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Original Article

Diagnosis and molecular characterization of three allexiviruses infecting garlic crop in Saudi Arabia

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Abstract

Forty-four samples of garlic plants showing virus-like symptoms were collected, during the growing season (2021-2022) from different locations in Qassim province, Saudi Arabia. These samples were analyzed by ELISA against the important *Allium* allexiviruses including garlic virus A (GarV-A), garlic virus B (GarV-B), garlic virus C (GarV-C), and Shallot virus X (ShVX). The obtained results showed that 22/44 (50%) samples were found to be infected with one of the tested viruses. Mixed infections were detected in 13/22 samples (59.1%) with more than one virus. However, 13.6%, 0% and 27.2% were detected as single infection with GarV-A, GarV-B and GarV-C respectively. RT-PCR amplification with general allexiviruses primer (750 bp) and specific primers for GarV-A (1330 bp), GarV-B (1216 bp) and GarV-C (1557 bp) were used to detect the respective viruses. The phylogenetic tree and nucleotide sequence analysis of one of each GarV-A (OQ397541), GarV-B (OQ397542) and GarV-C (OQ397543) with general primer for allexiviruses while one isolate of each GarV-A (ON155441), GarV-B (OR343811) and two isolates of GarV-C (ON155445, and ON155446) with specific primers showed their similarity with their respective viruses from GenBank. In host range study, *Chenopodium amaranticolor*, *Nicotiana benthamiana*, *N. tabacum* and *Allium cepa* expressed necrotic lesions, mosaic and yellowing symptoms respectively against GarV-A, GarV-B and GarV-C. To our knowledge, this is the first report of GarV-A, GarV-B, and GarV-C in Saudi Arabia.

Keywords: Allexiviruses, ELISA, RT-PCR, Sequence

1. Introduction

Allium crops are grown around the world and are one of the medicinal herbs that have anticancer, antibacterial, antithrombic, and cholesterol-lowering actions on animal cells [1]. In recent decades (1991 and 2012), the area planted with garlic declined by 38.0%, yet the production of garlic climbed by 32.7% as the yield more than doubled [2]. Garlic (*Allium sativum* L.) is a close relative of onion, shallot, leek, scallions, and chive, and was originally classified in the Alliaceae family [3, 4]. In addition, in 1972, 2.98 million tons were produced, while 15.8 million tons were produced in 2007 [5]. Globally, garlic production was predicted to be 25 million tons in 2015, with China accounting for 80% (20.0 million tons per year) of the total volume while the India contributed 1.25 million tons annually. Other countries such as South Korea, Russia, and Egypt produce less than 0.5 million tons annually. According to the available data from Food and Agriculture Organization Corporate Statistical Database from 2016 to 2018, [6] garlic production increased by 3.1% that was totaling 28,494,130 metric tons as compared to the previous year which was 27,648,023 million tons. Globally, China is the leading producer and exporter of garlic. In 2016, the production and exports were around 21.26 and 1.53 million tons, respectively, and accounted for more than 80% of the total worldwide [7].

At least twelve distinct viral infections belonging to *Potyvirus* [8, 9, 10] *Tospovirus* [11, 12] *Allexivirus* [13, 14] and *Carlavirus* [15] have been reported to infect *Allium* crops [16, 17]. Bereda et al., 2017 indicated that Allexiviruses have been detected in garlic plants from the Japan, USA, Russia, Poland, Korea, Australia, India, Greece, Brazil, Italy, Spain, Argentina ,New Zealand, Czech Republic, China, Ethiopia,and Iran.

In naturally occurring infections, allexiviruses only induce modest host symptoms such as yellow stripes, mild mosaic, stunted growth and transmitted by eriophyid mites [18, 19]. However, the worldwide proliferation of most of the viruses detected in these complexes is mostly owing to the transfer of infected bulbs or other vegetative parts of the plant without any phytosanitary inspections and they also transmit by mechanical means [9, 15, 20]. This study aimed to identify the occurrence of three allexiviruses and their distribution in commercially available garlic crops from different locations in Qassim province, Saudi Arabia using serological and molecular characterization to eva-

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luate the genetic diversity and phylogenetic connections between the identified viral species in accordance with their geographical origin and/or kind of propagation material.

2. Materials and Methods

2.1. Sample collection, serological detection, and Host range determination.

During the field survey (2021-2022), forty-four symptomatic garlic samples that showed virus-like symptoms like a mosaic, yellowing, leaf striping, stunting, and leaf deformation (Fig. 1) were collected from Qassim province, Saudi Arabia. The DAS-ELISA was used to test the collected samples against important *Allium* allexiviruses (Table 1) (DSMZ-German).

A microplate reader was used to check their absorbance value (405nm) [21]. According to the ELISA results, local lesion technique was used to isolate the positive samples of each *Allexivirus* species through *Chenopodium album* and *C. amaranticolor* as local lesion hosts. For the Host range experiment, five plant species of each *C. amranticolar, C. album* (*Chenopodiaceae*), *N. benthamiana*, *N. tabacum* (*Solanaceae*), and *Allium cepa* (*Alliaceaeae*) were used [22]. Whereas onion plants were utilized as propagative hosts for the following experiment of host range [23, 24, 25].

2.2. Total RNA Extraction and RT-PCR

Total RNA from all collected garlic samples (100 mg) was extracted using Gene JET Plant RNA Purification Mini Kit. RT-PCR reaction was performed using One-Step RT-PCR kit system (Thermo Scientific, USA), 25ul reaction mixture that consisted of 5 ul RNA and 20 ul reagents with previously reported degenerate and specific primers to detect the allexiviruses [10, 26] (Table 1). RT-PCR cycles were carried out using the following parameters: 10 min for 60℃ and 2 min for 98℃ to synthesize the cDNA, followed by 35 cycles, 45 sec at 94℃, 45 sec at 56℃, 1 min at 72℃ and the final extension was 72℃ for 10 min. The obtained products of PCR (5 ul) were analyzed on 1% agarose gel using gel electrophoresis and visualized by UV illuminator. GeneRuler100 bp and DNA Ladder of 1 kb from Thermo Scientific, USA were used to confirm the size of amplified fragments.

2.3. Nucleotide sequencing and phylogenetic tree analysis

RT-PCR products of garlic-positive samples obtained with degenerate (6 samples) and specific primers (5 samples) for allexiviruses were selected and sent to Macrogen Company, South Korea. Sequencing data comparisons and multiple sequence alignment analysis were performed using DNASTAR Lasergene, National Centre for Biotechnology Information (NCBI) and Sequence Demarcation Tool (SDT) [27]. The Maximum-Likelihood method with 1000 bootstrap replications was used to construct the phylogenetic trees from ClustalW-aligned sequences on MEGA-X.

Fig. 1. Symptoms observed on garlic plants under field conditions showing mild mosaic, leaf striping, depicted yellowing and deformation of leaves (A, B, C, and D).

3. Results

3.1. Sample collection, ELISA detection, and Host Range

Based on serological tests, the obtained results indicated that the incidence of GarV-A, GarV-B, and GarV-C were detected in 22/44 (50%) garlic samples. Whereas mixed infections with with more than one virus were observed in 13/22 (59%) samples. As far as single infection is concerned, the incidence of above three viruses was detected as 13.6%, 0%, and 27.2% respectively.

3.2. Detection of allexiviruses by RT-PCR

The infected samples were tested for RT-PCR amplification using degenerate primer (Fig. 2A). and specific primers (Fig. 2B). of allexiviruses. The obtained results revealed that 27 samples were positive with at least one of the allexiviruses using both degenerate primer (750 bp) and specific primers (Garv-A (1330 bp), GarV-B (1216 bp) and GarV-C (1557 bp), The amplified fragments of selected samples (6 samples with degenerate and 5 samples with specific primers) were visible when running on aga-

Table 1. List of primers for Allexiviruses that partially target their CP region of genome.

Virus Name	Primer Names	Primer Sequence 5`-3`	Amplification Size bp
Allxivirus general primers	Allexivirus-CP+- F	tggrcntgctaccacaaygg	750
	Allexivirus -NABP-R	ccyttcagcatatagcttagc	
Garlic virus A	GarV-A-F	tgtctcgcgctcctacatcagaa	1330
	GarV-A-R	tctggggacaatagttgttgcaaggt	
Garlic virus B	GarV-B-F	ttgtgttaagtttggayttgggttga	1216
	GarV-B-R	tgatatcaacagcatgggtgtctt	
Garlic virus C	GarV-C-F	agtgatttgsamccataycaagc	1557
	GarV-C-R	agtaatatcaacaagcatgggtgt	

rose gel electrophoresis (1%).

3.3. Nucleotide sequencing and phylogenetic tree analysis

Partial nucleotide sequence of RT-PCR amplified product obtained from the selected samples isolated from garlic using general allexiviruses primer showed the occurrence of GarV-A, GarV-B, and GarV-C. These sequences were blasted on the NCBI website, and they showed level of identity with their respective viruses which have already been registered in the NCBI database. The sequences of these viruses were submitted to GenBank with accession numbers OQ397541 (GarV-A-G-Qassim) OQ397542 (GarV-B-G-Qassim), and OQ397543 (GarV-C-G-Qassim), respectively. Phylogenetic tree of Saudi Arabian viral isolates showed three different clusters with their respective virus isolates that are posted in GenBank (Fig. 3). The nucleotide sequence percentage identities of these viruses indicated that GarV-A showed 81.9% (MN059317, MN059318, and MN059319, China) to 96.6% (MN059310, China) identity with the other available isolates in the NCBI (Fig. 4 A). GarV-B shared 82.6% (MN175534, Poland) to 93.9% (KX889770, China) identity with the other already published isolates from different countries (Fig. 4B). GarV-C showed 86.3% (MK814847 and MK814848, Serbia) to 96.6% (JX488644, Brazil) identity with the other isolates posted in the GenBank (Fig. 4C).

For specific primers of *Allexivirus*, the nucleotide sequence of one isolate of each GarV-A, GarV-B, and two isolates of GarV-C isolated from garlic were submitted to the GenBank under the following accession numbers ON155441 (GarV-A-Gar-QA2), OR343811 (GarV-B-G-Qassim), ON155446 (GarV-C-Gar-QC1), and ON155445 (GarV-C-Gar-QC2), respectively. Phylogenetic tree showed that all these three Saudi Arabian isolates made three different clusters with their respective virus isolates posted in the GenBank (Fig. 5). In addition, BLAST analysis of GarV-A Saudi Arabian isolate shared 96% (JX488632, Brazil) to 100% (MH686308, Australia) identity with the other isolates published in NCBI database. Moreover, seven Chinese isolates (MN059322, MN059310, MN059259, MN059256, MN059297, MN059314, and MN059279), two Poland isolates (KX889763 and KX889756), two Indian isolates (MT731489 and MT731490), two isolates of Korea (MN551281 and AF478197) one isolate of each Australia (JQ899446) and Japan (AB010300), showed 99% identity with GarV-A Saudi Arabian isolate. (Fig. 6A). On the other hand, analysis of GarV-B Saudi Arabian isolate shared 86.8% (OK330524, India) -100% (MN059177 and MN059174, China) with the other iso-

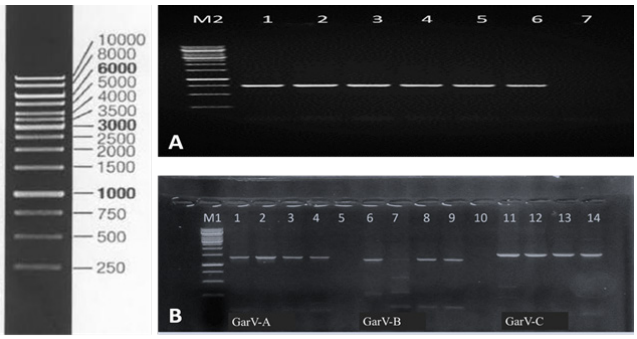

Fig. 2. Agarose gel (1%) electrophoreses show garlic-positive samples using general primers (**A**) and specific primers (**B**) from Qassim Region. Lanes: 1,2,3,4,5 and 6 are positive samples and show their bands on 750bp using general allexiviruses primer comparatively to the ladder. However, lane 7 is used as a negative control with healthy garlic plant in the RT-PCR reaction **(A).** Lanes 1,2, 3,4 with GarV-A, lanes 6,7, 8,9 with GarV-B and lanes 11,12, 13 and 14 with GarV-C showed their fragments at expected size of 1330 bp, 1216 bp and 1557 bp, respectively. Sample in lanes 5,10 are used as a negative control with healthy garlic plants for all three viruses **(B).** Lane M1 and M2GeneRuler 1 kb DNA Ladder; Thermo Scientific, USA.

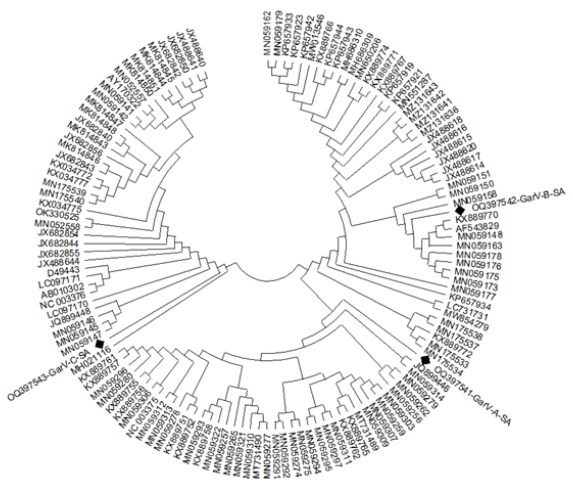

Fig. 3. Collective phylogenetic tree of GarV-A, GarV-B, and GarV-C Saudi isolates using general allexiviruses primer from garlic that shows their identity with the other respective isolates of different countries.

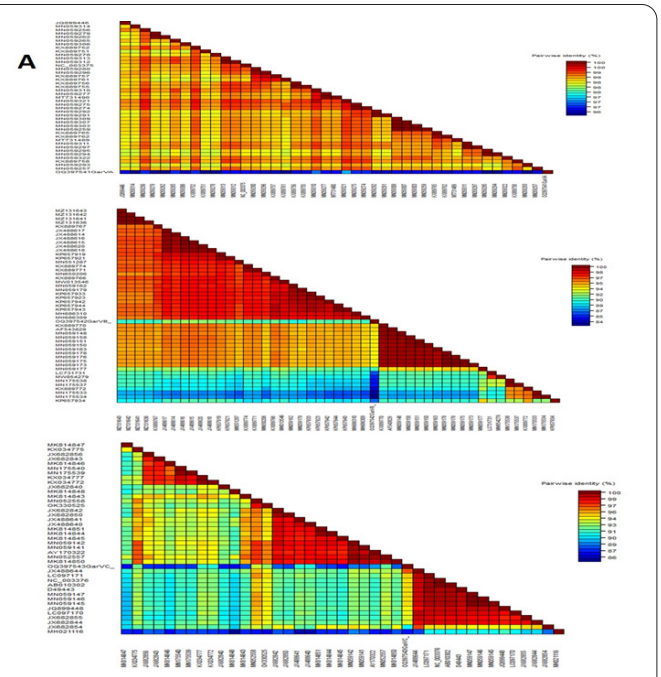

Fig. 4. Distance matrix illustrating the pairwise nucleotide identity percentage of GarV-A (**A**), GarV-B (**B**) and GarV-C (**C**) Saudi isolates using general primers as compared with other most similar sequences. A closely grouped isolates of GarV-A are indicated with red to yellow color. Closely grouped isolates of GarV-B are indicated with red to yellow color. Closely grouped isolates of GarV-C are indicated with red to yellow color. Whereas blue color shows lowest percentage identity.

lates in NCBI (Fig. 6 B). Likewise, both isolates of GarV-C shared 98-100% nucleotide identity among themselves and 85% to 98% with the other isolates in the GenBank. However, three Chinese isolates of GarV-C (MN059147, MN059146, MN059145) showed maximum percentage. The lowest percentage similarity was observed with the Brazilian isolate of GarV-C (KF955566) (Fig. 6C).

3.4. Mechanical inoculation and host range determination

Local lesion technique was used to isolate each *Allexivirus* species from ELISA-positive samples through *C. album* and *C. amranticolor* as a local lesion host. whereas onion plants were used as propagative hosts. After 14 days of inoculation, all the inoculated plants of each *Allexivirus* isolate produced similar symptoms on different host plants like local lesions and mosaic on inoculated *C. amranticolor*, and *N. benthamiana,* respectively. Yellowing with twisted leaves and mosaic with puckering were observed on inoculated onion and *N. tabacum* host plants sequentially (Fig. 7). *C. album* remained asymptomatic (no characteristic symptoms such as necrotic or chlorotic lesions were produced).

RT-PCR reactions of inoculated plants were done as above with the degenerate primers of allexiviruses. When running on agarose gel electrophoresis (1%), specific bands of the exact expected size (750 bp) appeared, and were visible in all tested samples, by comparing them with HyperLadder™ II DNA, 50 bp (Bioline, USA). Interestingly, *C. album* which remained asymptomatic during the host range experiment showed positive results in the exact expected size (Fig. 8).

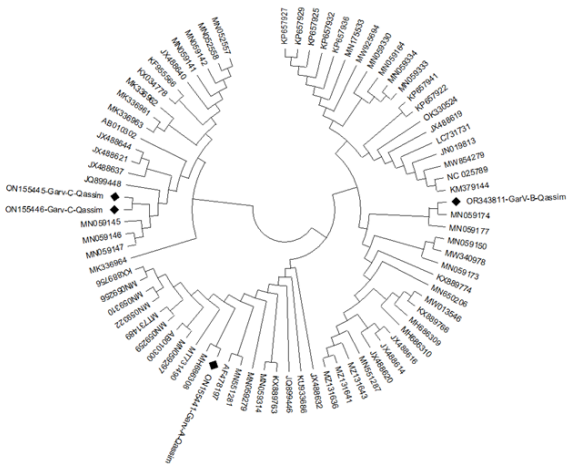

Fig. 5. Collective phylogenetic tree of one GarV-A, GarV-B and two GarV-C Saudi Arabian isolates using specific primers from garlic that show their similarity with their respective virus isolates posted in the GenBank.

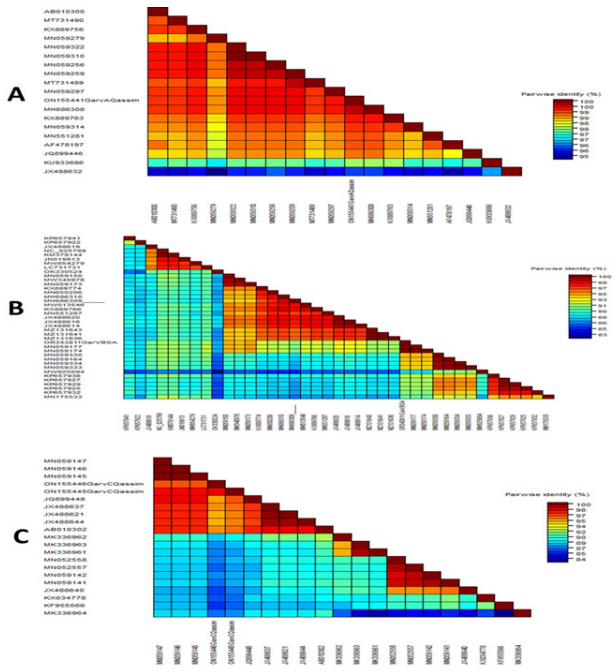

Fig. 6. Distance matrix illustrating the pairwise nucleotide identity percentage of GarV-A (A), GarV-B (B) and GarV-C (C) Saudi isolates using specific primers as compared with other most similar sequences. A closely grouped isolates of GarV-A are indicated with red to yellow color. Closely grouped isolates of GarV-B are indicated with red, yellowish green and light blue color. Closely grouped isolates of GarV-C are indicated with red to light blue color. Whereas blue color shows lowest percentage identity.

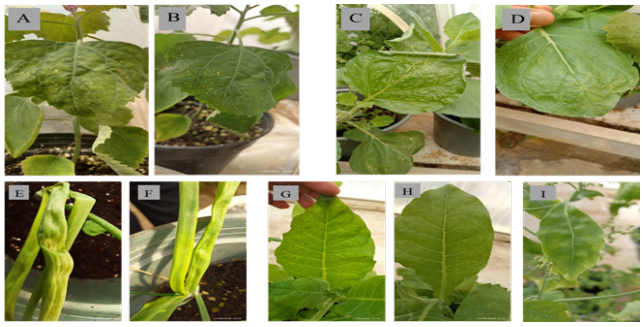

Fig. 7. Symptoms observed on selected host range as a result of inoculation with each virus isolate of GarV-A, GarV-C, and GarV-B. A, B, and C, D showed necrotic lesions and mosaics in inoculated *C. amranticolar* and *N. benthamiana*. Yellowing and twisted leaves in onion (E, F), light and dark green patches in *N. tabacum* (G, H), No characteristic symptoms developed in *C. album* (I).

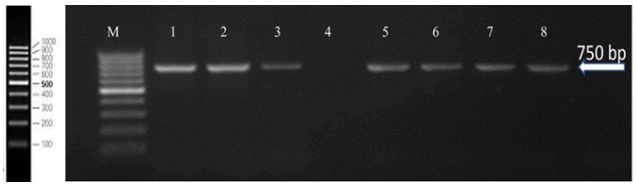

Fig. 8. Agarose gel electrophoresis (1%) depicted positive results of host range plants., *C. amaranticolar* (Lanes1,2), onion (Lane 3), *N. benthemiana* (lane 5), *N. tabacum* (lane 6,7) and *C. album* (lanes 8) are positive with general Allexivirus primers at the exact size 750bp. No results were obtained with negative control (lane 4), Lane, M1 represents 100 bp DNA Ladder GeneRuler; Thermo Scientific, USA.

4. Discussion

One of the most prominent crops in *Allium* family that is grown all over the world is garlic. Production and quality of the crop are significantly decreased by viral diseases causing a major economic issue [28]. Certain viruses, such as allexiviruses, potyviruses, tospoviruses, and carlaviruses, are commonly found to infect garlic plants [29, 30]. The garlic crop is propagated through bulbs; therefore, infected planting material is the main cause of disease spread and eliminating these viruses from the field has been a huge challenge for farmers and researchers [28]. Evidently, in

garlic, allexiviruses are a substantial problem because they comprise the eight viral pathogens and are responsible for the "garlic disease complex" although, their infection surges with the presence of potyviruses [31,32].

To understand garlic viral disease, the development of easy and robust diagnostic technology is important as well as genetic identification and molecular characterization of viruses are indispensable to adopt the appropriate management practices to produce virus-free garlic plant stock and reduce the viral load in the field [19]. Identification of the virus is also crucial to understanding the epidemiology of that virus and predicting the precautionary and preventive measures to protect the crop from the entry of the virus into the field. Collectively, serological, and host range experiments, to precisely identify and characterize garlic viruses, RT-PCR amplification and coat protein gene sequence data analysis are essential.

In this research, GarV-A, GarV-B, and GarV-C from the Qassim Region of Saudi Arabia have been characterized by using serological, general, and specific primers. However, no sample was found to be positive with ShVX.

The variation in the nucleotide identity could be due to the natural genetic variation of these isolates. Overall, phylogenetic analyses revealed that Saudi Arabian virus isolates from onion and garlic are typically grouped together in one cluster, and the phylogenetic relatedness relationship between them and their geographic origins are frequently unrelated. Numerous studies carried out in several countries have established a connection between the highly diverse and fast-evolving onion and garlic viruses and the international trade of vegetative planting materials [10, 33]. GarV-A, GarV-B, and GarV-C produced similar kinds of symptoms in all the host plants that were used in this study. However, *C. album* remained asymptomatic, due to the age of plants and handling errors during the inoculation or some inhibitor compound present in plant. As compared to our study, *C. quinoa* produced local lesions when inoculated with infected garlic sap and the samples were positive in ELISA and RT-PCR tests with at least one of the *Allium* viruses whereas tomato and tobacco did not exhibit any symptoms and remained negative in both ELISA and RT-PCR tests [34]. In *N. benthamiana* GarV-X produces granules in endoplasmic reticulum (ER) and the movement of virus to adjacent cells is assisted by p11protein which encodes by ORF3 [35].

Moreover, the occurrence of these viruses in Saudi Arabia has not been described but trading of garlic bulbs from different countries such as Egypt, India, and European countries to Saudi Arabia cannot be neglected because these viruses have been reported from these countries. Sharing of propagative material from one region to another play a vital role in the distribution of these viruses within the country and another important thing is, that eriophyid mites that efficiently transmit the allexiviruses within the field and storage condition, cause the wide spread of these viruses. Thus, the relation between the allexiviruses and eriophyid mites needs to be properly investigated and the identification of other viruses with advanced molecular techniques that are part of "garlic viral complex" is inevitable to minimize the losses.

5. Conclusion

In conclusion, future studies will include the characterization of other new viruses belonging to *Allexivirus* group infecting garlic plants growing in the Qassim province and other different Regions especially those associated with great production of garlic crops.

Conflicts of Interest

All authors have given their approval and agreed to submit the work for publication. They have no relevant conflicts of interest to disclose for this study.

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