

Antigenic differences within the species *Mycoplasma hominis*

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(Received 6 April 1970)

SUMMARY

Membrane and soluble fractions of one genital and two oral strains of *Mycoplasma hominis* were compared by immunodiffusion and polyacrylamide gel electrophoresis. Differences were demonstrated between the membrane antigens of the three strains by immunodiffusion, and the membrane proteins also gave dissimilar patterns in polyacrylamide gel electrophoresis. The soluble fractions gave identical lines in immunodiffusion tests and similar patterns in polyacrylamide gel electrophoresis.

When the strains were cross-titrated in metabolic inhibition (MI) and indirect haemagglutination (IHA) tests, statistical analysis of the results revealed significant differences between the strains. Previously, growth-inhibition, MI and IHA activity was shown to be associated with the membrane antigens of *M. hominis*, so the intraspecies differences revealed by MI and IHA correlate with the differences in the membrane antigens demonstrated by immunodiffusion. Growth-inhibition tests, which might also have been expected to show intraspecies differences, did not do so, probably because of the insensitivity of the test. In contrast to MI and IHA, complement-fixation (CF) tests revealed a high degree of relatedness between the strains. This is consistent with the observation that the soluble antigens of *M. hominis* participate in the CF reaction, and that the soluble antigens of different strains are identical in immunodiffusion tests.

INTRODUCTION

Lemcke & Hollingdale (1968) and Hollingdale & Lemcke (1969) showed that the membrane antigens of a genital strain of *Mycoplasma hominis* gave rise to antiserum active in growth-inhibition (GI), metabolic inhibition (MI) and indirect haemagglutination (IHA). Complement-fixing (CF) activity, on the other hand, was associated with antigens in the soluble cell fraction as well as the membrane. It was also possible, by immunodiffusion and immunoelectrophoresis, to distinguish the membrane antigens from those in the soluble fraction.

Since intraspecies differences between strains of *M. hominis* have been demonstrated by several serological techniques (Nicol & Edward, 1953; Taylor-Robinson, Ludwig *et al.* 1965; Purcell *et al.* 1967), the membrane and soluble fractions of three strains of *M. hominis* were compared by immunodiffusion to see if any

differences could be detected between the antigens of the three. The strains were also compared by MI, IHA and CF, and the results analysed statistically to determine the degree of relatedness.

MATERIALS AND METHODS

Cultures

The three *M. hominis* strains examined most fully were a genital strain, SC4 (Hollingdale & Lemcke, 1969), and two oral strains, DC63 and V2785 (Taylor-Robinson, Somerson, Turner & Chanock, 1963). Six other genital strains were included in growth-inhibition tests: H34 and 4387P (Lemcke, 1964) and four strains isolated by Nicol & Edward (1953)—H23 (PG25), H26 (PG23), H50 (PG21) and D419 (PG26).

Fractionation

To obtain membrane and soluble fractions, SC4, DC63 and V2785 were grown, disrupted by sonic treatment and fractionated as described by Hollingdale & Lemcke (1969). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) with crystallized bovine serum albumin (Puriss. grade, Koch-Light Laboratories, Colnbrook, Bucks.) as a standard.

Antisera

The rabbit antisera to whole cells of SC4, H34 and 4387P were those prepared by Hollingdale & Lemcke (1969) and Lemcke (1964, 1965). Antisera to whole cells of DC63 and V2785 were prepared by a regime of inoculation similar to that used for SC4, after the strains had been adapted to grow in liquid medium containing rabbit serum. Pre-immunization sera were obtained from all the rabbits and included as controls in every serological test.

Polyacrylamide gel electrophoresis

For membrane fractions, the method of Rottem & Razin (1967) was used except in the preparation of the membrane extract. Membranes washed six times and resuspended in dilute sodium chloride/tris buffer (0.0075 M sodium chloride, 0.0025 M tris) at pH 7.4 were mixed with phenol-acetic acid-water (2:1:0.5, w/v/v) to give a final protein concentration of 2 mg. per ml. After standing at room temperature for 30 min. and at 4° C. overnight, the mixture was centrifuged at 9500 g for 30 min. at 4° C. Two volumes of the clear supernatant was mixed with 1 volume of sucrose (40%, w/v) in acetic acid (35%, v/v) and 0.15 ml. of the mixture layered on the gel.

Soluble fractions were compared by a modification of the method of Davis (1964), in which solutions for analysis are run through a 7.5% polyacrylamide gel in a tris (3.0 M) buffer, pH 8.9, and the electrode buffer consists of tris/glycine (0.01 M tris, 0.077 M glycine) at pH 8.3. Soluble fractions in dilute sodium chloride/tris buffer were diluted with electrode buffer to a final concentration of 2.0 mg. protein/ml. This was then mixed with an equal volume of 60% (w/v) sucrose

solution in electrode buffer and 0.075 ml. layered on the gel. Electrophoresis was carried out for 90 min. at a constant current of 2 mA. per tube.

Serological tests

The methods for immunodiffusion, metabolic inhibition (MI) and indirect haemagglutination (IHA) were as described by Hollingdale & Lemcke (1969). For IHA, tanned erythrocytes were sensitized with sonicated suspensions of the mycoplasmas at 0.125 mg. protein per ml.

Growth-inhibition (GI) tests were carried out as described by Clyde (1964). For each strain the inoculum was a 10^{-1} dilution of an 18 hr. culture (0.02 ml. per 5 cm. plate). The 5 mm. disks (Whatman AA) applied to the agar surface contained 0.02 ml. of undiluted serum, and all the sera were tested simultaneously against the same strain.

For complement fixation (CF) tests, antigens were harvested from 6-day broth cultures, washed twice in saline (0.85%, w/v), resuspended in saline containing thiomersalate (0.0001%, v/v) and stored at 4° C. The method for CF was that of Bradstreet & Taylor (1962) except that the microtitre system with unit volumes of 0.025 ml. was used and complement was guinea-pig serum stored without preservative at -30° C. The optimal concentration of antigen, i.e. the dilution giving the maximum serum titre with its homologous antiserum, was the same for all three strains, namely 0.033 mg. cell protein per ml. All cross-titrations were carried out with antigens at this concentration.

Measurement of serological relationships

In metabolic inhibition, indirect haemagglutination and complement fixation, SC4, DC63 and V2785 were cross-titrated against the corresponding antisera in three replicate tests. The resulting mean titres were then analysed according to Alling (1967) and Purcell *et al.* (1967), so as to derive a numerical estimate, with confidence limits, for the degree of relatedness.

When two antigens are tested against the homologous and one heterologous antiserum to give four titres, T_{hom1} , T_{hom2} , T_{het1} , T_{het2} , each titre can be expressed in the form cb^y , where c is the reciprocal of the dilution of the least dilute serum used and b is the dilution factor for successive dilutions (e.g. for twofold dilutions, $b = 2$). Thus, for a titre of 1280 obtained from an initial dilution of 1/10 and a dilution factor of 1/2, $1280 = 10 \times 2^y$ and $y = 7$. The degree of serological relationship, R , is expressed in \log_b , here \log_2 .

$$R = \frac{T_{\text{het1}} T_{\text{het2}}}{T_{\text{hom1}} T_{\text{hom2}}}$$

Hence
$$\log_b R = \frac{y_{\text{het1}} y_{\text{het2}}}{y_{\text{hom1}} y_{\text{hom2}}}$$

When the antigens are identical, therefore, $R = 1$ and $\log_b R = 0$, whereas when they are completely unrelated, $R = 0$ and $\log_b R = -\infty$. Thus, the more $\log_b R$ deviates from 0, the greater the differences between the strains.

RESULTS

Immunodiffusion

The soluble fractions of the three strains, SC4, DC63 and V2785, gave very similar patterns with antisera to any of the three (Plate 1, *a-c*). In contrast, there were pronounced differences between the membrane antigens (Plate 1, *d-f*, and Table 1). The homologous membrane gave the most complex series of lines, the heterologous membranes sharing some but not all of the components. With all three sera, DC63 and V2785 membranes shared a line which was not present in SC 4.

Table 1. *Immunodiffusion tests with detergent-lysed membranes and antisera to whole cells of three strains of Mycoplasma hominis*

Plate no.	Anti-serum well	Precipitin lines*						Antigen well†
1d	SC4	-	+	(++)	++	+	.	SC4
		+	+	++	--	-	.	DC63
		+	-	++	+-	-	.	V2785
1e	DC63	++	+	+	-	++	.	DC63
		(++)	-	-	+	++	.	SC4
		++	+	-	-	+-	.	V2785
1f	V2785	+	+	?	+	+	+	V2785
		-	+	++	-	-	-	SC4
		+	+	++	-	-	-	DC63

* Scoring from left to right denotes order of lines from centre (antiserum) well to outer (antigen) well.

† Membranes lysed with 5 mg. Triton X-100 per mg. membrane protein; final concentration in wells, 2 mg. protein per ml.

+, Single line; ++, two lines, very close together, which may fuse into a single line (++); +-, one of two lines missing; -, --, one or two lines missing; ?, obscured by other precipitin lines.

Polyacrylamide gel electrophoresis

There were minor differences between the proteins of the soluble fractions but the general pattern was the same (Plate 2*a*). The membrane fractions gave more protein bands than the soluble fractions, but there were pronounced dissimilarities, especially among the slower-moving components in the upper part of the gels (Plate 2*b*).

Metabolic inhibition

The results of the titrations are shown in Table 2. All the relatedness (*R*) values fell outside the 95% confidence interval. This suggests that the strains were not identical with respect to the antigens reacting in this test, and confirms the results of Purcell *et al.* (1967).

Indirect haemagglutination

The results are shown in Table 3. As with MI tests, the relatedness values for the three strains were outside the 95% confidence interval.

Complement fixation

The results are shown in Table 4. All titres were within 25% of the homologous reaction. All the relatedness values fell within the 95% confidence interval. By this test, therefore, the three strains seem to be closely related.

Table 2. *Relationships among three strains of Mycoplasma hominis as shown by metabolic inhibition tests*

Antigen	Reciprocal of titre with antiserum		
	SC4	DC63	V2785
SC4	1280-2560	80	160-320
DC63	160-320	1280	160
V2785	160-320	80	1280

Homologous titres in bold type.

Relatedness ($\log_2 R$) from three replicate tests

	SC4	DC63
DC63	-7.67	
V2785	-6.33	-8.00

Ninety-five per cent confidence interval, -1.98 to +1.98.

Table 3. *Relationships among three strains of Mycoplasma hominis as shown by indirect haemagglutination tests*

Antigen*	Reciprocal of titre with antiserum		
	SC4	DC63	V2785
SC4	2560	640	320-640
DC63	160	1280	80-160
V2785	80-160	80	640-1280

Homologous titres in bold type.

* Tanned erythrocytes sensitized with sonicated suspensions of organisms at 0.125 mg. cell protein per ml.

Relatedness ($\log_2 R$) from three replicate tests

	SC4	DC63
DC63	-5.0	
V2785	-5.33	-6.66

Ninety-five per cent confidence interval, -1.62 to +1.62.

Growth inhibition

The results obtained with nine strains of *M. hominis* against antisera to five strains is shown in Table 5. All the strains were inhibited by the antisera. However, measurements of the zones of inhibition were within too small a range to permit differentiation of the strains on this basis.

Table 4. *Relationships among three strains of Mycoplasma hominis as shown by complement fixation*

Antigen	Reciprocal of titre with antiserum		
	SC4	DC63	V2785
SC4	640-1280	640-1280	640-1280
DC63	640	1280	640-2560
V2785	640-1280	640-1280	1280-2560

Homologous titres in bold type.

Relatedness ($\log_2 R$), from three replicate tests

	SC4	DC63
DC63	-1.0	
V2785	-0.66	-1.33

Ninety-five per cent confidence interval, -1.98 to +1.98.

Table 5. *Results of growth-inhibition tests on Mycoplasma hominis*

Strain	Zone of growth-inhibition (mm.) with antisera				
	SC4*	DC63	V2785	H34†	4387P
SC4	5	3	1-2	5	2
DC63	3	2-3	3	2	NR
V2785	3	3	5	4	3
H34	4	1-2	4	5	4-5
4387P	1-2	1-2	2-3	2	3
H23 (PG25)	2-3	2-3	2-3	3	2-3
H26 (PG23)	3	2-3	NR	3	2-3
H50 (PG21)	2	2-3	4	4-5	2
D419 (PG26)	1	2-3	3-4	1-2	1

Homologous reaction in bold type

NR = no result.

, † Representative of results with four () or three (†) other antisera against these strains.

DISCUSSION

Diversity within the species *M. hominis* has been demonstrated in various ways. Somerson *et al.* (1966), using a nucleic acid homology test, showed that eight strains were genetically heterogeneous. The cell proteins of five strains examined by Razin (1968) were also heterogeneous, in that they gave differing patterns in polyacrylamide gel electrophoresis. Serological differences between strains were noted by Nicol & Edward (1953) in agglutination tests and by Purcell *et al.* (1967) in metabolic inhibition tests. Taylor-Robinson, Ludwig *et al.* (1965) demonstrated differences between two strains in indirect haemagglutination tests with human sera, but not with hyperimmune rabbit sera.

We have shown that the serological intraspecies differences observed are attributable to differences in the membrane antigens. These differences were also

apparent in the electrophoretic patterns of the membrane proteins, which showed greater dissimilarities than the proteins of the soluble fractions from the same strains. It is noteworthy that the differences were demonstrable even with fractions prepared by sonic treatment, which often releases some membrane antigen into the soluble fraction (Hollingdale & Lemcke, 1969). Still clearer results might be expected with fractions prepared by alternate cycles of freezing and thawing.

Our results also showed that tests such as metabolic inhibition and indirect haemagglutination, which are dependent on membrane antigens, reveal the greatest intraspecies differences. In contrast, complement fixation tests, in which other antigens besides those in the membrane participate, tended to mask the differences. These results are consistent with those previously obtained by Card (1959) and Lemcke (1964) in which 73 genital strains of *M. hominis* were found to be closely related by CF.

If there is a similar heterogeneity within other *Mycoplasma* species, the choice of tests for identification and differentiation will influence any conclusions about the relatedness of the strains under test. Complement fixation tests will emphasize similarities between strains of the same species, and tests such as MI and IHA and other agglutination tests will tend to reveal intraspecies differences. Fluorescent antibody techniques, which are probably dependent on surface antigens, may also belong to the second category, although sensitivity may be a limiting factor in detecting strain differences, as it is in GI tests.

In our hands, GI, although previously shown, like MI, to be dependent on membrane antigens, was not sufficiently sensitive to detect intraspecies differences. Even D419 (PG26), which was found by Razin (1968) to be resistant to inhibition by three heterologous *M. hominis* antisera, was inhibited to some extent by all the antisera tested. There were differences between the sizes of the inhibition zones produced by different strains with any one antiserum, but in such an insensitive test it was impossible to evaluate their significance. Moreover, the size of the inhibition zone being related to the concentration of the colonies, the results obtained with any one antiserum are comparable only when the inoculum is the same for all strains, and this is difficult to ensure when a number are being tested simultaneously.

With immunodiffusion tests, the results are dependent on the mode of preparation of the antigen used. Thus, in gel-diffusion experiments, where mycoplasma suspensions disrupted by sonic treatment or alternate freezing and thawing were used, seven genital strains of *M. hominis* gave identical precipitin patterns (Lemcke, 1965). However, membranes must be lysed with detergents before the main precipitating components are released (Hollingdale & Lemcke, 1969), so that Lemcke's (1965) precipitating antigens were derived mainly from the soluble fraction and any dissimilarity due to membrane antigens would not have been evident. It is noteworthy that most gel-diffusion studies on mycoplasmas have been made with either frozen-thawed or sonic-treated antigens (Taylor-Robinson *et al.* 1963; Dinter, Danielsson & Bakos, 1965; Taylor-Robinson, Soběslavský & Chanock, 1965; Kenny, 1969). It is highly probable, therefore, that the antigens demonstrated in these studies were mainly soluble and not membrane antigens.

Both membrane and soluble antigens are present in mycoplasmas lysed with Triton X-100 (Hollingdale & Lemcke, 1969), but the precipitin patterns given by such lysates are rather complex.

Since MI and IHA tests reveal the greatest intraspecies differences in *M. hominis*, the use of any one strain to detect MI and IHA antibody in man may not give fully representative results. For example, Taylor-Robinson, Ludwig *et al.* (1965) found that DC63 was a more sensitive detector of IHA antibody than PG21. It is advisable, therefore, where the infecting strain is not available, to use more than one strain in screening human sera for MI and IHA antibody to *M. hominis*. The CF test should also be included, since it is dependent on antigens common to different strains of *M. hominis*. Moreover, CF antibody appears earlier in the infection than MI antibody (Jones & Tobin, 1969).

We do not consider it justifiable to separate *M. hominis* strains into more than one species as suggested by Razin (1968). The strain differences we have demonstrated in membrane proteins and antigens are comparable with those noted by Razin in the cell proteins, but the results of GI and CF tests indicate a basic relationship between the strains which does not obtain between distinct species of *Mycoplasma* (Lemcke, 1964; Clyde, 1964). There may be a case for establishing a number of types or subspecies of *M. hominis*, but the intraspecies differences which are demonstrable at present would not characterize them adequately. There are differences in the membrane antigens, but the possibility of extracting type-specific antigens, such as are used to characterize strains of *Streptococcus pyogenes*, seems remote. *M. hominis* membrane antigens appear to be proteins, and so far it has not been possible to prepare extracts which give a single precipitating component (Hollingdale & Lemcke, unpublished). Characterization of subspecies must await the definition of new properties by which strains of *M. hominis* can be distinguished.

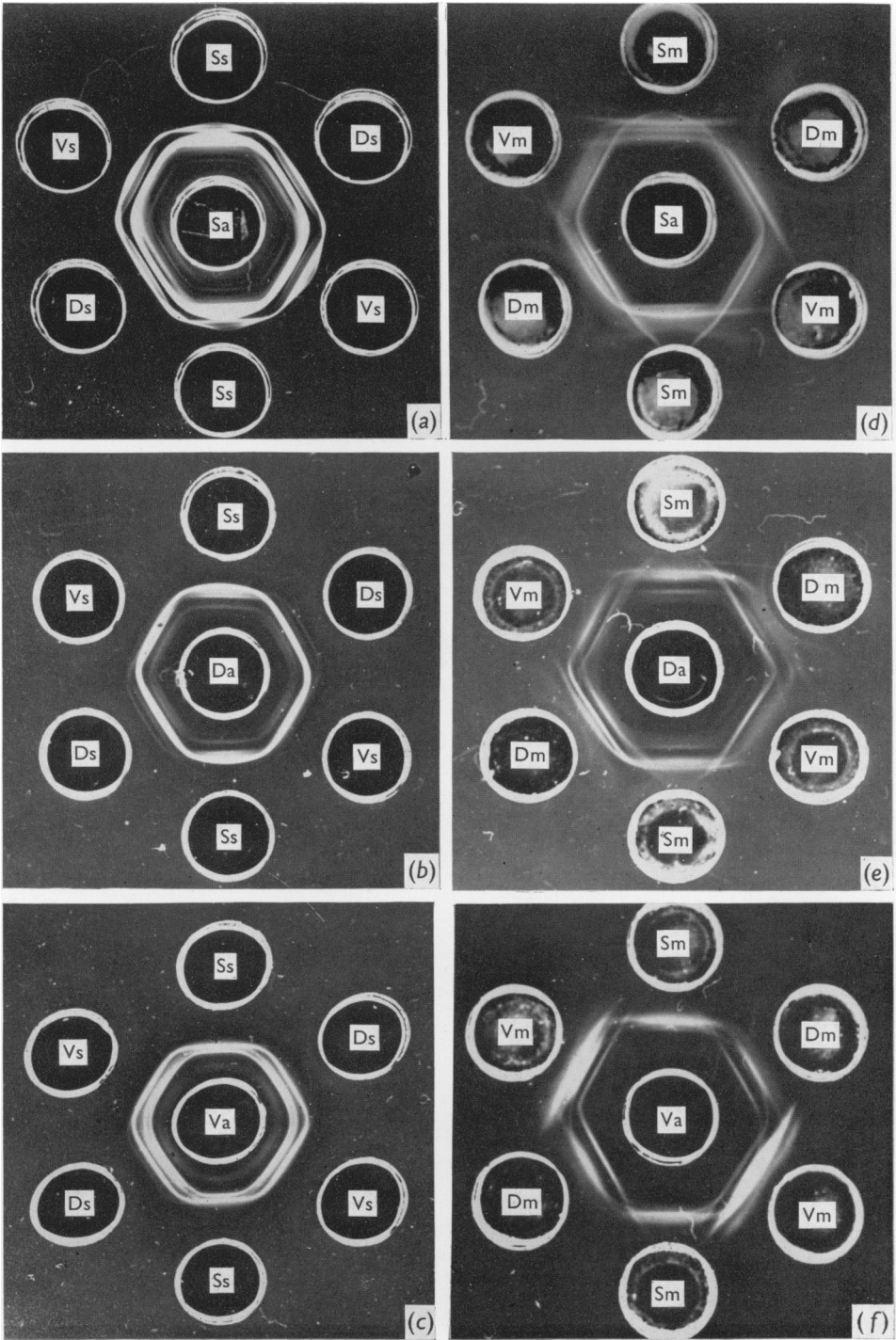
This work was supported by a Medical Research Council grant which provided the remuneration of MRH.

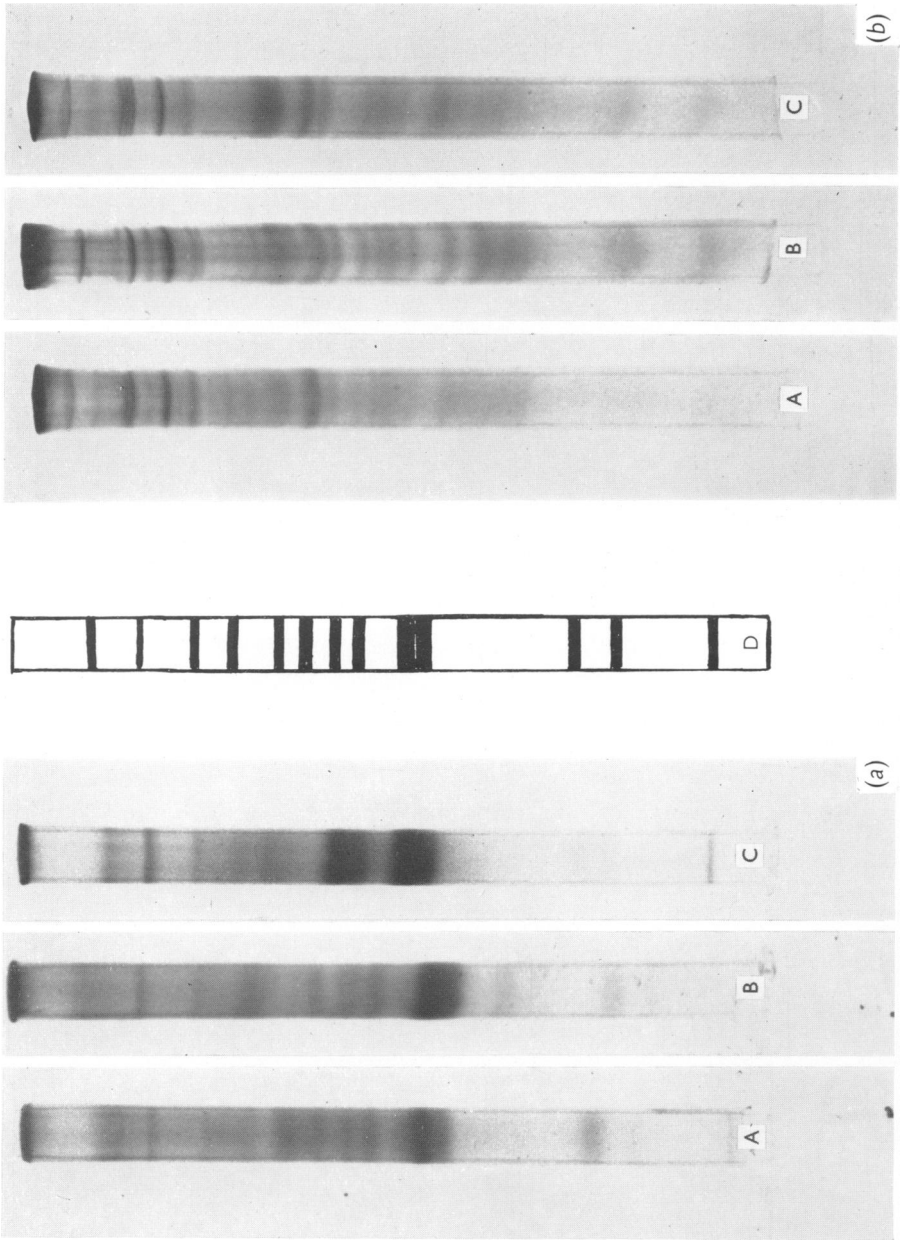
We are indebted to Dr D. W. Alling (National Institutes of Health, Bethesda, Md., U.S.A.) for giving us details of his method of statistical analysis.

We also thank Dr D. Taylor-Robinson (Clinical Research Centre, Harvard Hospital, Salisbury, Wilts.) and Dr D. G. ff. Edward (Public Health Laboratories, County Hall, London, S.E. 1) for supplying strains of *Mycoplasma hominis*.

REFERENCES

- ALLING, D. W. (1967). Tests of relatedness. *Biometrika* **54**, 459.
 BRADSTREET, C. M. P. & TAYLOR, C. E. D. (1962). Technique of complement-fixation test applicable to the diagnosis of virus diseases. *Monthly Bulletin of the Ministry of Health and the Public Health Laboratory Service* **21**, 82.
 CARD, D. H. (1959). PPLO of human genital origin. Serological classification of strains and antibody distribution in man. *British Journal of Venereal Diseases* **35**, 27.
 CLYDE, W. A. (1964). *Mycoplasma* species identification based upon growth inhibition by specific antisera. *Journal of Immunology* **92**, 958.
 DAVIS, B. J. (1964). Disc electrophoresis. II. Method and application to human serum proteins. *Annals of the New York Academy of Sciences* **121**, Art. 2, 404.





- DINTER, Z., DANIELSSON, D. & BAKOS, K. (1965). Differentiation of porcine *Mycoplasma* strains. *Journal of General Microbiology* **41**, 77.
- HOLLINGDALE, M. R. & LEMCKE, R. M. (1969). The antigens of *Mycoplasma hominis*. *Journal of Hygiene* **67**, 585.
- JONES, D. A. & TOBIN, B. M. (1969). Isolation of mycoplasmas and other organisms from the placenta after Caesarean section. *Journal of Medical Microbiology* **2**, 347.
- KENNY, G. E. (1969). Serological comparison of ten glycolytic *Mycoplasma* species. *Journal of Bacteriology* **98**, 1044.
- LEMCKE, R. M. (1964). The serological differentiation of *Mycoplasma* strains (pleuropneumonia-like organisms) from various sources. *Journal of Hygiene* **62**, 199.
- LEMCKE, R. M. (1965). A serological comparison of various species of mycoplasma by an agar gel double-diffusion technique. *Journal of General Microbiology* **38**, 91.
- LEMCKE, R. M. & HOLLINGDALE, M. R. (1968). Preliminary observations on the antigens of *Mycoplasma hominis*. Society for General Microbiology, Proceedings. *Journal of General Microbiology* **53**, ii.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurements with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265.
- NICOL, C. S. & EDWARD, D. G. ff. (1953). Role of organisms of the pleuropneumonia group in human genital infections. *British Journal of Venereal Diseases* **29**, 141.
- PURCELL, R. H., WONG, D., CHANOCK, R. M., TAYLOR-ROBINSON, D., CANCHOLA, J. & VALDESUSO, J. (1967). Significance of antibody to mycoplasma as measured by metabolic-inhibition techniques. *Annals of the New York Academy of Sciences* **143**, Art. 1. 664.
- RAZIN, S. (1968). *Mycoplasma* taxonomy studied by electrophoresis of cell proteins. *Journal of Bacteriology* **96**, 687.
- ROTTEM, S. & RAZIN, S. (1967). Electrophoretic patterns of membrane proteins of *Mycoplasma*. *Journal of Bacteriology* **94**, 359.
- SOMERSON, N. L., REICH, P. R., WALLS, B. E., CHANOCK, R. M. & WEISSMAN, S. M. (1966). Genetic differentiation by nucleic acid homology. II. Genotypic variations within two *Mycoplasma* species. *Journal of Bacteriology*, **92**, 311.
- TAYLOR-ROBINSON, D., SOMERSON, N. L., TURNER, H. C. & CHANOCK, R. M. (1963). Serological relationships among human mycoplasmas as shown by complement-fixation and gel diffusion. *Journal of Bacteriology* **85**, 1261.
- TAYLOR-ROBINSON, D., LUDWIG, W. M., PURCELL, R. H., MUFSON, M. A. & CHANOCK, R. M. (1965). Significance of antibody to *Mycoplasma hominis* type 1 as measured by indirect hemagglutination. *Proceedings of the Society for Experimental Biology and Medicine* **118**, 1073.
- TAYLOR-ROBINSON, D., SOBĚSLAVSKÝ, O. & CHANOCK, R. M. (1965). Relationship of *Mycoplasma pneumoniae* to other human *Mycoplasma* species studied by gel diffusion. *Journal of Bacteriology* **90**, 1432.

EXPLANATION OF PLATES

PLATE 1

Gel-diffusion reactions of fractions of three strains of *Mycoplasma hominis* with antisera against whole cells of the same strains. Antisera (undiluted) in centre wells. Antigens at final concentration of 2 mg. protein per ml.

(a-c) Soluble fractions of SC4 (Ss), DC63 (Ds) and V2785 (Vs) against antisera to SC4 (Sa), DC63 (Da) and V2785 (Va).

(d-f) Membranes of SC4 (Sm), DC63 (Dm) and V2785 (Vm) lysed with Triton X-100 (5 mg. per mg. membrane protein) against same antisera.

PLATE 2

Polyacrylamide gel electrophoresis of fractions of three strains of *Mycoplasma hominis*. a, Soluble fractions; b, membrane fractions. A, SC4; B, V2785; C, DC63; D, diagram of electrophoretic pattern of soluble fractions.