

Detection and partial sequence identification of grapevine leafroll-associated virus-1 in Egypt

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ABSTRACT

Grapevine leafroll-associated virus 1 (GLRaV-1) was detected in the grapevine plants collected from different cultivated areas in Egypt and tested using different serological and molecular tools. Double-antibody sandwich ELISA (DAS-ELISA) was successfully carried out using GLRaV-1 polyclonal antibodies to detect infected plants. PCR with primers designed at the heat shock protein 70 (HSP70) gene region, a fragment of 271 bp of GLRaV-1, was used. Molecular hybridization with non-radioactive probes was used to detect the presence of virus particles. The partial sequence of HSP70 fragment from the Egyptian isolate of GLRaV-1 was performed and showed high identity (95%) with the Australian isolate of GLRaV-1 sequence. The molecular methods used for viral diagnosis showed a higher sensitivity in the detection of GLRaV-1 compared to DAS-ELISA. These procedures may serve as an alternative method for GLRaV-1 detection, due to the weak sensitivity of ELISA test to differentiate between the different isolates.

Key words: Grapevine, GLRaV-1, ELISA, RT-PCR, sequence align, non-radioactive, labelling

INTRODUCTION

Grapevine (*Vitis vinifera* L.) is economically one of the most important cultivated fruit species in the world, not only because of the wine industry, but also due to the demand for fresh and dried fruits (Vivier and Pretorius, 2002). In Egypt, the area devoted for grapevine plantation in 2004 was about 65,000 hectares, which yielded 1.104.000 tons. Part of the crop is consumed locally as a fresh product, and the rest is processed and exported (FAO 2004 Statistical data). The most important governorates cultivating grape are Behera, Qualubia, Minufia, Giza and Beni-Swef. Grapes are attacked by some important virus diseases such as grapevine fanleaf virus

(GFLV), grapevine fleck virus (GFkV), grapevine virus A (GVA), grapevine virus B (GVB), and grapevine leafroll-associated viruses. Leafroll is a damaging disease of the grapevine causing yield loss of up to 40% (Woodham *et al.*, 1984). Grapevine leafroll-associated virus 1 (GLRaV-1) is one of the most important types (Martelli *et al.*, 1997). It is characterized by a down rolling of leaves and a reddening or a yellowing of limbs and its occurrence causes significant reduction in crop quantity and quality (Bovey *et al.*, 1980 and Woodham *et al.*, 1984). Leafroll is a worldwide graft-transmitted virus disease which adversely affects grape yield and fruit quality. The genome of GLRaV-1 was cloned and sequenced by Fazeli & Rezaian (2000).

They reported that it contains 10 major open reading frames (ORFs) and a non-coding region lacking a poly (A) tract. They identified the functions of 7 ORFs, among them ORF 3 which encodes a homologue of the HSP70 family of heat shock proteins. At least, seven serologically distinct closteroviruses have been isolated from leafroll diseased vines and designated as grapevine leafroll-associated closteroviruses-1 to -7 (Hu *et al.*, 1990; Zimmermann *et al.*, 1990; Gugerli and Ramel, 1993; Belli *et al.*, 1995; Choueiri *et al.*, 1996).

ELISA method can be used for testing multiple plants for a single virus using one well per plant sample, or alternatively a single plant can be simultaneously tested for many viruses on a single plate with different antibodies coated to each well in duplicate or triplicate for reproducibility (Craig *et al.*, 2004). PCR technology permits the detection of viruses at levels several orders of magnitude lower than is possible by other methods. This high sensitivity facilitates virus detection during early stages of infection of plants, in soil and in their vector samples (Fenby *et al.*, 1995). RT-PCR and PCR are popular techniques for detection and identification of RNA and DNA plant viruses, respectively. The procedures are extremely sensitive, fairly inexpensive and require minimal skill to perform. In the case of RNA viruses, a cDNA strand complementary to the virus is made with reverse transcriptase (RT). Oligonucleotide primers, flanking part of the genome of the virus, are extended by a thermo stable DNA polymerase in a series of denaturation and extension steps that exponentially increase the target DNA. For DNA viruses, the RT step is unnecessary (Craig *et al.*, 2004). Reverse transcriptase-polymerase chain reaction (RT-PCR) is a very sensitive and reliable molecular methodology which have been developed and used for the detection of many plant RNA viruses (viruses

which contain RNA as their genome constituent, which include all known viruses in grapevine) (Rowhani and Golino, 1995; Rowhani *et al.*, 2000). Nucleic acid hybridization of DNA or RNA probes has the advantage of being able to detect the nucleic acid of the virus in both forms; single and double-stranded. cRNA probes are preferable than cDNA probes when used in detecting RNA viruses, because RNA/RNA hybrids are more stable than DNA/RNA hybrids. Hybridization with digoxigenin-labelled cDNA permits the most sensitive detection of PCR products and is the most appropriate method for routine diagnosis (Fenby *et al.*, 1995 and Craig *et al.*, 2004). In this study, we developed an RT-PCR detection methodology and nucleic acid hybridization for the detection of GLRaV-I, in addition to partial sequence identification of the Egyptian isolate of GLRaV-I.

MATERIALS AND METHODS

Source of virus isolate and ELISA test

Field surveys were carried out in commercial grape orchards in different locations in Egypt. Leaf samples showed various types of virus and virus-like symptoms related to GLRaV and random samples from symptomless plants around them were collected from different areas for laboratory testing. Double antibody sandwich-enzyme linked immunosorbent assay (DAS-ELISA) was made according to Clark and Adams (1977). Using polyclonal antibodies against grapevine viruses (Agritest S.r.l., Italy). All samples, whose absorbance values were at least twice the absorbance values of the healthy control, were considered positive. Positive reactions were confirmed by using the polymerase chain reaction (PCR) with primers specific for the virus. The virus was isolated and maintained on grape (*Vitis vinifera* cv.

Tomson seedless) by graft transmission under standard greenhouse conditions.

Extraction of viral RNA

RNA was extracted from either frozen or fresh samples using RNeasy Plant Mini Kit (Qiagen, Inc). One hundred mg of tissue were ground under liquid nitrogen to a fine powder using a mortar and pestle, and then transferred to a sterile microfuge tube. Total RNA was extracted from each sample as described by the manufacturer.

Reverse transcription – polymerase chain reaction (RT-PCR)

RT-PCR was done using the One-Step RT-PCR Kit (Qiagen, Inc.). Following this protocol reverse transcription and PCR are carried out sequentially in the same tube. The protocol has been optimized for 1 pg – 2 µg of total RNA. Optimal reaction conditions, such as incubation times and temperatures during PCR amplification, were determined. The master mix typically contains all the components required for RT-PCR except the template RNA. The mix was prepared in a thin-walled 0.2 µl PCR tube by combining 10 µl of 5x QIAGEN OneStep RT-PCR buffer, 2.0 µl of dNTP (10 mM), 1 µl of each primer (GLRaV 1f, 5'-CATCGCAAGATGAGTCTGGG-3' and GLRaV 1r, 5'-TTCACATTGCCACGCTGCC-3' designed for GLRaV1 at the HSP70 ORF (Sefc *et al.*, 2000) to a final concentration of 0.6 µM, 2.0 µl of QIAGEN OneStep RT-PCR Enzyme Mix (Omniscript™ Reverse Transcriptase, Sensiscript™ Reverse Transc-riptase and HotStartTaq™ DNA Polymerase). To this mix, 5 µl of total RNA was added and RNase-free water was added to a final volume of 50 µl. First strand cDNA synthesis was done using the following parameters: 50°C for 30 min (1 cycle) to allow reverse transcription to be performed. Second strand cDNA synthesis and PCR amplification were done in the same

tube using DNA thermal cycler (Apollo ATC 401, CLP, Inc., USA) with the following parameters: 95°C for 15 min (1 cycle) to activate HotStarTaq DNA Polymerase, denaturation at 94°C for 45 sec, primer annealing at 60°C for 45 sec and extension at 72°C for 1 min. (40 cycles) with a final extension at 72°C for 10 min.

Electrophoresis

Five-microliters aliquots of the PCR product were analyzed on 1% agarose gel (6 x 8 cm), in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volt. The 1 Kb DNA Ladder molecular weight standard (GiBcoBRL, Inc.) was used to determine the size of RT-PCR products. Gels were stained with ethidium bromide (10 µg / ml) according to Sambrook *et al.* (1989), visualized under UV transilluminator and photographed using Gel Documentation System (AlphaInnotech Corporation).

Cloning and sequencing PCR products

The amplified PCR fragment was recovered from the agarose gel using the QIAquick Gel Extraction kit (Qiagen Inc.). Eluted PCR products were then cloned into pGEM-T (Promega Corp., Madison, WI) and transformed into *Escherichia coli* DH5α (max efficiency; Gibco BRL). Selected clones of GLRaV-1 were subjected to DNA sequencing by an automated sequencing facility / Center of Biotechnology, University of Florida by ABI Prism 377 Genetic Analyzer (Perkin-Elmer) following the manufacturer's instructions. Database searching of similarity to any nucleotide sequences found in that database were conducted by BLAST search programs of the National Center for Biotechnology Information (NCBI). The nucleotide sequences of other closteroviruses were obtained through the Entrez program at the NCBI and compared to other closterovirus sequences available in the Genbank using DNAMAN Sequence

Analysis Software (Lynnon Biosoft Quebec, Canada).

Probe preparation and dot blot hybridization

The cloned viral fragment was labeled by Digoxigenin non-radioactive labeling system (Roche, Boehringer Mannheim, Indianapolis) using PCR method. The PCR reaction was performed in 50 µl total volume containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01 % gelatin, 1 ng GLRaV-1 cDNA, 1X dNTPs labeling mixture (10 mM dATP, 10 mM dCTP, 10 mM dGTP, and 6.5 mM dTTP, 3.5 mM Dig-11dUTP, in Tris-HCl, pH 7.5), 1 µM of each of GLRav-1f and GLRaV-1r primers and 1.25 U *Taq* DNA polymerase and then the volume was completed to 50 µl with nuclease-free water. The PCR reagents were purchased from Roche (Boehringer Mannheim, Indianapolis). The PCR parameters were 40 cycles of denaturation at 94°C 45 sec, annealing at 60 °C for 45 sec and extension at 72 °C for 1 min. Total clarified sap preparations from infected plant materials were diluted ten-fold with 6X SSC buffer, heated to 95°C for 10 min and chilled on ice. A volume of 5 µl total RNA extracted from each plant sample was directly applied to nitrocellulose membrane (Roche). Membranes were air-dried and the nucleic acids were cross-linked to the membrane by exposure to UV irradiation from a transilluminator for 3 min between 2,500 and 10,000 µl/cm². Prehybridization, hybridization with single digoxigenin labeled probe and color detection with NBT and BCIP reagents as substrates were performed as recommended by the manufacturer (Roche, Boehringer Mannheim, Indianapolis).

RESULTS AND DISCUSSION

In order to identify the presence of virus infection in grapevine, samples were taken

from young affected plants in early April and were diagnosed using enzyme-linked immunosorbent assay (ELISA), RT-PCR and nucleic acid hybridization methods. In ELISA test, polyclonal antibodies specific to grapevine leafroll-associated virus 1 (GLRaV 1) were used for a total of 1,160 grapevine samples (representing 8 different cultivars and rootstocks) collected from different governorates in Egypt. Data summarised in Table 1 showed that closterovirus GLRaV 1 was found on cultivars Flam seedless and Thompson Seedless at Governorates Qualubia and Beni Swef, respectively.

RT-PCR was performed on total RNA extracted from 100 mg leaf tissue by the RNeasy Plant Mini Kit (Qiagen, Inc.). According to the designed primers, an amplification product of 271 bp was expected. The reaction yielded one cDNA fragment that was consistent with the anticipated size. Fig. (1) indicates the amplified products obtained from grapevine leaves collected from different location using RT-PCR. Most of the ELISA-positive samples from Beni swef and Qualubia governorate were able to amplify the expected size fragment (Lane 2). This product was not detected in samples collected from other locations. These results indicated the presence of GLRaV-1 in Egypt and demonstrated the success of RT-PCR methods to directly detect the GLRaV-1 in infected grapevine tissues. The specificity of the amplified RT-PCR fragment from viral genome is based on fragments detected from infected grapevine tissue but not from uninfected tissue (Habibi *et al.*, 1997) and can be used as a sensitive diagnostic tool. The rapid amplification of grapevine viruses cDNA transcribed *in vitro* from extracts of infected tissue is now possible with *Taq* DNA polymerase and viral specific primers in an RT-PCR assay (Minafra and Hadidi, 1994; Zhu *et al.*, 1998).

Table (1): Serological detection of different grapevine cultivars collected from different locations in Egypt using specific polyclonal antibody for GLRaV-1 in ELISA test.

Cultivars	Governorates	No. of tested samples	No. of infected samples	% of infection
Superior	Behera	150	0	0
	Giza	90	0	0
Flam seedless	Qualubia	90	35	38.8
	Giza	90	0	0
Thompson seedless	Behera	110	0	0
	Giza	60	0	0
	Beni Swef	120	60	50
Romy Ahmer	Beni Swef	110	0	0
Harmony (rootstock)	Beni Swef	50	0	0
Freedom (rootstock)	Behera	50	0	0
LN33 (rootstock)	Beni Swef	110	0	0
Dog Ridge (rootstock)	Beni Swef	130	0	0
Mean		1160	95	0.81

Fig. (1): Gel electrophoresis on RT-PCR amplification of a fragment from GLRaV-1 genome using specific primer pair designed to amplify 271 bp fragment of HSP70 gene. Lane M represent 1-kb DNA ladder (GIBCOBRL). Lanes 1 to 4 were loaded from different vine tissues infected with GLRaV-1 and processed with the RNeasy Plant Mini Kit (Qiagene, Inc). Lane 5, a healthy tissue control via RNeasy Mini kit. Lane 6, a water control.

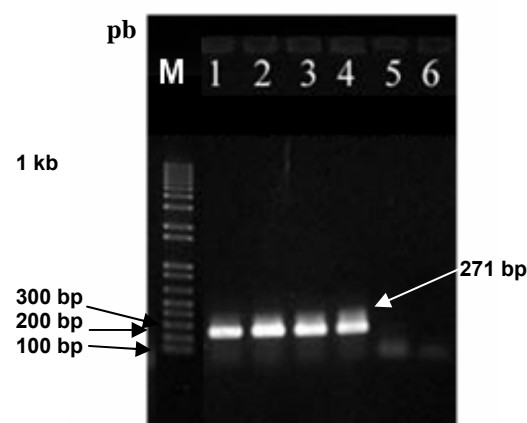
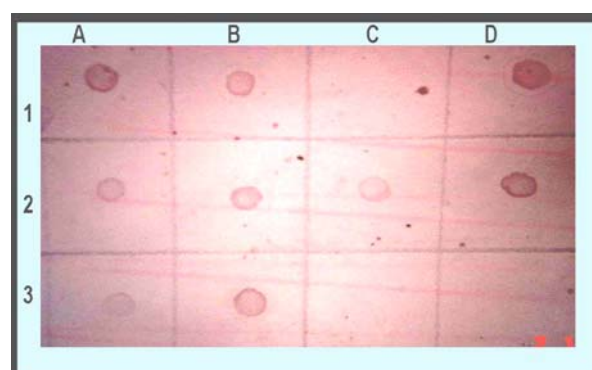


Fig. (2): Dot blot hybridization with GLRaV-1 DIG-cDNA probe of total RNA extracted from infected and uninfected grapevine samples. Row 1 (A, B, C & D) represent samples collected from Beni Swef governorate and Row 2 (A, B, C & D) represents samples collected from Qualubia governorate. Row 3 (A & B) grape represent infected samples used as positive control and no hybridization reaction was observed with uninfected samples Row 3 (C & D).



The nonradioactive labeled cDNA probe was prepared using primer specific for GLRaV-1 by PCR method. Dot blot hybridization with GLRaV-1 cDNA probe was used to detect the virus in infected plant materials. Fig. (2) showed a moderate reaction (blue signal) resulted using dot blot hybridization of DIG labeled probe with nucleic acids extracts collected from Beni swef (Thomson cultivar, Row 1: A, B, C and D) and Qualubia governorate (Flame cultivar, Row 2 : A, B, C and D). Hybridization was not observed between the probe and uninfected plant tissue (Row 3: C and D).

Although polyclonal and monoclonal antibodies have been raised to several filamentous phloem limited viruses (La Notte *et al.*, 1997), the erratic distribution and low concentration of the viruses in the host tissues, do not always enable their satisfactory and reproducible detection. On the other hand, the use of radioactive labeled (Minafra *et al.*, 1992; Saldarelli *et al.*, 1994 b) or digoxigenin labeled (Saldarelli *et al.*, 1994 a) molecular probes requires extensive manipulation of the samples for the removal of inhibiting compounds. RT-PCR, that was able to detect RNAs of grapevine trichoviruses A and B (GVA and GVB), and GLRaV-3 in femtogram amounts (Minafra *et al.*, 1992b; Minafra and Hadidi, 1994; Chevalier *et al.*, 1993), requires an extraction or immunocapture step that makes the procedure somewhat laborious.

Sequence alignment of GLRaV-1 derived sequence with previously published GLRaV HSP70 genes is shown in (Fig. 3). The phylogenetic tree for partial sequence of

HSP70 fragment (about 271 nucleotides) from the Egyptian isolate of GLRaV-1 showed high degree of homology with the GLRaV-1 sequence reported by Fazeli *et al.* (2000) (Australia Accession no. AF195822) with (95%) identity, followed by 92% identity with the sequence reported by Sefc *et al.* (2000) (Austria Accession no. AJ404738), Saldarelli *et al.* (1998) (Italy Accession no. Y15891), and Good and Monis (2000) with identity (91%) (USA Accession no. AF233935) (Fig. 4). It was concluded, from the putative GLRaV-1 sequence reported by Fazeli *et al.* (2000), which is similar to the Egyptian GLRaV-1 sequence with 95% identity, that both viruses are closely related.

It could be concluded from the obtained results that RT-PCR and nucleic acids hybridization meet the most important criteria of the reliable diagnostic system, because grapevine leaf roll is readily spread from infected to healthy grapevine through graft inoculation. In addition to bench grafting, field grafting and budding provide several means for the introduction of the disease into grapevine and like other viruses that affect grapevines, then the only control strategy is to ensure that healthy, certified virus-free propagation materials must be used to plant or graft-over grapevine orchards. Finally, the successful use of RT-PCR and nucleic acids hybridization to directly detect GLRaV1 in infected grapevine tissues were proven and results revealed the great sensitivity and specificity of PCR techniques with this respect.

Egypt	CATCGCAAGATGAGTCTGGGTGCGTGTCTGGTGAATTCTCCGTTTCGTTCCAACCGGAAA	60
Austria	CATCGCAAGATGAGTCTGGGTGTGTATCGTTGGTGAATTCTCCGTTTCGTTCCAACGCAGA	60
USA	CCACGCAAGATGATTCCGGATGTGTGTCTGGTGAATTCTCCGTTTCGTTCCAACGCAGA	60
Australia F	CCACGCAAGAAGATTCTGGGTGCGTGTCTGGTGAATTCTCCGTTTCGTTCCAACCGGAAA	60
Italy	CATCGCAAGATGATTCTGGGTGTGTATCGTTGGTGAATTCTCCGTTTCGTTCCAACGCAGA	60
Consensus	catcgcaagatgattctgggtgtgtctggtgaattctccgtttcgttccaacgcaga	
Egypt	TTTTTATAGGAAGCGACATGACGTACAGCATTGGTTCACAGAGCTTCTCCGATTTTGTGG	120
Austria	TTTTTATAGGAAGCGATGACGTATGACATTGGTTCACAGAGCTTACTCCGATTTCTGTAG	120
USA	TTTTTATAGGAAGCGACATGACGTACAGCATTGGTTCACAGAGCTTACTCCGATTTCTATGG	120
Australia F	TTTTTATAGGAAGCGACATGACGTACAGCATTGGTTCACAGAGCTTACTCCGATTTTGTGG	120
Italy	TTTTTATAGGAAGCGACATGACGTATGACATTGGTTCACAGAGCTTACTCCGATTTTGTAG	120
Consensus	tttttataggaagcgacatgacgtacagcattgg cacagagcttactccgattttgtgg	
Egypt	CTGGCAAACCGAGGTGGACTTTACATTAATCCGAAACGTTGGGTAGGTCTCGATAGCTTTA	180
Austria	CTGGCAAACCGGGGATTTTATCAATCCGAAACGTTGGGTGGGATCGACAGCTATA	180
USA	CTGGCAAACCGAGGTGGACTTTACATCAATCCGAAACGTTGGGTGGTGTGATAGCTTTA	180
Australia F	CTGGCAAACCGAGGTGGACTTTACATTAATCCGAAACGTTGGGTAGGTGTGATAGCTTTA	180
Italy	CTGGCAAACCGGGGACTTTTATCAATCCGAAACGTTGGGTAGGTCTCGACAGCTATA	180
Consensus	ctggcaaac ccggtggactttacatcaatccgaaacgttgggttaggtgtcgatagcttta	
Egypt	ACTTTCATGCTGTTAAGCGAAAGCTGAATCCGGAATACGAAGTTAAAATAAATAATGGCG	240
Austria	ACTTTCAGGCTGTGAAGCGAAAGCTGAATCCAGAATACGAAGTTAAAATGAGGATGGCG	240
USA	ACTTTCAGGCTGTTAAGCGAAAGCTGAATCCAGAATACGAAGTTAAAATAAATAATGGCG	240
Australia F	ACTTTCATGCTGTTAAGCGAAAGCTGAATCCGGAATACGAAGTTAAAATAAATAATGGCG	240
Italy	ACTTTCAGGCTGTGAAGCGAAAGCTGAATCCAGAATACGAAGTTAAAATGAGGATGGCG	240
Consensus	actttcaggctgttaagcgaagctgaatccagaatacgaagttaaa taataatggcg	
Egypt	AAATTTCTATAGGCAGCGTGGGCAATGTGAA	271
Austria	AAATCGTCTTATAGGCAGCGTGGGCAATGTGAA	271
USA	AAATTTCTATAGGCAGCGTGGGTAATCGGAA	271
Australia F	AAATTTCTATAGGCAGCGTGGGTAATCGGAA	271
Italy	AAATCGTCTTATAGGCAGCGTGGGCAATGTGAA	271
Consensus	aaatcttataggcagcgtgggcaatgtgaa	

Fig. (3): Sequence alignment among 5 different isolates of GLRaV-1 in which 4 of them represent sequences published in the GenBank. Consensus sequence indicates incomplete match among sequences. A total of 271 nucleotide (nt) from each isolate are compared.

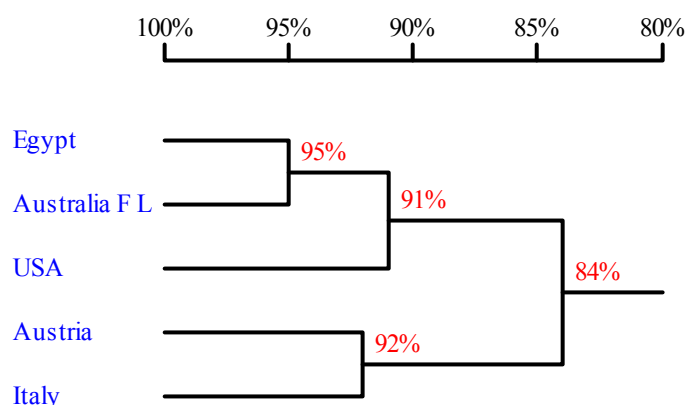


Fig. (4): Consensus phylogenetic tree constructed from the multiple alignment of the HSP70 region sequences of several major GLRaV-1 isolates, including GLRaV-1 from Egypt. Values at the nodes indicate significance in bootstrap analysis.

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الملخص العربي**تشخيص الإصابة و دراسة جزيئية للتتابع النيوتيدى لفيروس التفاف أوراق العنب السلالة 1
فى شجيرات العنب فى مصر**

تم تشخيص الإصابة بفيروس التفاف أوراق العنب السلالة 1 فى شجيرات العنب و التى جمعت من مناطق مختلفة مزروعة فى مصر باستخدام اختبار الاليزا واختبار الاستنساخ العكسى و تفاعل البلمرة المتسلسل و طريقة التهجين غير المشع للحمض النووى و كذلك تم دراسة التتابع النيوتيدى لجزء من الجينوم الخاص بالفيروس. و اظهرت دراسة التتابع ان العزله المصرية تتشابه مع عزلة استرالية فى الجزء من الجينوم تحت دراسته بنسبة 95% كما اثبتت الدراسة حساسية طرق تفاعل البلمرة المتسلسل و تهجين الحامض النووى فى الكشف عن الفيروس بالمقارنه بطريقة الاليزا. و هذه الطرق يمكن استخدامها بكفاءة عالية فى الكشف عن فيروسات العنب فى مصر.