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Immunofluorescence Identification of *Mycoplasma* on Agar by Use of Incident Illumination

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The fluorescent-antibody method has been employed for the rapid identification of *Mycoplasma* colonies growing on agar plates. The method was found to be effective for detection of mixtures of *Mycoplasma* serotypes growing on primary isolation plates. The technique also helped to define the presence of mycoplasmas which did not produce typical colonies. It was also possible to identify *Mycoplasma* colonies overgrown by bacterial or fungal contaminants. Conjugates directed against 10 distinct *Mycoplasma* serotypes have been successfully employed in this system. One of the serotypes is a human oral isolate which has not been previously characterized.

The definitive identification of mycoplasmas requires antigenic analysis. A number of techniques have been employed for this purpose. Complement fixation, agglutination, indirect hemagglutination, metabolic inhibition, and growth inhibition have all been used to define the antigenic relationships of mycoplasmas, but these techniques do not allow prompt serotyping of new isolates. The common occurrence of mixed cultures of different serotypes makes it necessary to clone all cultures and use a number of single colonies for the preparation of large stocks of antigen. Employing the standard procedure of cloning three to five colonies, there is little assurance that all strains in a mixed culture will be detected. These procedures are complicated by the fact that newly isolated mycoplasmas are frequently difficult to subculture. Furthermore, it has been reported that two species have been separated from a single colony (6). Immunofluorescence has been employed for the identification of mycoplasmas (1, 3, 9, 15) fixed to glass slides. This report describes a technique for direct fluorescent-antibody staining of mycoplasma colonies growing on agar plates, and for examination of the stained colonies in situ by use of incident ultraviolet illumination. This technique has been applied to primary isolation plates, and was found to be particularly well suited for the analysis of mixtures of serotypes.

MATERIALS AND METHODS

Preparation of antisera. The antisera employed in this study were obtained after the protracted immunization of animals (horses, mules, or goats) with large doses of concentrated live mycoplasma. It was found helpful when horses or mules were employed to cultivate immunizing antigen in broth media incorporating preimmune serum from the same animal subsequently used for immunization. A few antisera were prepared in goats by use of broth cultures containing horse serum. After the primary course of immunization, animals were rested for periods of 2 to 6 months and then given a booster of one or more doses of antigen. This cycle was repeated until desired titers were obtained. The longest total immunization period was 11 months (horse). The shortest period was 4 weeks (goat). Antigen doses consisted of 100 times concentrates from 500-ml broth cultures containing 10^7 to 10^{10} colony-producing units per dose.

Mycoplasma species. Seed cultures of the following *Mycoplasma* species were supplied by R. M. Chanock: *M. pneumoniae* (FH-Liu), *M. hominis* (PG-21), *M. salivarium* (PG-20), *M. fermentans* (PG-18), *M. arthritidis* (PG-27), *M. orale* type I (CH-19299), *M. orale* type II (CH-20247), *Mycoplasma* strain GDL (2, 12), and *Mycoplasma* strain S-880 (7, 11). The *Mycoplasma* strain herein designated as MaBy was isolated by one of us (R. A. D.) from the sputum of a pneumonia patient in Pittsburgh in October 1964.

Conjugation of antibody. The globulin from each antiserum was precipitated by half-saturation with neutral ammonium sulfate in an ice bath. The precipitate was dissolved in water and dialyzed against saline until free from sulfate. Labeling with fluorescein isothiocyanate of high chromatographic purity (8) was performed by use of 1 part fluorescein to 70 parts protein in a solution buffered at *p*H 8.8. The mixture was allowed to react overnight at 4 to 10 C. Unbound fluorescein was removed by molecular filtration through a Sephadex G-25 column adjusted to *p*H 7.2 to 7.4, with 0.01 M phosphate-buffered saline. Finally, each conjugate was dialyzed overnight against 0.01 M phosphate-buffered saline at *p*H 7.2 to 7.4.

Staining procedure. Mycoplasmas were grown on BBL Mycoplasma Agar Base supplemented with 20% horse serum (Tissue Culture Select, BBL) and 2% yeast extract dialysate (14). Plates (60 by 15 mm, Falcon polystyrene) containing microscopically detectable colonies on agar were flooded with 5 ml of buffered saline (*p*H 7.2) and held at room temperature for 20 min, after which the buffered saline was decanted. Staining was accomplished by the addition of 2 ml of the appropriate conjugate dilution. The working concentration was the highest dilution of conjugate which produced a 3+ or 4+ staining reaction in previous titrations. After incubation at room temperature for 30 min, the conjugate was decanted, and the agar surface was rinsed three times by flooding with buffered saline.

Fluorescence microscopy. Stained plates were examined by use of a Zeiss GFL 658 binocular microscope equipped with an Osram HBO 200 mercury vapor lamp. The microscope was fitted with an incident illumination attachment, thus avoiding the transmission of exciting ultraviolet light through the agar and the plastic plate. Staining titers presented in this report were obtained by use of a BG12 exciter filter with a no. 47 barrier filter and bright-field condenser.

RESULTS

Fluorescence of Mycoplasma on agar. Mycoplasma colonies stained with homologous conjugate displayed typical yellow-green fluorescence.

The conjugated antibody apparently permeated the colony, since staining was distributed throughout the granular center extending below the agar surface.

The colonial morphology as defined by immunofluorescence was similar to that seen with white light. The staining manipulations slightly disrupted some colonies. These alterations in colonial structure were minimal because of the tenacious mode of growth of mycoplasma on agar.

Heterotypic colonies were either not visible or displayed blue to purple autofluorescence. The agar exhibited a blue autofluorescence at conjugate dilutions greater than 1:5. At lower dilutions, the agar absorbed an excessive amount of conjugate and exhibited too much yellow-green fluorescence for satisfactory contrast with the specifically stained colonies.

Examination of stained colonies with 1,250 times magnification revealed "large bodies" displaying peripheral staining. The peripheral staining suggested that membrane antigens were primarily involved in the staining reaction.

No significant differences in staining reaction were noted with cultures incubated over a 3- to 10-day period. However, the central portion of colonies from 2- to 3-week-old cultures displayed diminished staining.

Plates stained with conjugated globulin obtained from goats immunized with antigen grown in medium containing horse serum exhibited stained granules scattered throughout the agar background. Some mycoplasma displayed an increased concentration of these stained granules around and under the colonies. These granules were apparently of horse serum origin, since they were stained by conjugated antihorse globulin; furthermore, this staining reaction was inhibited by incorporating 20% unlabeled horse serum in the conjugate diluent.

Immunofluorescence specificity. The staining specificity of the conjugates applied to mycoplasmas growing on agar was studied with the 10 strains listed in Table 1. Homologous staining titers ranged from 1:20 to 1:320. No heterologous staining was observed with any of the conjugates when employed at a 1:5 dilution.

Specificity of staining was further demonstrated with each conjugate by inhibition of staining when diluted in homologous broth cultures or concentrated antigen.

Observation of mixed cultures. The efficacy of specific immunofluorescence for the identification of the strains comprising mixed cultures was investigated by staining agar plates which had been inoculated with intentionally mixed *Mycoplasma* serotypes.

The cultures were mixed by adding drops from pure cultures to a plate and allowing the drops to run together on the plate. As many as seven different strains were inoculated onto the same plate in this manner. Each of the strains which had been inoculated on these plates was readily detected by conjugates directed against it. A mixed culture was always apparent on these plates, since heterotypic colonies visible with white light did not fluoresce when viewed with ultraviolet light (Fig. 1).

TABLE 1. *Fluorescent-antibody conjugate titer (reciprocal)*

Conjugate directed against	Homologous titer	Heterologous titer[a]
Mycoplasma arthritidis (PG-27)	20	<5
M. fermentans (PG-18)	160	<5
M. salivarium (PG-20)	320	<5
M. hominis (PG-21)	80	<5
M. pneumoniae (FH)	160	<5
M. orale type I (CH-19299)	160	<5
M. orale type II (CH-20247)	320	<5
Strain GDL	40	<5
Strain S-880	160	<5
Strain MaBy	20	<5

[a] Heterologous titer indicates the results obtained when the conjugate was reacted with all antigens listed in the table.

In another experiment, two different serotypes were mixed together in broth suspensions, incubated for 7 days, and then inoculated onto plates. From many mixed broth cultures, the two organisms were readily recovered on agar. On these plates, the picture was similar to that described above, in that the heterogeneity of the culture was clearly evident. When a plate was stained with a conjugate directed against either of the two strains, only a portion of the total colony population was reactive. Single colonies composed of segments of two different serotypes were discernible (Fig. 2).

In some mixed broth cultures, one strain interfered with the recovery of the other on agar. When these plates were stained with a conjugate directed against the predominant strain, the cultures appeared to be homogeneous. All of the colonies on the plate were stained by a single conjugate; however, staining a duplicate plate with a conjugate directed against the second strain revealed fluorescent microcolonies which were not detectable with white light. These microcolonies were distributed between and within colonies of the predominant strain.

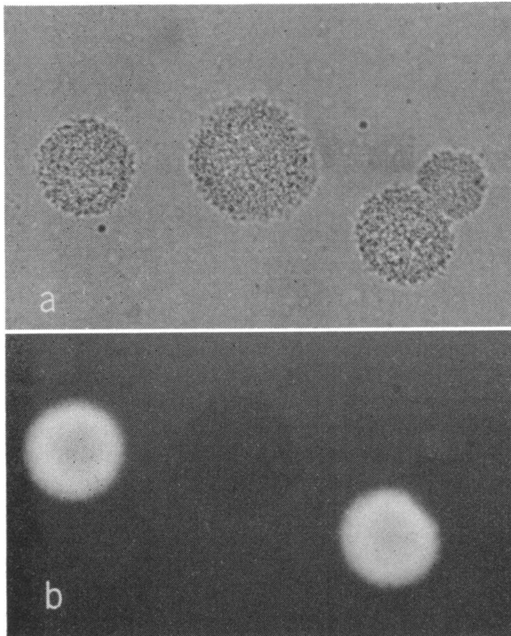

FIG. 1. *Mixed culture of two serotypes growing on the same agar plate. (a) Colonies of Mycoplasma orale type I and M. fermentans stained with a conjugate directed against M. orale type I and illuminated with white light. (b) The same field as (a), but excited with ultraviolet light.*

Occasionally, during the course of these experiments, bacterial or fungal overgrowth completely obscured the field. These contaminated plates were stained in the usual manner and examined. In these instances, the immunofluorescent *Mycoplasma* colonies could be seen through the overgrowth.

Immunofluorescence serotyping of Mycoplasma from fresh specimens. Specimens from a number of sources were cultivated on agar medium. All mycoplasma isolates were tentatively classified according to morphology and colonial hemadsorption (R. A. Del Giudice and R. Pavia, Bacteriol. Proc., p. 71, 1964). On the basis of this tentative classification, one of two duplicate plates was stained with the appropriate conjugate. When no reaction was apparent, the same plate was restained in succession with other conjugates, until immunofluorescent colonies were detected. The plate was then examined for the continued presence of unstained colonies. If none was found, the specimen was recorded as yielding only the organism against which the conjugate was directed. If unstained colonies were detected, a second plate was stained with additional conjugates, not including those already employed on the first plate. If staining was observed with another conjugate, the specimen was recorded as containing these two organisms. An alternative to the successive staining method was to inoculate a series of plates and stain each with a different conjugate.

Immunofluorescence typing results of the specimens yielding mycoplasmas are presented in Table 2. *Mycoplasma* cultures from 34 normal individuals were examined. Mixed cultures of *M.*

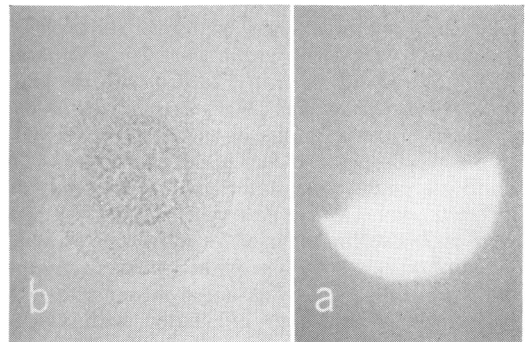

FIG. 2. *Single colony composed of segments of two different serotypes. A single colony growing on an agar plate, inoculated with a mixed culture of Mycoplasma orale type I and M. fermentans, and stained with a conjugate directed against M. orale type I. Viewed (a) with white light illumination and (b) with ultraviolet excitation.*

TABLE 2. *Immunofluorescence identification of Mycoplasma specimens*

Mycoplasma isolate		*Mycoplasma* species identification							
Source	No.	*salivarium* only	*orale* I only	*hominis* only	GDL only	*salivarium* and *orale* I	*pneumoniae* and *orale* I	GDL and *orale* I	Unidentified by FA
Normal human oropharynx	34	24	1	0	0	9	0	0	0
Patient with primary atypical pneumonia	1	0	0	0	0	0	1	0	0
Tissue culture and virus seed material	74	0	50	4	14	0	0	2	4

salivarium and *M. orale* type I were obtained from nine of the specimens. Twenty-four pure cultures of *M. salivarium* and one pure culture of *M. orale* type I were also found. One clinical case of primary atypical pneumonia with confirmatory X-ray and serological findings yielded a mixed culture of *M. pneumoniae* and *M. orale* type I.

Seventy-four cultures were obtained from tissue culture and virus seed material. Most of the isolates were stained by conjugates directed against *M. orale* type I or the GDL strain. Two specimens yielded both organisms. *M. hominis* was found in four specimens. Four isolates were not typable with any of the 10 conjugates. Three of these were identified by serum disc neutralization (5) as *M. laidlawii* A, *M. laidlawii* B, and *M. gallisepticum*. One isolate remains unidentified.

It was possible to estimate the ratio of *Mycoplasma* species in the original sample by counting stained and unstained colonies. In all of the mixed *M. salivarium* and *M. orale* type I cultures obtained from human throat washings, *M. salivarium* was predominant. The ratio ranged from 1:10 to 1:>500.

The appearance of stained and unstained colonies of primary isolates was similar to the laboratory mixtures described above. Some plates inoculated with broth cultures of human throat washings and subsequently stained with the conjugate directed against *M. salivarium* exhibited confluent growth of this organism only. No unstained colonies were detectable. However, when duplicate plates were stained with the conjugate directed against *M. orale* type I, stained colonies were visible within areas of *M. salivarium* growth.

Staining in association with deposited clumps of tissue culture cells was noted on many of the plates which had been inoculated with mycoplasma-contaminated material. These clumps of deposited cells exhibited granular interstitial fluorescence. The interstitial granules were similar in distribution and size to those seen on mycoplasma-infected tissue cultures on cover slip preparations (1, 3). This similarity in size suggests that little or no growth had taken place on the agar. Occasionally, larger masses of antigen were also evident in adjoining cells. This cell-associated antigen was not in the form of typical colonies. Such staining associated with deposited cells was observed on plates that contained only a few typical colonies, as well as on those displaying heavy growth. *Mycoplasma* colonies associated with epithelial cells from urethral scrapings have been observed by use of light microscopy (13). Fluorescent-antibody staining of plates inoculated with human throat washings also indicate an intimate association of *Mycoplasma* and epithelial cells.

DISCUSSION

The use of incident ultraviolet illumination permits the application of immunofluorescence to serotyping of *Mycoplasma* colonies growing on agar medium. This procedure can be applied to primary isolation plates, thus eliminating the need for blind cloning and the production of large quantities of antigen. Cross-reactions encountered in other serological tests because of mixed cultures are avoided with the fluorescent-antibody method. The technique is effective in the analysis of mixtures of serotypes, and provides an estimation of the relative numbers of different species in the mixture.

The efficacy of the system for detection of mixed infection is reflected in the high incidence (25%) of mixed *Mycoplasma* flora found in the normal human oropharynx in this study. The highest incidence of mixed flora from normal subjects previously reported (6) was approximately 12%. Other studies have indicated that mixed *Mycoplasma* flora occur in 0.3% of normal subjects (10). The probability of cloning a colony of a particular strain from a mixed culture is proportional to the number of background colonies.

Since the ratios of *M. orale* type I to *M. salivarium* encountered in the present study were as low as 1:>500, more than 500 colonies would have to be picked from some plates in order to find the *M. orale* type I colony. By the application

of fluorescent antibody to agar plates, the probability of detecting a particular colony is independent of the number of background colonies, since every colony of a selected strain on the plate can be serotyped. It has even been possible to discern antigenic heterogeneity of a single colony.

In addition to the rapid identification of visible colonies, immunofluorescence helps to define the presence of mycoplasmas not detectable by routine microscopic examination. *Mycoplasma* colonies which are obscured by bacterial or fungal overgrowth can be stained and visualized through the overgrowth. Immunofluorescence can also reveal the presence of otherwise inapparent microcolonies on agar plates which have been inoculated with suspensions overgrown by heterotypic mycoplasma. Staining of antigen in association with deposited host cells is another example of successful detection of mycoplasmas which were not detectable on agar by routine microscopic examination. It is uncertain whether these cell-associated stainable bodies indicate viable organisms which failed to adapt to artificial media or whether they represent clumps of nonviable mycoplasmas deposited on the agar. In either case, these findings suggest that fluorescent-antibody methods may be more sensitive for the detection of mycoplasmas than the demonstration of characteristic growth on agar. The mycoplasma strain MaBy is antigenically distinct from the other human oral mycoplasmas when tested by fluorescent antibody. This strain first appeared unique by its affinity for fat stain. Colonies of MaBy growing on media containing Oil Red-O are colored intensely red (*unpublished data*). All other mycoplasmas tested failed to take up the fat stain, or they reacted relatively weakly. MaBy will be further characterized and studied as a possible prototype candidate for a new species of human mycoplasma.

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