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Brief Report

A Sensitive Immunocapture-RT-PCR Assay for Detection of Maize Iranian Mosaic Virus in Individual Small Brown Planthopper Vector, *Laodelphax striatellus*

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Background: Sensitive detection of *Maize Iranian mosaic virus* (MIMV) in its insect vector, *Laodelphax striatellus* is essential for effective forecast and control of this viral disease.

Objectives: Three methods of ELISA, RT-PCR and IC-RT-PCR were compared regarding their sensitivity for detection of MIMV in the single planthopper with a series of various dilutions.

Materials and Methods: To detect MIMV from a single insect vector, the sensitivity of three methods including ELISA, RT-PCR and IC-RT-PCR was evaluated.

Results: Compared to the other methods, the IC-RT-PCR showed more sensitivity and detected virus at least at the 1:60 dilution. While, both ELISA and RT-PCR methods could detect up to the 1:20.

Conclusions: The results reported herein showed that IC-RT-PCR is a sensitive and simple method to detect MIMV from a single insect vector with high efficiency and reliability. These findings might be useful in the prediction of viral disease incidence in host plants and this method can also be effective to detect other viruses in their insect vectors.

Keywords: Immunocapture RT-PCR; Laodelphax striatellus; Maize Iranian mosaic virus; Sensitivity; Virus detection

1. Background

Insects are the most important vectors of plant viruses and are thus vital elements in the study of the epidemiology and molecular pathology of virus diseases (1,2). The small brown planthopper (SBPH), *Laodelphax striatellus* (*Delphacidae*; *Hemiptera*), is one of the most serious and destructive sap sucking pests that attacks and cause significant damage on a number of economically important crops including rice, wheat, barley, maize and sugarcane (3). SBPH as an insect vector transmits multiple plant viruses including a rhabdovirus, *Maize Iranian mosaic virus* (MIMV). MIMV is mainly transmitted by the *L. striatellus* in a persistent, circulative and propagative manner. This viral disease has been reported to be economically destructive in the barley and maize growing areas in Iran. It is one of the most widespread viruses infecting maize in many provinces of Iran (4,5). MIMV has become epidemic as a result of wide distribution of the insect vector since 2003 in temperate regions of Fars province, Iran. Because of small size, it is challenging to detect RNA viruses from a single SBPH. Several methods have been developed for the detection of RNA viruses in their insect vectors including biological inoculation of plants, electron microscopy, serological and molecular detection approaches (6–9). An alternative approach is immunocapture-reverse transcription-polymerase chain reaction (IC-RT-PCR) which is a combination of serology and RT-PCR technique (10). However, there is little information about a sensitive technique to detect viruses including MIMV in single insect vector, SBPH. Therefore, information of the number of virus-carrying SBPHs and accurate diagnosis of virus in a given local population of insect vector is very important for the viral disease forecasting and spray programs to warn farmers on the potential threat to their crops (11). In this study, the sensitivity of three different methods including ELISA, RT-PCR without RNA isolation

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and IC-RT-PCR were assessed for detecting MIMV in single SBPHs.

2. Objectives

In this study, by using MIMV and the *Laodelphax striatellus* (SBPH) vector, three methods of ELISA, RT-PCR and IC-RT-PCR were compared regarding their sensitivity for MIMV detection in the single planthopper (SBPH) with serial dilutions of 1, 1:10, 1:20, 1:40 and 1:60.

3. Materials and Methods

3.1. Insect Vector Rearing

SBPHs free MIMV and MIMV-viruliferous populations were collected from an infected maize field at the college of agriculture, Shiraz University, Shiraz, Iran, and reared on healthy barley seedlings under greenhouse conditions at 25 °C with a photoperiod of 16 h /8 h (light/dark), to produce viruliferous and non-viruliferous colonies. These colonies were tested with RT-PCR for ensure their healthy and infected conditions. The non-viruliferous SBPH population were used as negative controls.

3.2. Crude Extract Preparation from a Single Insect Vector

To evaluate the sensitivity of ELISA, RT-PCR and IC-RT-PCR for MIMV detection from young adult insect vector, each individual insect was crushed in 150 µl of distilled water (for RT-PCR and IC-RT-PCR) or ammonium citrate (for ELISA). All the experiments were repeated twice and each assay was conducted with five insect replications.

3.3. Indirect ELISA

ELISA was carried out using a method (12). A single young adult of SBPH was placed in a 1.5 mL centrifuge tube and ground in 150 µl of 0.1 M ammonium citrate. After centrifugation at 12,000 ×gravity for 1 min, the supernatant used for ELISA in a series of dilutions of 1, 1:10, 1:20, 1:40 and 1:60. One hundred µl of the supernatant added to a 96-well plate and incubated overnight at 4 °C. The plate was washed with 100 µl of Phosphate Buffered Saline with 0.2% Tween 20 (PBST), polyclonal antibody (13) was added at a dilution of 1:900 (v: v) in PBS containing 0.2% Tween 20 (PBST). The plate was incubated at 37°C for 4 h before washing and addition of 100 µl alkaline phosphatase-conjugated goat anti rabbit IgG (SIGMA company, A8025) (1mg: ml) at a dilution of 1:900 (v: v) in PBST. After an overnight incubation at 4 °C, final washing was performed, followed by addition (100 µl: well) of substrate buffer (0.6 mg: ml disodium p-nitrophenyl phosphate substrate in 10% diethanolamine). The plate was incubated for 30 min at room temperature. The absorbance was measured on an ELISA reader (BioTek™ ELx808™) at 405 nm. A sample was considered positive if its absorbance value was at least two times higher than that of the control non-viruliferous SBPHs.

3.4. RT-PCR

A single SBPH was placed in a 1.5 mL centrifuge tube and ground in 150 µl sterile H_2O. After centrifugation at 12,000 ×gravity for 1 min, the supernatant was precipitated with 3 M sodium acetate and 1:1 volume of isopropanol at -20°C. The pellet was used as a template for RT-PCR in a series of dilutions 1, 1:10, 1:20, 1:40 and 1:60.The first strand cDNA was synthesized using M-MuLV RT enzyme cDNA synthesis kit (Parstous Biotechnology, Iran) according to the manufacturer's protocol. The procedure is as follows: 11 µl pellet sample, 4 µl 5 ×M-MuLV reverse transcriptase buffer, 2 µl dNTP mix (10 mM), 2 µl reverse primer (10 pmol) and 1 µl M-MuLV RT (100 U/µl) were mixed and incubated at 42 °C for 1 h, followed by 70 °C for 10 min. Virus specific primers (forward primer: 5′-CAGCCCGAGTTGTAATGTG -3′ and reverse primer 5′- GGACCGCAGAGTATGACG -3′) were used based on the conserved nucleotide sequences of MIMV *glycoprotein* gene in GenBank (KP178685; Acc.No.) for PCR amplification to detect virus from a single SBPH. The PCR was carried out using a thermal cycler (Techne™ TC-312, UK) in a 20 µl total reaction volume by using 8 µl master mix (Ampliqon, Denmark), 1 µl of forward and reverse primers (10 pmol) and 1 µl DNA. The initial denaturation (94 °C, 5 min) was followed by 35 cycles of 94 °C for 30 s, 54 °C for 45s, and 72 °C for 1 min, and a final extension step at 72 °C for 10 min. Ten µl of each PCR product was evaluated by 1% agarose gel electrophoresis using ethidium bromide staining.

3.5. IC-RT-PCR

To do this, thin wall micro-tubes (0.2 ml) were coated with 100 µl (1:900 v: v) of MIMV polyclonal antibody (13) in blocking buffer (PBST containing 5% w/v nonfat dry milk). The micro-tubes were then incubated at 37 °C for 2 h and then washed with PBST. Planthopper samples were prepared by grinding SBPH tissue in 1.5-mL centrifuge tubes. After grinding and preparing

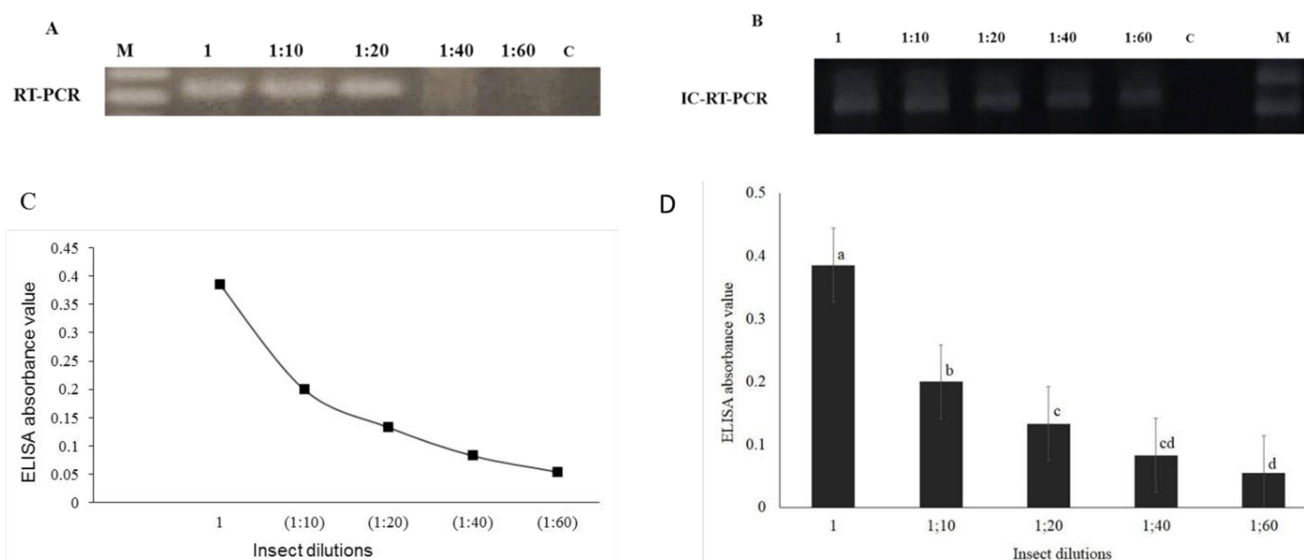

Figure 1. A: Electrophoretic pattern of RT-PCR, **B**: IC-RT-PCR products. **C**: detection of MIMV in individual SBPH by ELISA using serial dilutions of crude planthopper extract, 1, 1:10, 1:20, 1:40 and 1:60 dilutions. Ladder, Smobio-1 KB plus was used in this study. **D**: Absorbance values of indirect ELISA at 405nm for serial dilutions 1, 1:10, 1:20, 1:40 and 1:60 of crude planthopper extract. Data are mean absorbance values ± standard deviation (SD). Values followed by the common letter do not differ significantly at $p<0.01$ in the Duncan's multiple range test using SAS 9.4 software.

supernatant, 100 µl of the extract 1, 1:10, 1:20, 1:40 and 1:60 dilutions then added to each antibody-coated micro-tube. The micro-tubes were incubated at 4°C overnight to allow MIMV particles to be trapped to the micro-tubes and then washed with PBST. These micro-tubes were then used for cDNA synthesis (Parstous Biotechnology, Iran) with each reaction containing 4 µl 5X M-MuLV reverse transcriptase buffer, 2 µl dNTP mix (10 mM), 2 µl reverse primer and 1 µl M-MuLV RT (100 U/µl). The micro-tubes were subjected to thermal cycling as before, (consisting of 42 °C for 1 h, and 70 °C for 10 min). The PCR was carried out using a thermal cycler (Techne™ TC-312, UK) in a 20 µl total reaction volume by using 8 µl master mix (Ampliqon, Denmark), 1 µl of forward and reverse primers (10 pmol) and 1 µl DNA. The cycling parameters were as follow: the initial denaturation (94 °C, 5 min) followed by 35 cycles of 94 °C for 30 s, 54 °C for 45 s, 72 °C for 1 min, and a final extension step at 72 °C for 10 min. After RT-PCR, 10 µl of each reaction was loaded onto a 1% agarose gel. After electrophoresis, the gel was stained in ethidium bromide (1%) and visualized on a UV transilluminator.

3.6. Sequencing

To confirm the RT-PCR and IC-RT-PCR results, PCR products were sequenced (Bioneer, South Korea) in both forward and reverse directions. The sequences were subjected to nucleotide BLAST using NCBI BLAST tools.

4. Results

Agarose gel electrophoresis of the DNA products generated from RT-PCR and IC-RT-PCR revealed the presence of a specific 150 bp fragment in MIMV-viruliferous SBPH samples, while, no amplicon obtained for the non-viruliferous SBPH controls (**Fig. 1**), indicating that both methods successfully detected MIMV in a single MIMV-viruliferous SBPHs. The presence of no amplicon in non-viruliferous SBPH samples suggested that the antibody and primers had a high specificity in detecting MIMV in SBPH. BLAST search using nucleotide sequence showed that the PCR products had 98% nucleotide identity with *glycoprotein* (G) gene (Acc.No. KP178685) of MIMV, Shiraz isolate, which confirmed specific amplification of the viral gene.

The sensitivity of ELISA, RT-PCR and IC-RT-PCR was evaluated by testing 1, 1:10, 1:20, 1:40 and 1:60 dilutions of MIMV-viruliferous and non-viruliferous SBPH crude extracts. ELISA, RT-PCR and IC-RT-PCR methods could successfully detect MIMV up to

Table 1. Detection of MIMV in various dilutions of a MIMV-viruliferous SBPH sample by ELISA, RT-PCR and IC-RT-PCR.

Dilution	ELISA	RT-PCR	IC-RT-PCR
1	+	+	+
1:10	+	+	+
1:20	+	+	+
1:40	–	–	+
1:60	–	–	+

the 1:20, and at least 1:60, respectively (**Fig. 1**; **Table 1**). The detection sensitivity of IC-RT-PCR was thus at least three times higher than ELISA and RT-PCR methods. There was no difference in the detection sensitivities of ELISA and RT-PCR. Progress of ELISA absorbance value at different dilutions showed the most value (0.39) at 1 dilution followed by a reduction until (1:60) which showed the lowest ELISA absorbance value (0.05) (**Fig. 1**).

5. Discussion

Accurate and quick diagnosis of viruses in their insect vectors is necessary for the forecasting of viral disease epidemics (14). Viral disease incidence would be evaluated through sensitive MIMV detection in individual SBPH. Thus, there is need to a reliable diagnostic method for detecting MIMV from a single SBPH. In our recent study, we tested MIMV infection using RT-PCR and ELISA techniques in its plant and insect host (15). Here, we aimed to compare the potential of three different methods including ELISA, RT-PCR and IC-RT-PCR in terms of their sensitivity using MIMV-SBPH pathosystem, to detect MIMV from a single SBPH.

Our results suggest that IC-RT-PCR is at least three times more sensitive than ELISA and RT-PCR for the detection of MIMV in a single insect samples. The high sensitivity of the IC-RT-PCR assay might be due to the high copy number of the target sequence (*Glycoprotein* gene of MIMV). Immunocapture of virions may cause more separation of the virus from potential inhibitors to release RNA for the RT-PCR. IC-RT-PCR has been used to combine the advantages of PCR with ELISA (10,16). Thus, because of its simplicity, rapidity and sensitivity, IC-RT-PCR as a single-tube test would be an effective method to detect MIMV. This method shortens handling time for sample processing and risks of contamination.

Taken together, IC-RT-PCR constitutes a useful tool for the diagnosis and forecasting of epidemics of MIMV in the early stages of virus infection by enabling accurate detection in single insect vectors for a large number of planthopper samples. An accurate forecasting and control program is highly desirable to use the least amount of insecticide at the appropriate time for controlling of insect vectors (17). These findings might be useful in various studies including prediction of viral disease incidence in crop plants and this method can also be used for other plant virus-vector systems with high sensitivity.

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