Dot-Immunobinding Assay for Detecting *Xanthomonas campestris* pv. *holcicola* in Sorghum

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ABSTRACT

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The application of a dot-immunobinding assay (DIA) for detecting *Xanthomonas campestris* pv. *holcicola* (*X. c.* pv. *holcicola*), the bacterial streak pathogen of sorghum (*Sorghum bicolor*), is described. Leaf sections with symptoms were soaked in sterile distilled water, and the soaking solution containing the bacteria was spotted onto nitrocellulose membranes. Membranes containing the absorbed antigens were incubated in antiserum to glutaraldehyde-fixed cells of *X. c.* pv. *holcicola* and then in protein A-alkaline phosphatase conjugate. To visualize the antigen-antibody-protein A complexes, membranes were exposed to naphthol AS-MX phosphate substrate with fast-violet B stain; a violet precipitate indicated a positive response. The assay was rapid (about 4 hr) and sensitive, i.e., 400 bacterial cells per 4-μl sample could be detected (equivalent to 10^5 cfu/ml). Of 101 plant samples assayed, 46 of 48 known to be infected with *X. c.* pv. *holcicola* were detected by the DIA.

Pathogenic xanthomonads in plant tissue are typically detected by reisolation and identification of the bacteria using physiological and/or biochemical tests or by serological techniques. Immunofluorescent staining (8,15,18,19) and enzyme-linked immunosorbent assays (ELISA) (2,5) are normally rapid, sensitive, and specific. ELISA is desirable because of its simplicity; however, bacterial applications are limited by steric problems (i.e., bacteria are easily dislodged from plates during washing). Our objective was to adapt a simple, rapid, reliable, and inexpensive assay to detect the bacterial leaf blight pathogen, *X. campestris* pv. *holcicola* (*X. c.* pv. *holcicola*) of sorghum (*Sorghum bicolor* (L.) Moench) (10) in field surveys.

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Dot-immunobinding assays (DIA), which rely on the protein-binding properties of nitrocellulose membrane filters (13), have been used successfully for detecting viral antigens in plant tissues (3,11). Recently, a DIA was described for detecting *X. campestris* pv. *phaseoli* from dry bean seeds (14). The assay was specific, but its usefulness in detecting bacteria directly from bean seed extracts was limited by physical interference of bean constituents on the membranes. We describe a simple adaptation of the DIA for detecting *X. c.* pv. *holcicola* directly from sorghum leaves.

MATERIALS AND METHODS

Bacterial cultures. Bacterial strains were maintained at 28 C on yeast-extract, dextrose, calcium carbonate agar medium (YDCA [9]) or peptone-sucrose agar (PSA [21]). Bacteria were lyophilized for long-term storage. Sources of bacterial strains are described in Table 1.

Antiserum production. Bacteria (*X. c.* pv. *holcicola* strain ATCC 13461) were grown on YDCA for 96 hr at 28 C and washed with sterile 10 mM potassium phosphate-buffered saline (0.85% NaCl, pH 7.2) (PBS) by centrifuging three times at 17,000 *g* for 3 min. Cells were resuspended in PBS and fixed by dialyzing against a 2% glutaraldehyde solution for 3 hr at room temperature. The fixed cells were then dialyzed three times against 1 L of PBS to remove the glutaraldehyde. Glutaraldehyde-fixed bacteria (0.5 ml of 2×10^9 cfu/ml) were emulsified with 0.5 ml of Freund's incomplete adjuvant (Difco, Detroit, MI) and used to inject a New Zealand white rabbit. The injection schedule consisted of seven to 10 subcutaneous injections (0.1 ml each) on the back of each rabbit. Weekly test bleedings began after the fourth injection, and reactivity was tested by agglutination assays (4) and ELISA (6). The antiserum was stored at −20 C.

The antiserum was cross-absorbed with 1) *Pseudomonas syringae* pv. *syringae* (strain B359) and *P. andropogonis* (strain 74) to eliminate cross-reactivity with other bacterial pathogens of sorghum, 2) *Erwinia stewartii* (strain 369) to eliminate cross-reactions with members of this genus, and 3) *X. c.* pv. *oryzae* (strain PX040) to reduce reactions with other xanthomonads. One loopful of each bacterial strain (grown 3–5 days on PSA) was added to a 1.5-ml microfuge tube containing 1 ml of antiserum. After 2–4 hr at room temperature and 12 hr at 4 C, the mixture was centrifuged for 1 min at 13,000 *g*. The supernatant was transferred to a fresh tube, and the absorption procedure was repeated six times. Specificity of the antiserum was determined by DIA.

Preparation of bacterial antigens and tissue samples for DIA. Bacterial cells from 3-day-old PSA cultures were suspended and washed with distilled water three times by centrifugation (13,000 *g*) and adjusted to 10^8 cfu/ml (25 Klett units). Two techniques to extract bacterial antigens from lesions in sorghum leaf tissues were tested: 1) 1-cm^2 tissue samples were ground in 1 ml of distilled water with a mortar and pestle,

centrifuged for 10 sec at 13,000 *g*, and the supernatant collected for use in the assay; and 2) 1-cm^2 tissue sections were soaked in 1 ml of distilled water for 2, 4, 6, and 24 hr at room temperature to allow elution of the bacteria. The soaking solution was then used in the DIA.

Assay. Various procedures for DIA (7,12,16,20) were tested for speed and simplicity. The procedure selected for optimal detection of *X. c.* pv. *holcicola* from sorghum leaves was a modification of that reported by Jahn et al (12). Ten parameters were examined in detail:

1. Brands and types of filter paper: 0.2 and 0.47 μm nitrocellulose from Schleicher and Schuell Inc. (Keene, NH), and Millipore (Bedford, MA); nos. 1 and 4 filter papers from Whatman (Clifton, NJ).
2. Antigen fixation procedures: air-drying, heating to 80 C under vacuum, or acid/ethanol fixation.
3. Buffered salines: Tris-HCl or phosphate.
4. Blocking with 3% gelatin or 5% bovine serum albumin vs. no blocking.
5. Detergents: Triton X-100 or Tween 20.
6. Enzyme system: alkaline phosphatase- or peroxidase-labeled protein A.
7. Incubation times and temperatures for antibody and protein A-enzyme conjugate binding: 1, 2, 3, 4, 8, 12, and 24 hr at room temperature or 4 C.
8. Dilution of antiserum: 10-fold serial dilutions from 10^2 to 10^4, then 1:20,000, 1:40,000, 1:60,000, and 1:80,000.
9. Concentration of conjugate: 99, 33, and 11 ng/ml.
10. Reuse of antiserum solutions.

The most effective assay was as follows: Grids (squares 1 × 1 cm) were drawn on nitrocellulose membranes (5 × 3 cm, 0.2 μm, BA83; Schleicher and Schuell) with a ballpoint pen. All manipulations were done at room temperature unless otherwise specified. Membranes were rinsed in distilled water for 5 min and air-dried immediately before use. Samples (4 μl) of bacterial suspensions or the soaking solution from leaf sections were spotted onto the nitrocellulose strips and air-dried. Serial 10-fold dilutions of washed *X. c.* pv. *holcicola* cells (10^8–10^5 cfu/ml) and a 4-μl water sample were spotted on each strip as controls. Antigens were fixed to membranes by soaking in a 10% acetic acid and 25% ethanol solution for 15 min. After a brief rinse in distilled water, membranes were washed extensively in TBS-T100 (Tris-buffered saline [50 mM Tris-HCl, pH 7.4; 0.2 M NaCl] containing 0.1% Triton X-100). The membranes were then incubated for 2 hr at room temperature or overnight at 4 C in anti-*X. c.* pv. *holcicola* antiserum (diluted 1:60,000 in TBS-T100). After rinsing four times in TBS-T100 for 5 min, the strips were incubated for 2 hr in protein A-alkaline phosphatase conjugate (33 ng/ml, Sigma, St. Louis, MO) diluted in TBS-T100. The nitrocellulose membranes were finally rinsed four times in TBS-T100 followed by two rinses in Tris-buffered saline prior to addition of substrate.

Substrate was prepared immediately before use by dissolving 12 mg of fast-violet B salt (Sigma) in 2 ml of Napthol AS-MX phosphate alkaline solution (Sigma) and 48 ml of distilled water. The strips were incubated in substrate solution for 30 min in the dark. The enzymatic reaction was terminated by rinsing the strips in tap water for 5–10 min. The strips were air-dried for permanent storage. Reactions were visually assessed; violet color indicated the presence of bacterial antigens.

Storage. Nitrocellulose strips were spotted with 4-μl samples of bacterial cells and either fixed in acetic acid/ethanol or not fixed before storage in small cardboard boxes at 23, 4, or −20 C. The strips were processed biweekly to determine the effect of storage on detection of antigens by DIA.

Assay specificity and sensitivity. The specificity and sensitivity of the assay were determined using 10-fold serial dilutions of washed cells of *X. c.* pv. *holcicola* (10^8–10^0 cfu/ml), various *X. campestris* pathovars (10^8 cfu/ml), and other bacterial genera (10^8 cfu/ml) listed in Table 1. To compare the sensitivity of *X. c.* pv. *holcicola* detection by DIA with detection by reisolation from infected plant material, 1-cm^2 leaf sections were surface-sterilized in 20% (v/v) aqueous sodium hypochlorite (1.05% a.i.) solution for 1 min followed by two 1-min rinses in sterile distilled water and then placed in 1 ml of sterile distilled water. After 2, 4, 6, and 24 hr, 10-fold serial dilutions of the soak solution were plated on PSA containing cyclohexamide (75 mg/L). After incubation for 2–3 days at 28 C, *X. c.* pv. *holcicola* colonies were counted to determine the colony-forming units per

Table 1. Reactions of bacterial strains with antiserum prepared to *Xanthomonas campestris* pv. *holcicola* strain ATCC 13461 in the dot-immunobinding assay (DIA)

Bacteria tested	Strain (source)[a]	Strains showing DIA reaction (no.)	
		Negative	Positive
X. campestris			
pv. *alfalfae*	KX-1(2)	0	1
pv. *campestris*	KXccl(2), 528(6)	0	2
pv. *holcicola*	13461(1), 66(2), 86(2), 86S(2), 93(2), 415(2), TX-1(2), XH112(4), 3103(7), NEXH(11)	0	10
pv. *oryzae*	PX040(5), PX061(5), PX086(5), PX078(5)	0	4
pv. *oryzicola*	B910(8)	0	1
pv. *phaseoli*	1138(6), DRS-103(11), SC-4A(11)	0	3
pv. *translucens*	10771(1), 9000(1), KXctl(2)	0	3
pv. *vesicatoria*	75-3(10)	0	1
X. fragariae	10056(7)	0	1
Agrobacterium tumefaciens	A136(2)	1	0
Corynebacterium nebraskense	74-1(2)	1	0
Erwinia amylovora	EaP66(3), Ea303(3)	2	0
E. carotovora			
subsp. *atroseptica*	Ecal2(2)	1	0
subsp. *carotovora*	Ecc193(2)	1	0
subsp. *chrysanthemi*	Ec16(2)	1	0
E. herbicola	112(2)	1	0
E. stewartii	369(2)	1	0
Escherichia coli	HB101(2)	1	0
Pseudomonas andropogonis	23061(1), KPA1(2), KPA2(2), 74(2), PA133(4), PS186(4)	6	0
P. alboprecipitans	19860(1), PA134(4)	2	0
P. rubrilineans	19307(1), W6(2)	2	0
P. rubrisubalbicans	19308(1)	1	0
P. solanacearum	K60(9), B1(9)	2	0
P. syringae			
pv. *coronafaciens*	C163(2)	1	0
pv. *syringae*	B359(2), 7(2)	2	0
Unidentified yellow-leaf epiphyte of greenhouse sorghum	Kys(2)	1	0
Total strains tested		53	

[a] 1 = American Type Culture Collection, Rockville, MD; 2 = authors; 3 = E. Billing, East Malling Research Station, East Malling, England; 4 = International Collection of Phytopathogenic Bacteria, University of California, Davis; 5 = T. Mew, International Rice Research Institute, Philippines; 6 = National Collection of Plant Pathogenic Bacteria, Hertfordshire, England; 7 = Plant Diseases Division Culture Collection, Auckland, New Zealand; 8 = N. Schaad, University of Idaho, Moscow; 9 = L. Sequeira, University of Wisconsin, Madison; 10 = R. Stall, University of Florida, Gainesville; and 11 = A. Vidaver, University of Nebraska, Lincoln.

milliliter of soaking solution. Alternatively, a loopful of the soaking solution was streaked on YDCA medium for isolation of bacteria.

To assess the predictive value of the DIA, stems of 30-day-old seedlings of two sorghum inbreds (Tx433 and B35-6, obtained from D. T. Rosenow, Texas Agricultural Experiment Station, Lubbock) were injected above the third leaf with 0.5 ml of a bacterial suspension (10^9 cfu/ml in distilled water), using a syringe fitted with a 27-gauge needle. Five seedlings of each of the two inbreds were inoculated with each bacterial strain or water. Four strains of *X. c.* pv. *holcicola* and one strain each of *P. andropogonis* and *P. s.* pv. *syringae* were used. Inoculated plants were maintained in a greenhouse (22–28 C) with intermittent misting (6 min/hr for 12 hr/day). Symptoms were assessed 14 days after inoculation. One leaf from each plant was sampled for reisolation and for the DIA. Leaves were examined individually for infection with *X. c.* pv. *holcicola*, other pathogens (*P. andropogonis* and *P. s.* pv. *syringae*), or no infection (water-inoculated controls). The DIA was evaluated for its ability to distinguish the *X. c.* pv. *holcicola*-infected samples (positives) from healthy samples or those infected with other pathogens (negatives).

Field samples were collected from various locations in Kansas and Nebraska. Leaf samples (1 cm^2) were processed as described. The samples were examined individually as infected with *X. c.* pv. *holcicola* (based on reisolation of bright yellow colonies on YDCA and positive pathogenicity tests), infected with other bacterial pathogens (colony colors other than bright yellow on YDCA and symptoms), or healthy (leaf tissue from symptomless plants).

RESULTS AND DISCUSSION

DIA. Although we report the use of a specific membrane brand, other brands of nitrocellulose membranes work equally well. Nitrocellulose membranes with pore sizes of 0.2 μm gave more intense color readings than did filters of 0.47 μm. Reactions performed on Whatman Nos. 1 and 4 filter papers were more diffuse but were similar in intensity to those reactions performed on 0.47-μm nitrocellulose; however, the Whatman papers were more fragile during the manipulations of the assay. The Whatman papers may be useful where cost is a factor. Bacterial antigens did not adhere to the membranes throughout the assay unless they were either chemically or heat fixed; for simplicity, the membranes were fixed in acetic acid/ethanol. PBS can be used in place of TBS. Binding of the antibody and protein A-conjugates was essentially complete after 1 hr of incubation at room temperature. For convenience, incubation in antiserum was done overnight at 4 C and in the protein A-conjugates for 2 hr at room temperature. Antiserum solutions were reused as many as 12 times over a 2-wk period; however, the number of times for reuse depends on each antiserum and the concentrations used.

Other reports have indicated that a "blocking" solution was needed to saturate unoccupied binding sites on the nitrocellulose membrane after antigens were bound or fixed to membranes and before treatment with the antiserum (3,11,12,14,16,20). We and others (7) have found that adding a nonionic detergent such as Triton X-100 or Tween 20 was sufficient to prevent binding of proteins to unsaturated sites.

Of the two protein A-enzyme conjugate systems tested, alkaline phosphatase-labeled protein A was the most useful. Protein A-horseradish peroxidase conjugate was not acceptable for use because of high background staining, presumably from host-plant peroxidase activity. Membranes containing bacteria either soaked in fixative or not before storage at 23, 4, or −20 C were optimally reactive with *X. c.* pv. *holcicola* serum for up to 98 days. Pappas et al (17) reported that human visceral leishmaniasis antigens were optimally reactive with *Leishmania* serum when stored at −20 C for up to 270 days; storage at 4 and 23 C showed reproducible titer decreases at 60 days.

Plant-sampling technique. Grinding the infected plant tissue to release bacteria was less satisfactory than soaking because plant proteins saturated membrane binding sites, and also, plant pigments stained the membrane, making accurate detection difficult. Soaking plant tissue in water for 2 hr was usually sufficient for detecting *X. c.* pv. *holcicola*; however, if infection levels were low (i.e., less than 10^4 bacteria leached out of the tissues), longer soaking times were necessary (Table 2). We therefore sampled at 2 and at 24 hr to ensure detection.

Assay specificity and sensitivity. Specificity of the antiserum was enhanced by extensive cross-absorption with *P. s.* pv. *syringae, P. andropogonis, E. stewartii,* and *X. c.* pv. *oryzae*. At an antiserum dilution of 1:60,000, cross-reaction was only seen with other xanthomonads (Table 1, Fig. 1). At antiserum dilutions less than 1:10,000, slight cross-reactions with pseudomonads occurred. Adsorption with xanthomonads other than *X. c.* pv. *oryzae* was not attempted.

To further evaluate the specificity of the antiserum, leaf samples from

Table 2. Comparison of bacterial numbers and the dot-immunobinding assay (DIA) reactions from soaking solutions of *Xanthomonas campestris* pv. *holcicola*-infected sorghum leaf tissue samples

	Soaking time (hr)							
	2		4		6		24	
Sample[a]	cfu/ml[b]	DIA[c]	cfu/ml	DIA	cfu/ml	DIA	cfu/ml	DIA
1	5.4×10^6	+++	7.4×10^6	+++	9.2×10^6	+++	$>10^{10}$	++++
2	2.0×10^5	+++	6.6×10^5	+++	1.3×10^6	+++	1.9×10^8	++++
3	7.0×10^3	−	8.5×10^3	+	1.2×10^4	+	5.1×10^6	++
4	2.5×10^3	+	1.5×10^3	+	3.1×10^3	+	1.2×10^7	++
5	1.0×10^6	+++	1.8×10^6	+++	4.2×10^6	+++	2.4×10^6	++++
6	1.9×10^6	+++	6.1×10^6	+++	5.5×10^6	+++	6.1×10^6	++++
7	5.7×10^6	+++	6.6×10^6	+++	ND[d]	+++	6.8×10^5	++++
8	0	−	0	−	0	−	0	−
9	0	−	0	−	0	−	0	−

[a] Tissues from sorghum plants inoculated with *X. c.* pv. *holcicola* as described in Materials and Methods.

[b] Each leaf sample (1 cm^2) was surface-sterilized and suspended in 1 ml of sterile distilled water for 2, 4, 6, and 24 hr. Soaking solutions were serially diluted 10-fold in microtiter dishes, and 20-μl aliquots of 10^{-1} to 10^{-9} dilutions were spotted in duplicate on PSA medium containing 75 mg/ml cycloheximide.

[c] DIA results rated as (−) for no reaction through (++++) for an intense color reaction.

[d] Not determined.

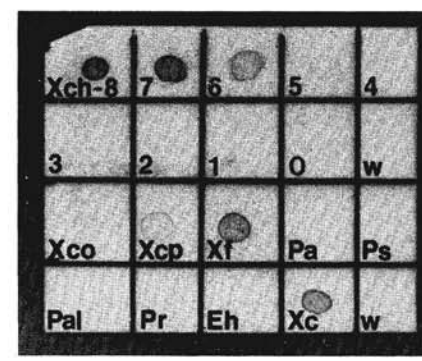

Fig. 1. Nitrocellulose membrane from a dot-immunobinding assay of *Xanthomonas campestris* pv. *holcicola* (Xch), *X. c.* pv. *oryzae* (Xco), *X. c.* pv. *phaseoli* (Xcp), *X. fragariae* (Xf), *Pseudomonas andropogonis* (Pa), *P. syringae* pv. *syringae* (Ps), *P. alboprecipitans* (Pap), *P. rubrilineans* (Pr), *Erwinia herbicola* (Eh), and water control (w). The numbers are the log of colony-forming units per milliliter of *X. c.* pv. *holcicola* (10^8–10^0). All other bacterial strains were adjusted to 10^8 cfu/ml.

Table 3. Detection of *Xanthomonas campestris* pv. *holcicola* in sorghum leaves from greenhouse and field samples by the dot-immunobinding assay (DIA)[a]

DIA reaction[b]	Number of sorghum leaves							
	Greenhouse				Field			
	Infected with *X. c.* pv. *holcicola*	Infected with other pathogens[c]	Healthy	Total	Infected with *X. c.* pv. *holcicola*	Infected with other pathogens	Healthy	Total
Positive	38	0	0	38	8	2	0	10
Negative	2	15	10	27	0	18	8	26
Total	40	15	10	65	8	20	8	36

[a] Samples were rated independently as infected (based on symptoms and reisolation) with *X. c.* pv. *holcicola*, with other pathogens, or healthy (water inoculated leaves). Inoculated leaves that were symptomless at 14 days, from which the pathogen could not be reisolated and which gave negative DIA reactions, were not included in this analysis.

[b] Tests were evaluated according to the ability to detect samples infected with *X. c.* pv. *holcicola* (positives) from either healthy samples or those infected with other pathogens (negatives).

[c] Other pathogens = *Pseudomonas andropogonis* and *P. syringae* pv. *syringae*.

greenhouse-inoculated plants were used as a source of bacterial antigens. Specificity and sensitivity of the DIA were 100% (35/35) and 95% (38/40), respectively (Table 3). The assay gave a 100% (38/38) predictive value for positive results and a 93% (25/27) predictive value for negative results. A limited number of field samples have been diagnosed with the DIA; the predictive value for positive and negative results were 80% (8/10) and 100% (26/26), respectively (Table 3). Together, the greenhouse and field samples indicate the DIA can correctly predict 95.8% (46/48) of the positives and 96.2% (51/53) of the negatives.

The sensitivity of the DIA was further evaluated with pure cultures of bacteria (Fig. 1). As few as 400 washed cells per 4-μl drop (10^5 cfu/ml) were detected with the assay. In comparison, the DIA detected fewer than 10^5 cfu/ml of *X. c.* pv. *holcicola* from the leachates of infected plants (Table 3). The discrepancy between this value and that observed for washed cells (10^5 cfu/ml) probably reflects the substantial numbers of dead bacterial cells in the plant tissue and extracellular bacterial products that may have been removed by washing steps in the preparation of cultured bacteria for the assay. The intensity of the DIA reaction roughly correlated with the number of bacteria present in the soaking solution.

Specificity and sensitivity of immunoassays are dependent on the antiserum used. For example, in the assay reported here, the dilution of antiserum used was the maximum dilution where color intensity would not be lost (i.e., sensitivity) but where cross-reaction with antigens of other bacterial genera would not be observed (specificity). To enhance sensitivity of detection from plant tissues where only slight infections are present, the leaf-soaking solution was sampled at 2 hr and then again at 24 hr, which allowed for further leaching of the bacteria from the leaf as well as bacterial growth. The potential problem of cross-reaction with other xanthomonads has been minimized in this assay by extensively washing the leaf sections before soaking. Thus, only those bacteria inhabiting vessels or intracellular spaces would be detected. The reaction with other pathovars of *Xanthomonas* could potentially be eliminated by the use of monoclonal antibodies specific to *X. c.* pv. *holcicola* (1).

The symptoms of bacterial streak (incited by *X. c.* pv. *holcicola*) are difficult to differentiate from the symptoms of bacterial stripe (incited by *P. andropogonis*). The DIA provides a means to accurately and rapidly detect *X. c.* pv. *holcicola* in sorghum tissues and thus is valuable as a diagnostic tool. The advantage of using the DIA rather than cultural methods to detect bacterial plant pathogens in host tissue is primarily the reduced assay time required to process large numbers of samples. Added features of the assay are that it is simple, inexpensive to perform, and requires little equipment. Because the bacterial antigens remain stable on the membranes for several months, samples from surveys can be collected over a season and processed together. The DIA is currently being used to survey bacterial pathogens of sorghum and millet in Africa.

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