Acta vet. scand. 1977, *18*, 176—186.

From the FAO/WHO Collaborating Centre for Animal Mycoplasmas, Institute of Medical Microbiology, University of Aarhus, Denmark.

MYCOPLASMAS: USE OF POLYVALENT ANTISERA FOR IDENTIFICATION BY INDIRECT IMMUNOFLUORESCENCE

By

Henning Ernø

ERNØ, HENNING: *Mycoplasmas: Use of polyvalent antisera for identification by indirect immunofluorescence.* Acta vet. scand. 1977, *18*, 176—186. — It would be an advantage, under many circumstances, to be able to make use of polyvalent antisera in the process of identifying mycoplasmas. As the indirect immunofluorescence test is sufficiently sensitive and also generally accepted as being rather specific, this technique was chosen to investigate whether polyvalent antisera are applicable in routine identification of mycoplasmas. Three polyvalent sera were used, each consisting of 9 or 10 rabbit antisera raised against 29 of the more common species of the genus Mycoplasma. Twenty-six field strains were examined. One strain did not react with any of the 3 polyvalent antisera although it was later identified as M. bovigenitalium. The remaining 25 strains reacted with 1 and only 1 of the polyvalent antisera and were subsequently identified by immunofluorescence utilizing monospecific antisera. Strains of the following species were identified: M. arginini, M. bovigenitalium, M. bovis, M. bovoculi, M. canis, M. capricolum, M. cynos, M. edwardii, M. hominis, M. hyorhinis, M. molare, M. mycoides subsp. mycoides and M. opalescens. It is concluded that polyvalent antisera may be used in identification procedures and thereby permit the use of a limited number of monospecific antisera without preceding biochemical testing.

mycoplasmas; identification; indirect immunofluorescence; polyvalent sera.

As a consequence of the general principles of mycoplasma classification the identification procedures usually include biochemical as well as serological methods. However, the final species identification is most often based on serology*. The results obtained by preliminary determination of biochemical characters

* Identification of the genera Spiroplasma and Anaeroplasma is outside the scope of this paper.

will provide guidance for a rational selection of the test sera, the number of which must be reduced as much as possible. As a first step in the biochemical testing the sensitivity to digitonin is determined (*Ernø & Stipkovits* 1973). The genera Mycoplasma and Ureaplasma are sensitive to digitonin, whereas strains of the genus Acholeplasma are resistant or only slightly sensitive. The urease activity, characteristic of ureaplasmas, may be demonstrated by a technique devised by *Shepard & Howard* (1970). Species differentiation within the genus Mycoplasma can be initiated by 2 tests, viz. catabolism of glucose and arginine. The antisera to be used for the subsequent serological testing are selected according to the results of these tests, which provide a reliable basis for subdivision of the genus into 4 biochemically defined groups.

The course described above has certain drawbacks: biochemical variants within a given species may remain unidentified; the procedure is time-consuming and the results of the glucose and arginine tests are not always easy to evaluate because of poor growth, simultaneous catabolism of glucose and arginine, or strong acidification independent of glucose metabolism.

In practice, the number of antisera for identification may be reduced on the basis of other criteria, such as the origin of the strain and cultural characteristics. On the other hand, situations will occur where it is necessary to use antisera against all known mycoplasma species (approx. 70), which is an almost overwhelming task. One way of facilitating the work might be to use polyvalent antisera. As routine identifications are most frequently based on growth inhibition or immunofluorescence, and as the latter test is much more sensitive than the former, the applicability of polyvalent antisera was examined using indirect immunofluorescence of unfixed mycoplasma colonies as described by *Rosendal & Black* (1972).

MATERIALS AND METHODS

Strains

a. The type strains of 29 species, divided into 3 groups:

1. M. arginini (G230), M. bovigenitalium (PG11), M. bovis (Donetta), M. bovirhinis (PG43), M. bovoculi (M165/69), M. dispar (462/2), M. hyorhinis (BTS-7), M. hyosynoviae (S16), M. mycoides subsp. mycoides (PG1).

2. M. alkalescens (D12), M. canis (PG14), M. cynos (H831), M. edwardii (PG24), M. feliminutum (Ben), M. felis (CO), M. maculosum (PG15), M. molare (H542), M. opalescens (MH5408), M. spumans (PG13).

3. M. agalactiae (PG2), M. buccale (CH20247), M. capricolum (California kid), M. fermentans (PG18), M. gateae (CS), M. hominis (PG21), M. mycoides subsp. capri (PG3), M. orale (CH19299), M. pneumoniae (FH), M. salivarium (PG20).

b. Field strains, numbering 26, isolated from different species of animals and sent to the laboratory for identification.

Monospecific antisera

Antisera against the type strains were produced in albino rabbits (*Ernø et al.* 1973).

Absorption of antisera

Some antigens used for the immunization of rabbits were prepared from cultures grown in media containing serum other than rabbit serum. In these cases, the antiserum was mixed with an equal amount of the heterologous serum concerned (horse or swine), and incubated first at 37°C for 1 hr. and then for 48 hrs. at 4°C followed by centrifugation at 15,000 × g.

Titration of fluorescein-conjugated anti-rabbit immunoglobulin

A commercial product (HAR-C, Centraal Laboratorium van de Bloedtransfusiedienst van het Nederlandsche Roode Kruis) was chessboard-titrated using 1 heterologous and 2 homologous systems. The homologous titrations included the strains PG1 and PG11 and the corresponding monospecific antisera, while the heterologous test was performed with the strain PG11 against G230 antiserum. In all 3 tests, the conjugate was diluted 1:10, 1:20, 1:30, 1:40, 1:50 and 1:100. The antisera were titrated in two-fold dilutions, from 1:10 up to 1:1280. All dilutions were performed with PBS (pH 7.4).

Homologous titrations of antisera

Antisera other than anti-PG1 and anti-PG11 were titrated against only 1 dilution of conjugate (1:20), chosen according to the results of the experiments described in the previous paragraph.

Composition of polyvalent antisera

On the basis of the results of the above-mentioned homologous titrations the monovalent antisera were diluted with PBS (pH 7.4) to give titres varying from 40 to 320. The 3 polyvalent antisera, made by the mixing of antisera (9 or 10) corresponding to the 3 groups of the type strains mentioned above thus had final titres from 4 to 32 against the strains involved.

Testing of type strains

All 29 type strains were tested against the 3 polyvalent sera as well as against each monovalent antiserum of the relevant group.

Identification of field strains

All of the 26 isolates were primarily tested against all 3 polyvalent antisera and then against each of the monovalent sera of the positive reacting multiserum. In those cases in which reaction with more than 1 monospecific serum was observed, the test was repeated with conjugate dilutions of both 1:50 and 1:20. The identifications were confirmed by growth-inhibition tests.

RESULTS

Titration of conjugated anti-rabbit immunoglobulin

In the homologous chessboard titrations with the strains PG1 and PG11 (Table 1), the optimum dilution of conjugate (i. e. the highest dilution giving maximum titres of antimycoplasma sera) was 1:10 and 1:20, respectively. With strain PG1, 1:40 was the highest dilution giving any fluorescence, while a dilution of 1:100 was still usable with strain PG11. The antimycoplasma titres were 80 and 640, respectively.

In the heterologous chessboard titration (strain PG11 against G230 antiserum) a titre of 80 was found with the conjugate in the dilution of 1:10 (Table 1), while dilutions of the conjugate from 1:20 to 1:50 gave only a titre of 10 as compared to a homologous titre of 320 (strain G230 against G230 antiserum).

On the basis of these results, a conjugate dilution of 1:20 was chosen as the standard dilution in homologous titrations, testings of type strains and identification of field strains.

Table 1. Titration of conjugated anti-rabbit immunoglobulin.

Dilution of conjugate	Strains	Dilutions of monovalent antisera 1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Sera
1:10	PG1	+	+	+	+	0	0	0	0	anti-PG1
	PG11	+	+	+	+	+	+	+	0	anti-PG11
	PG11	+	+	+	+	0	0	0	0	anti-G230
1:20 and 1:30	PG1	+	+	+	0	0	0	0	0	anti-PG1
	PG11	+	+	+	+	+	+	+	0	anti-PG11
	PG11	+	0	0	0	0	0	0	0	anti-G230
1:40	PG1	+	+	+	0	0	0	0	0	anti-PG1
	PG11	+	+	+	+	+	+	0	0	anti-PG11
	PG11	+	0	0	0	0	0	0	0	anti-G230
1:50	PG1	0	0	0	0	0	0	0	0	anti-PG1
	PG11	+	+	+	+	+	+	0	0	anti-PG11
	PG11	+	0	0	0	0	0	0	0	anti-G230
1:100	PG1	0	0	0	0	0	0	0	0	anti-PG1
	PG11	+	+	+	+	+	+	0	0	anti-PG11
	PG11	0	0	0	0	0	0	0	0	anti-G230

+: Fluorescence.
0 : No fluorescence.

Homologous titrations of antisera

The homologous titrations of monospecific antisera gave titres from 40 to 1280. In 2 sera the titres were 40 and 80, respectively; the titres were 160 in 4, 320 in 8, 640 in 6, and 1280 in the remaining 9 sera.

Testing of type strains

All strains reacted with the relevant polyvalent serum, but not with the other 2 sera. In 14 cases, the intensity of fluorescence was roughly on the same level whether polyvalent or monovalent serum was used. In 15 cases, a stronger reaction was seen with monospecific serum although the dilutions were intended to result in equal amounts of reactive antibody in both types of sera, as judged by immunofluorescence.

When tested against the monovalent antisera, one-way cross reactions were observed in 2 cases. Strain BTS-7 (M. hyorhinis) reacted with Donetta antiserum (M. bovis), and strain FH (M.

pneumoniae) reacted with M. agalactiae (PG2) antiserum. In both cases, a titre of 80 was seen as compared with homologous titres of 320 and 640, respectively. The cross reactions could not be confirmed by growth inhibition.

Identification of field strains

One out of the 27 strains did not react with any of the 3 polyvalent antisera although the isolate was later identified (through a growth-inhibition test) as M. bovigenitalium and did react with the monovalent antiserum in indirect immunofluorescence. The remaining 26 strains reacted with 1 and only 1 of the monovalent sera. They were subsequently identified by the monospecific antisera as representatives of the following species: M. arginini (1), M. bovis (1), M. bovigenitalium (2), M. bovoculi (3), M. canis (3), M. capricolum (2), M. cynos (3), M. edwardii (2), M. hominis (1), M. hyorhinis (2), M. molare (1), M. opalescens (3), M. mycoides subsp. mycoides (2).

In approximately half of the cases, a weaker fluorescence was seen with polyvalent sera as compared to the reaction observed with monovalent sera.

One strain of M. canis, identified by growth inhibition, also reacted with antiserum against M. spumans and M. edwardii. Another strain of the same species reacted with serum against M. cynos. A third cross reaction was observed with a strain of M. edwardii, which cross-reacted with M. felis and M. cynos. Using a conjugate dilution of 1:50 these non-species specific reactions were not observed. However, 1 of the 2 strains of M. edwardii would in this case have remained unidentified, as no fluorescence was observed with the monovalent serum using a conjugate dilution of 1:50. The above reactions were confirmed by growth inhibition only in 1 case; the first strain of M. canis mentioned above was partially inhibited with antiserum against M. edwardii.

DISCUSSION AND CONCLUSION

On the basis of the results, it may be concluded that the use of polyvalent sera can be of considerable practical value, but a few points must be emphasized concerning indirect immunofluorescence.

1. In considering the sensitivity and specificity in relation to indirect immunofluorescence as performed in this study, it is also necessary to stress the importance of the antigens. The use of living unfixed colonies implies that the native structure of the antigens involved in the reaction is maintained. But from this it cannot be deduced that all colonies of a certain culture are equally suitable for this kind of test. The author's experience suggests that the use of relatively small, young and growing colonies increases both the sensitivity and the specificity of the test.

2. Owing to the large number of mycoplasma species and the heterogeneity of genus Mycoplasma it would be unlikely that the optimum dilution of conjugated anti-rabbit immunoglobulin would be the same for all antigen-antibody complexes. It is also unlikely that the dilution stated as optimum by the manufacturer should be correct in all modifications of indirect immunofluorescence. Ideally, all monovalent antimycoplasma sera should be chessboard-titrated against the conjugate. However, as this is practically impossible a standard dilution of conjugate is chosen on the basis of at least 1 heterologous and 2 homologous chessboard titrations. To avoid false negative results it is important to choose a lower dilution of conjugate than any of the highest dilutions giving fluorescence, as determined by the homologous chessboard titrations. On the other hand the standard dilution must not result in too many false positive reactions, whether unspecific or due to minor interspecies antigenic relationships, as illustrated by the heterologous titration (Table 1).

3. The dilution of antimycoplasma sera for identification procedures must also be lower than the results of the homologous titration indicate in order to avoid false negative results. Above all, it must be lower because of intraspecies variations.

4. The finding that polyvalent sera often resulted in a weaker fluorescence than when monospecific sera were used is surprising, and the author shall refrain from suggesting explanations.

5. The use of polyvalent sera in the process of identification seems to be of considerable practical value as judged by the results obtained. Certain technical features still need to be de-

termined, such as whether or not the primary dilution of monovalent serum should be performed with PBS or normal rabbit serum, and whether or not there would in fact be a distinct advantage in preparing isolated immunoglobulins.

In the study presented here, the choice of the antisera to be pooled was based on 3 motives: (1) the identification was aimed at frequently occurring mycoplasmas (genus Mycoplasma); (2) the polyvalent sera should be directed against biochemically different mycoplasmas in order to demonstrate the possibility of identification without previous biochemical testing; (3) the grouping should also to some degree consider the host relationship as minor cross reactions would seem to be more likely between different mycoplasma species from the same animal species, if the concept of biological mimicry is real.

In general the choice of the antisera to be pooled also depends upon the diagnostic scope of the individual laboratories. Attention can be paid to the pathogenicity of the organisms, host relationship or biochemical characteristics.

A diagnostic laboratory concerned with diseases in cattle might, as an example, prefer to work with polyvalent antisera pooled according to host relationship. A system of 6 polyvalent antisera (I—VI), each consisting of 8 to 12 monovalent antisera, could be used. Table 2 gives the mycoplasmas corresponding to the monovalent antisera of each group. It is, naturally, necessary for such a laboratory to possess also monovalent antisera against the 12 bovine species (group I), whereas it should be unnecessary to hold antisera against the remaining 52 species or serogroups of genus Mycoplasma. In case a strain is reacting with group IV it would be more practical to have this isolate identified at a laboratory working with human mycoplasmas. Such a demonstration showing that a bovine isolate reacts with non-bovine polyvalent serum tends to diminish the establishment of new bovine "serogroups". This is an advantage in itself, since it might prevent unnecessary comparative taxonomical studies.

A reference laboratory for mycoplasmas would possibly prefer to use polyvalent antisera composed according to the results of 2 biochemical tests, viz. catabolism of glucose and arginine (Table 3). The identification, if necessary, can be accomplished within 24—48 hrs. if the strains are sent as plate cultures.

Table 2. Composition of polyvalent antisera pooled with regard to host relationship.

Polyvalent serum	Host(s)	Monovalent antisera
I	Cattle	M. alkalescens, M. alvi, M. arginini, M. bovigenitalium, M. bovirhinis, M. bovis, M. bovoculi, M. canadense, M. dispar, M. mycoides subsp. mycoides, M. verecundum, bovine group 7
II	Dogs and cats	M. canis, M. cynos, M. edwardii, M. feliminutum, M. felis, M. gateae, M. gateae group 1, M. gateae group 2, M. maculosum, M. molare, M. opalescens, M. spumans
III	Goats, sheep and horses	M. agalactiae, M. capricolum, M. conjunctivae, M. equirhinis, M. mycoides subsp. capri, M. ovipneumoniae, M. putrefaciens, ovine/caprine groups 5, 7, 8 and 11
IV	Man and monkeys	M. buccale, M. faucium, M. fermentans, M. hominis, M. lipophilum, M. moatsii, M. orale, M. pneumoniae, M. primatum, M. salivarium
V	Pigs and rodents	M. arthritidis, M. caviae, M. flocculare, M. hyorhinis, M. hyosynoviae, M. neurolyticum, M. pulmonis, M. suipneumoniae
VI	Poultry	M. anatis, M. gallinarum, M. gallisepticum, M. iners, M. meleagridis, M. synoviae, avian groups C, D, F, I and L

Finally it is pointed out that polyvalent antisera might be useful in controlling mycoplasmal contamination of cell cultures. It would probably also be worth while investigating the applicability of polyvalent antisera in a simple method such as the indirect immunoperoxidase test, which was recently introduced in mycoplasmology by *Polak-Vogelzang & Hagenaars* (1976). The use of intersecting and combinatorial pools as described for enteroviruses (*Schmidt et al.* 1971, *Lagercrantz & Espmark* 1973) is an attractive possibility for identification of mycoplasmas by immuno fluorescence as well as by the indirect immunoperoxydase test.

Table 3. Composition of polyvalent antisera pooled with regard to glucose and arginine catabolism.

Polyvalent serum	Biochemical reactions of the mycoplasmas	Monovalent antisera
A	Glucose positive and arginine negative	M. anatis, M. capricolum, M. cynos, M. feliminutum, M. felis, M. flocculare, M. hyorhinis, M. putrefaciens, M. suipneumoniae, M. synoviae
B	Glucose positive and arginine negative	M. bovirhinis, M. bovoculi, M. canis, M. conjunctivae, M. dispar, M. edwardii, M. molare, M. mycoides subsp. capri, M. mycoides subsp. mycoides, ovine/caprine group 8
C	Glucose positive and arginine negative	M. gallisepticum, M. neurolyticum, M. ovipneumoniae, M. pneumoniae, M. pulmonis, bovine group 7, avian groups C, D and F
D	Glucose positive and arginine positive	M. alvi, M. caviae, M. fermentans, M. moatsii, avian group I
	Glucose negative and arginine negative	M. agalactiae, M. bovigenitalium, M. bovis, M. verecundum, ovine/caprine groups 7 and 11
E	Glucose negative and arginine positive	M. alkalescens, M. arthritidis, M. buccale, M. canadense, M. lipophilum, M. maculosum, M. meleagridis, M. opalescens, M. primatum, M. spumans, avian group L, ovine/caprine group 5
F	Glucose negative and arginine positive	M. arginini, M. equirhinis, M. faucium, M. gallinarum, M. gateae, M. gateae group 1, M. gateae group 2, M. hominis, M. hyosynoviae, M. iners, M. orale, M. salivarium

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SAMMENDRAG

Mykoplasmer: Anvendelse af polyvalente antisera til identifikation ved indirekte immunofluorescens

Det ville i mange tilfælde være en fordel at kunne anvende polyvalente antisera som led i identifikationen af mykoplasmer. I nærværende arbejde refereres en undersøgelse af, hvorvidt sådanne sera er anvendelige til indirekte immunofluorescens. Tre polyvalente sera blev fremstillet, hver bestående af 9 eller 10 kaninantisera, repræsenterende 29 forskellige arter af Genus Mycoplasma. Der undersøgtes 26 vilde stammer. Een af disse reagerede ikke med nogen af de 3 polyvalente sera, endskønt den senere diagnosticeredes som M. bovigenitalium. De resterende 25 stammer reagerede med en og kun en af de polyvalente sera og kunne derefter diagnosticeres ved hjælp af de monovalente antisera. Stammer tilhørende følgende arter identificeredes: M. arginini, M. bovigenitalium, M. bovis, M. bovoculi, M. canis, M. capricolum, M. cynos, M. edwardii, M. hominis, M. hyorhinis, M. molare, M. mycoides subsp. mycoides og M. opalescens. Det konkluderes, at polyvalente antisera er brugbare og kan muliggøre anvendelse af et begrænset antal monospecifikke sera uden forudgående biokemiske undersøgelser.

(Received February 1, 1977).

Reprints may be requested from: H. Ernø, the Institute of Medical Microbiology, University of Aarhus, DK-8000 Aarhus C, Denmark.