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Screening *Rose* spp. from India for *Apple mosaic virus* and *Prunus necrotic ringspot virus* infection and designing a duplex RT- PCR assay for their simultaneous detection

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Abstract
During spring in the years 2017, 2018 and 2019, field surveys were conducted in the valley of Kashmir, New Delhi, Palampur (H.P), Khitoli (U.P) and Jhajra-Dehradun (U.K), to screen the rose species from India for *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV) infection. A total of 236 symptomatic and non-symptomatic leaf samples were collected and subjected to RNA isolation followed by cDNA synthesis. PCR amplification was carried out by using CP gene-specific primers for the detection of ApMV and PNRSV. Two samples from Kashmir and three from Dehradun were found infected with ApMV, sharing 96 - 100 % sequence identity with the CP gene of ApMV rose isolates from Poland and apple isolates from India. PNRSV was detected only in four samples from Kashmir sharing 98 - 99 % sequence identity with the CP gene of PNRSV apricot isolates from India and rose isolates from Poland. None of the samples from other sampling sites was found infected with any of these viruses and no infected sample was found to have a mixed infection. The duplex PCR assay designed for ApMV and PNRSV detection was 100 % specific to their targets and could simultaneously detect them up to 1 ng / µl concentration. This assay is more reliable, specific, and sensitive for early detection of these viruses and is worth using in plant quarantine for disease management and control.

Keywords: ApMV, PNRSV, duplex PCR, specificity, sensitivity

Introduction

Roses (*Rosa* spp.) belong to the family *Rosaceae* and are among the highly cherished flowering shrubs known for their ornamental and medicinal values. The roses have a socio-economic significance, as they are a rich source of aromatic oil and biologically active compounds [18]. In the year 2014 United States alone estimated rose production worth $204 million, and in New Zealand, NZ $270 million worth of wholesale value was generated from roses only [2, 11]. The viral diseases in roses put constraints on its essential oil production, degrade flower quality and reduce plant vigour [9].

The ApMV and PNRSV belong to the genus *Ilarvirus* and are reported to infect the rose plants globally [19, 25, 26]. Both these viruses possess a tripartite, positive sense, single-stranded, linear RNA genome with no report of any DNA stage [27]. The RNA 1 and RNA 2 of these viruses are monocistronic and translate into non-structural proteins involved in RNA replication [32]. The RNA 3 of both these viruses is bicistronic and encodes for the movement protein and coat protein. These viruses have a high potential for evolution due to a lack of proofreading activity in RNA-dependent RNA polymerase [6, 7, 20]. The high mutation rates make them more dynamic thus their detection based on host symptoms also becomes difficult [1, 3].

There is no natural vector reported for transmission of ApMV and PNRSV viruses. Therefore PNRSV mainly transmits via pollens, seeds, vegetative propagation and mechanical means [8]. While ApMV transmission mainly occurs through grafting and vegetative propagation from an infected source. The PNRSV infection may develop chlorotic to yellow line patterns, necrotic leaves and flowers, or spotted fruits [10, 37]. ApMV infection may develop mosaic symptoms in leaves [17] i.e., pale yellow to bright cream-coloured irregular spots appear along the major veins, oak-leaf patterns, premature leaf fall, and stunted growth of the host [5]. The symptom-based virus diagnosis has lost significance due to symptoms overlapping with biotic and abiotic stresses.

Serological methods like Enzyme-linked immunosorbent assay (ELISA) [40] and Tissue blot immunoassay (TIBA) are used for virus diagnosis but these methods have issues of non-specificity. The use of the latest molecular techniques like High-Throughput Sequencing / Next-generation Sequencing [24], molecular hybridization [28], and DNA arrays [35] are more reliable for detection in terms of specificity and sensitivity but are expensive techniques and need ample expertise for analysis. The conventional PCR / RT-PCR [14] have been routinely used for virus detection. However, mixed virus infection in crops makes RT- PCR more expensive and time-consuming to detect each virus individually. Therefore to solve this issue, researchers used the multiplex RT-PCR for simultaneous detection of multiple virus infections in a single reaction. This technique has more advantages over the conventional RT-PCR and other mentioned techniques because it has low cost-expenses and saves time in virus detection [29, 34].

Despite the known fact that India is holding approximately 46 % of the global land area under rose cultivation but accounts only 0.2 % of the world's cut rose export (Bhagat *et al*., 2019). This creates a huge trade imbalance thus becomes a matter of concern which need to be addressed as early as possible. From India, the available data elucidating the status of virus infection in rose is scanty. Therefore, this study was conducted with the aim to find the occurrence of ApMV and PNRSV infection in rose from India and design a reliable, sensitive and cost-effective duplex RT-PCR assay for their simultaneous detection.

Materials and Methods

Sample collection and maintenance

A total of 236 rose leaf samples showing symptoms of virus infection, were collected from the gardens of Kashmir, rose fields of Khitoli (Uttar Pradesh), Indo – African friendship rose garden New Delhi, Jamia Millia Islamia, main campus (New Delhi), Jhajra-Dehradun (Uttrakhand), and from the experimental plot of CSIR-Institute of Himalayan Bioresource Technology (Palampur, Himachal Pradesh). The collected samples were stored at -80^{o}c before proceeding for molecular analysis.

Total RNA extraction

Total RNA was extracted from the foliar tissues of rose plant by following CTAB method used by Zeng Y. and Yang T., 2002 [41] and by following TRIzoL method used by Meng L. and Feldman L., 2010 [21], total RNA extraction kit (Sigma Aldrich) and finally using the Option 2 of protocol designed by (Jordon-Thaden *et al* 2015) [12], which use both CTAB and TRIzol buffers for total RNA extraction. 100 mg of foliar tissue was crushed using DEPC treated double autoclaved mortar pestle by adding liquid nitrogen. The extracted RNA was finally dissolved in 40 µl Mili Q water (double autoclaved), quantified using Nanodrop 2000C spectrophotometer (Bio-Rad), and the integrity / intensity of bands was checked by loading 1 µl of the dissolved RNA on 2 % formaldehyde gel before proceeding for cDNA synthesis.

Reverse transcription

RNA extracted from each sample was reverse transcribed using the cDNA synthesis kit (iscript Bio Rad). For 20 µl reaction, 2 µl of RNA template (500 ng to 1000 ng), and other kit supplied components like 1 µl verso enzyme mix, 0.25 µl (100 ng) oligo dT and 0.75 µl (375 ng) random hexamer, 5X cDNA synthesis buffer (4 µl), 2 µl dNTP mix (5mM each), 1 µl RT- enhancer and 9 µl of nuclease-free water were used to set the final reaction. The reaction was carried out at 42^{o}C for 30 min. followed by enzyme deactivation at 95^{o}C for 2 min in a thermo cycler (Bio-Rad T100TM). The synthesized cDNA was stored at -20^{o}C for future use.

PCR amplification of ApMV and PNRSV CP genes

PCR was performed for detection of ApMV using, ApMV- CP gene-specific primer pair PAPMCP-5 (5`-TCAACATGGTCTGCAAGTAC-3`) and PAPMCP-3 (5`-CTAATCGCTCCATCATAATTC-3`) used by V. Lakshmi *et al.,* 2011 [16]. There was no positive control available for the detection of ApMV. The PNRSV detection was carried using primer pair CPu: (5`-AACTGCAGATGGTTTGCCGAATTTGCA-3`) and CPd: (5`-GCTCTAGA CTAGATCTCAAGCAGGTC-3`) used by S. Kulshrestha *et al.,* 2013 [15] during their study. Coat protein gene (accession no. FR773524.2) cloned within pGEM-T Easy vector (Promega, USA) (available in our lab) was used as positive control.

For preliminary screening 20 µl PCR reaction was set using 2 µl of 10x PCR buffer (Sigma Aldrich), 2 µl of cDNA (500 ng) of the infected sample, 1 µl forward primer (10 pM), 1 µl reverse primer (10 pM), 0.6 µl of 10 mM dNTPs, 0.6 µl of 25 mM $MgCl_2$, and 0.5 µl of *Taq* DNA polymerase (5 units /µl) (Sigma Aldrich) and, 11.9 µl of nuclease-free water [16]. ApMV CP gene amplification was carried out in thermo cycler (Bio-Rad T100^{TM}) with an initial denaturation at 95˚C for 5 min, followed by 35 cycles at 95˚C for 50s, 52˚C for 50 s and 72˚C for 1 min. Final extension was given at 72˚C for 10 min. Same cyclic thermal conditions were followed for PNRSV CP gene amplification, except its annealing at 55˚C for 50s. PCR products were resolved on 1.5% agarose gel (Invitrogen) stained with ethridium bromide (0.5 µg / ml) and observed in the Gel Doc system (Bio-Rad Molecular Imager Gel Doc^{TM} XR^{+} Imaging System).

Cloning and sequencing of PCR amplified products

PCR amplicons resolved on agarose gel were eluted using a gel elution kit (QIAGEN, Germany). The eluted PCR amplicons of the presumed ApMV- CP gene were ligated in a pJET1.2 cloning vector (Thermofisher), and the presumed PNRSV- CP gene amplicons were ligated in a pdrive cloning vector (QIAGEN, Germany). The *Escherichia coli* (E. coli) DH5α strain was transformed with ligated plasmid and cultured on Luria agar (LA) plates supplemented with ampicillin (100 µg / ml). The transformed colonies that appeared on the selection medium were confirmed through colony PCR and restriction digestion. The positive colonies were incubated overnight in a Luria broth (LB) medium supplemented with ampicillin (100 µg / ml) (supplementary data). Finally, the plasmid was isolated and confirmed by nucleotide sequencing by the dideoxy chain termination method [30] at Agrigenome Bangalore, India; Macrogen Inc. Soul South Korea and the University of Delhi, South Campus. The homology of retrieved sequences was checked online by using NCBI BLAST and after proper analysis; the confirmed sequences of ApMV and PNRSV were submitted to the NCBI GenBank repository.

Designing primers for duplex PCR assay

ApMV CP gene sequence (accession no. OK625807.1) and PNRSV CP gene sequence (accession no.OK625809.1) of our rose isolates were downloaded from the National Center for Biotechnology Information (NCBI) GenBank database (http://www.ncbi.nlm.nih.gov/blast.cgi) along with their respective ApMV CP gene isolates (MT678110.1, KY971019.1, MN822139.1, FN435316.1, KY883320.1, HG328270.1, HG328266.1, HG328265.1, HG328264.1, MT303163.1, GQ131805.1, HG328263.1, HG328268.1, HG328280.1) and PNRSV CP gene isolates (MF145112.1, MN656194.1, FJ610344.1, DQ983498.1, AY684271.1, DQ983497.1, AY948441.1, U57046.1, KF135206.1, MN656197.1, MF145096.1, KF135211.1, DQ003584.2, AY948440.1). The downloaded sequences were aligned using BioEdit software (bioedit.software.informer.com/7.2/) and primers were designed from the highly conserved region of the CP gene. The specificity of the designed primer sets was confirmed by *in-silico* analysis using NCBI BLASTn software (http://www.ncbi.nlm.nih.gov/blast.cgi). The possible amplification of the respective template strands and expected amplicon size were analyzed using the virtual PCR program (http://www.bioinformatics.org/sms2/pcr_products.html). The ideal properties for primer synthesis like; Tm range, G + C content, Primer length; ΔG plot values were ensured using the online integrated DNA technologies IDT Oligoanalyzer tool (idtdna.com/pages/tools/oligoanalyzer) before proceeding to their synthesis.

Table 1: The primer sequences designed for optimization of uniplex and duplex PCR for detection of *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV).

Target		Primer				Reference
Virus	Gene	Name	Sequence (5`- 3`)	Location (nt)	Amplicon size	
ApMV	CP	ApLaw-For.	CTCACCCTGGATCTTGCG	1150-1168[a]	616	This study
		ApLaw-Rev.	AACATTCGTCGGTATTTGCAC	1740-1761[a]		
PNRSV	CP	PnLaw-For.	GGTTTCGAGCGGTATAGGAC	1249-1268[b]	461	This study
		PnLaw-Rev.	ACAAATCCCTAACCAAGACCTT	1688-1709[b]		

The target is based on RNA 3 reference sequence of [a]ApMV isolate (accession number: NC_003480.1); and RNA 3 sequence of [b]PNRSV isolate (accession number: NC_004364.1) available in the NCBI GeneBank database.

Optimization of uniplex and duplex PCR assays for detection of ApMV and PNRSV

Plasmid vectors harbouring our cloned CP gene of ApMV and PNRSV were used as positive controls for uniplex or duplex PCR optimization. For uniplex PCR optimization, 25 µl reaction was set containing 2.5 µl of 10x PCR buffer (Sigma Aldrich), 4 ng of plasmid DNA as a template, 1 µl of each forward and reverse primers (each10 µM conc.), 0.6 µl of 10 mM dNTPs, 0.5 µl of 25 mM $MgCl_2$, 0.5 µl of *Taq* DNA polymerase (5 units/µl) (Sigma Aldrich) and 18.7 µl of nuclease-free water was added to make up the final volume.

For duplex PCR optimization, both the cloned plasmid vectors harbouring ApMV and PNRSV CP gene (4 ng / 1 µl each) were used as templates for amplification using primer cocktail ApLaw - for, ApLaw - rev, PNLaw - for and PNLaw - rev (each10 µM conc.) in the same reaction tube, with the same composition and concentration of other reagents as mentioned above. Seven uniplex PCR reactions for ApMV and PNRSV each and seven duplex PCR reactions were set at different annealing temperatures (Ta) for optimization, ranging from 48ºC to 60ºC (with an increment of 2ºC for each reaction). The gradient PCR reaction was carried at 95ºC for 5 min, 35 cycles of denaturation at 94 ºC for 40 s, primer annealing range from 48ºC to 60ºC for 45 s, elongation at 72ºC for 50 s, extension step at 72ºC for 5 min. To make our uniplex and duplex PCR assays more economical and rapid, a reaction carried out with a 2X master mix (GeneDirex) which contains a standard concentration of reagents.

Specificity of the designed duplex PCR assay for ApMV and PNRSV detection

After successful duplex PCR optimization, the specificity of the designed primers was experimentally confirmed by amplification of both the target viruses using primer cocktail and positive controls (cloned plasmids, harbouring the ApMV-CP gene; accession no.OK625807.1 and PNRSV-CP gene; accession no.OK625809.1). The uniplex PCR reaction for ApMV and PNRSV was also carried along with Non-Template Control (NTC) and negative control (2 µl cDNA from a healthy plant) reactions.

Duplex PCR based detection of ApMV and PNRSV in infected field-collected leaf samples

An Equimolar primer cocktail (designed in this study) was used to check the specificity of our duplex PCR. cDNA of ApMV and PNRSV infected field collected rose leaf tissues were used as templates for uniplex PCR. No mixed infection was reported during this study. Therefore the artificially mixed cDNA of both the ApMV and PNRSV infected rose leaf tissues were used for duplex PCR. The cDNA of samples that showed no PCR amplification during the preliminary screening were used as negative controls. Non-Template Control (NTC) was also set in the experiment. The amplified PCR products were sequenced and their identities were confirmed using BLASTn program.

Sensitivity of duplex PCR assay for detection of ApMV and PNRSV

In one experimental setup, 100ng of infected RNA (positive control of ApMV and PNRSV) were 10-fold serial diluted (10^0 - 10^{-5}), and cDNA was synthesized at each dilution. The equal volume of synthesized cDNA of ApMV and PNRSV (at the same dilution factor) was mixed and subjected to duplex PCR. Similarly, in another set of the experiment the sensitivity of designed duplex PCR was also checked by serial dilution of cDNA (10^0-10^{-5}) synthesized from undiluted total RNA. The sensitivity limit of duplex PCR was confirmed by agarose gel electrophoresis.

Results

The efficiency of the adopted RNA extraction methods

Option 2 of the protocol designed by Jordon-Thaden *et al* 2015 [12] was used for total RNA extraction from rose plants (rich in secondary metabolites and polysaccharides) and was highly efficient. The results showed an improvement in terms of quality, quantity, and integrity when compared with the results of the CTAB method by Zeng, Y. and T. Yang 2002 [41], the Trizol method by Meng, L. & Feldman, L. 2010 [21] and the total plant RNA isolation kit (Sigma Aldrich) (supplementary data).

RT-PCR based detection of ApMV and PNRSV viruses infecting rose samples

Among 80 rose leaf samples collected from the valley of Kashmir, four samples confirmed positive for PNRSV infection (Fig. 1A, Lane 3: 1B, Lane 4: 1C, Lane 2 and 4) with amplicon size of ~ 675 bp and two samples tested positive for ApMV infection with an amplicon size of ~ 688 bp (Fig. 2B, Lane 2 and 3). Among 35 samples collected from Jhajra-Dehradun in Uttrakhand, three samples tested positive for ApMV infection with an amplicon size of ~ 688 bp (Fig. 2A, Lane 1, 2 and 3). None of the samples collected from other sampling sites was found positive for ApMV or PNRSV infection. No sample was found to have a mixed infection of these viruses.

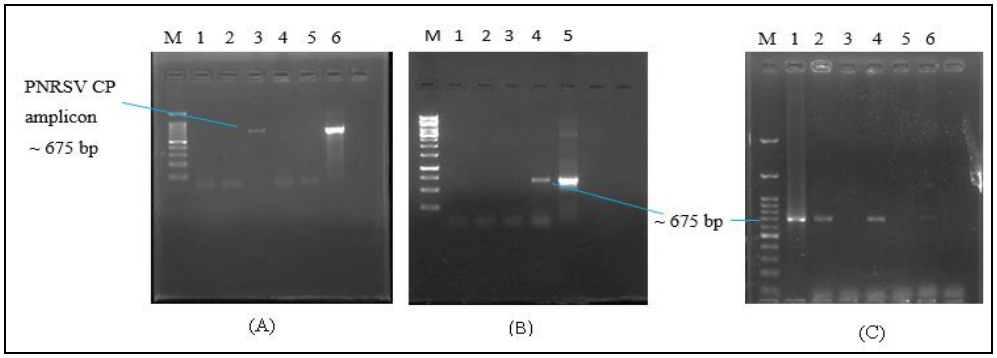

Fig 1: RT-PCR amplification (using PNRSV CP gene-specific primers) of rose samples collected from gardens of Srinagar and Kokernaag in Kashmir. (A): M = 100 bp DNA Marker; Lane 3, (KK10) amplicon size of ~ 675 bp. Lane 6 = Positive control. (B): M = 1 Kb DNA Marker; Lane 4 (KK27), amplicon size of ~ 675 bp, Lane 5 = positive control of PNRSV CP gene. (C): M = 100 bp DNA Marker; Lane 1 = Positive control, Lane 2 (KK46) and Lane 4 (KK50) amplicons size of ~ 675 bp.

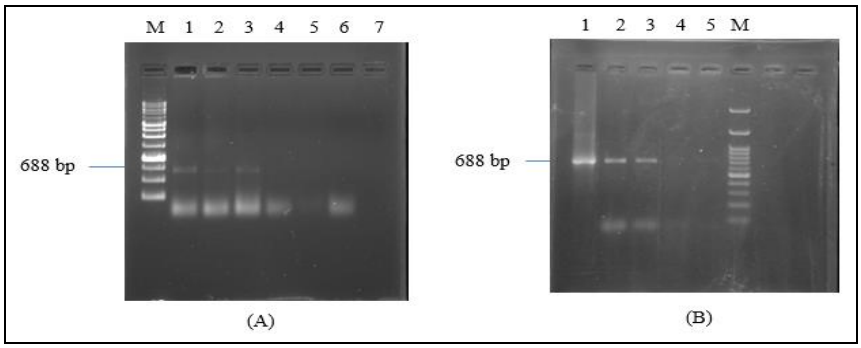

Fig 2: (A) RT-PCR amplification (using ApMV CP gene-specific primers) of rose samples collected from Jhajra, Dehradun, Uttrakhand. Lane: 1, 2 and 3, show the amplicon size of ~ 688 bp. M = 1 Kb DNA marker. (B): Lane: 2 and 3 show amplicon size of ~ 688 bp from rose-leaf samples collected from Kokarnaag Kashmir, Lane,1 = positive control, M = 100 bp DNA marker.

Sequence analysis of the PCR amplified products

After *in-silico* analysis, the retrieved sequences were submitted to the NCBI GenBank repository and are now available online with Accession numbers: OK625806.1, OK625807.1, OK625808.1, OK625813.1, OK625814.1 for ApMV rose isolates and Accession numbers: OK625809.1, OK625810.1, OK625811.1, OK625812.1 for PNRSV rose isolates. Our ApMV CP gene sequence of rose isolates shared more than 96 % sequence identity with the ApMV CP gene sequence from Warsaw, Poland (HG328271.1) and 100 % identity with the ApMV

apple isolate of India (MT678110.1). These sequences also share 98 – 99 % identity among themselves. PNRSV CP gene sequence of rose isolates from the valley of Kashmir, shared more than 98 % sequence identity with the PNRSV apricot isolate of India (LC382468.1) and shared > 99 % identity with PNRSV rose isolate of Poland (DQ983498.1). These sequences also share 99 – 100 % sequence identity among themselves.

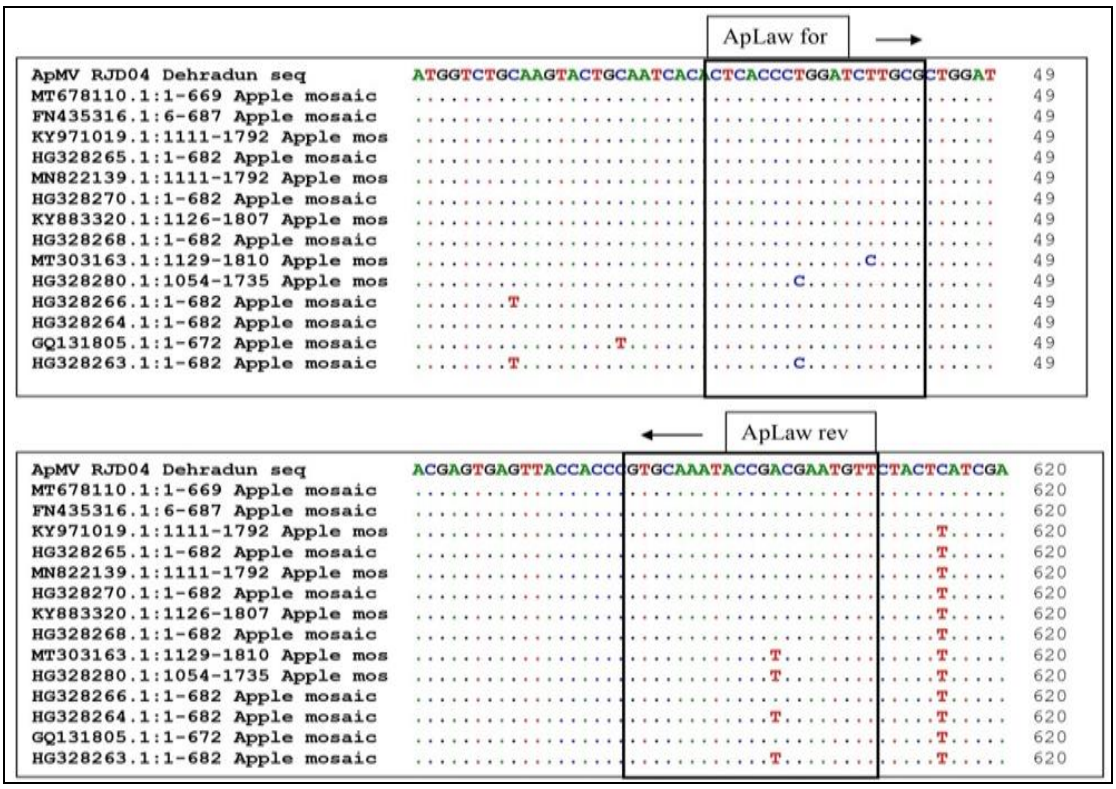

Fig 3(A): Specificity of ApMV CP gene-specific primer pair designed in this study. The NCBI accession no. of RJD04 Dehradun = OK625806.1

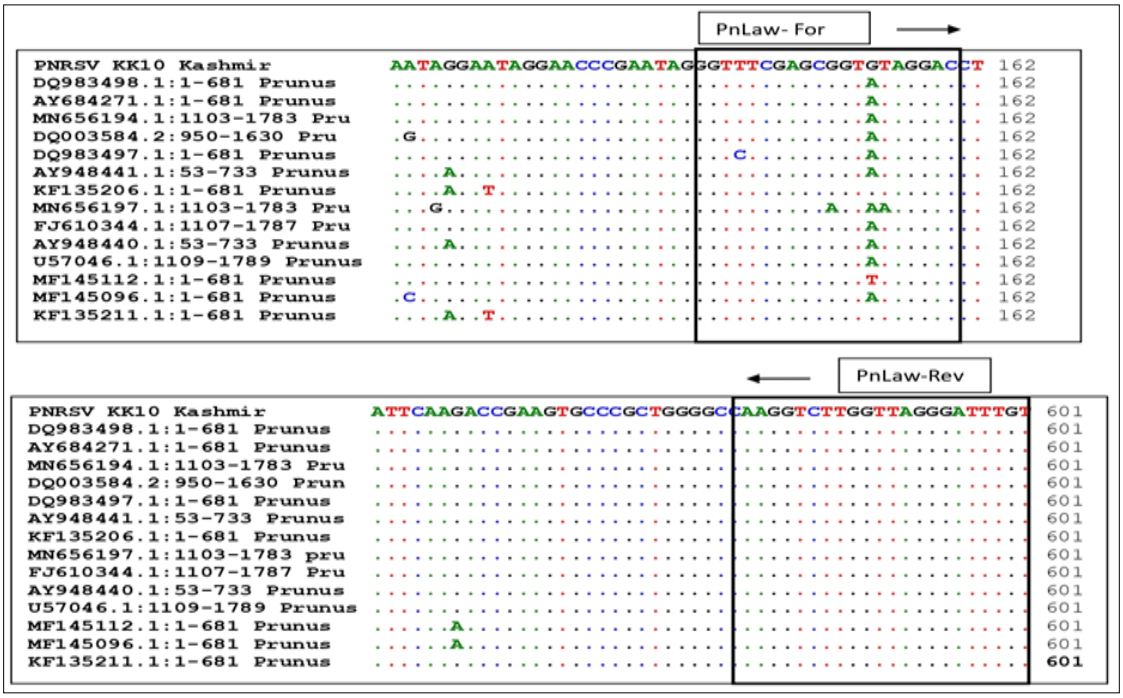

Fig 3(B): Specificity of PNRSV CP gene-specific primer pair designed in this study. The NCBI accession no. of KK10 Kashmir = OK625809.1

Uniplex and duplex PCR assays for ApMV and PNRSV detection

The uniplex gradient PCR was set to target the ApMV CP gene by ApLaw-for. and ApLaw-rev primer pair produced an amplicon size of ~ 616 bp (Fig. 4A: Lane 2 to 7), and uniplex gradient PCR targeting PNRSV CP gene by PnLaw-for and PnLaw-rev primer pair produced an amplicon size of 461 bp (Fig. 4B: Lane 2 to 7). For

duplex PCR plasmid amplification by primer cocktail of (ApLaw-for, ApLaw-rev, PnLaw-for, and PnLaw-rev) produced amplicon size of ~ 616 bp and 461 bp (Fig. 4C: Lane 2 to 7) as predetermined by *in-silico* analysis / virtual PCR results. The uniform high band intensity was observed throughout the Ta range from 48ºC to 60ºC. The uniplex and duplex PCR assays displayed optimum amplification at Ta values of 54ºC and 56ºC on agarose gel with high band intensities. Therefore, the Ta of 55ºC was selected as optimum for our duplex PCR. The same results were observed while using a ready-to-use master mix.

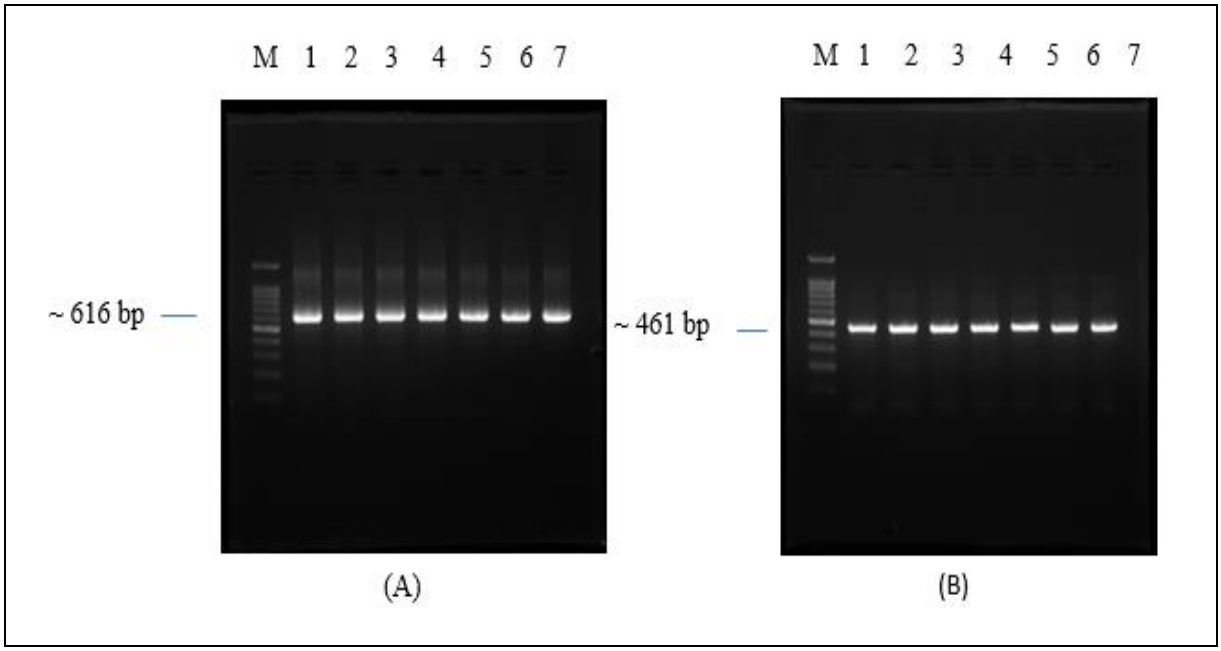

Fig 4: (A) Uniplex gradient PCR (Lane 1 - 7; Ta range from 48ºC to 60ºC) of ApMV CP gene cloned in a pJET1.2 plasmid vector). M = 100 bp DNA ladder. (B) Uniplex gradient PCR (Lane 1 - 7; Ta range from 48ºC to 60ºC, of PNRSV CP gene cloned in a pDrive plasmid vector). M = 100 bp DNA ladder.

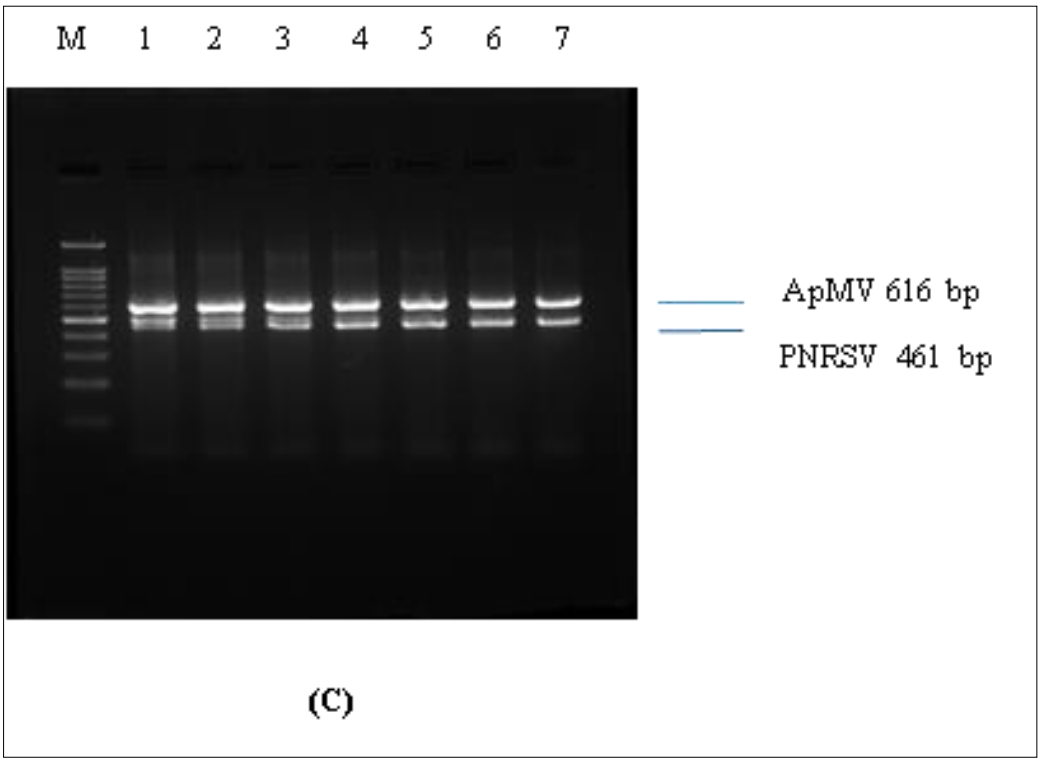

Fig 4(C): Duplex gradient PCR (Lane 1 - 7; Ta range from 48ºC to 60ºC of ApMV and PNRSV CP genes cloned in their respective plasmid vectors). M = 100 bp DNA ladder.

Specificity of our duplex PCR assay

Using primer cocktail (designed in this study), the uniplex PCR of the ApMV positive control produced only the amplicon size of ~ 616 bp (Fig. 5: Lane 1), and the uniplex PCR of the PNRSV positive control produced only

an amplicon size of ~ 461 bp (Fig. 5: Lane 2). The duplex PCR of cloned template strands of the ApMV CP gene and PNRSV CP gene successfully amplified both the gene targets of their respective size (Fig. 5: Lane 4). The reactions with negative control and Non-Template Control (NTC) both showed no amplification at all (Fig. 5: Lane 3 = -ve control, Lane 5 = NTC). The designed primer pairs showed no cross-amplification between ApMV and PNRSV despite belonging to the same genus of *Ilarvirus.*

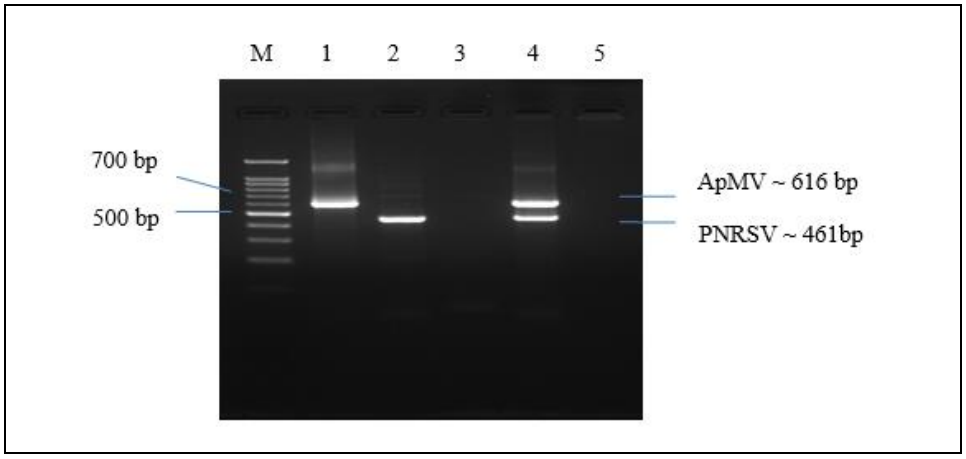

Fig 5: Specificity test using plasmid DNA of positive controls. Lane 1, shows the uniplex amplification of ApMV *CP* gene. Lane 2, denotes the uniplex amplification of the PNRSV-CP gene. Lane 3, negative control (cDNA from the healthy plant). Lane 4, duplex PCR amplification of ApMV and PNRSV CP genes. Lane 5 = Non Template Control (NTC); Lane M, 100 bp DNA ladder for amplicon size estimation.

Establishment of duplex PCR assay for ApMV and PNRSV detection in field collected samples

The duplex PCR was validated by using the artificially mixed ApMV, and PNRSV infected cDNA. The equimolar primer cocktail (ApLaw-for, ApLaw-rev, PnLaw-for and PnLaw-rev) designed in this study was used for duplex PCR amplification of ApMV and PNRSV infected cDNA samples and showed positive amplification of ~ 616 bp and 461 bp (Fig. 6: Lane 5). This primer cocktail was also used for uniplex PCR of the ApMV infected cDNA samples and it produced only an amplicon size of ~ 616 bp (Fig. 6: Lane 1) and the uniplex PCR of PNRSV infected cDNA samples amplified only an amplicon of ~ 461 bp (Fig. 6: Lane 2). The negative control and Non-template control (NTC) reactions were also set in the experiment, both showed no PCR amplification (Fig. 6A: Lane 3 = -ve control, Lane 4 = NTC).

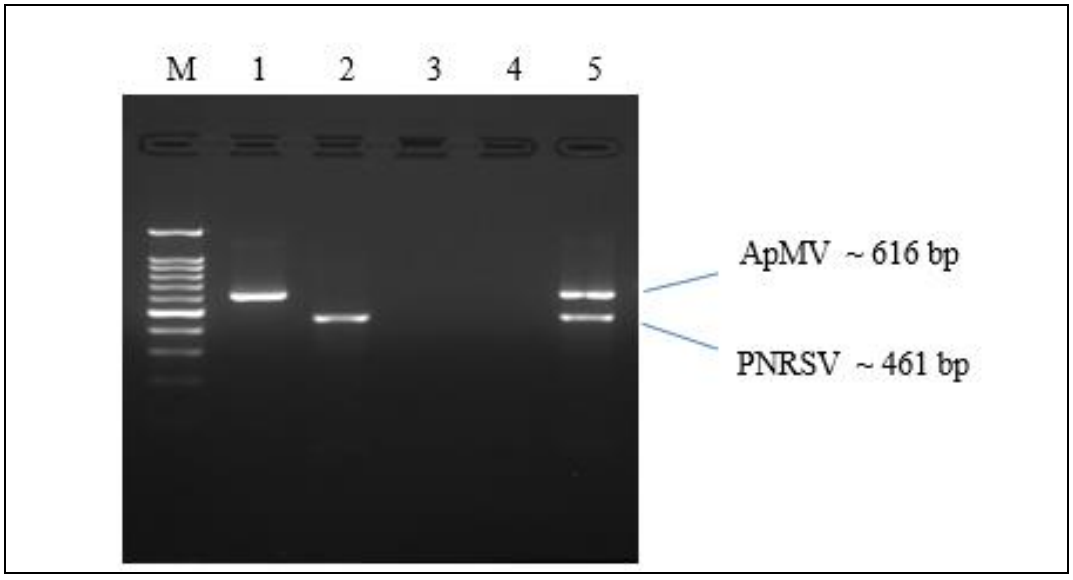

Fig 6: (A) Gel electrophoresis of the PCR products amplified from the cDNA of field-collected positive and negative controls of rose leaf tissues. Lane 1 = uniplex PCR of ApMV infected cDNA; Lane 2 = uniplex PCR of PNRSV infected cDNA; Lane 3 = negative control (cDNA of a healthy sample); Lane 4 = Non Template Control (NTC); Lane 5 = duplex PCR of artificially mixed cDNA of ApMV and PNRSV positive controls.

Confirmation of the duplex PCR products

The uniplex PCR products amplified by the primer cocktail were first resolved on agarose gel by electrophoresis to confirm the expected size of amplicons. Then amplified PCR products were sent for sequence determination.

The analysis of retrieved sequences confirmed the successful amplification of their respective CP gene sequences.

Sensitivity of designed duplex PCR assay for simultaneous detection of ApMV and PNRSV

The sensitivity of duplex RT- PCR assay from the diluted RNA showed the positive amplification of both the virus CP genes up to the dilution factor of 10^{-3} (1ng / µl) (Figure 7A: Lane 1 -3) and the ApMV sensitivity limit goes up to the dilution factor of 10^{-4} (100 pg / µl) visible on an agarose gel. Similarly, the agarose gel electrophoresis results of the duplex PCR from the diluted cDNA templates (synthesized from the undiluted RNA) showed the same results. The amplification of both the targets was visible with equal band intensity up to a dilution factor of 10^{-3} (Fig. 7B: Lane 1 to 3). The efficiency and sensitivity limit of the designed duplex PCR assay was found appreciably higher.

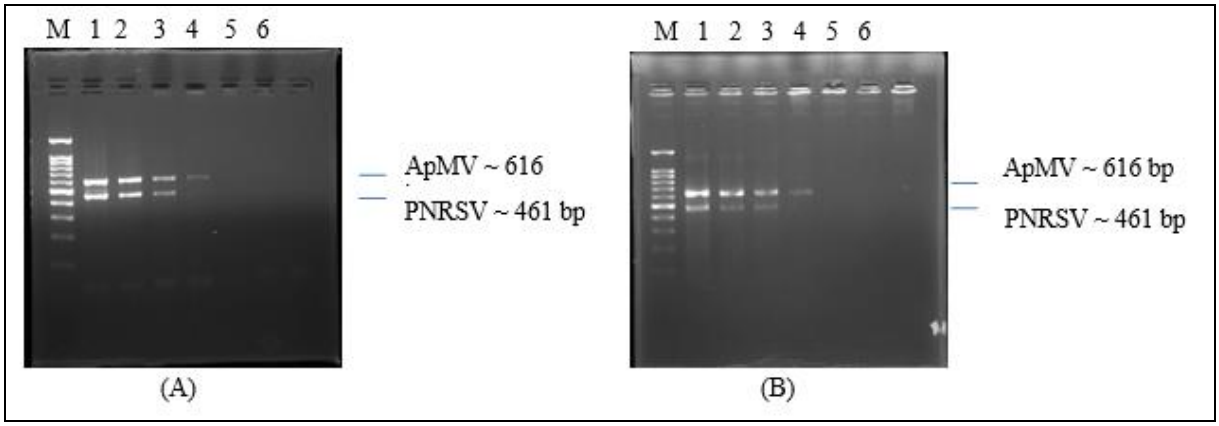

Fig 7: (A) The sensitivity limit of duplex RT-PCR for ApMV and PNRSV rose infecting viruses using 10-fold serial dilutions of cDNA prepared from undiluted RNA. Lanes 1–6: 10^{0} – 10^{-5} serial dilutions. Lane M: 100 bp DNA Marker. (B) Sensitivity limits of duplex RT-PCR assay using cDNA samples synthesized from a 10- fold serially diluted total RNA. Lanes 1–6: 10^{o} – 10^{-5} serial dilutions. Lane M: 100 bp DNA Marker.

Discussion

Virus disease in roses deteriorates flower quality and reduces crop yield, and plant growth [9]. *Apple mosaic virus* (ApMV) and *Prunus necrotic ringspot virus* (PNRSV) belonging to the genus *Ilarvirus* are reported to infect rose worldwide [13, 23]. In India the data available on virus infection in roses is very scanty, therefore in this study, we highlighted the incidence of ApMV and PNRSV infection in roses from India and attempted to design a duplex PCR assay for their simultaneous detection. The collected symptomatic foliar samples of rose were subjected to total RNA extraction using the CTAB method [41], TRIzol method [21] and using total RNA isolation kit (Sigma Aldrich). However, due to the abundance of secondary metabolites in rose plants, these extraction methods yielded a poor quality of RNA. Eventually, Option 2 of the protocol designed by Jordon-Thaden *et al*. 2015, [12] was used and the quality of extracted RNA was drastically improved. cDNA was synthesized and the PCR amplicons of the positively infected samples were cloned and sequenced.

During our study, a 2.5 % incidence rate of ApMV infection was found in the rose samples collected from the valley of Kashmir and an 8.57 % incidence rate was found in the rose samples collected from the Jhajra-Dehradun. Nearly the same ApMV incidence rate of 8 % infection was reported in rose from Turkey [39] and in India, a 7 % incidence rate was reported in the apple trees of Jammu and Kashmir and Himachal Pradesh [33]. We reported a 5 % per cent incidence rate of PNRSV infection in rose samples collected from the valley of Kashmir. These results closely comply with the 4 % incidence rate of PNRSV infection reported by researchers in rose plants from France [26], but this incidence rate is less than the incidence rate found in roses of New Zealand which is ~ 22% reported by E.J.M Milleza *et al* 2013 during their survey [23]. CP gene sequence of our ApMV rose isolates share >96 % sequence identity with ApMV rose isolate of Warsaw Poland (HG328271.1) and 100 % identity with an ApMV apple isolate of India (MT678110.1), and shared 98 – 99 % sequence identity among themselves. Our CP gene sequences of the PNRSV rose isolate shared > 98 % sequence identity with the PNRSV apricot isolate of India (LC382468.1) and shared > 99 % identity with a PNRSV rose isolate of Poland (DQ983498.1) and also shared 99 – 100 % sequence identity among themselves. None of the samples collected from other sampling sites was found positive for ApMV or PNRSV infection. Also, no infected sample was found to have a mixed infection.

Early detection of the virus is important for crop protection and disease management. To serve this purpose researchers use multiplex PCR which allows simultaneous detection of more than one target in a single reaction tube. This technique has proved specific, rapid and cost-effective for plant virus detection [4, 22, 31]. In this study, we designed a duplex PCR assay for simultaneous detection of ApMV and PNRSV viruses infecting rose plants. The CP gene sequences of our ApMV and PNRSV rose isolates were aligned with their respective sequences downloaded from the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/blast.cgi), and primers were

designed from a highly conserved region of CP gene to produce amplification size of 616 bp and 461 bp corresponding to ApMV and PNRSV.

The 25 µl reaction of uniplex as well as duplex PCR reaction was standardized with a final working concentration of primers (0.4 µM), 2 µl cDNA template (or 4 ng of plasmid DNA), Taq polymerase (2.5 U), dNTP (0.24 µM). The amplification was seen across the gradient PCR showing uniform band intensities both at Ta 54ºC and 56ºC. Therefore Ta 55ºC was selected as optimum for our duplex PCR reaction showing a clear band intensity of both the bands. The primer cocktail was used for uniplex PCR amplification of positively infected cDNA samples / positive plasmid clones of CP genes confirming the single band amplification of the desired size depending on the template strand added (Fig. 4 A and 4 B). Thus these results rule out the chance of non-specific amplification. The sensitivity test of our duplex PCR assay using primer cocktail showed the amplification of 616 bp and 461 bp up to 10^{-3} dilution factor (1ng / µl) for both ApMV and PNRSV CP gene targets (Fig. 7A and 7B: Lane 1–3). While the amplicon size of ApMV was visible up to 10^{-4} dilution factor (100 pg / µl) concentration. These results are in concordance with the earlier results of multiplex PCR carried out by different research groups to simultaneously detect different plant infecting viruses [36, 38]. None of the samples was found co-infected with both the ApMV and PNRSV viruses. Thus the duplex PCR assay's optimization was performed by artificially mixing the ApMV and PNRSV infected cDNA templates.

In conclusion, during our study, we successfully designed a duplex RT-PCR assay which proved more reliable, sensitive, highly specific, and economical for virus detection. This assay can help carry out epidemiological studies, plant certification and quarantine programs to design an effective strategy to control the spread of these viruses to roses and other economically important crops.

Author Contributions

Conceptualization, Prof. Jawaid A, Khan; Experiments performed by Latief A. Wani, Data curation, Latief A. Wani; Formal analysis, Prof. Jawaid Ahmad Khan and Priyanka Jawa; Supervision, Prof. Jawaid Ahmad Khan; original draft written by Latief A. Wani, Writing–review & editing, Latief A. Wani, and Prof. Jawaid A. Khan.

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Declarations

The authors declare no conflict of interest.

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