SHORT COMMUNICATION

USE OF MONOCLONAL ANTIBODIES TO CITRUS PSOROSIS VIRUS FOR DIAGNOSIS

O. Potere[1], D. Boscia[1], K. Djelouah[1], V. Elicio[2] and V. Savino[1]

[1] Dipartimento di Protezione delle Piante dalle Malattie, Università degli Studi and Centro di Studio del CNR sui Virus e le Virosi delle Colture Mediterranee, Via G. Amendola, 165/A, I-70126 Bari, Italy
[2] Agritest S.r.l., c/o Tecnopolis, I-70010 Valenzano, Bari, Italy

SUMMARY

Citrus Psorosis Virus (CPsV) can be detected by ELISA, but current protocols use polyclonal antisera. The availability of these antisera is limited, and new production is hampered by difficulties met with purification of the virus. Several specific monoclonal antibodies were recently produced, and we report how those have been successfully utilized for the development of an all-monoclonal antibody-based ELISA kit.

RIASSUNTO

IMPIEGO DI ANTICORPI MONOCLONALI PER LA DIAGNOSI DEL VIRUS DELLA PSOROSI DEGLI AGRUMI. Il virus della psorosi degli agrumi (CPsV) è rilevabile mediante ELISA, ma i protocolli attualmente in uso necessitano dell'impiego di antisieri policlonali, disponibili in quantità limitata. La produzione di nuovi antisieri è inoltre ostacolata dalla difficoltà a purificare il virus. Vista la disponibilità di numerosi anticorpi monoclonali specifici recentemente prodotti, questi sono stati utilizzati con successo per la messa a punto di un protocollo ELISA senza l'impiego di antisieri policlonali.

Key words: Citrus, Psorosis, *Ophiovirus*, ELISA, monoclonal antibodies.

Psorosis is an economically important and widespread disease of citrus whose putative causal agent, Citrus Psorosis Virus (CPsV), is a virus with thread-like particles assigned to the novel genus *Ophiovirus* (Milne *et al*., 1996). Several strains of this virus may exist, as suggested by differences in their biological behaviour (Roistacher, 1991).

CPsV can be detected by molecular (RT-PCR) and serological (ELISA) methods (Garcia *et al*., 1997; D'Onghia *et al*., 1998). ELISA with polyclonal antisera is suited for routine identification (D'Onghia *et al*., 1998), but Garcia *et al.* (1997) observed that heterologous isolates were less reliably detected than the homologous antigen. On the other hand, monoclonal antibodies (Mabs) raised to CPsV isolate CPV-4, the proposed type strain of CPsV (Barthe *et al.*, 1998), reacted only with the homologous antigen (Derrick *et al*., 1993; Barthe *et al*., 1998). According to Barthe *et al.* (1998), these Mabs are of little value for diagnostic purposes but are useful for the differential serological identification of CPsV strains. Recently, 24 Mabs were raised to the southern Italian CPsV isolate IAM-191Xa. These Mabs were tested singly by ELISA against a panel of 64 psorosis sources from different geographical areas. Sixteen different epitopes were identified and considerable serological variability, apparently associated with the geographical origin of the isolates, was found (Fig. 1) (Djelouah *et al*., 1999). For selection of these Mabs (Djelouah *et al*., 1999) and for routine ELISA (D'Onghia *et al*., 1998) a rabbit polyclonal antiserum (A-322) was used for plate coating. This antiserum had been raised to isolate CPV-4 at the Istituto di Fitovirologia Applicata del CNR, Torino, and is available in limited amounts. This prompted us to verify if antiserum A-322 could be effectively replaced by Mabs of which an unlimited supply is available.

The Mabs used in this study were selected among those obtained by Djelouah *et al.* (1999) and the antigen sources were citrus trees from the same collection previously tested, which carried serologically characterised virus isolates (Djelouah *et al*., 1999).

Before processing ascitic fluids for antibody purification, the isotype of each Mab was determined from culture supernatants using 'isotyping strips' (Sigma Immuno Type Kit ISO-1). The results showed that 16 out of 24 antibodies were IgG2a, 3 were IgG1, 2 IgG3 and 3 IgM (Table 1).

Only antibody isotypes IgG1 and IgG2a were selected for purification and subsequent enzyme conjugation, because previous experience indicated that these consistently gave higher yields. A further screening was made among these Mabs considering only those able to

Corresponding author: D. Boscia
Fax: +39.080.5442911
E-mail: csuudbo8@area.ba.cnr.it

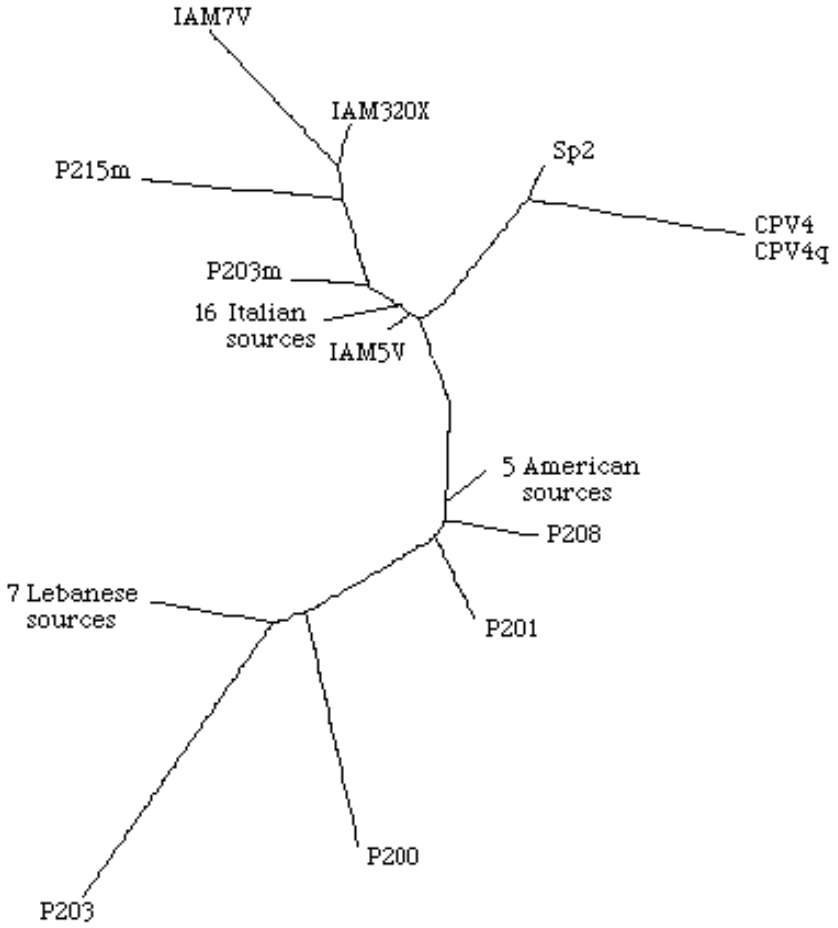

Fig. 1. Graphical representation of the serological relationship among 40 sources of CPsV, based on the distribution of the 16 epitopes identified in the isolate IAM-191Xa. All 'P' sources are of North American origin, IAM sources of Italian origin and the Sp source of Spanish origin (see Djelouah *et al.*, 1999).

Table 1. Isotype of monoclonal antibodies specific for CPsV.

Isotype	Mabs
IgG1	PS15, 23, 29
IgG2a	PS7, 11, 12, 13, 14, 17, 21, 22, 24, 25, 26, 27, 28, 31, 32, 34
IgG3	PS4, 8
IgM	PS1, 6, 18

detect all Mediterranean virus isolates, and at least 90% of all previously tested isolates (Djelouah *et al.*, 1999). Thus, seven Mabs were selected for purification: PS29 (IgG1) and PS22, 24, 25, 31, 32 and 34 (IgG2a).

The IgG1 (Mab PS29) was purified from 400 µl ascitic fluid by ion exchange chromatography using in FPLC apparatus (Pharmacia) an anion exchange column (1 ml Resource Q) containing 20 mM Tris, pH 8.2, and eluted with a linear 0-50% gradient of 20 mM Tris pH 8.2 + 1 M NaCl at a flow rate of 3 ml min^{-1}.

The IgG2a (Mabs PS 22, 24, 25, 31, 32, and 34) were purified from ascitic fluid (400 µl each) by affinity chromatography on protein A-sepharose columns (Clark and Bar-Joseph, 1984). All fractions (1 ml each) were tested by ELISA for identifying the IgG peak, whose purity was assessed visually by SDS-PAGE under denaturing conditions following the discontinuous system of Laemmli (1970). The highest IgG yield (10 mg ml^{-1} of ascitic fluid) was obtained with Mab PS29 which, therefore, was selected for conjugation with alkaline phosphatase (Avrameas, 1969), using aliquots of 1 mg of Mab in 1 ml of PBS, to which 5 mg of bovine serum albumin were added before storing at 4°C. Yields of all other Mabs ranged from 0.2 to 1.3 mg ml^{-1} of ascitic fluid. Considering the small volume of ascites, these yields were too low for effective enzyme conjugation.

For optimizing antigen trapping, a stock of antibodies was prepared by mixing 1 vol. each of ascitic fluid of 14 different Mabs (PS7, 11, 12, 13, 14, 15, 17, 18, 22, 23, 24, 25, 28, and 29), each specific for a different epitope (Djelouah *et al.*, 1999). This antibody cocktail was used for ELISA plate coating.

A total of 37 citrus trees growing under screenhouse conditions and known to be infected by CPsV isolates

Table 2. ELISA results (E 405 after 60 min of substrate incubation) of a comparative test between DAS-ELISA based on monoclonal antibodies and DASI-ELISA in which polyclonal antibodies were used for plate coating.

Sample	Isolate	DAS-ELISA (Coating Mabs)	DASI-ELISA (Coating Pabs)
1	IAM-317X	1.544*	0.378*
2	IAM-10V	0.043	0.082
3	IAM-36V	1.359*	1.519*
4	IAM-252X	Over 2.0*	0.855*
5	IAM-3V	0.019	0.077
6	AJ7	0.051	0.041
7	IAM-153X	0.653*	0.157*
8	P208	1.006*	0.177*
9	P213	0.239*	0.167*
10	P201	1.914*	0.897*
11	P216	1.344*	0.206*
12	P203m	0.477*	0.304*
13	P209	0.697*	0.212*
14	P205	Over 2.0*	0.815*
15	IAM-190X	Over 2.0*	1.850*
16	IAM-365X	0.120*	0.221*
17	IAM-197X	Over 2.0*	1.038*
18	IAM-195X	Over 2.0*	1.799*
19	IAM-254X	0.903*	0.550*
20	IAM-374X	Over 2.0*	1.659*
21	IAM-191Xm	Over 2.0*	1.275*
22	IAM-5V	1.642*	1.151*
23	IAM-9X	1.973*	1.769*
24	IAM-7V	0.889*	0.370*
25	IAM-165X	0.103*	0.076
26	IAM-39V	0.174*	0.054
27	IAM-160X	0.050	0.069
28	IAM-166X	0.033	0.063
28	250Ps	Over 2.0*	0.435*
30	IAM-191Xmv	0.499*	0.483*
31	9N36(2)	1.285*	0.520*
32	IAM-194X	Over 2.0*	1.449*
33	IAM-CG	0.015	0.073
34	IAM-191Xmvb	Over 2.0*	1.309*
35	9N56(1)	0.383*	0.168*
36	57N	0.890*	0.293*
37	PS-Ur.	Over 2.0*	0.910*
38	Healthy citrus	0.043	0.049
39	Buffer	0.000	0.000

* positive reactions.

detectable by Mab PS29 were compared by DAS-ELISA (trapping with the Mab cocktail) and DASI-ELISA (trapping with polyclonal antiserum A-322). Tests were carried out in spring using fully developed leaves. For DAS-ELISA, the Mab cocktail diluted 1:100,000 in carbonate buffer pH 9.6 was used for plate coating, and the alkaline phosphatase-conjugated Mab PS29 diluted 1:1000 in PBS was used for antigen detection. Other steps of the procedure were as described by D'Onghia *et al.* (1998). For DASI-ELISA coating was with antiserum A-322 and Mab PS29 was used as secondary antibody (Djelouah *et al.*, 1999). Samples were considered positives if their values were over 0.1 OD and 2 times or more higher than healthy citrus plant extracts used as control.

DAS-ELISA was better than DASI-ELISA in both sensitivity and specificity. A positive reaction was given by 31 (DAS-ELISA) versus 29 (DASI-ELISA) samples out of 39, and, in most cases, readings at 405 nm were consistently higher (Table 2). After 60 min of substrate incubation at room temperature, leaf extracts of 11 samples gave absorbance values at 405 nm over 2 OD (this number raised to 19 after 2 h), while no significant background was shown by the healthy control (Table 2). A thorough comparison with DAS-ELISA using A-322 for trapping and detection was not done because of shortage of antiserum. However, in a limited number of tests, the average time required for the development of the reaction was at least three times longer.

In Table 2, which reports the results of a single ELISA test, six samples (IAM-10V, IAM-3V, AJ7, IAM-160X, IAM-166X and IAM-CG), known to be infected with CPsV isolates detectable with Mab PS29 (Djelouah *et al.*, 1999), were negative with both ELISA protocols. When tests were repeated in the same season, each of the above six isolates gave ELISA readings above the positive threshold at least in one of three replicates, while the detection of most of 31 isolates that gave a positive response in the first test failed at least once. In general, failure of ELISA in detecting viruses in single tests, due to uneven distribution and low concentration of target antigen in infected plants, is far from being rare. Based on recent results, this applies also to CPsV, for which the risk of obtaining false negatives in single tests using citrus leaves is above 40%, regardless of whether Mabs or Pabs are used (Djelouah *et al.*, 1999).

Not with standing this shortcoming and considering the difficulty of purifying CPsV and producing good quality antisera, the development of an all-monoclonal antibody based ELISA kit represents in our opinion an important improvement for the serological detection of this virus. However, beside the identification of the optimal sampling conditions, not all problems are solved, for the widest range Mab available (Mab PS29) does not recognize three American isolates, among which is CPV-4 (Djeoulah *et al.*, 1999). Efforts are therefore underway for the identification of an universal Mab capable of detecting a wider range, if not the totality of CPsV isolates.

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