

Comparison of Different Immunosorbent Assays for Citrus Tristeza Virus (CTV) Using CTV-Specific Monoclonal and Polyclonal Antibodies

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ABSTRACT. Production of monoclonal antibodies specific to CTV has improved CTV detection and allowed the use of different immunoenzymatic techniques. In this study the indirect (I), DAS, and double antibody sandwich indirect (DASI) ELISA was compared for ability to detect CTV in plant extracts and in purified preparations. Twenty-four variations of ELISA were tested using CTV-specific monoclonal and polyclonal antibodies, anti-immunoglobulins, protein A and the biotin/avidin system. Alkaline phosphatase and peroxidase-labeled enzymes were also compared. The DASI ELISA variations with CTV-specific polyclonal antibodies used as coating antibodies and monoclonal antibodies used as the intermediate antibody detected 0.1 ng/ml CTV protein. The limit of detection for other variations was less than 100 ng/ml CTV protein.

Index words. virus detection, ELISA, biotin, streptavidin, conjugated antibody.

Use of the enzyme-linked immunosorbent assay (ELISA) to detect citrus tristeza virus (CTV) was first reported in 1979 (1, 9). During the next ten years, the conventional double antibody sandwich (DAS) ELISA (12) was used widely for routine detection of CTV, and for surveying large citrus areas for tristeza disease (3, 4, 5, 10, 11, 14, 16, 17, 18, 24, 25, 26, 27, 28, 29, 36). The change in CTV diagnosis due to ELISA is illustrated by the fact that approximately 2500 CTV tests were performed in Spain between 1959 and 1979 compared to over 900,000 tests in the subsequent 10 yr. In addition to CTV diagnosis, ELISA has been used in research laboratories for many different purposes, including monitoring CTV purification, monitoring translocation of the virus in plants (Cambra et al., unpublished data), detection of virus in aphids (8), and in plant material grown *in vitro* (15), to determine virus titer (6, 19), and for epidemiological studies (5, 10, 16, 26, 29).

The production of CTV-specific monoclonal antibodies (MAb) in Spain (34, 35), California (21), Florida (30), and Taiwan (32) has improved CTV diagnosis and provides a new tool to study the epitope variability in the CTV coat protein (20, 32, 36).

Different ELISA procedures have been described for plant pathogens (13, 23, 31, 33), but only a few have been used with MAb for CTV detection (28, 35). For this reason, the main objectives of this work were: 1) to explore the use of MAb in ELISA for detecting CTV; 2) to describe new detection techniques which use MAb and biotin-avidin and other systems; and 3) to compare the sensitivity of the different techniques.

MATERIALS AND METHODS

Antigens. Antigen sources used were freeze-dried crude extracts from sweet orange plants infected with the Florida CTV isolate T36 (30), a purified preparation of the Spanish CTV isolate T388 (6) (provided by Dr. P. Moreno, IVIA, Valencia, Spain), and crude extracts from healthy Pineapple sweet orange in phosphate-buffered saline (PBS), pH 7.2, containing 0.2% DIECA (1:20 dilution weight:vol). Serial dilutions (0.1 mg/ml to 0.1 ng/ml) of purified virus in either PBS, pH 7.2 (12), or in crude extracts of healthy tissue in PBS were prepared to calculate the sensitivity of different ELISA variations. And OD₂₆₀ of 2.0 was assumed for a 1 mg/ml concentration of purified CTV, and total protein was

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measured with a BIO-RAD (BIO-RAD Laboratories, Richmond, CA) protein determination kit.

Antibodies and conjugates.

Purified monoclonal and polyclonal immunoglobulins specific for CTV were used. The monoclonals were MAb 3DF1 and MAb 3CA5 (35) from Ingenasa, Hermanos García Noblejas 41, 28027 Madrid, Spain. These represent two different CTV epitopes (36). The polyclonal antibodies were from rabbit antiserum 879 (3) and from mouse antiserum M2 (Cambra et al., unpublished). The M2, 3DF1 and 3CA5 antibodies were against the Spanish isolate T308 (35) and the 879 antibodies were against the Florida isolate T-4 (1). Conjugates were prepared by linking alkaline phosphatase (ALKPHOS), peroxidase (PEROX), or biotin (12, 22) to purified immunoglobulins. Goat anti-rabbit immunoglobulins (GAR), GAR and goat anti-mouse immunoglobulins (GAM) conjugated to ALKPHOS and biotin, rabbit anti-goat immunoglobulins (RAG) conjugated to ALKPHOS, and streptavidin conjugated to ALKPHOS and PEROX were obtained from Boehringer Mannheim Biochemicals (P. O. Box 50414, Indianapolis, IN 46250). GAM conjugated to PEROX and protein A conjugated to ALKPHOS were obtained from Sigma Chemical Co. (P. O. box 14508, St. Louis, MO 63178) and GAR conjugated to PEROX was obtained from Nordic Immunological Lab, 55-61 Langestroat, Tilburg, The Netherlands.

ELISA variations. Variations of the indirect (I) or plate-trapped antigen, the double antibody sandwich (DAS) and the double antibody sandwich indirect (DASI) forms of ELISA were tested. All 24 variations compared in this study are illustrated in Fig. 1.











For ELISA-I variations 1 through 8 (Fig. 1), the antigens (extracts or of purified virus dilutions) were added to uncoated M129A microplates (Dynatech) and incubated overnight at 4°C. The plant extracts were prepared in 0.05M sodium carbonate buffer, pH

9.6 (coating buffer). Unlabeled MAb 3DF1 was used for variations 3 and 7, a 1:1 mixture of MAb 3DF1 + MAb 3CA5 for variation 8, M2 mouse polyclonal antibodies for variation 2, and rabbit polyclonal antibodies 879 for variation 1. All the intermediate immunoglobulins were added at 0.125 $\mu\text{g/ml}$. The enzyme-labeled antibodies specific to the unlabeled antibodies were added at a 0.1 $\mu\text{g/ml}$ concentration and included GAM-ALKPHOS and/or GAM-PEROX (variations 2 and 3), GAM-biotin (variation 6). A new variant (indirect double intermediate antibody) was created by adding an unlabeled GAR (20 $\mu\text{g/mg}$) to the specific unlabeled rabbit antibodies and finally a RAG-ALKPHOS conjugate (0.1 $\mu\text{g/ml}$) (variation 5). The conventional protein A-ALKPHOS conjugate (0.5 $\mu\text{g/ml}$) was used in variation 4 when immunoglobulins from 879 were added as unlabeled CTV-specific antibodies.

For ELISA-DAS variations, M129B microplates (Dynatech) were coated with purified rabbit polyclonal immunoglobulins 1 $\mu\text{g/ml}$ (variations 16, 21, and 22), 3DF1 MAb 1 $\mu\text{g/ml}$ (variations 17, 19, and 23), and a mixture of 3DF1 and 3CA5 MAbs each at 1 $\mu\text{g/ml}$ (variations 18, 20, and 24). All dilutions were in coating buffer and incubation was for 4 hr at 37°C. The plant extracts were prepared in PBS containing 0.2% DIECA, pH 7.2, and the purified CTV was diluted in the same buffer. The concentration of the rabbit polyclonal antibody-ALKPHOS conjugate was 0.1 $\mu\text{g/ml}$ (variations 16, 23, and 24) and 0.05 $\mu\text{g/ml}$ for 3DF1-ALKPHOS conjugate (variations, 17, 18, and 21) and 3DF1-PEROX conjugate (variation 17). When a mixture of the two MAb-ALKPHOS conjugates was used (variations 19, 20, and 22), the concentration was 0.05 $\mu\text{g/ml}$ for each.

For ELISA-DASI, M129B plates were coated with rabbit polyclonal immunoglobulins (variations 9, 14, and 15) with 3DF1 MAb (variations 10 and 12) or with a mixture of the two MAb (variations 11 and 13). Dilutions were

FIG. 1. COMPARISON OF 24 VARIATIONS OF ELISA FOR DETECTION OF CITRUS TRISTEZA VIRUS











VARIATION	STEPS	AP-OD ₄₀₅ ^z	PER-OD ₄₅₀ ^y
1. 	ANTIGEN / R-PAB / GAR-CONJ	0.92 f ^x	0.45 e
2. 	ANTIGEN / M-PAB / GAM-CONJ	0.63 g	--
3. 	ANTIGEN / 3DF1 MAB / GAM-CONJ	1.13 cde	0.44 e
4. 	ANTIGEN / R-PAB / PROTEIN-A-CONJ	0.19 h	--
5. 	ANTIGEN / R-PAB / GAR / RAG-CONJ	0.29 h	--
6. 	ANTIGEN / R-PAB / GAR-BIOTIN / SA-CONJ	--	0.64 d
7. 	ANTIGEN / 3DF1 MAB / GAM-BIOTIN / SA-CONJ	0.63 g	0.82 c
8. 	ANTIGEN / MAB MIX / GAM-BIOTIN / SA-CONJ	0.64 g	--
9. 	R-PAB / ANTIGEN / 3DF1 MAB / GAM-CONJ	1.67 b	0.99 b
10. 	3DF1 MAB / ANTIGEN / R-PAB / GAR-CONJ	1.19 cd	--
11. 	MAB MIX / ANTIGEN / R-PAB / GAR-CONJ	1.27 c	1.08 b
12. 	3DF1 MAB / ANTIGEN / R-PAB / GAR / RAG-CONJ	0.65 g	--
13. 	MAB MIX / ANTIGEN / R-PAB / GAR / RAG-CONJ	0.70 g	--
14. 	R-PAB / ANTIGEN / 3DF1 MAB / GAM-BIOTIN / SA-CONJ	2.00 a	1.55 a

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1 $\mu\text{g/ml}$ in coating buffer. The plant extracts and dilutions or purified virus were prepared as in ELISA-DAS. The intermediate unlabeled antibodies were used at 0.1 $\mu\text{g/ml}$ and incubated for 2 hr at 35C. Rabbit polyclonal im-

munoglobulins were used in variations 10, 11, 12, and 13; 3DF1 MAb in variations 9 and 14; and the mixture of MAb 3DF1 + 3CA5 in variation 15. The enzyme-labeled antibodies specific for the unlabeled intermediate antibodies

FIG. 1. Continued

VARIATION	STEPS	AP-OD ₄₀₅ ^z	PER-OD ₄₅₀ ^y
15.	 R-PAB / ANTIGEN / MAB MIX / GAM-BIOTIN / SA-CONJ	2.00 a ^x	—
16.	 R-PAB / ANTIGEN / R-PAB-CONJ	1.20 cd	—
17.	 3DF1 MAB / ANTIGEN / 3DF1 MAB-CONJ	0.37 h	—
18.	 MAB MIX / ANTIGEN / 3DF1 MAB-CONJ	0.32 h	0.95 b
19.	 3DF1 MAB / ANTIGEN / MAB MIX-CONJ	1.09 de	—
20.	 MAB MIX / ANTIGEN / MAB MIX-CONJ	0.92 f	—
21.	 R-PAB / ANTIGEN / 3DF1-CONJ	0.32 h	—
22.	 R-PAB / ANTIGEN / MAB MIX-CONJ	0.98 ef	—
23.	 3DF1 MAB / ANTIGEN / R-PAB-CONJ	1.59 b	—
24.	 MAB MIX / ANTIGEN / R-PAB-CONJ	1.74 b	—

● Antigen	□ Unlabeled Goat anti-rabbit (GAR) immunoglobulins
□ or ▭ Polyclonal antibodies, R-PAB = Rabbit M-PAB = Mouse	▣ Biotin labeled GAR
∧ or ∨ 3DF1 Monoclonal antibody (MAB)	■△ Biotin labeled Goat anti-mouse (GAM) immunoglobulins
∧ or ∨ Mixture of MAB 3DF1 and 3CA5	⊙ Alkaline phosphatase or peroxidase enzyme conjugates. R= GAR, M= GAM G= Rabbit-anti goat (RAG), A= Protein A, and SA= Streptavidin
⊙ or ⊙ Alkaline phosphatase or peroxidase conjugates of PAB, 3DF1, and MAB mix or	

x (P = 0.05) significance is within column, numbers with common letters do not differ significantly.
Residual standard error is 0.14.

z AP-OD 405 - Optical density at 405 nm for final alkaline phosphatase reaction.

y PER-OD 450 - Optical density at 450 nm for final peroxidase reaction.

were added at 0.1 μ g/ml. These were GAM-ALKPHOS and GAM-PEROX (variation 9), GAR-ALKPHOS and/or GAR-PEROX (variation 10 and 11),

and GAM-Biotin (variation 14 and 15). Variation 12 was prepared similarly to the "double intermediate" in ELISA-I (variation 5) by adding GAR and finally

RAG-ALKPHOS conjugate at the same concentrations indicated previously.

For all the ELISA variations and variants using biotin-labelled antibodies (variations 6, 7, 8, 14, and 15), the reaction was completed by adding streptavidin-ALKPHOS or streptavidin-PEROX conjugates at 1 μ g/ml and incubating for 30 min at room temperature.

Substrates, readings and statistical analysis. Freshly prepared substrate was used. For ALKPHOS, 1 μ g/ml p-nitrophenylphosphate in substrate buffer (31) was used and the plates were read (A405 nm) at 15 min intervals for 2 hr without stopping the reaction. For PEROX, 1 mg/ml orthophenyldiamine in substrate buffer (31) was used. The reaction was stopped after 5 min by adding 50 μ l of 3N sulfuric acid and read (A450 nm). Plate readings were made with a Titertek Multiscan (Flow) automatic plate reader zeroed on an empty plate.

Data were analyzed (separately for ALKPHOS and for PEROX plates), by an analysis of variance according the following model:

$$Y_{ijkl} = u + E_i + V_j(i) + A_k + EA_{ik} + VA_j(i)k + W_l(ijk)$$

where E_i represents the particular ELISA type (fixed) ($i = 1, 3$), $V_j(i)$ is the effect of different variations within each ELISA type (fixed), A_k is the effect (fixed) of the antigens ($K = 1, 3$), two letter effects are the corresponding interactions, and $W_l(ijk)$ is the within-error ($l = 1, 3$ (minimum) or 20 (maximum)).

Y_{ijkl} is the optical density (OD) obtained by subtracting the average OD of the control antigen (extract from healthy sweet orange) from the OD of the corresponding observations (variation and antigen type combination). The antigen combinations were the T36 extract, purified T388 diluted in healthy extract and purified T388 diluted in PBS. A second analysis did not consider ELISA type as a factor and stud-

ied all variations as a set of treatments. This allowed testing the differences between all variations disregarding the ELISA type.

RESULTS

The OD means for the I, DAS, and DAS I ELISA with both ALKPHOS and PEROX labeling enzymes are summarized in Fig. 1. The largest difference between CTV samples and healthy extracts were obtained with ELISA-DASI. All ELISA types studied discriminated between CTV-positive and healthy samples and detected concentration of viral protein ranging between 0.1 and 100 ng/ml.

Fig. 1 shows the OD means for the different ELISA variations. For ALKPHOS, DAS I variations 14 and 15 showed an extremely high OD followed by DAS variations 23 and 24 and DAS I variation 9. Conversely, DAS variations 17, 18, and 21 and ELISA-I variations 4 and 5 had the lowest OD values followed by some special forms of DAS I and I-ELISA. The top two groups were able to detect 0.1-0.5 ng/ml CTV antigen. For PEROX, DAS I variation 14 again showed the highest OD followed by DAS I variations 9 and 11 and DAS variation 18. Sensitivity data for DAS I variation 9 is shown in Table 2.

TABLE 1
MEANS AND RESIDUAL STANDARD ERROR (RES) FOR DIFFERENT TYPES OF ELISA

ELISA type ^z	Mean OD Readings	
	Alkaline phosphatase ^y	Peroxidase ^y
DAS I	1.068 a	1.150 a
DAS	0.842 b	0.950 b
I	0.570 c	0.513 c
(rse) ^x	0.156	0.145

^zCombined results obtained for variants shown in Fig. 1 for each type of ELISA.

^yMeans in columns followed by different letters are significantly different ($P = 0.05$).

^xResidual standard error.

TABLE 2
SENSITIVITY OF A DOUBLE ANTIBODY
SANDWICH INDIRECT (DASI) ELISA PRO-
CEDURE^z FOR DETECTION OF CITRUS
TRISTEZA VIRUS (CTV)

Virus Conc. ^x	OD ₄₀₅ ^y	
	Diluent ^w	
	Extract	PBS
100 µg/ml	----	----
10 µg/ml	1.676	1.602
1 µg/ml	1.120	1.203
100 ng/ml	0.527	0.612
10 ng/ml	0.392	0.422
1 ng/ml	0.251	0.309
0.1 ng/ml	0.201	0.205
Control	0.093	0.102

^zThe DASI procedure used was variation 9 in Fig. 1. Plates were coated with one rabbit polyclonal antibody, the intermediate antibody was monoclonal antibody 3DF1, and the labeled antibody was goat anti-mouse IgG conjugated to alkaline phosphatase.

^yOD₄₀₅ readings made after a 60-min incubation (mean of 4 wells).

^xVirus concentration calculated as 1 mg/ml = OD₂₆₀ 2.0.

^wPurified virus diluted in PBS or in a PBS extract of healthy citrus tissue.

DISCUSSION

ELISA-DASI had the highest reaction (OD) values. Results in treatments 9, 14, and 15 were especially high. This is probably due to the good trapping ability of the polyclonal immunoglobulins used, the specificity of the MAbs, and also to the amplification produced by the anti-mouse conjugates or the biotin/streptavidin system. This ELISA type is suitable for virus detection and for screening culture supernatants. The main disadvantage is the need to produce immunoglobulins in two different animal species (2). The ready availability of standard monoclonals to CTV, in effect, reduces the need to only a source of coating antibody.

The low OD values found for the DAS ELISA variations 17 and 18 compared with variation 16 may be explain-

ed by the fact that the conjugate concentration used for the monoclonals was 0.05 µg/ml rather than the 0.10 µg/ml used for the polyclonal conjugate. A similar increase was seen between variation 21 (0.05 µg/ml conjugate) and 22 (0.10 µg/ml conjugate).

The conventional ELISA-DAS is a very simple method and suitable for diagnosis and could be very convenient when using biotin-labeled MAbs (7). The biotin/avidin system also allows amplification of the reaction to increase sensitivity.

ELISA-I variations 3, 1, 7, and 8 are promising for CTV diagnosis because they demand less time and reactants. The sensitivity of the variants is in the range of 25-100 ng/ml of CTV antigen.

The sensitivity of different variations is a function of the specificity of the immunoglobulins used and of the degree of amplification. A good example of this is given by the ranking of the OD of the ELISA-I variations 3, 1, and 2. The best results were obtained with the CTV-specific monoclonal antibodies followed by the immunoglobulins from the 879 antiserum (which is also highly specific to CTV) and lastly with the immunoglobulins from the M2 polyclonal which is less specific. ELISA-DASI is more sensitive than ELISA-DAS because of the amplification that can be added.

In most cases, there were no significant differences between diluting the purified CTV solution in PBS buffer or in the extract of healthy tissue because using monoclonal antibodies ensures specificity. Use of reactive MAbs such as 3DF1 and 3CA5 increases the sensitivity of ELISA variations and allows the design of new variations. MAbs are also a stable source of well-characterized antibodies which make it easier to obtain reproducible results among different laboratories. They can be used in mixtures to increase the number of antigenic determinants involved in the reaction and increase the sensitivity and range of reaction.

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