Urease Color Test Medium U-9 for the Detection and Identification of "T" Mycoplasmas in Clinical Material

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A urease color test fluid medium (U-9) for the detection and identification of T (T-strain) mycoplasmas in clinical material is described which is sensitive and specific for this group of mycoplasmas. The medium was prepared from commercially available components and contained 95% half-strength, tryptic digest broth (pH 5.5), 4% unheated horse serum, 0.05% highest-purity urea, 0.001% sodium phenolsulfonphthalein, and 1,000 units of potassium penicillin G per ml. The final reaction of medium U-9 was pH 6.0. The overall agreement (positive and negative) between urease reactions in U-9 urease color test medium and culture findings in a standard agar primary culture system among 686 clinical specimens was 98.1%. The disagreement consisted of 13 false-positive urease reactions which were recognized visually as false-positive reactions due to other microorganisms. For specimens from the female genitourinary tract, the inclusion of 2.5 μg of amphotericin B (Fungizone) per ml of medium U-9 is recommended for the suppression of growth of *Candida* species and filamentous fungi.

As a result of continuing improvements in media for isolation of T (T-strain) mycoplasmas, recognition and identification of T mycoplasmas in primary agar cultures by means of characteristic small colony size and morphology alone are no longer completely reliable. In mixed mycoplasma cultures, it is often difficult to distinguish between large T mycoplasma colonies and colonies of classical mycoplasma species, especially if the latter have limited zones of surface growth or completely lack this feature due to crowding. However, it is now possible to utilize certain specific *biochemical* properties of T mycoplasma organisms (in addition to morphology, size, and staining reaction of agar colonies) to identify and further characterize this mycoplasma group (5–7). One of the most unique and characteristic biochemical properties of T mycoplasmas is their ability to hydrolyze urea with the production and accumulation of ammonia (1, 3, 5, 8). This metabolic capability was utilized to develop a sensitive, reliable urease color test fluid medium for the detection and identification of T mycoplasmas in clinical material.

MATERIALS AND METHODS

Test organisms. The following recognized species of human classical mycoplasmas were employed: *Mycoplasma hominis* PG-21, *M. fermentans* PG-18, *M. salivarium* PG-20, *M. pharyngis* NMFRL, *M. pneumoniae* NMFRL, and *M. lipophilum* MaBy. The following strains of T mycoplasmas, purified by three cycles of single-colony isolations, were also employed: 960, K12, K71, F354, K510 and 2K160. T strain F354 was obtained from D. K. Ford. Naturally occurring strains of T mycoplasmas among 686 clinical specimens from male nongonococcal urethritis patients were also employed; these included specimens of urethral exudate and sediments from centrifuged urine. Among the 686 specimens were included initial and repeated specimens for primary culture as well as post-treatment follow-up specimens.

Agar culture media. Agar medium A-3 described by Shepard (7) served as the standard reference culture medium for isolation of T mycoplasma organisms from clinical specimens and for cultivation of purified strains of T mycoplasmas. In the later phases of this study, an improved version of medium A-3 (designated A-5C) was also employed (*to be published*).

Fluid media. For cultivation of T mycoplasma organisms in broth cultures, A-3 fluid medium (7) was employed. Titers of 10^5 to 10^7 colony-forming units per ml were usually attained after 16 to 18 hr of incubation at 36 C in this fluid medium.

Urease color test medium U-9. (i) U-9 Basal Broth was first prepared which contained the following

ingredients: Tryptic Digest Broth powder [Fields, BBL no. 11754 or Difco no. 0977-01-8 (newly purchased, untried lots of Tryptic Digest Broth should be *pre-tested* against a lot of this product of known satisfactory performance in U-9 urease color test medium; BBL lot no. 710716 Tryptic Digest Broth should not be used in medium U-9 since its performance in this medium is unsatisfactory)], 0.75 g; sodium chloride (ACS), 0.5 g; monobasic potassium phosphate [KH_2PO_4, (ACS)], 0.02 g; and deionized water (resistance = 1.0 megohm or more), 100 ml. The ingredients were dissolved, the reaction was adjusted to *p*H 5.5 with 2 N HCl, and the basal broth was sterilized in the autoclave at 121 C for 15 min. (ii) Complete medium U-9 was prepared by combining the following sterile solutions: sterile basal broth, 95 ml; unheated normal horse serum, 4.0 ml; 10% urea stock solution, 0.5 ml; 1.0% phenol red stock solution, 0.1 ml; potassium penicillin G (100,000 units/ml of stock solution), 1.0 ml. The final reaction of complete medium U-9 was *p*H 6.0 ± 0.2.

Mann-9200 Ultra-Pure Urea was used to prepare the 10% urea stock solution in deionized water, and the solution was sterilized by filtration through a 0.22-μm porosity membrane filter. The 1.0% phenol red stock solution was prepared from Eastman Organic Chemicals 6131 sodium phenolsulfonphthalein in deionized water and sterilized in the autoclave at 121 C for 15 min. The penicillin stock solution was dispensed in convenient small volumes in screw-cap tubes or vials and stored in the frozen state at −20 C. If the medium was used primarily for specimens from females, amphotericin B (Fungizone), in a final concentration of 2.5 μg/ml, was added to medium U-9.

Medium U-9 was aseptically dispensed in 1.8-ml volumes in sterile screw-cap culture tubes (13 by 100 mm) or 1-dr vials (Wheaton 24342), employing an autoclave-sterilized Oxford Pipettor (400, model S-A). The medium was dispensed in 1.8-ml volumes so that 0.2 ml of sample could be diluted in 10-fold serial dilutions in tubes of medium U-9, if desired, in addition to its primary use as a medium for detection of T mycoplasmas. The preparation of basal broth, conversion to complete medium U-9, and aseptic dispensing into sterile tubes were always accomplished on the same day. Fresh medium U-9 was prepared each week, although medium U-9 prepared from satisfactory Tryptic Digest Broth powder may be stored in the refrigerator for periods of as long as 1 month without serious deterioration in performance. We do not recommend preparation and storage of multiple units of *basal* broth pending future use for cultivation of T mycoplasma organisms.

Inoculation of agar cultures and medium U-9. Urethral exudate from nongonococcal urethritis patients was collected by means of a 3-mm special bacteriological scraping loop made from no. 20 B&S gauge platinum alloy wire containing 15% iridium for increased stiffness. The loop was bent at a slight angle (10°) to increase scraping action. Exudate collected by scraping was suspended in 0.6 ml of sterile A-3 basal broth (7) carrying fluid (*p*H

6.0) and thoroughly mixed by means of a vortex stirrer. Such suspensions permitted uniform inoculation of several media under comparative evaluation from the same clinical specimen. Drops containing 0.01-ml volumes of exudate suspension were placed on the surface of standard A-3 or A-5C agar plates, and 0.1-ml volumes were added to tubes of medium U-9. First voided, 40-ml urine specimens were centrifuged at approximately 1,000 × *g* in a clinical-type centrifuge. After decanting the supernatant urine and mixing the sediment by means of a vortex stirrer, 0.01-ml volumes were placed on the surface of standard agar plates (as for exudate), and 0.1-ml volumes were added to tubes of medium U-9. Urine specimens were collected after the collection of urethral exudate.

RESULTS

Interpretation of urease reaction in medium U-9. Growth of T mycoplasma organisms in medium U-9 is accompanied by hydrolysis of urea and accumulation of ammonia. Since this is a weakly buffered medium, the reaction becomes progressively more alkaline and the color of the incorporated phenol red indicator changes from yellow to red (alkaline color). A positive urease reaction by T mycoplasma organisms in medium U-9, therefore, is indicated by a color change from yellow to red. The color change generally commences at the bottom of the tube, spreading throughout the medium during continued incubation at 36 C. It must be emphasized that growth and urease activity by T mycoplasma organisms in medium U-9 occur without detectable turbidity at any time. In our experience, specimens yielding positive urease reactions in nonturbid medium U-9, in the face of negative agar cultures, were nearly always confirmed positive for T mycoplasma organisms. This was routinely accomplished in our laboratory by inoculating a fresh tube of medium U-9 from the respective thawed clinical specimen which was stored in the frozen state (−85 C) pending further studies. At the first sign of color change in the freshly inoculated tube, subcultures to A-5C agar generally produced abundant outgrowth of typical T mycoplasma colonies. Employing a standardized inoculum of 0.1 ml of sediment from a centrifuged urine specimen (or 0.1 ml of urethral exudate suspended in 0.6 ml of A-3 basal broth-carrying fluid), positive urease reactions generally occurred after 18 to 24 hr of incubation if T mycoplasma organisms were present. Under other conditions of inoculation, longer incubation periods (48 to 72 hr) were sometimes required. In rare instances, delayed positive urease reactions due to T mycoplasmas occurred after 3 to 5 days of incubation.

Specificity. Medium U-9 is a restrictive urea medium developed specifically for the detection

of T mycoplasma organisms in specimens of clinical material and as an aid in the identification of T mycoplasmas in culture. Since T mycoplasmas are the only members of the mycoplasma group so far known to contain urease, the urease reaction in this medium is specific for T mycoplasmas (Table 1). The seven classical mycoplasma species tested were unreactive in this urease test medium, including *M. arginini*, which produces ammonia from arginine.

Proteus L-forms can be induced to hydrolyze urea in medium U-9 by inoculation with *Proteus* L colonies (via agar block) or with fluid cultures of *Proteus* L-forms. Generally, rather heavy inoculation is required, and an incubation period of 48 to 72 hr is needed to produce a positive urease reaction. Such observations were based upon laboratory manipulations by using laboratory-adapted strains of *Proteus* L-forms (strain no. 9, 18, 52, XK, and 2070, obtained from L. Dienes and R. M. Cole). In routine testing of clinical specimens from the genitourinary tract, false-positive reactions due to *Proteus* L-forms per se have not been observed. Instead, if *Proteus* is present in a clinical specimen, and succeeds in breaking through the penicillin barrier in medium U-9, it multiplies in the bacillary form, produces obvious bacterial-type turbidity, and yields a false-positive urease reaction for T mycoplasmas. In our laboratory, a separate, special A-4 culture plate without penicillin (*p*H 7.4) is also routinely

inoculated for detection of *Proteus* and *Neisseria gonorrhoeae* in clinical exudates.

Detection of T mycoplasmas in clinical specimens. Urease color test medium U-9 was compared to our standard agar culture system (A-3 and A-5C agar) for its ability to detect the presence of T mycoplasma organisms in clinical specimens. A total of 686 clinical specimens from untreated and treated male nongonococcal urethritis patients was employed in a performance evaluation. Specimens consisted of urethral exudate and sediment from centrifuged urine specimens from patients prior to treatment (initial and repeated specimens) and post-treatment follow-up specimens. Each specimen was planted on standard A-3 or A-5C agar and in medium U-9, respectively.

T mycoplasmas were isolated in primary cultures from 285 clinical specimens by employing the standard agar culture system. All of the 285 clinical specimens which were positive for T mycoplasma organisms in agar cultures also produced positive urease reactions in respective companion tubes of medium U-9. The remaining 401 clinical specimens were negative for T mycoplasmas in standard agar cultures. However, only 388 clinical specimens were urease negative in respective companion tubes of medium U-9. The difference in negative reactions observed was due to 13 false-positive reactions which occurred in medium U-9 (Table 2). These 13 reactions were all recognized visually as false-positive reactions and were caused by growth of bacteria (*Proteus*) or fungi (*Candida* and filamentous fungi). Thus, among 401 specimens negative for T mycoplasmas by standard agar cultures, agreement between the two detection systems was 96.8%. The overall agreement between urease reactions in urease color test medium U-9 and culture findings in the standard agar culture system among 686 clinical specimens (positives and negatives) was 98.1%.

False-positive reactions. Penicillin (1,000 units/

TABLE 1. *Urease reactions of human and other purified Mycoplasma species in urease color test medium U-9*

Mycoplasmas	Urease reaction in medium U-9
T (T-strain) mycoplasmas	
960[a]	+
K12[a]	+
K71[a]	+
F354[b]	+
K510[a]	+
2K160[a]	+
M. fermentans PG-18[c]	−
M. salivarium PG-20[c]	−
M. hominis PG-21[c]	−
M. pharyngis NMFRL	−
M. pneumoniae NMFRL	−
M. lipophilum MaBy[d]	−
M. arginini G-230[e]	−
M. arginini G-119[e]	−
M. arginini 88[e]	−

[a] Obtained from M. C. Shepard.
[b] Obtained from D. K. Ford.
[c] Obtained from D. G.ff Edward.
[d] Obtained from R. A. DelGiudice.
[e] Obtained from M. F. Barile.

TABLE 2. *Agreement between primary isolation of T mycoplasmas in standard agar cultures and urease reactions in medium U-9 among 686 clinical specimens*

T mycoplasmas	Standard agar cultures	Medium U-9	Differences	Agreement (%)
Positive	285	285	0	100
Negative	401	388	13[a]	97

[a] False-positive reactions due to *Proteus* sp., *Candida*, and filamentous fungi. Overall agreement among 686 clinical specimens was 98.1%.

ml), the antibacterial agent in medium U-9, was generally effective in preventing multiplication of all bacteria normally present in clinical specimens from the male genitourinary tract. However, *Proteus* may occasionally break through the penicillin barrier and produce false-positive T mycoplasma urease reactions. Such reactions are indicated by obvious bacterial-type turbidity. False-positive urease reactions in medium U-9 may also be produced by certain fungi, primarily species of *Candida* and certain filamentous fungi. These fungi (with the exception of *Candida humicola*; 4) are urease-negative. However, they are capable of multiplying in the presence of penicillin and releasing alkaline by-products from the basal medium alone to produce strong alkaline reactions and red color changes in the medium. False-positive reactions due to *Candida* are recognized by obvious turbidity, heavy sediment (or both), and the odor of yeast. False-positive reactions due to filamentous fungi are easily recognized by the development of characteristic fluffy, cotton-like balls of growth. The incorporation in medium U-9 of 2.5 µg of amphotericin B (Fungizone) per ml satisfactorily suppressed growth of *Candida* and other fungi occurring in genitourinary tract specimens, especially from females. A few strains of *Candida* failed to be inhibited by 2.5 µg of Fungizone per ml. In our experience, this level of Fungizone may delay the appearance of positive urease reactions due to T mycoplasmas by 3 to 4 hr but has never been observed to completely inhibit growth.

Recovery of viable T mycoplasmas from medium U-9. Although this urease color test medium was not designed as a growth medium per se, T mycoplasma organisms can be readily recovered from the medium by subculture to A-3 agar medium, provided that subcultures are made at the time red color change is first detected. Subcultures made to agar after development of full red color in medium U-9 will generally yield negative agar cultures due to the high alkalinity (*p*H 8.0 to 9.0) reached in this medium which is lethal to T mycoplasma organisms (1).

DISCUSSION

Initially, we attempted to develop a urease color test medium that would serve equally well as a growth medium for T mycoplasmas and as a sensitive indicator of the presence of T mycoplasma organisms in clinical material. This approach proved to be impractical, since the basic requirements of the two objectives are diametrically opposed. The basic requirement of a sensitive indicator medium is to signal a specific

change in the shortest practical incubation time. In the case of medium U-9, this meant stimulating urease activity by T mycoplasma organisms in the inoculum and promoting a rapid rise in *p*H due to accumulation of ammonia in the medium. Such requirements precluded incorporation of a buffer system. Earlier versions of this medium included 0.067 M Sorensen's phosphate buffer. However, in the presence of urea concentrations below 1.0%, this level of phosphate proved to be completely inhibitory to initiation of growth, and phosphate buffer was therefore omitted. The rapid rise in *p*H in medium U-9 due to urea hydrolysis by T mycoplasma organisms in the absence of a buffer soon results in a reaction of *p*H 8.0 or more (full red color of the phenol red indicator) which is lethal for T mycoplasmas. A low level of phosphate (0.0015 M KH_2PO_4) was incorporated in medium U-9, however, for the special purpose of *p*H stabilization during storage in the refrigerator. In the absence of this small amount of added phosphate, the reaction of the medium slowly climbed to *p*H 6.8 to 7.0 during storage at 4 C.

Commercially available urease test media (9) are unsatisfactory for use with T mycoplasmas because of high (2.0%) urea content, high phosphate buffer content, and other factors. Urea levels in the region of 0.05% and a *p*H of 6.0 in medium U-9 appear to be optimal in stimulating early and most active rates of urease activity by T mycoplasma organisms. Initially, we attempted to develop a serum-free urease color test medium, and one of the most satisfactory of these media was formula U-7A, in which the "Boston" T strain was first isolated. (2) It was found, however, that sensitivity of the medium could be increased by incorporation of low levels of horse serum, and all subsequent versions of this medium contained 4.0% unheated horse serum. Supplementation with 40 µg of calcium pantothenate per ml, found in earlier experiments to extend T mycoplasma viability in agar culture systems (Shepard, *unpublished data*), resulted in frequent breakthrough of the penicillin barrier by *Proteus* and was, therefore, abandoned.

Best performance of medium U-9 for the detection and identification of T mycoplasma organisms in specimens from the genitourinary tract was achieved by inoculating the medium directly with a 3.0-mm loopful of urethral exudate (obtained by urethral scraping) or 0.1 ml of sediment from a 30- to 40-ml centrifuged urine specimen. Highest purity reagent grade chemicals containing minimal traces of heavy metals should be employed. Heavy-metal ions, even in very

low concentrations, are notorious inhibitors of urease, and precautions should be taken to avoid such contamination of media and of glassware.

Medium U-9 was found useful in determining whether certain classical, large-colony mycoplasma species were free of contamination by T mycoplasmas. Medium U-9 has also proved to be a sensitive indicator fluid medium for the titration of T mycoplasma organisms in culture fluids and preserved materials, employing 10-fold serial dilutions directly in medium U-9. The end point was expressed as color-changing units per ml, as proposed by Purcell et al. (3). The medium is similarly useful in metabolic studies of T mycoplasmas, including ureolysis inhibition by antiserum, specific biochemicals, or chemotherapeutic agents.

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ADDENDUM IN PROOF

Subsequent to the preparation of this manuscript, we observed that the standard antimicrobial complement of penicillin and amphotericin B in medium U-9 was ineffective in controlling bacterial and fungal contaminants in certain types of clinical specimens. Under these adverse conditions, the incorporation of an additional antimicrobial combination, V-C-N Inhibitor (BBL), effectively suppressed both bacterial and fungal growth in medium U-9. The triple combination of penicillin (1,000 units/ml), amphotericin B (2.5 μg/ml), and V-C-N Inhibitor (1.0%) is not inhibitory to growth of T mycoplasmas in medium U-9 and is recommended under special conditions.

LITERATURE CITED

1. Ford, D. K., and J. MacDonald. 1967. Influence of urea on the growth of T-strain mycoplasmas. J. Bacteriol. 93:1509–1512.
2. Kundsin, R. B., S. G. Driscoll, and P. L. Ming. 1967. A strain of mycoplasma associated with human reproductive failure. Science 157:1573–1574.
3. Purcell, R. H., D. Taylor-Robinson, D. Wong, and R. M. Chanock. 1966. Color test for the measurement of antibody to T-strain mycoplasmas. J. Bacteriol. 92:6–12.
4. Seeliger, H. P. R. 1956. Use of a urease test for the screening and identification of cryptococci. J. Bacteriol. 72:127–131.
5. Shepard, M. C. 1966. Human mycoplasma infections. Health Lab. Sci. 3:163–169.
6. Shepard, M. C. 1967. Cultivation and properties of T-strains of mycoplasma associated with nongonococcal urethritis. Ann. N.Y. Acad. Sci. 143:505–514.
7. Shepard, M. C. 1969. Fundamental biology of the T-strains. In L. Hayflick (ed.), The mycoplasmatales and the L-phase of bacteria. Appleton-Century-Crofts, New York.
8. Shepard, M. C., and C. D. Lunceford. 1967. Occurrence of urease in T-strains of Mycoplasma. J. Bacteriol. 93:1513–1520.
9. Stuart, C. A., E. Van Stratum, and R. Rustigian. 1945. Further studies on urease production by Proteus and related organisms. J. Bacteriol. 49:437–444.