Immunofluorescence of Mycoplasma Colonies Grown on Coverslips[1] (34809)

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(Introduced by Henry G. Cramblett)

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Immunofluorescence has proved useful in the identification of mycoplasmas and detection of antibodies against them (1–3). For *Mycoplasma pneumoniae,* the indirect fluorescent antibody test has been found to be highly specific and high fluorescent antibody titers to *M. pneumoniae* have been correlated with resistance to clinical illness (4). The purpose of this study was to develop antigenic preparations for a variety of mycoplasma species which would be both reliable and convenient to use in fluorescent antibody tests. Two methods of preparing mycoplasma antigens for fluorescence tests have thus far been reported. One, as described by Liu (5), employs frozen sections of chick embryo lung infected with *M. pneumoniae.* In the second, and most commonly used precedure (6), mycoplasma colonies growing on agar are transferred to microscope slides and fixed in a hot water bath. The Liu technique employing egg-grown antigens is technically difficult and time consuming; the procedure utilizing hot water fixation gives nonspecific fluorescence primarily because of the presence of contaminating agar.

Broth-grown mycoplasmas have been shown to adhere to glass surfaces (7). We have exploited this property by using glass coverslips on which mycoplasmas have been grown for use in both direct and indirect fluorescent antibody tests. Our method eliminates (1) the necessity of transferring agar-grown organisms, (2) centrifugation to concentrate broth-grown mycoplasmas for smear preparations, and (3) the use of chick embryos.

Materials and Methods. Medium. The broth medium has been described previously (8), but was modified to contain 3.0% PPLO Serum Fraction. Briefly, the medium contained PPLO broth base, Eagle's minimum essential medium, yeast extract, glucose, phenol red, HEPES buffer and PPLO Serum Fraction A.

Organisms. The sources of human mycoplasma species used in this study have been described elsewhere (9). The strain of *M. pulmonis,* a rodent species, was supplied by Dr. Nelson at Rockefeller University.

Reagents. Mycoplasma antisera conjugated with fluorescein isothiocyanate were supplied by Richard Del Giudice at Baltimore Biological Laboratories (BBL), Baltimore, Maryland. Guinea pig complement, anti-human and anti-rabbit globulins (caprine) conjugated with fluorescein (isothiocyanate) also were obtained from BBL. Some human sera containing *M. pneumoniae* antibody and unlabeled rabbit antisera for the human mycoplasmas species were purchased as "reference serums for CF antigens" from Robbins Labs, Chapel Hill, North Carolina. Other human antisera containing antibody to *M. pneumoniae* were kindly supplied by Dr. Robert Chanock of NIH; Dr. Joseph Tully of NIH generously donated human serum con-

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taining antibody to *M. hominis* (10, 11). Rabbit hyperimmune antiserum to *Mycoplasma orale* type 2 was obtained from Microbiological Associates, Bethesda, Maryland. Phosphate-buffered saline (PBS) at pH 7.8 was used for diluting antisera and reagents.

Inoculum for coverslips. Representative strains of each mycoplasma species were grown in 25 ml of medium contained in a 6-oz prescription bottle (8). Cultures were incubated at 37° until a confluent layer of organisms was obtained. The culture fluid was decanted, and the layer of organisms attached to the glass was removed by scraping. The organisms were suspended in 1 ml of mycoplasma broth medium.

Attachment of mycoplasma to coverslips. Sterile coverslips were placed in a disposable tissue culture dish (60 × 15-mm Falcon) containing 5 ml of mycoplasma broth medium. After addition of 0.1 ml of mycoplasma suspension to each dish, cultures were incubated at 37° and examined daily with an inverted microscope. When fermenting mycoplasmas were grown on coverslips, the pH of the broth was closely monitored for potential correlations between colony adhesion an acidity. In all cases, the coverslips were removed when they contained colonies large enough to be readily visible under low-power microscopic examination (100×). The coverslips were air-dried and fixed for 3 min in acetone. Prior to use, each 9 × 22-mm coverslip was sectioned with a diamond point into 12 to 14 pieces. A microscope slide was used as a straight edge for the sectioning procedure. After staining, as many as 36 sections were mounted on a single standard-size slide as a matter of convenience in grading fluorescence.

Coverslips were examined with a Reichert Zetopan fluorescent microscope using a Binolux ultraviolet light source (Mercury vapor arc bulb HBO 200) and OG4 and KG2-5970 filters. Serum titers were defined as the reciprocal of the highest dilution giving an unequivocal positive reaction (bright green fluorescence). All tests were performed in duplicate and included positive and negative control antigen coverslips and control sera. For comparative purposes, smears were made of the same organisms grown in broth and concentrated by centrifugation.

Staining with fluorescein conjugate. Direct test. Coverslip preparations (sections) were placed in wells of a disposable plastic tray (Model No. 965C Linbro Chemical Co., New Haven, Conn.) and 1 drop of fluorescein-conjugated antiserum was evenly distributed over each coverslip section. Trays were covered with plastic sheets cut to size and placed in a humid atmosphere at 37° for 30 min. After removal of excess conjugate by suction, coverslips were washed three times with 1 ml of PBS. As a counterstain, 1 drop of 1:200 dilution of eriochrome black (12) was added. After 10 sec, the coverslips were washed in 1 ml of PBS, inverted, and mounted either wet or dry in 1 drop of elvanol (13).

Blocking test. Sera were placed on the coverslips, incubated for 30 min at 37° and washed with 1 ml of PBS. The rest of the procedure was as described above under direct test.

Indirect test. Appropriate dilutions of sera were spread evenly over coverslip preparations and incubated at 37° for 1 hr. After three washes with PBS, a 1:10 dilution of the conjugated antiglobulin was added. After incubation for 1 hr, the preparation was washed, counterstained with eriochrome black, and mounted as described above. For controls, known positive and negative human sera were used for *M. pneumoniae* and *M. hominis*. Commercially obtained hyperimmune rabbit sera were used for other mycoplasmas.

Results. Preparations of seven mycoplasma species grown on coverslips were evaluated for their adequacy as antigenic preparations for fluorescent tests. Of these, six consistently produced satisfactory colony densities. One species, *M. salivarium* failed to produce colonies of sufficient size to fluoresce distinctly when grown in the manner described. With this organism, smears obtained by centrifugation of broth cultures were found satisfactory for homologous reactions. The incubation time required to produce adequate colony size and even distribution on the coverslips varied among the species and ranged from a minimum of 1 day for *M. pneumoniae* and *M. pulmonis* to a maximum

TABLE I. Direct Fluorescent Antibody Test with Human and Rat Mycoplasmas Grown on Coverslips.

Organisms (strain)	Rabbit antisera						
	M. pneumoniae	*M. orale I*	*M. orale II*	*M. hominis*	*M. fermentans*	*M. salivarium*	*M. pulmonis*
M. pneumoniae (FH)	*40*[a]	<2.5	2.5	2.5	<2.5	2.5	2.5
M. orale I (CH19299)	<2.5	*20*	<2.5	2.5	2.5	2.5	<2.5
M. orale II (CH20247)	<2.5	<2.5	*80*	2.5	<2.5	2.5	<2.5
M. hominis (V2785)	<2.5	<2.5	<2.5	*20*	2.5	<2.5	<2.5
M. fermentans (PG18)	<2.5	<2.5	2.5	<2.5	*20*	<2.5	<2.5
M. pulmonis (N3)	<2.5	<2.5	<2.5	<2.5	<2.5	<2.5	*10*

[a] Reciprocal of fluorescent antibody dilution.

of 5 days for the others. In the case of *M. pneumoniae* and *M. pulmonis,* it was observed that the coverslips must be removed before medium pH falls below 6.7 otherwise colonies do not adhere to the coverslips. When coverslips were harvested before a fall in pH below 6.7, the colonies of these acid-producers adhered as strongly as the others, permitting thorough washing without loss of antigen.

Coverslip preparations were used to test for cross-fluorescence among six species. Species-specific reactions were detected at dilutions between 1:10 and 1:80 (Table I). Cross-reactions between species were minimal by the direct test, with positive fluorescence occurring only at serum dilutions of 1:2.5 or less.

With both the direct and indirect staining procedures, there is a clear distinction between the weak fluorescence of heterologous reactions and the bright-green fluorescence of homologous reactions. Figure 1 illustrates this contrasting fluorescence in the direct test. On the left (Fig. 1A) a coverslip-grown antigenic preparation of *M. pneumoniae* has been reacted with fluorescein-tagged antiserum to *M. hominis* (but not counterstained). The colonies fluoresced with a dull yellowish-green. Subsequently, the same slide was washed and stained again using conjugated homologous antiserum (Fig. 1B). The colonies fluoresced bright green (4+) indicative of a specific reaction of the antigen with the homologous antiserum.

Commercially prepared rabbit antisera were used for positive controls in the indirect test. The sera had been heat-inactivated for use in complement-fixation tests. Even when used undiluted, these sera were nonreactive. After the addition of one part guinea pig complement to three parts control serum, strongly positive species-specific reactions were obtained with these same sera (Table II). For all antigens, titers obtained with homologous antisera were at least 4-fold higher than heterologous sera.

TABLE II. Indirect Fluorescent Antibody Test with Human Mycoplasmas Grown on Coverslips.

Organisms (strain)	Antisera				
	Human		Rabbit		
	Known negative	Known positive	*M. pneumoniae* C-F serum with complement	*M. hominis* C-F serum with complement	*M. fermentans* C-F serum with complement
M. pneumoniae (FH)	<2.5[a]	20	10	2.5	<2.5
M. hominis (V2785)	<2.5	40	<2.5	40	<2.5
M. fermentans (PG18)	ND[b]	ND[b]	<2.5	2.5	20

[a] Reciprocal of fluorescent antibody dilution.

[b] ND = not done.

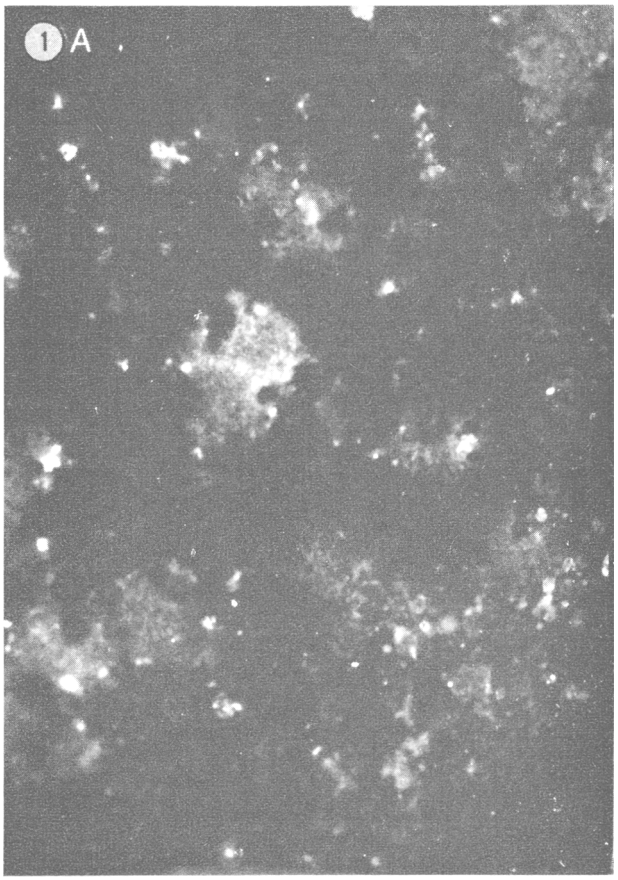
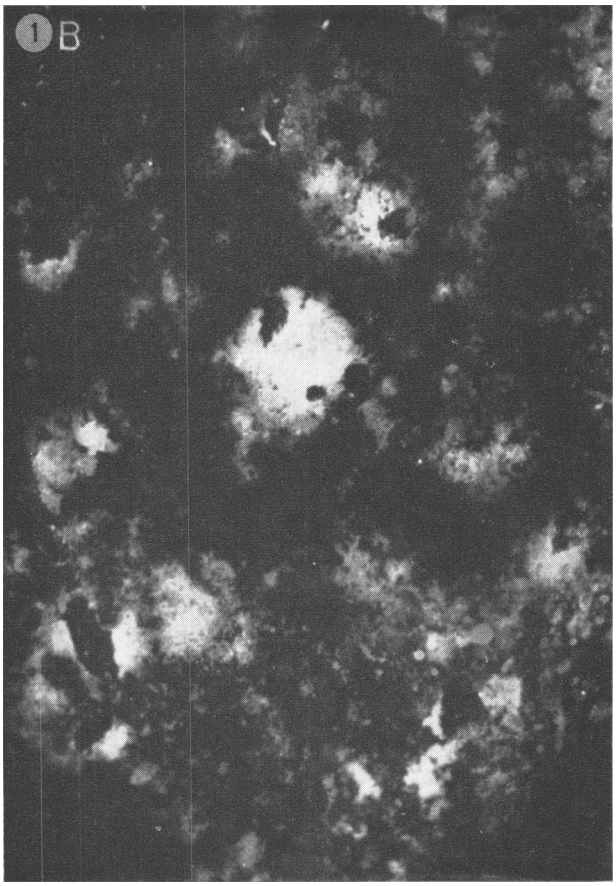


FIG. 1. A. Method: direct test; antigen: coverslip-grown colonies of *M. pneumoniae*; antiserum: conjugated *M. hominis*; fluorescence: dull yellowish-green (±); magnification: ×312.

Interpretation of fluorescence. The intensity of fluorescence and serum dilution end-points were consistent and reproducible for duplicate coverslip-grown preparations of all organisms in both the direct and the indirect tests. Fixed coverslip preparations showed no loss in antigenicity after 12 months of storage at 4°.

In all tests, the negative controls were found to fluoresce with an intensity of grade 0 – 1+, and positive controls were graded 3 – 4+. All test specimens which fluoresced in 1 – 2+ range were repeated for confirmation. Less than 8% of test specimens stained by the indirect method and fewer than 7% of those stained by the direct method required repetition. In the blocking test, however, 40% of all readings required retesting to obtain agreement among four or more samples before interpretation was deemed reliable. A difficulty encountered with smear preparations in both direct and indirect procedures was the difference in staining characteristics of small colonies compared to large clumps of mycoplasma. On the same smear, large clumps fluoresced brightly at serum dilution end-points 2- to 3- fold higher than individual colonies or small clumps. As a result, the determination of serum dilution end-points with smear preparations as antigens was equivocal.

Discussion. The utilization of coverslip-grown antigens in the direct immunofluorescent test provides a relatively rapid and simple method for identifying mycoplasma. Moreover, use of coverslip preparations for the indirect test appears to be a practical means of screening human sera for evidence



B. Method: direct test; antigen: *M. pneumoniae* (same slide as Fig. 1A, re-stained); antiserum: conjugated *M. pneumoniae*; fluorescence: bright green (4+); magnification: ×312.

of an individual's experience with mycoplasmas. The indirect test required only 0.3 ml of conjugated human antiserum to determine antibody levels for six mycoplasma species. Smear preparations of these same organisms required a much larger antigen sample (an entire coverslip) with an attendant greater expenditure of reagents. The large sample is necessary since colony distribution is uneven and antigenic material may be missing over large areas of the coverslip surface. The advantages of our modification of the indirect test do not extend to the blocking test, however, since a positive reaction requires an unequivocal demonstration of the absence of fluorescence. This required the visualization of unstained colonies which was found to be difficult and time consuming.

Titer end-points in the indirect test were found to be slightly, but consistently, lower than those obtained by fluorescent techniques employing smears or agar-grown antigens. Although this might have been due to conservative grading of fluorescence, it's more likely because the fluorescence of larger colony aggregates is more striking than that of smaller colonies. With smear preparations, it was even possible to obtain two separate serum dilution end-points related to colony size. Antibody titers based upon fluorescence of small colonies were similar to titers obtained with coverslip-grown preparations. However, larger clumps on the same smears gave titers 4-fold higher. This colony size factor seems to influence the serum dilution end-point of potent antisera only. Both the smear and coverslip techniques were capable of detecting serum dilution end-

points as low as 2.5.

The technical advantages of preparing antigens by direct growth on coverslips may be summarized as follows: (1) rapid growth with a high yield of antigenically reactive organisms, (2) concentration of organisms on glass coverslips without centrifugation, (3) growth on coverslips produces colonies which are small, uniform in size, evenly distributed, and which stain with uniform fluorescent intensity, (4) antigen preparations are free of metabolic products, medium components, cellular debris, and other contaminants, (5) cross-reactivity is minimized, (6) commercially prepared conjugates can be used without further purification, and (7) there is an economy of reagents.

Summary. A technique is described for employing coverslip antigenic preparations of six species of mycoplasmas in immunofluorescent tests. When grown directly on coverslips in broth media, the colonies produced were small, uniform in size, evenly distributed over the coverslips, and fluoresced uniformly with a high degree of specificity. These factors reduced the size of antigen samples required for the unequivocal demonstration of serum dilution end-points with an attendant economy of time and reagents. Direct growth on coverslips simplified the preparation of antigens and eliminated the introduction of media components or other contaminants into the preparations. The resultant purified antigens exhibited minimal cross-reactions between species, retained their reactivity after prolonged storage, and were adaptable to batch testing. Utilized in the direct fluorescent test, coverslip grown antigens provided a simple and relatively rapid means of identifying mycoplasmas. Their use in the indirect test appeared to be a practical means of screening human sera for evidence of immunologic experience with a variety of mycoplasmas.

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