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MOLECULAR DETECTION OF NEPOVIRUSES IN PAKISTANI GRAPEVINE GERMPLASM USING REAL TIME qRT-PCR AND CONVENTIONAL RT-PCR

A. Younas[1] and S. Naz[1]

[1]Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan Corresponding Author's email: drsnaz31@hotmail.com

ABSTRACT

The aim of the present work was to investigate the molecular identification and characterization of the three different viruses including Grapevine Fanleaf virus (GFLV), Arabis Mosaic virus (ArMV) and Tobacco ringspot virus (TRSV) from Nepovirus group in the main grape producing regions of Pakistan by using reverse transcription polymerase reaction (RT-PCR) and real time polymerase reaction (qRT-PCR). Total 370 symptomatic and asymptomatic samples were screened for these viruses. Total RNA of all samples was extracted by guanidine isothiocyanate and CTAB method and the efficient one was further used as a template for the amplification by RT-PCR. 28 out of 370 were detected as positive for GFLV while none of other viruses were found positive in the screened samples. The results from the analysis of the conventional PCR confirmed the presence of only GFLV as the gene fragment was amplified by PCR at desired size of 322 bp. One of the positive PCR amplified product of GFLV was subjected to Sanger sequencing and phylogenetic relationship using MEGA 7.0. The phylogenetic analysis showed 90% homology with American isolates. The real-time TaqMan® RT-PCR assay was compared to the conventional RT-PCR assay for the detection of viruses using purified total RNA. This study showed that TaqMan® RT-PCR was more sensitive than conventional RT-PCR for testing different isolates of these viruses present in low titer and it also gave the amount of virus present in each sample. The current study produces a significant preliminary data showing the current status of Nepoviruses that can be used for the establishment of a pathogen tested grapevine germplasm program for grape cultivation in Pakistan.

Keywords: GFLV, ArMV, Pakistani grapevine germplasm, RT-PCR, TRSV, Phylogenetic analysis

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INTRODUCTION

Grapevine (*Vitis* spp.) is a major vegetative propagated fruit crop with high socioeconomic importance worldwide. Grapes are economically one of the most important cultivated fruit species, not only because of wine production but also due to consumption as dried and fresh fruit (Vivier and Pretorius, 2002). It is the crop that has been infected with the highest number of viruses. According to the Organization of vine and wine, 7.4 million hectares is the global area under vines with 77.8 metric tonnes grape production in 2018 (FAO,2019). The climatic diversity of Pakistan is suitable for the cultivation of nearly all types of fruits-tropical, temperate and subtropical. Pakistan ranks 58th in the world in terms of grapes production (FAO, 2017). Grapes are the 10th most produced fruits in Pakistan with annual production of 65811 tonnes having an area of 14729 hectares under cultivation (GoP, 2017). With approximation,57% of total world grapes production is used for wine, 36% as fresh fruits while only 7% is used as dried fruit (FAO, 2019).

Grape is the crop which is infected with the maximum number of known viruses (Martelli, 2014). Currently, almost 65 viruses, 8 viroids and 4 satellite RNAs from different families have been reported that infect grapevine (Chiumenti *et al*., 2016; Martelli, 2014). Although the pathogenicity of these viruses has not been well-documented, most of them are considered as detrimental pathogens. These severe pathogens include emerging grapevine Pinot Gris virus and the well-described viruses responsible for leafroll and grapevine fanleaf diseases (Basso *et al.,* 2017). The latter one is the most widespread and severe grapevine disease as it affects the high added value crop throughout the world (Basso *et al*., 2017).

Three distinct Nepoviruses, Grapevine Fanleaf virus (GFLV), Tobacco ringspot virus (TRSV) and Arabis mosaic virus (ArMV)) are implicated in the genesis of this disease. The viruses present in this group have serological variants except ToRSV that has two distinguishable strains (Piazzolla *et al.*, 1985) that has the capability to infect the grapevines by inducing different diseases. There are biological variants that produce symptoms in artificially and naturally inoculated hosts. The viruses of Nepovirus group have isometric particles about 30 nm in diameter and bipartite genome (Martelli and Boudon-Padieu, 2006).

From the previous years, bioassays and serological techniques have been widely utilized as an important and fundamental assay for the identification of grapevine viruses, but recently, molecular biology-based techniques have been used (Pacifico *et al*., 2011).

Detection of grapevine viruses by nucleic acid-based methods has been proven more sensitive and reliable method. Molecular method (polymerase chain reaction) is the utmost specific, accurate and reliable technique for the screening of grapevine viruses as compared to biological indexing and serological assays (Osman *et al*., 2008). Real time quantitative reverse transcription PCR (RT-qPCR) is very sensitive method to determine and quantify the low titer of pathogens in the infected tissue (Agindotan *et al*., 2007; Osman *et al*., 2007). In Pakistan, grapes yield is low as compare to the developed and developing countries especially our neighboring country India. This needs to categorize the features responsible for the low productivity of grapes. The present study was conducted to identify the most prevalent grapevine Nepoviruses in Pakistani germplasm and to determine the genetic relationship of Pakistani Nepovirus isolate with the rest of the world. This report shows that conventional PCR tests using published primers are inadequate for detecting all Nepoviruses present in Pakistani germplasm. To improve virus detection, a qRT-PCR assay was developed which was capable of detecting more positive isolates.

MATERIALS AND METHODS

Pathogens and plant materials: Viruses targeted in this study included Grapevine Fanleaf virus (GFLV), Tobacco ringspot virus (TRSV) and Arabis mosaic virus (ArMV). Different local and commercial vineyards were surveyed for the sample collection. A total of 370 samples were collected from Sillanwali, Lahore, Fortabbas, Quetta, Pishin, Kila Abdullah, Khuzdaar and Kalaam during the months of July and August, 2017 to study the occurrence of these pathogens. The grapevine leaves were collected from plants showing vein yellowing, yellow speckles or no apparent symptoms. Considering the possible even distribution of viruses within a given plant, samples from at least five different shoots or canes of the same plant were mixed. Samples were taken from all sides of vine.

Sample processing: The collected leaf samples were cleaned, labelled and packed in zip lock plastic bags and were transported in ice box to Plant tissue culture Laboratory, Lahore College for women university, Lahore. The samples were stored in freezer (-20 °C) until the RNA extractions were performed. Total RNA was extracted from 1 g of grapevine tissues (petiole and mid rib). Two methods were evaluated to select a reliable one for RNA extraction i.e CTAB method (Iandolino *et al*., 2004) and Guanidine isothiocyanate method (Osman *et al*., 2008). To assess the quality of extracted RNA, 1% agarose gel was prepared in TBE buffer (Sambrook *et al*., 1989) and stained with ethidium bromide. 5ul of extracted RNA was mixed with 3ul of loading dye and then loaded into the wells and visualized under UV light. RNA purity and concentration were assessed by determining the absorbance of the samples by NanoDropTM-1000 (Thermo Fisher Scientific Inc) at 260 and 280 nm.

cDNA synthesis: First-strand cDNA synthesis was performed using 1 µg of total RNA treated with DNase following manufacturer's instructions (Thermo Scientific RevertAid). 3ul of RNA, 1ul of random hexamers and 7ul of RNAase free water were incubated at 70°C for 5min. After incubation, a cocktail containing 4 ul of 5X RT buffer, 2.5µl of 10 mM dNTPs, 1 µl of 50mM DTT, 0.5 µl of 40 U inhibitor (RiboLockTM RNase inhibitor kit) and 1µl of 200U Moloney murine leukemia virus (Mu-MLV) Reverse transcriptase was added in the reaction tube. The mix for reverse transcriptase (20 µl) was incubated for 10 min at 25°C, 60 min at 42°C and 10 minutes at 70°C.

RT-PCR: The PCR cocktail (25 µl) contained 2 µl of cDNA (10% of the first strand reaction, corresponding to about 100 ng of total RNA), 1× (2.5 µl) PCR buffer (Invitrogen Life Technologies), 2.5 mM dNTPs, 10 mM each primer, 2.5 mM MgCl2, and 1 U of Taq polymerase (Thermo Scientific Fisher). Cycling conditions for all primer pairs consisted of initial denaturation at 95°C for 2 min followed by 35 cycles at 95°C for 1 min, 58°C for 1 min, and 72°C for 1 min. A final extension of 7 min at 72℃ was part of PCR amplification prior to holding the samples at 4℃. The specific primers used for the analysis of GFLV, ArMV and TRSVare shown in Table 1. Reaction products were analyzed by electrophoresis on 1.5% agarose gels and visualized on gel documentation system after staining with ethidium bromide.

TaqMan RT-PCR: Single-tube TaqMan® RT-PCR reactions were set up in 394-well reaction plates using a TaqMan® Fast virus one step Master mix kit (ABI) as follows: 2.5 ul one-step RT-PCR Master Mix, 0.5 primer/probe mix (250 nM probe and 900 nM primer), and 2ul of total RNA template in 10ul reaction. Reactions were carried out in a thermocycler Quant Studio™ 7 Flex Real-Time PCR System (Thermo Fisher Scientific) in a one-step reaction as recommended by ABI (RT-PCR Master Mix procedure). Reverse transcription and amplification conditions were as follows: 45 ◦C for 35 min, 95 ◦C for 10 min, followed by 40 cycles of 95 ◦C for 30 s and 60 ◦C for 30 s. The data was analyzed quantitatively by measuring the threshold cycles (CT) in a Microsoft Excel program and graphically by an amplification plot using Quant Studio™ 7 Flex Real-Time PCR System Software. The threshold cycle (CT) is the cycle at which a significant increase in fluorescence occurs; hence a CT value below 30 indicates a positive result in this setup. The Primers and probes used for

GFLV, ArMV and TRSV amplification are shown in Table 2.

Agarose gel purification and Sequence Analysis: To purify the expected amplicon size on gel, Thermo Scientific Gene Jet PCR Gel Extraction Kit was used. The expected amplicon from the PCR was cut out of the gel and purified. Thermo Scientific Gene JET PCR Purification Kit purified the PCR products and sequencing was done using primer pair in both orientations using the same set of primer as in amplification. The obtained nucleotide sequence was analyzed by using BLAST with default parameters. The consensus coat protein sequence of the positive isolate was compared and analyzed with coat protein sequences obtained from Genbank databases. The phylogenetic relationship was determined by using neighbor joining method using MEGA 7.

RESULTS

Total nucleic acid Extraction: Total RNA was extracted from both symptomatic and asymptomatic grapevine samples to check the presence of Nepoviruses in the Pakistani grapevine germplasm. Two different extraction methods were used to extract RNA. The quality and quantity of extracted RNA by both methods were analyzed by using agarose gel electrophoresis and Nanodrop. The sharp bands of RNA extracted by Guanidine isothiocyanate method showed better quality as compare CTAB to as shown in Fig 1 (A) and 1 (B). The quantity of RNA extracted by Guanidine isothiocyanate method was in the range (A260/280= 1.56-2.29) while with CTAB method the range was low (A260/280= 1.21-1.83). The results showed that the Guanidine isothiocyanate method showed better quality RNA as compare to CTAB. So, for the detection of concerned Nepoviruses by RT-PCR, RNA extracted by Guanidine isothiocyanate method was used.

Nepoviruses detection by RT-PCR: All samples were tested by conventional PCR for the presence of Grapevine Fanleaf virus (GFLV), Tobacco ringspot virus (TRSV) and Arabis Mosaic Virus (ArMV) using specific primers given in Table 1. Only 18 samples were found positive for the GFLV while none came up positive for other two viruses except their positive controls. Analysis by agarose gel electrophoresis of the PCR products (Figure 3: A) revealed the presence of the expected band of 322 bp in GFLV isolates while in Fig 3 (B and C) the PCR amplification of ArMV and TRSV showed that none of the isolate came up positive except positive control.

Fig 1: Map of Pakistan showing areas of Grapevine sampling

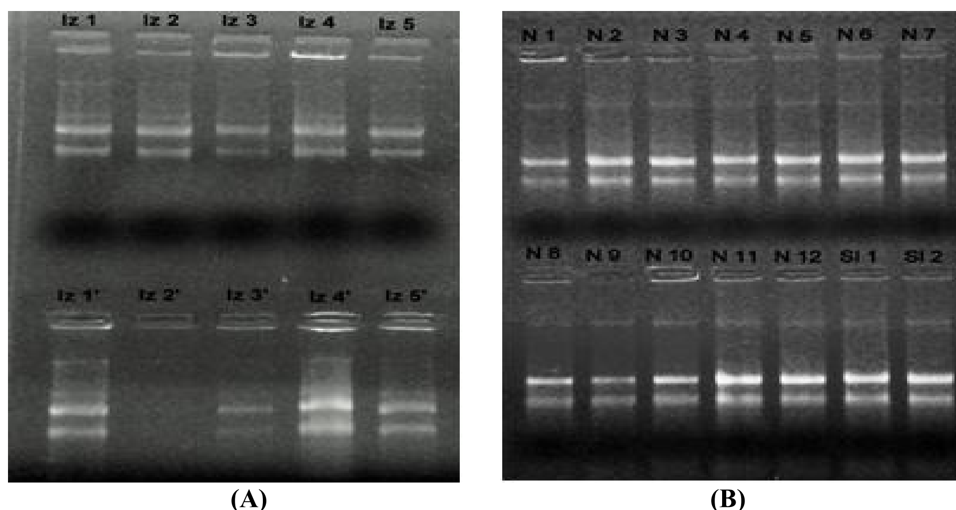

Fig. 2. Comparison of total RNA isolation by two different methods (A) by CTAB method (B) by Guanidine isothiocyanate method.

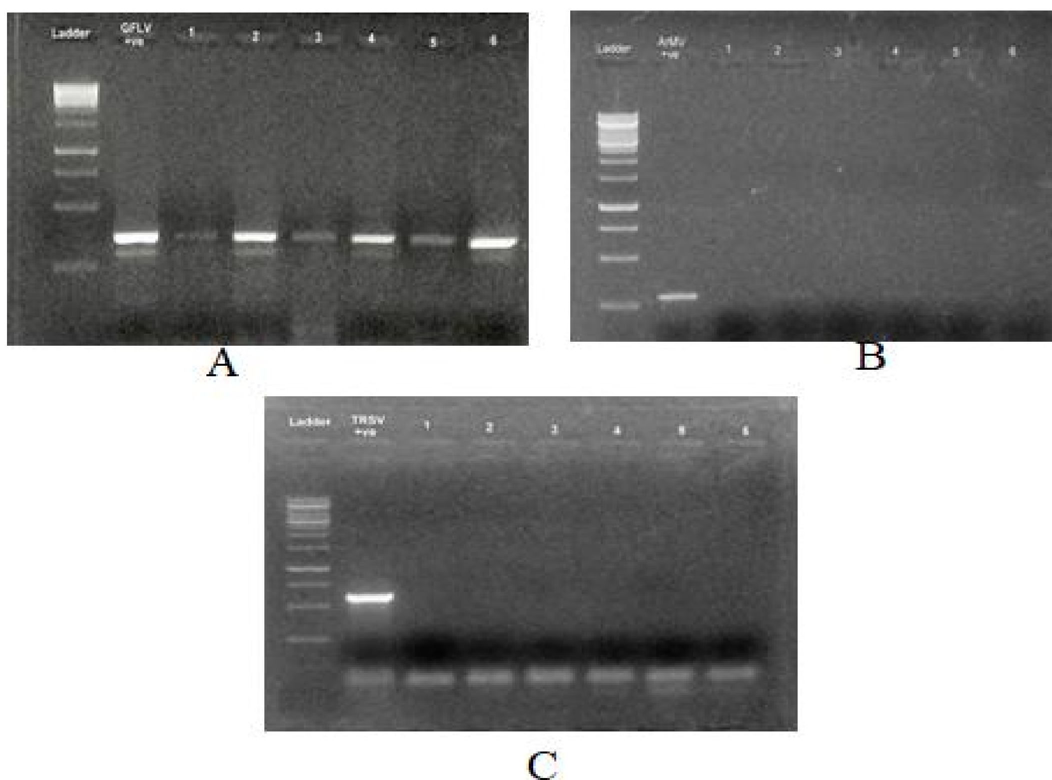

Fig 3: PCR amplification of Nepoviruses (A) GFLV (B) ArMV (C) TRSV from different isolates of Pakistani Grapevine Germplasm.

Table 1. Primer set used for Nepoviruses amplification by RT-PCR

Primer	Sequence	Size
GFLV C1	5’-CCAAAGTTCGTTTCCCAAGA-3’	322bp
GFLV V1	5’-ACCGGATTGACGTGGGTGAT-3’	
ArMV CP	5’-CTGTGCCATCGTTCCCCAATGAT-3’	290 bp
ArMVCP	5’-GAGATGCTCCATCCATGCCAGT-3’	
TRSV CP	5’-CTGGACATGGACTGTGCAACTG-3’	590 bp
TRSV CP	5’-CAGGAGCTATAGGCCCGGAGA -3’	

Nepoviruses detection by TaqMan RT-PCR: Three main viruses (GFLV, ArMV and TRSV) from the Nepovirus group was detected by TaqMan RT-PCR. Only Grapevine fanleaf virus (GFLV) was the frequently encountered virus in the grape growing region of Pakistan. Previously reported TaqMan primers and probes were used for the diagnosis of these viruses. Total 28 isolates were positive with GFLV and showed 7.5% infection rate. The distribution of GFLV was observed in Sillanwali, Qila Abdullah and Pishin. The widespread infection of GFLV was seen in 19 cultivars of Sillanwali region. The CT values of these samples were ranged from 22-32 (Table 3). Negative and positive control was also run. The amplification curve by Rn and ΔRn vs cycle for each virus is shown in Fig 4 (A-C).

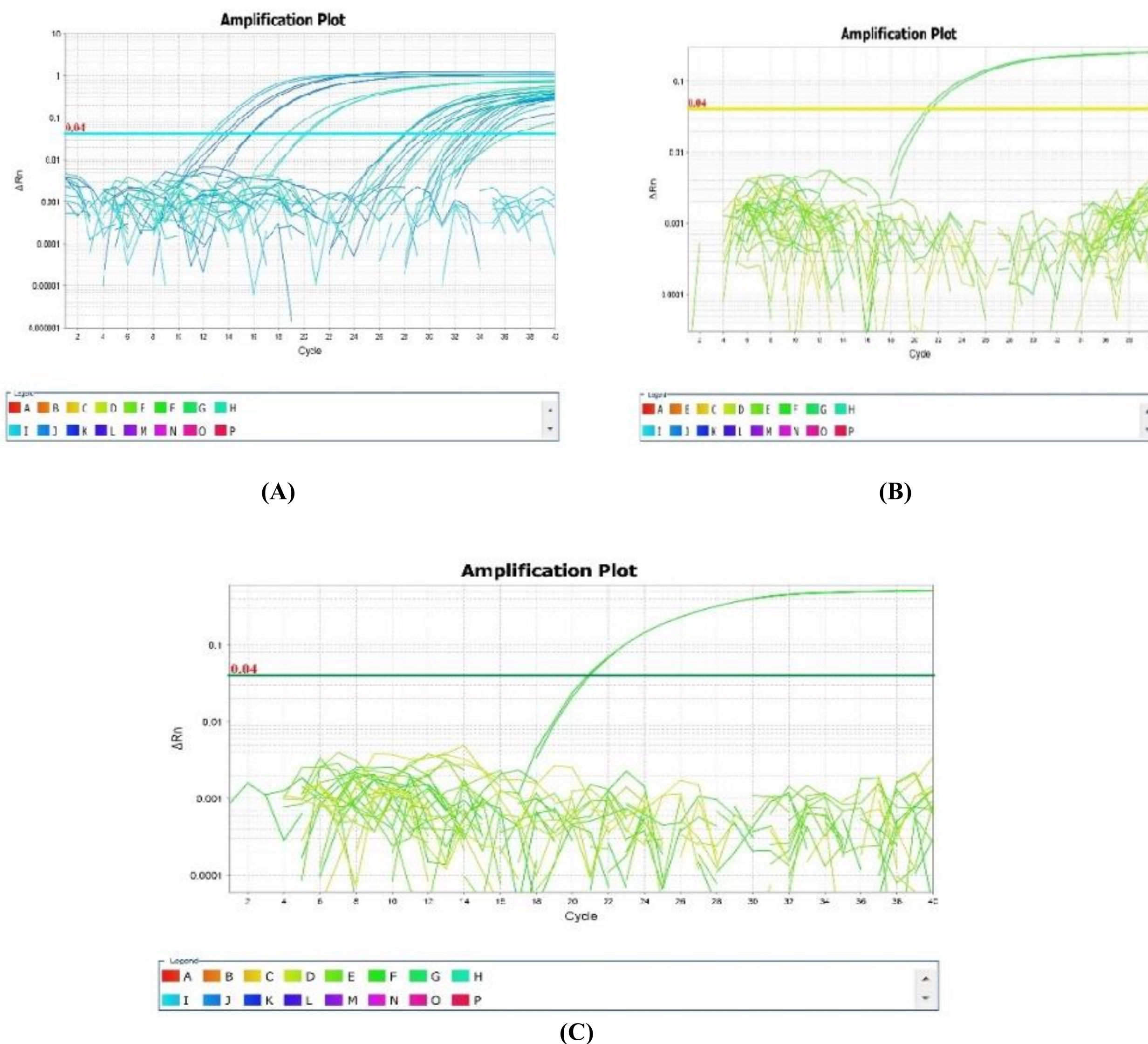

Fig4. Amplification plot of Relative grapevine Nepoviruses quantification of the TaqMan RT-PCR assay.

Table 2. Real time RT-PCR primers and probes information for Grapevine Fanleaf Virus (GFLV), Arabis mosaic virus (ArMV) and Tobacco ringspot virus (TRSV).

Virus	Primer/Probe Name	Primer/ Probe Sequence (5'-3')	Location Size	Size (bp)
GFLV	GFLV 2801F	GTTAGTGAGTGGAACGGGACC	Coat protein	151
	GFLV 2822R	TTCCACATACACCCCGGGATA		
	GFLV 2825P	CTATGGATTGGAATGAA		
ArMV	ArMV cp F	TAGCCCTTGGAGACAATCCT	Coat protein	

	ArMV cp R	CCTCCAAATCCCACATTAAC		
	ArMVcp P	TGCCCATATGATAGCTTGTCATGGAC		
TRSV	TRSV-1710F	AGGATGGTCCGGCTCTTAGG	Coat Protein	93
	TRSV-1795R	GCTGACAGACAGACATTCTTGGG		
	TRSV-1743P	TGTATAAACTCAGCTTCTTGGG		

Real-time RT-PCR primers and probes information for Arabis mosaic virus (ArMV), Cucumber mosaic virus (CMV), Lily symptomless
virus (LSV), Tobacco rattle virus (TRV) and Tulip virus X (TVX
Real-time RT-PCR primers and probes information for Arabis mosaic virus (ArMV), Cucumber mosaic virus (CMV), Lily symptomless
virus (LSV), Tobacco rattle virus (TRV) and Tulip virus X (TVX

Table 3. Quantitative detection of GFLV by TaqMan RT-PCR

Sr. No.	Variety Name	Sample Code	Target Name	Reporter	Quencher	CT value
1.	Midgerly's Purple	SL 1	GFLV	FAM	NFQ-MGB	25.22
2.	Lake Mount	SL 2	GFLV	FAM	NFQ-MGB	27.54
3.	Delight	SL 3	GFLV	FAM	NFQ-MGB	26.39
4.	Lomanto	SL 4	GFLV	FAM	NFQ-MGB	29.09
5.	Kings Ruby L2 P1	PSH 22	GFLV	FAM	NFQ-MGB	28.87
6.	Toran L1 P2	ARI 2	GFLV	FAM	NFQ-MGB	28.53
7.	Thomcord	SL 7	GFLV	FAM	NFQ-MGB	29.77
8.	Perlette	SL 8	GFLV	FAM	NFQ-MGB	30.11
9.	Red Globe	PSH 23	GFLV	FAM	NFQ-MGB	27.68
10.	Thompson Seedless	SL 10	GFLV	FAM	NFQ-MGB	31.01

Phylogenetic Analysis of GFLV CP: In order to understand the relationship between the Pakistani and the other GFLV isolates available at the GenBank, a phylogenetic tree was established. The newly characterized nucleotide sequence of the Pakistani isolate was blasted with previously reported GFLV isolates in NCBI. It appeared that the newly generated sequence corresponds to the CP gene of GFLV. When the sequence of new isolate of GFLV was subjected to BLAST analysis, more than 90% similarity was shown between the new isolate and other GFLV isolates previously reported from USA, Chile, China, Poland and Iran. Little cherry virus CP is used an as outgroup while determining the evolutionary relationship within the group.

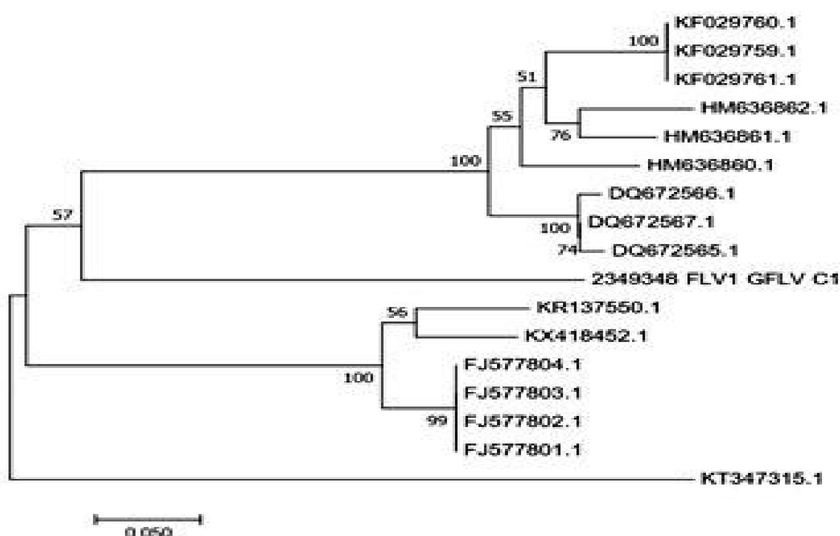

Fig. 3. Evolutionary relationships of taxa: Neighbor joining phylogenetic tree based on nucleic acid sequences of CP gene of GFLV isolate generated by MEGA 7. The optimal tree with the sum of branch length= 1.429598 is shown. The p-distance method is used to compute the evolutionary distance. Total 17 nucleotides sequences are involved in the analysis. Branches with bootstrap value of <50% are unrevealed. Pakistani isolate is shown by bold letters.

DISCUSSION

The annual production of grapevine in Pakistan is lower as compared to other countries. There are certain factors responsible for this loss, one of which is the viruses present in grapevine as reported by Rasool *et al.,* (2019). The extraction of high-quality RNA is a crucial and important aspect of molecular assay performance. Grape is a woody perennial crop and comprises of large amounts of polysaccharides and polyphenols. Isolation of good-quality RNA from grapevine tissue is difficult because of these compounds (Vasanthaiah *et al*., 2008). In this study, two methods of extraction; CTAB and Guanidine buffer with the use of the extraction kit were used. The quality and quantity of the RNA from two different extraction techniques was evaluated which suggested that Guanidine was a better RNA extraction method than CTAB. Gambino and Gribaudo, (2006) compared two extraction methods having objective of increasing the grapevine extracts quality for use in RT-PCR and extracting RNA from different tissues (leaves, phloem from mature canes, and whole *in vitro* plantlets). But they could not have produced extracts that can be effectively amplified. With the RNeasy kit MacKenzie *et al.* (1997) successfully extracted RNA from grapevine. Nassuth *et al.* (2000) obtained similar findings but found problems with old tissue RNA extraction. Our findings were similar to those of Osman *et al.* (2012), who compared various RNA extraction methods and considered method with guanidine buffer preferable to other procedures of extraction. In the present study, GFLV was not detected in all symptomatic leaf samples, which can be explained by the low titer of the virus to be detected by conventional PCR or may indicate that observed symptoms might be due to infection with others viruses. The occurrence of GFLV, ArMV and TRSV isolates in major grape growing regions of Pakistan was determined and confirmed by conventional PCR. The rate of infection of GFLV was 7.6% based on RT-PCR results, among which the highest infection rate was found in Sillanwali region from Punjab province. Our results are in agreement with Ghomi *et al.* (2007) who also randomly surveyed for presence of Grapevine fanleaf virus (GFLV) along with two other viruses in north-eastern provinces of Iran using RT-PCR. The results showed the presence of GFLV in 7% of the collected samples. Similarly, Palomares-Rius *et al.* (2012), investigated the occurrence of Grapevine fanleaf virus (GFLV) in 74 vineyards in grapevine-growing areas of southern Spain and found that the overall prevalence of GFLV was 24%. Rakhshandehroo *et al.* (2009) detected and identified GLRaV-3, GFLV, GVA, TRSV, ArMV and ToRSV in leaf samples taken from various Iranian vineyards. All the vineyards surveyed consider GFLV to be the most prevalent virus. Realtime PCR is very sensitive, reproducible and has the low risk of contamination carry-over because the output of the amplicon is observed in a closed tube. In the present study, GFLV detected in more samples by TaqMan real time PCR as compared to conventional PCR. Osman *et al.* (2008) also discussed that quantitative PCR could identify viruses at 32-fold higher dilutions from purified RNA than conventional PCR. Osman *et al*., (2007) also studied that real time RT-PCR identified 100% of the studied viruses whereas RT-PCR identified 89% studied viruses.

The Phylogenetic analysis allowed us to recognize the relationships around the world between Pakistani isolates and geographically distant isolates. GFLV Pakistani isolate (SLW 7, Thomcord) exists in an isolated position, but it was closely related to American, Chinese and Iranian isolates and sharing a nucleotide sequence identity of 92%. The BLAST analysis showed that the Pakistani isolate CP gene shared higher identities of nucleotides that show clear evidence of taxonomic rank. (Gouveia *et al.,* 2011). The phylogenetic history of the Pakistani isolate displayed polyphyletic behaviour. The fact that the spread of the viruses happens primarily due to uncontrolled trade and distribution of budwood material from these nations is consistent with this data.

Conclusion: The present study reports the identification of Nepoviruses in different vineyards of Pakistan. RT-PCR and qRT-PCR was used as a diagnostic tool for the detection. Although the applied diagnostic method is efficient but the use of more reliable and sensitive diagnostic tools like TaqMan RT-PCR proved an essential step to identify the virus that have less titer in order to maintain the sanitary conditions of the vineyards.

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