European Journal of Plant Pathology (2005) 111: 77–84

Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR

J.A. Sánchez-Navarro[1], F. Aparicio[1], M.C. Herranz[1], A. Minafra[2], A. Myrta[3] and V. Pallás[1,*]
[1]*Instituto de Biología Molecular y Celular de Plantas, Universidad Politecnica de Valencia-CSIC, Avenida de los Naranjos s/n, 46022 Valencia, Spain;* [2]*Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi and Istituto di Virologia Vegetale, Sezione di Bari, Via Amendola 165/A, 70126 Bari, Italy;* [3]*Istituto Agronomico Mediterraneo, Via Ceglie 9, 70010 Valenzano, Bari, Italy; *Author for correspondence (Phone: +34-96-387-7877; Fax: +34-96-387-7859; E-mail: vpallas@ibmcp.upv.es)*

Accepted 8 July 2004

Key words: multiplex, one-step RT-PCR, RNA extraction method, stone fruit tree viruses

Abstract

A sensitive and reliable one step RT-PCR reaction with an internal control has been developed to detect and differentiate eight important viruses that affect stone fruit tress: *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV), *American plum line pattern virus* (APLPV), *Plum pox virus* (PPV), *Apple chlorotic leaf spot virus* (ACLSV), Apricot latent virus (ApLV) and Plum bark necrosis stem pitting associated virus (PBNSPaV). In addition, we investigated the detection limit and the efficiency of three different nucleic acid extraction methods that avoid the use of organic solvents, for both multiplex RT-PCR and dot-blot hybridisation assays. The primer cocktail was used to analyse 38 stone fruits originating from nine different countries and six species. A large number of virus combinations was detected and up to three different viruses were observed in five samples. A decrease in sensitivity was observed when the primer cocktail contained more than five different pair primers. However, comparative analyses showed that the multiplex RT-PCR containing the eight virus pair primers was even more sensitive than the ELISA or molecular hybridisation assays. The use of the multiplex RT-PCR technology in routine diagnosis of stone fruit tree viruses is discussed.

Stone fruit trees are hosts for a large number of economically important and common RNA plant viruses belonging to different genera. Relevant viruses include members of the genus *Ilarvirus*: *Apple mosaic virus* (ApMV), *Prunus necrotic ringspot virus* (PNRSV), *Prune dwarf virus* (PDV) and *American plum line pattern virus* (APLPV); the potyvirus responsible for the 'Sharka' disease, *Plum pox virus* (PPV), and the Trichovirus *Apple chlorotic leaf spot virus* (ACLSV), which is considered to be responsible for the 'Viruela' disorder (Peña-Iglesias and Ayuso-Gonzalez, 1975). Recently, two new viruses affecting stone fruit crops have been partially characterised: Apricot latent virus (ApLV, *Foveavirus*) and Plum bark necrosis stem pitting associated virus (PBNSPaV, *Ampelovirus*). High incidence of some of the above viruses, e.g. 25–100% for ACLSV or PNRSV was reported by Dominguez et al. (1998) and Varveri and Bem (1995), respectively. Moreover, Myrta et al. (2003) reported frequent mixed infections in the Mediterranean stone fruit industry (8–22% of the infected samples depending on the species). High infection rate together with the significant yield reduction (up to 57%), especially for mixed infections (Pusey and Yadava, 1991; Hilsendegen, 1999), underline the importance of propagating virus-free material. Testing of stone fruit trees has been performed mainly by grafting on woody indicator plants and/or serological methods (ELISA). The first method is time consuming (ranging from months up to 2–3 years), expensive

and, in most of the cases, the interpretation of the positive results may require confirmation by another detection method. ELISA test although reliable and very appropriate for routine diagnosis, lacks of the required sensitivity when pathogens are present at very low titer. Detection methods based on the nucleic acid analysis, alone or in combination, with serological methods (e.g. molecular hybridisation, RT-PCR, RT-PCR–ELISA, nested RT-PCR; co-PCR, etc.) offer a practical real alternative to the previous methods, due to their high specificity and detection limits (Sánchez-Navarro et al., 1998; Pallás et al., 1998a, b; Foissac et al., 2001; Scott and Zimmerman, 2001; Marini et al., 2002; Menzel et al., 2002; Olmos et al., 2002; Saade et al., 2000).

Procedures that allow the simultaneous detection and/or identification of different viruses are desirable for routine diagnosis because they require less time, labour and cost. In this sense, the multiplex reverse transcriptase polymerase chain reaction (RT-PCR) has been used successfully for routine diagnosis of plant viruses and viroids (Bertolini et al., 2001; Ito et al., 2002; Saade et al., 2000; Thompson et al., 2003). However, simultaneous detection of eight different viruses of woody crops has never been performed. In the present study we have developed a one-step multiplex RT-PCR reaction for the simultaneous detection of ApLV, ACLSV, APLPV, PDV, ApMV, PNRSV, PPV and PBNSPaV viruses, plus an internal control, using an easy, economical and fast RNA extraction procedure. In addition, we present data concerning the evidence of the influence of the number of different primer pairs on the detection limit of multiplex RT-PCR.

Infected leaves from different woody plants were previously tested by ELISA and/or molecular hybridization (MH) (e.g. PBNSPaV and ApLV) to confirm the presence of the corresponding pathogen. Three different nucleic acids extraction methods (NAEM) were evaluated for their reliability and their limit of detection in the multiplex RT-PCR: the Qiagen RNeasy Kit (NAEM1), a procedure that avoids the use of organic solvents previously adapted from the method described by Dellaporta et al. (1983) to virus RNAs (NAEM2; Astruc et al., 1996) and a modified protocol of the silica extraction procedure (NAME3; Thompson et al., 2003). The different samples analysed were first grounded with liquid nitrogen and then aliquots of 0.1 g used for each extraction method. In NAEM2, the tissue was homogenised with 2.5 ml of extraction buffer (100 mM Tris–HCl, 50 mM EDTA, pH 8.0, 500 mM NaCl, 10 mM β-mercaptoethanol). 0.5 ml of the homogenate were transferred to a 1.5 ml tube and incubated with 25 µl of 20% SDS at 65 °C for 20 min. Then,

Table 1. List of primers used in the one-step multiplex RT-PCR

Virus		Sequence 5′-3′	Product size (nt)	Location	Acc. no.
ApLV	s[a]	GGGAATAGAGCCCCAAGAAG	717	(RNA 3) 667–1393	AF057035
	a	TGCCGTCCCGATTAGGTTG			
ACLSV	s	CCATCTTCGCGAACATAGC	632	6902–7537	NC001409
	a	GTCTACAGGCTATTTATTATAAG			
APLPV	s	GGTCGTCAAGGGAGAGGC	563	(RNA 3) 1490–2053	NC003453
	a	GGCCCCTAAGGGTCATTTC			
PDV	s	CAACGTAGGAAGTTCACAG	517	(RNA 3) 1610–2129	L28145
	a	GCATCCCTTAAAGGGGCATC			
ApMV	s	CGTGAGGAAGTTTAGGTTG	417	(RNA 3) 1638–2056	U15608
	a	GCCTCCTAATCGGGGCATCAA			
PNRSV	s	GAACCTCCTTCCGATTTAG	346	(RNA 4) 527–884	AJ133208
	a	GCTTCCCTAACGGGGCATCCAC			
PPV	s	CAATAAAGCCATTGTTGGATC	313	9196–9506	M92280
	a	CTCTGTGTCCTCTTCTTGTG			
PBNSPaV	s	TACCGAAGAGGGTTTGGATG	270	149–421	AF195501
	a	TAGTCCGCTGGTACGCTACA			
Rbcl gene	s	TACTTGAACGCTACTGCAG	186	685–868	AF206813
(internal control)	a	CTGCATGCATTGCACGGTG			

[a]s: sense primer; a: antisense primer.

125 µl of 5 M KOAc were added and the mixture was maintained at 0 °C for 20 min. The tubes were then centrifuged at 10 K for 15 min to remove unwanted plant debris. Nucleic acids were recovered from the supernatant by ethanol precipitation and resuspended in 50 µl of sterile water. 0.5 µl of total nucleic acids from the different extraction procedures were used to perform a 10 µl multiplex RT-PCR reaction using the SuperScript III one-step RT-PCR system with Platinum Taq DNA polymerase kit (Invitrogen). The reaction was performed with the mixture of the all primer pairs listed in Table 1 at a final concentration of 0.25 pmol/µl except primers of APLPV (0.75 pmol/µl), ApMV (1 pmol/µl) and the internal control Rbcl gene (0.05 pmol/µl) corresponding to partial sequence of the ribulose 1,5-bisphosphate carboxylase chloroplast gene (AF206813). All primers matched perfectly with all isolate sequences available in the database except two (out of 20) sequences of PDV and three (out of 56) sequences of PPV, representing the 97% of all the target sequences. The one-step multiplex RT-PCR was optimised in order to obtain robust simultaneous amplification of all expected DNA fragments. Best results were achieved by performing an initial incubation at 50 °C for 30 min for cDNA synthesis, followed by denaturation of the RT enzyme by incubation 2 min at 94 °C and then, 40 cycles of denaturation at 94 °C for 15 s, primer annealing at 50 °C for 30 s and primer extension at 68 °C for 1 min. A final incubation at 68 °C for 7 min was introduced to finish incomplete PCR fragments. The amplified PCR products were analysed on 2% agarose/TAE gels stained with ethidium bromide.

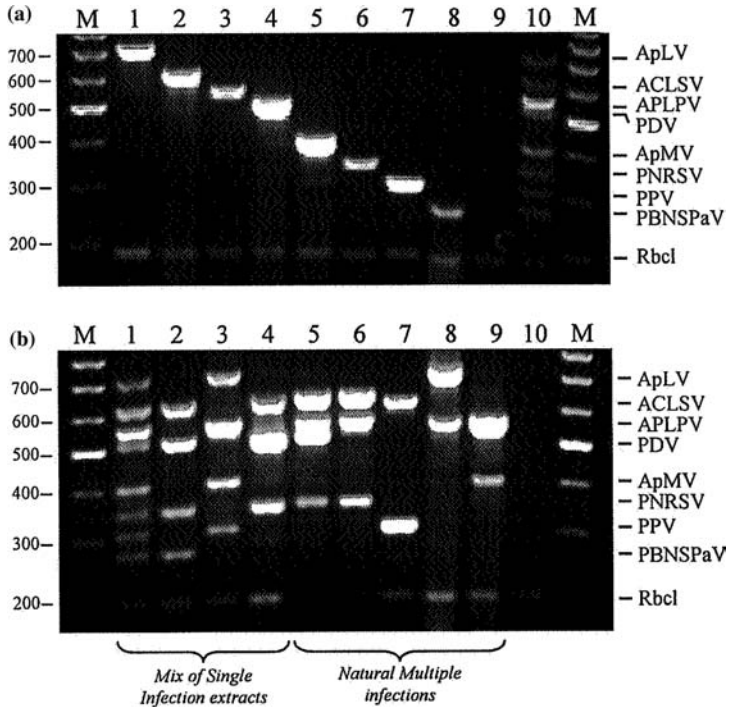

Figure 1. (a) One-step multiplex RT-PCR analysis of single (lane 1–8) or simulated multiple (lane 10) infected stone fruit trees. Lane 1: GF305-ApLV; lane 2: plum-ACLSV; lane 3: plum-APLPV; lane 4: Peach-PDV; lane 5: apricot-ApMV; lane 6: cherry-PNRSV; lane 7: GF-305-PPV; lane 8: plum-PBNSPaV; lane 9: healthy plum; lane 10: mix of total nucleic acids analysed in lanes 1–8. (b) Multiple RT-PCR analysis of simulated (lanes 1–4) or natural (lanes 5–9) multiple infections. Lane 1: ApLV, ACLSV, APLPV, PDV, ApMV, PNRSV, PPV and PBNSPaV; lane 2: ACLSV, PDV, PNRSV and PBNSPaV; lane 3: ApLV, APLPV, ApMV and PPV; lane 4: ACLSV, PDV and PNRSV; lane 5: ACLSV, APLPV, PDV and PNRSV; lane 6: ACLSV, APLPV and PNRSV; lane 7: ACLSV and PPV; Lane 8: ApLV and APLPV; lane 9: APLPV and ApMV; lane 10: healthy cherry. M, 300 ng of 100 bp molecular weight marker. The expected PCR fragment for each virus is indicated.

Analysis of total nucleic acids by non-isotopic dot-blot hybridisation was performed as described previously by Sánchez-Navarro et al. (1998) using a dig-RNA probe complementary to RNA 4 of PNRSV.

Multiplex RT-PCR with a cocktail of all primers listed in Table 1 was used first to analyse different woody tissues infected with each virus individually. Figure 1a shows the specific amplification of the expected viral DNA fragment, plus the internal control, from leaf tissue infected with ApLV (lane 1), ACLSV (lane 2), APLPV (lane 3), PDV (lane 4), ApMV (lane 5), PNRSV (lane 6), PPV (lane 7) and PBNSPaV (lane 8). No extra bands interfering with the specific virus DNA fragment were detected. To confirm the viral origin, amplified PCR products were extracted from the gel and subjected to direct sequence using the specific primer for each virus. In all cases, the obtained DNA sequence was that expected for each virus (data not shown). The expected fragment of the internal PCR control was detected in all stone fruit trees analysed indicating its broad-spectrum. Amplification of a single PCR fragment in single-infected tissue using the cocktail of all primers demonstrates the specificity of the multiplex RT-PCR reaction. The nine expected PCR fragments were detected when a simulated infection of eight different viruses was performed by mixing total nucleic acids extracted from single infected tissues (Figure 1a, lane 10). To confirm the specificity of the multiplex RT-PCR procedure in different virus combinations, simulated or natural multiple infections were analysed (Figure 1b). The expected viral PCR fragments plus the internal control were observed in all cases. In naturally occurring mixed infections, up to four viruses (PNRSV, PDV, APLPV and ACLSV) were simultaneously detected (Figure 1b, lane 5).

The nucleic acid extraction method could represent a critical step in routine diagnosis in terms of time, cost and reagents required. Three procedures were analysed regarding time, reliability and sensitivity of the multiplex analysis: Qiagen RNeasy Kit (NAEM1), a procedure that avoids the use of organic solvents (NAEM2) and a method based on the use of the silica chemical (NAEM3). All procedures allowed the extraction of total RNA (NAME1 and NAEM3) or nucleic acids (NAEM2) in less than 90 min. Comparable amounts of plum tissue infected with PNRSV were first extracted with the three methods, serially diluted in total nucleic acids extracted from plum tissue infected with PPV and the different dilutions were subjected to viral analysis by dot-blot, single and multiplex RT-PCR, respectively. No differences in terms of end point dilution limit were observed between NAEM1 and NAEM3, using any of the three detection methods (data not shown). Figure 2 shows the detection limit obtained with multiplex RT-PCR and dot-blot hybridisation using a PNRSV-specific dig-RNA probe. The specific PCR fragment corresponding to PNRSV was detected until dilution 5^{-4} (NAEM1) and 5^{-6} (NAEM2) (Figure 2a and b, respectively). When the serial dilutions were analysed by dot-blot hybridisation, which is a detection method that gives a positive signal proportional to the amount of RNA target, the end point dilution limit observed in infected tissue extracted with NAEM2 was 5^{-4}, 25 times higher

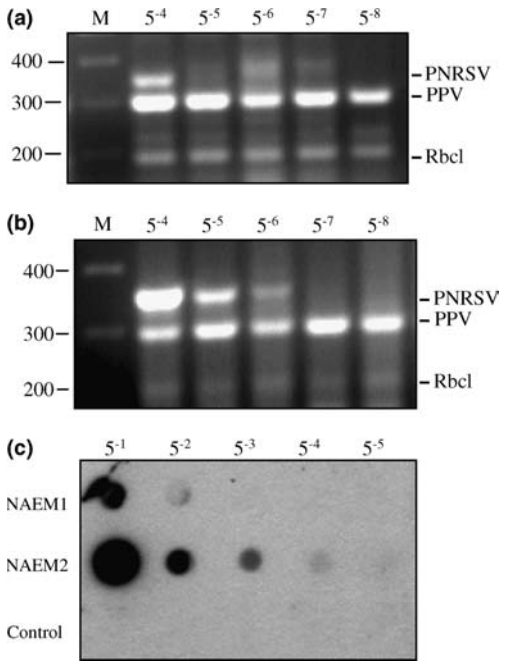

Figure 2. Comparison of two nucleic acid extraction protocols: NAEM1 and NAEM2. Serial five time dilutions of PNRSV infected plum extract in PPV infected plum extract were analysed by one-step multiplex RT-PCR (a: NAEM1 and b: NAEM2) and non-radioactive molecular hybridisation (c: PNRSV probe). Control sample correspond to an undiluted mix of PPV infected plum extracts performed with both methods. M, 300 ng of 100 bp molecular weight marker. The expected PCR fragment for each virus is indicated.

than NAEM1 (5^{-2}; Figure 2c). This result suggests that the higher sensitivity observed in NAEM2 is due to the higher amounts of target viral RNA rendered by NAEM2 versus NAEM1.

The detection limit of multiplex RT-PCR in both extraction methods was only 25 times higher than the dot-blot hybridisation (compare Figure 2a and b with c). The detection limit was not increased by doubling the amount of enzyme used in the multiplex RT-PCR. However, a previous study performed with nectarine tissue infected with PNRSV rendered a difference for the end point detection limit of 625 times between dot-blot hybridisation and single RT-PCR (Sánchez-Navarro et al., 1998). In order to check if the presence of nine pairs of primers in the RT-PCR cocktail reaction could influence the detection limit of the multiplex RT-PCR, the same PNRSV infected extract analysed in Figure 2b was serially diluted in plum tissue infected with PPV and/or APLPV viruses and the RT-PCR performed by adding either only PNRSV primers (Figure 3a) or a cocktail of 5 (PNRSV, PPV, APLPV, ApMV and PDV; Figure 3b) and 7 (PNRSV, PPV, APLPV, ApMV, PDV, ACLSV and PBNSPaV; Figure 3c) different pairs of primers. The expected PCR fragments were clearly observed until dilution 5^{-10} (Figure 3a and b) and 5^{-8} (Figure 3c). The detection limit of the single RT-PCR reaction was not affected using a total amount of primers equivalent to the multiplex RT-PCR performed with seven pairs of primers (data not shown). Thus, these detection

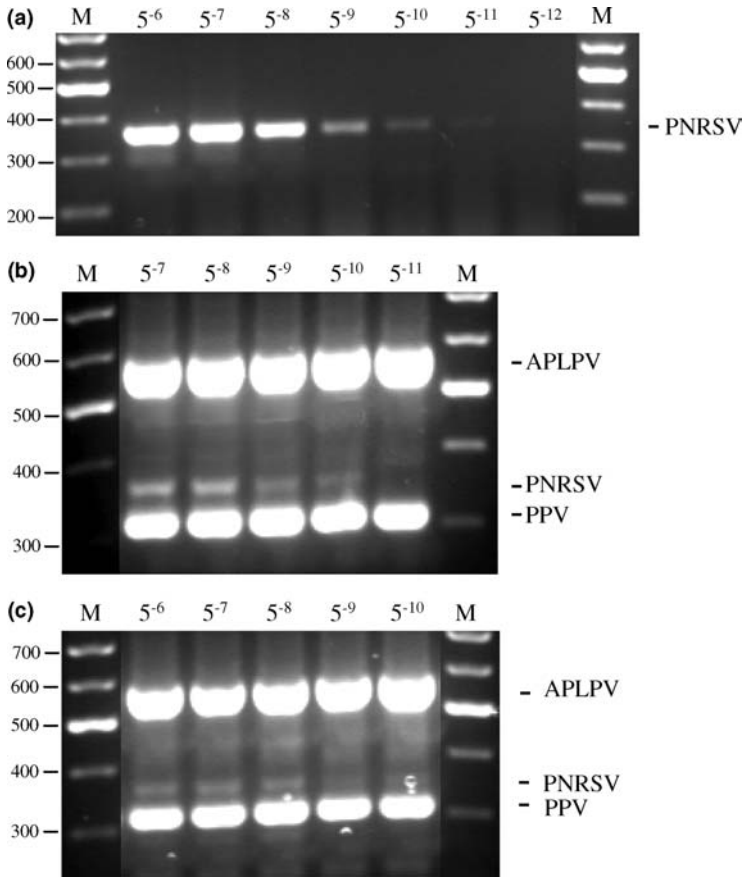

Figure 3. Influence of the number of primer pairs on the one-step multiplex RT-PCR detection limit. Plum extract infected with PNRSV was serially diluted (five times) in Plum extract infected with PPV and APLPV. Dilutions were analysed by multiplex RT-PCR containing a cocktail of one (a: PNRSV), five (b: PNRSV, PPV, ApMV, PDV and APLPV) or seven (c: PNRSV, PPV, APLPV, ApMV, PDV, ACLSV and PBNSPaV) primer pairs. Dilutions are represented on the top of the gel. M, 300 ng of 100 bp molecular weight marker. The expected PCR fragment for each virus is indicated.

Table 2. Comparative analysis for the presence of eight stone fruit viruses made by DAS-ELISA (E)[a] and multiplex RT-PCR (M)

	Country	Host	PBNSPaV[a]		PPV		PNRSV		ApMV		PDV		APLPV		ACLSV		ApLV[a]	
			E	M	E	M	E	M	E	M	E	M	E	M	E	M	E	M
1	Italy	Peach					+	+		+		+						+
2	Italy	Peach					+	+	+	+								
3	Italy	Plum					+	+				+						
4	Italy	Plum											+	+				
5	Italy	Almond							+	+								
6	Italy	Almond								+	+	+						
7	Italy	Apricot							+	+								
8	Tunisia	Plum										+	+	+				
9	Palestine	GF305															+	+
10	USA	Plum											+	+				
11	Palestine	Plum					+	+										
12	Italy	Plum														+		
13	Palestine	Plum					+	+										
14	Chile	Plum					+	+										
15	Jordan	Plum		+		+												
16	Jordan	GF305					+	+		+								
17	Iran	Apricot						+									+	
18	Iran	Plum						+									+	
19	USA	Plum											+	+				
20	Palestine	Plum										+	+	+				
21	USA	Cherry					+	+										
22	USA	Cherry					+	+										
23	USA	shirofugen									+	+						
24	Italy	GF305					+	+			+	+	+	+	+	+		
25	Italy	GF305					+	+					+	+	+	+		
26	Italy	GF305					+	+					+	+				
27	Italy	GF305						+		+			+	+	+	+		
28	Chile	Apricot			+	+												
29	Unknown	GF305			+	+												
30	Greece	GF305			+	+												
31	Polonia	GF305			+	+		+										
32	Jordan	Plum			+	+										+		
33	Unknown	GF305																
34	Unknown	Peach																
35	Unknown	Plum																
36	Unknown	Apricot																
37	Unknown	Cherry														+		
38	Unknown	Almond																
Total ELISA: 35			0		5		12		3		3		9		3		3	
Total multiplex: 52				1		6		16		7		6		9		6		1

[a]Except for PBNSPaV and ApLV that, since no antisera are available, were tested by Molecular Hybridization (MH).

limit values suggest that the sensitivity of the reaction is influenced by the number of different primer pairs instead of the total amount of primer present in the cocktail. A similar variation of the detection limit was observed using ACLSV infected tissue diluted in extracts of tissue infected with PPV and APLPV using different cocktails of primers (data not shown). The results presented in this study show that the use of a cocktail of five pairs of primers does not affect the detection limit of multiplex RT-PCR, while seven pairs does. In agreement with this observation, multiplex RT-PCR performed to amplify 3 (Saade et al., 2000; Lee et al., 2002) or 6 (Bertolini et al., 2001) different DNA fragments did not have any influence on the detection limit when compared to a single assay. Simultaneous amplification of more than six PCR fragments by multiplex RT-PCR has been

reported recently (Ito et al., 2002), although no data related to the detection limit were presented. Despite the reduction observed, the detection limit of the multiplex RT-PCR was 25 times higher than the dot-blot hybridisation (Figure 2), a method that has been demonstrated to have a detection limit 25 times higher than that of DAS-ELISA (Sánchez-Navarro et al., 1998). To check for the reliability and the robustness of the new multiplex RT-PCR developed a total of 38 field samples from the virus collection kept at the 'Instituto Agronómico Mediterráneo' were analysed using both DAS-ELISA and multiplex RT-PCR. Samples included six different hosts (peach, almond, cherry, plum, apricot and GF305) and nine geographic origins as well as different virus combinations. Table 2 shows the results obtained with both detection methods. Except for two samples originating from Iran and infected with ApLV (numbers 17 and 18; Table 2) the rest of the ELISA positive samples were detected with the multiplex procedure. Apparently, the ApLV strain present in Iran is not recognised with the primer used in the present study. In the other hand, the multiplex technique rendered 20 positives more than the serological method confirming the higher sensitivity of the molecular detection technique. The multiplex RT-PCR was able to identify reliably a large number of virus combinations and up to three viruses were simultaneously detected in five fruit trees.

The multiplex RT-PCR reaction presented in this study represents a significant advance for routine diagnosis of stone fruit viruses, reducing cost, time and handling. The multiplex technology, together with an easy and fast total nucleic acid extraction method, could permit the routine and reliable analysis of large number of stone fruit trees for the presence of eight different pathogens. Furthermore, since multiple infections are present in the field, this methodology will facilitate the correlation between tree disorders and different virus combinations.

Acknowledgements

We thank Verónica Navarro for her technical assistance. This work was supported by grant BIO02-04099 from the Spanish granting agency DGICYT and the CSIC-CNR agreement. F.A. and M.C. were the recipient of a fellowship from the Ministerio de Educación, Cultura y Deporte of Spain. J.A. was the recipient of a contract from the Ramón y Cajal program of the Ministerio de Educación, Cultura y Deporte of Spain.

References

Astruc N, Marcos JF, Macquaire G, Candresse T and Pallas V (1996) Studies on the diagnosis of hop stunt viroid in fruit trees: identification of new hosts and application of a nucleic acid extraction procedure based on non-organic solvents. European Journal of Plant Pathology 5: 1–10

Bertolini E, Olmos A, Martinez MC, Gorris MT and Cambra M (2001) Single-step multiplex RT-PCR for simultaneous and colourimetric detection of six RNA viruses in olive trees. Journal of Virological Methods 96: 33–41

Dellaporta S, Woods H and Hicks J (1983) A plant DNA minipreparation: version II. Plant Molecular Biology Reporter 1: 19–21

Dominguez S, Aparicio F, Sanchez-Navarro JA, Cano A, Garcia-Brunton J and Pallas V (1998) Studies on the incidence of ilarviruses and apple chlorotic leaf spot virus (ACLSV) in apricot trees in the Murcia region (Spain) using serological and molecular hybridisation methods. Acta Horticulturae 472: 203–210

Foissac X, Svanella-Dumas L, Dulucq MJ, Candresse T, Gentit P and Clark MF (2001) Polyvalent detection of fruit tree tricho, capillo and foveaviruses by nested RT-PCR using degenerated and inosine containing primers (PDO RT-PCR). Acta Horticulturae 550: 37–43

Hilsendegen P (1999) Results of the tolerance of sour cherry cultivars to prunus necrotic ringspot virus (PNRV). Erwerbsobstbau 41: 192–197

Ito T, Ieki H and Ozaki K (2002) Simultaneous detection of six citrus viroids and Apple stem grooving virus from citrus plants by multiplex reverse transcription polymerase chain reaction. Journal of Virological Methods 106: 235–239

Lee JH, Tennessen K, Lilley BG and Unnasch TR (2002) Simultaneous detection of three mosquito-borne encephalitis viruses (eastern equine, La Crosse, and St. Louis) with a single-tube multiplex reverse transcriptase polymerase chain reaction assay. Journal of the American Mosquito Control Association 18: 26–31

Marini DB, Zhang YP, Rowhani A and Uyemoto JK (2002) Etiology and host range of a Closterovirus associated with plum bark necrosis-stem pitting disease. Plant Disease 86: 415–417

Menzel W, Jelkmann W and Maiss E (2002) Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. Journal of Virological Methods 99: 81–92

Myrta A, Di Terlizzi B, Savino V and Martelli GP (2003) Virus diseases affecting the Mediterranean stone fruit industry: a decade of surveys. In: [Myrta A, Di Terlizzi B and Savino V (eds)] Option Méditerranéennes, Sér. B/n° 45 – Virus and Virus-like Diseases of Stone Fruits, with Particular Reference to the Mediterranean Region (pp 15–23)

Olmos A, Bertolini E and Cambra M (2002) Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses. Journal of Virological Methods 106: 51–59

Pallás V, Más P and Sanchez-Navarro JA (1998a) Detection of plant RNA viruses by nonisotopic dot-blot hybridization. Methods in Molecular Biology 81: 461–468

Pallás V, Sánchez-Navarro JA, Más P, Cañizares MC, Aparicio F and Marcos JF (1998b) Molecular diagnostic techniques and their potential role in stone fruit certification schemes. Options Méditerranéennes 19: 191–208

Peña-Iglesias A and Ayuso-Gonzalez P (1975) Preliminary identification of the viruses producing Spanish apricot pseudopox (viruela) and apricot mosaic diseases. Acta Horticulturae 44: 255–265

Pusey PL and Yadava UL (1991) Influence of Prunus ringspot virus on growth, productivity and longevity of peach trees. Plant Disease 75: 847–851

Saade M, Aparicio F, Sanchez-Navarro JA, Herranz MC, Myrta A, Di-Terlizzi B and Pallas V (2000) Simultaneous detection of the three ilarviruses affecting stone fruit trees by nonisotopic molecular hybridisation and multiplex reverse-transcription polymerase chain reaction. Phytopathology 90: 1330–1336

Sanchez-Navarro JA, Aparicio F, Rowhani A and Pallas V (1998) Comparative analysis of ELISA, nonradioactive molecular hybridisation and PCR for the detection of prunus necrotic ringsport virus in herbaceous and Prunus hosts. Plant Pathology 47: 780–786

Scott SW and Zimmerman MT (2001) American plum line pattern virus is a distinct ilarvirus. Acta Horticulturae 550: 221–227

Thompson JR, Wetzel S, Klerks MM, Vaskova D, Schoen CD, Spak J and Jelkmann W (2003) Multiplex RT-PCR detection of four aphid-borne strawberry viruses in Fragaria spp. in combination with a plant mRNA specific internal control. Journal of Virological Methods 111: 85–93

Varveri C and Bem F (1995) Viruses of stone and pome-fruit mother-tree plantations in Greece. Acta Horticulturae 386: 431–438