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Filter Paper Dot-Immunobinding Assay for Detection of *Spiroplasma citri*

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A rapid filter paper dot-immunobinding assay was adapted to detect the wall-less mollicute *Spiroplasma citri* in medium, plants, or insects. Filter paper spotted with sample was incubated first in dilute antiserum, then in protein A-peroxidase, and finally in a substrate of 4-chloro-1-naphthol plus hydrogen peroxide. The detection limit averaged 2.3 × 10^{10} CFU/ml in cultures, and *S. citri* was detected in single infected leafhoppers. This assay was less sensitive but more rapid and economical than an enzyme-linked immunosorbent assay.

The dot-immunobinding assay (DIMA) was developed as a rapid, simple, and economical method for detection of proteins (2), plant viruses (8; J. Sherwood, J. Phytopathol. [Berlin], in press), phytopathogenic bacteria (J. E. Leach, B. A. Raymundo, and L. E. Claflin, Abstr. Int. Conf. Plant Pathol. Bacteriol., p. 43, 1985), and, recently, mycoplasmas (5, 6). Most reports of DIMAs suggest the use of nitrocellulose membranes as a substrate for the binding of antigens. Recently, however, Haber (S. Haber, Can. J. Phytopathol., in press) adapted the procedure of Esen (2) for detection of plant viruses. With this method, filter paper is substituted for the costly nitrocellulose. In the present study, we describe a simple filter paper DIMA for detection of the wall-less mollicute *Spiroplasma citri* in culture medium and host tissues.

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S. citri BR3 and BR6 (originally cultivated from Illinois horseradish affected by brittle root disease) (4), and *S. melliferum* AS576 and *S. floricola* 23-6 (both provided by R. E. Davis, U.S. Department of Agriculture, Agricultural Research Service, Beltsville, Md.), were maintained in LD8 broth (7) at 31°C. *S. kunkelii*, the corn stunt spiroplasma, isolated from infected corn leaves supplied by L. R. Nault, Ohio State University, Wooster, Ohio, was maintained under anaerobic conditions (GasPak Anaerobic Jar; BBL Microbiology Systems, Cockeysville, Md.) at 31°C in C3-G broth (1).

Infected and healthy test plants included turnip (*Brassica rapa* L.), periwinkle (*Catharanthus roseus* L.), and horseradish (*Armoracia rusticana* Gaertn., Mey., and Scherb.). Infected and healthy leafhoppers (*Circulifer tenellus* Baker) were provided by C. E. Eastman, Illinois Natural History Survey.

Antiserum against *S. citri* BR3 was produced in a New Zealand White rabbit as described previously (3). The antiserum titer was 1:16,000 as determined by the serological deformation test (9).

Spiroplasmas grown in culture medium either were applied directly from culture tubes to filter paper or were pelleted (25,300 × *g*, 20 min) and suspended in a smaller volume of 1.7% HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.5) containing 7.5% sucrose (HEPES-S). The titer of the spiroplasmas in the culture medium was determined by direct counts prior to centrifugation. In an experiment designed to determine whether plant sap had an inhibitory effect on detection of *S. citri*, pelleted cells were initially divided into two groups. One group was suspended in HEPES-S, and the other was suspended in sap from healthy turnip leaves ground in 2 volumes of HEPES-S.

Young leaf or root tissue of infected or healthy plants was usually ground in 2 volumes of HEPES-S, although 0.1 M Tris hydrochloride (pH 7.4)–0.85% NaCl–0.1% sodium azide (TBS) plus 0.2% ovalbumin (TBS-O), 70% ethanol, and 350 mM sorbitol were also tested as extraction liquids. The slurry was clarified (5 min, 5,000 × *g*), and the supernatant was spotted onto filter paper disks (see below).

Infected or healthy leafhoppers were collected from feeding hosts and stored at −80°C. Insects were thawed and ground (either singly or in groups) in 0.1 ml of HEPES-S.

Autoclaved Whatman no. 1 filter paper disks (diameter, 7 mm) were blocked for 15 min in a solution of either 1% powdered skim milk (Carnation) or TBS-O.

Samples of 4 μl were applied (2-μl aliquots) in the center of blocked filter paper disks. On one occasion, disks were spotted with a total of 10 μl. The disks were allowed to dry between the 2-μl applications. In several experiments with plant tissue, disks were soaked briefly in 70% ethanol after sample application to remove green pigments and then washed (as described below) before the assay was continued.

Disks were each covered with 150 μl of normal serum or antiserum diluted in TBS-O (1:100, 1:200, 1:400, 1:600, or 1:800) and shaken at 60 rpm for 30 to 90 min; the serum was removed by aspiration. The disks were first washed with enough TBS-O to cover each disk; the TBS-O was aspirated off immediately. During the second wash in TBS-O, the disks were shaken at 120 rpm for 15 min. Protein A-peroxidase (2 μg/μl) (no. P 8651; Sigma Chemical Co., St. Louis, Mo.) in TBS-O was added in 150-μl volumes to each disk. After the disks were shaken at 60 rpm for 15 to 60 min, the protein A-peroxidase was removed and the disks were washed twice as described above. Fresh substrate was prepared by mixing 5 parts of TBS, 1 part of 4-chloro-1-naphthol (no. C 8890; Sigma) (3 mg/ml in methanol), and 0.018 part of 3% H_2O_2. A volume of 150 μl was added to each disk, which was then incubated with shaking (60 rpm); the color developed maximally within 10 min. Washing was repeated as described above, with water instead of TBS-O to stop the reaction. Disks were air dried, and the results were evaluated visually.

Color development with cultured *S. citri* cells was optimum when 4-μl samples were prepared in TBS or HEPES-S

TABLE 1. Specificity of DIMA with *S. citri* BR3 antiserum for detection of spiroplasmas

Spiroplasma	Serogroup	Detection limit (CFU/ml)			No. of trials
		Minimum	Maximum	Avg	
S. citri BR3	I	1.2×10^9	6.5×10^{10}	2.3×10^{10}	10
S. melliferum	I	1.1×10^{10}	9.3×10^{10}	5.0×10^{10}	4
S. kunkelii	I	1.8×10^{11}	5.0×10^{11}	2.6×10^{11}	4
S. floricola	II	ND[a]	ND	ND	2

[a] ND, Not detected.

and then applied to disks blocked with TBS-O, with complete drying of spots between applications. After drying, best results were obtained with a brief wash in 70% ethanol followed by a 60-min incubation in antiserum (diluted 1:200). Protein A-peroxidase incubation was optimum at 30 min.

DIMA detected *S. citri* BR3, homologous to the antiserum, at levels as low as 1.2×10^9 CFU/ml, but the detection level averaged 2.3×10^{10} CFU/ml; this is equivalent to detection of 10^8 cells, since the sample size was 4 μl. The test did not distinguish between serogroup I species *S. citri* and *S. melliferum* (Table 1). However, although *S. kunkelii* reacted with BR3 antiserum, the detection limit for this serogroup I spiroplasma was much higher (Table 1). The test did not detect *S. floricola*, a member of serogroup II.

When leafhoppers fed on *S. citri*-infected plants were processed singly or in groups, the percentage of samples correctly reacting positively ranged from 87% for single leafhoppers to 100% for groups of 10 or 25 (Table 2). No positive samples were observed for healthy control insects.

Turnip samples infected with *S. citri* for approximately 3 weeks tested positive with DIMA. Healthy plants showed no reaction. However, the spiroplasma was not detected in periwinkle, horseradish, and turnip that had been infected for a longer time.

Plant sap apparently inhibited color reaction. When equal numbers of *S. citri* cells were pelleted and suspended in either sap from healthy turnip leaves or TBS-O, the color development was much greater in those suspended in buffer.

In three parallel tests with *S. citri* BR3 (and BR3 antiserum), the detection limits for DIMA and an enzyme-linked immunosorbent assay averaged 1.1×10^{10} and 1.9×10^9 CFU/ml, respectively. Results for strain BR6 with BR3 antiserum were similar (9.2×10^9 and 2.4×10^9 CFU/ml, respectively). Neither *S. melliferum* nor *S. kunkelii* was detected by enzyme-linked immunosorbent assay with BR3 antiserum; their detection limits with DIMA were 5.2×10^{10} and 2.3×10^{11} CFU/ml, respectively.

TABLE 2. Detection of *S. citri* BR3 in leafhopper samples[a] with *S. citri* antiserum in DIMA

No. of insects/ group[b]	No. positive/ no. tested	% Positive	No. of controls positive/ no. tested[c]
1	50/58	86	0/27
3	34/35	97	0/13
5	18/19	94	0/5
10	3/3	100	0/1
25	1/1	100	0/1

[a] Frozen at −80°C and processed for testing either singly or in groups of 3, 5, 10, or 25.
[b] Leafhoppers were fed on *S. citri*-infected turnip plants.
[c] Leafhoppers were fed on healthy turnip plants.

DIMA is a rapid assay, requiring 2 to 3 h to process a small number of samples, as compared with 1 to 2 days for the enzyme-linked immunosorbent assay. Neither purified immunoglobulin G nor conjugated alkaline phosphatase is required. The filter paper adaptation of DIMA is especially economical when the cost of filter paper is compared with that of nitrocellulose (2). The detection limits, averaging 10^{10} CFU/ml, are within the range of *S. citri* titers in the host plant turnip (*Brassica rapa*), which reach 10^{10} to 10^{11} CFU/ml (3).

There are a number of useful applications for DIMA. It would be much faster than the growth inhibition test for designating serogroup affiliation for an unknown, cultured spiroplasma; it could not, however, be used to accurately designate species. DIMA could be used to quickly determine the location of spiroplasma-containing fractions in a number of laboratory procedures. Spiroplasma detection in plant samples is a possible application but is limited at the present to plant samples for which stringent controls are available. This assay is also ideal for rapid screening of leafhopper samples (even when processed as single insects) for the presence of spiroplasmas.

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LITERATURE CITED

1. **Chen, T. A., and R. E. Davis.** 1979. Cultivation of spiroplasmas, p. 65–82. *In* R. F. Whitcomb and J. G. Tully (ed.), The mycoplasmas, vol. III. Academic Press, Inc., New York.
2. **Esen, A., J. M. Conroy, and S.-Z. Wang.** 1983. A simple and rapid dot-immunobinding assay for zein and other prolamins. Anal. Biochem. **132:**462–467.
3. **Fletcher, J., and C. E. Eastman.** 1984. Translocation and multiplication of *Spiroplasma citri* in turnip (*Brassica rapa*). Curr. Microbiol. **11:**289–292.
4. **Fletcher, J., G. A. Schultz, R. E. Davis, C. E. Eastman, and R. M. Goodman.** 1981. Brittle root disease of horseradish: evidence for an etiological role of *Spiroplasma citri*. Phytopathology **71:**1073–1080.
5. **Kotani, H., and G. J. McGarrity.** 1985. Rapid and simple identification of mycoplasmas by immunobinding. J. Immunol. Methods **85:**257–267.
6. **Kotani, H., and G. J. McGarrity.** 1986. Identification of mycoplasma colonies by immunobinding. J. Clin. Microbiol. **23:**783–785.
7. **Lee, I. M., and R. E. Davis.** 1984. New media for rapid growth of *Spiroplasma citri* and corn stunt spiroplasma. Phytopathology **74:**84–89.
8. **Parent, J.-G., F. Belanger, S. Desjardins, and J. D. Brisson.** 1985. Dot-immunobinding for detection of tomato mosaic virus and potato virus X infecting greenhouse tomatoes. Phytoprotection **66:**53–57.
9. **Williamson, D. L., R. F. Whitcomb, and J. G. Tully.** 1978. The spiroplasma deformation test, a new serological method. Curr. Microbiol. **1:**203–207.