A Serological Comparison of Various Species of *Mycoplasma* by an Agar Gel Double-Diffusion Technique

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SUMMARY

Sixteen serological types of *Mycoplasma* previously distinguished by complement-fixation tests were compared by means of a gel precipitin (Ouchterlony) technique. Each of the sixteen gave a consistent pattern of three to seven precipitation lines with its homologous antiserum. Cross-reactions occurred between heterologous strains, especially with antisera produced with the aid of an adjuvant. Homologous reactions were complex and sufficiently distinctive for the technique to be used to identify unknown strains. Two of the sixteen types, *Mycoplasma mycoides* var. *mycoides* and *M. mycoides* var. *capri*, had certain antigenic components in common. The appearance of other cross-reactions suggested that the majority were due to the presence in different species of partly related rather than identical antigens.

INTRODUCTION

A previous communication (Lemcke, 1964*a*) reported the results of using a complement-fixation (CF) test to characterize mycoplasmas from a variety of sources. Among 82 strains, 17 serological types were distinguished, although two of these, *Mycoplasma arthritidis* and strain R38, both from rats, were partly related. Although these serological types were easily distinguishable by complement-fixation tests, cross-reactions of the order of 1/16 to 1/32 of the homologous titres occurred between all of them. This phenomenon was also noted in complement-fixation tests by Card (1959) and Coriell, Fabrizio & Wilson (1960). Non-specific reactions with certain constituents of the medium were responsible at least in part, but it was not clear whether certain antigenic components common to different species were also involved (Lemcke, 1964*a*). To clarify this point, an agar gel double-diffusion technique was used to examine the reactions of various *Mycoplasma* strains with homologous and heterologous antisera.

METHODS

Organisms. Details of the organisms used are given in Table 1. The sources of all the strains except BRA were described in earlier reports (Lemcke, 1964*a, b*). Strain BRA was freshly isolated from the rectum of a woman with vaginal cervicitis.

Media and growth conditions. The liquid medium used for cultures from which the gel diffusion antigens were prepared consisted of Hartley's digest broth (Oxoid)

supplemented with: unheated horse serum 20% (v/v); yeast extract (Oxoid), 0.5% (w/v); deoxyribonucleic acid, 0.002% (w/v); penicillin, 50 units/ml. For the growth of cultures for immunizing rabbits, yeast extract was omitted and unheated pooled normal rabbit serum was substituted for horse serum. All strains were incubated at 37°, *Mycoplasma salivarium*, *M. orale* and *M. fermentans* anaerobically and the rest aerobically with continuous shaking.

Table 1. *Mycoplasma* strains

Designation of strain	Source	Serological type or species
H 34,* H 33, MJW, 4387 P, H 27 H 68, BRA	Man (genital tract)	<i>Mycoplasma hominis</i> type 1
B 1, B 3,* B 5	Man (mouth)	<i>M. salivarium</i>
887,* E	Tissue culture {	<i>M. orale</i> †
S, BM, 898/2	Man (throat) }	
G 2*	Man (genital mucosa)	<i>M. fermentans</i>
Navel*	Man (umbilicus)	Umbilical
FH	Man (primary atypical pneumonia)	<i>M. pneumoniae</i>
Campo (PG 27)*, O 7	Man (genital tract)	' <i>M. hominis</i> type 2'
Jasmin*	Rat (polyarthritidis)	<i>M. arthritidis</i>
R 38	Rat (rhinitis)	Partly related to <i>M. arthritidis</i>
M 1*	Mouse (lung)	<i>M. pulmonis</i>
KSA*	Mouse ('rolling disease')	<i>M. neurolyticum</i>
PG 11*	Cattle (genital tract)	<i>M. bovis genitalium</i>
Gladysdale*	Cattle (contagious pleuro-pneumonia)	<i>M. mycoides</i> var. <i>mycoides</i>
G 1/61*	Goat (contagious pleuro-pneumonia)	
pp. goat*	Goat (contagious pleuro-pneumonia)	<i>M. mycoides</i> var. <i>capri</i>
Agalactia*	Goat (agalactia)	<i>M. agalactiae</i>
Fowl*	Chicken (upper respiratory tract)	<i>M. gallinarum</i>
A 36*	Chicken (tracheal exudate)	Non-pathogenic avian
T*	Embryonated egg	<i>M. gallisepticum</i> (Nelson's coccobacilliform bodies)
Laidlaw A*	Sewage	<i>M. laidlawii</i>

* Precipitating antisera available against these strains.

† Name assigned to new oral type (Dr D. Herderscheê, personal communication; Taylor-Robinson, Canchola, Fox & Chanock, 1964).

Preparation of antigens. Cultures were grown for 2–4 days since it was found that fewer precipitation lines were obtained with strain H 34 when incubation was continued for 6–14 days. Organisms were harvested by centrifugation at 10,000g for 30 min. at 4°, washed twice in 0.85% (w/v) saline and finally suspended in saline to a concentration 100 times that of the original culture. Although some precipitation lines were formed with suspensions of whole organisms, more were obtained with suspensions which had been cooled in ice and water and treated for 10 min. with a Branson S-75 Sonifier (Branson Instruments Inc., Stamford, Connecticut, U.S.A.) at 20 kcyc./sec. Suspensions in de-ionized water, which were frozen and thawed ten times in solid carbon dioxide + ethanol at –25°, gave precipitation patterns similar to those given by organisms disrupted ultrasonically. The freeze/thaw method was suitable for small quantities of culture. In most of the work reported below

suspensions of organism from 250 or 500 ml. medium were disrupted ultrasonically. Suspensions were stored at -25° to -30° in volumes of 0.5–1.0 ml. Storage in large volumes was impracticable because some antigens deteriorated during the repeated thawing and freezing required for the removal of samples for test. Suspensions preserved at 4° with the addition of thiomersalate (the procedure for storing complement fixation test antigens) gave fewer precipitation lines than those stored frozen. To detect non-specific reactions with the medium, a control 'antigen' consisting of the resuspended residue obtained by centrifugation of sterile broth was prepared.

Antisera. Most of the antisera used had been prepared for use in complement-fixation tests (Lemcke, 1964*a*). They were selected for their ability to give precipitation lines with a suspension of homologous organism and were tested for reactivity with the medium and the control 'antigen'.

Antisera induced by antigen + Freund's adjuvant gave the best results in gel diffusion tests and new antisera were prepared by this method. The adjuvant consisted of a mixture of 9 parts Drakeol 6-VR (Pennsylvania Refining Co., Butler, Pa., U.S.A.) and 1 part Arlacel (Atlas Chemical Industries Inc., Wilmington, Del., U.S.A.). The organisms from 500 ml. of a 3- to 4-day culture were harvested by centrifugation, washed, resuspended in 2 ml. normal saline and homogenized with an equal volume of adjuvant. Rabbits were inoculated subcutaneously in the flank. Those receiving samples of strains B3, G2 and Campo were given one injection, those receiving strains 837 and Navel two injections separated by an interval of 3 weeks. Three weeks after the last subcutaneous inoculation, all the rabbits were given a course of six intravenous injections of the appropriate organism suspended in saline. The best antisera were obtained 5–7 days after the last intravenous injection.

Double-diffusion tests. The medium and pattern of the reservoirs used were those described by Lemcke (1964*a*). Precipitation was allowed to take place at room temperature; at 4° fewer lines developed and at 30 – 37° the lines became very diffuse. Optimal development occurred between 2 and 5 days, depending on the antiserum.

RESULTS

Of the stock antisera tested for ability to give precipitation lines with homologous antigen preparations, only those against *Mycoplasma pneumoniae* and strain R38 did not react. Thus, precipitating antisera were available against 15 of the 17 serological types previously described (Lemcke, 1964*a*) and against *M. gallinarum*, another 'avian' species which proved to be serologically distinct by the complement-fixation test (Table 1). Of these 16 type antisera, 14 gave no sign of precipitation with the medium or control 'antigen'. The remaining two, an antiserum against strain KSA (*Mycoplasma neurolyticum*) and one against strain Gladysdale (*M. mycoides* var. *mycoides*) gave diffuse zones of precipitation with both the medium and the control 'antigen'. With both, the reaction was eliminated by absorption of the sera with an equal volume of medium. The absorbed sera, even though diluted 1/2 by this procedure, gave several clear precipitation lines with the homologous antigen; only the rather diffuse non-specific band disappeared. In the case of strain KSA, the absorbed antiserum did not react with suspensions of any other strains.

Adjuvant-induced antisera gave more precipitation lines with their homologous antigens than did antisera produced by the intravenous inoculation of saline suspensions (Table 2).

The results of cross-testing suspensions with 16 antisera against strains representing different serological types are shown in Table 3. When two antisera were available against any one strain, as was the case for strains H34, B3, 837, G2, Navel and Campo, the results obtained with the adjuvant-induced serum are given. Although heterologous cross-reactions were frequent, the homologous reaction in each case was always more complex and characteristic of the particular strain. To make sure that none of the cross-reactions represented non-specific reactions, all

Table 2. *Comparison of antisera produced by suspensions of mycoplasmas, with and without adjuvants*

Mycoplasma strain	No. of precipitation lines with homologous suspensions		Cross-reactions with heterologous suspensions	
	With adjuvant	No adjuvant	With adjuvant	No adjuvant
H34	6	1-2	+	—
B3	4-5	2	+	—
837	6-7	2	+	—
G2	5-7	2	+	—
Navel	3	1	—	—
Campo (PG27)	5-6	2	+	+

cross-reacting sera were tested against concentrations of the complete medium up to twenty-five times and against two-fold dilutions down to 1/4096 of the complete medium, Hartley's digest broth, undiluted horse serum, yeast extract (10 %, w/v), and deoxyribonucleic acid (0.2 %, w/v). No precipitation lines were detected in any of these tests.

There was a substantial degree of cross-reaction between the strains H34 (*Mycoplasma hominis* type 1), B3 (*M. salivarium*), 837 (*M. orale*), G2 (*M. fermentans*) and Navel, which represented five different serological types of human origin (Table 3). Of these, only antiserum to strain Navel was entirely monospecific, despite the use of adjuvant in its preparation; suspensions of this strain did, however, react with antisera to the other serotypes of human origin. Table 3 also shows extensive cross-reactions between the five strains of human origin and Campo, a strain of the so-called '*Mycoplasma hominis* type 2'. Although strain Campo and another 'type 2' strain, o7, were isolated from the human genital tract, complement-fixation tests have shown them to be indistinguishable from strains of *M. arthritidis* associated with polyarthritis in rats (Lemcke & Csonka, 1962; Lemcke, 1964*a*). Gel diffusion tests confirmed that strains Campo and o7 were very closely related to *M. arthritidis* strain Jasmin. With antiserum to strain Campo, strain Jasmin shared at least four precipitation lines with strain Campo, but lacked another line (A) given by the latter (Fig. 1*a*). With antiserum to strain Jasmin, strains Campo, o7 and Jasmin shared three lines of precipitation (Fig. 1*b*). In addition strains Campo and Jasmin had a fourth line, nearest the antigen well, which formed a spur at the point of junction (B) and was therefore not quite identical in the two organisms; this component was apparently missing from o7.

Table 3. Number of gel precipitation lines obtained in homologous and heterologous reactions with *Mycoplasma* strains

Antigens	Antisera from strains														
	H 34*	B 3*	837*	G 2*	Navel*	Campo* (PG 27)	M 1	KSA*	PG 11*	Gladys- dale*†	pp. goat	Agal- actia	Fowl	A 36*	Laidlaw A*
H 34	6	2	2	2	—	2	—	—	—	—	—	—	—	—	—
B 3	2	4-5	2	1	—	2	—	—	—	1	—	—	—	—	—
837	1	2	6-7	1	—	1	—	—	—	—	—	—	—	—	—
G 2	1	2	2	5-7	—	2	—	—	—	—	—	—	—	—	—
Navel	1	1	2	3	3	1	—	—	—	—	—	—	—	—	—
Campo	2	1	2	2	—	5-6	—	—	—	—	—	—	—	—	—
M 1	—	—	1	—	—	—	3	—	—	—	—	—	—	—	—
KSA	—	—	1	1	—	—	1	5-6	—	—	—	—	—	—	—
PG 11	—	—	2	2	—	—	—	—	4	—	—	—	—	—	—
Gladysdale	—	—	—	—	—	—	—	—	1	4-5	2	—	—	—	—
pp. goat	—	—	2	—	—	—	1	—	1	3	3	—	—	—	—
Agalactia	—	—	3	3	—	—	1	—	—	—	—	4	—	—	—
Fowl	2	1	2	3	—	1	—	—	1	—	—	—	4	—	—
A 36	—	—	2-3	1	—	—	—	—	—	—	—	—	—	4-5	—
T	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3
Laidlaw A	—	—	—	—	—	—	—	—	—	—	—	—	—	—	3-4

* Adjuvant used in antiserum production.
 † Antisera absorbed with medium.
 — = no precipitation lines.

The technique thus revealed a slight difference, not apparent from the results of complement-fixation tests, between the 'type 2' strains from man and *M. arthritidis* from a rat. Nevertheless, since strains from both sources shared the majority of their antigens, there seems to be no justification for regarding '*M. hominis* type 2' as a species distinct from *M. arthritidis* (Lemcke, 1964*a*).

Fig. 1. Agar gel precipitin reactions of (a) antiserum to '*Mycoplasma hominis* type 2' strain Campo with homologous strain and *M. arthritidis* strain Jasmin (A, extra band formed by homologous strain), and (b) antiserum to *M. arthritidis* strain Jasmin with homologous strain and '*M. hominis* type 2' strains Campo and o7 (B, partial intersection of lines from strains Jasmin and Campo). as. = antiserum well.

Fig. 2. Agar gel precipitin reactions of (a) antiserum to *Mycoplasma mycoides* var. *capri* strain pp. goat with homologous strain and *M. mycoides* var. *mycoides* strain Gladysdale (A, line common to both strains; B, line partly intersecting with one of homologous lines), and (b) antiserum to *M. mycoides* var. *mycoides* strain Gladysdale with homologous strain and *M. mycoides* var. *capri* strain pp. goat (C, D, E, lines common to both strains). as. = antiserum well.

With regard to the nature of the cross-reactions between the sixteen serological types, two of them, *Mycoplasma mycoides* var. *mycoides* from contagious bovine pleuropneumonia and *M. mycoides* var. *capri* from contagious pleuropneumonia in a goat, clearly had common antigens. With antiserum to *M. mycoides* var. *capri* strain pp. goat, *M. mycoides* var. *mycoides* strain Gladysdale gave one precipitation line which fused completely with one produced by the homologous strain (Fig. 2*a*, line A). A second line partially intersected with one of the other two homologous lines giving spur formation (Fig. 2*a*, line B). This suggested the presence of one identical and one partly related component in the two organisms. The reciprocal

test with antiserum to *M. mycoides* var. *mycoides* strain Gladysdale (Fig. 2*b*), showed that three of the precipitation lines (C, D, E) formed by 'mycoides' and 'capri' were identical. Tests with an antiserum to *M. mycoides* var. *mycoides* strain G1/61 (a strain from goat pleuropneumonia but indistinguishable from strain Gladysdale) showed that 'mycoides' and 'capri' had one antigenic component in common and one partly related. The rather more complex picture obtained with antiserum to strain Gladysdale was probably due to the use of adjuvant in its production; strains pp. goat and G1/61 antisera were prepared without adjuvant. 'Mycoides' and 'capri' also had an identical line in their cross-reaction with antiserum to *M. bovis genitalium*, suggesting an antigenic component common to both.

Fig. 3. Agar gel precipitin reactions of (a) antiserum to *Mycoplasma hominis* type 1 strain H34 with homologous strain, *M. salivarium* strain B3, '*M. hominis* type 2' strain Campo, *M. orale* strain 837, *M. pulmonis* strain M1 and strain Navel, and (b) antiserum, obtained after one subcutaneous inoculation, to '*M. hominis* type 2' strain Campo with homologous strain, *M. salivarium* strain B3, *M. hominis* type 1 strain H34, *M. fermentans* strain G2, *M. orale* strain 837 and strain Navel. as. = antiserum well.

Fig. 4. Agar gel precipitin reactions of antiserum to *Mycoplasma orale* strain 837 with homologous strain and *M. hominis* type 1 strain H34 (A, diffuse band apparently fusing with one of homologous lines; B, line partly intersecting with one of homologous lines). as. = antiserum well.

In most of the other cross-reactions spur formation occurred where the heterologous precipitation lines joined one or other of the homologous lines (Fig. 3*a, b* and Fig. 4, line B). In some cases, the complexity of the homologous pattern tended to obscure the junction of homologous and heterologous lines. Nevertheless, reactions with less complex sera obtained after a single subcutaneous injection suggested that spur formation did indeed occur (Fig. 3*b*). Both Figs. 3*a* and *b*

illustrate reactions with several different heterologous strains. Spur formation occurred not only between the homologous and the adjacent heterologous systems, but also between different heterologous strains.

Besides the partly related antigens indicated by spur formation, a few strains may have identical antigens in common. With *Mycoplasma orale* strain 837 antiserum, *M. hominis* type 1, *M. salivarium*, *M. fermentans*, Navel, 'M. hominis type 2' and *M. gallinarum* all showed a rather diffuse precipitation band which apparently fused with the homologous line nearest the antiserum well. The width and diffuseness of the bands may however give a false appearance of fusion (Fig. 4, line A). Similar reactions were observed between *M. salivarium* strain B3 antiserum and *M. orale*, and between *M. fermentans* strain G2 antiserum, and *M. orale* and *M. gallinarum*.

The technique was also used to compare strains which were indistinguishable by the complement-fixation test. Thus, with any one antiserum to *Mycoplasma hominis* type 1, strains from a post-operation wound infection (H34), non-gonococcal urethritis (H33, MJW, 4387 P), Reiter's disease (H27), Bartholin's abscess (H68) and the rectum (BRA) gave similar reactions. No difference was detectable between the freshly isolated strain BRA and the others, which had been maintained in the laboratory for some time. The only differences noted between strains were in the intensity of certain of the weaker precipitation lines. Such differences were probably due to the deterioration during storage of the more labile antigenic components, since the same lines were found to be missing when suspensions of strain H34 were stored at 4° with thiomersalate and when cultures to be used as antigens were incubated for 6-14 days instead of 2-4 days.

Apart from differences in intensity, the precipitation reactions of these seven human genital strains were indistinguishable and the results thus confirm those obtained by the complement-fixation test (Lemcke, 1964*a*). Some earlier observations (Nicol & Edward, 1953; Card, 1959; Oates, Whittington & Wilkinson, 1959; Lemcke & Csonka, 1962 and Taylor-Robinson, Somerson, Turner & Chanock, 1963) suggested that there might be slight antigenic differences between strains of *Mycoplasma hominis* type 1. There was, however, no evidence from the strains examined here that some possessed antigenic components not present in others.

Three strains from the human mouth (B1, B3, B5; Card, 1959) appeared identical when tested with antiserum to strain B3, just as in complement-fixation tests.

The similarity of certain tissue culture contaminants isolated by Hayflick to a new oral type (Herderscheê, Ruys & van Rhijn, 1963) was reported previously (Lemcke, 1964*b*). This type has been named *Mycoplasma orale* (Dr D. Herderscheê, personal communication; Taylor-Robinson, Canchola, Fox & Chanock, 1964). The precipitation patterns of the strains from tissue cultures (strains 837, E) and from the human throat (strains S, BM, 898/2) were closely similar, the only difference being in the intensity of the precipitation band nearest the antigen wells. This may have been due to the better preservation of a labile antigenic component in some suspensions than in others or it may have reflected differences in the amount of a particular antigen present in different strains.

DISCUSSION

It is clear that in many of the *Mycoplasma* species examined here there was a complexity of antigenic components not revealed in previous immunodiffusion studies (e.g. Villemot & Provost, 1959; Taylor-Robinson *et al.* 1963). Clearly, much depends on the quality of the antiserum used; some of the best antisera were produced with the aid of adjuvant in the primary subcutaneous inoculation of rabbits, followed by a secondary course of intravenous injections. The disrupted cell suspensions need to be concentrated, and the contained antigens (some of which are labile) preserved from deterioration. Homologous reactions were always clearly distinguishable from the cross-reactions. The gel diffusion technique therefore constitutes a relatively simple method of identification, provided the appropriate antisera are available. It has been successfully used in this laboratory for identifying mycoplasmas from human genital infections.

However, the results are probably of greater significance as a basis for further work on the separation and purification of the various antigenic components. Immunochemical studies of this kind have so far been concerned mainly with the contagious bovine pleuropneumonia organism *M. mycoides* var. *mycoides* (Dafaalla, 1957; Yoshida, 1961; Plackett, Buttery & Cottew, 1963). Similar work on species of human origin would be of value in improving serological methods for diagnosing mycoplasma infections in man.

There was no evidence from the present work, after exhaustive tests, that the cross-reactions observed between different species in gel diffusion tests were non-specific. Cross-reactions in the complement-fixation test were thought to be associated, at least in part, with the broth medium (tryptic digest of horse meat) used to grow cultures for both the complement-fixation test antigen and for immunizing rabbits (Lemcke, 1964*a*). In fact, a different broth medium (Hartley's digest of beef heart muscle) was used to grow the suspensions used in gel diffusion tests. It seems clear from these tests that cross-reactions between species of *Mycoplasma* are due to specific antigenic components. More cross-reactions between species would probably have been detected if potent sera against each strain had been available.

The cross-reacting antigenic components were sometimes identical. *Mycoplasma mycoides* var. *mycoides* and *capri* probably possessed three antigens in common. Because they can be distinguished serologically, the author previously thought that there was little justification for regarding the goat pleuropneumonia organism as a variant of the bovine pleuropneumonia organism and for naming them both as varieties of *M. mycoides* (Lemcke, 1964*a*). However, the precipitation patterns show that these two organisms are more closely related than any of the other serological types examined. Most of the other cross-reactions appeared to be due to partly related rather than identical antigens. There was no evidence of the widespread distribution of the same antigens among very diverse species of *Mycoplasma*, as suggested by Villemot & Provost (1959). Nevertheless, the use of complex mixtures of antigens which comprise the disrupted suspensions makes it difficult to determine the exact relationship between some of the cross-reacting components. These need further study, with cross-absorbed antisera and with, if possible, the separate individual components.

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