Differential Agar Medium (A7) for Identification of *Ureaplasma urealyticum* (Human T Mycoplasmas) in Primary Cultures of Clinical Material

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Received for publication 10 December 1975

A differential agar medium for the identification of *Ureaplasma urealyticum* in primary cultures of clinical specimens is described. The differential medium (no. A7) is specific for the identification of *U. urealyticum* and other members of the genus *Ureaplasma*. Large-colony, classical *Mycoplasma* and *Acholeplasma* species and *Proteus* L colonies are unreactive on this differential medium. The medium incorporates the biochemical principle of the direct spot test for urease in colonies of *Ureaplasma* and contains added urea and a sensitive indicator of ammonia, manganous sulfate. *Ureaplasma* colonies on this medium are identified as dark golden-brown or rich deep-brown colonies, in sharp contrast to the light background of the medium, when viewed by direct transmitted illumination under the low power of the microscope.

The identification of *Ureaplasma urealyticum* (23) in primary agar cultures of clinical specimens has become increasingly more difficult with the development of improved agar media. Under certain conditions of cultivation, *U. urealyticum* colonies may develop surface growth zones (6, 11, 20), which mimic those of classical *Mycoplasma* colonies in agar cultures. Conversely, under conditions of crowding in mixed *Mycoplasma* cultures, colonies of classical *Mycoplasma* frequently grow without zones of surface growth and may resemble colonies of *Ureaplasma*.

The discovery in *U. urealyticum* of a urease enzyme system capable of hydrolyzing urea with the production of ammonia (5, 12, 14, 21) made possible the development of new methods for recognition and identification of this organism, based upon detection of urease (17). The simplest method of detecting *U. urealyticum* in clinical specimens is by means of a sensitive urease color test fluid medium (22). Such a medium (medium U9) has been in routine use for this purpose in our laboratory since 1970 and is also useful in quantifying *U. urealyticum* in urine specimens (18, 19), urethral exudates, and broth cultures by direct titration in this color test medium.

The problem of positive identification of *U. urealyticum* in primary agar cultures, however, remained to be resolved. The first significant progress in this direction was the develop-

ment of a direct biochemical (spot) test for urease in colonies of *U. urealyticum* in standard agar cultures for this organism (17, 20). In this test, a reagent solution was applied directly to colonies of suspected *U. urealyticum* growing in agar cultures. The reagent solution contained the enzyme substrate (urea) and a sensitive indicator (manganous chloride) for detection of ammonia produced by hydrolysis of urea by *U. urealyticum*. The direct test for urease proved to be a simple, rapid, and reliable test for the identification of *U. urealyticum* colonies. After application of enzyme substrate and indicator reagent to *Ureaplasma* colonies, ammonia was rapidly evolved from the reaction and was quickly detected by the sensitive manganese indicator. This indicator in turn produced a stable reaction product (manganese dioxide), which developed in and on the surface of the individual *U. urealyticum* colonies. The reaction product imparted a dark golden- to deep-brown color to the colony when normally viewed under low power of the microscope by direct transmitted light. In essence, the test produced an accretion of manganese dioxide on the *U. urealyticum* colony. The direct test for urease was reactive only with colonies of *Ureaplasma*. Colonies of the recognized classical human *Mycoplasma* and *Acholeplasma* species and *Proteus* L-forms were unreactive to the direct test (20). The biochemical principle of the direct test for urease was subsequently applied to the development of a differential agar medium for the specific identification of

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colonies of *U. urealyticum* in primary agar cultures and is the subject of this report.

MATERIALS AND METHODS

Test organisms. The following 2,891 test organisms, including purified stock strains and naturally occurring fresh isolates from clinical specimens over a period of 4 years, were used: 13 purified stock strains of *U. urealyticum* originally isolated from male nongonococcal urethritis (NGU) patients, including serotypes I through VIII (1, 2, 4, 23); the "Boston T-strain" isolated from fetal membranes of a spontaneously aborted human fetus (8); 2,848 unpurified fresh clinical isolates of *U. urealyticum* in primary cultures of urethral exudates and urine specimens from male NGU patients; 10 *Ureaplasma* sp. isolates from lower animals, including four bovine strains (three lung, one genital), three canine genital strains, and three non-human primate female genital strains (9); seven recognized, large-colony, classical human *Mycoplasma* species; five species of *Acholeplasma*; and seven *Proteus* L-form strains. The name, history, and source of the various test organisms used in this study are given in Table 1.

Culture media. (i) **Urease color test medium.** The urease color test fluid medium used as an aid in the detection of *U. urealyticum* in clinical materials was medium U9, described previously (22), further supplemented by the addition of L-cysteine hydrochloride to 0.01% (0.00057 M) final concentration (= medium U9B). Tubes of medium U9B were stored in screw-capped tubes in the frozen state at -20°C prior to use.

(ii) **Carrying fluid.** The carrying fluid used for collection, transport, and storage (-60 to -85°C) of clinical exudates from NGU patients was A3XB solution and was developed in our laboratory for preserving the viability of *U. urealyticum* in urethral epithelial scrapings, exudates, and sediment from centrifuged urine specimens. It contained the following ingredients: sterile Trypticase soy broth (BBL no. 11768), adjusted to pH 6.0 with 2 N HCl, 100 ml; sterile L-cysteine hydrochloride (4.0% filter-sterilized stock solution), 0.25 ml; and sterile bovine albumin (30% solution), 3.3 ml. The final concentrations of L-cysteine hydrochloride and bovine albumin were 0.01 and 1.0%, respectively.

(iii) **Standard agar medium.** The standard agar medium used for primary isolation of *U. urealyticum* from exudates and urine specimens, and for cultivation of *Ureaplasma* strains, was medium A5H. A5H basal medium was first prepared and contained: Trypticase soy broth (BBL no. 11768) or tryptic soy broth (Difco no. 0370), 4.8 g; deionized distilled water, 165 ml (the broth powder was dissolved and the reaction was adjusted to pH 5.5 with 2 N HCl); GIBCO no. 1400000, bacteriological grade agar, 2.1 g. Without heating to dissolve the agar, the bottle was placed in the autoclave and sterilized at 121°C for 15 min. This both dissolved the agar and sterilized the basal medium in a single operation. After cooling to approximately 56°C in a water bath, the contents of the bottle were mixed thoroughly by 10- to 15-s rotation on a flat surface to disperse the

melted agar at the bottom. Without further delay, 160 ml of the sterile basal medium was converted to complete medium A5H by addition of the following sterile supplements: unheated normal horse serum, 40 ml; CVA Enrichment (GIBCO chemically defined enrichment containing cofactors, vitamins, and amino acids), 1.0 ml; yeast extract, pH 6.0 (fresh 25% aqueous extract of pure dry yeast), 2.0 ml; 10% urea stock solution, 0.4 ml; 4% L-cysteine hydrochloride stock solution, 0.5 ml; and penicillin G potassium (100,000 U/ml of stock solution), 2.0 ml. We found it convenient to combine aseptically all of the six sterile supplements (45.9 ml) in a separate, sterile, screw-cap bottle and then to add the combined supplements at one time to the melted sterile basal agar medium. The complete, supplemented medium A5H was carefully mixed by rotation of the vessel on a flat surface. This unit volume (206 ml) was sufficient to pour 10 agar plates (20 ml/plate) in standard 15-by 100-mm petri plates. After hardening, the poured plates were allowed to remain inverted on the workbench at room temperature overnight. They were then placed in the refrigerator in sealed plastic bags to reduce loss of moisture during storage. Fresh plates of medium A5H were prepared each week, using the single batch method outlined above. The final concentrations of the various enrichments were: horse serum, 20%; CVA, 0.5%; yeast extract, 1%; urea, 0.02%; L-cysteine hydrochloride, 0.01%; penicillin, 1,000 U/ml. The practice of preparing sterile basal medium in advance and storing it for subsequent use was not used and is not recommended in preparing media for isolation and cultivation of *U. urealyticum*.

Source of chemicals and ingredients. Trypticase soy broth, BBL no. 11768, was obtained from the BioQuest Division of Becton-Dickinson and Co., Cockeysville, Md. Tryptic soy broth, Difco no. 0370, was obtained from Difco Laboratories, Detroit, Mich. GIBCO no. 1400000 bacteriological grade agar (lot no. 48757 or 50015) was obtained from GIBCO Diagnostics, Madison, Wis. This is an acceptable substitute for the Ionagar No. 2 formerly used. Manganese(ous) sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), no. M-113, was obtained from Fisher Scientific Co. CVA Enrichment, no. 1401582, was obtained from GIBCO Diagnostics. The brewer's yeast was Fleischmann's type 2040 pure dry yeast, Standard Brands, Inc., Industrial Division, Peekskill, N.Y. A 25% water extract, adjusted to pH 6.0, was prepared from this yeast using the method of Hayflick (7). Ultrapure grade urea, no. 9200, was obtained from the Schwarz/Mann Division of Becton-Dickinson and Co., Rockville, Md. L-Cysteine hydrochloride, no. 2430, was obtained from Calbiochem, Atlanta, Ga. Bovine albumin, Pentex, 30% sterile solution in Tyrode's buffer, (no. 82-007-1), was obtained from Research Products, Miles Laboratories, Inc., Elkhart, Ind.

Collection and inoculation of clinical specimens.

(i) **Urethral exudates.** Urethral exudates from male NGU patients were collected by means of an epithelial scraping of the anterior urethra. The instrument used was a 3-mm bacteriological loop made in our laboratory from B&S gauge 20 platinum alloy

TABLE 1. Name, history, and source of test organisms used

Organism	Strain	Source
<i>U. urealyticum</i> (human)	K12	M. C. Shepard
<i>U. urealyticum</i> (human)	K42	M. C. Shepard
<i>U. urealyticum</i> (human)	K71	M. C. Shepard
<i>U. urealyticum</i> (human)	K510	M. C. Shepard
<i>U. urealyticum</i> (human)	2K160	M. C. Shepard
<i>U. urealyticum</i> (human)	7 (I) ^a	D. K. Ford
<i>U. urealyticum</i> (human)	23 (II)	D. K. Ford
<i>U. urealyticum</i> (human)	27 (III)	D. K. Ford
<i>U. urealyticum</i> (human)	58 (IV)	D. K. Ford
<i>U. urealyticum</i> (human)	354 (V)	D. K. Ford
<i>U. urealyticum</i> (human)	Pi (VI)	D. K. Ford
<i>U. urealyticum</i> (human)	Co (VII)	D. K. Ford
<i>U. urealyticum</i> (human)	960 (VIII)	M. C. Shepard
<i>U. urealyticum</i> (human)	"Boston T-strain"	R. B. Kundsin
<i>U. urealyticum</i> (human)	Clinical isolates (2,848)	M. C. Shepard
<i>Ureaplasma</i> sp. (bovine)	A417	R. N. Gourlay
<i>Ureaplasma</i> sp. (bovine)	D32	R. N. Gourlay
<i>Ureaplasma</i> sp. (bovine)	U20	D. Taylor-Robinson
<i>Ureaplasma</i> sp. (bovine)	95	C. W. Livingston, Jr.
<i>Ureaplasma</i> sp. (canine)	19	C. D. Lunceford
<i>Ureaplasma</i> sp. (canine)	66	C. D. Lunceford
<i>Ureaplasma</i> sp. (canine)	239	C. D. Lunceford
<i>Ureaplasma</i> sp. (primate)	Talapoin PR-1	R. B. Kundsin/T. Rowell
<i>Ureaplasma</i> sp. (primate)	Talapoin PR-2	R. B. Kundsin/T. Rowell
<i>Ureaplasma</i> sp. (primate)	Talapoin PR-3	R. B. Kundsin/T. Rowell
<i>M. hominis</i>	PG-21	D. G. ff Edward
<i>M. fermentans</i>	PG-18	D. G. ff Edward
<i>M. salivarium</i>	PG-20	D. G. ff Edward
<i>M. orale</i> , type 1	No. 1	W. A. Clyde, Jr.
<i>M. lipophilum</i>	MaBy	R. A. DelGiudice
<i>M. arginini</i>	119	M. F. Barile
<i>M. pneumoniae</i>	NMFRL	M. C. Shepard
<i>A. laidlawii</i> A	PG-8	J. G. Tully
<i>A. modicum</i>	PG-49	J. G. Tully
<i>A. oculusi</i>	19-L	J. G. Tully
<i>A. granularum</i>	BTS-39	J. G. Tully
<i>A. axanthum</i>	S-743	J. G. Tully
<i>Proteus</i> sp. (L-form)	9-L	R. M. Cole
<i>Proteus</i> sp. (L-form)	52-L	R. M. Cole
<i>Proteus</i> sp. (L-form)	18-L	S. Madoff
<i>Proteus</i> sp. (L-form)	2070-L	S. Madoff
<i>Proteus</i> sp. (L-form)	(Fresh, clinical)	C. D. Lunceford
<i>Proteus</i> sp. (L-form)	(Fresh, clinical)	C. D. Lunceford
<i>Proteus</i> sp. (L-form)	(Fresh, clinical)	C. D. Lunceford

^a The Roman numerals indicate the serotype designation. The first seven of these serotypes were originally isolated from NGU patients by Ford (4) and were subsequently studied and given the above serotype designations by Black (1, 2, 23).

wire, 75 mm in length, containing 15% iridium for increased stiffness (Englehard Industries, Carteret, N.J.). The loop was formed around a 3-mm polished nail, using a simple contact closure without twist or solder, and bent at a slight angle (10°) to facilitate scraping action. It was freed of burrs by polishing with emery cloth and rouge cloth. The patient was instructed to assume the supine position on an examination table. The epithelial scraping was accom-

plished by inserting the specially made wire loop (flame-sterilized and cooled) a distance of 3.5 to 5.0 cm up the urethra and slowly withdrawing it along the urethral ceiling. The external meatus was slightly parted and held open during the procedure. Urethral exudates collected in this manner were immediately streaked to culture plates. Exudate was also collected for inoculation of a tube of U9B medium (medium [i]) and for storage in the frozen

state (-85°C) in 1.0 ml of carrying fluid (medium (ii)). The urethra was re-entered for collection of multiple specimens.

(ii) **Urine specimens.** First-voided morning urine specimens were collected from male NGU patients after digital stripping of the urethra three times by the patient. Digital stripping expresses fresh exudate from the periurethral mucous glands and ducts. This step is important since the urine specimen for this purpose is a urethral washout specimen. The specimens were centrifuged at 3,000 rpm for 10 to 20 min and aseptically decanted, and the sediment was mixed with the remaining few milliliters of urine by agitation in a Vortex mixer. The urine sediments were then streaked without delay on the same agar media used for exudates and also inoculated into medium U9B (0.4 ml/3.6 ml of medium).

(iii) **Specimens from females.** Females (wives or consorts of male NGU patients) were asked to furnish, with materials provided, a first-voided morning urine specimen and a vaginal specimen. The vaginal specimen was collected by means of a sterile cotton or alginate wool swab and transported to the laboratory in medium (ii) without delay, together with the separate urine specimen.

Incubation of agar culture plates. All agar cultures were incubated at 36°C in Anaero-Jars (Pfizer Diagnostics, Clifton, N.J.), containing moistened gauze or blotting paper at the bottom to provide a saturated moisture content. The jars were evacuated to 25 inches (ca. 62.0 cm) of Hg and gassed one time with a gaseous mixture of 10% carbon dioxide and 90% nitrogen from a compressed gas cylinder. Higher levels of carbon dioxide (such as obtained from two or three cycles of evacuation and gas mixture replacement) were less satisfactory and are not recommended. The modified Fortner method of incubation (15) was also used for incubation of agar cultures when several observations were to be made of individual culture plates during incubation. Incubation of agar plates in a candle jar or in a tissue culture incubator gassed with flowing 5% carbon dioxide may also be used but is less satisfactory.

RESULTS

Preliminary studies. The feasibility of incorporating the reagents of the direct test for urease (20) into an agar growth medium to produce a differential agar medium for identification of *U. urealyticum* was explored. The usefulness of the simple, rapid, highly specific direct test for urease in colonies of *U. urealyticum* has been amply substantiated in laboratory practice. However, the test must be performed on *Ureaplasma* colonies that are not older than 48 h or the test may be variable or completely unreactive due to loss of urease activity in such colonies. A differential agar medium containing the essential reagents for detection of urease would effectively eliminate the incubation time limitation imposed by the direct test for urease. Furthermore, the direct

test for urease would be performed automatically, as it were, in situ.

The concentrations of the test reagents as used in the direct test for urease, when incorporated in agar medium (iii), were found to be completely inhibitory to multiplication and growth of all strains of *U. urealyticum* tested. The concentration of enzyme substrate (urea) was subsequently reduced 100-fold from the original value of 10% to a final concentration of 0.1%.

The concentration of the manganese reagent in the differential agar was found to be critical and to require a 27-fold reduction from the toxic 0.8% concentration used in the direct test. In the early developmental work with this medium, a final concentration of 0.03% manganous chloride appeared to yield optimal results. Concentrations lower than 0.03% yielded excellent colony numbers but were inconsistent and produced unsatisfactory levels of reaction product. Concentrations higher than 0.03% were toxic and resulted in a sharp reduction in colony numbers. It was further observed that the sulfate salt of manganese permitted better colony growth than the chloride salt. Manganous sulfate in a final concentration of 0.03% was, therefore, selected as the ammonia-detecting reagent of choice in this original (A6) version of the differential medium.

In view of recent improvements in this medium (24; Shepard and Lunceford, unpublished data), it was felt to be essential to re-examine the optimal manganous sulfate concentration. The increased performance of the present medium (due to supplementation with L-cysteine hydrochloride and CVA Enrichment) permitted a 50% reduction in manganous sulfate concentration, from 0.03 to 0.015%. At the latter concentration, colony size and numbers were greatest and reaction product was intense. *U. urealyticum* colony growth became established by 24 h with many strains. Furthermore, the sensitivity of the medium was increased by approximately 35% (Table 2). At the lowest concentration of manganous sulfate tested (0.01%), a slightly higher sensitivity was achieved, but colony size and intensity of reaction product suffered.

Several types of commercially available media were tested for suitability as basal medium in this differential medium (Table 3). Of the 12 combinations tested, tryptic soy broth (Difco) solidified with Ionagar No. 2 and Trypticase soy broth (BBL) solidified with Ionagar No. 2 proved to be the most satisfactory commercial bases for use in a differential agar medium for specific identification of *Ureaplasma* species.

Eugonagar (BBL) and Trypticase soya broth (Oxoid) solidified with Ionagar No. 2 were considered unsatisfactory for this purpose and are not recommended. Oxoid Trypticase soya agar proved to be equally unsatisfactory.

Differential agar medium A7. Standard agar medium A5H was converted to differential agar medium A7 by (i) incorporation of manganous sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$) to yield a final concentration of 0.015% and (ii) incorporation of ultrapure urea to yield a final concentration of 0.1%. The preparation of the differential medium follows the procedure outlined for medium (iii) (standard medium A5H) in the preceding section. A7 basal medium was first prepared and contained Trypticase soya broth (BBL

no. 11768, or Difco tryptic soy broth no. 0370), 4.8 g; manganese(ous) sulfate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$), 0.031 g; deionized distilled water, 165 ml (the ingredients were dissolved and the reaction was adjusted to pH 5.5 with 2 N HCl or 2 N NaOH, depending upon the reaction produced by the particular lot of manganous sulfate used); (GIBCO no. 1400000 bacteriological grade agar, 2.1 g (final agar concentration, 1.0%). Ionagar No. 2, which was used earlier, is no longer commercially available. The GIBCO product in this formula is a satisfactory, tested substitute.

(Note: A commercial version of differential agar medium A7 (Shepard-Lunceford) will be available from GIBCO Diagnostics, Madison Wis. 53711.)

After sterilization and cooling of the A7 basal agar to 56 C in the manner described for A5H standard agar medium, 160 ml of sterile basal agar was converted to complete A7 differential agar medium by the addition of the following sterile supplements (first combined aseptically in a separate sterile bottle): unheated normal horse serum, 40 ml; CVA Enrichment, 1.0 ml; yeast extract, pH 6.0 (fresh 25% aqueous extract of pure dry yeast), 2.0 ml; 10% urea stock solution, 2.0 ml; 4% L-cysteine hydrochloride stock solution, 0.5 ml; and penicillin G potassium (100,000 U/ml of stock solution), 2.0 ml.

Complete differential agar medium A7 was compounded in the same manner and was enriched with the same sterile supplements as described for standard agar medium A5H (medium [iii]). (Note: The final concentration of manganese(ous) sulfate given in Abstract M61, M. C. Shepard and C. D. Lunceford, Bacteriol. Proc. 70:83, 1970, is a printer's error [0.3%] and should be disregarded.)

Examination. Plates of inoculated differen-

TABLE 2. Effect of manganous sulfate concentration in differential medium A7 on growth of *U. urealyticum* and generation of manganese reaction product

Manganous sulfate ^a (%)	Colony growth and reaction product ^b	Colony counts ^c
0.01	3+	25.0 ± 8.1
0.015	4+	22.4 ± 5.2
0.02	3+	19.8 ± 6.8
0.03	2+	16.6 ± 1.9
0.04	2+	15.9 ± 5.8
0.05	1+	14.1 ± 3.7

^a Manganous sulfate was added to the basal agar to yield the final concentrations shown.

^b Colony size and intensity of manganese reaction product among 41 test specimens (25 fresh clinical specimens and 16 purified stock strains). Values are averages from three separate experiments.

^c Mean *U. urealyticum* colony counts per 0.01-ml volume of sediment from 25 different *U. urealyticum*-positive, centrifuged, freshly voided urine specimens from urethritis patients.

TABLE 3. Comparison of several commercial media for possible use as a basal medium in differential medium A7

Commercial medium	<i>Ureaplasma</i> colony growth	Intensity of reaction product
Trypticase soya broth (BBL 11768) + Ionagar No. 2 ^a	++++	++++
Tryptic soya broth (Difco 0370) + Ionagar No. 2	++++	++++
Spirolate broth (BBL 11636) + Ionagar No. 2	++++	+++
Brucella agar (Difco 0964)	+++	+++
Trypticase soya agar (BBL 11043)	+++	+++
PPLO broth without CV (Difco 0554) + Ionagar No. 2	++	++++
PPLO agar (Difco 0412)	++	++++
BYE agar (BBL 11017)	++	+++
Brain heart infusion (BBL 11059) + Ionagar No. 2	++	++++
Tryptone soya broth (Oxoid CM129) + Ionagar No. 2	+	+++
Tryptone soya agar (Oxoid CM131)	+	+++
Eugonagar (BBL 11230)	+	+++

^a Ionagar No. 2 is no longer available in the United States. However, GIBCO bacteriological grade agar no. 1400000 (lot 48757 or 50015) is an acceptable substitute in differential agar medium A7.

tial medium A7 were examined after 48 h of incubation. They were examined in the usual manner for colonies of *U. urealyticum* with the culture plate inverted on the microscope stage, under low power ($\times 100$), using direct transmitted light through the substage condenser. As with all members of *Mycoplasmatales*, colonies of *U. urealyticum* are found growing at or slightly subsurface to the plane of inoculation. The morphology and growth characteristics of *U. urealyticum* colonies have been described previously (13, 15, 16, 23). The characteristic appearance of normal *U. urealyticum* colonies on standard agar medium A5H is illustrated in Fig. 1 and 2.

Interpretation. On differential medium A7, colonies of *U. urealyticum* are identified as dark golden-brown or rich deep-brown colonies of variable size (depending upon the degree of crowding), in sharp contrast to the clear, light background of the medium (Fig. 3 and 4). Under certain conditions, the colonies may look more metallic gray than brown in color, usually when an error has been made in preparation of the medium. We frequently observed subtle, variegated colors on the surface of both young and older, larger colonies. These colors were enhanced in agar block preparations stained by Dienes' stain (3, 10). The dark golden-brown or rich deep-brown colonies are manganese reaction product (manganese dioxide) within and directly on the surface of *Ureaplasma* colonies. The biochemical reaction and the microscopic picture are similar to those obtained in the direct test for urease (20). If these same dark, manganese accretion colonies are examined under low power by indirect transmitted or oblique light, the colonies appear white (Fig. 5 and 6). Similarly, if a plate of differential medium A7 bearing *Ureaplasma* colonies is held up to strong light (in the beam of a microscope illuminator, for example) and examined macroscopically in an oblique, indirect manner, the minute colonies also appear white.

Clinical specimens containing mixtures of *U. urealyticum* and classical, large-colony *Mycoplasma* species (e.g., *M. hominis*) yield both types of colonies on differential medium A7. In such mixtures, however, classical *Mycoplasma* colonies are unreactive and fail to produce the manganese reaction product, since they are urease negative and cannot hydrolyze urea (Fig. 7). Staining such mixtures of *Ureaplasma* and *Mycoplasma* colonies with Dienes' stain by the wet-stained agar block technique (3, 10) presents a striking picture when examined under the low power ($\times 100$) of the microscope (Fig. 8). Colonies of all classical *Mycoplasma*

species in such preparations stain blue in color, in sharp contrast to the dark golden-brown or rich deep-brown color of the intermixed, manganese accretion colonies of *Ureaplasma*. In either stained or unstained agar preparations containing mixtures of *Mycoplasma* and *Ureaplasma* colonies on medium A7, the classical *Mycoplasma* colonies may have deep-brown centers containing manganese reaction product. This unusual appearance is the result of *Ureaplasma* organisms (or a colony) growing immediately beneath or within the central growth core of the classical *Mycoplasma* colony (Fig. 9 and 10). In addition, *Ureaplasma* organisms sometimes can be observed growing around an *M. hominis* colony, and this unusual manner of growth is detected on A7 medium by development of encircling manganese reaction product (Fig. 11). In rare instances, a fan-shaped sector of *Ureaplasma* growth may occur within the surface growth zone of a classical *Mycoplasma* colony on differential medium A7 (17). Such a sector (like the two unusual modes of *Ureaplasma* growth described above) indicates urease activity and production of ammonia by *U. urealyticum*, and the resultant manganese reaction product effectively unmasks the presence of a second organism within such classical *Mycoplasma* colonies (Fig. 12).

Under certain conditions of cultivation (6, 11, 20) and from certain clinical specimens, *U. urealyticum* colonies develop zones of surface growth that mimic those of classical *Mycoplasma* species. Such zones of *Ureaplasma* surface growth possess strong urease activity. They produce the characteristic manganese reaction product by application of the direct test for urease (20) and on differential medium A7, resulting in large (to 130 μ m in diameter) manganese accretion colonies (Fig. 13).

In cultures of female urine specimens on differential medium A7, we have often observed squamous epithelial cells that exhibited characteristic manganese reaction product, suggesting intracellular or cell-associated urease activity (Fig. 14). Such epithelial cells were observed only in association with positive cultures for *U. urealyticum*, and they were rarely observed in urine cultures from male NGU patients. The observed cellular urease activity was produced by intracellular or cell-associated *U. urealyticum* organisms and suggests that *U. urealyticum* may persist in the asymptomatic female carrier by parasitizing certain squamous epithelial cells of the genitourinary tract.

There is often a close association of *U. urealyticum* colonial growth with epithelial cells. This is especially true of squamous and meta-

FIG. 1. Normal colonies of *U. urealyticum* on standard agar medium A5H, showing sharply diminished size due to crowding. $\times 90$.

FIG. 2. Same as Fig. 1, but well-separated colonies of normal size. $\times 90$.

FIG. 3. *U. urealyticum* colonies on differential agar medium A7 showing typical manganese accretion colonies and sharply diminished size due to crowding. $\times 90$.

FIG. 4. Same as Fig. 3, but well-separated manganese accretion colonies of large size. $\times 90$.

FIG. 5. *U. urealyticum* colony growth on differential agar medium A7 inoculated by means of the agar push-block method, visualized by standard low-power microscopy under direct transmitted light; characteristic manganese accretion colonies. $\times 115$.

FIG. 6. Same as Fig. 5, but visualized under oblique, indirect illumination from above against a dark background. Under these conditions of illumination, manganese accretion colonies of *U. urealyticum* appear white. $\times 115$.

FIG. 7. Mixture of *M. hominis* and *U. urealyticum* colonies on differential agar medium A7. Unstained, natural appearance. Colonies of *U. urealyticum* are unmistakably identified by their deep golden-brown color on this A7 medium, whereas colonies of *M. hominis* are unreactive. $\times 100$.

FIG. 8. Mixture of *M. hominis* and *U. urealyticum* colonies on differential agar medium A7. The preparation was stained by Dienes' wet-stained agar block method. The unreactive, urease-negative colonies of *M. hominis* stain blue, whereas the manganese accretion colonies of *U. urealyticum* remain essentially unchanged (deep brown). $\times 90$.

plastic epithelial cells from the urethral mucosa of male NGU patients and from certain females. *U. urealyticum* colonial growth associated with such cells often completely covers the epithelial cell. The resulting strong urease action produces a large, single accretion colony, which may partially or completely obliterate the epithelial cell with manganese reaction product. Occasionally, several adjacent epithelial cells parasitized by *Ureaplasma* organisms produce giant manganese accretion colonies in which the reaction product has fused, resulting in large, irregular, odd-shaped, "lumpy" masses of reaction product (Fig. 15). Urethral exudates often yield large masses of *U. urealyticum* growth on A7 differential medium, as indicated by the occurrence of large masses of manganese reaction product (Fig. 16). Such extensive outgrowth of *Ureaplasma* organisms is nearly always associated with clinical exudates containing large numbers of polymorphonuclear leukocytes and epithelial cells.

During inoculation of differential medium A7 with clinical material, the surface is sometimes cut by the loop. Such cuts often entrap *Ureaplasma* organisms. Their subsequent urease activity during incubation produces intense streaks of manganese reaction product in the agar.

Urine specimens. The identification of *U. urealyticum* in primary agar cultures of sediment from centrifuged urine specimens has always been a difficult and frustrating task on standard agar media. Sediments from centrifuged urine specimens planted on differential medium A7 produce a microscopic picture essentially identical to that obtained by the direct test for urease (20) in the presence of *U. urealyticum* colonies. On A5H standard agar (medium [iii]) inoculated with urine sediments, small colonies of *U. urealyticum* are often nearly obliterated by urine cellular deposits and may be unrecognizable by even experienced observers (Fig. 17). However, colonies from such a urine specimen planted on differential agar medium A7 are immediately and positively identified as those of *U. urealyticum* (Fig. 18). Although urine specimens are satisfactory for the primary isolation of *U. urealyticum*, colony numbers may be sharply reduced in agar cultures due to the presence of inhibitory substances. Far larger numbers of *U. urealyticum* colonies are generally isolated in primary agar cultures of urethral exudate than from a urine specimen from the same urethritis patient, in our experience.

Urine mucous threads. The presence of mucous threads in first-glass urine specimens from

urethritis patients is a significant finding in the laboratory diagnosis of NGU. After specific treatment, their disappearance from urine specimens is similarly important in assessing cure of the disease. The presence and extent of *U. urealyticum* infection of mucous threads from NGU patients can be easily and directly demonstrated on differential medium A7. Indi-

vidual mucous threads planted on the surface of A7 agar medium frequently yield specific regions or entire areas of intense reaction product, signifying strong urease activity by *U. urealyticum*, similar to that illustrated in Fig. 19. *U. urealyticum*-infected mucous threads were nearly always associated with a strong leukocytic response within the mucous threads.

FIG. 9. Growth of *U. urealyticum* associated with the central growth core of an *M. hominis* colony (arrow) on differential agar medium A7. The presence of *U. urealyticum* is revealed on this medium by the intense manganese reaction product in the central growth core of this *M. hominis* colony. $\times 180$.

FIG. 10. Similar occurrence of associated growth of two different mycoplasma organisms, as in Fig. 9. The manganese reaction product produced by growth of *U. urealyticum* in this case is much larger, very irregular, and more obvious. From an Agfachrome original. $\times 90$.

FIG. 11. Growth of *U. urealyticum* almost completely encircling a colony of *M. hominis* on differential agar medium A7, indicated by encircling manganese reaction product. In addition, *U. urealyticum* growth is revealed within or under the central growth core of the *M. hominis* colony. $\times 200$.

FIG. 12. A fan-shaped sector of intense manganese reaction product unmask the presence of *U. urealyticum* within an *M. hominis* colony developing on differential agar medium A7. $\times 190$ (from reference 17).

FIG. 13. *U. urealyticum* colonies on differential agar medium A7 that have produced zones of surface growth in the manner of classical *Mycoplasma* colonies. However, the zones produce the same characteristic manganese reaction product as the more usual colonies of *U. urealyticum* without zones on this differential agar medium. $\times 200$.

FIG. 14. *U. urealyticum*-infected urethral epithelial cell showing formation of manganese reaction product on differential agar medium A7. Urine culture from wife of a man with *U. urealyticum*-associated NGU. Compare with adjacent (upper) normal epithelial cell. $\times 400$.

FIG. 15. Fused manganese accretion colonies of *U. urealyticum* that are closely cell associated and exhibit a "lumpy" appearance in primary cultures of exudate or urine sediment on differential agar medium A7. $\times 200$.

FIG. 16. Mass of small manganese accretion colonies of *U. urealyticum* on medium A7. These colonies are closely associated with epithelial cell groups. Urine culture from wife of a man with *U. urealyticum*-associated NGU. $\times 180$.

In contrast, normal urinary mucous from asymptomatic carriers of *U. urealyticum* showed no mucous thread formation and no leukocyte response, and colonies of the organisms showed a random distribution in such cultures.

Specificity. The direct test for urease in colonies of *U. urealyticum* on standard agar medium was specific for urease. The development of a stable manganese reaction product positively identified areas of urease activity due to colonies of *U. urealyticum* or unorganized

masses of the organism (20). Differential agar medium A7 was shown to be equally specific for *U. urealyticum* urease, since it uses the same biochemical principle used in the direct test and the same sensitive manganese indicator. Bizarre manifestations of *U. urealyticum* growth on medium A7, including growth in association with colonies of other species of mycoplasmas

and growth associated with single or grouped urethral epithelial cells, urethral mucous threads, and exudates (as illustrated in Fig. 9-19), are not artifacts. Their numbers are reduced in direct proportion to the degree of dilution of *U. urealyticum*-containing inoculum. Furthermore, the incorporation of 25 μ g of erythromycin per ml (23) to medium A7 com-

FIG. 17. Primary culture of sediment from centrifuged urine specimen from a male NGU patient. Standard agar medium A5H. Although this culture was positive for *U. urealyticum*, the colonies of this organism were extremely difficult to discern and identify in the presence of cellular exudate. $\times 180$.

FIG. 18. Primary culture of same urine specimen as shown in Fig. 17, but inoculated onto a companion plate of differential agar medium A7. Areas of manganese reaction product and manganese accretion colonies provide positive, specific identification of *U. urealyticum* in such a culture. $\times 180$.

FIG. 19. Serpentine mass of intense manganese reaction product resulting from strong urease activity by *U. urealyticum* in this particular area of urethral exudate from a male NGU patient. $\times 120$.

pletely inhibited *U. urealyticum* growth, urease activity, and development of manganese reaction product in culture plates inoculated with known *U. urealyticum*-positive clinical exudates, urine sediments, or purified cultures of the organism. The specificity for urease of differential agar medium A7 was further established by comparative testing against (i) urease color test medium U9B (medium [i]) and (ii) the direct test for urease in colonies of *U. urealyticum* isolated on standard agar medium A5H (medium [iii]). All 2,872 strains of *Ureaplasma* tested yielded positive reactions for urease in each of the three test systems used.

In contrast, seven different large-colony, classical *Mycoplasma* species, including *M. hominis*, *M. fermentans*, *M. salivarium*, *M. orale* (type 1), *M. lipophilum*, *M. arginini*, and *M. pneumoniae*, and five species of *Acholeplasma*, including *A. laidlawii*, *A. modicum*, *A. oculusi*, *A. granularum*, and *A. axanthum*, yielded negative reactions for urease by the three different test methods used and produced normal colonies on medium A7. Growth of *Acholeplasma* colonies was poor on medium A7.

It was of interest to determine whether L-phase colonies of *Proteus* would be reactive and produce manganese accretion colonies on differential medium A7. *Proteus* species are actively urealytic, and urease activity has been observed in an altered type B *Proteus* L-form (L. Dienes and S. Madoff, personal communication). L-form colonies of six different strains of *Proteus* were previously found to be unreactive to the direct test for urease (20). Seven different strains of *Proteus* L-forms were tested on differential medium A7 by agar push-block method of inoculation. With the exception of *Proteus* L-form strain 9-L, all mature, well-isolated *Proteus* L colonies tested were unreactive and failed to produce manganese accretion colonies on differential medium A7. However, *Proteus* L-form strain 9-L produced variable amounts of manganese reaction product in the center of certain areas of heavy, confluent growth on medium A7, which indicated that a limited degree of urease activity was retained by this *Proteus* L-form. It should be emphasized, however, that the large L-form colonies of *Proteus* would never be confused with manganese accretion colonies of *Ureaplasma* on medium A7 (or with *Ureaplasma* colonies on any standard agar medium).

DISCUSSION

A urea-containing agar medium, buffered to pH 6.5 with a suitable mixture of phosphate salts, has recently been reported (25) for the

identification of *U. urealyticum*. This medium uses a buffer to enhance colony size, in the manner reported by previous workers (11). In the case of phosphate mixtures, however, colonies of *U. urealyticum* produced enlarged colonies containing a white precipitate. Caution must be exercised, however, when using phosphate salts in media for primary isolation of *U. urealyticum*. Phosphate ions are toxic to ureaplasmas, and the optimal concentration is critical in certain formulations, especially when used for primary isolation of *U. urealyticum* directly from clinical material.

In the manner of the direct test for urease in colonies of *U. urealyticum* on standard agar media (20), differential medium A7 is of special usefulness for detection and identification of *U. urealyticum* in primary cultures of urine specimens, in addition to urethral exudates per se. As indicated earlier, differential medium A7 in fact performs the direct test in situ as the colonies grow during incubation, without the incubation time limitation imposed by the direct test. Further advantages of medium A7 are that it provides a means of readily identifying and visualizing *U. urealyticum* colony growth in assay procedures, such as testing antibiotic sensitivity and inhibition by immune serum (Shepard and Lunceford, manuscript in preparation). Differential medium A7 is specific for all members of the genus *Ureaplasma* (23) (including strains from lower animals). Species of *Ureaplasma* are the only *Mycoplasmatales* known to contain urease, upon which development of the specific reaction product in this medium depends.

ACKNOWLEDGMENTS

We are deeply indebted to M. F. Barile, F. T. Black, W. A. Clyde, Jr., R. M. Cole, R. A. DelGiudice, D. G. ff Edward, D. K. Ford, R. N. Gourlay, R. B. Kundsins, C. W. Livingston, S. Madoff, T. Rowell, D. Taylor-Robinson, and J. G. Tully for generously making available the several strains of *Mycoplasma*, *Ureaplasma*, *Acholeplasma*, and *Proteus* L-forms used in this study, and to B. W. Foepfel for his excellent technical assistance.

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