JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 1981, p. 582-584
0095-1137/81/110582-03$02.00/0

Vol. 14, No. 5

Modified Colony Indirect Epifluorescence Test for Serotyping *Ureaplasma urealyticum* and an Adaption to Detect Common Antigenic Specificity

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Received 9 February 1981/Accepted 12 June 1981

A microtiter method for efficiently serotyping colonies of *Ureaplasma urealyticum* by immunofluorescence is described. Prior detergent treatment allowed identification of common group determinants.

A number of years ago, Rosendal and Black (5) utilized a method which allowed indirect immunofluorescence to be applied to mycoplasmas. Entire unfixed mycoplasma colonies on agar blocks were reacted first with primary rabbit antiserum and then, after being washed, with fluorescein-conjugated antibody to the primary antibody. The washed colonies were examined by epifluorescence microscopy. Colonies of *Ureaplasma urealyticum* serotyped by this method yielded results comparable to those obtained by a metabolic inhibition test (1). Moreover, the immunofluorescence method had two advantages: it could be applied to primary growth on agar and could be used for identifying cultures of mixed serotypes. The modified colony epifluorescence procedure described here is performed in a microtiter plate, which makes it less cumbersome, faster, and, consequently, more suitable for examining large numbers of cultures.

The species and strains of mycoplasmas that we examined are described in Table 1. Dilutions of broth cultures of each organism were spread onto appropriate agar media. (A 5-ml volume of agar per 60-mm petri plate provided an agar layer 2 mm thick.) The inoculated plates were incubated for 2 to 3 days for full development of the colonies. Plates with between 15 and 100 colonies per field at ×40 magnification were selected for use, and cylindrical plugs (diameter, 6 mm) were cut with a punch.

Meanwhile, alternate wells of a microtiter plate (catalog no. IS-MRC-90; Linbro Scientific Co. Inc., New Haven, Conn.) received 0.15 ml of melted 1% (wt/vol) agar (Noble agar; Difco Laboratories, Detroit, Mich.). When the gel was firm, the plugs containing mycoplasma colonies were transferred to the surface of the agar in the wells, with care taken to seat the plugs properly.

Rabbit anti-*U. urealyticum* sera prepared for earlier studies (4) were diluted in a buffer solution that contained 0.85% (wt/vol) NaCl, 0.05 M potassium phosphate (pH 7.5) with 5% (vol/vol) calf serum, and 0.02% (wt/vol) NaN_3. Each antiserum was used at the greatest dilution which gave bright, whole-colony fluorescence. These dilutions ranged between 1:100 and 1:800. Two drops of diluted antiserum (or normal rabbit serum as a control) were added to the top of each plug, which was then incubated at room temperature for 15 min. The antiserum was removed by aspiration with a hypodermic syringe fitted with a blunt needle. Immediately, the surface of each plug was washed twice with 2 drops of buffer and then covered with 2 more drops of buffer. After 10 min, this buffer was removed and replaced with fresh buffer for a second 10-min extraction. Each plug was then covered with 2 drops of diluted fluorescein-conjugated goat anti-rabbit immunoglobulin G (IgG), incubated for another 15 min, and washed as before. This second reagent was used away from bright light. After the second 10-min wash, the plugs were usually covered again with buffer and extracted overnight at 4°C for further reducing the background.

We prepared the anti-rabbit IgG by injecting goats with diethylaminoethyl cellulose-purified rabbit IgG in complete Freund adjuvant and boosting after 1 month with a second injection, also in complete Freund adjuvant. The hyperimmune serum was precipitated with 45% saturated ammonium sulfate for obtaining a crude immune globulin. This fraction was conjugated with fluorescein isothiocyanate by the method of Clark and Shepard as described by Goldman (2) and was found to contain about 20 mg of protein per ml with 1.5 fluorescein groups per protein molecule. The conjugate was used at a

TABLE 1. *Species and strains of Mycoplasmatales examined by detergent-colony epifluorescence*

Species	Strain(s)	Source
U. urealyticum	Serotypes 1-9	Human[a]
U. urealyticum	2065-8-LR	Bovine[b]
U. urealyticum	1762	Bovine[c]
U. urealyticum	1763	Bovine[c]
U. urealyticum	1966	Bovine[c]
U. urealyticum	1611	Ovine[c]
U. urealyticum	1643	Ovine[c]
U. urealyticum	1655	Ovine[c]
Acholeplasma laidlawii	B	R. N. McElhaney[d]
Mycoplasma pneumoniae	15531	ATCC[e]
M. fermentans	19989	ATCC
M. hominis	14027	ATCC
M. pulmonis	19612	ATCC

[a] See reference 4.
[b] From B. Kingscote, Animal Diseases Research Institute, Lethbridge, Alberta, Canada.
[c] From L. Ruhnke, Veterinary Services Laboratory, Guelph, Ontario, Canada.
[d] From R. N. McElhaney, Department of Biochemistry, University of Alberta, Canada.
[e] American Type Culture Collection, Rockville, Md.

1:40 dilution.

After the final wash, the buffer was removed, and the plugs were examined with a Dialux microscope equipped with Ploem epiillumination with a 50-W mercury light source and an I2 filter block (Ernst Leitz Wetzlar GMBH; Wetzlar, West Germany). With a ×10 objective lens (numerical aperture, 0.25; mechanical tube length, 170 mm), the total magnification was ×125; this objective lens has a working distance that allowed it to be focused on the colonies in the wells of the microtiter plate. Colonies first were located with visible, transmitted light which was then interrupted for examination for fluorescence. We considered observed fluorescence positive if it was as bright as the positive control (the homologous serotype antigen and its antiserum); otherwise, it was described as weak or, if there was no fluorescence, negative. Normal rabbit serum diluted 1:25 to 1:400 was always negative. Occasionally, we found plugs which had been inverted; i.e., the colonies were on the lower surface of the agar cylinders. These tests and those in which the intensity of the staining fell between positive and weak were always repeated, along with the positive controls.

In addition to the nine standard antigens (4), we serotyped about 300 clinical isolates on primary cultures. We found the method both convenient and reliable, providing the plugs were washed adequately after exposure to the primary antiserum. If this is not done, false-negative results may be obtained. Presumably, excess immunoglobulin on the plug reacts with and therefore blocks the fluorescent anti-immunoglobulin. For instance, colonies of serotype 2 stained with anti-2 at a dilution of 1:400 were strongly positive. However, if 10% (vol/vol) normal rabbit serum was added to the antiserum, the fluorescence intensity decreased to the point at which it would be judged weak. Since this problem was associated only with weak primary reagents (i.e., those diluted less than 1:30), and most of our antisera were used at relatively high dilutions (i.e., 1:200 to 1:800), this was not a significant problem.

The advantage of the modification over the original method is economy of operations. The original method requires several times the 0.1-ml volumes of primary and secondary reagents now needed for each test. Moreover, instead of using a separate slide for each test, technologists can test an isolate against all typing antisera in a single microtiter plate. Because the tests are both performed and examined in the plates, handling is decreased significantly. However, the initial reason for devising the modification was for providing a tight fit for the plugs so that proper orientation could be maintained throughout the manipulations. With the original methodology, plugs commonly were inverted; with the modification, inversions are rare.

U. urealyticum is the only species of its genus. Among isolates from humans, at least 14 serotypes have been identified (unpublished data). Antiserum to any one ureaplasma serotype reacted strongly with the homologous strain and weakly, if at all, with the other serotype standards. On many occasions, a simple assay for ureaplasmal group determinants could supply useful information. Because of the large number of serotypes, such a test would allow a considerable saving of both time and money. We found that if we let the ureaplasma colonies react with a weak detergent solution (1% [vol/vol] Triton X-100 in the buffer described above) for 3 min before being tested, type specificity was lost. With this detergent-colony epifluorescence test, antiserum to any serotype of human ureaplasma reacted with every immunotype strain. Furthermore, the endpoints of these reactions generally equalled those of the particular serotype specificity; e.g., the endpoint for antiserum to serotype 6 was 1:400 by both the colony epifluorescence and the detergent-colony epifluorescence procedures. The loss of type specificity was also demonstrated with all seven animal ureaplasmas tested (three ovine and four bovine isolates;

Table 1). However, detergent-treated colonies of a species of *Acholeplasma* or four diverse species of *Mycoplasma* (of human and animal origin) reacted with neither the normal rabbit serum control nor anti-ureaplasmal rabbit sera. Thus, although it eliminated type specificity, the detergent-colony epifluorescence test revealed common ureaplasmal group determinants. Primary cultures of 30 out of 33 clinical isolates of *U. urealyticum*, none of which was serotype 8, were positive when the detergent-colony epifluorescence test was performed by using anti-serotype 8 antiserum as the primary reagent; the other three isolates were judged weak.

Our assumption is that one or more specificities located within the membrane or cytoplasm of the ureaplasma are exposed by the detergent treatment. Recently, Nagatomo et al. (3) described a precipitin band common to ureaplasmas but not found in other mycoplasmas. We may have identified the same constituent(s) and speculate that it might include the urease unique to this species. As determined by immunoelectrophoresis, all of the eight human serotypes of *U. urealyticum* examined demonstrated a common antigenic peak. This antigen gave a positive reaction with a stain specific for urease activity (I. A. Sayed and G. E. Kenny, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, G23, p. 80). Nonprimate strains were not included in that study.

Using fluorescein-labeled goat anti-human immunoglobulin as the second reagent, we are now using the detergent-colony epifluorescence procedure to screen human sera for antibodies to ureaplasmas. The method may also have wide application in veterinary medicine.

We thank Errol Prasad and Adrian Wills for skilled technical assistance.

This work was supported by grants A7320 from the National Science and Engineering Research Council, Ottawa, Canada (to G.W.S.) and MA 5414 from the Medical Research Council, Ottawa, Canada (to J.A.R.).

LITERATURE CITED

1. **Black, F. T., and A. Krogsgaard-Jensen.** 1974. Application of indirect immunofluorescence, indirect haemagglutination and polyacrylamide-gel electrophoresis to human T-mycoplasmas. Acta Pathol. Microbiol. Scand. Sect. B **82**:345–353.
2. **Goldman, M.** 1968. Fluorescent antibody methods, p. 102 and 125. Academic Press, Inc., New York.
3. **Nagatomo, H., H. Kotani, and M. Ogata.** 1980. Serological studies of bovine ureaplasmas by agar-gel precipitation test. Jpn. J. Vet. Sci. **42**:9–17.
4. **Robertson, J. A., and G. W. Stemke.** 1979. A modified metabolic inhibition test for serotyping strains of *Ureaplasma urealyticum* (T-strain mycoplasma). J. Clin. Microbiol. **9**:673–676.
5. **Rosendal, S., and F. T. Black.** 1972. Direct and indirect immunofluorescence of unfixed and fixed mycoplasma colonies. Acta Pathol Microbiol. Scand. Sect. B **80**:615–622.