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Molecular Identification of *Strawberry Latent Ring Spot Virus* (SLRSV) In Egypt

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ABSTRACT

Strawberry latent ringspot virus (SLRSV) isolated from symptoms and symptomless strawberry plants and identified with a specific antiserum using Double Antibody Sandwich ELISA (DAS-ELISA). Survey was conducted through three successive seasons during 2013-20l6 on commercial strawberry fields in four governorates in Egypt. The percentages of infection which recorded from the survey were 7.5, 6.3, 5.9% (Al-Dair-region) and 9.4, 10.6, 10.5% (Kafer Al-Shapy-region) in El-Qalubia governorate. In El-Behera governorate the percentage was 5.3, 6.0, 5.7% (Badrregion) and 4.0, 4.4, 5.2% (Al-Nobaria- region). El- Monufyia (Quesna-region) recorded 8.8, 8.9, and 9.2 %. Ismailia (fayed-region) was 4.7, 5.0, and 5.5% respectively. SLRSV mechanically transmitted from infected strawberry plants onto 16 host species belonging to seven families. Mottling, local lesion, leaf deformation, systemic and dwarf plants were appeared two weeks' post inoculation on indicator host *Chenopodium quinoa* and *Chenopodium amaranticolor*. Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify 497 bp fragment using specific primers for the viral coat protein gene, as a tool for molecular diagnosis. The amplified PCR fragments were cloned, sequenced and compared with other of those sequences available in GenBank. Results observed that, the Egyptian SLRSV isolate was A+T, G+C (251, 246) identical content with Poland and USA isolates. Obtained sequence comparison with the isolates available in the GenBank indicated that SLRSV Egyptian isolate shared 99% identity with Poland isolate followed by 84% USA isolate, 83% UK isolate and 80% Newzeland isolate.

Keywords: Strawberry, SLRSV, Host range, Survey, DAS-ELISA, RT-PCR, Sequence

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1. INTRODUCTION

The cultivated strawberry, *F.* × *ananassa*, originated ~250 years ago, and is among the youngest crop species [8]. Botanically, it is neither a berry nor a true fruit, as the actual fruit consists of the abundant dry achene's (or seeds) that dot the surface of a fleshy modified shoot tip, the receptacle. Unlike other Rosaceae family crops such as apple and peach, the strawberry is nonclimacteric because the flesh does not ripen in response to ethylene. Genomically, *F.* × *ananassa* is among the most complex of crop plants, harboring eight sets of chromosomes ($2n = 8x = 56$) derived from as many as four different diploid ancestors. Paradoxically, the small basic ($x = 7$) genome size of the strawberry genus, ~240 Mb, offers substantial advantages for genomic research. An international consortium selected *F. vesca* ($2n = 2x = 14$) for sequencing as a genomic reference for the genus [24]. The so-called 'semperflorens' or 'alpine' forms of *F. vesca* ssp. *vesca* have been cultivated for centuries in European gardens [8]. Their widespread temperate growing range, self-compatibility and long history of cultivation, coupled with selection for favorable recessive traits such as day neutrality, non-runnering and yellow fruited forms offer extensive genotypic diversity.

Strawberry (Fragaria x ananassa Duch.) is one of the most economic vegetable crops in Egypt and considered the main cash crop for strawberry growers in some governorates. It is one of the most favorite and delicious fruits of which the demand has been increased in Egypt for local consumption

and for exportation. Strawberry production is increasing annually, the world production exceeding 4 million tons, [9]. The total cultivated area of strawberry in world (241109 Ha) and total world production of strawberries about (4516810) per ton. Egypt Occupies the fourth position of strawberries production after USA, Turkey and Spain. The total area of

Strawberries orchards in Egypt are (13999.2) feddan with an approximate yearly production (242297) per ton according to the statistics of [7]. Strawberry can be infected by more than 30 viruses [18]. Many viruses form a major threat to the strawberry industry in Egypt, causing severe economic losses.

Strawberry latent ringspot virus (SLRSV) genus Nepovirus, in the family Comoviridae was first identified more than 40 years ago, [17]. Has been listed as quarantine past in the United States prior to 2004, SLRSV has a host range that exceeds 125 plant species belonging to 27 families of both monocots and dicots [14] and [3]. The virus occurs naturally in many species of wild and cultivated plants and infects, often symptomless, a wide range of commonly used herbaceous test plants, SLRSV was isolated from leaves and flowers parts, can be transmitted with plant sap by mechanical inoculation. SLRSV is a positive-sense single-stranded RNA virus [6].

Strawberry latent ringspot virus was detected, isolated and characterized on hosts' plants and by DAS-ELISA, among molecular methods, RT-PCR has proved to be the most rapid, sensitive and reliable technique for detecting RNA, viruses in infected plants [1]. Thus, the use of PCR technology is an important step to optimize and speed up strawberry viruses' diagnosis.

The present work aimed to study several aspects of SLRSV including disease distribution in some Egyptian Governorates, identification and characterization of the Egyptian isolate by host range, symptomatology, serologically, biologically and molecular studies. In addition to, sequence analysis to measure the similarities and differences between the Egyptian isolate and the isolates available in the Gen Bank.

2. MATERIALS AND METHODS

Source of samples and filed inspection:

Plant samples showing symptoms and symptomless were collected from four governorates (El-Qalubia, El-Behera, El-Monofiya and El-Ismailia) during the spring and early summer of three successive seasons (2013/2014, 2014/2015 and 2015/2016). 3009 samples were collected from different localities of strawberry cv. (Festival, Camarosa, Florida, montakhab, Fortuna, winter star and Rosa Linda) plants carrying different external symptoms like that caused by viruses, and tested using ELISA.

Enzyme-linked immune sorbent assay (DAS ELISA):

The samples were tested serologically using DASELISA technique demonstrated by [15]. using SLRSV polyclonal antibodies, according to the manufacturer's instructions. Positive and negative control were used, absorbance was measured at 405 nm using ELISA reader (BioTeK-ELX808).

Isolation of SLRSV

Samples of infected plants which gave positive results with ELISA were used for isolating these targeted viruses under greenhouse conditions using specific indicator host plant for SLRSV virus.

Mechanical transmission and Host rang:

Strawberry plants which give positive reaction with ELISA were used as a source of mechanical infection to the indicator host rang (Strawberry, *Chenopodium* and *Cucumber*). Sixteen species and varieties belonging to families (*Rosaceae*, *Solanaceae*, *Cucurbitaceae*, *Chenopodiaceae*, *Leguminosae*, *Lamiaceae* and *Compositae*) were mechanical inoculated using SLRSV and maintained under greenhouse conditions for 20 days. Plants showed symptoms and symptomless were checked by back inoculation to the indicator hosts, results was confirmed by DAS-ELISA.

Molecular detection

Total RNA Extraction and Reverse transcription (RT-PCR):

Total RNA was extracted from leaves and flowers of infected strawberry plants using plant total RNA Mini kit (RBC) as described in the manual instruction. Reverse transcription polymerase chain Reaction was carried out on RNA preparations with Reverse Verso™ one-Step RTPCR Reddy Mix Kit (Thermo scientific). RT-PCR was performed in 25 µl total volume containing 5.5 µl of nuclease - free water, 4 µl (1ng) of total RNA, 12.5 µl of one step PCR master mix, 0.5 µl (10µM) specific forward and reverse primers (Table 1), 0.5 µl Verso enzyme mix and 1.5 µl RT-Enhancer. Synthesis of cDNA was performed at 50°C for 15 min, followed by denaturation at 95°C for 2 min. Amplification was carried out for 35 cycles under the following conditions: denaturation at 95°C for 20 sec, annealing at 52°C, 30 sec. extension at 72°C for 30 sec, followed by a final extension for 5 min at 72°C. Amplified products were detected by agarose gel electrophoresis.

Electrophoresis analysis:

The PCR product were electrophoresed in 1% agarose gel in -0.5x TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5) at 120 volt. 100bp DNA molecular weight markers (ABgene, UK) for 1 hour and stained with ethidium bromide (0.5 µl/ml) [11]. The fragments were photographed using UV lamp in geldocumentation (Bio Rad, Gel Doc XR system 170-8170).

Table (1): Primer sequences and the expected size of amplified coat protein gene of SLRV cDNA

Virus	Primer sequence	Expected size	Reference
SLRVF	5'- CCTCTCCAACCTGCTAGACT -3'	497bp	Martin *et al*., 2004
SLRVR	5'- AAGCGCATGAAGGTGTAACT -3'		

Cloning of PCR amplified fragments of SLRV:

The amplified 497 bp of SLRSV cDNA product was SLRSV isolated from cloning using mini-preparation directly cloned into pCR™ TOPO vector using TA™ were sequenced in Macrogen Inc. (Seoul, Korea). Cloning system (Invitrogen, Carlsbad, CA).

Sequence and analysis:

Similarities and differences were analyzed between SLRSV Egyptian isolate and the isolates available on the GenBank using DNAMAN software.

3. RESULTS AND DISCUSSION

Source of samples and filed inspection:

Fig (1): Naturally infected strawberry showing different viral like external symptoms. (A) mottling, (B) yellowing, (C) vein banding, (D) ring spot in flower, (E) cup shape and leaf crinkle, (F) leaf deformation, (G) leaf crinkle, (H) stunting, (I) Yellowing edges.

A survey of strawberry latent ringspot virus nepoviruses (SLRSV) disease was conducted in some Egyptian governorates during the growing seasons of 2013/2014, 2014/2015 and 2015/2016. The strawberry plants were randomly taken from different regions following an imaginary line from the fields' outer border to the center. Collected samples were tested by ELISA during the three seasons for the presence of viral infections. External symptoms were observed on some strawberry samples suspected to be virus infection such as mottling, yellowing, leaf curling, leaf deformation, cup shape, stunting, ring spot in flower and vein banding in as observed in Fig(1).

SLRSV isolates were detected in all surveyed governorates, varied from region to region and from season to season. Survey was conducted through three successive seasons during 2013-20l6 on commercial strawberry fields in four governorates in Egypt. The

Monofiya (Quesna-region) recorded 8.8, 8.9, and 9.2 %. Ismailia (fayed-region) was 4.7, 5.0, and 5.5% respectively Table (2) and Fig (2). In general, from a total of 1070 strawberry samples collected during the first season 6.60% were SLRSV positive, the second season samples 974 and the percentage of was 6.80%, while from a total of 965 samples collected during the third season, 7.00% of samples

percentages of infection which recorded from the survey were 7.5, 6.3, 5.9% (Al-Dair-region) and 9.4, 10.6, 10.5% (Kafer Al-Shapy-region) in El-Qalubia governorate. In El-Behera governorate the percentage was 5.3, 6.0, 5.7% (Badr-region) and 4.0, 4.4, 5.2% (Al-Nobaria- region). El-

were SLRSV positive Surveys of *Strawberry Latent ringspot virus* (SLRSV) disease were conducted in Egypt during the 2013-2016 growing seasons. Plant samples were collected from strawberry cultivars, in commercial field in different locations including Qalubia, Beheira, Monufyia and Ismailia governorates.

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Table (2): Survey of SLRSV in strawberry plants from different locations in Egypt, during 2013-2016.

Governorate	Percentage of SLRSV infection using DAS ELISA								
	2013/2014			2014/2015			2015/2016		
	tested	infected	infection %	tested	infected	infection %	tested	infected	infection %
EL-Qalubia	380	32	8.42%	350	29	8.28%	360	30	8.33%
El-Beheira	340	16	4.70%	380	20	5.26%	275	15	5.45%
EL-Monufyia	160	14	8.75%	124	11	8.87%	130	12	9.23%
Ismailia	190	9	4.73%	120	6	5.00%	200	11	5.50%
Total	1070	71	6.6%	974	66	6.80%	965	68	7.00%

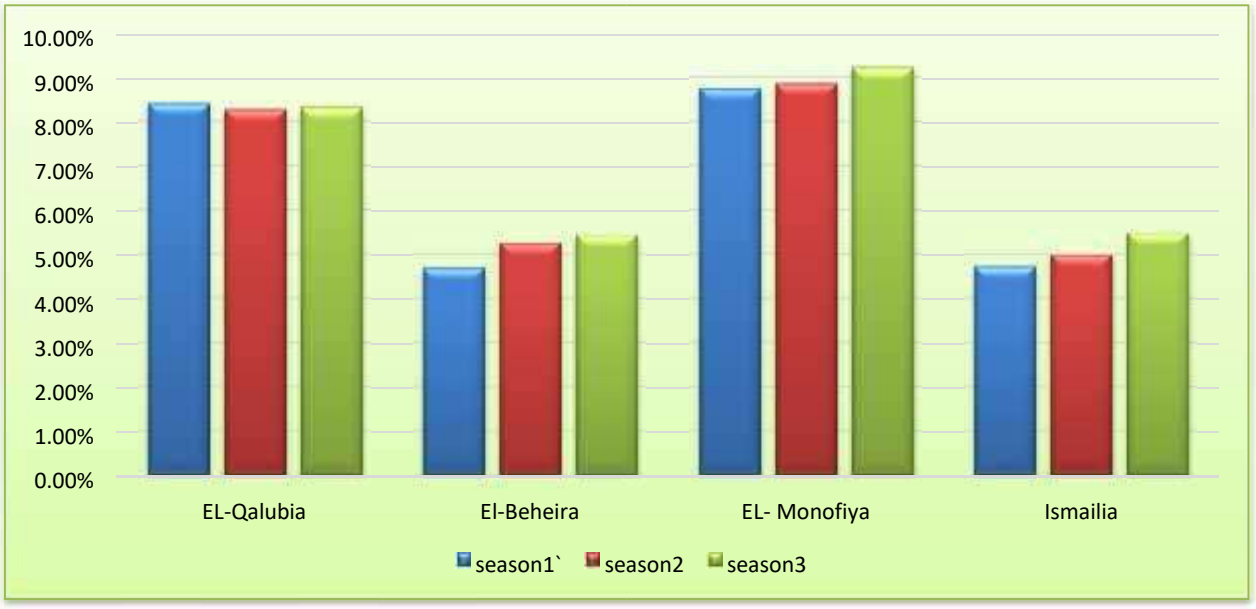

Fig (2): Histogram showing incidence of SLRSV virus in strawberry plants collected from different Governorates in Egypt during 2013-2016.

The total tested samples were 3009 using DAS-ELISA. Data obtained from the survey in the main strawberry production areas showed that the virus was recorded in all surveyed Governorates. From a total of 3009 samples collected during the three seasons, 6.82% of samples were SLRSV positive. The highest infection rates were recorded in Monofiya, Qalubia and Behera respectively, the lowest infection rate was recorded in El- Behera and Ismailia respectively. These percentages of infection were less than the results recorded by [2] who identified the SLRSV in Egypt. The survey results showed that SLRSV has a wide distribution in all the regions where strawberry grown in Egypt. Although, infection rates were varying from one locality to another. The obtained results agree with [20] which detect of SLSRV in leaves of olives trees in Egypt using PCR protocol with the percentage of infection reached to 2.3% of tested samples. Also, the results are in agreement with [16]. who recorded that SLRSV infection in olive trees in Italy and in a varietal collection with the infection rate of 19%. The previous results are in agreement with [9] who recorded that SLRSV is an economically important virus due to its extensive host range and the yield losses it can cause.

Mechanical transmission and Host rang of SLRSV:

The obtained results from mechanical inoculation by rubbing finger in one-direction on cotyledon or primary 26 leaves of hosts using sap inoculations for SLRSV indicated that, symptoms on infected plants differ according to the plant species. From 16 tested host plants, SLRSV infected 16 hosts. Mottling systemic local lesion and dwarf plants on *Chenopodium quinoa*, mottling and leaf deformation on *Chenopodium amaranticolor*, systemic mottling on *Nicotiana glutinosa*, local lesion and systemic necrotic on *Nicotiana occidentalis*, systemic yellow mosaic infection on *Phaseolus Vulgaris*, mosaic on *Vicia faba* and vein clearing and leaf deformation on *Cucumis sativus*, mottling and leaf crinkling on *Capsicum annum*. Yellow vein banding was observed on *Mentha spicata* Table (3) and Fig (3).

Table (3): Host range and symptoms recorded after SLRSV mechanical inoculation and ELISA test

Family	Hosts	Symptoms infectivity	Incubation period (days)	ELISA test
Rosacea	*Fragaria ananassa*	No symptoms	20 days	+
Chenopodiaceae	*Ch. amaranticolour*	Mottling systemic local lesion	12 days	+
	Ch. quinoa	Mottling and leaf deformation	12days	+
Leguminosae	*Ph. vulgaris*	systemic yellow mosaic	14 days	+
	Vicia faba	mosaic	14 days	+
Cucurbitaceae	*Cucumis sativus*	Vein clearing, yellow, crinkle and leaf deformation	15 days	+
	Cucurbita pepo	No symptoms	15 days	-
Lamiaceae	*Mentha spicata*	yellow vein banding	17 days	+
Solanaceae	*Capsicum annuum*	mottling and leaf crinkling	15 days	+
	Datura metel	No symptoms	20 days	-
	Datura stramonium	No symptoms	20 days	-
	Solanum melongena	No symptoms	20 days	-
	Lycopersicon esculentum	No symptoms	20 days	-
	Nicotiana glutinosa	systemic mottling	13 days	+
	Nicotiana occidentalis	local lesions	15 days	+
	Petunia hybrid	No symptoms	20 days	-

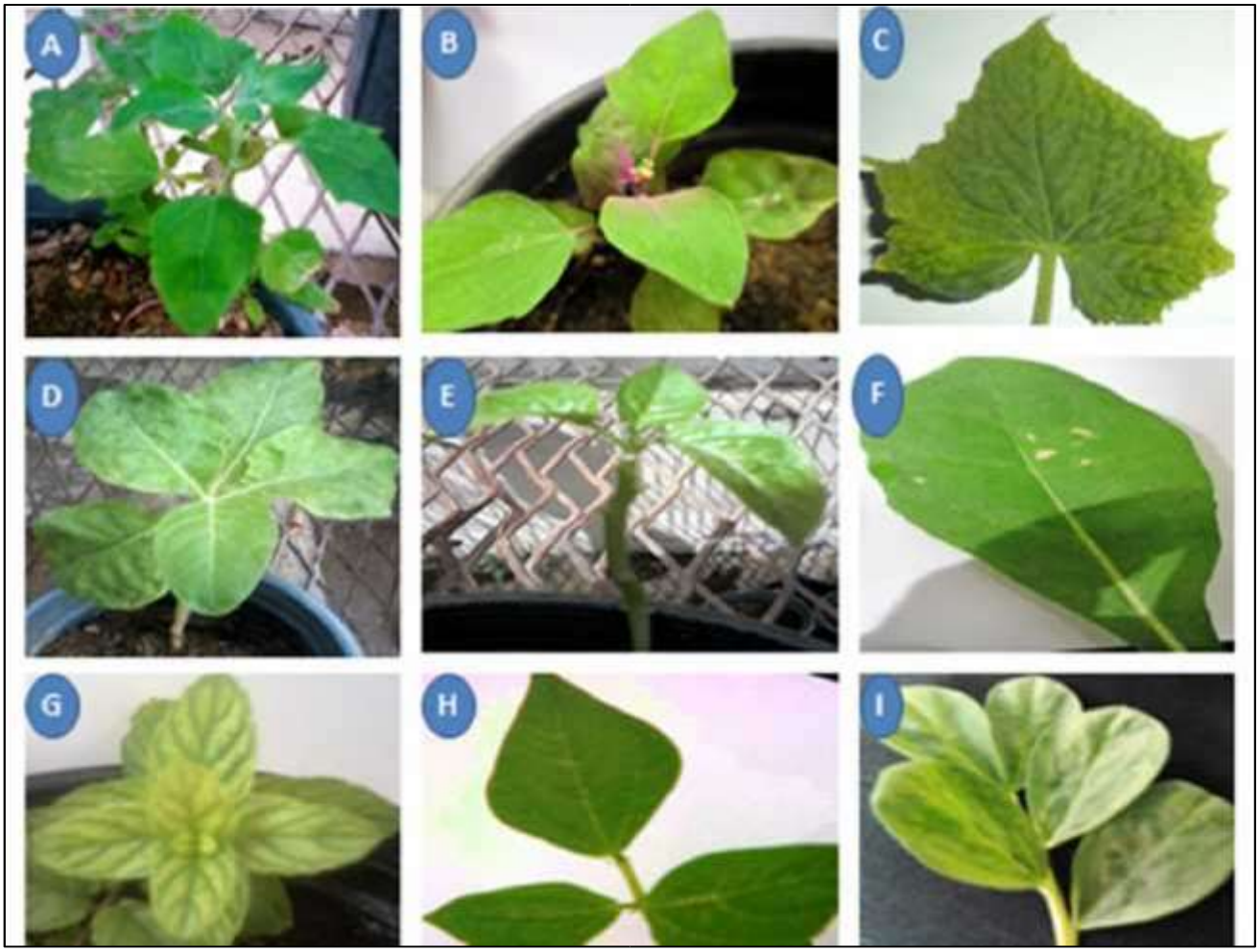

Fig (3): SLRSV symptoms on herbaceous indicator plants. (A), leaf deformation and plant dwarf on *Chenopodium quinoa*, (B), systemic mottling on *Chenopodium amaranticolour*, (C), mosaic and vein clearing on *Cucumis sativus*, (D), systemic mottling on *Nicotiana glutinosa*, (E), mottling and leaf crinkling on *Capsicum annuum*, (F), local lesions on *Nicotiana occidentalis*, (G), yellow vein banding on *Mentha spicata*, (H), mosaic on *Phaseolus vulgaris*, (I), vein clearing and chlorosis on *Vicia faba*.

The obtained results were confirmed by ELISA test. The obtained data reviled that SLRSV Egyptian isolate had limited host range these results agreed with [17] which reported that SLRSV infects restricted species in and outside family *Rosaceae*. Same results were confirmed by [12] and [2] who used the same techniques for virus transmission and host range. Samples of infected plants which gave positive results with DAS-ELISA using specific polyclonal antibodies for SLRSV were used for isolating these targeted viruses under greenhouse conditions using specific indicator host plant for SLRSV virus. The finding is consistent with Biological detection as well as serological detection by ELISA is reliable from April to June and in September and October. It is more difficult in July and August [10].

Molecular detection: -

Total RNA Extraction and Reverse transcription (RTPCR):

Samples of infected plants which gave positive results with DAS-ELISA were used for total RNA isolated using one step RT PCR amplification. The one step RT-PCR protocol is rapid and sensitive in the diagnosis of SLRV [22]. Electrophoresis analysis of RT-PCR product showed a single amplified fragment at 497 bp Fig. (4). the size of PCR product was expected 497 bp. similar results confirmed by [19].

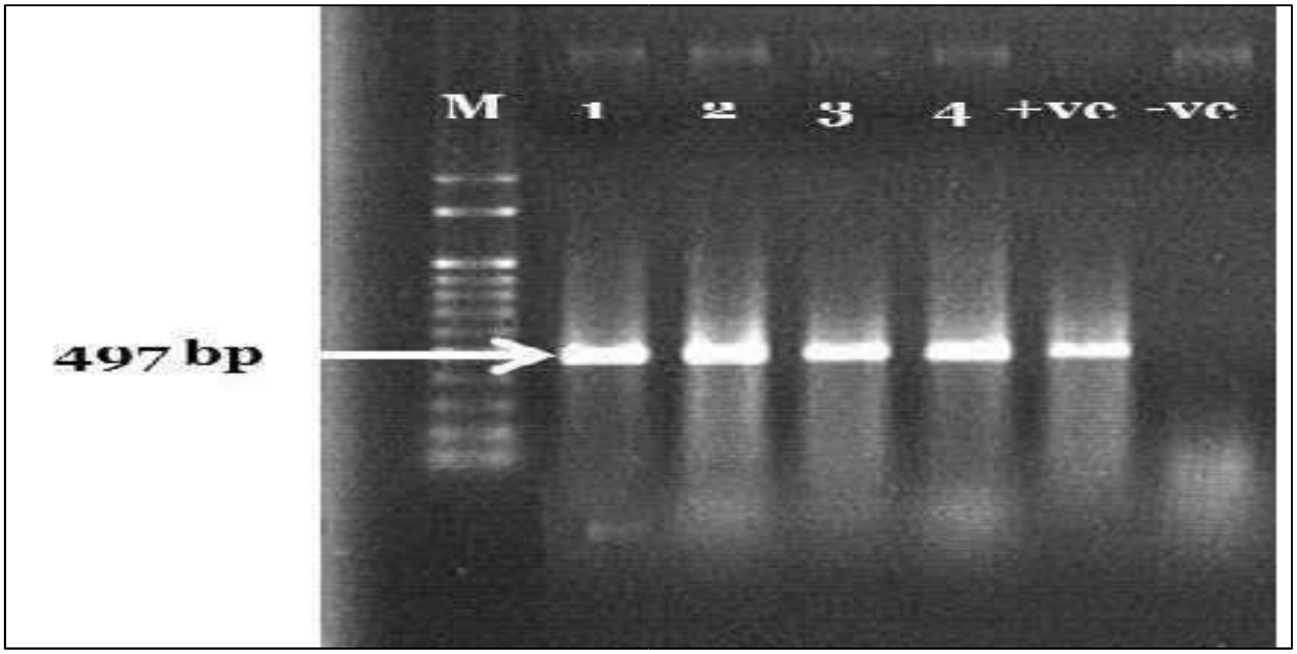

Fig (5): 1% Agarose gel electrophoresis analysis of RT-PCR showing the amplified fragment of strawberry latent ringspot virus (SLRSV) from strawberry tissues. Lanes 1, 2, 3, 4, +ve positive results, –ve Negative and M 100bp DNA molecular weight marker (ABgene, UK).

The obtained results are in agreement with [2] and in contrast with [12], who proved that, No. bands were amplified from the healthy plant controls that reflected the sensitivity as well as the specificity of the PCR primers used in this study, [6]. Developed and used a one-step reverse transcription-polymerase chain reaction (RT-PCR) protocol, for the detection of Strawberry latent ring spot virus (SLRSV) in leaves of olive trees in Italy and proved that the one-step RT-PCR protocol is rapid and sensitive and has the potential to be used on a mass scale for the diagnosis of SLRSV in olive, [23] developed and used a reverse transcription nested polymerase chain reaction (RT-n-PCR) and applied to the detection of Strawberry latent ringspot virus (SLRV). The method was more sensitive and reliable than conventional RT-PCR. This increased sensitivity allowed virus detection in samples that were negative to the first round of RT-PCR amplification, [4] mentioned that the multiplex RT-PCR approach was developed for simultaneous detections of Strawberry latent ringspot virus (SLRSV) from the imported lily bulbs. The results indicated that good specificity and sensitivity for virus detection were obtained, [5] used molecular methods; PCR (Polymerase Chain Reaction) and RT-PCR (Reverse Transcription and Polymerase Chain Reaction) are extensively developed in recent twenty years, and used for the detection of most small fruit virus diseases.

Nucleotide sequence analysis:

Molecular characterization of SLRSV genome has been done. Fresh leaf tissues of *F. ananassa* were applied for extraction and PCR amplification. The amplified PCR fragments were used for cloning, sequencing and comparing the sequencing with other of those sequencing available in GenBank. The nucleotide sequence of PCR amplified fragments for the SLRSV Egyptian isolate were completed to determine if the PCR fragments are belonging to SLRSV. The DNA sequence was performed using the PCR product 497 bp of the coat [protein gene obtained when primers specific to virus under study were used and cloned. Nucleotide sequence analysis of several clones of amplified SLRSV cDNA obtained from RNA extracts of infected leaves from different geographical locations were determined. The nucleotide sequence SLRSV Egyptian isolates Fig (5) Was aligned by using DNAMAN program with four isolates for SLRSV available in Gen Bank Comparison between bases composition of coat protein gene sequence for SLRSV Egyptian isolates with four isolates for SLRSV available in GenBank, was done to determine A+T, G+C content as shown in Table (4), the Egyptian SLRSV isolate was A+T, G+C identical content with SLRSV Poland and SLRSV USA which recorded 251, 246 respectively.

Fig (5): Nucleotide sequence of cloned DNA of 497 bp from the coat protein gene of SLRSV Egyptian isolate.

Table (4): Comparison between bases composition of coat protein gene sequence for SLRSV Egyptian isolate and four SLRSV isolates available in GenBank.

SLRSV isolates	Total base pair	Bases											
		A		C		G		T		A + T		C+G	
		No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
Egypt	497	112	22.53	138	27.76	108	21.73	139	27.96	251	50.50	246	49.49
Poland	497	112	22.53	136	27.36	110	22.13	139	27.96	251	50.50	246	49.49
USA	497	122	24.54	137	27.56	109	21.93	129	25.95	251	50.50	246	49.49
UK	497	114	22.39	139	27.96	114	22.93	130	26.15	244	49.09	253	50.90
Newzeland	497	107	21.52	130	26.15	111	22.33	144	29.97	256	51.50	241	48.49

(A): Adenine (C): Cytosine (G): Guanine (T): Thymine

The results showed that the Egyptian isolate nucleotides arranged with the highest number of Adenine 112 (22.53%) and Guanine (G) 108 (21.73%), then Thymine (T) 139 (27.96%) and Cytosine (C) 138 (27.76%) respectively.

Nucleotide sequence analysis for 497bp amplified fragment from the coat protein gene of the SLRSV genome showed similarity ranged from 99%, 84%, 83% and 80% when it compared with the other SLRSV sequence isolates available in the GenBank. SLRSV Egyptian isolate shared 99% identity with Poland isolate followed by 84% USA isolate, 83% UK isolate and 80% Newzelanda isolate Fig. (6). Phylogenetic tree was constructed for the different

sequences comparison and the amino acid translation using DNAMAn software (Fig.7 &8).

The obtained results are in agreement with [2]. Showed that Sequence analysis and the phylogenetic tree among the DNA nucleotide sequences ranged of 80 to 85 % similarity with the different SLRV isolates in the GenBank, [13]. Nucleotide sequence of the coat protein genes of Strawberry latent ringspot virus (SLRSV) where located in RNA-2. [21], analyzed the genomic RNA of Strawberry latent ringspot Nepovirus (SLRSV) isolate Hampshire H. The product PCR was 98.8 K polyprotein. The gene specific for coding capsid protein within RNA2 has 3824 pb nucleotides.

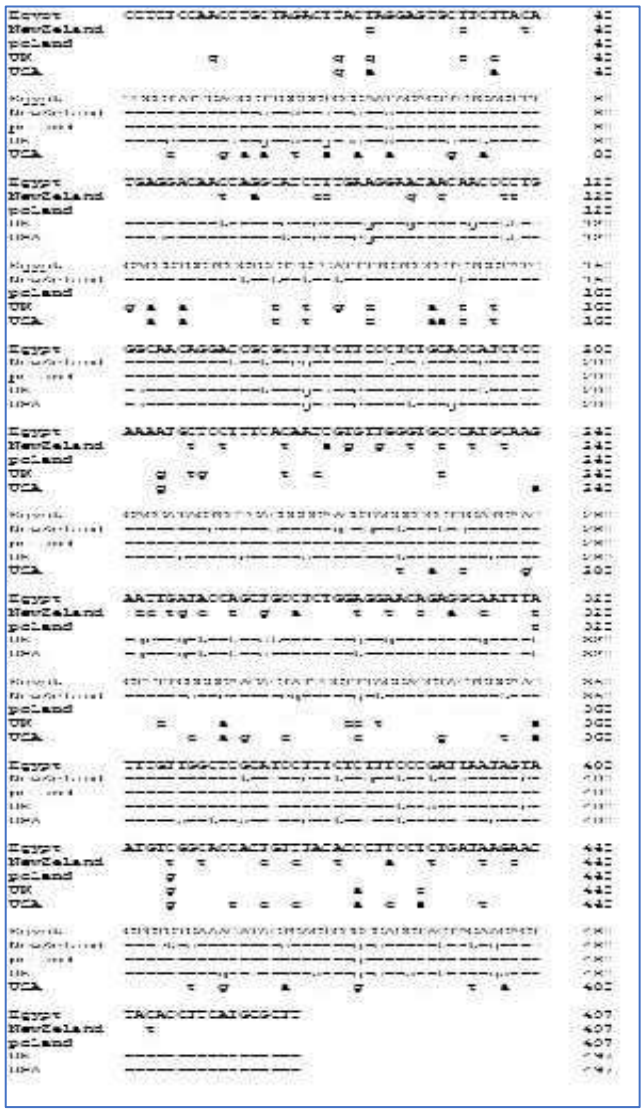

Fig (6): Multiple sequence alignment of coat protein gene of the Egyptian SLRSV isolate with corresponding sequence of the four-major different SLRSV isolates available in the GenBank.

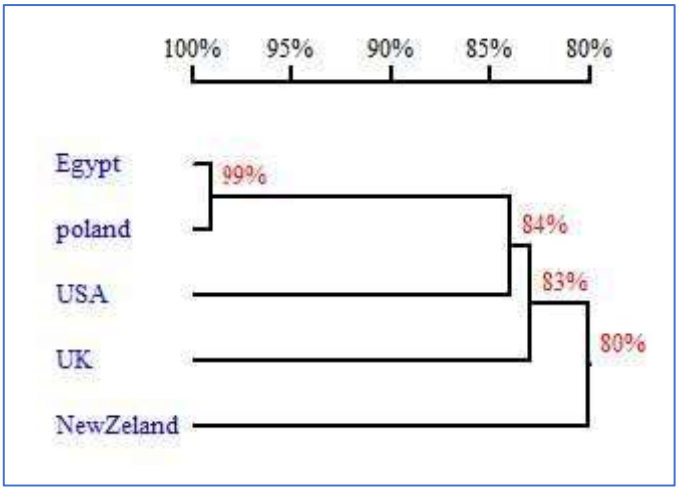

Fig (7): Phylogenetic tree constructed from multiple sequence alignment of coat protein gene of the Egyptian SLRSV isolate with corresponding sequence of the four-major different SLRSV isolates available in the GenBank.

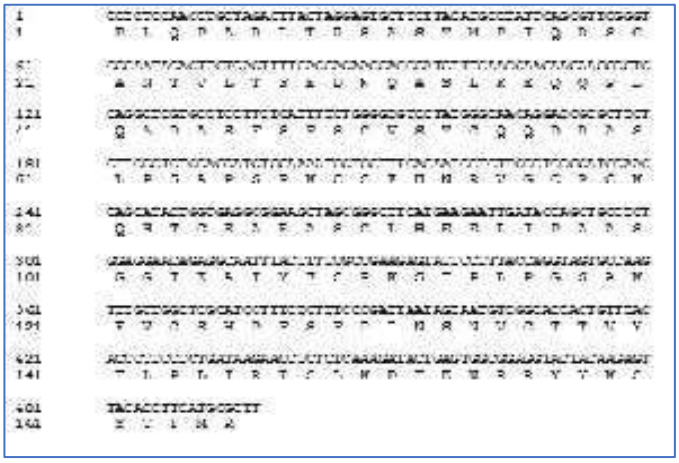

Fig (8): Nucleotide sequences and predicted amino acid sequence of the cDNA of the SLRSV CP gene.

4. CONCLUSION

In conclusion, the present data confirmed that SLRSV should be considered an important pathogen of strawberry, even if its presence in the surveyed regions is limited to well defined areas. Moreover, the establishment of a reliable, sensitive and quick molecular diagnostic method, such as the one-step RT–PCR will allow detection and identification of SLRSV in the early stages of strawberry cultivation. Thus, SLRSV control will be simpler and easier than previously and knowledge of the health status of strawberry for commercial and agricultural purposes will be improved.

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