentas Co, cat. no. EP0641) were mixed. The mixture was put for incubation for 1 h at 60 °C. Next step was to carrying out PCR reaction on a Thermal Cycler (iCycler, BIO RAD, CA, USA) in a 25 µL volume containing 1.5 mM $MgCl_2$, 1 × PCR buffer (50 mM KCl and 10 mM Tris-HCl, pH 8.3), 20 pmol of each B and F primers, 0.625 U of *Taq* DNA polymerase (Cinagen Co, Cat. No TA7505C), 0.2 mM of each dNTP and 2 µL cDNA. Then, amplification was done on the mixture for 3 min at 94 °C and further 35 cycles followed by for 1 min at 94 °C, 1 min at 54 °C, 1 min at 72 °C, and finally for 10 min at 72 °C. After amplification, electrophoresis was done on the mixture using 1.5 % agarose gel, stained with ethidium bromide; it was photographed under UV light using Gel Documentation System (GELDOC 2000, Bio-Rad, USA).

RT-LAMP assay: The mentioned positive control was employed to examine and optimize the impacts of temperature, dNTP, $MgSO_4$, betaine, and Bst DNA polymerase concentrations. Afterwards, the isolated RNA was involved in the one-step RT-LAMP reaction as well as was described by ALMASI (2015). 1 µL HNB (Lemongreen, Shanghai, China), before the amplification, was separately added to the RT-LAMP master mix to provide a visual detection of the products. The color changes occurring in the tubes were monitored by simply watching them.

Immunocapture (IC)-RT-LAMP assay: As ALMASI (2015) specified, IC-RT-LAMP was performed. IC-RT-LAMP was done in two steps. First (IC step), using GFkV -specific IgG diluted in coating buffer, the polystyrene microtiter plate was coated. Second, RT-LAMP in a total volume of 100 µL was conducted and the color of each well was studied visually with no aid.

Results

Serological test: Out of 38 samples, four samples (10.5 %) were marked as infected because they were positive after performing DAS-ELISA including 1 sample from East Azerbaijan province and 3 samples of Ardebil province (Figure B). Interestingly, due to observing a clear colour change in the wells containing positive reactions (yellow colour as an indicator), no attempt was accordingly made to employ an ELISA Reader.

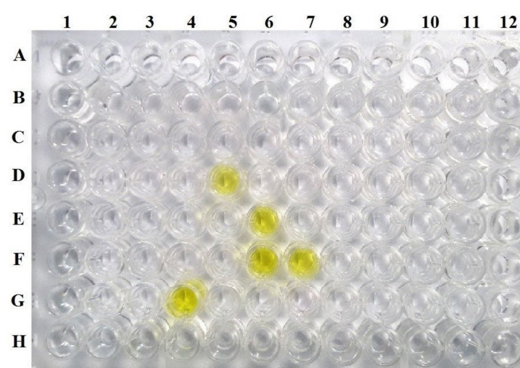

Figure B: Verification of DAS-ELISA assay results. Wells 4G, 5D, 6E and 6F, GFkV positive samples; well 7F, positive control; well 7G, negative control.

Molecular assays: As regards RT-PCR, the amplification occurred via both backward and forward primers to generate ultimate products. RT-PCR assay confirmed the positive samples as well and a predictable fragment (427 bp) was observed on agarose gel (Figure C). Using the RNA, positive samples could be detected by one-step RT-LAMP after performing the reaction optimization. The reaction was performed in a water bath for temperature control. Using a 1.5 % agarose gel, RT-LAMP amplicons were electrophoresed and consequently several fragments (a ladder-like pattern) were observed from positive samples, but not from any negative samples (Figure D).

Visual detection: After addition of HNB, the RT-LAMP products can be visualized directly by the naked eye; it was possible to differentiate the positive (sky blue) and negative (violet) samples clearly using HNB stain (Figure E). Contrary to RT-LAMP, positive samples without RNA extraction by IC-RT-LAMP visually in a water bath (Figure F). IC-RTLAMP results were able to be detected with the naked eye by adding visual dye followed by colour changing in the solutions.

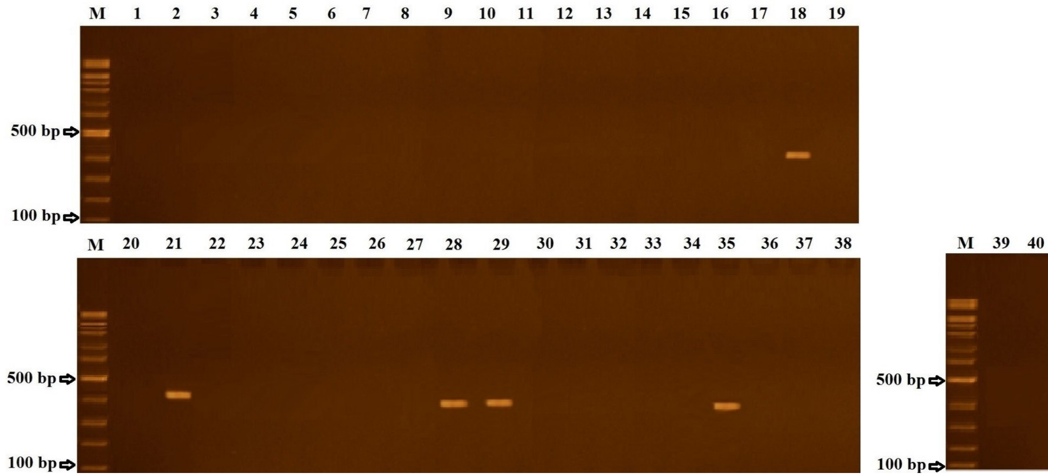

Figure C: RT-PCR results. M, DNA size marker (100 bp); lanes and tubes 18, 21, 28 and 29 positive samples; lane and tube 35, positive control; lane and tube 36, negative control.

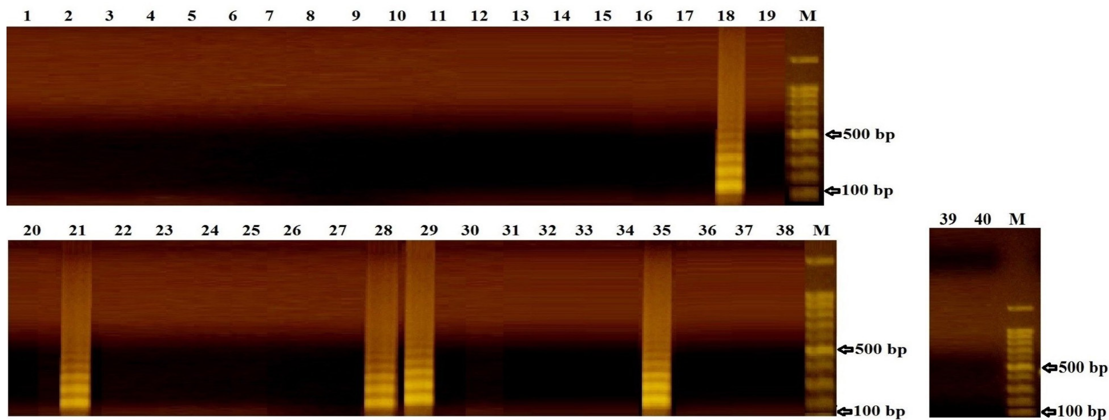

Figure D: RT-LAMP results.

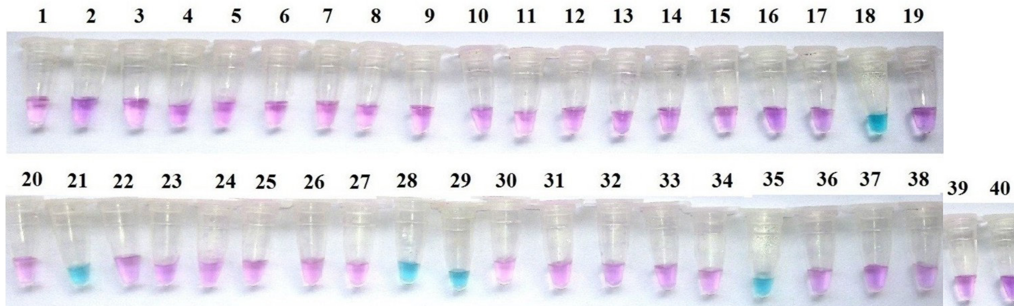

Figure E: Visual detection of RT-LAMP by HNB.

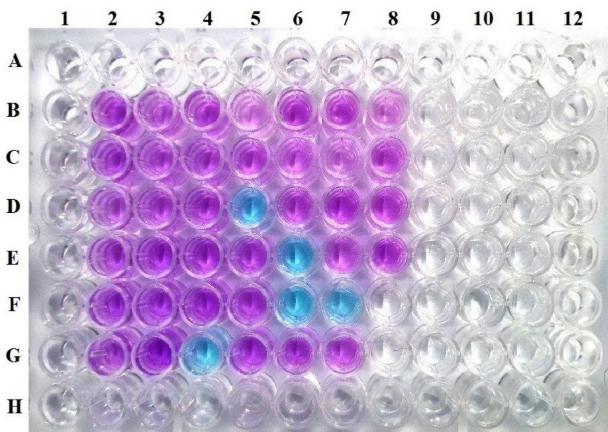

Figure F: IC-RT-LAMP results.

Discussion

Four detection methods including DAS-ELISA, RT-PCR, RT-LAMP, and IC-RT-LAMP have been evaluated in terms of GFkV detection in this study. All these techniques were potentially able to detect the infection accurately, but it was shown that IC-RT-LAMP outperforms other methods in terms of time, safety, simplicity, cost and being user friendly. Contrary to other methods, DAS-ELISA usually takes longer time to detect infections (two or more days). Excluding step one (taking equal time), RT-PCR and RT-LAMP (or IC-RT-LAMP) took just 3 h and 60 min to be done (as the least demanding detection method) respectively; but DAS-ELISA requires at least 1 d. Thus, the detection procedure will be simplified and the time needed to

separate the amplified products on a gel and data analysis is decreased. The usual procedure for observing amplicons is using gel electrophoresis systems. Color indicators can be used to detect LAMP amplified products easily without additional staining systems; thus, toxic staining materials would be avoided considerably (Goto *et al*. 2009). DAS-ELISA and RT-PCR assays are expensive methods because its needs equipped labs with some molecular instruments as well as trained staff; however, LAMP is done easily only in a water bath or temperature block without thermocycler and gel electrophoresis (Notomi *et al*. 2000). It should be highlighted that RNA extraction must be done in many RT-based methods; this requires going through several time-consuming protocol(s) and optimization processes to reach purified RNA; but IC-RT-LAMP is done easily without the need to isolate RNA. Thus, the detection procedure is simplified and time is saved (Fukuta *et al*. 2003). Assessment was made possible by the addition of HNB dye; thus, it was not necessary to perform post-amplification processes such as electrophoresis. The additions of dye can be done before amplification; hence, opening the assayed samples to add the dye is avoided and the risk of cross-contamination is decreased. Following 14-d exposure to peripheral light, HNB dye lasts into the positive/negative reactions.

Conclusion

The new colorimetric assay for LAMP (RT and IC-RT) can be carried out by only adding HNB to the reaction solution. This colorimetric LAMP assay using HNB in both tubes and 96-well plates has some benefits compared with other techniques: easy operation, no need for special equipment, superior sensitivity and speed, low contamination risk, and suitability for high-throughput DNA and RNA detection. The current detection approach may be used satisfactorily for diagnoses of other plant viruses in addition to laboratory research.

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