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Development of an Efficient and Highly Sensitive Multiplex RT-PCR Assay for Concurrent Detection of Viruses Associated with Stone Fruits

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Abstract

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Stone fruits | PNRSV | ACLSV | CVA | Multiplex RT-PCR

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Abstract

Stone fruit production is immensely threatened by viral diseases worldwide. In recent years, the spread of stone fruit viruses like apple chlorotic leaf spot virus (ACLSV), prunus necrotic ringspot virus (PNRSV), plum pox virus (PPV), prune dwarf virus (PDV), and cherry virus A (CVA) has been reported to cause devastating losses worldwide. In this study, three viruses that infect stone fruits, i.e., apple chlorotic leaf spot virus (ACLSV), cherry virus A (CVA), and prunus necrotic ringspot virus (PNRSV), were simultaneously detected and differentiated using a multiplex reverse transcription polymerase chain reaction assay. The optimized parameters were the annealing temperature, extension duration, and primer concentration. The multiplex RT-PCR assay detected ACLSV, CVA, and PNRSV, yielding specific amplicons of 677 bp, 1089 bp, and 346 bp, respectively. The assay demonstrated a detection limit of 10 pg of total RNA for ACLSV, 1 pg for PNRSV, and 100 pg for CVA. The devised technique detected all three viruses in single or multiple infections of the stone fruits (apricot, cherry, peach, plum, and nectarine) after the multiplex RT-PCR assay was validated using samples collected in the field. This study developed a multiplex RT-PCR assay that can be further utilized for indexing and diagnosing stone fruit viruses in field-grown populations.

Keywords: *Stone fruits, PNRSV, ACLSV, CVA, Multiplex RT-PCR*

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1. Introduction

The term "stone fruits" generally refers to certain species of the genus, which belong to the family *Rosaceae* (Kole and Abbott, 2012; Pedrelli *et al*., 2024). The major commercial stone fruits include apricots (*Prunus armeniaca*), sweet cherries (*Prunus. avium*), plums, primarily Asian or Japanese plums *(P. salicina*) and European plums (*Prunus. domestica*), peaches, and nectarines (*Prunus. persica*) (Famiani *et al*., 2020; Chawla and Kumar Sharma, 2024). These temperate fruits have a diverse geographic origin than pome fruits, with many species extending from Eastern Europe to China (Ogawa, 1991; Faust *et al*., 2011). The majority of stone fruit trees are reproduced asexually using techniques like root cuttings, tissue culture, grafting or budding on rootstocks (Hartmann and Kester, 1975; Iqbal and Singh, 2020; Nabi *et al*., 2024; Mandal *et al*., 2025). These propagation techniques have contributed to the international and national spread of numerous viral diseases (Bettoni *et al*., 2024). Stone fruits play a crucial role in the agricultural economies of major stone fruit-producing countries, including the United States, Australia, Afghanistan, China, Greece, France, Iran, Italy, New Zealand, Portugal, the Central Asian countries of the former USSR, and India (Khan *et al*., 2021; Pedrelli *et al*., 2024). Major stone fruit-producing states in India include Jammu and Kashmir, Himachal Pradesh, Punjab, and Uttarakhand(Wani *et al*., 2024). In Jammu and Kashmir, the Kashmir valley is blessed with the production of a large variety of temperate fruit crops and among them, stone fruits are occupied a major commercial venture after apple because of higher remuneration per unit area as well as the realization of fruit consumption (Askary *et al*., 2012; Askary *et al*., 2014). In Jammu and Kashmir, the total cultivated area under major stone fruit is about 13,545 hectares, with 55,118 metric tons of annual production (Wani *et al*., 2024).

Throughout history, stone fruits have been prone to various fungal, bacterial, and viral diseases. Most of the diseases are common among stone fruits as they are closely related, making these diseases one of the main constraints in their overall production (Desvignes *et al*., 1999; Myrta *et al*., 2003; Nabi *et al*., 2018; Wani *et al*., 2024). Among the various diseases of stone fruits, viruses constitute a major threat to fruit production in orchards worldwide (Barba *et al*., 2015; Umer *et al*., 2019). In addition to reducing yield, viral infections can impact fruit size, shape, and quality, leading to substantial economic losses across all sectors of the production chain (Hadidi *et al*., 2011; Jones and Naidu, 2019; Wani *et al*., 2021; Wani *et al*., 2025;Wani et al. 2026)). Some of the economically important viruses of stone fruits include plum pox virus (PPV), prune dwarf virus (PDV), peach mosaic virus (PMV), prunus necrotic ringspot virus (PNRSV), cherry leaf roll virus (CLRV), apple mosaic virus (ApMV), apple chlorotic leaf spot virus (ACLSV), apple stem pitting virus (ASPV), cherry virus A (CVA), apple stem grooving virus (ASGV), american plum line pattern virus (APLPV), little cherry virus 1(LChV1) and plum

bark necrosis stem pitting-associated virus (PBNSPV) etc. (Aparicio *et al*., 1998; Rouag *et al*., 2008; Zagrai *et al*., 2022; Khan *et al*., 2024; Wani *et al*., 2024; García *et al*., 2025). Viruses infecting stone fruits are widely distributed in India, causing huge losses in production and yield (Golnaraghi and Gaur, 2024; Wani *et al*., 2024). These viruses are often insidious, frequently remaining undetected and untreated. In some cases, they exhibit latent infections without visible symptoms. Yet, they can significantly affect plant health by causing stunted growth, reduced fruit production, smaller fruit size, shortened lifespan, altered product composition, and other subtle impacts that may go unnoticed (Hadidi *et al*., 2011; Sokhandan-Bashir and Ighani, 2024; García *et al*., 2025; Wani *et al*., 2025). These viruses can cause various symptoms, from asymptomatic (latent) infections to a general decline in plant vigour and productivity (Hull, 2018). Leaf symptoms may include distortion, twisting, mottling, rolling, necrotic spots, shot holes, and unusual colour patterns (Rybicki and Foster, 2024; Wani *et al*., 2025). Affected fruits often exhibit reduced size and quality, shape distortions, and characteristic alterations such as ringspots, mottling, and line patterns (Hadidi *et al*., 2011; Jevremović and Paunović, 2024; Wani *et al*., 2025). Moreover, viral infections cannot be cured, often inflicting more harm on perennial crops than annual crops (Chandel *et al*., 2013; Singhal *et al*., 2021). Early detection and accurate diagnosis of viral diseases are essential for developing effective and sustainable management strategies to limit their spread (Ibaba and Gubba, 2020; Nabi *et al*., 2020; Kim *et al*., 2025). Therefore, this study aimed to develop a highly sensitive and specific multiplex RT-PCR assay to detect three commercially significant stone fruit viruses simultaneously.

Various diagnostic methods have been established for detecting ACLSV, PNRSV, and CVA, including reverse transcription-polymerase chain reaction (RT-PCR), reverse transcription loop-mediated isothermal amplification (RT-LAMP), and serological techniques (Marais *et al*., 2012; Hu *et al*., 2014; Lu *et al*., 2018; Canales *et al*., 2021; Raguseo *et al*., 2021; Iralu *et al*., 2025; Wani *et al*., 2025; Wani *et al*., 2025). However, these approaches typically detect only a single virus per reaction. In contrast, multiplex RT-PCR, an advanced variant of PCR, offers a rapid, reliable, and cost-effective method for simultaneously detecting multiple viruses in a single assay (Cao *et al*., 2022; Cao *et al*., 2023; Cayak and Fidan, 2024; Wani *et al*., 2025). This technique has been widely applied for diagnosing viral infections in apple, cherry, peach, and papaya (Wani; Noorani *et al*., 2013; Hao *et al*., 2016; Nabi *et al*., 2022; Wani *et al*., 2025). However, there haven't been any reports of ACLSV, CVA, and PNRSV being detected simultaneously from infected stone fruits. Thus, the primary objective of this study was to develop a multiplex RT-PCR assay with high sensitivity and specificity for the simultaneous detection of three economically significant viruses. The detection of these viruses in field samples was used to assess the optimized multiplex RT-PCR test, which demonstrated the reliability and sensitivity of the assay.

2. Materials and methods

2.1. Sample Collection

From spring 2020 to 2023, stone fruit orchards were surveyed in the major fruit-growing districts Anantnag (L1), Srinagar (L 2), Budgam (L3), Ganderbal (L4) and Baramulla (L5) of Jammu and Kashmir (J&K) and suspected symptoms of the virus were visually inspected. Symptoms of the virus, such as mosaic, shot holes, necrotic spots, and chlorotic spots, were noticeable on apricots, cherries, nectarines, plums, and peaches in different orchards. Symptomatic leaf samples suspected to be virus-infected along with the corresponding asymptomatic leaves were collected in polythene bags. These samples were properly labelled and stored in an ultra-low temperature freezer at -80 °C for further analysis.

2.2. Total RNA Isolation and cDNA Synthesis

Total RNA was extracted from both symptomatic and asymptomatic leaf samples of all species using the TriZol method (Vennapusa *et al*., 2020; Wani *et al*., 2025; Wani *et al*., 2025). Approximately 100 mg of leaf sample was finely crushed using liquid nitrogen, and 1 ml of TriZol® reagent per 100 mg of leaf tissue was added, and the subsequent procedure was performed as per manufacturer's instruction (Invitrogen, Thermo Scientific). RNA was precipitated overnight at -80 °C using ice-cold 70% ethanol. The resulting RNA pellet was then dissolved in nuclease-free water (Promega). RNA was quantified using a spectrophotometer Nanodrop (Thermo Scientific, USA). To verify RNA integrity, samples were run on a 1% agarose gel and visualized with ethidium bromide staining in UV-transilluminator. First-strand cDNA was synthesized using a Revert-Aid cDNA synthesis kit (Thermo Scientific). The reaction mixture was incubated at 65 °C for 5 minutes, 42 °C for 60 minutes, and 70 °C for 5 minutes and the resulting cDNA was used for PCR amplification. The details of the primers used for simplex and multiplex RT-PCR assays were previously reported (60-62) and were listed in Table 1 and their specificity was indeed verified through BLAST analysis against the NCBI database to ensure their ability to detect the targeted viruses.

2.3. Reverse Transcription-PCR (RT-PCR)

2.3.1. Simplex RT-PCR Assays

For simplex RT-PCR, 25 µl volume of reaction mixture containing 12.5 µl master mix (GoTaq Green, Promega), 1 µl cDNA, 9.5 µl of nuclease-free water (Promega), 2 µl each of (10 µM) specific forward and reverse primers of CP (coat protein) for ACLSV, CVA and PNRSV(Sanchez-Navarro *et al*., 2005; Noorani *et al*., 2010; Gospodaryk *et al*., 2013 ; Wani *et al*., 2024). PCR reactions were performed in Eppendorf Master cycler (Eppendorf, USA) and the conditions were 35 cycles of denaturation: 95 °C for 30 seconds, annealing: 51°C (ACLSV), 54 °C (CVA), 53 °C (PNRSV) for 30 seconds, extension:72 °C for 30 seconds and final elongation: 72 °C for 10 minutes. The PCR products were electrophoresed in 1% agarose gel in 1X TAE buffer (pH 8.0) at 80V. A 1 kb DNA molecular ladder was used to estimate the amplicon size (NEB Applied Science, New England Biolabs). Electrophoresis was carried out for 60 minutes and the results were visualized under a gel-documentation system (Chemi Doc- BioRad).

2.3.2. Optimization of Multiplex RT-PCR

Optimization of the multiplex RT-PCR assay involved sequential testing of primer concentration, annealing temperature, and extension time, with RNA isolated from virus-infected plants. The three primer pairs in combinations of different concentrations were tested from 10 to 25 µM. The annealing temperature was set at 50-57 °C in increments of 1°C. The extension times tested were 30 seconds, 45 seconds, 60 seconds, and 75 seconds. PCR reactions were performed in 25 µl of reaction volume containing 12.5 µl GoTaq Green master mix (Promega), 2 µl cDNA, 4.5 µl nuclease-free water, and 0.5 µl each of (10 µM) specific forward and reverse primers of CP for ACLSV, CVA and PNRSV. PCR assays were performed in a thermocycler (Eppendorf Master cycler, USA). The program was set up for 30-40 cycles with a 5-minute initial denaturation at 95 °C, 30 seconds denaturation at 95 °C, 30-45 seconds annealing at 50-57 °C followed by 30 seconds extension at 72 °C and a 10-minute final elongation at

72 °C. The best results were achieved at 53 °C for 45 seconds, repeated for 40 cycles. PCR products were separated on a 1.5% agarose gel, and amplifications were visualized using a gel-documentation system (Chemi Doc- BioRad).

2.3.3. Detection Limits of Multiplex RT-PCR

The sensitivity of the multiplex RT-PCR assay for all three viruses (ACLSV, CVA, and PNRSV) was compared to that of individual simplex RT-PCR assays. This comparison was performed using 10-fold serial dilutions of plant total RNA from virus-infected source plants (100 ng/µl to 1 pg/µl).

2.3.4. Multiplex RT-PCR Assay

Between 2021 and 2023, a total of 448 leaf samples exhibiting symptoms such as chlorosis, necrosis, mosaic, leaf puckering, or no visible symptoms were collected from orchards of different locations L1 to L5. Total RNA was extracted from the collected samples as described earlier. Nuclease free water served as a negative control, while RNA from previously confirmed stone fruit plants was used as a positive control. The multiplex RT-PCR assay results for the field samples were further validated using simplex RT-PCR assays.

3. Results

3.1. Symptoms

During the survey conducted in various districts of Jammu and Kashmir, cherry trees were identified with the symptoms of mosaic, puckering and necrosis (Fig.1 A-C), Nectarine with mosaic and leaf deformation (Fig.1 D-E), severe mosaic and shot holes were identified on peach and plum cultivars (Fig.1 F, H, I) and deformed leaves on apricot (Fig.1G). As the extent of cherry cultivation across the surveyed districts increased, a high virus incidence was observed in cherry farms in the Budgam, Ganderbal, Srinagar, and Baramulla districts. Although the cultivation of apricots, peaches, and plums was limited to a few orchards, they exhibited unique symptoms of viral infection across all the surveyed locations. However, nectarine trees revealed a remarkable dissimilarity of symptoms in each surveyed district.

3.2. Specificity and Compatibility of Primers Using Simplex RT-PCR

Symptomatic leaf samples were initially tested in a simplex RT-PCR assay with specific primers, each resulting in the detection of PNRSV, ACLSV, and CVA. In the simplex RT-PCR assay, the three tested virus-specific primers amplified coat protein genes of all three viruses (ACLSV, CVA, and PNRSV). The expected amplicons of 346 bp for PNRSV, 677 bp for ACLSV and 1089 bp for CVA, and were readily obtained in the PCR assay (Fig.2, Lane 1–3).

The tested primers were later combined in an optimized multiplex RT-PCR test that simultaneously detected all three target viruses and amplified specifically to the length of expected amplicons to each virus. PCR products corresponding to each target coat protein gene were purified and sanger sequenced. The consensus sequence of each virus was deposited in GenBank (Table 2). Pairwise nucleotide sequence comparison was performed by using the Basic Local Alignment Search Tool (BLAST) to identify the phylogenetically closest neighbours based on the nucleotide identities at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The partial coat protein sequence of identified viral strains revealed a maximum identity to PNRSV, ACLSV, and CVA isolates already available in the GenBank. These results further confirmed the successful amplification of the target fragments. The selected primer combinations were subsequently optimized for use in the multiplex RT-PCR assay (Fig. 2, Lanes 4–10). No significant primer interactions or primer-dimer formations were observed (Fig. 2, Lane N).

3.3. Viruses Detected in Different Stone Fruits Using Simplex PCR

In simplex PCR, PNRSV was detected in 149 symptomatic samples collected from cherry, peach, plum, apricot and nectarine orchards and was identified as the most widespread virus infecting stone fruit crops. The virus was detected in all the surveyed locations, but its occurrence was significantly higher in the Srinagar district. The presence of PNRSV and its high incidence was evident, as most of the isolates in the RT-PCR assay were tested positive for PNRSV, followed by ACLSV. Following the prevalence of PNRSV, ACLSV was also distributed in a higher number of samples, indicating 110 samples were tested positive for its presence. However, CVA was identified in a few symptomatic samples (36), and its presence was prevalent in the Srinagar and Budgam districts. The presence of CVA has not been identified in the symptomatic samples collected from different locations. Table 3 describes in detail the total symptomatic samples tested for the presence of the virus in simplex, duplex, and multiplex RT-PCR assays.

3.4. Optimization of Multiplex RT-PCR

After confirming specific amplification in simplex PCR, these primers were used to optimize multiplex PCR. To optimize the reaction, primer concentrations were tested for each virus. A mixture containing 3 µl of forward and reverse primer for each virus could not achieve stable amplification for all the viruses. As a result, a 1.5 µl mixture of forward and reverse primer (final concentration of 1 µM) containing 0.5 µl of ACLSV, CVA and PNRSV each could successfully amplify three fragments with the expected amplicon sizes (677 bp for ACLSV, 1089 bp for CVA, and 346 bp for PNRSV). To further optimize the reaction, the annealing temperature was varied from 50 °C to 57 °C within a 40-cycle PCR protocol, which included an initial denaturation at 95 °C, followed by denaturation at 95 °C, extension at 72 °C, and a final extension at 72 °C. The results presented in Fig. 3 indicate that annealing temperatures within the 50 °C to 57 °C range produced three distinct amplicons of the expected sizes: 677 bp, 1089 bp, and 346 bp.

3.5. Detection Limit of the Simplex and Multiplex RT-PCR Assays

The sensitivity of the multiplex RT-PCR assay was evaluated using 10-fold serial dilutions of 100 ng total RNA. The assay detected PNRSV (346 bp) at a 10^{-5} RNA dilution and ACLSV (677 bp) at a 10^{-4} dilution, while CVA (1089 bp) was detectable only at a 10^{-3} dilution (Fig. 4). Nuclease free water was used as negative control was not amplified. The simplex RT-PCR assays achieved detection limits of 10 pg of total RNA for ACLSV, 1 pg for PNRSV, and 100 pg for CVA .((Fig. 4, A–C). In the multiplex RT-PCR assay, the detection limit for PNRSV, ACLSV and CVA was same as simplex PCR (Fig. 4D). The results revealed that the multiplex RT-PCR assay exhibited same sensitivity when compared to the simplex RT-PCR assay.

3.6. Validation of the Multiplex RT-PCR

To validate the multiplex RT-PCR assay's reliability, 448 leaf samples collected during the survey were tested by RT-PCR and multiplex RT-PCR assays. The results of the RT -PCR assay demonstrated that PNRSV was positive in 149 samples (33.2%), ACLSV in 110 samples (24.5%) and CVA in 36 samples (8.03%) (Table 4). From the test results, PNRSV has the predominant distribution on all the stone fruit crops collected from the L1, L2 and L3 locations (Table 2). The

symptoms of necrotic spots and mosaic on all stone fruit crops of surveyed locations were consistently amplified in the PCR assay. The incidence of CVA was almost limited to the isolates collected from the L2 and L3 districts, and its incidence was not recorded from fruit crops of the location L1, L4 and L5. The mosaic and leaf puckering symptoms observed on cherry, peach and nectarine were tested positive for CVA in PCR assays. The presence of ACLSV was equally distributed on all fruit crops and is a prominent virus infecting all the orchards of the surveyed districts. ACLSV associated with the symptoms of chlorosis in apricot, nectarine and leaf spots on cherry, peach, plum cultivars are tested positive in PCR. Table 4 presents the viral profiles of stone fruit samples from various districts of Jammu and Kashmir that tested positive in PCR assays. Most symptomatic samples were infected with a single virus (PNRSV). At the same time, a smaller number exhibited co-infection with two viruses (PNRSV and ACLSV), and a few showed infections with all three viruses (PNRSV, ACLSV and CVA) (Fig. 5).

4. Discussion

Stone fruits are economically important, and there has been a spike in the planting area of these fruits in North Western Himalayan regions in recent years. Viral diseases are one of the major constraints for the quality production of stone fruits (Umer *et al.*, 2019; Costa *et al.*, 2022; Wani *et al.*, 2024), and mixed infection of viruses often causes rigorous damage to the trees(Costa *et al.*, 2022). Plant viruses, including those affecting stone fruits, are now spreading to and infecting previously non-host species, resulting in the association of new hosts to the virus. Routine PCR assay relying on a single primer pair cannot identify multiple viruses infecting the host plant. Also, the high cost of genome sequencing limits its accessibility as a universal solution, and nucleic-acid-based techniques, such as duplex/multiplex PCR, still allow for detecting multiple viruses in a single assay. In this study, we developed a multiplex RT-PCR assay for the detection of three viruses infecting stone fruit species. This assay successfully identified PNRSV, ACLSV, and CVA in both single and mixed infections and was validated on 448 field samples. A common challenge in multiplex RT-PCR is the unbalanced amplification of target viruses due to the simultaneous presence of multiple gene targets in a single reaction. This can lead to competition for enzymes and nucleotides, affecting primer compatibility and overall assay efficiency (Wei *et al.*, 2023). This multiplex RT-PCR assay showed weak amplification of the CVA fragment when equal concentrations of all three primer sets were used. A 10 μM primer concentration was used for all targets in the multiplex RT-PCR to achieve uniform amplification and consistent band intensities. To optimize amplification efficiency and achieve uniform band intensities for all three viruses, a relatively higher primer concentration (10 μM) was used. This adjustment ensured consistent amplification and comparable amplicon intensities within the same reaction. Also, the multiplex RT-PCR was approximately 10-fold less sensitive than the simplex RT-PCR for detecting PNRSV. Consistent with previous studies (Wei *et al.*, 2009; Yao *et al.*, 2019), high oligonucleotide and primer concentrations likely contribute to reduced multiplex RT-PCR efficiency. The multiplex RT-PCR assay's reliability for field applications was validated by testing 448 stone fruit samples from five Kashmir districts. The multiplex RT-PCR assay revealed single infection rates of 33.2% (149/448) for PNRSV, 24.5% (110/448) for ACLSV, and 8.0% (36/448) for CVA. Additionally, 18.75% (84/448) of the samples showed coinfection with 2-3 viruses and 65.8% (295/448) were infected with at least one virus. According to this result, PNRSV is highly prevalent and is the major virus infecting the stone fruits of Kashmir. The high infection rates observed suggest that viruses infecting stone fruit crops of Jammu and Kashmir can be a major limiting factor in the quality production of temperate fruits. To the best of our knowledge, this is the first multiplex RT-PCR assay developed for the simultaneous detection of PNRSV, ACLSV, and CVA in stone fruits. The optimized multiplex RT-PCR assay developed here offers a practical and sensitive tool for efficiently detecting PNRSV, ACLSV, and CVA coinfections in temperate fruit crops. Compared to single-virus detection through routine RT-PCR assays, this technique provides a more time-efficient and cost-effective approach for detecting coinfections of these three viruses, especially in large-scale surveys

5. Conclusion

This study successfully developed and optimized a sensitive and specific multiplex RT-PCR assay for the simultaneous detection and differentiation of three major stone fruit viruses—ACLSV, CVA, and PNRSV. The assay demonstrated high efficiency with clearly distinguishable amplicon sizes and high detection limits, enabling reliable identification of both single and mixed infections of viruses infecting apricot, cherry, peach, plum, and nectarine. We found that the multiplex RT-PCR assay offers an efficient method for detecting viruses in stone fruit samples from large-scale field surveys. The multiplex RT-PCR was optimized by evaluating primer pair compatibility, primer concentration, annealing temperature, and extension time, resulting in a highly sensitive and specific method of detecting viruses with an equal sensitivity as of simplex PCR.

DECLARATIONS

Author Contribution

S.W: Conceptualization, methodology and writing-original draft preparation, **S.U.N and A.H;M.D.S:** formal analysis, validation, investigation, resources and writing-review and editing, **D.M:** Methodology, data curation, visualization, supervision, **SW; SN**: Formal analysis, validation, investigation Project administration, **S.N;A.H;M.D.S**: funding acquisition and writing-review and editing, **M.D.S, S.U.N** : Conceptualization, supervision, writing-review and editing All authors have read and agreed to the published version of the manuscript.

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