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Short communication

# Simultaneous detection of stone fruit tree viruses by one-step multiplex RT-PCR

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ABSTRACT

A protocol of multiplex RT-PCR in a one-tube system for the detection of the most common stone fruit trees viruses [e.g., plum pox virus (PPV), prune dwarf virus (PDV), and *Prunus* necrotic ringspot virus (PNRSV)], including the internal control of NADH dehydrogenase subunit 5 (*nad*5) gene are described here. The method specificity was tested on more than 80 different samples with various isolates and strains of the viruses. It showed that the targeted viruses produced the expected amplicons, whereas all other related viruses produced only the *nad*5 internal control amplicon. The method sensitivity was evaluated by comparing it with Simplex RT-PCR with the same primers; no significant differences in detection limits were recorded. Furthermore, the competitiveness of the primers in the assay was tested by serial RNA dilutions of samples with mixed and single infections. The least competitive was the internal control *nad*5 gene primer pair; therefore, there is a reduced risk of false negatives as all the other primers tend to be more efficient in the given primer cocktail than in the primers for internal control.



## 1. Introduction

*Prunus* spp. are affected by many viruses, and most frequently occur in the genera *Ilarvirus*, *Potyvirus,* and *Trichovirus*. The following viruses are the most important for stone fruit trees: plum pox virus (PPV), *Prunus* necrotic ringspot virus (PNRSV), prune dwarf virus (PDV), and apple chlorotic leaf spot virus (ACLSV) (Németh, 1986). Currently, these pathogens may be detected by biological indexing (Di Terlizi, 2000; Bertozzi et al., 2002; Gentit, 2006), immunological methods (Clark and Adams, 1977; Cambra et al., 1994; Spiegel et al., 1998; Mekuria et al., 2003), molecular hybridization (Palkovics et al., 1994; Saade et al., 2000), and reverse transcription polymerase chain reaction (RT-PCR) (Wetzel et al., 1991, 1992; Scott et al., 1992; Olmos et al., 1997; Rosner et al., 1998; Spiegel et al., 1999). In all these assays, usually one pathogen is detected per assay (Hadidi et al., 2004). Procedures that allow simultaneous detection and/or identification of different viruses are desirable for routine diagnosis because they require less time, labor, and cost than single RT-PCR. Several multiplex systems are available for routine detection of multiple viruses and viroids (Grieco and Gallitelli, 1999; Bertolini et al., 2001; Ito et al., 2002; Menzel et al., 2002; Ragozzino et al., 2004; Roy et al., 2005) in fruit trees. However, the efficiency of multiplex assays is variable with respect to combination and number of tested viruses or viroids (Sánchez-Navarro et al., 2005).

We describe here a protocol of multiplex RT-PCR in a one-tube system for the detection of the most common viruses (e.g., PNRSV, PDV, and PPV) in stone fruit trees. The detection specificity of the assay was also performed by sequence analysis of the PCR fragments.

## 2. Materials and methods

### 2.1. Virus source

Leaf samples (three–five leaves per sample) were collected randomly from plum (*Prunus domestica* L.), cherry (*P. avium* (L.) L.), sour cherry (*P. cerasus* L.), Nanking cherry (*P. tomentosa* Thunb.), peach (*P. persica* (L.) Batsch), and blackthorn (*P. spinosa* L.) trees known or suspected to be PPV-, PDV-, and PNRSV-positive grown in research and commercial orchards and in the wild in the Czech Republic. Two PDV isolates from Romania, BN51125-2/8 and BN51125-2/4 from *P. domestica,* were used for comparison with the Czech isolates.

### 2.2. Oligonucleotides

Current PDV and PNRSV coat protein nucleotide sequences from the GenBank database were aligned using the program Clustal W version 1.7 (Thompson et al., 1997) and used to identify oligonucleotide primer sequences that may be used for universal detection of PDV and PNRSV. Additional primers used in this study include the PPV-RR and F3 primers described by Varga and James (2005, 2006) and *nad5* mRNA-specific primers Nad5-F and Nad5-R (mRNA coding mitochondrial gene of higher plants encoding subunit 5 of the NADH ubiquinone oxidoreductase complex) described by

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**Table 1**
Primer used in this study.

| Primer name | Sequence (5′-3′) | Nucleotide position | Fragment size | Target | Reference |
|---|---|---|---|---|---|
| PPV-RR | CTCTTCTTGTGTTCCGACGTTTC | 9475-9497[a] | 345 bp | PPV | Varga and James (2005) |
| F3 | GGAATGTGGGTGATGATGG | 9153-9171[b] | | | Varga and James (2006) |
| PDVdpR | CCTTTAATGAGTCCGT | 1572-1557[c] | 220 bp | PDV | This study |
| PDVdpuF | CCGAGTGGATGCTTCACG | 1353-1370[c] | | | This study |
| PNcpR | CTTTCCATTCGGAGAAATTCG | 1821-1801[d] | 425 bp | PNRSV | This study |
| PNcpinF | GAGTATTGACTTCACGACCAC | 1396-1416[d] | | | This study |
| Nad5-R | CTCCAGTCACCAACATTGGCATAA | 968-987 and 1836-1838[e] | 181 bp | Plant | Menzel et al. (2002) |
| Nad5-F | GATGCTTCTTGGGGCTTCTTGTT | 1973-1995[e] | | | |

[a] Genome position on PPV accessions D (X16415) and M (M92280).
[b] PPV Fantasia, accession AY912056.1.
[c] PDV ch-137 accession L28145.
[d] PNRSV PV32 accession Y07568.
[e] nad5 accession D37958.

Menzel et al. (2002) as an internal control. Primers are described in Table 1.

### 2.3. RNA preparation

RNA was isolated from the leaves of all the above mentioned plants by using a commercially available extraction kit, RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) with a modification according to Mekuria et al. (2003). Two hundred milligrams of either fresh or frozen leaf tissue was ground into a fine powder in liquid nitrogen, mixed with 2.0 ml of extraction buffer containing 4.4% (w/v) PVP-40 (Sigma, MO, USA) and 1% (w/v) sodium metabisulphite, and briefly vortexed. Five hundred microliters of the homogenate was mixed with 60 μl of 20% (w/v) sarkosyl (*N*-lauroyl-sarcosine, Sigma) and incubated at 70 °C with agitation for 10 min. The contents were then transferred to a QIA shredder mini column and centrifuged at 14,000 rpm for 5 min. The column flowthrough (350 μl) was mixed with 315 μl of 95% ethanol, and the remainder of the protocol was carried out according to the manufacturer's instructions. RNA was stored at −20 °C (or at −80 °C for long-term storage). The quality and quantity of isolated RNA were determined by spectrophotometry at 260, 230, and 280 nm.

### 2.4. One-step RT-PCR for multiplex virus detection

One-step-RT-PCR was performed with the One-Step-RT-PCR kit (Qiagen) as described by Kundu (2003). The One-Step-RT-PCR mixture containing 5 μl of the 5× Qiagen One-Step-RT-PCR buffer, 10 nM of each dNTP, 1 μl of the Qiagen One-Step-RT-PCR enzyme mixture, 1 μl of Q solution, and 6 pM of reverse and forward primers (Table 1) were prepared in 2 μl of RNA and the mixture was adjusted to 25 μl with RNase-free water. The reaction was carried out in a thermocycler (MJ Research) as follows: a RT step at 50 °C for 30 min and an initial PCR activation step at 95 °C for 15 min, then 33 cycles of 94 °C for 30 s (denaturation), 51 °C for 45 s (annealing), and 72 °C for 80 s (extension). After the last cycle, a final extension step at 72 °C for 10 min was added.

The PCR products were analyzed in 2% agarose gel electrophoresis; staining was done by SYBR Green (Invitrogen, CA, USA). PCR fragments were also analyzed by sequencing of amplicons using reverse and forward primers of each virus of several isolates. Sequences were analyzed by using software Clustal W version 1.7 (Thompson et al., 1997), Sequencher 4.8 (Gene Codes Corporation, MI, USA) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

## 3. Results

### 3.1. Virus detection by simple and multiplex RT-PCR

A multiplex RT-PCR was developed for the identification of PPV, PDV, and PNRSV in stone fruit tree tissues. Included in the method were the PPV-specific primers developed by Varga and James (2005, 2006), which resulted in a 345 bp amplicon; universal primers used to amplify a 181 -p fragment of the *nad*5 mRNA, serving as an internal positive control for the RT-PCR (Menzel et al., 2002); and novel primers designed to amplify a 425-bp fragment of the coat protein gene characteristic of PNRSV and a 220-bp fragment of the coat protein gene of PDV (Fig. 1A). The primers in this reaction were chosen among many candidates because of their specificity and ability to produce only the targeted amplicons (Fig. 1A). The thermal gradients of annealing temperature were run for some samples (the $T_a$ between 48 and 63 °C) and the efficiency of the assay was confirmed to be optimal between 50 and 55 °C. However, the reaction is so robust that the targeted sequences amplified under all tested temperatures—nevertheless, the products were weaker under annealing temperatures higher than 55 °C.

The method specificity was tested on more than 80 different samples with various isolates and strains of the viruses and showed that the targeted viruses resulted in the expected amplicons (Fig. 1B), while all other related viruses produced only the *nad*5 internal control amplicon (data not shown). In the study, three PPV strains common in Europe were included—PPV-D (Dideron), PPV-M (Marcus), and PPV-Rec (Recombinant) (Candresse et al., 1998; Glasa et al., 2004). A simple one-step-RT-PCR was carried out for each of the tested samples and for each of the tested viruses to compare the specificity of the reaction. The primers used in the multiplex RT-PCR were used in the simple RT-PCR. There were no differences recorded for specificity and robustness of these two methods. There were an equal number of positive samples detected by both methods (data not shown).

The specificity of the detection assay was further proved by sequence analysis of PCR fragments of some isolates of detected viruses. The Genbank accession numbers of the sequences of the virus isolate are as follows: PPV (isolates cz1cp = FJ842715, cz2cp = FJ842716, cz3cp = FJ842717, cz4cp = FJ842718, cz6cp = FJ842719, z8cp = FJ842720); PDV (isolates cz1cp–cz9cp = FJ842698–FJ842706); and PNRSV (isolates cz1cp–cz8cp = FJ842707–FJ842714).

### 3.2. Multiplex RT-PCR with/or without internal control

Multiplex RT-PCR including and excluding internal control was carried out and compared. Serial dilution tests compared the sensitivity of multiplex RT-PCR including and excluding the internal control. The exclusion of the internal control from the reaction did not have any effect on the sensitivity of PDV detection. On the other hand, the sensitivity of PPV and PNRSV detection was even increased by the addition of the internal control primers into the reaction mix—in this case, on average, the differences in RNA dilutions, in which the viruses were detected, varied from 10 to 100 times (Fig. 1C and D). The multiplex RT-PCR method can therefore