Xanthomonas campestris pv. *citri* Detection and Identification by Enzyme-Linked Immunosorbent Assay

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ABSTRACT

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The double antibody sandwich enzyme-linked immunosorbent assay was evaluated for the specific detection and identification of *Xanthomonas campestris* pv. *citri*. Alkaline phosphatase conjugates were prepared with immunoglobulin partially purified by ammonium sulfate precipitation from antisera to intact, live *X. c.* pv. *citri* cells. *X. c.* pv. *citri* antigens were detected in heated (100 C for 30 min) cell suspensions containing 10^3–10^4 colony-forming units per milliliter. *X. c.* pv. *citri* was also detected by enzyme-linked immunosorbent assay when added to extracts of healthy grapefruit seedling leaves and in extracts of lesions from artificially inoculated leaves. No detectable reactions occurred with several other xanthomonad nomenspecies and one saprophytic bacterium. Positive reactions occurred with five of six *X. campestris* pv. *manihotis* strains using antiserum to an Asian (pathotype A) strain of *X. c.* pv. *citri* but not with antisera prepared against *X. c.* pv. *citri* strains from Argentina and Brazil.

Citrus bacterial canker disease (CBCD), caused by *Xanthomonas campestris* pv. *citri* (7), is a serious disease of most major citrus varieties. CBCD is not endemic to the United States but was introduced into Florida and other Gulf states via contaminated plants about 1910 (8). CBCD was eradicated in the United States over a long period of time and at considerable expense (9). The widespread occurrence of CBCD in foreign citrus-growing areas presents a continuous hazard for reintroduction of *X. c.* pv. *citri* and establishment of CBCD in the United States. Reduction of the potentially serious economic threat to the U.S. citrus industry depends upon exclusion of the bacterium and diseased plant material through strict quarantine regulations and practices. However, there is a need to develop rapid, convenient diagnostic procedures to detect and identify *X. c.* pv. *citri*.

At least three pathotypes of *X. c.* pv. *citri* are distinguished by host reaction (9). The Asian or A pathotype, the most common widespread form, is extremely virulent on grapefruit and orange but also infects other citrus varieties. The B pathotype occurs primarily on lemon in Argentina and Uruguay. The C pathotype infects Mexican lime in Brazil and is synonymous with *X. c.* pv. *citri* n. f. sp. *aurantifolia* (19,20). In addition, three groups or strains among 15 isolates of *X. c.* pv. *citri* were differentiated on the basis of virulence on *Murraya exotica* (14).

Pathotype A strains are serologically related to *X. campestris* pv. *manihotis* (2,28) but not to *X. c.* pv. *citri* pathotypes B or C (2,17,19,20). Pathotypes B and C have been distinguished serologically (16). Pathotype C strains are serologically related to *X. campestris* pv. *campestris* (2) but serologically distinct from *X. c.* pv. *citri* pathotype A strains and *X. c.* pv. *manihotis* (2,19).

Enzyme-linked immunosorbent assay (ELISA) techniques have been used extensively to detect clinically a wide range of organisms including protozoa, bacteria, and viruses (5), but they have been used only to a limited extent for phytopathogenic bacteria (1,4,6,8,15, 18,21,23,25,26). Application of the double antibody sandwich ELISA for detecting and identifying *X. c.* pv. *citri* strains is described herein.

MATERIALS AND METHODS

Bacteria. Nineteen strains of seven bacterial nomenspecies were used (Tables 1 and 2). Six *X. c.* pv. *citri* strains representing different pathotypes, serotypes, and lysotypes were obtained from Japan and South America (Table 1). Twelve strains of five additional xanthomonad pathovars and nomenspecies were also used (Table 2). A yellow, rapidly growing bacterium was isolated from a lesion on a lemon leaf from Argentina. This leaf was provided by R. Stall and designated as affected by the B form of CBCD. The bacterium was identified as *Enterobacter agglomerans* at the American Type Culture Collection in Rockville, MD (Contract 297). This name is a synonym of *Erwinia herbicola,* and the bacterium is designated EH1 (3). All strains were used as provided without further purification.

X. c. pv. *citri* strains and strain EH1 were maintained on Wakimoto's potato semisynthetic medium (16). *X. fragariae* strains were maintained on medium B containing 1.5% agar (13). All other stains were maintained on nutrient agar (Difco) supplemented with 2% glucose. All cultures were maintained at 3 C and subcultured weekly on fresh media.

For cell suspension preparations, bacteria were grown at 28–29 C with rotary shaking in nutrient broth (Difco) supplemented with 0.2% glucose and 0.5% sodium chloride (NaCl) (NGSB). Cell suspension titers were expressed as colony-forming units (CFU) per milliliter and were determined by standard dilution plating on nutrient agar supplemented with glucose, Wakimoto's potato semisynthetic medium (16), or medium B (13). The virulence of each *X. c.* pv. *citri* isolate was verified by the development of CBCD lesions on leaves of Duncan grapefruit seedlings, Eureka lemon seedlings, Mexican lime seedlings, and *Citrus natsudaidai* seedlings following inoculation by leaf injection-infiltration (10).

Bacteriophages. Phages CP1 and CP2, received from M. Koizumi, and phage CP3, received from M. Goto, were propagated in strains XC62, XC63, and XC64, respectively. The sensitivity of *X. c.* pv. *citri* strains to lysis by these phages was determined by plaque formation following spotting of droplets of phage-containing preparations on Wakimoto's potato semisynthetic medium with 0.7% agar seeded with 0.5 ml of cells from 16–20 hr NGSB shake cultures at 28–29 C. The phage-containing preparations in NGSB were tested at 0.1, 1, and 10 times the routine test dilution, which is defined as the highest dilution at which confluent lysis of the homologous host occurs.

Antisera production. Immunogen preparations were suspensions of intact, live cells from cultures grown in NGSB at 28–29 C overnight with gyrotary shaking. Cells were collected by low-speed centrifugation and washed once in

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phosphate-buffered saline (PBS). White New Zealand female rabbits (4–6 kg) were injected intravenously once with 0.5 ml of the appropriate *X. c.* pv. *citri* cell suspension, followed by four or five intramuscular injections at weekly intervals. For intramuscular injection, each immunogen preparation ($1–3 \times 10^8$ CFU/ml) was emulsified with an equal volume of Freund's incomplete adjuvant. Blood was collected 7–10 days after the last injection. Antisera titers were determined by agglutination tests. Twofold dilutions of antisera were made in sterile 0.85% NaCl with 0.02% sodium azide. A 0.5-ml aliquot of diluted antiserum was mixed with an equal volume of cell suspension, incubated at 37 C for 2 hr, and then incubated at room temperature for an additional 2–3 hr. The turbidity of each cell suspension was adjusted to 65–80% transmittance at 620 nm ($1–5 \times 10^8$ CFU/ml). Each antiserum was tested against antigens from homologous and several heterologous strains.

Immunoglobulin (Ig) was precipitated from rabbit sera with ammonium sulfate, followed by two additional precipitations and resuspension and dialysis in 0.15 M NaCl (27). Immunoglobulin protein concentration was based on E_{280} mg/ml = 1.8. The enzyme-conjugated Ig was prepared using alkaline phosphatase (Sigma, Type VII, P-4502) (5).

Antigen preparation. Bacteria were grown in liquid media as for immunogen preparation, and cells were collected from log phase cultures by low-speed centrifugation at 5,000 *g* for 15–20 min. Cells were resuspended in sterile PBS and adjusted turbidimetrically to contain about $1–2 \times 10^8$ CFU/ml (75–80% T at 620 nm). For ELISA tests, appropriate dilutions were made in PBS + 0.1% Tween 20. Extracts of leaves from healthy Duncan grapefruit seedlings were prepared by homogenizing leaf tissue in 5–10 volumes of PBS + 0.1% Tween. *X. c.* pv. *citri* lesion antigens in artificially inoculated leaf tissue were prepared by thoroughly triturating individual lesions or pieces of tissue with several lesions in small volumes (1 ml or less) of sterile PBS + 0.1% Tween 20. Culture and tissue antigens were either heated at 100 C for 30 min or unheated.

Detection and measurement of antigen. The double antibody sandwich ELISA (5,24) was used for antigen detection and measurement. In a checkerboard design, the optimum concentration of each Ig and dilution of Ig-enzyme conjugate were determined experimentally using homologous pure culture antigens. The optimum concentrations of Ig preparations varied from 8–25 μg of protein per milliliter. Conjugate preparations were used at 1/5 to 1/25 dilutions. Generally, microtiter plates (Dynatech) were coated for several hours or overnight at 3 C with 200 μl of partially purified Ig diluted in 0.05 M carbonate buffer, pH 9.6.

After washing the plates successively three times for 3 min each time with PBS + 0.1% Tween 20, 200 μl of antigen-containing preparation was added to each well and incubated overnight at 3 C. The plates were washed as before, and 200 μl of alkaline phosphatase-Ig conjugate was added to each well and incubated for 3–4 hr at room temperature in a humid atmosphere. After again washing the plates, 300 μl of para-nitrophenyl-phosphate (1 mg/ml in 10% diethanolamine) substrate was added to each well and incubated for 1 hr at room temperature. Fifty microliters of 3 M sodium hydroxide was added to each well, the contents of three to six wells were combined, and the results were quantitated by determining the absorbance at 405 nm.

Table 1. *Xanthomonas campestris* pv. *citri* strains used in enzyme-linked immunosorbent assays

Lab strain[a]	Source strain no.	CBCD pathotype[b]	Citriphage type[c]	Host of origin	Source[d]
XC59	IBBF-164	A	$CP1^R$, $CP2^S$, $CP3^R$	*Citrus aurantifolia* (Brazil)	1
XC62	6501	A	$CP1^S$, $CP2^R$, $CP3^R$	*Citrus* sp. (Japan)	2
XC63	7801	A	$CP1^R$, $CP2^S$, $CP3^R$	*Citrus* sp. (Japan)	2
XC64	B-4	B	$CP1^R$, $CP2^R$, $CP3^S$	LaMagrugada lemon (Argentina)	3
XC69	Xc-11 ("B" type-4)	B	$CP1^R$, $CP2^R$, $CP3^S$	Lemon (Argentina)	4
XC70	IBBF-512	C	$CP1^R$, $CP2^R$, $CP3^R$	*Citrus* sp. (Brazil)	1

[a] Strain designations assigned in the Fruit Laboratory, Horticultural Science Institute, Beltsville, MD 20705.
[b] CBCD = citrus bacterial canker disease. Pathotype A is the causal agent of the type A canker—Asian canker, cancrosis A, or true canker form of CBCD. Pathotype B is the causal agent of the type B canker—cancrosis B, canker B, or false canker form of CBCD. Pathotype C is the causal agent of the Mexican lime cancrosis in Brazil.
[c] Citriphages CP1 and CP2 received from M. Koizumi. Citriphage CP3 received from M. Goto. S = susceptible to lysis; R = resistant to lysis.
[d] 1 = V. Rossetti, Divisao de Patologia Vegetale, Instituto Biologico, São Paulo, Brazil. 2 = M. Koizumi, Fruit Tree Research Station, Kuchninotsu, Nagasaki, Japan. 3 = M. Goto, Shizuoka University, Shizuoka, Japan. 4 = J. W. Miller, I.N.T.A., Bella Vista, Argentina.

Table 2. Bacterial strains other than *Xanthomonas campestris* pv. *citri* used in enzyme-linked immunosorbent assays

	Received as			
Lab strain[a]	Pathovar name	Source strain no.	Host of origin	Source[b]
XCml	*campestris*	B-24		1
XF3	*fragariae*	NCPPB-2473	*Fragaria vesca*	2
XF4	*fragariae*	ICPB-102	*Fragaria x. ananassa*	3
XM1	*manihotis*	CBB-8	*Manihot* sp.	4
XM2	*manihotis*	CBB-10	*Manihot* sp.	4
XM3	*manihotis*	CBB-13	*Manihot* sp.	4
XM4	*manihotis*	CIAT-1060	*Manihot* sp. (Colombia)	4
XM5	*manihotis*	CIAT-1088	*Manihot* sp. (Africa)	4
XM6	*manihotis*	CIAT-1105	*Manihot* sp. (native Nigerian variety)	4
XP1	*pruni*	Original	*Prunus armeniaca* cv. Blenril	5
XV1	*vesicatoria*	XV24 (pepper strain)	Pepper	6
XV2	*vesicatoria*	XV26 (tomato strain)	Tomato	6
EH1	*Erwinia herbicola*	Original	Lemon	7

[a] Strain designations assigned in the Fruit Laboratory, Horticultural Science Institute, Beltsville, MD 20705.
[b] 1 = N. W. Schaad, Department of Plant Pathology, Georgia Experiment Station, Experiment 30212. 2 = National Collection of Plant Pathogenic Bacteria, Harpenden, England. 3 = International Collection of Phytopathogenic Bacteria, Davis, CA (M. P. Starr, Curator). 4 = W. E. Fry, Department of Plant Pathology, Cornell University, Ithaca, NY 14853. 5 = E. L. Civerolo, Fruit Laboratory, Horticultural Science Institute, Beltsville, MD 20705. 6 = M. Sasser, Department of Plant Science, University of Delaware, Newark 19711. 7 = Isolation from lemon leaf naturally infected with *X. campestris* pv. *citri*. This leaf was provided by R. Stall and designated as being affected with the cancrosis B form of CBCD.

RESULTS

Bacteriophage reactions. Generally, pathotype A strains were susceptible to phages CP1 and CP2, and pathotype B strains were susceptible to phage CP3. Pathotype C strain was not lysed by any of these phages (Table 1).

Agglutination tests. In several tests, cross-agglutination reactions of the six *X. c.* pv. *citri* strains in homologous and

heterologous antisera were variable. Only two broad serological groupings of the *X. c.* pv. *citri* strains were apparent (Table 3). However, only a limited number of pathotype B and C strains were available. In general, strong agglutination of cells of pathotype A, B, and C strains occurred in the homologous antisera. In addition, there were strong cross-agglutination reactions between strains XC64 (pathotype B) and XC70 (pathotype C) in the heterologous antisera.

Strain XC69 (pathotype B) was not strongly agglutinated in any of the heterologous antisera. Weak agglutination occurred only in the anti-pathotype B (strain XC64) and anti-pathotype C (strain XC70) sera. Weak cross-agglutination reactions also occurred with cells of strains XC62 and XC70 in the heterologous antisera. Very weak agglutination of strain XC64 cells occurred in anti-XC62 serum, but no apparent agglutination of strain XC62 cells occurred in anti-XC64 serum. Strain EH1 was not agglutinated in any of the *X. c.* pv. *citri* antisera, but it was strongly agglutinated in the homologous antiserum.

In Ouchterlony agar gel double diffusion tests using the unfractionated anti-XC62 (pathotype A) serum, reactions occurred only with antigens extracted by mild acid (0.03 *N* acetic acid) and heat (100 C for 45 min) from pathotype A strains of *X. c.* pv. *citri* from Brazil and New Zealand, but not with similarly extracted antigens from a pathotype C strain of *X. c.* pv. *citri* from Brazil (A. P. C. Alba, Instituto Biologico, São Paulo, Brazil, *personal communication*).

ELISA. *X. c.* pv. *citri* was detected by ELISA in both heated (100 C for 30 min) and unheated cell suspensions. A_{405} values of heated suspensions were generally 1.2 to about 4 times higher than those of unheated preparations containing approximately 10^2–10^6 *X. c.* pv. *citri* colony-forming units per milliliter.

The sensitivity of ELISA to detect *X. c.* pv. *citri* was increased approximately tenfold by heating the antigen preparations. In general, approximately 10^4 CFU/ml were detected routinely by ELISA when the antigen preparations were heated. Occasionally, *X. c.* pv. *citri* was detected in heated cell suspensions containing 10^2–10^3 CFU/ml. In contrast, *X. c.* pv. *citri* was positively detected only in unheated antigen preparations containing more than approximately 10^4 CFU/ml. In unheated preparations containing less than 10^4 CFU/ml, positive reactions occurred only when the concentration of coating Ig was increased.

Sensitivity. In comparative ELISA tests using anti-XC62 and anti-XC70 sera, positive reactions with pure culture cell suspensions of the homologous strains occurred when cell suspensions contained 10^4–10^5 CFU/ml (Fig. 1). In two tests, the A_{405} values for 2.4×10^4 and 1.52×10^4 XC62 colony-forming units per milliliter were 0.08 and 0.33, respectively, compared with 0.03 and 0.20 for the PBS-Tween controls. The same general results were obtained in seven additional tests with XC62 only and in one test with XC62, XC63, and XC59. In one test, the A_{405} value for pure culture XC70 at approximately 3.2–6.4 CFU/ml using anti-XC70 serum was 0.27 compared with 0.16 for the PBS-Tween control.

Specificity. In general, the specificity of ELISA to detect strains of *X. c.* pv. *citri* pathotypes was similar to that in agglutination tests (Table 4). Strong positive reactions occurred in ELISA tests using anti-XC62 serum with pure culture cells of pathotype A strains XC62 and XC63 from Japan and XC59 from Brazil. Specific reactions of the heterologous pathotype A strains in ELISA using anti-XC62 serum were not affected by increasing the concentration of coating Ig protein twofold or by heating the antigen preparations at 100 C for 30 min.

No significant reactions occurred with cells of the B and C pathotypes using the anti-pathotype A (strain XC62) serum. Similarly, no positive reactions occurred

Table 3. Cross-agglutination reactions of six *Xanthomonas campestris* pv. *citri* strains and an *Erwinia herbicola* strain isolated from *Citrus* species

Strain	Agglutination titer in antiserum against[a]			
	XC62	XC64	XC70	EH1
XC59	1,280	–	–	–
XC62	1,280	–	(80)	–
XC63	1,280	–	–	–
XC64	(20)	1,280	640	–
XC69	–	(40)	(80)	–
XC70	(80)	640	1,280	–
EH1	–	–	–	1,280

[a] Each number is the reciprocal of the highest dilution in which distinct agglutination of cells occurred. The numbers in parentheses are for weak reactions. Minus sign indicates no apparent agglutination of cells in 1/20 dilution of antiserum.

Table 4. Absorbance values for enzyme-linked immunosorbent assay of six *Xanthomonas campestris* pv. *citri* strains

Strain[a]	A_{405}[b] when using antiserum to	
	XC62	XC70
XC59	2.75 ± 1.76	
XC62	2.50 ± 1.12	0.19 ± 0.16
XC63	2.80 ± 1.70	
XC64	0.17 ± 0.22	0.96 ± 0.90
XC69		1.01 ± 0.86
XC70	0.08 ± 0.03	1.10 ± 0.47
PBS-Tween	0.10 ± 0.07	0.13 ± 0.11

[a] Suspensions of cells from overnight shake cultures of nutrient broth supplemented with glucose and sodium chloride at 28–29 C were adjusted in PBS-Tween to contain about 10^6 CFU/ml. Cell suspensions were heated in boiling water bath (100 C) for 30 min.

[b] Values for the combined contents of three wells in two (XC59, XC63), five (XC70), six (XC64), or eight (XC62, PBS-Tween) tests using anti-XC62 serum and in three tests (XC62, XC64, XC69, XC70, PBS-Tween) using anti-XC70 serum.

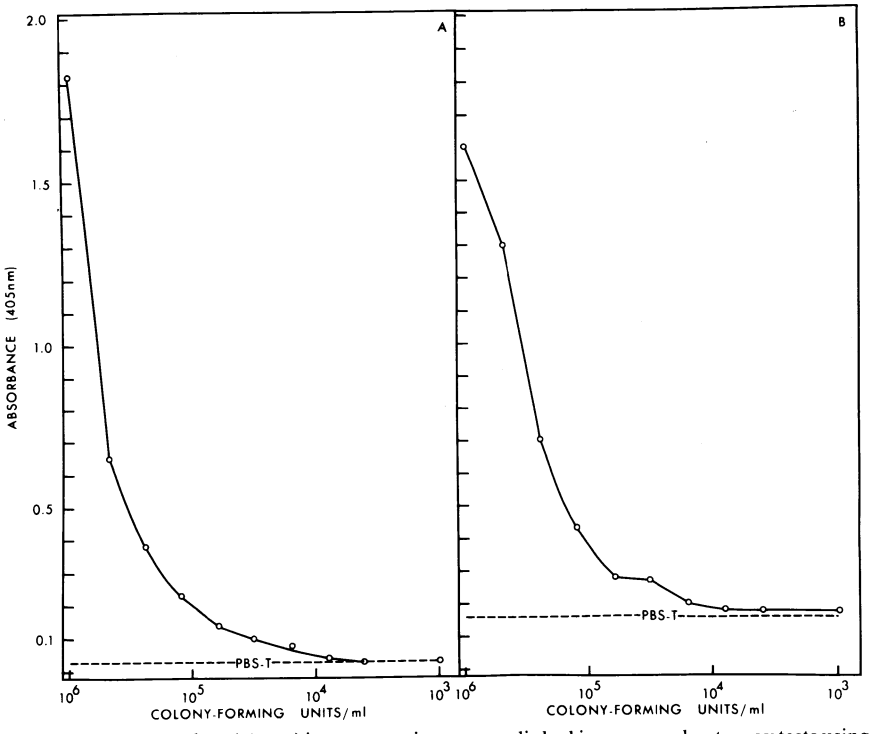

Fig. 1. Absorbance values (A_{405nm}) in comparative enzyme-linked immunosorbent assay tests using antisera prepared against intact, live cells of *Xanthomonas campestris* pv. *citri* and various concentrations of homologous *X. c.* pv. *citri* antigen. Each point is the value obtained for the combined contents of six wells. PBS-T = phosphate-buffered saline plus Tween 20. **(A)** Plate coated with XC62 immunoglobulin at 8 μg/ml. Enzyme-conjugate XC62 used at 1/25 dilution. **(B)** Plate coated with XC70 immunoglobulin at 19 μg/ml. Enzyme-conjugate XC70 used at 1/10 dilution.

when cells of the pathotype A strain (XC62) were tested against anti-pathotype B (strain XC64) and anti-pathotype C (strain XC70) sera. In contrast, positive reactions occurred with pathotype B (strains XC64 and XC69) cells using anti-pathotype C (strain XC70) serum. However, in some comparative ELISA tests, the A_{405} values for the homologous pathotype C strain-antiserum combinations were 1.7–2.2 and 1.5–1.8 times higher than those of pathotype B strains XC64 and XC69, respectively, using anti-pathotype C (strain XC70) serum.

Because of the previously reported serological relationship between the pathotype A strains of *X. c.* pv. *citri* and *X. c.* pv. *manihotis* strains (2), the comparative reactions of these bacteria in ELISA tests using anti-XC62 serum were determined. Positive reactions occurred with five of six *X. c.* pv. *manihotis* strains when tested against anti-XC62 (pathotype A) serum. The A_{405} values for these five *X. c.* pv. *manihotis* strains ranged from about 0.3 to 0.5 times that of the homologous (*X. c.* pv. *citri*) strain XC62 when cell suspensions containing approximately 10^5 or 10^6 CFU/ml were used. When anti-XC70 serum was used, there were no positive reactions with cells of any of the six *X. c.* pv. *manihotis* strains in ELISA.

With various homologous and heterologous strain-antiserum combinations, a weak positive reaction occurred only with *X. c.* pv. *campestris* (strain B-24) at $10^5 \times 10^6$ CFU/ml and anti-XC62 serum. In two comparative tests, the average A_{405} values for *X. c.* pv. *citri* (strain XC62) and *X. c.* pv. *campestris* (strain B-24) were 1.54 ± 8 and 0.33 ± 0.30, respectively. No positive reactions occurred with *X. c.* pv. *campestris* (strain B-24) and anti-XC70 serum. No positive reactions occurred with two strains of *X. fragariae*, one strain of *X. c.* pv. *pruni*, two strains of *X. c.* pv. *vesicatoria*, and one strain of *E. herbicola* using anti-XC62 and anti-XC70 sera.

Mixed populations. Each of several strains at approximately 10^6 CFU/ml was mixed separately with strain XC62 at 10^6 CFU/ml. Each mixture was then assayed by ELISA using anti-XC62 serum. There was no significant absorbance at 405 nm with antigen preparations from cell suspensions of heterologous strains containing 0.5 or 1×10^6 CFU/ml. Extinction values with XC62 antigen preparations were 1.33 and 0.54 for about 1×10^6 and 0.5×10^6 CFU/ml, respectively. When the homologous strain (XC62) was mixed with an equal number of cells of strains XC64, XC69, XC70, and EHl, the A_{405} values were 0.50, 0.49, 0.46, and 0.51, respectively.

Detection of *X. c.* pv. *citri* in tissue extracts. *X. c.* pv. *citri* was also detected by ELISA after addition to PBS-Tween extracts of healthy, greenhouse-grown Duncan grapefruit seedling leaves. In general, the A_{405} values for unheated citrus leaf extracts were more variable and lower than those for heated extracts. The A_{405} values of heated citrus leaf extracts containing *X. c.* pv. *citri* were generally the same as for similar concentrations of *X. c.* pv. *citri* cells in PBS-Tween. In three tests, the average A_{405} values of preparations containing approximately 10^3, 10^4, 10^5, and 10^6 CFU/ml, respectively, were 0.21 ± 0.12, 0.26 ± 0.15, 0.92 ± 0.63, and 1.60 ± 0.69 for XC62 cells in PBS-Tween; the values were 0.24 ± 0.17, 0.34 ± 0.17, 0.75 ± 0.30, and 1.77 ± 0.39 for XC62 cells in citrus leaf extracts. In the same tests, the average A_{405} value was 0.16 ± 0.08 for healthy citrus leaf extracts. In two of these tests, the average A_{405} value for healthy citrus leaf extracts containing XF3 at 10^6 CFU/ml was 0.12 ± 0.05. In the third test, the A_{405} values for citrus leaf extracts containing XC64 or XC70 at 10^6 CFU/ml were 0.12 and 0.13, respectively.

The sensitivity of ELISA to detect *X. c.* pv. *citri* in heated preparations containing cells added to citrus leaf extracts, as from pure culture, was about 10^4 to 10^5 CFU/ml. The minimum specific detection level of *X. c.* pv. *citri* in unheated preparations was variable but appeared to be greater than 10^5 CFU/ml. Variable nonspecific reactions, presumably caused by normal host components in different healthy citrus leaf extracts, precluded conclusive determination of the minimum specific detection level of *X. c.* pv. *citri* in unheated preparations.

No reactions developed in ELISA with anti-XC62 serum when citrus leaf extracts containing the heterologous, serologically distinct strains XC64 and XC70 at 10^6 CFU/ml were tested.

ELISA with anti-XC62 serum was also used to detect *X. c.* pv. *citri* in lesions on Duncan grapefruit seedling leaves artificially inoculated by leaf injection-infiltration with strain XC62 (Table 5). The A_{405} values for various concentrations of the lesion extract were two to 25 times higher than those for the healthy citrus leaf tissue extract. Positive detection of *X. c.* pv. *citri* was possible with the lesion extract diluted to 10^{-4}. The *X. c.* pv. *citri*-specific A_{405} value at this sample dilution was equivalent to *X. c.* pv. *citri* at about 10^4–10^5 CFU/ml of extract sample, as determined by comparison with the reactions obtained at the same time with various concentrations of XC62 cells from pure culture. This is based on the assumption that the A_{405} difference between similarly diluted extracts of healthy leaf tissue and lesions is specific for *X. c.* pv. *citri*. This is equivalent to an average of 2×10^3 to 2×10^4 CFU/lesion. This is consistent with the minimum concentration of cells detected by ELISA with pure culture cells suspended in PBS-Tween or added to healthy citrus leaf tissue extracts. In another test, the A_{405} values for undiluted, 10^{-1} diluted, and 10^{-2} diluted extracts of lesions were 1.22, 1.12, and 0.72; the values for healthy citrus leaf tissue extracts were 0.15, 0.13, and 0.14, respectively. The A_{405} of the PBS-Tween control was 0.12.

Table 5. Double antibody sandwich enzyme-linked immunosorbent assay using anti-XC62 serum to detect *Xanthomonas campestris* pv. *citri* in CBCD-A lesions on artificially inoculated Duncan grapefruit seedling leaves

Sample[a] Dilution	A_{405} CBCD-A lesions extract (A)	A_{405} Healthy leaf tissue extract (B)	(A)/(B)
Undiluted	3.67	0.81	4.53
10^{-1}	3.46	0.34	10.18
10^{-2}	2.80	0.11	25.45
10^{-3}	0.84	0.06	14.00
10^{-4}	0.67	0.09	7.44
10^{-5}	0.13	0.06	2.17
PBS-Tween		0.04	

[a] Ten 7-mm-diameter leaf disks (0.5–0.6 g) were excised from healthy or XC62-inoculated Duncan grapefruit seedling leaves and triturated in 2 ml of PBS-Tween. From artificially inoculated leaves, each leaf disk contained a single lesion. The leaves were not surface disinfested. After heating 1 ml of homogenate, serial tenfold dilutions were made in PBS-Tween. Each A_{405} value was obtained for the combined contents of three wells.

DISCUSSION

Based on the results of these tests collectively, the double antibody sandwich ELISA can be used to detect and identify strains of *X. c.* pv. *citri*. Preparations containing 10^4–10^5 CFU/ml were routinely detected by ELISA. The sensitivity of ELISA in these tests was not as high as that reported for detecting *X. c.* pv. *citri* based on pathogenicity and phage tests (10,11,16,22). However, the reportedly higher sensitivity of pathogenicity tests depends upon enrichment of samples with nutrients or large numbers of inoculations and upon extended periods of incubation. For detecting *X. c.* pv. *citri* at 10^4 to 10^5 CFU/ml, ELISA is more rapid than pathogenicity tests.

X. c. pv. *citri* was detected in both heated and unheated cell suspensions of leaf tissue extracts. The *X. c.* pv. *citri*-specific reactions of heated preparations were generally greater than those of unheated preparations. This may be

caused by the release of heat-stable antigens released from the killed cells (15). In addition, nonspecific reactions, apparently from normal citrus tissue components, were reduced with heated preparations.

The coating preparations were obtained by three successive precipitations with ammonium sulfate and are heterogeneous Ig mixtures. The sensitivity of ELISA for detection and identification of *X. c.* pv. *citri* may be increased by further purification of the Ig fraction containing antibody specific to *X. c.* pv. *citri.*

Alternatively, more specific antisera with higher titers might be produced with different *X. c.* pv. *citri* immunogens. In addition, use of additives such as polyvinylpyrrolidone or protein (ovalbumin or bovine serum albumin) in the PBS-Tween plant tissue extraction medium might reduce nonspecific reactions caused by the presence of normal plant components (23). Alternatively, adaptation or modification of other forms of ELISA (24) might be more sensitive.

The sensitivity of ELISA for *X. c.* pv. *citri* detection here is about the same as that reported for other phytopathogenic bacteria (1,8,15,21,23,25). ELISA tests using these antisera were generally specific for *X. c.* pv. *citri.* Six strains of four other phytopathogenic xanthomonads and of one saprophytic strain of *E. herbicola* isolated from a lemon leaf lesion did not react in ELISA tests using antisera against strains XC62 and XC70. The presence of serologically unrelated strains in mixtures with strain XC62 did not affect the specific reaction in ELISA using anti-XC62 serum. Strain EH1, a saprophytic species that commonly occurs in CBCD lesions (12), did not react in agglutination or ELISA tests with either *X. c.* pv. *citri* antiserum used here.

Five of six *X. c.* pv. *manihotis* strains reacted in ELISA tests using anti-XC62 serum, but not when anti-XC70 serum was used. This is consistent with a previous report (2) that pathotype A strains of *X. c.* pv. *citri* are serologically related to *X. c.* pv. *manihotis* based on results in Ouchterlony agar gel double diffusion tests. However, in these ELISA using anti-XC62 serum, the reactions of heterologous *X. c.* pv. *manihotis* strains were quantitatively less than the reactions with the homologous XC62 and heterologous XC59 and XC63 pathotype A strains. In indirect hemagglutination tests, sheep red blood cells sensitized with *X. c.* pv. *citri* (strain IB-30) exopolysaccharide extracts were agglutinated by anti-*X. c.* pv. *manihotis* (strain ENA-975) serum (28). However, no agglutination of sheep red blood cells sensitized with *X. c.* pv. *manihotis* exopolysaccharide occurred in anti-*X. c.* pv. *citri* serum (28).

The apparent weak reaction of *X. c.* pv. *campestris* (strain B-24) in ELISA with anti-XC62 serum and the lack of an apparent reaction with anti-XC70 serum are not consistent with a previous report (2) that a pathotype C variant of *X. c.* pv. *citri* (culture IBBF-503) is serologically related to *X. c.* pv. *campestris* (culture IBSP-134) but is serologically unrelated to pathotype A variants (cultures IBSP-132, IBBF-160, IBBF-140, IBBF-501, IBSP-130). These results were based on Ouchterlony double diffusion tests in agar gels with antigens extracted by heating for 45 min in 0.03 *N* acetic acid (2).

In general, the ELISA reactions in these tests were similar to the cross-agglutination reactions in precipitin tests. Strains XC59, XC62, and XC63, representing the pathotype A form of *X. c.* pv. *citri*, formed a distinct ELISA reaction group with anti-XC62 serum. These were readily distinguished from strains XC64, XC69 (pathotype B variants), and XC70 (pathotype C variant). Strains XC64, XC69, and XC70 did not react in ELISA using anti-XC62 serum. Similarly, strains XC64, XC69, and XC70 formed a separate ELISA reaction group with anti-XC70 serum. Strain XC62 did not react in ELISA tests with anti-XC70 serum. These results are also consistent with a previous report (2) that pathotype A strains of *X. c.* pv. *citri* are serologically distinct from a pathotype C strain.

ELISA is potentially useful for the rapid diagnosis of *X. c.* pv. *citri* in infected citrus tissue. Preliminary diagnosis based on ELISA can be confirmed by subsequent pathogenicity tests. In addition, ELISA tests may be useful in epidemiologic studies for the rapid detection of *X. c.* pv. *citri* associated with symptomless tissue and with alternate host plants. In preliminary tests using anti-XC62 serum, *X. c.* pv. *citri* antigens were readily and quantitatively detected in aqueous soil suspensions to which XC62 cells were added. Serologically distinct *X. c.* pv. *citri* pathotypes may be identified by ELISA techniques.

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