Iranian Journal of Virology 2009;3(1): 7-11
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Original Article

A New Sensitive Method for Detection of Viroids

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Abstract

Background and Aims: Viroids are smallest known plant pathogens and cause several economically significant diseases. Until recently, viroid detection relied mainly on biological tests and indexing. Today various diagnostic techniques such as nucleic acid hybridization, southern blot and reverse transcription coupled with polymerase chain reaction (RT-PCR) are being used for detection and diagnosis of viroids.

Methods: This paper describes a new method for detection of citrus viroids, based on a combination of RT-PCR and dot blot hybridization (RT-PCR-DBH). In this method instead of using nucleic acid extracted directly from the plants, RT-PCR products are subjected to dot-blot hybridization.

Results: The results showed that the above mentioned method has some advantages compared with the other methods. It is more sensitive, relatively simple, cost-effective, rapid and easy to apply. It was about 1000 times more sensitive than southern blot and about 100 times more sensitive than PCR in detecting hop stunt viroid in citrus.

Conclusion: The introduced method here has a high potential in diagnosis of viroids and is suitable for detection of low concentrations of the agent.

Keywords: Viroid; HSVd;CEVd;RT-PCR-DBH;Southern blot hybridization

Introduction

Viroids are small circular single-stranded RNA molecules with sizes ranging between 246 and 401 nucleotides. They cause several economically significant crop diseases. Rapid and specific detection of viroids is a prerequisite for their management and control (1). Until recently, viroid detection has relied mainly on biological indexing which precludes the detection of viroids prior to symptom expression. In this method inoculated indicator plants must be incubated at 27–32ºC for 3–6 months before symptoms appear. This procedure is also very time consuming and expensive due to the additional costs of maintaining greenhouses at elevated temperatures (2). Today, other diagnostic techniques such as nucleic acid hybridization and reverse transcription coupled with polymerase chain reaction (RT-PCR) have become popular for the practical diagnosis of viroids because of their relative simplicity and high sensitivity when compared with the conventional diagnostic methods (3). In particular, dot-blot hybridization (DBH) is widely applied as specific test, using a probe labeled with a non-radioisotopic reagent (such as biotin or digoxigenin), or radioisotopic reagent. Despite higher sensitivity of these molecular methods, detection of viruses and viroids by any single molecular method may be

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[DOI: 10.21859/isv.3.1.7]

jeopardized if their concentration is too low. This often happens in detection of viral and viroidal agents in fruit trees. This paper reports a new method for detection of citrus viroids, which, in comparison with other methods, has some advantages, being considerably more sensitive and allows detection of low concentration of viroids.

RT-PCR-DBH incorporates both the RT-PCR technology and dot-blot hybridization for confirmation of results, with the combination of both techniques potentially augmenting the sensitivity of this assay.

Methods

Plant samples and Extraction of nucleic acids

Plant samples used in this study consisted of Washington navel orange (*Citrus sinensis*) leaves affected by yellow corky vein variant of hop stunt viroid disease (HSVd-sycv) and citrus exocortis viroid (CEVd) from Jahrom and Darab in the Fars province of Iran. The samples were known to contain a strain of HSVd and CEVd (Bagherian & Izadpanah 2009, unpublished). Extraction of nucleic acids from samples was conducted as standard viroid extraction method (4) designed to yield high viroid titers. The total nucleic acids were partitioned in 2M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris–HCl; 10 mM KCl; 0.1 mM MgCl2; pH 7.4).

RT-PCR, cloning and sequencing of the amplified fragments

Nucleic acid samples were subjected to RT-PCR in order to detect HSVd and CEVd following the method described previously (5) using primer pairs 5′-GGGGCAACTCTTCTCAGAATCC-3′/5′-GGGGCTCCTTTCTCAGGTAAGTC-3′ for HSVd and 5′-GGAAA CCTGGAGGAAGTCG-3′/5′-CCGGGGATCCCTGAAGG A-3′ for CEVd (6) designed to amplify the full length of each target viroid.

Electrophoretic analysis in 1% agarose gels was used to confirm the synthesis of a DNA product of the expected size. The PCR-amplified product was ligated in the vector pTZ57R/T (Fermentas) and the recombinant plasmids were used to transform DH5α E. coli cells. Plasmids from transformed cells were purified using the Plasmid Extraction Kit (Bioneer). PCR analysis using the above-

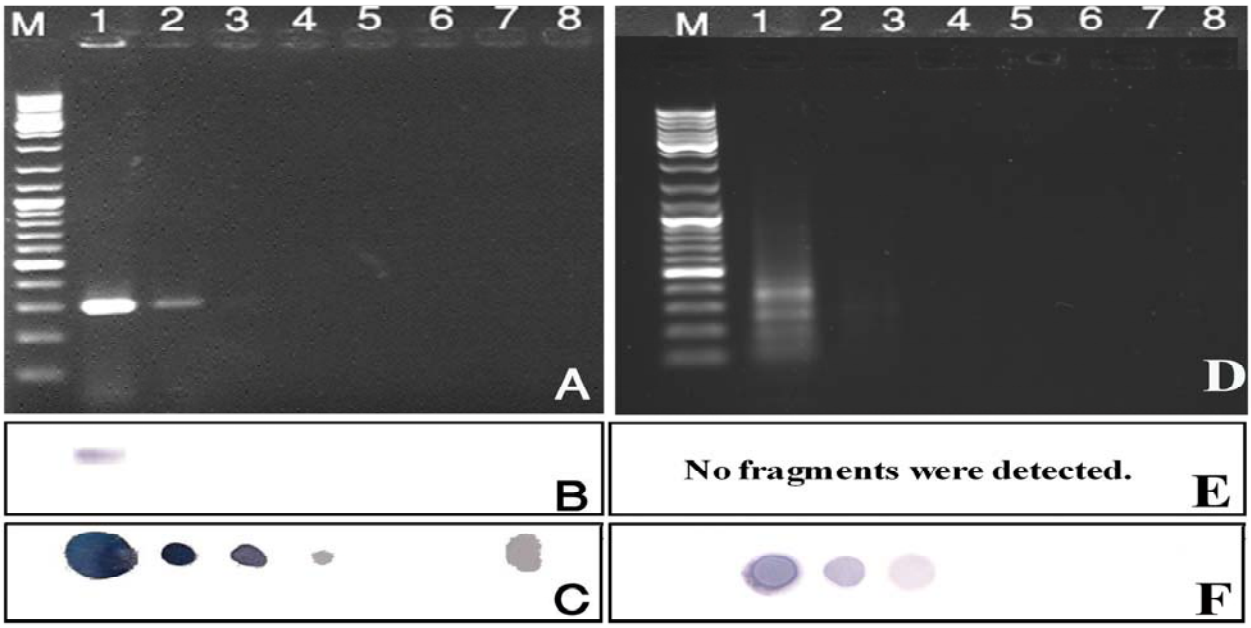


Fig. 1: Comparison of the detection sensitivity of purified HSVd (A-C) and CEVd (D-F) by (A, D) Electrophoresis, (B, E) Southern blot hybridization, (C, F) RT-PCR-DBH. (M) 100 bp marker (Promega); (1–5) Serial dilution of positive control 10^0 – 10^{-4}; (4) Negative control without a template; (5) RT product; (6) Extracted RNA.

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[DOI: 10.21859/isv.3.1.7]

mentioned primer pairs demonstrated the presence of an insert of the expected size and PCR product analyzed in 1% agarose gels.
Sequencing was performed by Macrogen Inc. (Seoul, South Korea). Sequence data were analyzed and compared with those available in GenBank, using NCBI/BLAST, to search for related sequences.
Electrophoretic analysis of 5 µL of 10-fold serial dilutions of PCR product, positive control $10^0 – 10^{-4}$, negative control, RT product and extracted RNA was performed in 1 % agarose gel (Fig. 1A, D).

Detection of viroids by southern blot hybridization

5 µL of 10^{-fold} serial dilutions ($10^0 – 10^{-4}$) of PCR product positive control, negative control, RT product and extracted RNA were loaded on to 1% agarose gel. After electrophoresis, nucleic acids bands were transferred to nylon membrane by blotting (Fig. 1B, E) (7).
Hybridization analysis was carried out using digoxigenin (Dig) labeled probes.

Detection of viroids by dot-blot hybridization and RT-PCR-DBH

Full-length cDNAs of HSVd and CEVd were cloned into plasmid vector pTZ57R/T (Fermentas). DIG-labeled cDNA probes were prepared from these plasmid DNAs according to the method described by Li et al (8).
Dot-blot hybridization of 5 µL of 10-fold serial dilutions of PCR product positive control $10^0 – 10^{-4}$ negative control, RT product, and extracted RNA was performed according to carried out the method of Li et al (6, 8) with slight modifcations. Hybridization was at 50°C and the membranes were washed at 60°C. The signals were detected by a chromogenic assay.

Results

We examined whether viroids could be detected from their natural host tissues by dot-blot hybridization RT-PCR, southern blot and RT-PCR-DBH. We then compared the sensitivity of these four methods. 5 µL of 10-fold serial dilutions ($10^0 – 10^{-4}$) of PCR product positive control, negative control, RT product and extracted RNA were spotted and hybridized with DIG-labeled cDNA probes. Each probe hybridized only to its complementary sequence in the amplified products (Fig. 1). As shown in figure 1B, only 100 dilution of HSVd PCR product could be detected by southern blot hybridization. Therefore, RT-PCR was 10-fold more sensitive than southern blot hybridization. However, 10^{-1} dilution of HSVd and 100 CEVd PCR products could be detected by electrophoresis of RT-PCR product (Fig. 1B, E).
Detection of DIG-labeled HSVd and CEVd cDNA products by probe capture hybridization was also 100–1000 more sensitive than detection by agarose gel electrophoresis analysis. Compared with dot-blot hybridization, RT-PCR was more sensitive for detecting viroids in total nucleic acids from infected plant tissues. However, RT-PCR required more highly purified nucleic acids in the case of citrus plants. It is important to adopt the most suitable method that fits to the purpose and the materials (9). Table 1 shows the quality and quantity of nucleic acids required for detecting viroids in their natural host by four methods. This study demonstrated that RT-PCR-DBH was sensitive and specific

Table 1: Comparison of the detection sensitivity of purified HSVd by (A) Electrophoresis, (B) Southern blot hybridization, (C) RT-PCR-DBH.

	10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	Negative control	RT product	Extracted RNA
RT-PCR	++	+	---	---	---	---	---	---
Southern blot	+	---	---	---	---	---	---	---
RT-PCR-DBH	++++	+++	++	+	---	---		

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[DOI: 10.21859/isv.3.1.7]

for the detection of the targeted nucleic acid. RT-PCR-DBH assay for viroid detection have two levels of specificity based on RT-PCR primers and an internal hybridization probe. RT-PCR-DBH assay with the specificity, sensitivity, visual examination, adaptation to automation and speed of the assay, make this an attractive tool for diagnosis of certain plant pathogens. The development of RT-PCR-DBHs for HSVd, CEVd should greatly assist in controlling this pathogen for certification and quarantine programs.

Discussion

In our study, RT-PCR-DBH detected hop stunt viroid (HSVd), Citrus exocortis viroid (CEVd) and additional positive samples which would otherwise have been missed by routine methods. These results highlight the superiority of the sensitivity and specificity of RT-PCR-DBH compared with those of conventional methods.

This paper reports new protocol for detection of citrus viroids that has many advantages compared with conventional methods. This method designated RT-PCR-DBH is a combination of RT-PCR and dot-blot hybridization tests. It is over 1000 folds more sensitive than southern blot and over 100 folds more sensitive than electrophoresis of RT-PCR product (Fig. 1). The technique permits routine viroid detection. It avoids the use of ethidium bromide, a carcinogenic dye, used in electrophoresis of PCR products. Transferring amplified band from electrophoresis gel to nitrocellulose membrane inevitably leads to some loss in the resolving power of gel electrophoresis (3). Potential misinterpretation of results using polyacrylamide or agarose gel electrophoresis analyses is completely avoided by RT-PCR-DBH. Since RT-PCR-DBH was able to detect more positive samples, which would otherwise have been missed by routine methods, we suggest that this method provides a highly sensitive and specific means of diagnostic detection of viroids. RT-PCR-DBH constitutes a more specific and sensitive alternative to conventional methods, making this assay well suited for use in epidemiological studies and beneficial for a disease diagnostic service, that will aid in the production of viroid-free citrus plants for certification programs. Generally, the RT-PCR-DBH assay developed here is a simple, reliable, rapid, sensitive, specific and cost-effective diagnostic for viroids.

Acknowledgement

The authors wish to thank the Council of Center of Excellence and the Iranian Chapter of TWAS (ISMO) for supporting this research.

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