



Molecular Confirmation of Plant Viruses Detected by High-Throughput Sequencing (HTS) in Fruit Trees Using RT-PCR

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Abstract

The aim of the study was to validate the existence of Citrus Exocortis Viroid (CEVd) in *Ficus carica* (fig) tree in the Al-Kufa and Al-Hilla area of Iraq by using a molecular diagnostic method. The first indication of CEVd incidence in symptomatic fig leaf samples was derived from high-throughput sequencing (HTS) data. To confirm these results total RNA were isolated on field samples and followed by reverse transcription and polymerase chain reaction (RT-PCR) with CEVd-specific primers. The RT-PCR findings proved the presence of the viroid in 30% of the studied samples in Al-Kufa and 28% in Al-Hilla. This molecular confirmation does not only confirm the HTS data but also is the first RT-PCR-based detection of CEVd in Iraq fig trees. The findings indicate the importance of systematic monitoring of the molecular background and provide the possible role of CEVd in the emergence of fig mosaic disease in the research areas.

Keywords: CEVd, RT-PCR, High-Throughput Sequencing (HTS), Plant Viroid detection.

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Introduction

Ficus carica (fig) *Ficus carica* (fig) has been regarded as one of the significant fruit-bearing trees in Iraq because of its nutritional value and also its ability to adapt to the local environmental conditions especially in the central and southern part of the country. Fig growing is common in the provinces of Najaf and Babylon, in the region of Al-Kufa and Al-Hilla, which are the locations where fig growing is a major element of local agricultural production. Nevertheless, over the last several years, plant diseases, especially those involving microscopic pathogens, e.g., viruses and viroids have become an issue in fig cultivation (Sarkhosh et al., 2022; Sabanadzovic et al., 2010). One of the new phytopathogens that is likely to give a serious challenge to fig trees is the Citrus Exocortis Viroid (CEVd) which is a species of Pospiviroidae. One feature of CEVd is the exceptionally simple molecular architecture, which is comprised of a small circular, single-stranded RNA molecule lacking a protein coat. This structural simplicity notwithstanding, it has shown a large replication proficiency in host plant cells and causes specific pathological symptoms capable of leading to stunted growth and decreased productivity. Although CEVd has always been linked to citrus and tomato plants, recent reports indicate, that it may also be transmitted into other plants, such as fig trees. This is a growing body of evidence highlighting the necessity of additional studies aimed at the improved comprehension of the host range of the viroid and its possible influence on non-traditional hosts. (Góra-Sochacka et al., 2024). The traditional diagnostic procedures including observing the phenotypic symptoms or the biological culturing would not be very effective because the viroid symptoms can be similar to those of the other diseases or environmental factors. Thus, molecular diagnostic methods, i.e., reverse transcription followed by polymerase chain reaction (RT-PCR), are now counted on because they offer



an accurate and fast method of finding the presence of viroids in plant tissue even in a disease in its initial stages or in cases without symptoms. (Alsharksi et al., 2024). Against this background, this research paper seeks to examine whether Citrus exocortis viroid (CEVd) can be present in fig trees in the areas of Kufa (Najaf Governorate) and Hillah (Babel Governorate) by use of reverse transcription polymerase chain reaction (RT-PCR). The analysis also aims at determining the level of the viroid distribution across these agriculturally important regions. The study will be a first move in the knowledge of CEVd infection prevalence among fig trees in Iraq and will establish a scientific base on the development of preventive measures that will protect fig orchards and hence stability and sustainability of local agricultural production. (Al-Kaeath et al., 2024).

Materials and Methods

Ten fig leaf samples were gathered at the Hillah area, and 10 fig leaf samples at the Kufa area and subjected to RNA extraction and RT-PCR analysis.

RNA Purification Protocol Procedure

Sample Preparation

- a) Fresh or frozen plant tissue was weighed and then frozen in liquid nitrogen up to 100mg. The material was centrifuged into a 1.5 or 2 ml centrifuge tube after fine powdering. 10 μ l of β -mercaptoethanol, 100 μ l of PVR solution, and 1 ml of PVR solution were added. Following mixing, a Thermomixer was used to incubate the sample for 10 minutes at 70°C. The sample was centrifuged for five minutes at 14,000 to 16,000 $\times$ g after incubation.
- b) A bead beater was filled with up to 100 mg of frozen or fresh plant tissue. After adding 1 milliliter of PVR solution, 100 microliters of PVR solution, and 10 microliters of β -mercaptoethanol to the sample, the combination was well mixed. After being moved to a 1.5 or 2 ml centrifuge tube, the sample was incubated for 10 minutes at 70°C with stirring every three minutes or with a Thermomixer. Following incubation, the sample was centrifuged for five minutes at 14,000–16,000 $\times$ g.
- c) Fresh or frozen plant tissue weighing up to 100 mg was put into a homogenization bag. After adding 20 μ l of β -mercaptoethanol and 2 ml of PVR solution, the material was well combined until it was homogenized. Next, 100 μ L of PVR solution was added to a 1.5 or 2 mL centrifuge tube containing 1 mL of the homogenized mixture. The next step was to use a Thermomixer or incubate at 70°C for 10 minutes, stirring occasionally every three minutes. Following incubation, the sample was centrifuged for five minutes at 14,000–16,000 $\times$ g.

RNA Binding

A clean 1.5 or 2 mL centrifuge tube was filled with 450 μ L of the supernatant. After that, 225 μ L of 95–100% ethanol was added.

The liquid with ethanol added was poured onto the PV column, which was then set inside a 2 mL collecting tube.

For one minute, centrifugation was carried out at 14,000–16,000 $\times$ g. After centrifugation, the centrifugation period could be increased until full passage of the material through the PV column membrane was obtained.

The PV column was moved to a fresh collection tube, and the effluent was disposed of. Option 1 for DNA removal: 100 μ L of DNase I enzyme (at a concentration of 2 KU/ml) was added after being dissolved in the proper reaction buffer (for example, 50 mM Tris-HCl pH 7.5, 10 mM MnCl₂, and 50 μ g/ml BSA at 25°C or using the enzyme's solution). For ten minutes, the sample was allowed to react at room temperature.



RNA Wash

1. Add 400 µl of W1 Buffer into the center of the PV Column then centrifuge at 14-16,000 x g for 1 minute.
2. Discard the flow-through then place the PV Column back in the 2 ml Collection Tube.
3. Add 600 µl of Wash Buffer (make sure ethanol was added) to the CENTER of the PV Column.
4. Centrifuge at 14-16,000 x g for 1 minute then discard the flow-through and place the PV Column back in the 2 ml Collection Tube.
5. Add 600 µl of Wash Buffer (make sure ethanol was added) to the CENTER of the PV Column.
6. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through and place the PV Column back in the 2 ml Collection Tube.
7. Centrifuge at 14-16,000 x g for 2 minutes to dry the column matrix.

RNA Elution

1. Place the dried PV Column in a clean 1.5 ml microcentrifuge tube (RNase-free).
2. Add 50 µl of RNase-free Water or TE (RNase-free) to the CENTER of the column matrix.
3. Let stand for 2 minutes or until the RNase-free Water or TE (RNase-free) is absorbed completely by the matrix.
4. Centrifuge at 14-16,000 x g for 1 minute to elute the purified RNA

Preparation of Buffers, Solutions

All the buffers and solutions were prepared according to Sam brook and Russell, 2001.

Table 1. Oligonucleotide Primers Sequence Used for RT. PCR Amplification of Specific Gene

Primer's name	Primer's sequences 5' _____ 3'	PCR products	Reference
CEVd-F1	ACTGCCGGTGATAAACCGGA	310bp	AL KAEATH <i>et al.</i> , 2024
CEVd-F1	ACTGCCAGTGATATACTGGA		

Preparation of PCR primers

The primers are prepared depending on the manufacturing instruction by dissolving the lyophilized primers with TE (Tris-EDTA) buffer to make stock solution of concentration of 100 pmole/mL.

RNA Electrophoresis

Gel Electrophoresis for PCR Product

Agarose gel electrophoresis was performed on the amplified products of several genes using a standardized procedure that was modified from Sambrook and Russell (2001). Using a water bath kept at 100°C for about 15 minutes, agarose was first dissolved in 1X TBE buffer to create a 2% (w/v) agarose gel. After allowing the solution to cool to between 50 and 60 degrees Celsius, 2 µL of ethidium bromide (EtBr) was added to the molten gel to help see the DNA.

After pouring the gel mixture into a casting tray with a comb positioned correctly to create wells, the gel was allowed to set for fifteen minutes at room temperature. Ten microliters of each PCR result were poured into separate wells after the comb was carefully removed once it had set. Additionally, as a molecular size marker, 5 µL of a 1500 bp DNA ladder was placed into a different well.



After that, the gel was put in an electrophoresis chamber containing 1X TBE buffer inside of it. To improve resolution, an electric current of 110 volts was applied for 30 minutes during the electrophoretic separation process, and then 75 volts for another 60 minutes. Lastly, a transilluminator device was used to see DNA bands under ultraviolet (UV) light.

RT-PCR Procedure

To simplify reaction setup and lower the risk of contamination, One-Step RT-PCR combines PCR and first-strand cDNA synthesis in the same tube. One-Step RT-PCR requires only gene-specific primers. The kit contains TransScript® II RT and TransTaq® HiFi DNA Polymerase. fragment amplification up to 8 kb. Table (2).

Table 2. RT-PCR Procedure Components

Component	AH411-02 (200 rxns)
TransScript® II One-Step Enzyme Mix	80 µl
2×One-Step Reaction Mix	2×1 ml
RNase-free Water	2×1 ml

Table 3. Reaction Components

Component	volume	Final concentration
Total RNA	1pg~1µg	As required
Forward Primer	0.4µl	0.2µl
Reverse Primer	0.4µl	0.2µl
2x One-step Reaction Mix	10µl	1x
TransScript® II One-Step Enzyme Mix	0.4µl	-
RNase-free Water	Variable Up To 20µl	-
Total volume	20 µl	-

PCR Thermocycling Condition

Thermal cycling conditions for the amplification of each target gene were performed using a conventional PCR thermocycler, as detailed in Table (4). Following the preparation of the PCR master mix, the reaction components were transferred into standard PCR tubes pre-loaded with lyophilized RT-PCR reagents. These lyophilized formulations contained all essential elements required for successful amplification, including reverse transcriptase enzyme, reaction buffer, specific primers, and the RNA template. The tubes were then briefly mixed and centrifuged using an Exispin vortex centrifuge for 3 minutes to ensure proper homogenization. Subsequently, the tubes were placed into the thermocycler to initiate the amplification process.

Table 4. ONE STEP RT-PCR Conditions

PCR step	Temperature	Time	Repeat cycle
cDNA synthesis	45 °C	5 min	1
Initial Denaturation	94 °C	4 min	1
Denaturation	94 °C	30 sec	40
Annealing	50-60 °C	30 sec	
Extension	72°C	1 min	
Final Extension	72°C	7min	1
Hold	4°C	∞	



Results

In this study, a collection of plant samples was obtained from fig tree (*Ficus carica*) leaves across two major agricultural regions in Iraq: Al-Kufa in Najaf Province and Al-Hilla in Babylon Province. The sampling included both symptomatic specimens—exhibiting fig mosaic-like patterns—and asymptomatic ones. This work was conducted during the current growing season with the aim of detecting the presence of Citrus Exocortis Viroid (CEVd) using the reverse transcription polymerase chain reaction (RT-PCR) technique.



Figure 1. Fig Leaves Collected from the Hillah and Kufa Regions in Southern Iraq Show Symptoms Similar to Those of Fig Mosaic Disease



All samples underwent total RNA extraction using a plant tissue-specific extraction protocol, followed by reverse transcription to synthesize complementary DNA (cDNA). Subsequently, a PCR reaction was performed using specific primers targeting the CEVd sequence. Figure 2 shows the CEVd product, which is 310 base pairs in length, amplified using the TM primer at an annealing temperature of 59°C (Figure 3).

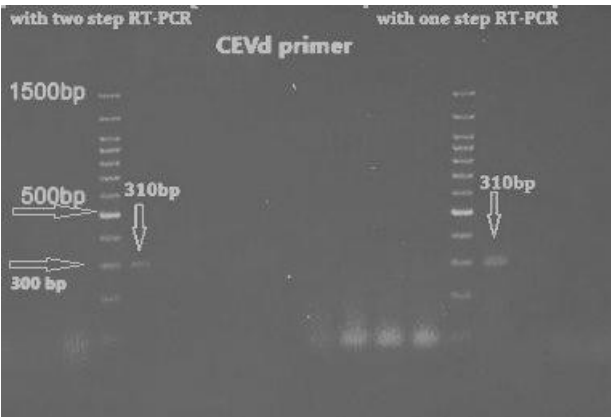


Figure 2. Gel Electrophoresis was Performed to Optimize the PCR Product of Primer Set (CEVd) at Various Temperatures

CEVd exhibited a band of 310 bp. The gel was prepared using a 2% agarose gel and electrophoresed at 110 volts for 15 minutes, followed by a reduction to 75 volts for an additional 60 minutes. Subsequently, the gel was visualized under UV light after staining with Ethidium bromide. Lane L contained a DNA ladder ranging from 100 to 1500 bp.

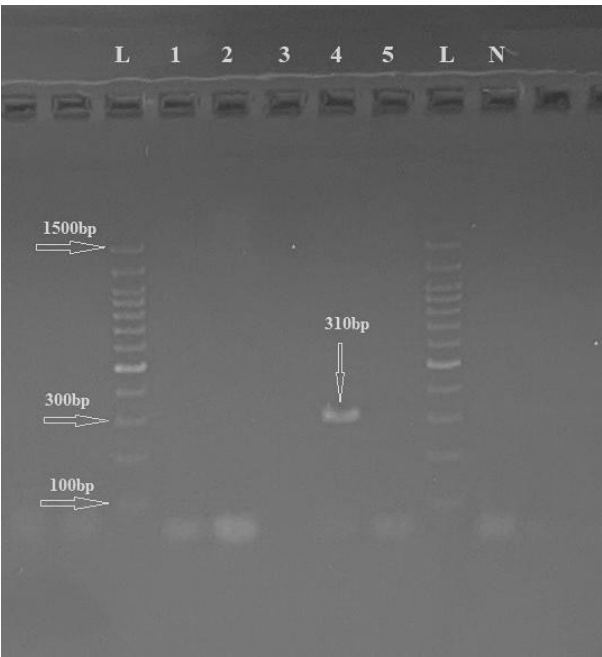


Figure 3. Electrophoretic Visualization of PCR Amplicons Using CEVd-Specific Primers

Agarose gel electrophoresis analysis of PCR products amplified with CEVd-specific primers targeting a 310 bp fragment. Amplification was performed with an annealing temperature of 59°C. The separation was conducted on a 1.5% agarose gel for 15 minutes at 110 V, followed by 60 minutes at 75 V. DNA bands were visualized under ultraviolet light after staining with ethidium bromide. Lane L contains the DNA size marker (ranging from 100 to 1500 bp). Lanes 4 and 5 exhibit positive amplification signals, while lanes 1 to 3 show no detectable bands, indicating negative results. Lane N corresponds to the negative control reaction.

Molecular amplification assays (RT-PCR) revealed that approximately 30% of the samples collected from the Kufa region tested positive for the presence of the viroid, with 6 out of 20 analyzed specimens exhibiting amplification signals. In comparison, the infection rate in the Hilla region was estimated at 28%, as viroid-specific sequences were identified in 5 out of 18 tested samples (Table 5).

Table 5. Number of Samples Examined and the Number of Samples Infected with CEVd Viroid Using the RT-PCR Technique

Geographical Area	Number of Samples	Infected Samples	Infection percentage
Hillah	18	5	% 27.5
Kufa	20	6	%30

The positive results were validated through agarose gel electrophoresis, which revealed a distinct amplification band corresponding to the expected size of the CEVd sequence. This provides clear evidence of the viroid's genetic material in the analyzed samples. These findings represent a significant indication of CEVd presence in Iraqi fig orchards, even though some infected samples exhibited mild or non-specific symptoms. This highlights the significance of molecular diagnostics that is more effective as a tool than using visual field tests alone. Interestingly, some positive samples were found in trees that showed no clear symptoms like stunting, or cracks in the stems an indication that there are latent infections or emergence of the disease. These findings are in line with the result of Negahban et al. (2024) and Al Kaeath et al. (2024).

It should be mentioned that the current paper is the first scientific report where RT-PCR was used to identify CEVd in fig plants in Iraq. The above reports have largely been founded on the high-throughput sequencing (NGS) technologies that although highly accurate is generally costly and labor intensive to regularly implement in a diagnostic setting (Al Kaeath et al., 2024). Introduction of RTPCR in this research is a tremendous step towards development of feasible diagnostic guidelines. These protocols might also be embedded in the agricultural surveillance and disease control initiatives, part of which Kuan Wu highlighted in 2024.

Discussion

The results that were achieved in this research prove the real presence of the Citrus Exocortis Viroid (CEVd) infection in fig trees (*Ficus carica*) at Kufa and Hilla regions with the rates of infection being 30% and 28 respectively. This finding is quite important not only because of the established presence of CEVd in Iraqi fig orchards, but also because it is the first case of the viroid in Iraq detected with RT-PCR. This makes RT-PCR a fast and dependable molecular diagnostic device. These results follow the same findings of Al Kaeath et al. (2024), which used next-generation sequencing (NGS) in their study in the same year.

The current results support the presence of the viroid in the Iraqi agricultural setting when compared with previous investigations that applied NGS in the detection of CEVd. Nevertheless, this paper also shows that RT-PCR presents a viable and cost-efficient substitute to viroid monitoring, especially in regular surveys and diagnostics laboratories of small scale. The capability of RT-PCR to uniquely detect infections in any plant



sample that has no symptoms highlights the importance of the method in the early detection and management of diseases.

Remarkably, the viroid was observed in trees with pronounced symptoms as well as seemingly healthy trees. This is indicative of the latent infections or low viroid titers that cannot be detected using field observations only. These findings demonstrate the drawbacks of visual diagnosis and support the need to use the method of molecular diagnosis in plant health monitoring programs.

The incidence of CEVd in an economically important crop like fig can have long term effects on the fruit quality and general productivity particularly in the absence of a successful preventive control. The possible infections sources are the traditional propagation methods, non-sterilized farming equipment (e.g., pruning machinery), and planting of infected seedlings.

In line with this, the findings of this paper go beyond a simple identification; it highlights the essence of the national framework that incorporates the early diagnosis tactics, regular monitoring, and adoption of adequate farming practices. This is particularly important because the cultivation of figs still goes forth in different parts of Iraq.

Conclusions

The sample of figs in Kufa and Hilla was tested by RT-PCR that identified Citrus Exocortis Viroid (CEVd), the first use in Iraq of this molecular method to identify a viroid. These disease rates of Kufa and Hilla were 30 percent and 28 percent, and show that the viroid is not a solitary organism, but present in the general agricultural environment. Even RT-PCR could be used to detect the disease in the plants that were asymptomatic hence a practical method in the diagnosis of the disease at the initial stages. This highlights the importance of attacking source such as propagation material that is already contaminated and instruments that are not sterilized that can be contributing to the spread of viroid between trees. Through these findings, this research proposes RT-PCR to be used as a national program of health surveillance diagnostic tool. The bigger parts of the agriculture areas should also be surveyed regularly to get to know more about the spreading of the viroid and where infection hot spots are located. They are also to suggest the establishment of a national viroid infection database of the horticultural crops as so to inform the decision making process and to guide the strategic disease management undertakings.

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