Identification of Mycoplasma: Procedures for Both Characterization and Purification

THOMAS L. BARBER* AND JULIUS FABRICANT

Department of Avian Diseases, New York State Veterinary College, Cornell University, Ithaca, New York 14850

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Procedures with potential value for identification and purification of Mycoplasma were applied to over 240 cultures representing 11 of the 12 known avian serotypes. Growth was tested at pH 5.5 and 9.5, at 25 and 42 C, in serum-free media, and in the presence of (i) 1% bile salts, (ii) 3% NaCl, and (iii) 0.02% methylene blue. One avian Mycoplasma serotype grew in broth containing 1% bile salts. Two of 11 avian serotypes were resistant to 0.02% methylene blue. A number of Mycoplasma strains grew at 42 C or in a pH 9.5 medium. Usefulness of these procedures for purifying cultures of Mycoplasma is discussed.

Improved techniques for identification of Mycoplasma are needed (7, 9, 10). Mixtures in cultures at the time of primary isolation (1, 3, 8, 22) and in stock laboratory cultures (9, 13, 14) have complicated the classification of Mycoplasma. Major impediments to identification have been the lack of practical characterization procedures and the lack of effective methods for purifying mixed Mycoplasma cultures. Single, well-separated colonies of Mycoplasma may contain more than one species (3, 4, 9, 10); J. M. Al-Aubaidi, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969). Consequently, reliance on cloning of single colonies for purification seems unwarranted. Replacement or at least reinforcement of this technique through use of selective media appears logical. Our objectives were to develop or adapt biochemical or biophysical procedures useful in purification and characterization of Mycoplasma and to determine their usefulness with representative avian Mycoplasma serotypes.

MATERIALS AND METHODS

Organisms. The more than 240 avian Mycoplasma cultures used were previously described (T. L. Barber, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969; T. L. Barber and J. Fabricant, Avian Dis., in press). They represented all known avian serotypes except M. synoviae. Prior to the start of this work, most cultures were purified by repeated cloning of single colonies and were partially characterized and tentatively identified.

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A stock 1% solution of methylene blue in distilled water was sterilized by autoclaving. The methylene blue solution was added to BS to 0.2% final concentration. (vii) Sodium azide (Fisher Scientific Co., Chicago, Ill.) prepared as a 1% stock solution was added to BS at final concentrations of 0.1 to 2.0% and to BA at 0.02%. (viii) Crystal violet (Will Corp., Rochester, N.Y.), prepared as a 1% stock solution, was added to BS medium to final concentrations of 0.01 and 0.001% and to BA in final concentrations of 0.001 and 0.0001%. (ix) Calcium chloride (Mallinckrodt, St. Louis, Mo.) was...
added to BS at final concentrations of 0.5 or 1.0% and to BA to final concentrations of 0.1, 0.5, and 1.0%. (x) Phenethyl alcohol (Eastman Organic Chemicals, Rochester, N.Y.) was added to BS growth medium to final concentrations of 0.5 or 0.25%. (xi) Merthiolate (thimerosal; Eli Lilly, Greenfield, Ind.) was added to physiological saline or to Sorenson's buffered saline (pH 7.6) at final concentrations of 1:1,000 and 1:10,000.

**Inoculation of test media.** Inocula were 0.01 ml of a 3- to 4-day-old broth culture for all procedures. For serial transfer in test media, 0.01 ml was transferred every 3 or 4 days. No attempt was made to standardize the number of organisms in inocula. The same volume was plated for final assays. As a control, identical volumes of inocula were transferred to growth medium and assayed for viability.

**Test procedures.** All inoculated media, unless otherwise noted, were incubated at 37 C. Inoculated plates were observed during 6 days of incubation.

Ability to grow in different hydrogen ion concentrations was determined by inoculating broths at pH 5.5 or 9.5. After 3 to 4 days of incubation, 0.01 ml was transferred to a BA plate having the same pH. Colony formation on this plate was the assay for ability to grow or survive in media with altered pH.

Growth at 25 or 42 C was assessed by three subcultures (at 3- to 4-day intervals) in liquid media. The third subculture was streaked on a BA plate, incubated at 37 C, and examined for colony formation.

Evaluation of growth without serum was done by three subcultures in BS and in RYE without serum enrichment. The third subculture was streaked to a plate of corresponding medium enriched with serum to detect growth or survival.

Methylene blue medium was inoculated and, after 3 to 4 days of incubation, subcultured to a tube of BS medium. Three to 4 days later, they were streaked on BA plates to assess growth.

For determination of sensitivity to antibiotics, BA growth medium was prepared in plastic petri dishes (100 by 15 mm) with four compartments. Undiluted, 10^-1, 10^-2, and 10^-3 dilutions were individually placed in sectors and spread by tilting. After inocula were dried at 37 C for 2 hr, antibiotic discs (Difco) were placed on each sector. High level concentrations of kanamycin (30 μg) and erythromycin (15 μg) were used.

Liquid media containing bile salts, extra NaCl, sodium azide, crystal violet, calcium chloride, or phenethyl alcohol were tested by inoculation and, after 3 to 4 days of incubation, were subcultured onto BA growth medium for assay. Certain of these inhibitory chemicals were also included in agar medium. Inhibitory agars were streaked from a culture in BS medium without inhibitors. Plates were then examined in the usual manner. Inoculated tubes of merthiolated saline were held overnight at 4 C and streaked on BA for assay.

**RESULTS**

Table 1 gives results with the avian Mycoplasma serotypes and indicates the most useful differential procedures. Results with serotypes F, H, *M. laidlawii* var. inocuum, and *M. anatis* are included only for comparison because too few representative strains were tested to draw final conclusions. A serotype was marked positive or negative when 80 to 100% of the cultures survived or died. A serotype was marked variable if fewer than 80% of the strains gave one reaction. Figures in parentheses show the extent of variation.

Growth at pH 5.5 and 9.5 was a more variable characteristic within serotypes than other procedures. At pH 5.5, only serotypes A, F, and *M. laidlawii* var. inocuum were totally eliminated, but in serotypes C-O and D-P most cultures did not grow. All other serotypes were varied in their resistance to pH 5.5. At pH 9.5, only serotypes F and H were eliminated; serotypes A, B, L, *M. laidlawii* var. inocuum, and *M. anatis*, and the complex I, J, K, N, Q, R group grew.

Only the *M. laidlawii* var. inocuum culture grew during three subcultures at 25 C. At 42 C, 80% of cultures belonging to the complex avian I, J, K, N, Q, R group continued to grow during three subcultures. Some strains of six other serotypes grew at 42 C. All strains of four serotypes (A, C-O, F, H) were eliminated.

*Mycoplasma laidlawii* var. inocuum grew without serum. The *M. anatis* duck-source culture grew in RYE without serum but did not grow in serum-free BS. Nine other avian *Mycoplasma* serotypes were eliminated by three serial subcultures in the two serum-free media.

Growth in the presence of bile salts was a characteristic unique to the complex avian I, J, K, N, Q, R group. Growth of this serotype was virtually diminished in the presence of 1% bile salts. Identical results were obtained with either sodium glycocholate or sodium glyco- taurocholate (Table 1). Similar results were obtained whether 0.5, 1.0, or 2.0% bile salts were used. No bile-tolerant cultures were found among the other avian *Mycoplasma* serotypes. Avian group I, J, K, N, Q, R was also resistant to 1% bile salts in Sorenson's phosphate buffer (pH 7.6) at 5 C for 24 hr. However, most I, J, K, N, Q, R strains were reduced in number if not eliminated by exposure to 1% bile salts in this buffer for 24 hr at 37 C.

Avian *Mycoplasma* serotypes E, L, *M. laidlawii* var. inocuum, and the I, J, K, N, Q, R group were the most resistant to an added 3% NaCl in BS growth medium; serotype B was resistant to a lesser degree (Table 1). Four of the 11 serotypes studied were totally eliminated by this treatment, and two other serotypes were drastically reduced.

Methylene blue resistance was found in three avian *Mycoplasma* serotypes (Table 1). Resistance was most distinct in serotype A and in the complex avian serotype group I, J, K, N, Q, R.
The table below shows the results of growth studies with avian Mycoplasma serotypes.

| Avian Mycoplasma serotype | No. of cultures tested | Growth at pH 5.5 | pH 9.5 | 25°C | 42°C | Growth in BS | RYE | 1% Bile salts | 3% NaCl | 0.02% Methylen blue |
|---------------------------|------------------------|------------------|--------|------|------|---------------|-----|----------------|--------|---------------------|
| A (M. gallisepticum)      | 20                     | -                | +      | -    | -    | -             | -   | -              | -      | +                   |
| B (M. gallinarum)         | 26                     | V (13/26+)       | + (22/26+) | V (18/26+) | -    | -             | -   | -              | (18/26+) | -                   |
| C-O                      | 22                     | -                | -      | -    | -    | -             | -   | -              | -      | -                   |
| D-P                      | 23                     | V (2/22+)        | V      | -    | -    | -             | -   | -              | -      | -                   |
| E (M. iners)             | 28                     | V (4/23+)        | V      | -    | -    | -             | -   | -              | -      | -                   |
| F                        | 4                      | V (8/28+)        | V      | -    | -    | -             | -   | -              | -      | -                   |
| H (M. meleagridis)       | 3                      | V (1/3+)         | V      | -    | -    | -             | -   | -              | -      | -                   |
| L                        | 11                     | V (8/11+)        | V      | -    | -    | -             | -   | -              | -      | -                   |
| M. laidlawii var. inocuam | 4                      | -                | -      | +    | +    | (7/11+)       | V   | +              | -      | (10/11+)            |
| M. anatis                | 3                      | V (2/3+)         | V      | -    | -    | (2/4+)        | V   | +              | -      | (3/11+)             |
| I, J, K, N, Q, R group   | 100                    | V (56/100+)      | +      | -    | -    | (98/100+)     | V   | +              | (97/100+) | +                  |

* Symbols: + = growth, - = no growth or survival, V = variable.

** BS, Heart Infusion Broth supplemented with swine serum and bacterial inhibitors. RYE, rabbit meat infusion broth enriched with rabbit serum, yeast extract, and peptone and containing bacterial inhibitors.

Three antibiotics resulted in pronounced sensitivity differences between avian *Mycoplasma* serotypes. Only serotype A and the complex I, J, K, N, Q, R group were sensitive to erythromycin and resistant to kanamycin. The pattern was precisely reversed with serotypes C-O, D-P, E, F, and H. Serotype L was resistant to erythromycin and kanamycin, but *M. laidlawii* var. *inocuam* was sensitive to both. Only serotype B cultures varied within the serotype in response to these antibiotics.

Colony formation by avian *Mycoplasma* cultures was not inhibited on BA medium with 0.02% sodium azide. In BS broth, 0.1% sodium azide was not inhibitory, but toxicity was evident at 0.5% and greater concentrations. Several cultures, however, were not totally eliminated by the 2.0% concentration. Resistance of differential value was shown only by *M. laidlawii* var. *inocuam* which appeared unaffected by sodium azide at concentrations of 0.5 to 2.0%.

Although crystal violet at 0.001% in BA medium inhibited only a few *Mycoplasma* cultures, at 0.001% concentration most did not grow. None survived subculture in BS medium containing 0.01% crystal violet. In BS with 0.001% crystal violet, many cultures survived but no useful differential pattern was established.

Differential inhibition patterns were not obtained with calcium chloride in agar or liquid media. Most avian *Mycoplasma* were inhibited by 0.5% phenethyl alcohol but were unaffected by 0.25%. Avian *Mycoplasma* survived well in control tubes of both Sorenson’s phosphate buffer (pH 7.6) and physiological saline. One serotype (*M. laidlawii* var. *inocuam*) was unaffected by 1:10,000 thimerosal under these conditions, whereas all others failed to survive.

**DISCUSSION**

This study confirms evidence (19, 20) that *Mycoplasma* serotypes have a wide range of biophysical and biochemical characteristics. Such differences are of value in selective primary isolation of pathogenic species as has been done with *M. pneumoniae* (3), in purification of mixed cultures, and in taxonomic grouping.

Our procedures were established after testing a
range of possibilities, i.e., media were tested at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, and 10.0; various concentrations of salt were tried; preparations of two bile salts included 0.5, 1.0, and 2.0% concentrations; methylene blue was tried at 0.01, 0.02, 0.001, 0.002, and 0.0001%. Efforts were made to find the optimal procedure for each test medium or condition, i.e., the number of passages under test conditions or in inhibitory media, and whether to incorporate the various inhibitors into liquid or solid media. Procedures and test conditions were selected to demonstrate sufficient differences among the avian Mycoplasma serotypes.

The procedures were used to purify certain cultures before using them as antigens for immunization or serology. They were also used to detect and to separate mixed cultures on a very limited scale. For example, culture DUD had been grouped into I, J, K, N, Q, R on the basis of its biochemical and serological properties before a contaminant Mycoplasma, later identified as M. laidlawii, was revealed by three serial subcultures at 25 C. The DUD culture more appropriately fit the I, J, K, N, Q, R pattern after subculture in BS medium with 1% bile salts which eliminated the M. laidlawii contaminant. When the mixed culture was tested serologically by metabolic inhibition with arginine as an indicator, no irregularity was seen because arginine was not split by M. laidlawii. Thus, the mixture was detected only by serial subculture at 25 C and this technique, together with the bile salt medium, enabled the components of the mixture to be separated.

Table 1 shows that these characteristics will differentiate the 11 avian Mycoplasma serotypes. The two most nearly identical serotypes, B and E, are distinguishable by tetrathionate reduction and by serology (9; M. M. Sabry, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1968; T. L. Barber and J. Fabricant, Avian Dis., in press).

Mycoplasma were reported (6) to grow over a pH range of 6.8 to 9.2, and a pH of 8.0 was recommended for routine use. The T strains grew best at pH 5.5 to 6.0 in agar and liquid media (16). We found in working with media of altered pH that one broth passage followed by subculture on agar would eliminate or suppress certain avian serotypes. Because we used many strains of serotype A (M. gallisepticum) and because there was no variation among the strains, we are confident that this serotype is eliminated from mixtures by growth at pH 5.5. At pH 9.5, growth was usually reduced, but none of the adequately represented avian Mycoplasma serotypes was eliminated. Growth at various pH levels, although a variable trait, did serve as a useful alternative to terminal dilution for reducing populations before cloning. The same single colony cloned from agar at high or low pH would very likely not be the one persisting when a culture is diluted and plated at the end point of growth.

By using these same procedures, 13 bovine Mycoplasma serotypes (J. M. Al-Aubaidi, Ph.D. Thesis, Cornell University, Ithaca, N.Y., 1969) grew in a differential pattern. Some bovine serotypes grew at both pH 5.5 and 9.5, some at neither pH extreme, some only at 9.5, and one serotype grew at pH 5.5 but not 9.5.

The optimum temperature for growth of most Mycoplasma is 37 C. Only M. laidlawii has been reported to grow between 22 and 37 C, with 30 C the optimum temperature for freshly isolated strains (7). In limited studies with mammalian-source cultures, M. mycoides var. capri (three representative strains) grew at 25 C, and 6 of 13 bovine serotypes grew through three subcultures at 25 C. One of the bovine Mycoplasmas growing at 25 C was M. laidlawii (PG-8, sewage A), but five others were serologically and biochemically distinguishable from M. laidlawii (PG-8).

Growth at 42 C was also helpful in characterization of Mycoplasma. Among the adequately represented avian serotypes, only A and C-O were eliminated at 42 C; serotypes B, D-P, and E persisted but with wide variation between strains. Three strains of M. mycoides var. capri survived three subcultures at 42 C as did 6 of 13 bovine Mycoplasma serotypes. Like M. mycoides var. capri, three of the six bovine serotypes grew at 25 and 42 C in contrast to the avian serotypes, none of which grew at both temperatures.

The growth of M. laidlawii var. innocuum in serum-free media was expected, but the replication of the duck-source M. anatis in RYE without serum was surprising. There is no report of this species being tested for ability to grow without serum. In initial descriptions (13), the culture was reported to grow at both 30 and 27 C and to ferment carbohydrates. Among mammalian-source cultures, four bovine serotypes other than M. laidlawii (PG-8) grew without serum enrichment. Only two of the bovine serotypes grew consistently in serum-free BS, whereas two others and M. laidlawii (PG-8) grew in serum-free RYE. Not all Mycoplasma that grew without supplemental serum grew at 25 C nor did all ferment dextrose.

Composition of media in which cultures grew without serum enrichment appreciably affected the results. Even two of four strains of M. laidlawii var. innocuum failed to grow in serum-free BS. The serum-free BS consisted of only HIB and inhibitors. The serum-free RYE was freshly prepared infusion from rabbit meat enriched with
peptone (Difco) and yeast extract, but all ingredients except penicillin had been autoclaved. Since cholesterol is in animal tissues, and not all forms of cholesterol have a melting point below the autoclave temperature of 121 °C, sterols may have remained in the RYE. The usefulness of the results was that 9 of 11 avian *Mycoplasma* serotypes and 8 of 13 bovine serotypes also failed to grow under these conditions. Although the scope of our trials do not substantiate the assumption that *Mycoplasma* growing in serum-free RYE do not require sterols, the procedure does conform to our objectives for purification and characterization methods. More specific examination of sterol requirements of cultures which grew on serum-free RYE is indicated.

Tolerance to 1% bile salts in the growth medium by the complex I, J, K, N, Q, R avian group was an excellent marker. The bile salts completely eliminated 10 other avian serotypes, 13 bovine serotypes, 3 strains of *M. mycoides* var. *capri*, and 1 strain of *M. agalactiae*. One *Mycoplasma* serotype from dogs grew in the presence of bile salts under these test conditions (J. Fabricant, unpublished data).

Both *M. mycoides* var. *mycoides* (18) and *M. laidlawii* (Seiffert, 1935, cited in reference 11) are decomposed by bile salts. The bile solubility test (5) for pneumococci was applied to eight avian strains (21). Only one of the eight was visibly lysed by bile, and no mention was made of viability of the *Mycoplasma*. None of the eight strains in that study was a member of the complex I, J, K, N, Q, R avian group. The activity of three bile acids upon four *Mycoplasma* strains from human and one from avian sources has been reported (17). No cultures replicated, but some survived better in certain bile acids than in others.

Resistance to bile salts is a reliable differentiating characteristic for a complex avian serotype (I, J, K, N, Q, R) and gives a method for separating it from mixtures with at least 10 other avian serotypes. It would appear that this characteristic is a taxonomic criterion of primary importance.

Resistance to increased levels of sodium chloride was a variable characteristic of limited value in grouping avian serotypes. This characteristic was a part of a pattern of similarity among avian *Mycoplasma* serotypes B, E, L, and the complex I, J, K, N, Q, R group. Each of these four serotypes had some ability to grow at pH 5.5 and 9.5, at 42 °C, and in the high salt medium. This combination of similarities was notable among these serologically unrelated groups. Nine of 13 bovine serotypes also grew in BS with 3% added NaCl; however, not enough representative cultures were used to establish properly variability among serotypes.

Resistance of the human pathogen *M. pneumoniae* to 0.002% methylene blue in agar medium was reported (12). We selected a 0.02% concentration of methylene blue in liquid media after using several variations in preliminary trials. A high concentration (0.02%) in liquid was preferred because this medium most clearly differentiated avian serotypes. Subculture in liquid might be better for purification because an agar block with one visible colony might possibly contain other viable noncolony-forming *Mycoplasma*. Usually avian *Mycoplasma* within a serotype reacted uniformly to 0.02% methylene blue, but those from pigeons (serotype L) did not. Some bovine *Mycoplasma* serotypes survived, some did not, and a few were variable on repeated tests in 0.02% methylene blue medium.

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