Latex Agglutination as a Rapid Detection Assay for *Spiroplasma citri*

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ABSTRACT

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The latex agglutination test (LAT) was effective in detecting the wall-less mollicute *Spiroplasma citri*. The minimum detection level in culture medium or turnip samples enriched with spiroplasma cells was 10^7 cells per milliliter. Cross-reactions were not detected with corn stunt spiroplasma, honeybee spiroplasma strain AS576, or flower surface spiroplasma strain 23-6. *S. citri* was accurately detected in preparations of some plant species, with negative results from uninfected controls. However, false positives occurred in other uninfected plant species, especially weeds. Spiroplasmas were detected in single leafhoppers (*Circulifer tenellus*) as well as in groups of 3, 5, 10, 25, and 41 insects, but false positives were also detected (in about 25% of cases) with healthy leafhoppers. LAT can be used as a rapid and simple qualitative assay for detecting and identifying *S. citri* in culture; its application to experimental uses with infected plant or insect tissue is limited to those for which stringent controls are available.

Since their discovery as a distinct type of microorganism in 1972 (3), spiroplasmas have been detected in plant hosts and insect vectors by symptomatology, light and electron microscopy, remission of symptoms after tetracycline treatments, cultivation in artificial medium, and serology. The latter approach has proven to be the most specific and reliable. Enzyme-linked immunosorbent assay (ELISA) has been shown to be reliable and sensitive for detection of *Spiroplasma citri* (1) and the corn stunt spiroplasma (4) and useful for quantifying the pathogen in specific tissues (5,15). However, a faster and simpler assay would be welcome, especially where small numbers of samples are being evaluated and spiroplasma detection is the primary concern.

The latex agglutination test (LAT) has been reported to be a useful detection method for plant-pathogenic viruses (9,10,13,14) and bacteria (16). In this paper, we demonstrate that LAT is a rapid, sensitive, and simple serological test for detecting the wall-less mollicute *S. citri* in cultured samples and many infected plant species. False-positive reactions with some plant species limit its use to previously tested species or those for which adequate controls are available. A preliminary report has been published (7).

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MATERIALS AND METHODS

Sources and maintenance of spiroplasma cultures and infected tissues. *S. citri* isolate BR6 was originally cultivated from Illinois horseradish affected by brittle root disease (8), and spiroplasma strains AS576 and 23-6 were kindly provided by R. E. Davis (USDA/ARS, Beltsville, MD). These spiroplasmas were maintained in LD8 broth (11) at 31 C. Corn stunt spiroplasma was isolated from infected corn leaves supplied by L. R. Nault (Ohio Agricultural Research and Development Center, Wooster, OH) and was maintained under anaerobic conditions (Gas-Pak Anaerobic Jar, Baltimore Biological Laboratories, Cockeysville, MD) at 31 C in C3-G broth (2). Infected and healthy plant materials tested included horseradish (*Armoracia rusticana* Gaertn., Mey., & Scherb.), turnip (*Brassica rapa* L.), shepherd's purse (*Capsella bursa-pastoris* (L.) Medic.), wild mustard (*B. kaber* (DC.) L. C. Wheeler), yellow rocket (*Barbarea vulgaris* R. Br.), China aster (*Callistephus chinensis* Nees), and periwinkle (*Catharanthus roseus* (L.) G. Don), provided by C. E. Eastman (Illinois Natural History Survey, Champaign, IL), common bean (*Phaseolus vulgaris* L.), a weed (*Macroptilium lathyroides* (L.) Urban) supplied by S. Haber (University of Illinois), and a number of weed samples collected from horseradish field borders in southern Illinois (Table 1). Infected and healthy beet leafhoppers (*Circulifer tenellus* Baker) were supplied by C. E. Eastman.

Antiserum production. Antiserum was produced in New Zealand white rabbits against Illinois horseradish isolates BR3 and BR6 as described (5). Antiserum titer was 1:16,000 by the spiroplasma deformation test (18).

LAT. The LAT was performed as described by Khan and Slack (10) and modified by Slack et al (16). Antiserum was serially diluted in twofold increments (1:100–800) in 100 mM Tris (hydroxymethyl) aminomethane-HCl (Tris), pH 7.4, and conjugated with latex (polystyrene) spheres 0.794 μm in diameter, 10% concentration, w/v (Sigma) diluted 1:50 (v/v) in 0.85% NaCl. Sensitized latex was washed twice in 0.02% polyvinylpyrrolidone (mol wt 40,000) in 0.85% NaCl and resuspended in Tris containing 0.02% sodium azide. Bovine serum albumin (0.4%, w/v) was added to the suspension to reduce nonspecific background. Samples were tested in capillary tubes by rotating at seven cycles per minute for 15 min before scoring.

For each assay, samples were tested with two to four antiserum dilutions of sensitized latex and reactions were rated visually with a dissecting microscope on a scale of 0–5 (0 = no agglutination, 5 = complete agglutination). Values $\geq$2.0 were considered positive (a rank of 1.0 indicates clouding of the background, 2.0 represents clear agglutination). Positive (*S. citri* cultures) and negative (no antigen) controls were included in each test.

Test sensitivity was determined by evaluating *S. citri* dilution series against dilutions of sensitized latex. *S. citri* concentrations were determined by dilution plating on agar-solidified LD8.

Test antigens were prepared either from spiroplasmas maintained in liquid (LD8 or C3-G) culture, from leaf tissue of plants, or from whole-body extracts of insects. From culture, spiroplasma suspensions were diluted twofold in 0.1 M Tris, pH 7.4, containing 0.85% NaCl and 0.1% sodium azide (TBS). In cell-washing experiments, cultures were pelleted at 14,500 × *g* for 20 min and resuspended in an equal volume of LD8. Washing was repeated twice. Cells were counted by direct dark-field microscopic observation. Plant leaf material (0.3 g) was minced with a sterile scalpel in 1–2 ml TBS except where indicated and either ground with a mortar and pestle or homogenized using an electric tissue homogenizer (Rotary Mechanical Homogenizer Model TSO-45, Heat

System-Ultrasonics, Inc., Plainview, NY). Samples were clarified for 2.5 min in a Microfuge (Beckman Model 11, Beckman Instruments, Palo Alto, CA). For some experiments, known quantities of cultured spiroplasma were added to healthy plant samples before mincing to simulate the effect of using infected samples. Leafhoppers, which were provided frozen at −20 C, were placed singly or in groups into 0.25 ml of TBS, homogenized with the electric tissue homogenizer, and clarified. Blind tests were performed in which one investigator prepared coded samples and a second investigator evaluated these samples.

RESULTS

LAT was successful in detecting *S. citri* from culture medium and living tissue. Optimum antiserum dilutions varied among preparations but were generally best at 1:400–1:600 for about 8 wk after preparation and 1:200–1:400 after 8 wk. Sensitized latex was stored at 4 C. Based on four tests in which twofold dilutions of cultured material were tested, the minimum detection level for *S. citri*, using the optimum antiserum preparation, was 10^7 cells per milliliter. Washing cells had no effect on detection levels.

Test specificity was evaluated by examining reactions of other spiroplasmas from broth culture. Illinois horseradish isolates of *S. citri* could not be distinguished from each other by the test. No agglutination was detected with the corn stunt spiroplasma, which is in the same serogroup as *S. citri* (17). With honeybee spiroplasma (strain AS576), also in the same serogroup as *S. citri*, occasional low agglutination values (0.5–1.5) were recorded, but these values are not considered positive. Spiroplasma strain 23-6 from flower surfaces, which belongs to a different serogroup, caused significant agglutination in some tests but not in others. The precipitant was associated with old cultures, and because it looked very different from the homologous reaction, it would not be mistaken for a normal positive reaction.

Latex agglutination proved effective with some limitations for detecting *S. citri* in infected plant hosts (Table 1). Positive ratings (≥2.0) were assigned to almost all plants designated infected by visual evaluation and/or by ELISA or spiroplasma isolation. However, false positives (agglutination with samples from which spiroplasmas had not been recovered in isolation attempts) occurred with one turnip sample when concentrated (1:200) latex was used, with one of three healthy horseradish leaf samples, and with more than half of the weed samples collected from the field. Adding 0.2% ovalbumin, 0.001 M dithiothreitol (Cleland's reagent), 0.02% polyvinylpyrrolidone (mol wt 40,000), or 0.1% Tween 20 to the grinding buffer or grinding in 0.35 M sorbitol did not eliminate the false-positive reactions.

When applied to individual leafhoppers (*C. tenellus*) fed on infected turnip plants, the test was positive for 19 of 36 samples. When leafhoppers were bulked in groups of 3, 5, 10, 25 and 45, the ratios of positives to numbers of samples tested were 12/20, 10/11, 6/11, 2/2, and 1/1, respectively. However, false positives (agglutination occurring on samples of leafhoppers fed only on uninfected food plants) were frequent. In bulked groups of 1, 3, 5, 10, 25, and 41, these uninfected insect samples were positive, respectively, in 4/16, 3/11, 4/10, 1/6, 0/2, 0/1, and 0/1 cases.

DISCUSSION

A number of serological procedures have been used successfully to detect spiroplasmas in plant and insect tissues. Each is useful in circumstances determined by factors such as need for speed, reliability, sensitivity, or lack of complexity. LAT, though slightly less sensitive than ELISA (13,14), has been used to detect plant viruses in plants (14) and insects (13). Slack et al (16) developed the test as a diagnostic assay tool for detecting *C. sepedonicum*, the bacterial pathogen causing ring rot of potatoes.

We have shown that LAT is useful in detecting the wall-less mollicute *S. citri* under some conditions. Using latex beads sensitized with antiserum against *S. citri*, a minimum of 10^7 cfu/ml was required for detection of this spiroplasma. This level is well below the peak titers of *S. citri* in turnip (*B. rapa*), which reach 10^{10}–10^{11} cfu/g as described by Fletcher and Eastman (5). Soluble antigens, which can increase detection sensitivity considerably (S. A. Slack, *unpublished*), were not detected in the cell-washing experiment. Normal agglutination did not occur when other spiroplasmas, even those in the same serogroup, were tested.

We found the test, when used on plant samples, to be sensitive and dependable for detecting *S. citri* in turnip, periwinkle, and several weed species, with no agglutination in healthy samples from these species. However, one cultivated horseradish plant and several weed species, apparently not infected with *S. citri*, gave a positive agglutination. Spiroplasmas could not be cultured from any of these plants. Nonspecific serological reactions are not unique to LAT; horseradish and many of the same weed species also produced false positives in ELISA using IgG prepared from the same antiserum (6). Other researchers (12) have also reported false positives in ELISA when testing for the presence of viruses in many of the same weed species. Thus LAT, like other serological tests, should be used only on acceptable plant species or those for which adequate controls are available.

The LAT was tested on only one leafhopper species, *C. tenellus*. Single leafhoppers fed on infected turnip plants were rated positive in 53% of the cases, and detection success generally increased as the number of insects in composite

Table 1. Use of latex agglutination for detection of *Spiroplasma citri* in plants

Plant	Condition[a]	Samples positive/samples tested (no.)	Mean av.[b]
Shepherd's purse	Healthy	0/2	0.4
	Infected	3/3	3.6
Wild mustard	Healthy	0/2	0.0
	Infected	3/3	3.2
Yellow rocket	Healthy	0/5	0.0
	Infected	4/5	2.0
Turnip	Healthy	0/8[c]	0.4
	Infected	6/6	2.8
Horseradish	Healthy	1/3[d]	1.5
	Infected	10/11	4.1
Periwinkle	Healthy	0/8	0.0
	Infected	11/11	3.2
Aster	Healthy	0/2	0.3
	Infected	4/4	4.0
Weeds[e]	Field-collected, presumed healthy[f]	8/15	1.5[g]

[a] Condition of plants was determined by visual rating and/or by ELISA or isolation of spiroplasmas.
[b] Mean agglutination value at latex dilution of 1:400, 1:600, or 1:800, from a scale of 1–5, where 0 = no agglutination and 5.0 = maximum agglutination.
[c] Healthy turnip samples gave positive reactions on three occasions when a 1:200 dilution of latex was used; this dilution was later dropped.
[d] A false-positive reaction occurred at a latex dilution of 1:400.
[e] Ten weed species were collected adjacent to horseradish fields in southern Illinois, and each species was separately tested for *S. citri*. Included were *Plantago lanceolata* L. (plantain), *Solidago* L. sp. (goldenrod), *Taraxacum officinale* Weber (dandelion), *Erigeron annuus* (L.) Pers. (daisy fleabane), *Cirsium* Mill. sp. (thistle), *Rumex* L. sp. (dock), *Vicia* L. sp. (vetch), *Hippuris vulgaris* L. (mares' tail), and *Chrysanthemum leucanthemum* L. (daisy).
[f] None of the weeds showed characteristic symptoms. None yielded spiroplasmas when normal isolation procedures were followed.
[g] Mean average of the weeds is an average of the readings of all 15 weed samples tested.

tests increased. Nonspecific agglutination was observed, but the percentage of positives from healthy leafhopper samples was always less than half of that from infected insects. Omura et al (13) found that adding Tween 20 to their insect-grinding buffer dispersed nonspecific aggregates, but in our experience, this did not solve the problem. Although sample treatments may be developed that will minimize or eliminate nonspecific reactions, current data suggest that the application of LAT to testing samples for the presence of spiroplasma is limited and must include stringent control samples.

Although LAT lacks the precise, quantifiable features of some other serological techniques, it has become a valuable aid for simple and economical detection of pathogens such as viruses and bacteria. We have shown that it is applicable for use in detecting the plant-pathogenic mollicute *S. citri*. Thus, LAT might be applied in purification attempts or other laboratory procedures for which it would be helpful to know which fractions contained spiroplasma. Because of its specificity, it could also be used as a very quick serological identification method for cultured spiroplasmas.

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