Identification of Mycoplasmas of Human Origin

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SUMMARY

Fifty-five strains of the genus *Mycoplasma* were isolated from sputum of 95 patients suffering from various respiratory tract diseases. The polyacrylamide gel electrophoretic method was used to identify the isolated strains, in addition to the conventional biochemical methods and growth-inhibition by antisera. The electrophoretic patterns of the isolated strains were compared with those of known human mycoplasmas. The electrophoretic patterns obtained were species specific and highly reproducible. The results of the identification of the isolated strains by the gel electrophoretic method correlated well with those of the growth inhibition test. It is suggested that polyacrylamide gel electrophoresis of whole cell proteins might become a useful routine method for identification of mycoplasmas.

The most prevalent *Mycoplasma* found in the sputa was *Mycoplasma salivarium*, only four strains were identified as *M. orale* type 1 and one strain as *M. pneumoniae*. One strain was similar to, but not identical with, *M. salivarium*. The three oral anaerobic mycoplasmas (*M. salivarium*, *M. orale* types 1 and 2) showed a certain similarity when examined by the gel electrophoretic method, they may be genetically related to each other.

INTRODUCTION

Since it was established that the aetiological agent of primary atypical pneumonia associated with cold agglutinins is not a virus but a mycoplasma (Chanock, Hayflick & Barile, 1962), interest in these organisms has markedly increased. Recent reports suggest that mycoplasmas may be associated with some diseases of as yet unknown aetiology. Hayflick & Koprowsky (1965) reported the recovery of a mycoplasma directly from human leukaemic bone marrow, and their isolation has been reported from patients with Reiter's syndrome, rheumatoid arthritis and systemic lupus erythematosus (Bartholomew, 1965). There are indications that certain mycoplasmas, the T-strains, may be a cause of non-gonococcal urethritis (Csonka, Williams & Corse, 1966; Shepard, 1967). Several *Mycoplasma* species are common inhabitants of the human oral cavity. *Mycoplasma salivarium* was isolated from the gingival sulci of almost all persons having natural dentitions, and it was concluded that this mycoplasma is a normal constituent of the anaerobic flora of the human oral cavity (Razin, Michmann & Shimshoni, 1964). A strain more recently classified as *M. orale* type 1 is also a common inhabitant of the oropharynx; less common are *M. orale* types 2 and 3 and *M. hominis* type 1 (Taylor-Robinson, Canchola, Fox & Chanock, 1964; Taylor-Robinson, Fox & Chanock, 1965; Purcell & Chanock, 1967). The relationship of these strains to respiratory tract diseases has been investigated, but there is as yet no con-

clusive evidence that they are able to produce disease in man (Glezen, Thornburg, Chin & Wenner, 1967). The pathogenicity of *M. pneumoniae*, on the other hand, has been proved beyond doubt and it is rarely recovered from the oropharynx of healthy people (Chanock, 1965). *M. pneumoniae* also differs from all the other human mycoplasmas in its biochemical characteristics and in its antigenic structure.

The species identification of Mycoplasma strains, and in particular those isolated from the human oropharynx, is a frequent laboratory problem. The few biochemical tests available are of little help in species identification and at present this is based mainly on serological methods, which are time consuming and some of which frequently yield non-specific results. Nucleic acid homology techniques recently applied to Mycoplasma classification (Reich, Somerson, Rose & Weissman, 1966) are still too complicated to be done in most laboratories. Rottem & Razin (1967) showed that the membrane proteins of animal and saprophytic Mycoplasma strains produce electrophoretic patterns in polyacrylamide gels which are species specific and reproducible and can be used for identification purposes. Further studies (Razin & Rottem, 1967) have shown that the electrophoretic patterns of whole-cell proteins can serve the same purpose, simplifying the procedure considerably. The present report deals with the results of a survey of the Mycoplasma species isolated from sputa of patients suffering from various respiratory diseases, with special emphasis on the identification of the isolates by the new electrophoretic method as compared with the conventional biochemical and serological methods.

METHODS

Organisms. *Mycoplasma pneumoniae*, 15531; *M. fermentans*, 15474; *M. hominis*, type 1, 15056; *M. salivarium*, 14277; *M. orale* type 1, 15539 were obtained from the American Type Culture Collection (Rockville, Md.). *Mycoplasma orale* type 2 was kindly provided by R. M. Chanock (National Institutes of Health, Bethesda, Md.). Locally isolated strains were obtained as described below.

Cultural conditions. The medium used for the isolation and growth of the mycoplasmas was that of Chanock, Hayflick & Barile (1962). Sputum specimens collected from 95 patients with respiratory tract infections, mainly bronchitis, were inoculated on two agar plates; one was incubated aerobically and the other anaerobically at 37°. The plates were inspected microscopically for Mycoplasma colonies every 2 days for up to 21 days. For polyacrylamide gel electrophoretic studies the reference and some of the isolated strains were grown in 200–500 ml. of liquid medium, and incubated for 5–10 days at 37°. In those cases where the local strains did not appear to be pure cultures on the basis of disc growth-inhibition tests, pure cultures were obtained from single colonies. The organisms were harvested by centrifugation at 13,000 g for 20 min., washed three times and resuspended in 1 to 2 ml. of 0.25 M-NaCl. The amount of cell protein was determined according to Lowry, Rosebrough, Farr & Randall (1951). One mg. of cell protein was sufficient for polyacrylamide gel electrophoresis.

Biochemical characters. Glucose fermentation was examined on agar plates containing 1 % (w/v) glucose and 0.0025 % (w/v) phenol red. Reduction of 2,3,5-triphenyl-tetrazolium chloride (TTC) and growth inhibition by methylene blue were tested according to Kraybill & Crawford (1965). Haemolytic activity of the mycoplasmas was tested by the method described by Somerson, Taylor-Robinson & Chanock (1963).

Serological characterization. Immune sera against the six reference *Mycoplasma* strains were prepared in rabbits by the method of Clyde (1964). All sera were stored at -20° until used. Growth inhibition tests were done with impregnated filter-paper discs (Clyde, 1964). Each isolate was tested for growth inhibition by antiserum against each of the reference strains. Inhibition was recorded when colony-free zones were observed around the filter-paper discs; these zones usually measured 5–12 mm. from the edge of the disc.

Polyacrylamide gel electrophoresis. Washed whole organisms were dissolved in phenol+acetic acid+water (2+1+0.5, by vol.) and the dissolved material was run in polyacrylamide gels containing 35 % acetic acid and 5 M-urea (Rottem & Razin, 1967).

RESULTS

Fifty-five of the 95 sputum specimens, were positive for *Mycoplasma* (57.9 %). One of the isolated strains grew aerobically, fermented glucose, reduced TTC aerobically, haemolysed guinea-pig erythrocytes and was not inhibited by 0.02 % methylene blue,

Table 1. *Mycoplasma* strains isolated from sputa of patients with various respiratory diseases as identified by growth-inhibition with monospecific antisera

Species	No. of strains
<i>Mycoplasma salivarium</i>	48
<i>M. orale</i> type 1	4
<i>M. pneumoniae</i>	1
identification doubtful	2
Total	55

presenting the biochemical characters of *Mycoplasma pneumoniae*. All the other 54 isolates grew only anaerobically and did not show any of the characters described above. Table 1 summarizes the results of the growth inhibition tests by immune antisera. Forty-eight of the isolates (87 %) were identified by this method as *M. salivarium* and only four isolates (7.2 %) as *M. orale* type 1. The strain which presented the biochemical properties of *M. pneumoniae* was inhibited by the antiserum against *M. pneumoniae* only. Two strains could not be definitely identified because the zones of inhibition which they showed with antiserum against *M. salivarium* were very small compared to that obtained with the reference *M. salivarium* strain. They were not inhibited at all by any of the other immune sera.

The electrophoretic patterns of the cell proteins of the six reference strains were compared. Each of the species exhibited a characteristic pattern. Although the patterns of *Mycoplasma salivarium* and those of the *M. orale* type 1 and type 2 showed a certain similarity, a well-defined band near the top of the gel of *M. salivarium* which is characteristic for this species did not appear in either of the latter two (Fig. 1). Nineteen of the local isolates were selected and their electrophoretic patterns compared to those of the reference strains. Thirteen locally isolated strains which were inhibited by *M. salivarium* antiserum were examined by polyacrylamide gel electrophoresis and showed patterns identical to each other and to that of *M. salivarium*. All the isolates inhibited by *M. orale* type 1 antiserum showed a pattern identical with that of the reference *M. orale* type 1 strain. The electrophoretic patterns of *M. orale* type 1

always lacked the well-defined upper band characteristic of *M. salivarium*. Of the two *Mycoplasma* isolates that were only slightly inhibited by antiserum against *M. salivarium*, one exhibited a pattern identical with that of the reference strain of *M. salivarium*, whereas the other showed a somewhat different pattern. The electrophoretic pattern of the isolate identified biochemically and serologically as *M. pneumoniae* was identical to that of the reference *M. pneumoniae* strain.

Fig. 1. Schematic representation electrophoretic patterns of the cell proteins of type strains of human mycoplasmas. A = *M. pneumoniae*, ATCC 15531; B = *M. salivarium*, ATCC 14277; C = *M. orale* type 1, ATCC 15539; D = *M. orale* type 2 (Chanock); E = *M. fermentans*, ATCC 15474; F = *M. hominis* type 1, ATCC 15056. The arrow indicates the characteristic upper band of *M. salivarium*.

The polyacrylamide gel electrophoresis patterns of the various mycoplasmas were reproducible in that identical patterns were obtained when the electrophoresis was repeated several times with different pools of the same strains.

DISCUSSION

The isolation of *Mycoplasma* strains other than *Mycoplasma pneumoniae* from the respiratory tract in health and disease has often been reported, but their role in

producing disease is as yet unconfirmed. *Mycoplasma hominis* type 1, however, was found to produce exudative pharyngitis in volunteers (Mufson *et al.* 1965). The mycoplasmas most frequently found in the oropharynx are *M. salivarium* and *M. orale* type 1. According to some authors, *M. orale* type 1 is found much more frequently than *M. salivarium* (Glezen, Thornburg, Chin & Wenner, 1967; Clyde, 1964), while others (Del Giudice, Robillard & Carski, 1967) found, as we did, a higher incidence of *M. salivarium* than of *M. orale* type 1. *Mycoplasma pneumoniae* is rarely found in the normal oropharynx. Its isolation is usually associated with disease. The strain isolated in the present study exhibited all the characteristics of *M. pneumoniae*. The person from whom it was isolated had complement-fixing antibodies against *M. pneumoniae* in a serum dilution of 1/100, but the diagnosis of primary atypical pneumonia was not confirmed clinically.

It is likely that the strains of *Mycoplasma salivarium* and *Mycoplasma orale* present in the sputum are salivary contaminants. The fact that they require an anaerobic or micro-aerophilic atmosphere for growth suggests that they might form part of the normal anaerobic flora of the gingival sulci. Evidence for this suggestion was presented before (Razin *et al.* 1964). Organick (1967) showed that the percentage of *Mycoplasma* isolates in bronchial swabs obtained by bronchoscopy was much lower than in pharyngeal swabs. Since the *Mycoplasma* isolates belonged to the same species in both cases, they suggested that the mycoplasmas occurring in the bronchial swabs resulted from contamination by pharyngeal secretions. Organick (1967) also claimed that the mycoplasmas do not play any causative role in chronic bronchitis.

Growth inhibition by specific antiserum yields highly specific results in the identification of *Mycoplasma* species (Hayflick & Chanock, 1965). Unfortunately this method has some disadvantages. It requires the preparation of potent immune sera and the results of the growth inhibition depend to some extent on the size of the inoculum. The polyacrylamide gel electrophoretic method used in this study seems to be adequate for the rapid identification of new isolates and eliminates the need for expensive antisera. The results obtained by the gel electrophoretic method are reproducible and correlate well with those obtained by the growth inhibition test. The electrophoretic pattern of *Mycoplasma salivarium* and *M. orale* types 1 and 2 resembled each other and formed a distinct group in comparison with the other human mycoplasmas. This finding suggests that the oral anaerobic mycoplasmas may be genetically related to each other, since the composition of cell proteins may reflect genetic relatedness. Complement-fixation tests showed a high level one way cross reaction between *M. salivarium* and *M. orale* (Taylor-Robinson *et al.* 1964). On the other hand, the few available results of nucleic acid homology determinations showed only a low degree of cross-reaction between the anaerobic oral mycoplasmas (Reich *et al.* 1966). The one strain which could not be definitely identified as *M. salivarium* by the growth inhibition test and which also differed from *M. salivarium* in its electrophoretic pattern seems nevertheless to be more closely related to *M. salivarium* than to the other human mycoplasmas. The possibility that this was a mixed culture cannot be ruled out, although the disc growth inhibition test did not indicate the presence of more than one type. These findings suggest the existence of different types of *M. salivarium* as was found for *M. hominis* type 1 (Reich, Somerson, Rose & Weissman, 1966). Further study is needed before any conclusion can be reached in this matter.

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