Serological Classification of Spiroplasmas: Current Status

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Data concerning serological classification of spiroplasmas are in good agreement, but slightly different numerical designations have been given to existing groups. It is proposed that a standardized system be adopted based on information developed mainly by the IRPCM working team on spiroplasmas. The type species (Spiroplasma citri) should be redefined to include only the agent of citrus stubborn disease (subgroup I-1). Six other subgroups, including three proposed by Bové et al. in this volume (I-5, I-6, and I-7), are members of the Group I complex. Because subgroups I-1, I-2, and I-3 (1) show significant reciprocal differences in DNA-DNA homology and two-dimensional electrophoretic protein profiles, (2) occupy exclusive habitats, (3) are each associated with important diseases, and (4) consist of clusters of very similar or identical strains, it is suggested that Latin binomials could be assigned to subgroups I-2 and I-3. It is proposed that those criteria could serve as general guidelines for consideration of subgroups for species status in the class Mollicutes. The I-4 subgroup is assigned an uncertain status, pending comparisons with the LB-12 (I-5), M55 (I-6), and N525 (I-7) subgroups. To previously described serogroups we add the CN-5 Cotinus beetle spiroplasma (IX), the AES-1 mosquito strain (X), and the MQ-4 Monobia strain (XI).

INTRODUCTION

Although spiroplasma serology had its beginnings in early studies on the SRO [1] and SMCA [2] organisms, it was not until 1973 that comparative studies [3] were initiated to assess strain relatedness. Since that time, many studies have been performed and three major serological methods—growth inhibition [4], deformation [5, 6], and metabolic inhibition [6]—have proved to be of crucial importance in determining spiroplasma interrelatedness.

Studies on spiroplasma serology have been so numerous that competing classification have emerged. One of these arose from the efforts of international working teams organized by the Subcommittee on Taxonomy of Mollicutes [7] and the International Research Programme on Comparative Mycoplasmology (IRPCM), in which serological results were correlated with data on the spiroplasma genome [8] and polyacrylamide gel electrophoresis (PAGE) [9, 10]. In 1980, this effort culminated in an original proposal [11] for recognition of five major groups and four subgroups. In 1982 this classification was updated [12]. A similar effort [15] was mounted in response to the discovery of spiroplasmas in flower habitats [13, 14] and was later complemented with molecular genetic data [16]. Fortunately, data

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from these studies have been mutually supportive, although there have been differences in interpretation in terms of spiroplasma taxonomy.

In this report we propose that the scheme of Junca et al. [11], as amended and updated in [12] and herein, be adopted as a single standard system. We propose certain new criteria for recognition of major groups and candidate species heretofore recognized only as subgroups, recognition of three new subgroups of the S. citri complex, and recognition of three new serogroups.

METHODS

Deformation

The deformation (DF) test was performed essentially as described by Williamson and his colleagues [5,6]. However, modified procedures for reading results were employed for certain spiroplasmas. Endpoints in classical DF tests are assessed by estimating the dilution of antiserum at which 50 percent of helical, otherwise nondeformed filaments are modified by specific antiserum. At low serum concentrations, blebs are formed on the helical filaments whereas at higher concentrations all traces of helicity are destroyed. Deformation of helical spiroplasmas may also involve formation of aggregates, which may have blebs as their nuclei. Unfortunately, in serotyping isolates that are growing poorly, or in performing any test on organisms such as the Y32 spiroplasma (group VI) that are essentially nonhelical [19], the tests must be interpreted in a different way. Nonhelical filaments, such as those produced by S. citri in stationary phase, or Y32 at all phases, are also deformed by antiserum. At low titer, single blebs are observed; at high titer, multiple blebs are formed, so that the cells may appear "bunchy," much larger than normal, and completely nonfilamentous. Considerable experience may be required in reading results with these, or certain other spiroplasmas, which (while never completely losing helicity) may nevertheless fail to form typical helices or may rapidly lose the helical morphology characteristic of the logarithmic phase of growth. In all of these cases, we were able, with experience, to read and interpret DF endpoints. Although endpoints involving nonhelical cells can be determined, helical cells in logarithmic growth are still greatly preferred for deformation tests.

Growth Inhibition

Growth inhibition (GI) tests were performed on appropriate solid media (MID, SP-4, or conventional mycoplasma media) that depended on the cultured organisms [4]. Ideal conditions for the test were provided by cultures diluted to about 10^6 colony-forming units/ml. In practice, however, difficulties were encountered with spiroplasmas that formed diffuse colonies. In some cases, as with group VII organisms, motile filaments moved readily through the solid medium and the vaguely defined diffuse colonies could be discerned only by observing the plates with light transmitted through the agar. Since single colonies of such spiroplasmas could be as wide as 3-4 mm, many fewer colonies were required on each plate to provide a satisfactory reading. Such fast-growing spiroplasmas were inhibited by specific antibodies as effectively as slower growing organisms, and the inhibition zones were always clear. Organisms that were poorly helical or nonhelical in liquid medium, such as Y32 and related strains, tended to form umbonate colonies and could be studied by classical means without modification. For strains that formed poor colonies or often failed to form colonies e.g., group I-5 (LB-12), it was important to use clones that had been selected for their colony-forming ability.
Metabolism Inhibition

Techniques for performing metabolism inhibition (MI) tests, in conjunction with deformation, were described by Williamson et al. [6]. Classical procedures, involving the use of guinea pig complement, proved to be appropriate for all but one of the spiroplasmas studied. Complement has apparently not been used by all workers but has been shown to greatly enhance cellular killing of spiroplasmas mediated by specific antibody [6]. In the case of the Y32 organisms, complement itself had high antispiroplasmal activity, so tests involving this organism were performed with antibody alone.

RESULTS

Levels of Discrimination

The GI, DF, and MI tests all discriminated between serogroups (and therefore species [12]), but had different levels of discrimination. These could be best interpreted by estimating relationships in terms of the fraction of observed homologous reactivity of each of the antigens. Results for the seven subgroups of Group I are given in Table 1. The growth inhibition test was the least discriminatory, in that zones of substantial size [4] were obtained with organisms such as the corn stunt and citrus stubborn spiroplasmas, which are known, by independent analysis of the genome [8] and protein profiles [9,10], to be disparate. It was recently suggested [4] that the GI test was therefore suitable for discrimination at the species level. Within strains of the Group I complex, relative zone widths [4] reflected the amount of DNA-DNA hybridization [8] that had been observed among the organisms. Of course, the spiroplasmal genomes studied in hybridization experiments control many phenotypic characters other than membrane protein antigenicity. The defor-

| Anti-serum | Growth Inhibition | Deformation | Metabolic Inhibition |
|------------|-------------------|-------------|---------------------|
|            | I-1   I-2  I-3  I-4 | I-1   I-2  I-3  I-4 | I-1   I-2  I-3  I-4 |
| Subgroups 1-4* |       |             |                   |
| I-1        | 1.00  .79* .57  .06 | 1.00  .25* n*  n   |
|            |       |             |                   |
| I-2        | .89   1.00 .79  .14 | .12   1.00  .03  .01 |
|            |       |             |                   |
| I-3        | .68   .40  1.00 .61 | .03   .02  1.00  .03 |
|            |       |             |                   |
| I-4        | .09   .00  .17  1.00 | n     n     .02  1.00 |
|            |       |             |                   |
| I-4        |       |             |                   |
| I-5        |       |             |                   |
| I-6        |       |             |                   |
| I-7        |       |             |                   |
| I-4        | 1.00  .50  .77  .54 | 1.00  .03  .25  .13 |
|            |       |             |                   |
| I-5        |       |             |                   |
| I-6        |       |             |                   |
| I-7        |       |             |                   |
| I-4        |       |             |                   |
| I-5        |       |             |                   |
| I-6        |       |             |                   |
| I-7        |       |             |                   |

*Representative strains: R8A2 (I-1); BC-3 (I-2); E275 (I-3); 277F (I-4); LB-12 (I-5); M55 (I-6); N525 (I-7)

a Ratio of zone widths in cross between indicated antigen and heterologous antiserum; homologous cross assigned ratio of 1.00

b Ratio of titers in cross between indicated antigen and heterologous antiserum; homologous cross assigned ratio of 1.00

c n = negative. Defined in this table as <0.005
mation test was of intermediate discriminatory value. Homologous reactions were invariably much stronger than heterologous crosses, which were not more than 25 percent of homologous crosses in any case. Metabolic inhibition provided the finest levels of discrimination. No more than 4 percent cross-reactivity was observed in any heterologous cross among the Group I strains. This circumstance might be explained by the very high homologous titers (as high as 353,000) that were obtained. In comparison with such high homologous values, even titers as high as 1,458 could represent minor heterologous reactions. In certain cases, titers of the magnitude of 162–1,458 were observed between spiroplasmas with no other evident affinity. We do not know whether such cross-reactions, which were often only one way, represent clues to interspecific groupings or were nonspecific.

Present Serological Classification

An updated serological classification, with brief descriptions of representative strains and appropriate literature citations, has recently been published [12]. In this paper, we present serological data (Table 1) that supports the recognition of three new subgroups proposed by Bové et al. in this volume (I-5, I-6, and I-7). We also present new serological data (Table 2), which permit recognition of three new serogroups (IX, X, and XI). The revised classification is as follows:

Group I. *Spiroplasma citri* complex. We suggest use of the word “complex” when dealing with subgroups other than I-1.

Group I-1. *S. citri*: Classical subgroup. *Citrus stubborn spiroplasma*. Type strain R8A2 = Maroc = ATCC 27556

Group I-2. *Honeybee spiroplasma*. Representative strain BC-3 (ATCC 33219). The AS 576 strain (ATCC 29416) is similar or identical.

Group I-3. *Corn stunt spiroplasma*. Representative strains E275 (ATCC 29320) and I-747 (ATCC 29051)

Group I-4. Representative strain 277F (ATCC 29761)

Group I-5. *Green leaf bug spiroplasma*. Representative strain LB-12 (ATCC 33469) is from the green leaf bug, *Trigonotylus ruficornis*, in Taiwan.

Table 2

| Antiserum | Group | Strain | I-VIII | IX | X | XI |
|-----------|-------|--------|--------|----|----|----|
|           | I-VIII|        |        |    |    |    |
|           | IX    | CN-5   | p*     | n* | n  | n  |
|           |       |        |        | 8;| n  | n  |
|           |       |        |        | 640|    |    |
|           |       |        |        | 13,000|   |    |
|           | X     | AES-1  | n      | n  | 5;| n  |
|           |       |        |        | 640|    |    |
|           |       |        |        | 4,374|   |    |
|           | XI    | MQ-4   | n      | n  | n  | 6;|
|           |       |        |        | 640|    |    |
|           |       |        |        | 4,374|   |    |

*Results from reciprocal crosses with serogroups I-(VIII) were published [11].

*n* = negative. All crosses between group (IX), (X), and (XI) antigens or antisera with the previously described serogroups were negative.

†Homologous titers expressed as: mm of growth inhibition/deformation endpoint titer/metabolism inhibition titer.
Group I-6. *Maryland flower spiroplasma.* Representative strain M55 (ATCC 33502). Isolated from fall flowers in Maryland

Group I-7. *Cocos spiroplasma.* Isolated from surfaces of coconut palm in Jamaica. Representative strain N525 (ATCC 33287)

Group II. *Sex ratio organisms* (SROs). Noncultivable agents that induce the "sex-ratio" trait in *Drosophila*

Group III. *Spiroplasma floricola.* Type strain 23-6 (ATCC 29989). From flowers in Maryland, and "Lethargie" diseased *Melolontha* beetles

Group IV. *Spiroplasma apis.* Type strain B31 (ATCC 33834). Causes natural disease in the honeybee in France [17,18]. The SR 3 strain (ATCC 33095) was from flowers in Connecticut, but most strains are from Mediterranean or tropical regions. The PPS1 strain (ATCC 33450) is from flowers in Florida. There is considerable serological heterogeneity among Group IV strains.

Group V. *Spiroplasma mirum.* The suckling mouse cataract agent (SMCA) type strain (ATCC 29335) is experimentally pathogenic for some vertebrates.

Group (VI). A group of eight strains from *Ixodes pacificus* in Oregon [19]. The representative strain is Y32 (ATCC 33835).

Group (VII). Representative strain MQ-1 (ATCC 33825). Isolated from the hemolymph of the wasp *Monobia quadridens* in Maryland [20]

Group (VIII). Representative strain EA-1 (ATCC 33826). Isolated from the syrphid fly *Eristalis arbustorum* in Maryland [20]

Group (IX). Representative strain CN-5 (ATCC 33827). Isolated from the gut of the beetle *Cotinus nitida* in Maryland. Several isolates have been obtained.

Group (X). Representative strain AES-1. Isolated from insects in New Jersey by one of us [Chen T-A: unpublished data]

Group (XI). Representative strain MQ-4. Isolated from *Monobia quadridens* in Maryland by one of us [Clark TB: unpublished data]

**DISCUSSION**

At its 1980 sessions in Custer, South Dakota, the Subcommittee discussed [21] the taxonomic status of the organisms of citrus stubborn (I-1), corn stunt (I-2), and honeybee spiroplasmosis (I-3) diseases. Some members felt that the status of the organisms as genetically and serologically distinguishable strains and the fact that they were important disease agents provided a good case for assigning Latin binomials. Other members felt that the levels of reported homology and the significant crossing of the organisms in growth inhibition tests were too high to justify their distinction as species. Because the Subcommittee had previously urged great restraint in naming of spiroplasma species, it was concluded that this policy was still advisable.

Since that time, further serological tests have been performed on members of the Group I complex [12], and concepts regarding the relationship between host relationship and genetic heterogeneity in *Mollicutes* have begun to develop [22]. On one hand, observations of genetic homogeneity in mollicutes suggest that "clonality," or something closely approaching it, may be characteristic of organisms from a single habitat in which selection pressures may act to favor a particular "wild type." On the other hand, our serological data from comparisons of Group I strains raise the possibility that a gradient of strains that have minimal natural discontinuity in the degree of genetic and serological relatedness could eventually emerge. If that were true, it might be necessary to retain the concept of *S. citri* for a large group of
strains, just as *Acholeplasma axanthum* now describes a large complex of strains with different degrees of relatedness.

Although *S. citri*, technically, is the currently accepted binomial for a citrus pathogen (Subgroup I-1), a honeybee pathogen (Subgroup I-2), and a corn pathogen (Subgroup I-3), most workers are justifiably confused when the name is appended to subgroups other than the I-1 subgroup. Since the levels of homology among subgroups I-1, I-2, and I-3 do not exceed generally accepted levels for naming of bacterial species [23], a strong case exists for assigning specific names to these disease agents. In contrast, however, a proposal [24] for designation of separate species for members of the Group IV complex that are closely related or identical in GI tests, that have unknown ecologies, and whose genetic interrelationships have not been determined, has no merit. Prerequisites for elevation of existing subgroups to species level should be (a) demonstration of a strong pattern of genetic homogeneity in an assortment of strains from different geographical locations and, to the extent possible, different niches or habitats; (b) economic or basic importance of the organisms; (c) less than 70 percent DNA-DNA homology with all known subgroups; (d) substantially different protein patterns in inter-subgroup comparisons of their two-dimensional PAGE profiles; and (e) complete fulfillment of the minimal standards requirements.

Discovery that a diversity of spiroplasma species are widespread in the insect world [20] suggests that further work in characterization and study of the organisms should take into account the overall importance of the organisms to health, agriculture, and basic microbiological science.

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