COMPARATIVE SEROLOGY OF SEVERAL ISOLATES OF
SPIROPLASMA CITRI

by

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ABSTRACT

Spiroplasma citri is a mycoplasma-like organism (Class: Mollicutes; Order: Mycoplasmatales) associated with "stubborn" disease, an important malady of citrus in Arizona and several other citrus production areas.

Three isolates of *Spiroplasma citri* from Arizona citrus were compared serologically with Morocco and California strains by three parameters: growth inhibition on solid medium, metabolic inhibition in broth medium, and Ouchterlony double diffusion technique. In growth inhibition tests, all isolates yielded similar reactions and are therefore considered to be closely related serologically.

Results of metabolic inhibition tests showed one Arizona isolate (No. 4) to be closely related to the Morocco and California strains. Two Arizona isolates (No. 103 and No. 169) showed significant differences from all other isolates studied.

Differences found by the Ouchterlony double diffusion technique suggest that even though isolates are serologically interrelated they probably are not identical.

INTRODUCTION

Stubborn disease of citrus is an important malady in many citrus areas of the world (9, 21, 22). A recent report from California estimated that two million orange (*Citrus sinensis*(L.)Osbeck), grapefruit (*C. paradisi* Macf.), and tangelo (*C. reticulata* x *C. paradisi*) trees were severely affected (31). In 1963 (1), stubborn disease in Arizona was estimated to cause $640,000 annual production losses of oranges and grapefruit with navel oranges being most severely affected. Currently, it is estimated that stubborn effects a 20-30 percent reduction in the yield of navel oranges for a loss of more than 1.1 million dollars (personal communication from Dr. Ross M. Allen, Department of Plant Pathology, The University of Arizona, Tucson).

Fawcett (20, 22) concluded that stubborn disease was caused by a virus because the causal entity was graft transmissible and symptoms were similar to those produced by viruses in other plants. In 1970, however, Igwegbe and Calavan (29) demonstrated, by electron microscopy, that sieve tubes of stubborn-affected citrus leaves contained many units of a mycoplasma-like organism. Since then, various workers (2, 3, 24, 25, 39, 40) have successfully isolated and cultured this organism in cell-free media. Studies of two isolates in particular (California 189, available as American Type Culture Collection No. 27563, and Morocco R8-A2, American Type Culture Collection No. 27556) have led to a characterization of the organism (7, 8, 23, 38, 41, 42) and its assignment to the class Mollicutes of the order Mycoplasmatales (42). Saglio et al. (42) named the stubborn organism Spiroplasma citri, with the Morocco isolate designated as the type strain. Pathogenicity of Spiroplasma citri, as transmitted by leafhoppers, has been shown (15, 31, 34).

Allen (2, 3) has obtained Spiroplasma citri isolates from several cultivars of stubborn-affected citrus grown in different areas of Arizona. Several of these Arizona isolates, when compared with the Morocco and California isolates, showed differences in various metabolic and other cultural tests (4, 5). These tests have suggested the existence of distinct strains of Spiroplasma citri.

Evidence for occurrence of strains, or even species, of Spiroplasma has increased with recent discoveries relating to corn stunt disease. The etiological agent associated with corn stunt (CS) has the morphology of a Spiroplasma (16, 17) and, like the isolate from citrus, has been transmitted by leafhoppers (11, 52). However, it grew poorly in media supporting good growth of Spiroplasma citri (11, 43, 52).

Serological studies for the identification and classification of Mycoplasmatales of animal origin have been extensive (12, 18, 26, 27, 32, 33, 35, 36, 37, 45, 46, 47, 53) but few such studies have been reported concerning mycoplasmas of plant origin. Bové et al. (8) found that the Morocco and California isolates of Spiroplasma had a very close serological relationship but were not homologous. These workers also showed that this organism was serologically unrelated to any of the previously described animal mycoplasmas or acholeplasmas. Similar negative results were obtained for several spirochetes which have some similarities to the stubborn organism on the basis of ultrastructure and shape. Tully et al. (48) reported serological similarities for the Morocco and California isolates and also that both isolates were serologically related to the organisms found in CS-affected corn. Chen and Liao (11) and Williamson and Whitcomb (52), in recent, separate but simultaneous reports, noted serological relationships and differences between the CS and citrus spiroplasmas.

The research reported in this thesis was undertaken because differences in metabolic activity between isolates found in Arizona, and those from Morocco and California (4, 5) suggested the probable existence of different enzyme systems in the several isolates. Because enzymes are proteins, and proteins are the active sites of antibody-antigen reactions, it was thought that serological differences could be demonstrated.

The objectives of this research were,

1. To develop antisera to important isolates of Spiroplasma citri, including several from Arizona and those from Morocco and California.
2. To determine by serological methods whether the isolates of Spiroplasma from different geographical locations and host species are of the same or different strains.

MATERIALS AND METHODS

Isolates

Five isolates of Spiroplasma citri were compared serologically. These included the Morocco and California strains and three obtained from different host cultivars and locations in Arizona (Table 1).

Media

Organisms used for immunization and growth inhibition tests (described below) were grown in a modified complete sorbitol medium (SMC)(39). This medium consisted of 0.1 percent glucose, 0.1 percent Bacto-D-fructose, 1.0 percent sucrose, 0.2 percent Bacto-yeastolate, 1.0 percent Bacto-tryptone, 2.1 percent Bacto-PPLO broth, 7.0 percent sorbitol, 0.002 percent phenol red, all added (w/v) to 1 liter of sterile distilled water (SDW). About 0.6 percent of 1N NaOH was added to the medium to adjust the pH to 7.7-7.8. After autoclaving at 15 lbs for 30 minutes, the medium was cooled and 10 ml sterile PPLO bovine serum fraction (Difco) was added. Pencillin, usually included in SMC medium, was omitted.

When maltose medium was used for metabolic inhibition and Ouchterlony double diffusion tests, 1.0 percent maltose (w/v) was substituted for 1.0 percent sucrose; 25 ml horse serum (GIBCO) (v/v), previously heated for 30 minutes at 56 C, was added to supplement the PPLO bovine serum fraction.

Table 1. Isolates of Spiroplasma citri compared for serological relationships.

Isolate Identity	Host	Inoculum Source
103	Frost Washington navel (N)	Tempe, Arizona
4	Hamlin sweet orange*	Tucson, Arizona
Morocco**	Washington navel	R8-A2, ATCC No. 27556
California**	Madam Vinous sweet orange	C-189, ATCC No. 27563
169	Frost Marsh grapefruit (N)	Phoenix, Arizona

* Hamlin orange seedling was grown in a glasshouse, University Farm, Tucson; infection by Spiroplasma resulted by graft transmission from an infected Frost Washington navel tree grown at Yuma, Arizona.

** Isolates obtained from American Type Culture Collection (ATCC).

The agar medium used for growth inhibition tests consisted of 1.0 percent Bacto-agar, 0.2 percent Bacto-yeastolate, 1.0 percent maltose, 1.0 percent Bacto-tryptone, 2.1 percent Bacto-PPLO broth; all were added on a w/v basis. Heat treated horse serum, 20 percent (v/v), and 800 ml of SDW completed this formulation.

Culture of Organisms

Cultures for routine propagation and maintenance were prepared by transferring 0.5 ml of a 3-4 day-old culture of *Spiroplasma* isolate into each of four or more screw-capped vials (4 dram) containing 5.0 ml of modified SMC broth medium. Cultures were then incubated at 32 C for 3-4 days.

Antigen Preparation

Cultures, 3-4 days old, having populations of 10^{8}-10^{10} colony forming units (CFU) as determined by estimation under a phase contrast microscope, were used as inocula. Nine ml of inoculum were transferred aseptically to each of one or more 500 ml Ehrlenmeyer flasks, each containing 200 ml of modified SMC. These cultures were then incubated at 32 C for 3-4 days to obtain populations of 10^{8}-10^{10} CFU. Samples of each culture were examined by phase microscopy to verify the purity of the culture before harvesting it by centrifugation at 4100 x g for 1 hour at 4 C (Lourdes Beta-Fuge Centrifuge, Model No. A-2). Resulting pellets were washed and recentrifuged twice with phosphate-buffered saline (PBS), pH 7.2 (50), and subsequently resuspended in PBS to 1/200 of the original volume. Tubes of concentrated antigen suspensions were cooled in an ice bath before disruption of the antigens by sonication for 30 seconds with a Branson S 125 Sonifier (Branson Instruments, Inc.) at 45 amperes/minute.

Protein Analysis

Protein content of the sonicated antigen suspensions was determined by Chaykin's modification of the Lowry method (10). This procedure required two reagents: Reagent A consisted of one part 1.0 percent $CuSO_4 \cdot 5H_2O$, one part 2.0 percent $Na_2C_4H_4O_6 \cdot 2H_2O$, and 100 parts 2.0 percent Na_2CO_3; reagent B contained one part Folin's phenol reagent and two parts SDW.

A standard curve was established by using bovine albumin stock solution (Sigma Chemical Co.) containing 1.0 mg/ml of protein. Stock solution and various dilutions (using PBS as a dilutent) ranging from 1.0 μg to 1.0 mg/ml protein content were prepared. Each solution (0.5 ml) was mixed by agitation with 5.0 ml of reagent A and allowed to incubate for 10 minutes at room temperature (RT; ± 25 C), after which time 0.5 ml of reagent B was added and the resulting solution was again mixed thoroughly. These preparations were incubated at RT for 10, 70, and 100 minutes to allow completion of the reaction. Solutions were then poured into Spectronic 20 colorimeter test tubes (1.27 x 10 cm) and light transmittance was read at 600 nm with a Spectronic 20 spectrophotometer (Bausch and Lomb). Results after the 10-minute incubation period were so variable (as much as ten percent difference when compared with results at 70 or 100 minutes) that subsequent determinations were based on a 70-minute incubation period. Transmittance of each dilution was recorded as the abscissa on semi-log paper and protein content/ml as the ordinate. Three separate trials for establishment of a standard curve were in general agreement within ± 1-2 percent. Therefore, arbitrarily, one curve was used for the standard.

Each sample of Spiroplasma antigen to be spectrophotometrically analyzed for protein content was diluted 1:5, 1:10, and 1:50 with PBS (pH 7.2) and reacted with the reagents described above. The transmittance of each dilution was compared with the standard curve for determination of the protein content of the antigen.

Preparation of Antisera

Antisera for five isolates of Spiroplasma were prepared using New Zealand white doe rabbits. Two animals, each weighing approximately 2.0 kg, were used for antiserum production for each Spiroplasma isolate. Two rabbits, not injected, were retained as controls for production of normal serum. Immunization methods included two foot-pad and one intramuscular injection of each rabbit at 3-week intervals, with a mixture of equal volumes of sonicated suspensions of the several isolates and incomplete adjuvant (Difco). Each rabbit received 2 ml of the mixture containing approximately 1.0 mg/ml of Spiroplasma protein at each injection. Serum from each rabbit was checked for normality prior to initiation of the injection series by use of the growth inhibition test to be described below. One month after the last injection, antisera were collected at weekly intervals by the heart puncture technique for as long as 12 weeks. Processed antisera were stored by freezing at -20 C until needed.

All antisera, normal sera, and SDW used, were sterilized by filtration through sterile 0.22 μm Millipore filters (Millipore Corp.) immediately prior to use in growth inhibition and metabolic inhibition tests.

Growth Inhibition Test

The procedure described by Clyde (12) was followed with some modifications. In this test, the antisera were examined for their capability of inhibiting formation of Spiroplasma colonies on agar medium.

Heated agar medium, as described previously under Media, was poured into plastic petri plates (60 x 15 mm; Falcon Plastics) 2 days prior to use to allow the agar surface to dry partially. Each Spiroplasma isolate, having a population of approximately 10^8 CFU/ml, was diluted $1:10^3$. Diluted culture, 0.2 ml, was transferred to each of 36 to 42 plates and spread evenly over the agar by swirling.

Antisera, in duplicate for each antigen, were used in both homologous and cross-reaction (or heterologous) tests.

For the homologous test, antisera and normal sera were diluted in a two-fold series, starting with undiluted material and ending at 1:1024. Sterilized filter paper discs (12.7 mm; Schleicher and Schuell Inc.) were impregnated individually with 50 μl of each diluted antiserum or with normal serum. Each plate received two antiserum impregnated discs and, as controls, two normal serum impregnated discs. These were placed alternately and equidistant from one another on the agar.

Plates, in duplicate, were incubated in a moist chamber at 32 C. Results were read after 10-14 days incubation period as colonies of the different isolates matured. Plates were examined using a B and L dissecting microscope with 0.7-3.0 x zoom objectives and 15 x eyepieces, and zones of inhibition around discs were measured from the edge of each disc to the margin of colonial development. Zones less than 1.0 mm were not considered significant. For this test, the reciprocal of the highest dilution of each antiserum producing an inhibition zone of 1.0 mm or more was considered to be an expression of titer of the antiserum.

For the cross-reaction test, each antiserum, undiluted in this case, was tested against each of the antigens. Discs impregnated with undiluted normal serum or SDW served as controls on each plate. Incubation, examination, and measurement of inhibition zones were as described for the homologous test.

Antibody Titer Ratio (Percent Relationship)

It was difficult to interpret the antigenic relationship between the five *Spiroplasma* isolates directly from the results obtained from the growth inhibition tests. Therefore, the formula of Archetti and Horsfall (6) as modified by Clyde (12) was used to analyze the results. In this way the magnitude of the relationship among the isolates can be more simply expressed. This formula states that percent relationship (r) equals $\sqrt{r_1 \times r_2} \times 100$ percent, where

$$r_1 = \frac{\text{heterologous titer, antigen 2}}{\text{homologous titer, antigen 1}}, \quad r_2 = \frac{\text{heterologous titer, antigen 1}}{\text{homologous titer, antigen 2}}$$

As a single figure, the percent relationship reflects the degree of interaction between two antigens and their respective antisera. By definition, a homologous antiserum-antigen reaction receives a rating of 100 percent. A relationship of less than 50 percent indicates a distinct antigenic difference between two isolates.

Metabolic Inhibition Test

This test is based upon the premise that *Spiroplasma citri*, when grown in heat sterilized media containing glucose, will utilize the glucose by fermentation with resultant production of lactic acid. When acid is produced the pH of the liquid medium is lowered. Phenol red is added to the medium as a pH indicator. Color of the medium is changed from red to orange or yellow with a reduction in pH. Thus, the color change indicates that growth of *Spiroplasma citri*, which is barely visible to the naked eye, has taken place. If an antiserum reactive to a particular isolate of *Spiroplasma* is added to the liquid medium, growth of the *Spiroplasma* is inhibited. As a consequence, no acid is produced and the color of the medium does not change.

For the conduct of this test, the procedure described by Taylor-Robinson et al. (47) and Taylor-Robinson and Berry (46) was slightly modified.

Cultures of isolates for the metabolic inhibition test were prepared essentially as described under Culture of Organisms. Maltose medium (described under Media), however, was substituted for SMC to encourage rapid and nearly-synchronous development of large populations of organisms.

It was necessary to standardize the populations of *Spiroplasma* units in each of the five isolates for reaction with specific dilutions of each antiserum. The populations of recognizable *Spiroplasma* units in each isolate were estimated by obtaining an average of the number of *Spiroplasma* units seen by dark field microscopy in ten rectangular fields of view (70 x 100 μm) on a single slide. Approximate uniformity of preparation of microscope slides bearing the several isolates (3-4 days' age) was obtained by placing 10 μl of each broth culture on identical glass slides (25 x 75 mm) and covering the droplets with 22 x 22 mm cover glasses (No. 1 thickness). Prepared slides were maintained at RT for 30 minutes prior to examination to minimize movement of the units during the counting process.

Populations of each isolate were adjusted to approximately $5.0\text{-}6.0 \times 10^7$ units by transfer of a calculated quantity of each isolate to vials containing a known volume of maltose broth medium. Two successive dilution transfers were then made to obtain populations of approximately $5.0\text{-}6.0 \times 10^5$ of each isolate for use as inoculum. Each metabolic inhibition test involved the use of 100 vials (each containing 4.5 ml of maltose broth medium) plus control vials for each isolate. Each vial received 0.5 ml of inoculum so that each contained $5.0\text{-}6.0 \times 10^4$ units of *Spiroplasma*.

Antisera from one set of rabbits were used because, in the growth inhibition tests, minor variations in the sizes of inhibition zones between the pairs of rabbit antisera were found. Each of the five antisera was inactivated by heating to 56 C for 30 minutes and sterilized by filtration through 0.22 μm filters. Serial, two-fold dilutions, starting with a 1:100 preparation, were made with SDW to obtain antiserum dilutions from 1:100 to 1:51,200.

Duplicate vials of each _Spiroplasma_ isolate received 50 μl of each dilution of antiserum. Controls were in duplicate and included vials containing only maltose broth medium (for pH and medium purity control), vials containing medium and _Spiroplasma_ isolate, and vials containing medium, _Spiroplasma_ isolate and normal serum. All cultures and controls were incubated at 32 C for 7-10 days. When each control containing medium plus _Spiroplasma_ isolate showed a color change from red to yellow the pH of the medium in each test vial pertaining to that isolate was determined by using a pH meter (Corning Digital 110 Expanded Scale). The highest dilution of antiserum preventing a change of 0.5 pH unit, compared with the pH of the controls of medium only, was recorded as the titer of that antiserum.

The metabolic inhibition test was performed three times.

The results obtained from these tests also were analyzed in terms of antibody titer ratios as described previously for the Growth Inhibition Test.

Ouchterlony Double Diffusion Tests

Standard Test

The method described by Lemcke was followed (32). These tests involved slightly different techniques to compare the relationships of various _Spiroplasma_ isolates with each of the antisera.

Tests were made using plastic petri plates (100 x 15 mm; Falcon Plastics) containing, per liter, 7.5 g Noble agar (Difco), 9 g sodium chloride, 200 ml sodium azide (0.1 percent) and 800 ml SDW. Wells were formed by removing agar with a 0.5 cm diameter cork borer; distance between wells was 1.0 cm. In each agar plate there were three sets of wells. Each set contained one central well and six outer wells.

Preparation of sonicated antigen suspensions was as described previously. Protein content of each antigen suspension was determined and adjusted to contain 1.0 mg protein per ml.

Antisera were inactivated by heating at 56 C for 30 minutes. Each antiserum (50 μl) was placed individually in a central well and usually two of the sonicated antigen suspensions (50 μl) were placed separately and alternately in the six surrounding wells. Plates were incubated in a moist chamber at RT for 24-48 hours to allow diffusion of the antigens and antibodies through the neutral agar. Precipitin lines were formed where antigens and antibodies met in optimum proportions. The number of precipitin lines and their shape and location between wells depended upon the characteristics of the particular antigen-antibody reaction.

Cross-Absorption Test

For further demonstration of differences between isolates, the cross-absorption test as described by Van Regenmortel (49) was employed. This test has been described as useful for detecting monospecific precipitin lines between two closely related strains.

General methods described for plates, medium, well arrangement, antigen preparation, and antiserum treatment in the standard test were used. The principal differences between the standard test and the cross-absorption test relate to timing of placement of antigens and antisera and selection of wells into which they were placed. For the cross-absorption test antigen A, for example, was placed into a central well and the plate was incubated in a moist chamber at RT for 18 hours to allow for diffusion of the antigen. Antiserum B was then placed into the same central well while antigen A and B were placed separately in alternate outer wells. Plates were again incubated in a moist chamber at RT for 24-48 hours before reading the results. The above procedures were followed in two separate tests utilizing all possible combinations of antisera and antigens.

Attempted Detection of Bacteriophage Antibodies

Cole et al. (14) and Cole, Tully, and Popkin (13) found that most isolates of *Spiroplasma citri*, including several from Arizona, sporadically showed infection by one or more of three distinct forms of bacteriophage. Bacteriophages are considered good antigens; they induce animals to produce antibodies against them. Therefore, it was considered that the antisera used in this study possibly contained antibodies against bacteriophages, if such were present in the sonicated *Spiroplasma* preparations used for immunizing the rabbits. In order to determine whether the antisera used in this study contained antibodies to bacteriophages the standard Ouchterlony double diffusion test was employed.

An isolate of *Spiroplasma* (No. 551) containing all three forms of bacteriophage (13) was used for this study. A purified bacteriophage suspension from the isolate was provided by Dr. R. M. Allen, Department of Plant Pathology, The University of Arizona, Tucson.

Protein content of the bacteriophage suspension and each of the *Spiroplasma* isolates was determined and each was adjusted to contain 1 mg protein per ml. However, the relative proportions of the three phages in the sample were not known.

All five antisera were used individually to react with the bacteriophage suspension. As controls, each of the five *Spiroplasma* isolates (sonicated antigen) was used at least twice against each antiserum and the bacteriophage suspension. This test was performed twice with two replicate plates per treatment.

The entire test was repeated with a slight modification. In this trial the Noble agar, described under Ouchterlony double diffusion Standard Test, was changed by adding sodium dodecyl sulfate (SDS) at the rate of 1 percent (v/v); SDS was added to facilitate degradation of any large molecule antigens into diffusible soluble antigen.

RESULTS

Growth Inhibition Test

Results of the homologous tests, in which antigens were tested against various dilutions of their homologous antisera, are shown in Table 2. These data are expressed as arithmetic means of width of zones of inhibition produced by duplicate sets of rabbit antisera. Minor variations were found in the sizes of inhibition zones between the pairs of rabbit antisera. The titer of the antiserum to isolate 4 was 128. Titers of Arizona isolate 103 and 169 and those of Morocco and California strains were 256. The width of the inhibition zones for each antigen was generally, with few exceptions, directly proportional to the antiserum concentration, as shown in Table 2. Figure 1 shows representative reactions, using isolate 4 as an example.

In the cross-reaction test, in which antigens were tested against their undiluted heterologous antisera, two antisera (from different rabbits) against each antigen also were used. There were inhibition zones around all the discs impregnated with undiluted antiserum. No inhibition zones were observed around discs impregnated with normal serum or SDW. Representative reactions are shown in Figure 2. Since results obtained from the two sets of antisera (different rabbits) were approximately the same, the data shown in Table 3 represent averages of zone widths obtained in 3-5 replicate tests for both sets of antisera.

Table 2. Relationship between antiserum dilution and width of inhibition zone in growth inhibition tests for five isolates of *Spiroplasma citri*.

Antigen Identity	Reciprocals of Homologous Antiserum Dilution for Impregnation of Discs											
	0*	1	2	4	8	16	32	64	128	256	512	1024
	Width of Inhibition Zone (mm)**											
103	9.0	7.5	7.5	7.0	7.0	6.5	6.5	6.0	4.0	2.0	0	0
4	7.0	5.5	4.5	4.5	3.5	2.5	2.5	1.5	1.0	0	0	0
Morocco	7.0	7.5	5.5	5.5	6.0	5.5	4.0	3.0	3.0	3.0	0	0
California	8.5	8.0	6.5	6.5	6.0	5.5	6.0	6.5	5.0	3.5	0	0
169	9.0	7.5	7.0	6.0	4.5	4.0	2.5	2.5	2.0	1.5	0	0

* Undiluted antiserum.

** Measurement from disc edge to margin of *Spiroplasma* growth. Averages from two sets of rabbit antisera.

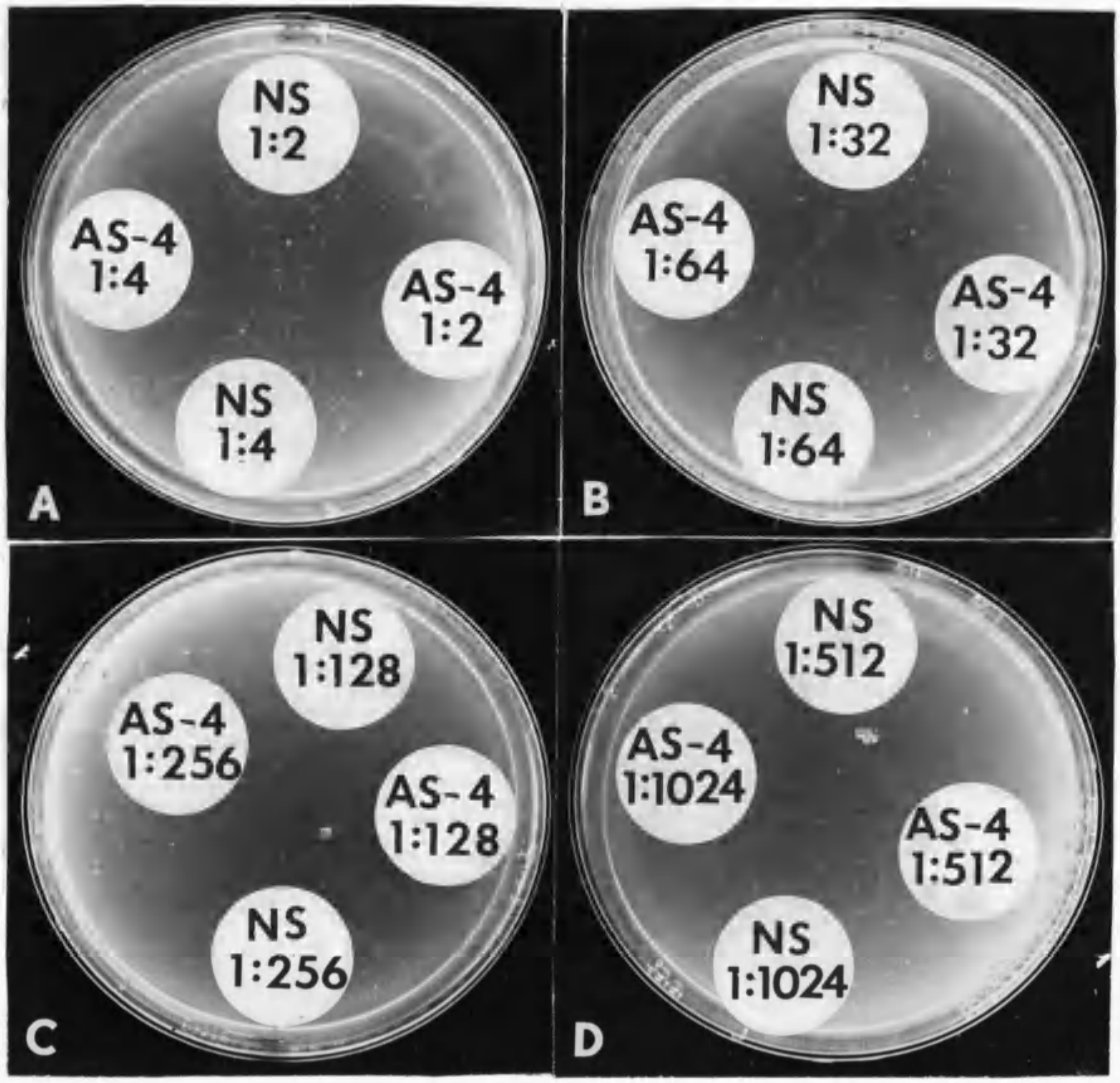

Figure 1. Photographs of growth inhibition test plates showing the results of homologous trials. -- These agar plates were inoculated with 0.2 ml (2 x 10^4 CFU) of a 3-4 day-old culture of isolate 4 of Spiroplasma. Discs were impregnated with 50 μl of antiserum to isolate 4 (AS-4) or normal serum (NS) at indicated dilutions. Plates were incubated 10-14 days at 32 C.

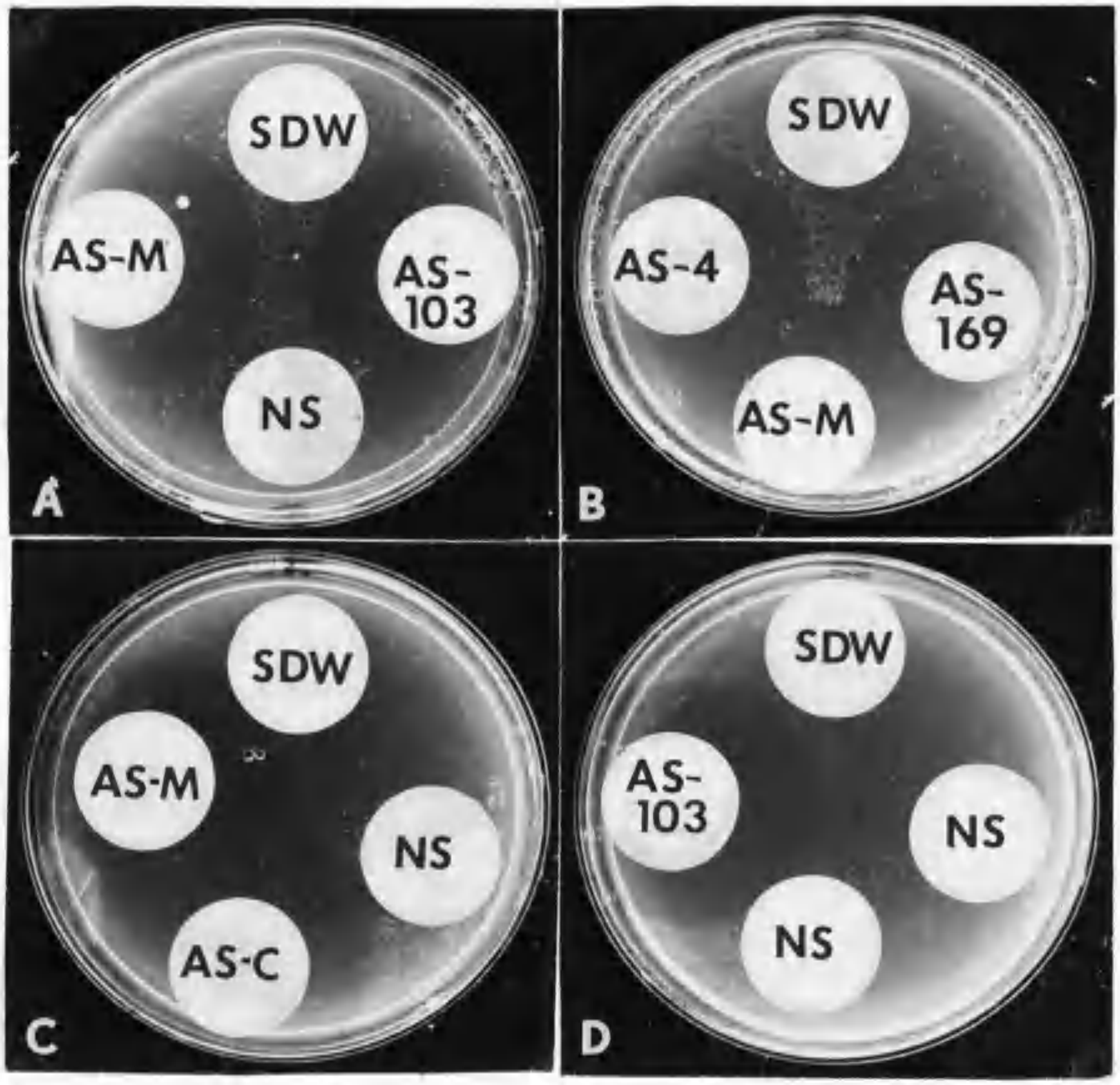

Figure 2. Photographs of growth inhibition test plates showing the results of cross-reaction trials. -- These agar plates were inoculated with 0.2 ml (2 x 10^4 CFU) of a 3-4 day-old culture of isolate 4 (A, B) or the California strain (C, D) of Spiroplasma. Discs were impregnated individually with 50 μl of antiserum (AS), normal serum (NS) or sterile distilled water (SDW) as indicated. The antisera used were those to: isolate 103 (AS-103), Morocco strain (AS-M), California strain (AS-C), isolate 169 (AS-169), and isolate 4 (AS-4). Plates were incubated 10-14 days at 32 C.

Table 3. Inhibition of growth of five *Spiroplasma* isolates by their homologous and heterologous antisera from one set of rabbits.

Isolate Identity	Undiluted Antisera to *Spiroplasma* Isolates Used to Impregnate Discs				
	103	4	Morocco	California	169
	Mean Width of Inhibition Zone (mm)*				
103	12.5(±2.2)**	11.4(±0.9)	13.0(±2.3)	13.0(±2.0)	12.7(±2.0)
4	8.0(±0.8)	8.2(±1.3)	7.8(±0.9)	7.7(±0.8)	7.9(±0.7)
Morocco	8.7(±1.3)	8.0(±0.7)	8.1(±0.6)	8.3(±0.5)	8.3(±1.1)
California	8.7(±0.4)	7.7(±0.4)	7.8(±0.4)	7.8(±0.6)	7.7(±0.8)
169	9.0(±0.7)	8.5(±0.6)	7.8(±1.2)	8.3(±1.1)	8.0(±1.0)

* Measurement from disc edge to margin of *Spiroplasma* growth. Averages from 3-5 replicates.

** Standard deviation.

These data show that all of the antisera, regardless of antigen strain, were capable of inhibiting growth of all the isolates tested. Inhibition zones ranged between 7.7-13.0 mm. All five antisera caused similar zones of inhibition when tested against four of the isolates (4, 169, and the Morocco and California strains) but these same antisera caused 34-68 percent larger zones of inhibition for isolate 103.

For further emphasis of the relationship among the five *Spiroplasma* isolates, the results in Table 3 were calculated by the formula of Archetti and Horsfall (6) as modified by Clyde (12). The degrees of relationship among these five isolates are shown in Table 4. The antigenic relatedness among five *Spiroplasma* isolates ranged between 94-107 percent, indicating no antigenic differences among them. Therefore, the five *Spiroplasma* isolates appear to be closely related antigenically, as judged by results from the growth inhibition tests.

Metabolic Inhibition Test

The medium in control vials containing either isolate 103 or 169 without antiserum always showed significant color change occurring 2-3 days later than those of isolate 4 or the Morocco and California strains. Therefore, isolates 103 and 169 were considered as slow growing organisms.

Growth of *Spiroplasma citri* was not inhibited by any of the various concentrations of normal serum. In fact, the growth of the organisms was apparently accelerated by normal serum because the media containing *Spiroplasma* and normal serum showed color changes occurring

Table 4. Antigenic relatedness among five *Spiroplasma* isolates indicated by results of growth inhibition tests converted to antibody titer ratios and expressed as percent relationships.*

Antigen Identity	Antiserum Identity: 103	4	Morocco	California	169
103	(100)**				
4	94	(100)			
Morocco	105	97	(100)		
California	107	96	100	(100)	
169	106	100	99	100	(100)

* The relationships among isolates were calculated by the formula of Archetti and Horsfall (6) as modified by Clyde (12).

** By definition, a homologous antiserum-antigen reaction receives a rating of 100 percent. Parentheses indicate homologous titers.

earlier (24-48 hours) than the controls containing *Spiroplasma* without normal serum.

In these metabolic inhibition tests, vials of medium showing orange to yellow color indicated that the added antiserum at that particular dilution failed to inhibit growth of *Spiroplasma*. Vials of medium remaining red (the original color of the medium) indicated that the added antiserum at that particular dilution contained sufficient and appropriate antibodies to inhibit growth of the organism. For example, reactions of isolates 4 and 103, both from Arizona sources, in the presence of different antisera at various antiserum dilutions, are shown in Figures 3 and 4. The highest dilution of antiserum preventing a color change from red to orange or yellow represented the titer of the antiserum. For example, as shown in Figure 3(A), isolate 4 was reacted with various dilutions of antiserum to isolate 103. The vials of medium containing antiserum 103 at dilutions less than 1:400 remained the original red color. Media in vials containing antiserum 103 at dilutions of 1:800 to 1:51,200 became yellow. Therefore, the heterologous titer of isolate 4 against antiserum 103 was 400.

The data obtained from three repeated tests were approximately the same. The geometric means (geometric mean of three numbers is the cube root of their product) of the results of these three tests are shown in Table 5. These results demonstrate that isolate 4 and the Morocco and California strains are closely related because their homologous titers, and the heterologous titer obtained from

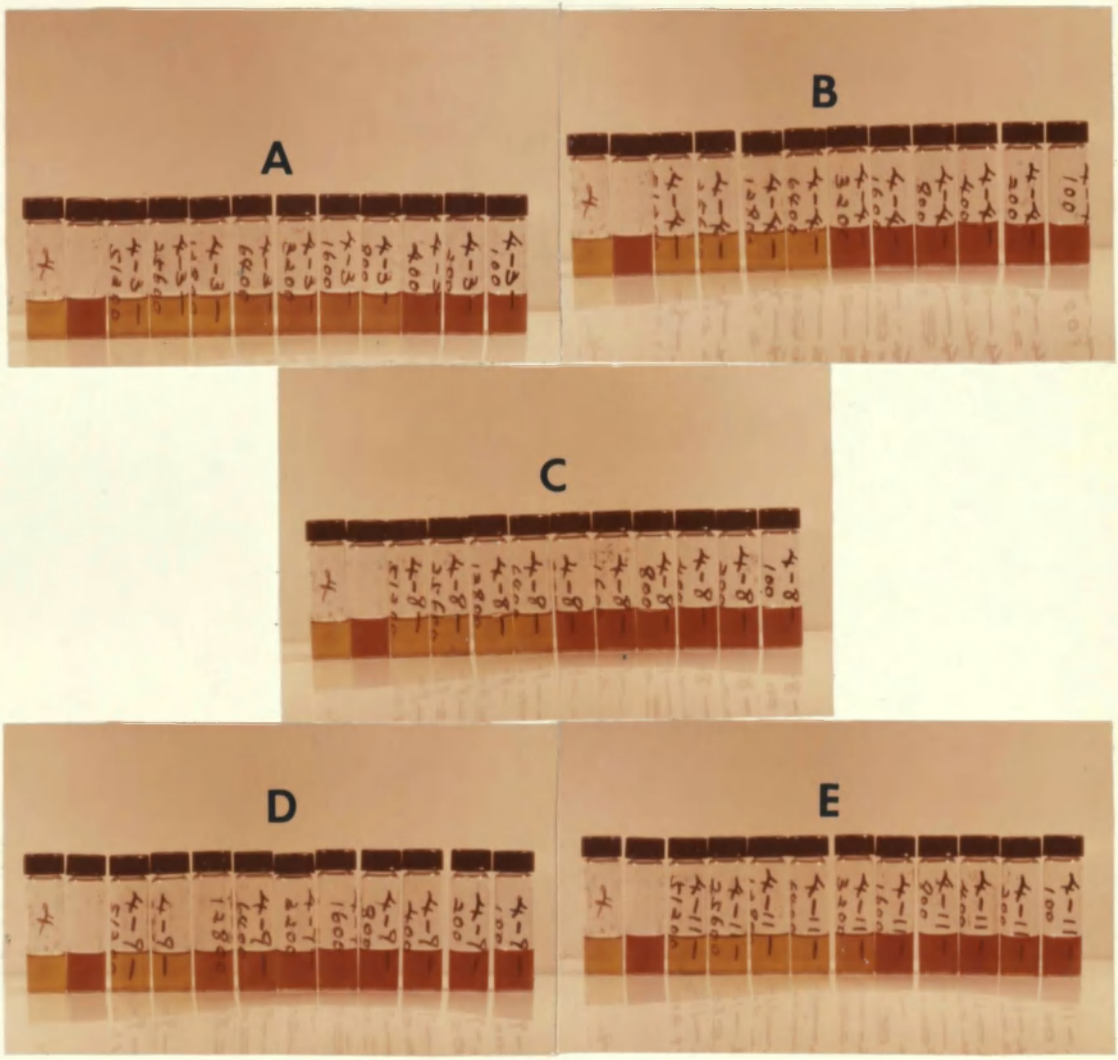

Figure 3. Photographs of metabolic inhibition test vials showing the results of homologous and heterologous reactions using isolate 4. -- Vials containing 4.5 ml of medium were inoculated with 0.5 ml (5.0-6.0 x 10^5 CFU) of a 3-4 day-old culture of isolate 4. In addition, 50 μl of serial two-fold dilutions of homologous or heterologous antiserum were added in each vial. (A) AG-4 with AS-103; (B) AG-4 with AS-4; (C) AG-4 with AS-M; (D) AG-4 with AS-C; (E) AG-4 with AS-169. Vials were incubated 7-10 days at 32 C.

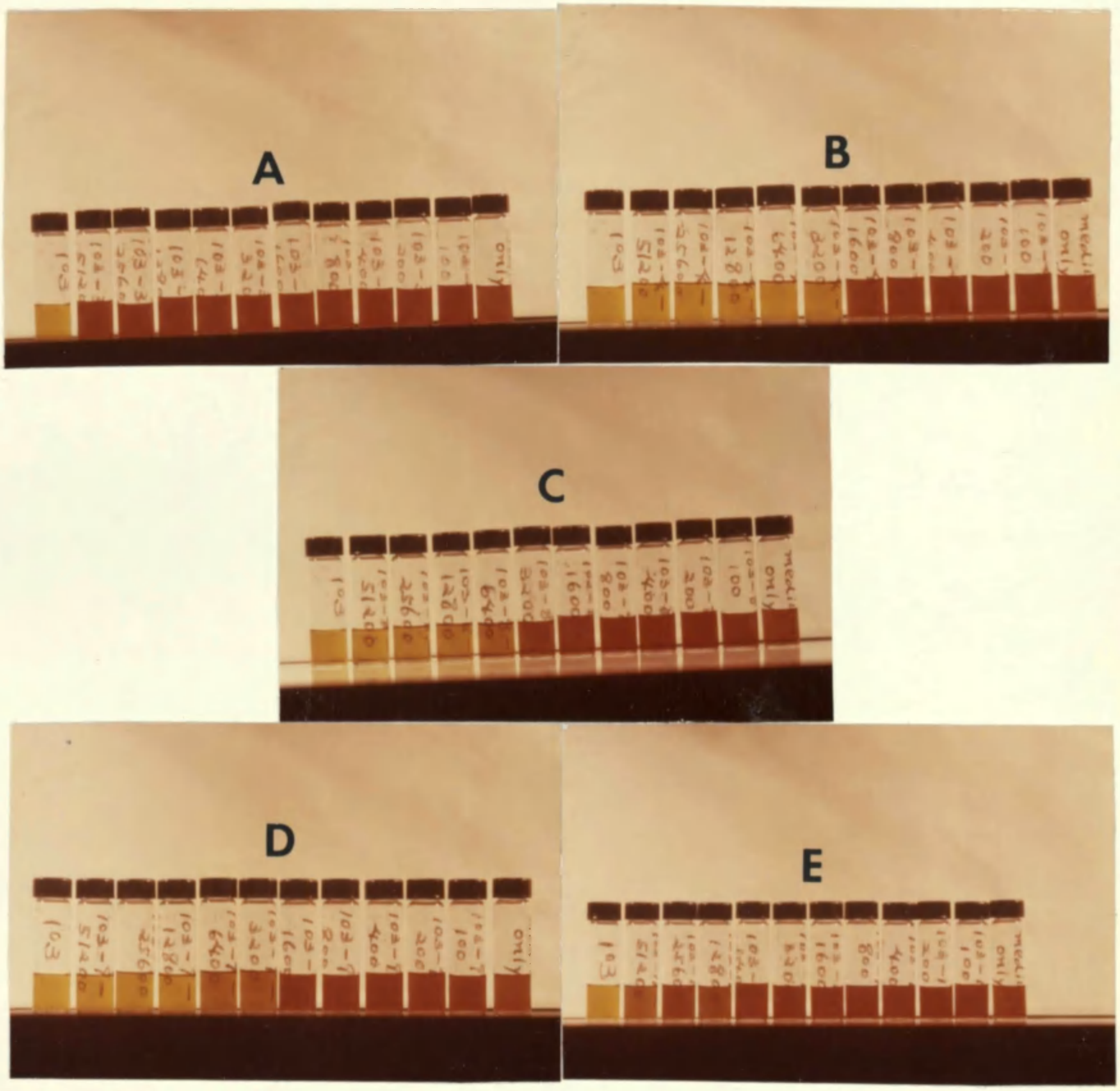

Figure 4. Photographs of metabolic inhibition test vials showing the results of homologous and heterologous reactions using isolate 103. -- Vials containing 4.5 ml of medium were inoculated with 0.5 ml ($5.0\text{-}6.0 \times 10^5$ CFU) of a 3-4 day-old culture of isolate 103. In addition, 50 μl of serial two-fold dilutions of homologous or heterologous antiserum were added in each vial. (A) AG-103 with AS-103; (B) AG-103 with AS-4; (C) AG-103 with AS-M; (D) AG-103 with AS-C; (E) AG-103 with AS-169. Vials were incubated 7-10 days at 32 C.

Table 5. Metabolic inhibition titers of antisera from five isolates of *Spiroplasma citri* against their homologous and heterologous antigens.

Antigen Identity	Antiserum Titers* 103	4	Morocco	California	169
103	(51200)**	1008	1600	2016	6400
4	504	(3200)	2016	2016	2016
Morocco	400	1600	(2262)	1600	1600
California	400	3200	1600	(2262)	2262
169	1628	1600	2016	1600	(20314)

* Each titer represents the geometric mean of titers determined in three separate but identical trials.

** Parentheses indicate homologous titers.

cross-reaction to each other, ranged between 1600 and 3200; these differences are not considered meaningful.

Isolate 103, however, appears to be different from all other tested isolates because antiserum to isolate 103 inhibited its homologous antigen at a titer of 51,200 or more. In tests with other isolates, the antiserum to isolate 103 inhibited other heterologous antigens at relatively low titers of 400 to 1628.

Rather variable reactivity was shown by isolate 169. Antiserum to this isolate inhibited its homologous antigen at a titer of 20,314. However, when tested against other isolates much higher concentrations of antiserum were required (titers ranging from 1600-2262) to inhibit growth of isolate 4 or the Morocco and California strains. The considerable difference between the titers required for the homologous reaction and the heterologous reactions, suggests that isolate 169 differs serologically from the others. However, antisera to isolate 4 or the Morocco and California strains inhibited the growth of their homologous antigens as well as isolate 169 at approximately the same titers, 1600-3200 (Table 5). From these results, isolate 169 appears to be closely related to isolate 4, and the Morocco and California strains. However, when the results of both test series are compared, isolate 169 appears to be an unstable isolate.

Results of both homologous and heterologous metabolic inhibition tests, as shown by Table 5, were converted to antibody titer ratios by using the formula of Archetti and Horsfall (6) as modified by Clyde (12). These conversions, expressed by percent relationship, are shown in

Table 6. These data demonstrate not only similarities of relationship among the five isolates but also some striking differences. Table 6 shows that isolate 4 and the California strain have a 94 percent relationship and, therefore, lack antigenic differences. The Morocco strain is 67 percent related to isolate 4, and 71 percent related to the California strain. The Morocco strain, therefore, is related to both isolate 4 and the California strain but less closely than the latter two are to each other. Greater degrees of antigenic differences are shown for other pairs of isolates: 169-103 (32 percent), 169-4 (18 percent), 169-Morocco strain (27 percent), 169-California strain (28 percent). Even more marked differences are noted when isolate 103 is compared with isolate 4 and the Morocco and California strains; these relationships are quite low and are expressed as 6 percent, 7 percent, and 8 percent, respectively.

Ouchterlony Double Diffusion Tests

Standard Test

Close serological relationships among all isolates tested are shown by Figures 5 and 6. In each comparison of pairs of isolates (i.e., isolates in adjacent wells) there are shared precipitin lines. In each instance, however, each isolate, when reacted against each of the other isolates, showed one or more antigenic differences by (a) forming at least one precipitin line not formed by its counterpart, (b) forming lines of partial identity (spurs), and/or (c) forming lines of non-identity. Sharing of precipitin lines indicates commonness of

Table 6. Antigenic relatedness among five <u>Spiroplasma</u> isolates indicated by results of metabolic inhibition tests converted to antibody titer ratios and expressed as percent relationships.*

Antigen Identity	Antiserum Identity				
	103	4	Morocco	California	169
103	(100)**				
4	6	(100)			
Morocco	7	67	(100)		
California	8	94	71	(100)	
169	32	18	27	28	(100)

* The relationship among isolates were calculated by the formula of Archetti and Horsfall (6) as modified by Clyde (12).

** By definition, a homologous antiserum-antigen reaction receives a rating of 100 percent. Parentheses indicate homologous titers.

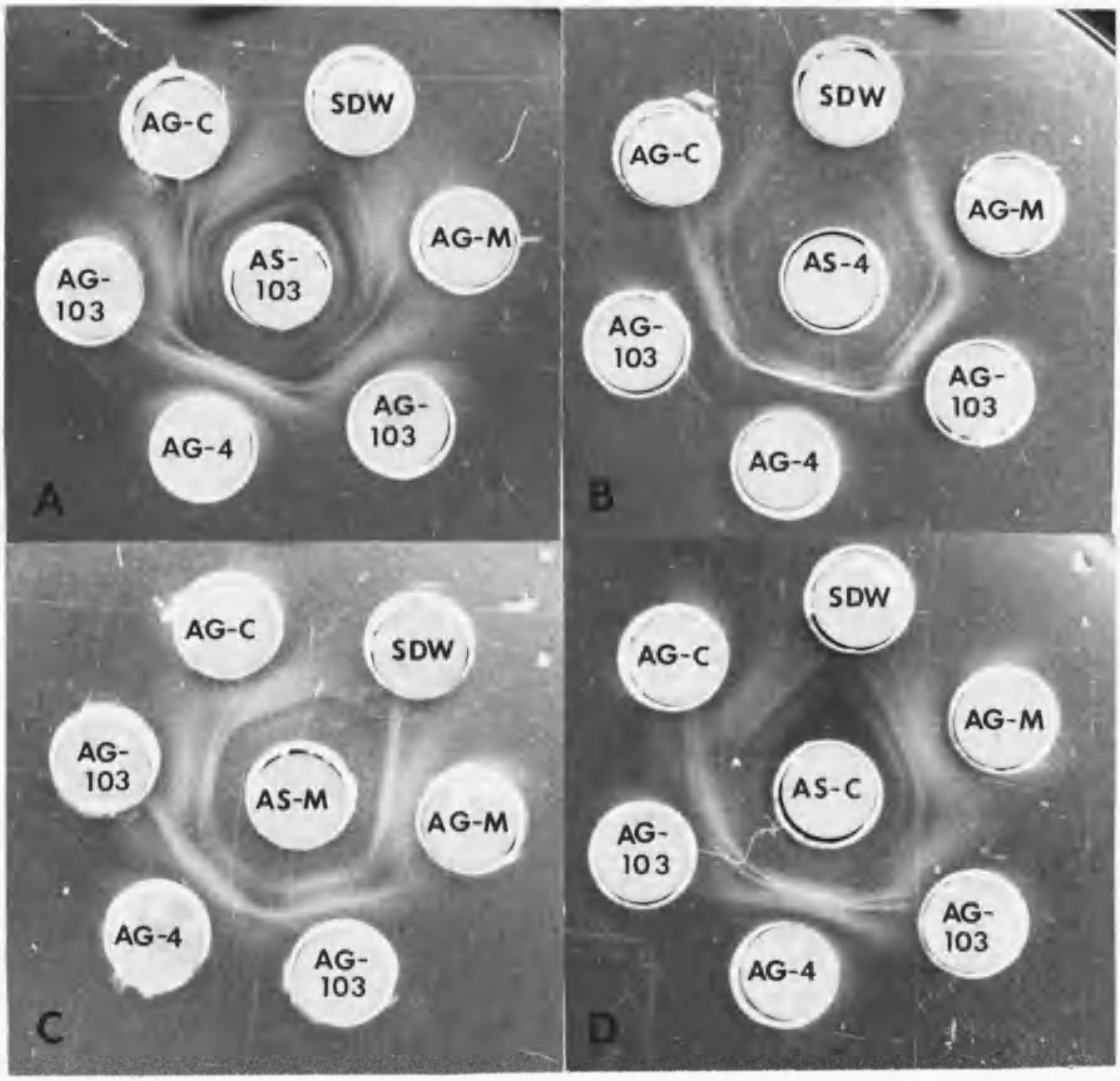

Figure 5. Photographs of precipitin line patterns developed in standard Ouchterlony double diffusion tests (A through D) after 24-48 hours incubation at 25 C. -- Undiluted antiserum (AS) to the indicated <u>Spiroplasma</u> isolate was placed (50 μl) in the central well; sonicated antigen (AG) suspensions (1.0 mg protein/ml), as indicated, were placed (50 μl) into outer wells.

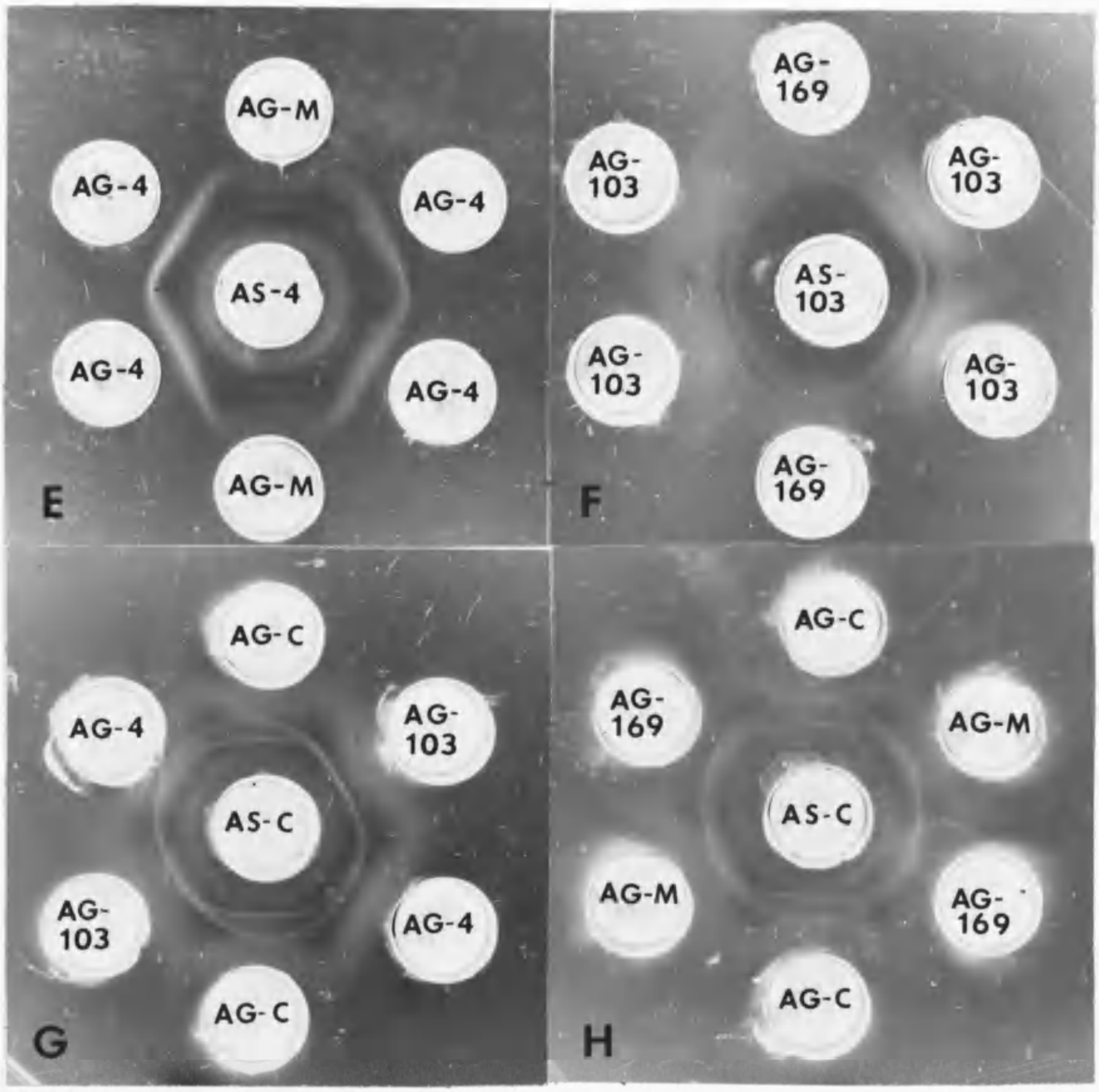

Figure 6. Photographs of precipitin line patterns developed in standard Ouchterlony double diffusion tests (E through H) after 24-48 hours incubation at 25 C. -- Undiluted antiserum (AS) to the indicated Spiroplasma isolate was placed (50 μl) in the central well; sonicated antigen (AG) suspensions (1.0 mg protein/ml), as indicated, were placed (50 μl) into outer wells.

antigenic components and, therefore, provides evidence of relatedness. Since each isolate, however, had one or more antigenic components not found in any of the other isolates each must be considered a different entity.

With antiserum to isolate 103 (AS-103), isolate 4 (AG-4) shares one precipitin line with AG-103 but both antigens each produced an unshared line. Morocco strain (AG-M) shares one line with AG-103; one line of partial identity was formed. AG-103 and the California strain (AG-C) formed one identical line and one of partial identity; AG-103 formed one line more than AG-C (Figure 5A). The reciprocal test (Figure 5B), with AS-4, shows AG-4 and AG-103 forming one identical line and one of partial identity; AG-4 produced one line more than AG-103. In Figure 5C, with AS-M, AG-103 and AG-M formed one identical line and one of partial identity. With AS-C, AG-103 shares one precipitin line with AG-C but AG-103 lacks one line near the central well (Figure 5D). These results indicate AG-103, AG-4, AG-M and AG-C are related but are not identical.

With AS-4, AS-M shares one precipitin line with AG-4; AG-4 formed one line more than AG-M (Figure 6E). With AS-103, AG-169 shares one precipitin line with AG-103 but lacks another line produced by AG-103 (Figure 6F). These results indicate AG-4 and AG-M; AG-103 and AG-169 also are related but are not identical.

With AS-C, AG-4 and AG-103 share one precipitin line with AG-C; AG-C, however, had one extra line near the central well (Figure 6G). With AS-C, AG-M and AG-169 share one precipitin line with AG-C; AG-C formed one line more than AG-M and AG-169 (Figure 6H). These results indicate AG-C, AG-M and AG-169; AG-C, AG-103 and AG-4 are related but lack identicalness.

Cross-Absorption Test

While the major portion of the complex antigen-antibody system for each Spiroplasm isolate appears to be common to the others, each isolate possesses a unique, though apparently minor, antigen-antibody system that is not cross reactive to the other isolates. This is illustrated in Figures 7 and 8 where all common antibodies are removed by cross-absorption leaving those unique to each isolate free to react specifically with the homologous antigen.

In Figure 7A, AG-4 possessed one precipitin line which was not present in AG-M. In Figure 7B, AG-C possessed two precipitin lines which were not shared with AG-M. These results indicate that AG-M, AG-4, and AG-C are different.

In Figure 7C, AG-103 possessed one precipitin line which was not present in AG-C (the faint lines near the three wells containing AG-C, are caused by the bending of two adjacent precipitin lines). In Figure 7D, AG-103 possessed one precipitin line which was not shared with AG-M. These results indicate that AG-103, AG-C, and AG-M also are different.

One precipitin line unique to AG-M was not associated with AG-169 (Figure 8E). AG-103 had one specific precipitin line which was not present for AG-169 (Figure 8F). These results indicate differences between AG-169, AG-M, and AG-103.

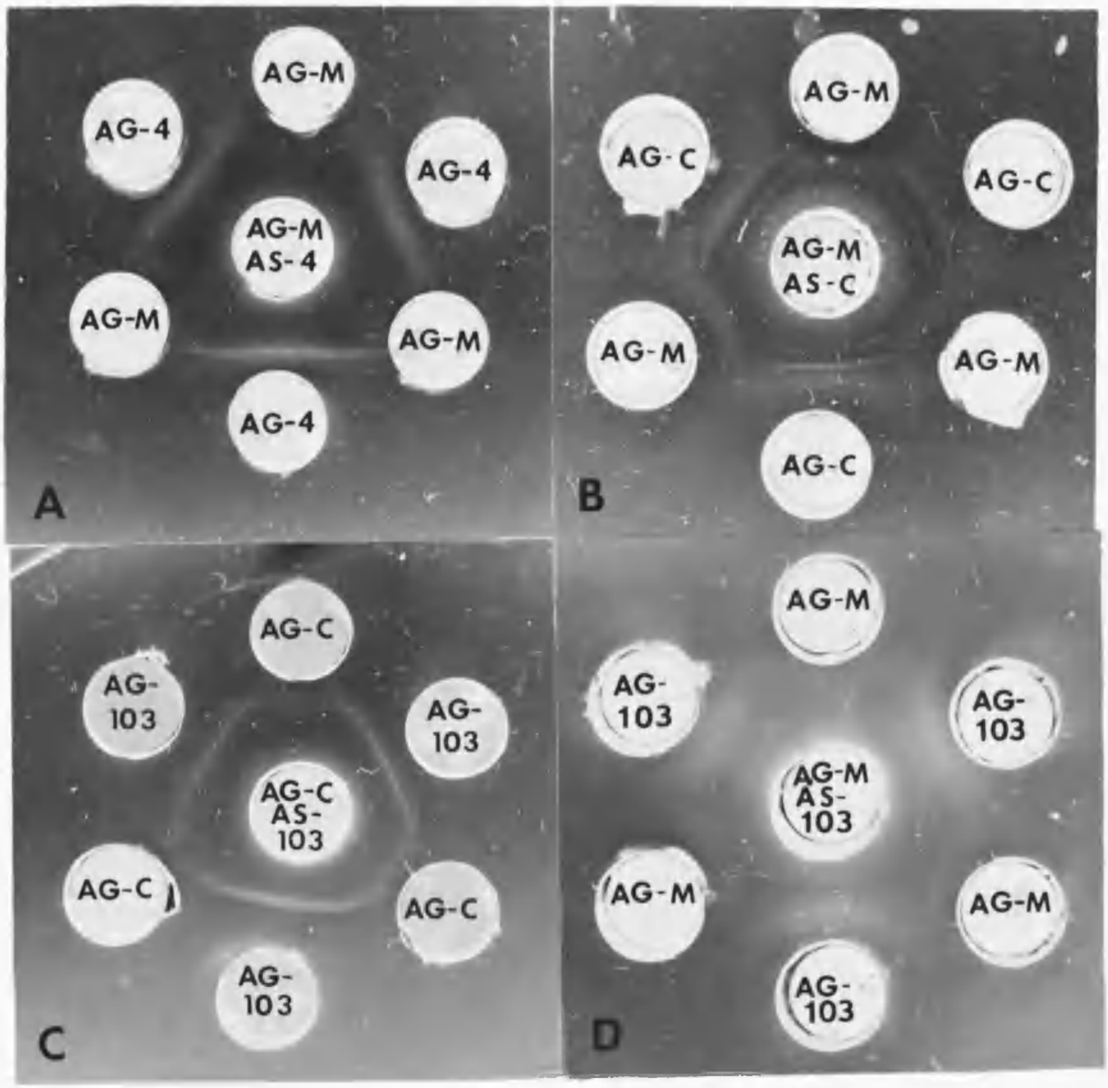

Figure 7. Photographs of precipitin line patterns developed in cross-absorption tests (A through D) after 24-48 hours incubation at 25 C. -- Sonicated antigen (AG) suspensions (1.0 mg protein/ml), as indicated, were initially placed (50 μl) into central well; then 18 hours later, undiluted antiserum (AS) to the indicated Spiroplasma isolate was placed (50 μl) in the central well; meanwhile, sonicated antigen suspensions, as indicated, were placed (50 μl) into outer wells.

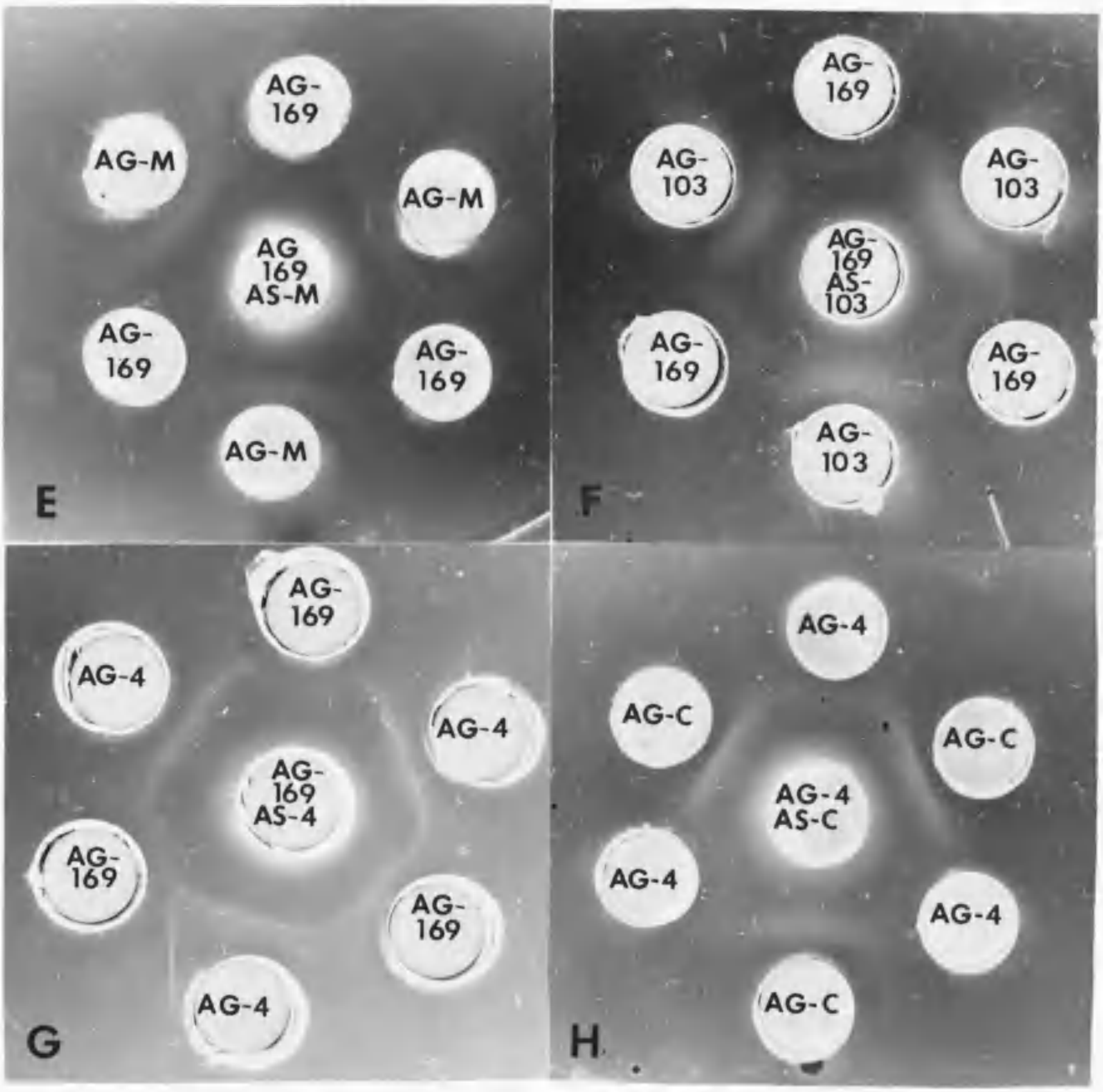

Figure 8. Photographs of precipitin line patterns developed in cross-absorption tests (E through H) after 24-48 hours incubation at 25 C. -- Sonicated antigen (AG) suspensions (1.0 mg protein/ml), as indicated, were initially placed (50 μl) into central well; then 18 hours later, undiluted antiserum (AS) to the indicated <u>Spiroplasma</u> isolate was placed (50 μl) in the central well; meanwhile, sonicated antigen suspensions, as indicated, were placed (50 μl) into outer wells.

In Figure 8G, AG-4 possessed one precipitin line which was not present in AG-169 (lower right, near the AG-169 showed a faint line which is caused by the bending of two adjacent precipitin lines). There is one precipitin line unique to AG-C which was not present for AG-4 (Figure 8H). These results indicate AG-169,AG-4, and AG-C are different.

The results obtained from combined standard and cross-absorption tests, therefore, show that each of the five Spiroplasma isolates possesses different antigenic components and must be considered to have serologically distinct antigenic sites.

Attempted Detection of Bacteriophage Antibodies

Purified bacteriophage suspension and each of the five Spiroplasma isolates were used at least twice against each antiserum. In no case was a precipitin line observed near the well that contained bacteriophage suspension although there always was a precipitin line near each of the control wells which contained the sonicated antigen of each of the five Spiroplasmas (Figure 9).

SDS added to the Noble agar had no effect upon the bacteriophage suspension; no precipitin line was ever observed near the well that contained bacteriophage.

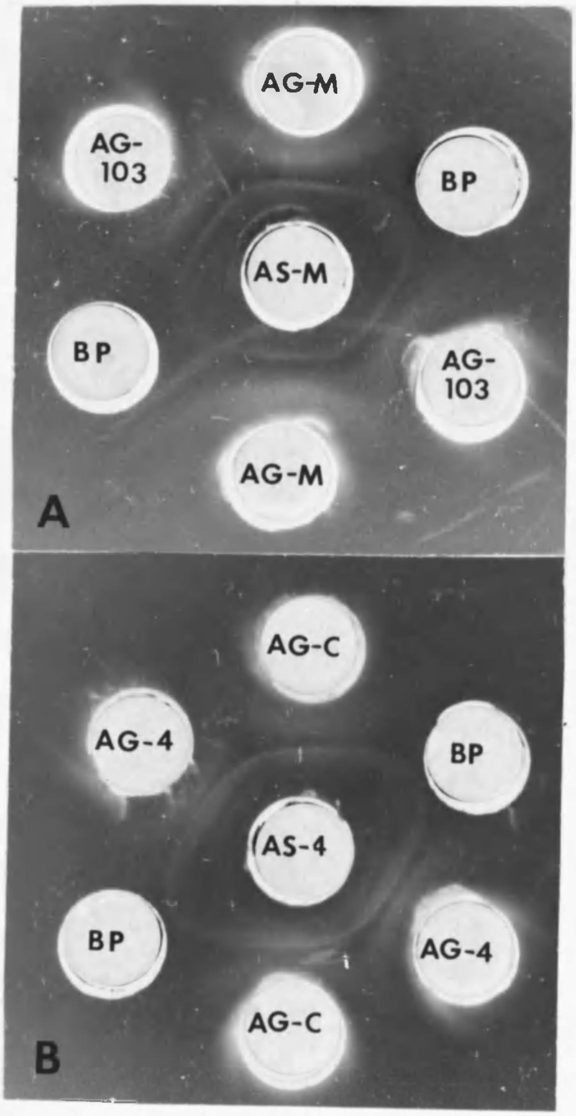

Figure 9. Photographs of precipitin line patterns showing the results of attempted detection of bacteriophage antibodies after 24-48 hours incubation at 25 C. -- A. and B.: Undiluted antiserum (AS) to the indicated Spiroplasma isolate was placed (50 µl) in the central well; sonicated antigen (AG) and bacteriophage (BP) suspensions (1.0 mg protein/ml), as indicated, were placed (50 µl) into outer wells.

DISCUSSION

The five isolates of *Spiroplasma citri* obtained from different geographical areas and host species were found to be serologically related but probably antigenically different from one another.

Results obtained from growth inhibition tests showed that all five antisera caused similar zones of inhibition when tested against four of the isolates (4, 169, and the Morocco, and California strains) but these same antisera caused 34-68 percent larger zones of inhibition against isolate 103. The reason is unknown. However, when these results were analyzed by the method of Archetti and Horsfall (6) as modified by Clyde (12), the antigenic relationships among the five isolates ranged between 94-107 percent. These differences are not considered significant. Therefore, the results of the growth inhibition tests revealed no appreciable differences among the five *Spiroplasma* isolates.

Results of the metabolic inhibition tests emphasized the antigenic differences among isolates 4, 103, 169, and those strains from Morocco and California. The results obtained by the metabolic inhibition tests, when expressed as percent relationships, showed that the antigenic differences among *Spiroplasma* isolates ranged from slight to profound. On the basis of these data the five *Spiroplasma* isolates may be sorted into three main sets. Isolate 4 and the isolates from Morocco and California constitute one set because the antigenic relationships of these three isolates are between 67 to 94 percent. This set may be further divided into two subsets. Isolate 4 and the California strain showed 94 percent antigenic relationship whereas the Morocco strain was 67 percent related to isolate 4 and was 71 percent related to the California strain. These data show that the Morocco strain is less closely related to either isolate 4 or the California strain than the latter two are to each other. Therefore, isolate 4 and the California strain may be paired in one subset and the Morocco strain alone represents another subset.

Distinct differences for relationship were shown by isolate 103 when comparisons were made with isolate 4, 169, the Morocco and the California strains (6-32 percent). Isolate 103, therefore, was unique in three tests and represents a second main set. Isolate 169 also showed low antigenic relationship with isolate 4, 103, and those from Morocco and California. Thus, isolate 169 is considered to represent a third set. Strikingly enough, these data are similar to those of metabolic tests performed in this laboratory by Allen and Donndelinger (personal communication). According to their data, isolate 103 could be differentiated from isolate 4, and the isolates from Morocco and California by both arginine and glucose tests. Similarly, in those tests, isolate 169 showed a high degree of variability in reactions.

In the Ouchterlony double diffusion tests at least one precipitin line was common to all five isolates. However, lines of non-identity were also observed between these isolates in cross-absorption tests. Therefore, the five *Spiroplasma* isolates are closely related, but probably are not identical. Since it appears that critical antigenic differences between the five Spiroplasma isolates are best revealed by cross-absorption, this procedure should be included in future serological studies of these microbes.

The Morocco and California strains have been reported to be closely related as shown by growth inhibition, metabolic inhibition, precipitin, and fluorescent-antibody tests (7, 48). Bové et al. (7) also reported that the Morocco and California strains reacted differently in both hemadsorption and tetrazolium reduction tests. In my tests, the Morocco and California strains showed no significant differences in growth inhibition tests. In the metabolic inhibition tests, these two strains appeared slightly different. However, these two strains, when subjected to the Ouchterlony double diffusion tests, formed lines of non-identity. Therefore, it is concluded that while these two isolates are closely related, they are not identical.

Razin et al. (38) have reported that membranes of Spiroplasma citri contain proteins and lipids amounting to more than 80 percent of the total dry weight of the membranes. Other works have shown that much antigenic activity is associated with lipids (38, 44). Therefore, the differences among the five Spiroplasma isolates, as shown by the metabolic inhibition and Ouchterlony double diffusion tests, may be caused by the differences in membrane composition of each isolate.

The five isolates, when tested by growth inhibition, metabolic inhibition, and Ouchterlony double diffusion techniques, yielded different results. In the growth inhibition tests, the five isolates appeared to be closely related. By the metabolic inhibition tests, apparently the five Spiroplasma isolates can be divided into three sets. However, in Ouchterlony double diffusion tests, all five isolates seem to differ from one another.

The differences shown by the results of these three tests may be a matter of what particular antigen-antibody reaction is measured by these specific techniques. First, when the same antiserum was measured by both growth inhibition and metabolic inhibition techniques, the metabolic inhibition method appeared to be much more sensitive than the growth inhibition technique. The titer of each of the antisera was approximately 10 times higher in the metabolic inhibition than in the growth inhibition test. This means that fewer antibodies were needed to inhibit the metabolic activity of the five Spiroplasma isolates than were needed to inhibit colony development. Therefore, the growth inhibition test is considered to be a much less sensitive test. Such a conclusion is in agreement with that mentioned by other workers (27, 33, 46). Second, the most plausible reason for the difference in sensitivity of the two tests is that in the metabolic inhibition test the antiserum is in direct contact with Spiroplasma units, whereas in the growth inhibition test the antiserum effect is reduced because it must diffuse through the agar in order to react with the antigen. Third, the reactive sites of inhibitory action for both growth inhibition and metabolic inhibition antibodies are on the cell membrane of the organism (28, 30, 35, 51, 53). Purcell et al. (35) suggested, however, that it is possible that metabolic inhibition antibodies may enter the organism and exert an inhibitory effect at certain intracellular sites. Similar conclusions were reached by Eagle (19), who studied contact inhibition among human diploid fibroblasts in tissue culture. He suggested that changes occurring at the cell surface eventually affect the metabolism of the inner cell by decreasing the rate of macromolecular synthesis, and consequently inhibiting the cell metabolism. Therefore, the results of the metabolic inhibition reaction must be more specific than those in the growth inhibition test. Fourth, Purcell et al. (35) have suggested that the Ouchterlony double diffusion tests involve both internal and surface antigens because, for that test, sonicated antigens are used. In growth inhibition and metabolic inhibition tests whole Spiroplasma cells were used to react with the antiserum.

Therefore, it is my conclusion that the results obtained by each of these techniques vary because of substantive differences in the test systems themselves.

In the attempted detection of bacteriophage antibodies, no reaction occurred between the bacteriophage suspension and each of the five antisera. Accordingly, it is difficult to tell if there are actually antibodies to bacteriophages in the antisera or not because I did not have antiserum produced by purified bacteriophage suspension as a control. However, based upon the foregoing results, preliminary indications are that the antisera used in this study probably do not contain antibodies to any of the three reported forms of bacteriophage (13, 14). Therefore, the differences among the five Spiroplasma isolates, as shown in the metabolic inhibition and Ouchterlony double diffusion tests, are most likely directly related to specific antigenic components of the isolates themselves.

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