Research Article

# Developed One-step Reverse Transcription Loop-Mediated Isothermal Amplification (Rt-Lamp) Assay for Detection of Strawberry Latent Ring Spot Virus

**Ahmed A. Kheder**

*Virus and Phytoplasma Research Department, Plant Pathology Research Institute, AgriculturalResearch Center, Giza, Egypt.*

**Abstract** | Strawberry latent ring spot virus (SLRSV) is an important virus which is responsible for considerable losses in horticulture and vegetable crops production including strawberry plants. SLRSV was detected and characterized using serological (DAS-ELISA), biological and molecular diagnostic techniques. Virus incidence was performed in different locations in five governorates during 2018-2020. Forty-one samples out of 693(5.94%) reacted positively to SLRSV. Specifically, the percentages of infection were 7.3, 6.7, 5.4, 5.16 and 2.63% in El-Qalyubia, El-Beheira, El- Menoufia, El-Sharkia and El-Ismailia governorates respectively. SLRSV was mechanically transmitted from infected strawberry plants onto ten herbaceous plant species, different types of symptoms have been observed associated to virus infection on indicator hosts; *Mentha spicata, Chenopodium quinoa, Nicotiana tabacum cv.* White Burley and *Vicia faba* after 15, 18, 20 and 22dpi respectively. Reverse transcription polymerase chain reaction (RT-PCR) was used to amplify ~497bp for coat protein gene in RNA 2, the amplified product was confirmed with direct sequences. Phylogenetic analysis results indicated that SLRSV-Eg isolate under study (acc. no. MT648777.1), showed 65.9 – 99.5% nucleotide similarity with available homologous sequences from other crops. Reverse-transcription loop-mediated isothermal amplification (RT-LAMP) assay which is one of the most promising molecular diagnostic techniques was applied. The amplified products were analyzed using amplification curves and electrophoresis. In this study SLRSV was characterized and detected with DAS-ELISA and molecular diagnostic techniques the obtained results concluded that the virus was varied due to locations, also all tested cultivars were susceptible to virus infection and the sensitivity of the RT-LAMP protocol was tenfold higher than that of conventional RT-PCR. Finally it is recommended to use LAMP assay which is useful for researchers, seed production specialists and was suggested to the Egyptian quarantine system which is interested in determining strawberry virus infections by using a single assay.

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***Correspondence** | Ahmed A. Kheder, Virus and Phytoplasma Research Department, Plant Pathology Research Institute, Agricultural Research Center, Giza, Egypt; **Email:** ahmed.zakaria@arc.sci.eg
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## Introduction

Strawberry (*Fragaria ananassa* Duch) is widely grown hybrid species of the genus *Fragaria* spp, it is considered one of the most important members of the family *Rosaceae*. Its fruit is rich in vitamins A, B and C, potassium, calcium, magnesium, iron, sulfur and phosphorus. Strawberry plants were first bred in France in the 1750s via a cross of *Fragaria virginiana* and *Fragaria chiloensis*. *Fragaria vesca* woodland strawberry was the first strawberry species cultivated in 17th century, have been replaced with *Fragaria ananassa* in commercial production (Welsh, 2008). Egypt is among the great's countries that produce strawberry and is considered as the fourth-largest producer in the world after the United States, Turkey and Spain by yields per hectare of about 390966 hg/ha the total harvested area reached 11772 hectares with production about 460245 tons in 2019 (FAO, 2019), the planting area has been annually increased. Strawberry has become one of the most economically important vegetable crops in El-Menoufia, El-Qalyubia, El-Sharkia, El-Beheira and El-Ismailia governorates. Strawberry plants are propagated vegetatively and it attacked with many viruses and virus-like disease during plant propagation and fruit production stages. Strawberry plants are usually symptomless when infected with one virus and often in mixed infections having serious effects on vigor and yield (Pisi, 1986; Martin, 2004 and Tzanetakis, 2006). *Strawberry latent ring spot virus* (SLRSV), first characterized in Scotland from strawberry and raspberry by Lister (1964) and is conceded one of the most common viruses of cultivated strawberry plants and occurs where ever they are grown and was classified as unassigned species in family Secoviridae (Mayo and Jones, 1999). Recently, this virus is classified as a member of genus *Stralarivirus* belonging to family *Secoviridae* (Dullemans *et al*., 2020) with a worldwide distribution, producing symptoms that includes mottling, mosaic, ring spot and systemic necrosis; often particularly in the late stage infections are symptomless (Murant *et al*., 1981). SLRSV infects a wide range of hosts including many economically crops such as small fruit (strawberry, blackberry and raspberry), stone fruit (plum, cherry and peach), vegetables (celery and parsley) grapevine, olive, ornamental plants (lily and rose) and tree species (*Aesculus* and *Robinia* spp.) (Schmelzer, 1969; belli *et al*., 1980; Credi *et al*., 1981; Faggioli *et al*., 2005; Mazyad *et al*., 2012 and El-Morsy *et al.,* 2017). The virus can be transmitted with plant sap by mechanical inoculation, through infected seeds, pollen and by soil-inhabiting nematodes *Xiphinema diversicaudatum and X. Coxi* (Murant, 1974). SLRSV appears as isometric particles 30nm in diameter (Faggioli *et al*., 2002), has a bipartite genome composed of two liner positive-sense single-stranded RNA molecules, RNA 1 ($M_r$ 2.9 x $10^6$), it encodes the proteins that are important in replication and RNA 2 ($M_r$ 1.4 x $10^6$), which encodes the proteins that are important in cell-cell movement and evasion of cellular defenses (Mayo *et al.,* 1982). In Egypt, SLRSV has been detected using ELISA and reverse transcription-polymerase chain reaction (RT-PCR) (Mazyad *et al.,* 2012 and El-Morsy *et al.,* 2017). Due to ELISA technique and/or RT-PCR reaction, the reaction time for these methods is time-consuming and laborious, requiring at lease 10hrs to obtain results, also gradient of cycling temperature between three amplification steps as a consequence, they are inconvenient for rapid diagnosis and point-of-care applications (Parida *et al*., 2008 and Sidoti *et al*., 2013). Attempts to find differences in coat protein of the different isolates of SLRSV were limited due to the lack of data in GenBank (Borodynko, *et al.,* 2007). In general, Loop-mediated isothermal amplification (LAMP) technique was found to be 10-100 fold more sensitive than PCR with a detection limit of 0.01-10 pfu of the virus (Thai *et al*., 2004) and The specificity of the LAMP amplification is referred to the six sets of primers spanning eight distinct sequences of the target gene that are being used for amplification. In this study, SLRSV was isolated and characterized based on serological, biological, and molecular studies and developed molecular tools based on RT-LAMP for rapid and specific SLRSV detection compared with standard RT-PCR detection method.

## Materials and methods

*Source of virus isolate and virus incidence*

Strawberry leaves showing virus-like symptoms and others symptomless were collected from commercial strawberry fields located in El-Menoufia, El-Qalyubia, El-Sharkia, El-Beheira and El-Ismailia governorates during the spring and early summer seasons of 2018- 2020. About 693 samples were collected from different locations of strawberry cvs. (Festival, Selva, Camarosa and Florida). The collected leaf samples were tested by direct enzyme-linked immunosorbent assay (DAS-ELISA) as described by Clark and Adams (1977) using commercial antisera

**Table 1:** *Sequence of degenerate primers for subgroup detection and sense and antisense specific primers used in RT-PCR and RT-LAMP and their nucleotide positions in the genome.*

| Primer Code | Sequences ('5-3') | Subgroup/ Virus | Location / position | Amplification product | References |
|---|---|---|---|---|---|
| Conventional RT-PCR | | | | | |
| Nepo-A (s/a) | GGHDTBCAKTMYSARRARTGG | Subgroup A | CP-RNA2 | 255 bp | Digiaro et al. (2007) |
| | TGDCCASWVARYTCYCCATA | | | | |
| Nepo-B (s/a) | ATGTGYGCHACYACWGGHATGCA | Subgroup B | CP-RNA2 | 390 bp | |
| | TTCTCTDHAAGAAATGCCTAAGA | | | | |
| Nepo-C (s/a) | TTRKDYTGGYKAAMYYCCA | Subgroup C | UTR-RNA1 | 640 bp | |
| | TMATCSWASCRHGTGSKKGCCA | | | | |
| SLRSV-F(sp) | CCTCTCCAACCTGCTAGACT | SLRSV | CP-RNA2 | 497 bp | Martin et al., (2004) |
| SLRSV-R(sp) | AAGCGCATGAAGGTGTAACT | | | | |
| Loop-mediated isothermal amplification primers | | | | | |
| SLRSV F3 | TTCTTATGGACAACAGGATCG | SLRSV | CP-RNA2 | | This study |
| SLRSV B3 | CCACATTACTGTTGATCGGA | | | | |
| FIP(F1c+F2) | TCCACTAGCTTCCGCCTCA-TCCAAA-GTGCTCCTTTCAC | | | | |
| BIP(B1c+B2) | AGTTGGTTCCTGCAGCCTCCCTGGTA-GAGGAATGCTCT | | | | |
| LoopF | CCAGTATGTTGTTTGCATGGG | | | | |
| LoopB | TGGTGGAACAGAGGCAATC | | | | |

**sp: specific; s: sense; a: antisense; B (C+G+T); D (A+G+T); H (A+C+T); M (A+C); R (A+G); S (C+G); W (A+T); Y (C+T); K (G+T).*

(Loewe Biochemica, Sauerlach, Germany). Positive samples were confirmed by RT-PCR.

*Mechanical transmission and host rang*

Strawberry leaf samples which reacted positively with DAS-ELISA were used as source of virus and used for mechanical inoculation onto ten different plant species belonging to families *Amaranthaceae, Chenopodiaceae, Cucurbitaceace, Lamiaceae, Leguminosae, Rosaceae and Solanaceae* and kept under greenhouse conditions 25˚C±5 and 65±5% RH for 2-3weeks and were observed for symptoms development. All mechanically inoculated plants were examined by ELISA and confirmed by both RT-PCR and RT-LAMP. Isolate used in this study was obtained by the single local lesion isolate method, a single local lesion formed on *Ch. quinoa* was used to inoculate *Mentha spicata* leaves. Infected *Mentha spicata* plants which reacted positively to SLRSV were served as source of virus inoculum (Boonham and wood, 1998).

*Molecular detection*

*Viral RNA extraction and one-step RT-PCR amplification*

Total RNA was extracted from about 150mg fresh leaf tissues of healthy and infected strawberry plants as well as mechanically inoculated plants of *Gomphrena globose, Ch. quinoa* wild, *Cucurbita pepo*, *Mentha spicata, Vicia faba, Fragaria ananassa* cv. Festival, *Datura stramonium, Lycopersicon esculentum c.v* Castle Rock, *Petunia hybrida* and *N. tabacum cv.* White Burley, then re-suspended in 50µl of RNase-free water as described in the manufacture instructions of Geneaid RNA extraction mini Kit (Geneaid - Taiwan). The RNA quality and quantity were assessed using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts, USA), and the final concentration was adjusted to approximately 150 ng/µl. Reverse transcription-polymerase chain reaction (RT-PCR) was done with reverse Verso™ one-step RT-PCR Reddy Mix Kit (Thermo Scientific) according to the manufactory instruction. RT-PCR assay with degenerate primers for detection of *nopovirus* subgroups were performed in 25µl final volume containing 12.5µl of one-step RT-PCR master mix, 5µl of nuclease-free water, 3µl of total RNA, 1.5µl (10µM) sense and antisense of each degenerate primers (Table 1), 1.25µl RT-Enhancer and 0.25µl Verso enzyme. Different combinations of denaturing and annealing conditions (temperature and time of treatment)


**Figure 1:** *Targeted amplification region on the coat protein gene RNA2 of Strawberry latent ring spot virus (MT648777.1) for RT-LAMP assay.*

were used with each set of primers for determining the optimal operative conditions for PCR amplification. Complementary DNA (cDNA) synthesis was done at 50˚C for 15min, 94˚C for 5 min; followed by 35 cycles of 94˚C, 35 sec, 50˚C, for 45 sec and 72˚C for 1min and a final extension for 7 min at 72˚C for subgroups A, B and C. *Grapevine fanl eaf virus* (GFLV) and *Tomato ring spot virus* (ToRSV) were provided from Plant Pathology Research Institute, ARC, Egypt, and were used as positive control for *nepovirus* subgroups A and C respectively. RT-PCR assay with specific primer was done in 25µl total volume containing 12.5µl of one-step PCR master mix, 5µl of nuclease-free water, 3µl of total RNA, 1.5µl (10µM) specific forward and reverse primers in Table (1), 0.25µl Verso enzyme and 1.25µl RT-Enhancer. Synthesis of (cDNA) was done at 50˚C for 15min, 94˚C for 3 min; followed by 40 cycles of 94˚C, 20 sec, 52˚C, for 30 sec and 72˚C for 30 sec and a final extension for 5 min at 72˚C. 6µl of the amplified products were loaded in 1.5% agarose gel prepared in TAE buffer (1x), stained with EZview stain (Biomatik- USA) and visualized using a UV transilluminator. The size of the amplified products was estimated using 100bp DNA Ladder (Biomatik - USA).

*Sequencing and phylogenetic analysis*

The RT-PCR product was purified using Gel/PCR DNA Fragments Kit (Genaid –Taiwan), and direct sequenced on an ABI automatic sequencer (Applied Biosystem, NJ, USA). Nucleotide and amino acid sequences were compared with the sequences available in the GenBank database. The multiple sequence alignments were performed using ClustalW (Thompson et al., 1994). The alignments were used to calculate pairwise genetic distances by Kimura's two-parameter method. Phylogenetic analysis was carried using the Maximum Likelihood method implemented with Molecular Evolutionary Genetics Analysis software (MEGA) version X (Kumar et al., 2018). Bootstrap values for phylogenetic comparisons are based on 1000 pseudoreplicates.

Real-time loop-mediated isothermal amplification (RT-LAMP) assay LAMP primers were designed using LAMP Designer 1.12 software program. Based on the alignment of five isolates of SLRSV coat protein sequence available in GenBank (Accession Nos. X77466, DQ324374.1, MF796996.1, AY438666.1 and AY860979.1) with Egyptian isolate SLRSV-Eg (Accession No. MT648777.1). Alignment was constructed with MEGA X software to identify conserved SLRSV genomic regions. A set of six primers for the viral coat protein gene comprising two outer (F3 and B3), two inner (FIP and BIP) and two loop primers (FLP and BLP), that recognize eight distinct regions on the target sequence was designed (Figure 1). The primer sets used in this study are listed in Table 1. RT-LAMP was carried out in 25µl reaction containing 15µl Isothermal Master Mix (OptiGene, Horsham, UK) contains fluorescence dye, 200 nM each external primer (F3 and B3), 2µM each internal primer (FIP and BIP), 1µM each loop primer (F-loop and B-loop), 1.2 units Thermo-Script reverse transcriptase (Life Technologies, CA, USA) and 1 µl RNA. Reaction mixtures were incubated at 50˚C for 10 min, then 60˚C for 30 min, the reactions were subjected to a slow annealing step (0.05˚C/s) from 95˚C to 75˚C with fluorescence monitoring. Reactions containing GFLV, ToRSV and water instead of SLRSV-RNA were used as the negative control in each run (Tomlinson et al., 2013). To assess the sensitivities of

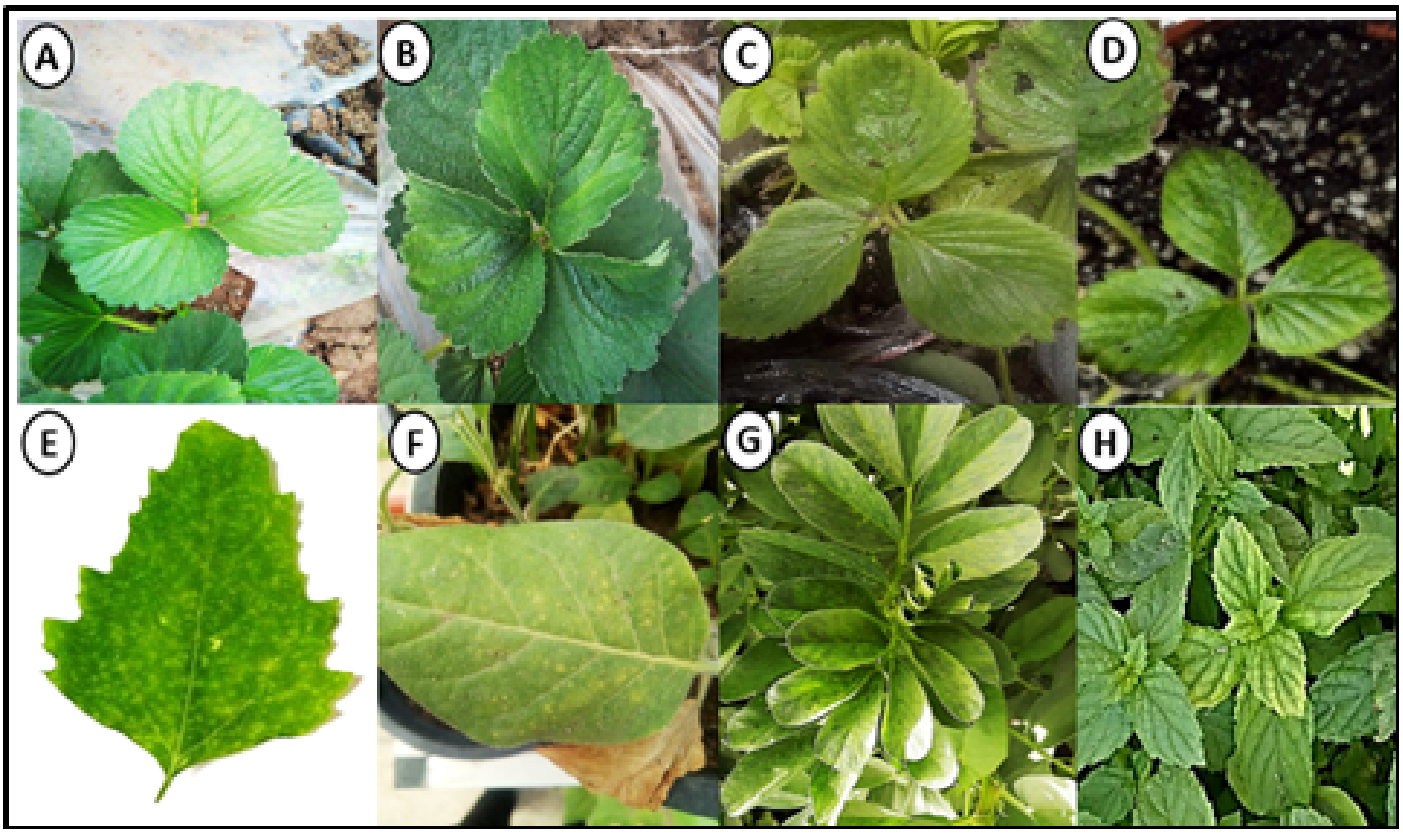


**Figure 2:** *Symptoms of Strawberry latent ring spot virus (SLRSV). Naturally infected strawberry plants showing viral like symptoms (A) symptomless plant (B) showing mottling. Mechanically inoculated herbaceous hosts: (C and D) mechanically inoculated Fragaria ananassa cv. Festival leaves showed yellowing and mottling, (E) systemic local lesion on Chenopodium quinoa, (F) Chlorotic local lesion on Nicotiana tabacum cv. White Burley, (G) mosaic vein clearing and chlorosis on Vicia faba plant and (H) vein banding on Mentha spicata.*

**Table 2:** *Incidence of Strawberry latent ringspot virus (SLRSV) infection in strawberry plants in different location in Egypt.*

| Location / Cultivar | El-Qalyubia | | | El-Beheira | | | El-Menoufia | | | El-Sharkia | | | El-Ismailia | | | Total | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *Tested | Infected | % | *Tested | Infected | % | *Tested | Infected | % | *Tested | Infected | % | *Tested | Infected | % | *Tested | Infected | % |
| **Festival** | 48 | 4 | 8.3 | 46 | 3 | 6.5 | 50 | 4 | 8 | 32 | 2 | 6.3 | 22 | 1 | 4.55 | 198 | 14 | 7.07 |
| **Selva** | 46 | 3 | 6.5 | 32 | 2 | 6.3 | 47 | 3 | 6.4 | - | - | - | - | - | - | 125 | 8 | 6.4 |
| **Camarosa** | 33 | 3 | 9.1 | 55 | 4 | 7.3 | 35 | 1 | 2.9 | 30 | 1 | 3.3 | 30 | 1 | 3.33 | 183 | 10 | 5.46 |
| **Florida** | 37 | 2 | 5.4 | 44 | 3 | 6.8 | 43 | 2 | 4.7 | 34 | 2 | 5.9 | 29 | 0 | 0 | 187 | 9 | 4.81 |
| **Total** | 164 | 12 | 7.3 | 177 | 12 | 6.7 | 175 | 10 | 5.5 | 96 | 5 | 5.2 | 81 | 2 | 2.63 | 693 | 41 | 5.94 |

* Data are based on enzyme-linked immunosorbent assay (DAS-ELISA) of collections during 2018-2020. ELISA value absorbance at 405nm.
* No. of tested plants in 3 years from **2018 to 2020**.
* Positive samples were confirmed with RT-PCR and RT-LAMP

the RT-LAMP assay and the standard RT-PCR approach, the concentration of total RNA from highly infected strawberry plants with SLRSV was adjusted to 100ng/µl then serially diluted. RT-LAMP reactions were done using 1µl of serially diluted templates as well as conventional one-step RT-PCR. The amplified products of RT-LAMP and RT-PCR were analyzed by electrophoresis using 1.5% agarose gels containing EZview stain (Biomatik - USA) and visualized using a UV transilluminator. All RT-LAMP runs were performed at least twice.

## Results and Discussion

The virus under investigation was isolated from naturally infected strawberry plants in Egypt showing virus-like symptoms and others symptomless (Figure 2 A and B) and identified on the basis of symptomatology, mechanical transmission, serology, and molecular assays. All data obtained confirmed that the virus under investigation was *Strawberry latent ring spot virus* (SLRSV).

*Source of virus isolate and virus incidence*

Virus incidence was checked during the spring and early summer seasons of 2018- 2020 in strawberry fields in five different governorates, the viruses infection were detected using DAS-ELISA, among those

**Table 3:** *Reaction of some inoculated host plants to SLRSV infection.*

| Family/ tested host | Reaction/infectivity | ELISA test | RT-PCR | RT-LAMP |
|---|---|---|---|---|
| **Amaranthaceae** | | | | |
| *Gomphrena globose* | N.S/ (0/10) | - | - | - |
| **Chenopodiaceace** | | | | |
| *Ch. quinoa* wild | SLL/ (9/10) | + | + | + |
| **Cucurbitaceace** | | | | |
| *Cucurbita pepo* | N.S /(0/10) | - | - | - |
| **Lamiaceae** | | | | |
| *Mentha spicata* | V.B/(8/10) | + | + | + |
| **Leguminosae** | | | | |
| *Vicia faba* | M /(6/10) | + | + | + |
| **Rosacea** | | | | |
| *Fragaria ananassa* cv. Festival | N.S /(5/10) | + | + | + |
| **Solanaceae** | | | | |
| *Datura stramonium* | N.S / (0/10) | - | - | - |
| *Lycopersicon esculentum* cv. Castle Rock | N.S / (0/10) | - | - | - |
| *N. tabacum* cv. White Burley | LL / (7/10) | + | + | + |
| *Petunia hybrida* | N.S / (0/10) | - | - | - |

* LL: local lesion, * M: Mosaic, * N.S: No Symptoms, * SLL: systemic local lesion, * V.B: Vein banding,* - : Negative reaction and * +: Positive reaction.

viruses, SLRSV was found to be the most prevalent virus in strawberry mean cultivated areas, data in (Table 2) indicated that in total, 41 out of 693 (5.94%) strawberry plants tested positive to SLRSV and the average of infection in strawberry cultivars Festival, Selva, Camarosa and Florida were about 7.0%, 6.4%, 5.4% and 4.8% respectively. Also, the percentages of infection were about 7.3%, 6.7%, 5.4%, 5.16% and 2.63% in El-Qalyubia, El-Beheira, El- Menoufia, El-Sharkia and El-Ismailia in tested samples respectively. The samples which gave positive results with DAS-ELISA were confirmed by RT-PCR.

*Mechanical transmission and Host rang*

Ten plant species belonging to seven families: *Amaranthaceae, Chenopodiaceae, Cucurbitaceace, Lamiaceae, Leguminosae, Rosaceae and Solanaceae,* were mechanically inoculated with the virus preparation to determine the host range. Data presented in Table (3) and (Figure 2), illustrated the reaction of mechanically inoculated plants for SLRSV-Eg infection under greenhouse conditions (25˚C±5 and 65±5% RH). SLRSV-Eg isolate produced vein banding on *Mentha spicata*; mottling systemic local lesion on inoculated leaves of *Ch. quinoa* wild; chlorotic local lesion on *Nicotiana tabacum cv.* White Burley and mosaic on the inoculated leaves of *Vicia faba* plants these symptoms were observed after 15, 18, 20 and 22 days post-inoculation (dpi) respectively with different infection rates. Results were confirmed by DAS-ELISA, RT-PCR and RT-LAMP assays. Moreover, inoculated *Fragaria ananassa cv.* Festival leaves showed no symptoms as well as ELISA, RT-PCR and RT-LAMP assays succeeded to detect the virus (20dpi). while, inoculated *Datura stramonium*, *Gomphrena globose, Cucurbita pepo, Petunia hybrid* and *Lycopersicon esculentum cv.* Castle Rock were not infected with the virus and ELISA, RT-PCR and RT-LAMP assays failed to detect the virus from these hosts 30dpi.

*Molecular detection*

*RT-PCR and sequence analysis*

The extracted RNA from naturally infected strawberry plant and all the inoculated plants were tested for SLRSV infection by RT-PCR. Amplicon of 497bp was obtained using specific primers SLRSV-F and SLRSV-R for detection of SLRSV, designed to amplify fragment of CP-RNA2 of SLRSV-Eg isolate. RT-PCR product of the expected size were obtained from four herbaceous plant species, as well as symptomatic and asymptomatic strawberry plants (Table 3 and Figure 3). Electrophoresis analysis of RT-PCR product showed a single amplified fragment of about 497bp presumably from the part of coat protein gene of SLRSV (Figure 3). Direct sequencing of the amplicon confirmed that is SLRSV and the consensus sequence obtained was deposited in GenBank (accession number MT648777.1). Moreover, No amplicons were obtained when using the *nepovirus* degenerate primers Nepo-A (s/a), Nepo-B (s/a) and Nepo-C (s/a) while, the one-step RT-PCR reactions generat-

ed the expected amplicons for all positive controls (Figure 3). Data confirmed that no mixed infection with *nepovirus* group such as *Grapevine fanleaf virus* (GFLV), *Tomato ring spot virus* (ToRSV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV) and *Raspberry ring spot virus* (RPRSV).

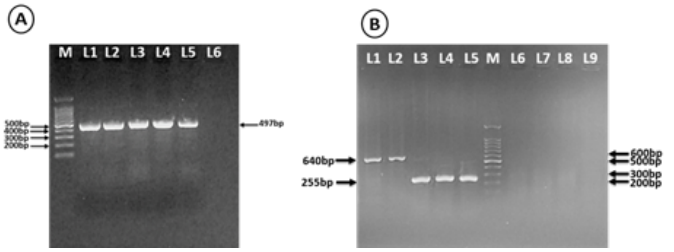


**Figure 3:** *Agarose gel electrophoresis analysis of one-step RT-PCR amplified products of naturally infected and mechanically inoculated plants. (A) Using CP-RNA2 specific primer, Lane 1: Naturally infected strawberry plants, Lane 2: inoculated Ch. quinoa wild, Lane 3: inoculated Vicia faba, Lane 4: inoculated Fragaria ananassa cv. Festival, Lane 5: inoculated N. tabacum cv. White Burley, Lane 6: empty and M: 100pb Marker (Biomatic-USA). (B) using nepovirus degenerate primers, Lane1: ToRSV as a positive control of group C, Lane 2: naturally infected strawberry plant detection with Nepo-C (s/a) Lane 3: GFLV as a positive control of group A, Lane 4-5: Naturally infected strawberry plant detection with Nepo-A (s/a), Lane 6, 7 and 8: Naturally infected strawberry plant with SLRSV detected with Nepo-A (s/a), Nepo-B (s/a) and Nepo-C (s/a) respectively, Lane 9: Negative control and M: 100pb Marker (Biomatic-USA).*

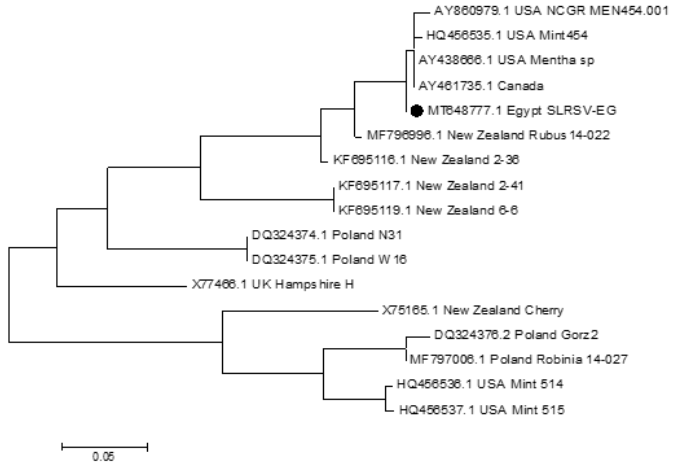


**Figure 4:** *Phylogenetic relation of Strawberry latent ring spot virus isolates based on a fragment of RNA2. The numbers at each branch point indicate bootstrap values, branch values shown are also 99.5 – 65.9%. Nucleotide sequences used for above analysis available in GenBank.*

Phylogenetic analysis was carried out to compare sequences across the region of *CP-RNA2* (497bp) from the Egyptian isolate (SLRSV-Eg) with other SLRSV isolates overseas as shown in (Figure 4 and Table 4), a Pairwise comparisons of the 497bp nucleotide sequence revealed that the USA (MEN454) and Canada isolates indicate 99.5% homology and 97.8% amino acid sequence identities. In comparison to Poland isolate (Robinia 14-027) it shared 65.9% nucleotide 90.4% amino acid sequence identities. Pairwise comparisons showed that the USA isolates (SLRSV- Mint454 and NCGR_MEN454.001) had 99% and 98.6% nucleotide identities and 96.4 and 97.8% amino acid sequence identities respectively. While, New Zealand isolates Rubus14-022, 2-36, 2-41 and 6-6 were distinct from the Egyptian isolate and shared 96.5%, 94.5%, 82.9% and 82.9% nucleotide identities respectively, both Polish isolates N31 and W16 shared 76.6%.

*Detection of SLRSV by RT-LAMP using the designed primer sets*

The *CP-RNA2* gene of SLRSV was chosen as the amplification target. SLRSV detection was carried out successfully using real-time RT-LAMP assay on healthy and infected strawberry plants as well as mechanically inoculated plants, the obtained results revealed that the *CP-RNA2* gene of SLRSV isolate can be amplify rapidly within 15-40 minutes based on virus concentration (Figure 5), the sensitivity was gradually boosted by using 50°C for 10 min for cDNA synthesis followed by a single incubation temperature of 60°C for 30 min then slow annealing step (0.05°C/s) from 95°C to 75°C. Amplification products were visualized by 1.5% agarose gel electrophoresis exhibited typical ladder-like pattern of LAMP products and no visible amplification product was observed from RNA isolated from GFLV, ToRSV and healthy strawberry plants as well as water controls as shown in (Figure 5 a and b), demonstrating the high specificity of RT-LAMP primers for detection of the virus. Obtained results confirmed that the RT-LAMP assay allows detection of SLRSV in $10^{-4}$ dilution of total RNA, while the detection limit of the conventional RT-PCR was about $10^{-3}$ as seen in Figure 5b. Therefore, the RT-LAMP assay is 10 times more sensitive than conventional RT-PCR in addition to the important advantages of the RT-LAMP assay as the very short detection time furthermore it doesn't require expensive equipment.

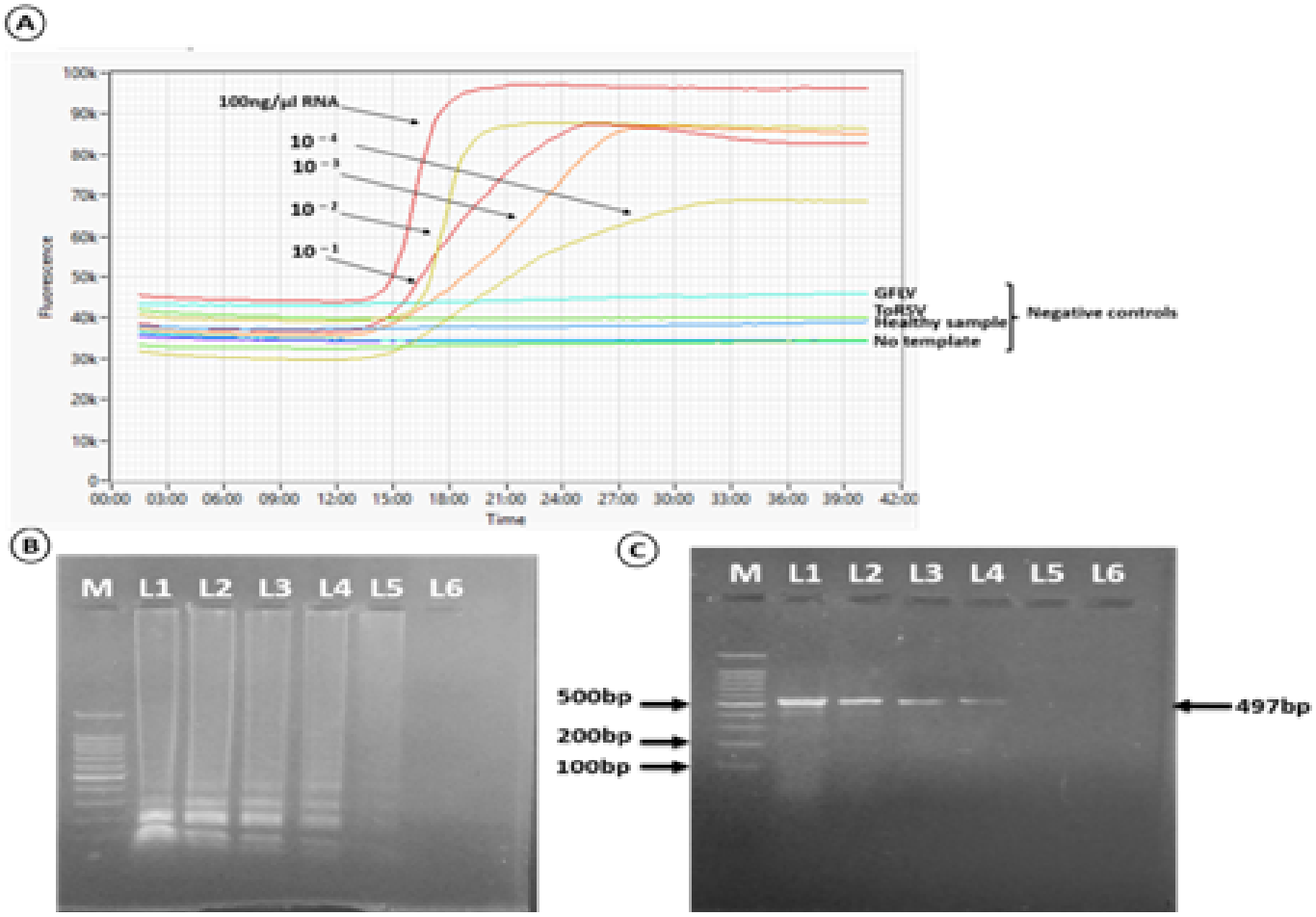


**Figure 5:** *Sensitivity comparison between the RT-LAMP assay and the RT-PCR detection method. The detection limit of the RT-LAMP assay was determined based on real-time amplification plots and no visible amplification of GFLV, ToRSV and healthy samples as well as water control (A), Gel electrophoresis (b) and one-step RT-PCR (C). M. 100pb molecular marker (Biomatic-USA); Lane 1, total RNA 100ng/µl; Lane 2-6 tenfold serial dilutions of total RNA. The order of samples in panel C is the same as in panel B.*

The current study dealt with characterization and detection of isolate of *Strawberry latent ring spot virus* (SLRSV) in strawberry plants in Egypt showing virus-like symptoms and others symptomless and identified on the basis of symptomatology, mechanical transmission, serology, and molecular assays. During the spring and early summer seasons of 2018- 2020 in strawberry fields in five different governorates, the viruses infection were detected using DAS-ELISA. Although, the serological methods are simple and easy to perform, false results can occur due to the instability and altered distribution of the virus in the host plants, Moury *et al.* (2000), reported that RT-PCR can exceed DAS-ELISA in sensitivity to detect plant viruses especially with symptomless hosts, as well as the reaction time for these methods is time-consuming and laborious, requiring at lease 10hrs to obtain results reported by Parida *et al.* (2008). The LAMP assay has been used for the molecular detection and diagnosis of many pathogens, including bacteria, viruses, *Spiroplasma* (Haj Ali, M. 2020), fungi, and parasites responsible for plant, animal, and human diseases (Przewodowska et al., 2015). During the spring and early summer seasons of 2018- 2020 virus incidence was checked in strawberry fields in five different governorates using DAS-ELISA, among those viruses, SLRSV was found to be the most prevalent virus in strawberry mean cultivated areas. The incidence results showed that dissemination of the virus was varied due to locations, moreover all tested cultivars were susceptible to infection. These results are in agreement with Mazyad *et al.* (2014) and El-Morsy *et al.* (2017) in Egypt, which found that SLRSV is limited in the main strawberry growing areas checked by DAS-ELISA and the virus infection varied from region to region as well as from season to season. Also, the obtained results contrasted with Everett *et al.* (1994) they identified SLRSV in a single sweet cherry trees and was thought to be rare in New Zealand. This virus was detected with low incidence

**Table 4:** *Comparison of the nucleotide and Amino acid sequences identities of Strawberry latent ring spot virus (SLRSV) isolated in the present study (SLRSV-EG, Acc. No. MT648777.1) with the corresponding sequences available in the GenBank database.*

| Accession No. | Country | Isolate | Host Plant | N.A Id % | A.A Id% |
|---|---|---|---|---|---|
| MT648777.1 | Egypt | SLRSV-EG | *Fragaria ananassa cv.* Festival | | |
| AY438666.1 | USA | MEN454 | *Mentha x gentilis* 'Variegata | 99.5% | 97.8% |
| AY461735.1 | Canada | Strawberry isolate | Fragaria X ananassa cv. Totem | 99.5% | 97.8% |
| HQ456535.1 | USA | Mint454 | *Mentha x gracilis* Sole | 99.0% | 96.4% |
| AY860979.1 | USA | NCGR_MEN454.001 | *Mentha x gentilis* 'Variegata | 98.6% | 97.8% |
| MF796996.1 | New Zealand | Rubus14-022 | Cucumis sativus | 96.5% | 97.8% |
| KF695116.1 | New Zealand | 2-36 | Vaccinium darrowii | 94.5% | 97.8% |
| KF695117.1 | New Zealand | 2-41 | Vaccinium darrowii | 82.9% | 97.8% |
| KF695119.1 | New Zealand | 6-6 | Vaccinium darrowii | 82.9% | 97.8% |
| DQ324374.1 | Poland | N31 | Robinia pseudoacacia | 76.6% | 97.1% |
| DQ324375 | Poland | W16 | Robinia pseudoacacia | 76.6% | 97.1% |
| X77466 | UK | Hampshire H | Cherry | 73.3% | 94.2% |
| X75165 | New Zealand | flowering cherry | Cherry | 71.3% | 93.8% |
| HQ456537 | USA | Mint 515 | Mentha arvensis L. | 67.1% | 91.2% |
| DQ324376.2 | Poland | Gorz2 | Robinia pseudoacacia | 66.3% | 90.4% |
| HQ456536 | USA | Mint 514 | Mentha arvensis L. | 66.9% | 91.2% |
| MF797006.1 | Poland | Robinia_14-027 | Robinia pseudoacacia | 65.9% | 90.4% |

**N.A Id% = Nucleotide Acid sequence identities % * A.A Id% = Amino Acid sequence identities %*

in olive plants (Godena *et al*., 2016), while incidence of varied from 0.3% in Lebanon (Fadel *et al*., 2005), 6.7% in Croatia (Bjeliš *et al.,* 2007) and 29.2% in Italy (Saponari *et al*., 2002). The severity of the virus was assessed visually by developing symptoms, different types of symptoms have been observed associated to virus infection. Several studies have confirmed the previously described symptom pattern on different host plants. Previous studies cleared that the virus infects a broad range of hosts, including many economically important crops and is easy to be transmitted via infected sap to the tested host plants and induced symptoms, also, provided that SLRSV is an economically important virus due to its extensive host range and the yield losses it can cause. Similar results were obtained by Borodynko *et al.* (2007), Mazyad *et al*. (2014) and El-Morsy *et al*. (2017), who used the same techniques (DAS-ELISA and RT-PCR) for virus detection and host range. Also, These results are in agreement with those mentioned by Richmond *et al*. (1998), Martin *et al*. (2004), Postman *et al*. (2004) and Tzanetakis *et al*. (2006), study the mechanical transmission which reported that SLRSV was considered a quarantine virus in the United States until its recent identification in both strawberry and mint nurseries where the soil was treated with methyl bromide to eliminate nematode vestors and it had thought that the virus may be transmitted by insect vector but transmission studies failed to demonstrate transmission of the virus. Everett *et al*. (1994), suggested that an alternative mode of spread may be due to cultivation practices (e.g., grafting or vegetative propagation by cutting and or using infected materials or tools). Moreover, SLRSV was detected in a much wider range of hosts belong to different plant families, including *Ranunculaceae, Rosaceae, Balsaminaceae, Solanaceae, and Melastomataceae*. Tang *et al*. (2013) found that the reverse inoculation from SLRSV-infected *C. quinoa* to healthy impatiens plants resulted in a symptomless infection and reported that the possibility of latent infections may explain why SLRSV has gone undetected in a wider host range and were also found to be easier to transmit onto indicator plants and induce symptoms. In the present study obtained data confirmed that no mixed infection with *nepovirus* group such as *Grapevine fanleaf virus* (GFLV), *Tomato ring spot virus* (ToRSV), *Arabis mosaic virus* (ArMV), *Tomato black ring virus* (TBRV) and *Raspberry ring spot virus* (RPRSV). This result was similar to the previous results obtained Digiaro *et al.* (2007) who used the same primer sets and reported that, none of the three *nepovirus* degenerate primer sets were able to detect SLRSV in infected tissues, these data confirming the molecular distance of this virus

from nepovirus species. Also, agreed with Dullemans *et al.* (2020) who supported the assignment of SLRSV to a new genus in the family *Secoviridae*, for which the name "*Stralarivirus*" is proposed based on high-throughput sequencing (HTS) data analysis. Moreover, RT-PCR product of 497bp was obtained using specific primers SLRSV-F and SLRSV-R for detection of SLRSV, designed to amplify fragment of CP-RNA2 of SLRSV-Eg isolate. These result are in agreement with Martin *et al*. (2004), Mazyad *et al*. (2014) and El-Morsy *et al*. (2017), who had used the same primer and got same results. Direct sequencing of the amplicon confirmed that is SLRSV. Moreover, phylogenetic analyses of sequences of SLRSV-CP showed a high level of diversity about 65.9% – 99.5% nucleotide sequences and 90.4% - 97.8% amino acid sequence identities among the sixteen SLRSV-isolates from different parts of the world. Obtained results are in contrast with Mazyad *et al*. (2012) who reported that SLRSV Egyptian isolate showed a very low identity that ranged between 80-85% when compared with different SLRSV isolates available in GenBank. In contrast El-Morsy *et al*. (2017). Stated that SLRSV Egyptian isolate shard 99%, 84%, 83% and 80% identity with Poland, USA, UK, and New Zealand isolates respectively. Attempts to find differences in coat protein of the different isolates were limited due to the lack of data in GenBank. Also, both of the two Egyptian isolates which previously detected by Mazyad *et al*. (2012) and El-Morsy *et al*. (2017) are not available in GenBank database. Therefore, further studies, comprising of high-throughput sequencing (HTS) and/or complete nucleotide sequence are recommended to obtain more accurate homology between our isolate with other available SLRSV isolates. SLRSV control strategies have focused on the development of effective diagnostic methods aimed at early detection. Recently, nucleic acid-based amplification methods crave low expenses, rapid, specific and simple in comparison with traditional methods. LAMP does not require a thermal cycler or analysis software and can be done using water bath (Parida *et al*., 2008 and Sriworarat *et al*., 2015). These features of LAMP amplification assay make it efficient for detecting many pathogenic organisms including virus, bacteria, protozoa, *Spiroplasma* and fungi (Karanis and Ongerth, 2009; Mori and Notomi, 2009 and Haj Ali M., 2020). Therefore, LAMP assay will be a useful tool for on-site, rapid, reliable, and sensitive, detection of SLRSV in infected plants and diagnosis in quarantine. RT-LAMP is a powerful and flexible tool for monitoring microbial pathogens and shown to have superior sensitivity to DAS-ELISA and RT-PCR, in addition to symptoms or identification of infected materials in a wide range of hosts. Obtained results clearly concluded that the sensitivity of the RT-LAMP protocol was tenfold higher than that of conventional RT-PCR. These results corresponds with Liu *et al*. (2010), who reported that the detection limit of the RT-LAMP method for tobacco mosaic virus detection was 100 times higher than that of RT-PCR and demonstrate that the method is stable, sensitive and specific. These data in agreement with RT-LAMP sensitivity reports for other plant viruses of Zong *et al*. (2014), Przewodowska *et al*. (2015), Budziszewska *et al*. (2016), Kim *et al*. (2016) and Haj Ali M. (2020), these data indicate that the detection limit for these methods may be comparable. In contrast with Soliman and El-Matbouli (2006) who found that the detection limit of the RT-LAMP assay was similar to the commonly used RT-PCR method and concluded that both methods detected viral hemorrhagic septicaemia virus (VHS) RNA at a dilution of $10^{-6}$.

## Conclusion

The present study showed that SLRSV detected in five governorates, associated with asymptomatic movement of infected plants from nurseries production that already have a problem related with that current SLRSV-Eg isolate. Symptoms development of SLRSV varied depending on the host plants, vein banding, mottling systemic local lesion and mosaic are some of symptoms exhibited by the current isolated virus. The coat protein gene of the four SLRSV-Eg isolate was submitted to GenBank under the accession number MT648777.1. RT-LAMP assay for detection of SLRSV is cost-effective, efficient, easy to perform, rapid and appropriate for early detection and could be successful tool for monitoring of plant virus diseases and in quarantine. The sensitivity of RT-LAMP is equivalent to or better than that of one-step RT-PCR. In addition to RT-LAMP doesn't require expensive equipment's (e.g., thermal cycler, and it may be done successfully using water bath or thermal block.

*Conflict of interest*

The authors have declared no conflict of interest.

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