

RELIABILITY OF DETECTION OF *CITRUS TRISTEZA VIRUS* BY AN IMMUNOCHROMATOGRAPHIC LATERAL FLOW ASSAY IN COMPARISON WITH ELISA

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SUMMARY

Citrus tristeza virus (CTV) is one of the most dangerous pathogens for *Citrus* spp.. Early diagnosis is essential for containment of the disease and many diagnostic techniques have been developed. But these need skilled personnel and laboratory equipment, and it takes some days for results to be obtained. We applied the easy-to-use technology known as Lateral Flow (LF) to detect CTV in orchard trees within few minutes of samples being taken. We compared LF results from leaf and fruit samples with those obtained by using ELISA and found a good correlation between these two techniques.

Key words: Citrus, diagnosis, immunochromatography, Tristeza, Lateral Flow.

INTRODUCTION

Citrus tristeza virus (CTV) causes serious disease whenever citrus is grown, and recently a serious outbreak of CTV has been reported in Italy for the first time (Davino *et al.*, 2003; D'Onghia and Savino, unpublished). Effective control of the virus needs rapid and reliable diagnostic methods. CTV is listed in most citrus-growing countries as a quarantine pathogen, and the almost exclusive use as rootstock of sour orange (*Citrus aurantium* L.), which is very sensitive to the disease, requires a high level of attention to avoid the establishment of epidemic foci of the pathogen. For this reason, the control of CTV is mandatory, requiring, among others, systematic inspections and analysis of citrus trees and immediate eradication of infected plants.

The use of ELISA for CTV provides easy and rapid detection compared to biological indexing, so that official methods, including the programme of mandatory control, now rely on it. Polyclonal antibodies are normally used, and they can detect all virus strains but not discriminate among them. Some monoclonal antibodies

can detect all isolates (Vela *et al.*, 1986; Cambra *et al.*, 2000), or are specific for different strains (Permar *et al.*, 1990; Garnsey *et al.*, 1998). Other immunochemical techniques, such as direct tissue blot immunoassay (DT-BIA) provide results similar to ELISA (Garnsey *et al.*, 1993; Cambra *et al.*, 2000).

All these methods are strictly laboratory tests, requiring some equipment, trained people and the transport of the samples or blotted membranes to laboratories. Furthermore, results are obtained only after some days. Recently, rapid immunochromatographic techniques like Lateral Flow (LF) have been applied to plant virus detection (Danks and Barker, 2000; Salomone and Roggero, 2002; Salomone *et al.*, 2002), mainly for viruses of vegetable and ornamental plants. The main advantage of LF is that detection can be done anywhere, with a simple device, operated by personnel with very little or no training, and results are obtained within minutes. This allows an easier approach to the diagnosis of any pathogen if the kits are reliable. We have developed a lateral flow device based on polyclonal antibodies to CTV and compared its performance with that of ELISA in different situations.

In diagnostic systems, the assay is intended mainly to distinguish between negative and positive responses. Therefore, in our study, terms like sensitivity and specificity refer to the reliability of the diagnostic test and are termed "diagnostic sensitivity" and "diagnostic specificity" (Galen and Gambino, 1975). The quantity of the pathogen detected has not been considered as a major factor.

MATERIALS AND METHODS

Polyclonal antiserum and purification of antibodies.

A rabbit polyclonal antiserum, raised against isolate CTV-0002 of the collection of the Department of Plant Protection and Applied Microbiology, University of Bari (Barbarossa L., Potere O., and Castellano M.A., unpublished) was used. The antiserum was cross-absorbed with preparations of healthy citrus leaf. Twenty grams of leaves were triturated in 50 ml of 0.5 M phosphate buffer pH 8 containing 10 mM Na-DIECA and

20 mM Na₂SO₃. After filtration through nylon stocking, the extract was centrifuged at 3,000 g for 10 min and the supernatant fraction was centrifuged at 235,000 g for 45 min. Pellets were resuspended with 3 ml of PBS and 1 ml of the serum was added. After overnight incubation at 4°C on a shaker, the preparation was centrifuged at 300,000 g for 90 min and the supernatant used for antibody purification. Antibodies were affinity purified with Protein G Hitrap Column (Amersham Biosciences, Little Chalfont, UK) and dialysed overnight against PBS. Antibody concentration was determined by spectrophotometry at 280 nm and then adjusted to 1 mg ml⁻¹.

DAS ELISA was performed using standard procedures (Clark and Adams, 1977). One mg of antibodies was used in a single step procedure with 1,000 Units of alkaline phosphatase and 0.05% glutaraldehyde for conjugate preparation. Plates (Falcon 3911) were coated with antibodies at 1.4 µg ml⁻¹ and the conjugate preparation was diluted 1:1,000. Leaf and fruit samples were homogenized 1:10 (w/v) with PBS-Tween-20 containing 2% polyvinylpyrrolidone (MW about 24,000). Each sample was put in duplicate wells and each plate contained 4 wells filled with healthy citrus samples. Final absorbance at 405 nm was read with automatic plate readers and samples having an absorbance at least 3 times higher than the mean of healthy controls were considered positive.

Lateral flow assay. Colloidal gold was prepared by the authors by reduction of tetrachloroauric acid with trisodium citrate (Frens, 1973); particles were about 30 nm in diameter as determined by electron microscopy and spectrophotometric scan. The pH of the colloidal gold solution was adjusted to 7.0 with 0.1 M carbonate buffer pH 9.6 and stored at 4°C.

The minimum quantity of antibodies that are able to prevent aggregation of colloidal gold solution by NaCl was chosen for gold conjugation. For titration of 1 ml of gold, 100 µl of 50 mM borate buffer pH 8, different amount of antibodies and 100 µl of 10% NaCl were added and the suspensions were observed for blue colour as evidence of aggregation.

Conjugation of antibodies to colloidal gold was made by incubating the chosen amount of antibodies and gold for 1 h at room temperature with gentle stirring. After blocking with 20 mM borate buffer pH 8 containing 1% BSA, the preparation was centrifuged at 39,000 g for 30 min. The pellet was resuspended in the same buffer, centrifuged again and finally resuspended in 2 mM borate buffer, pH 8 containing 0.1% BSA. After a final centrifugation, the pellet was resuspended in PBS containing 2% sucrose, 1% BSA and 0.25% Tween-20 to give an optical density of about 3 at 540 nm.

Test lines were made with 1 mg ml⁻¹ anti-virus antibody solution and control lines with affinity purified an-

ti-rabbit antibodies from goat (Sigma-Aldrich, MO, USA) at 0.5 mg ml⁻¹ and were dispensed at 6°C with an EasyPrinter (Advanced Microdevices, Ambala, India) at 1 µl cm⁻¹ flow rate on HF240 membranes (Millipore, MA, USA) supplied in laminated cards. After deposition, membranes were dried at 37°C for 2 h. Absorbent pad and sample pad (both AP22) and glass fiber for the conjugate pad were also from Millipore. The glass fiber pad was soaked in conjugate-gold with O.D. = 3 and then dried at 37°C for 4 hours. After assembling all the components, cards were cut into 0.5 mm wide strips with a hand cutter and every strip was inserted in a plastic housing. Devices were stored in sealed aluminium bags with desiccant. Kits for diagnosis distributed to final operators included the device described, a plastic bag for extraction, a plastic pipette and 5 ml of extraction buffer consisting of PBS, 0.02% Tween-20, 2% PVP (MW 24,000) and 0.5% Triton X-100.

Samples of leaf and fruit (white part of the peel) were homogenised by hand in a w/v ratio of about 1:10 and 3-4 drops of the extract were added to the sample hole for testing. Results were evaluated within 10 min by eye. A sample was considered negative for CTV when only the control line appeared whereas a sample was considered positive when 2 lines appeared. The intensity of the 2 lines was not considered.

Comparison between ELISA and Lateral flow assay.

To compare ELISA and LF, leaf and fruit samples were analysed in parallel. Most of the samples were collected directly from commercial citrus orchards in Apulia (south-eastern Italy) in Spring 2003, from 3 different sites: site A in an area known to be heavily affected by Tristeza; site B on the border of the same area and, therefore, presumably recently infected; site C in an area known to be free of CTV. Samples were from orchards of sweet orange, cv Navelina, grafted on sour orange (sites A and B) or a mixture of *Citrus* spp. (sour orange, mandarin and clementine, site C). Other samples were from commercial material from Spain sold as fruit in Torino in winter 2003. In this case, samples consisted of almost dry leaves and fruits of mandarin and sweet orange cv Navelina.

ELISA and LF results were compared through a series of parameters previously assessed for medical diagnoses (Galen and Gambino, 1975; Scassellati, 2000), which are defined in Table 1. It was assumed that if the parameters are over 90%, the correlation between the two techniques was good.

RESULTS

In preliminary LF, no test line appeared with extracts of citrus leaves known to be healthy. The control line appeared within 3-5 min of applying the sample. Sam-

Table 1. Parameters for comparative analysis between two diagnostic methods, as used in medical practice (Galen and Gambino, 1975)^a.

Sensitivity	$(TP/TP+FN) \times 100$	Percent positivity in disease
Specificity	$(TN/TN+FP) \times 100$	Percent negativity in the absence of the disease
Positive Predictive Value	$(TP/TP+FP) \times 100$	Percent of positive results that are true positive
Negative Predictive Value	$(TN/TN+FN) \times 100$	Percent of negative results that are true negatives
Efficiency	$(TP+TN)/(TP+FP+TN+FN) \times 100$	

^a TP (True Positives): number of sick subjects who are correctly classified by the test; FP (False Positives): number of subjects free of the disease who are misclassified by the test; TN (True Negatives): number of subjects free of the disease who are correctly classified by the test; FN (False Negatives): Number of sick subjects who are misclassified by the test.

ples from healthy mandarin, sweet orange, clementine and sour orange were tested and all gave negative results. When LF was tested with greenhouse leaf samples from infected plants at the University of Bari, both the test and control lines appeared within 3-5 min. A typical result is shown in Fig. 1. Use of a higher concentration

of the samples resulted in conjugate migration difficulties and a high green background on the membranes; for this reason the w/v ratio of 1:10 was used in further experiments. The limit of detection was evaluated from dried leaf and fresh tissue using two-fold dilution steps: in both cases, it was found to be 1:320. These encouraging preliminary results prompted us to carry out a wider comparison between ELISA and the LF tests.

From commercial material in Torino, CTV was detected in some leaf and fruit peel samples by ELISA. Twenty samples from leaves and 6 from fruits were tested in parallel by ELISA and lateral flow and the two methods gave identical results. A larger comparison was made with samples collected in Apulia in spring 2003 from naturally infected citrus orchards.

ELISA, carried out on leaf samples collected from individual plants, showed that 18 of 32 samples from site A were positive; 16 out of 52 samples from site B were also positive whereas none out of 11 samples from site C was positive. A comparison was done for CTV detection on mature fruit samples collected from the same plants previously tested using leaves. From site A, 31 out of 32 samples were positive; from site B, 7 out of 10 were positive whereas none from site C (Table 2). Thus a greater proportion of samples gave positive results by analysing fruits rather than leaves. To confirm the better sensitivity of the peel compared with leaf tissues, more samples from site A and B were tested by ELISA. Among these, 35 samples were positive both in fruits and leaves; 33 were negative in leaves but positive in fruits, whereas only 5 were negative in fruits but positive in leaves. Moreover, the virus titre was much higher in peel than in leaves, as indicated by the ratio of absorbance (infected/healthy) at 405 nm after 1 h of substrate development: 12.2 for peel *versus* 6.0 for leaves.

A total of 145 samples from leaves and fruits were tested in parallel by ELISA and LF. Of the 34 leaf samples testing positive by ELISA, 28 were also positive by

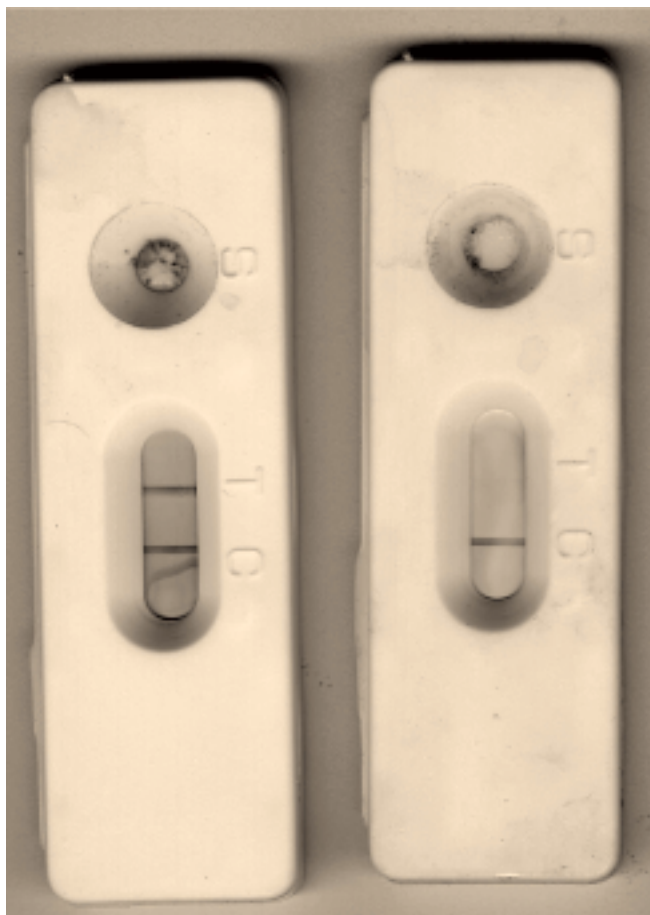


Fig. 1. On the left, a positive sample results in two coloured lines. On the right, a negative sample results in one line.

Table 2. Comparison between ELISA and lateral flow (LF) assay for the detection of CTV on leaves and fruits, choosing ELISA as reference test to discriminate healthy and infected samples.

		Leaves			Fruits			Leaves and fruits		
		ELISA	LF +	LF -	ELISA	LF +	LF -	ELISA	LF +	LF -
Site A	Healthy	14	2	12	1	1	0	15	3	12
	Infected	18	16	2	31	30	1	49	46	3
Site B	Healthy	36	1	35	3	0	3	39	1	38
	Infected	16	12	4	7	7	0	23	19	4
Site C	Healthy	11	0	11	8	0	8	19	0	19
	Infected	0	0	0	0	0	0	0	0	0
Total	Healthy	61	3	58	12	1	11	73	4	69
	Infected	34	28	6	38	37	1	72	65	7

Table 3. Efficiency performance of the Lateral Flow assay compared to ELISA as reference test (data derived from Table 2).

Parameters	Leaves	Fruits	Total
Diagnostic Sensitivity (%)	82	97	90
Diagnostic Specificity (%)	95	92	95
Predictive Value of True Positive (%)	90	97	94
Predictive Value of True Negative (%)	91	92	91
Efficiency (%)	90	96	92

LF and of the 38 fruit samples testing positive by ELISA, 37 were also positive by LF. Of the 61 negative leaves ELISA samples, 58 were also negative in LF; for fruit samples of the 12 negative, 11 were also negative in the LF. Thus LF appeared to give 3 and 1 false positive, respectively, in leaves and fruits, with respect to ELISA. Details of the comparison are shown in Table 2. Parameters illustrating efficiency of diagnosis are given in Table 3. All considered parameters except diagnostic sensitivity for leaves were over 90% and better results were obtained by the analysis of fruits rather than leaves.

DISCUSSION

Among laboratory methods for the diagnosis of plant viruses, ELISA is still the most widespread and accepted. It is also the official method used in certification

schemes. However, LF offers many advantages over ELISA, as listed below, and it gives reliable detection of some important viruses infecting herbaceous vegetables and ornamental plants (Danks and Barker, 2000; Salomone and Roggero, 2002; Salomone *et al.*, 2002). Tests of woody plants are more difficult because viruses are usually present in low concentrations and tissues are rich in oxidizing or, more generally, in interfering compounds. Therefore, we wished to see if LF could be used to detect a major virus like CTV with high confidence.

For sample extraction, especially with hard tissues like citrus leaves, we found that using a plastic bag with a net inside to be the easiest method. If one considers the cost of a single analysis, the LF tests are probably more expensive than the ELISA tests, if applied to large-scale diagnosis.

The situations in which LF may have advantages over ELISA are the following:

- it can be performed by untrained people and results are unequivocal and easily interpreted;
- we found that the kits were stable for some months when sealed in aluminium bags with desiccant and stored at room temperature, thus facilitating their transport;
- the need to run a test for a very limited number of samples increases the cost of ELISA but not of LF;
- the mandatory control of CTV according to legislation requires large scale inspections and laboratory analysis every year. In some cases of trees strongly suspected of infection, immediate action taken by the phytosanitary inspector is needed (i.e.: to avoid the germplasm movement from the suspect orchard or

nursery); this is not compatible with the time required for ELISA testing. In this case, LF may give an immediate confirmation of the infection, helping the inspectorate in making its decision;

- the immediate answer given by LF may be helpful in training inspectors or technicians with little experience of field symptoms;
- field application of tests avoids the cost of shipping samples to specialized laboratories;
- use of LF may eliminate the need to label the plants and the need to make a second visit to communicate assay results;
- direct analysis is psychologically more cogent than a formal written result coming days after sampling;
- frontier controls require a rapid diagnosis.

On the other hand, as in other field applications, LF can give only indicative results, sufficient to take safeguarding action. Confirmation by using official assays such as ELISA is needed before enacting eradication. In our trials, we detected 5% of false positives and this level is, in general, acceptable, except in cases of mandatory eradication. Among the samples found to be positive by ELISA, 90% were also positive by LF and both diagnostic sensitivity and predictive value were even higher when considering only fruits. Thus, both diagnostic sensitivity and diagnostic specificity are acceptable if LF is used only as a screening method.

Although our comparison was based on samples taken over a limited time of the year, we believe that the new assay has a good reliability since good results were also obtained with poorly stored leaf samples. Also of interest is the possibility of testing fruit samples directly: results were similar or even better than those obtained by testing leaves, although fruit is present for a short time and we used samples taken only when the fruit was ripen. This has a practical importance in some European countries, such as Italy, that are classified as a “protected zone” and, for this reason, cannot import citrus fruit with leaves from countries where CTV is present.

To our knowledge, this is the first time that a comparison between diagnostic methods is carried out through the use of parameters such as diagnostic sensitivity and diagnostic specificity in plant virology. Although these parameters were first introduced in medical diagnoses, they should be used whenever a diagnostic method is evaluated. For this purpose, the diagnostic parameters can be even more important than the analytical ones, like limit of detection or strain selectivity, to make a complete comparison between diagnostic tools.

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