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Comparison of Media for Isolation of *Ureaplasma urealyticum* and Genital *Mycoplasma* Species

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A total of 484 frozen patient specimens originally positive for *Ureaplasma urealyticum* or *Mycoplasma* spp. or both were recultured, and the results were compared on the following media: Shepard's A7 agar, modified phenol red-urea, SP-4–urea, modified phenol red-arginine, and SP-4–arginine broths. Of 351 specimens positive for *U. urealyticum*, 30 (8.5%) were detected only in one or more of the broth media, whereas 117 (33%) were positive only on A7 agar. Separate use of the SP-4–urea broth or modified phenol red-urea broth isolated all but 1 and 2, respectively, of the negative A7 agar cultures. Of the 76 specimens positive for large colony *Mycoplasma* spp., 58 (76.3%) were not grown on the primary plating (A7) agar. Of 73 broth isolates, 73 (100%) grew in SP-4–arginine broth, and 64 (87.7%) grew in modified phenol red-arginine broth. Modified SP-4 broth appears to be a useful supplement to the A7 plating medium commonly used in the diagnostic laboratory for the isolation of *U. urealyticum* and *Mycoplasma* spp.

Ureaplasma urealyticum and *Mycoplasma hominis* are two mycoplasmas frequently isolated from the genitourinary tract. *U. urealyticum* is presently accepted as an etiological agent of nongonococcal urethritis and has been implicated in reproductive problems, including chorioamnionitis and low birth weight (15). This species includes all the human strains of *Mycoplasmataceae* that possess urease and therefore are capable of producing ammonia from the breakdown of urea. This property prompted the development of a reliable differential agar medium, Shepard A7 agar (12). *M. hominis* has been associated with pelvic inflammatory disease, postabortal and postpartum fevers, and pyelonephritis (15) and accounts for more than 95% of the large colony *Mycoplasma* spp. found in the genitourinary tract (1, 7).

Numerous media formulations have been advocated for enhanced growth of genital mycoplasmas (8, 11, 12, 16, 18, 20, 21). It is currently recommended that cultivation systems include a solid agar medium as well as supplemental broths for optimal isolation of ureaplasmas and genital mycoplasmas from clinical specimens (2, 4, 5, 8, 11, 14). In this investigation we assessed the need for a supplemental broth to be used in conjunction with A7 agar, and we sought to determine the best base formulation for the isolation, detection, and identification of genital mycoplasmas. Shepard A7 agar was compared with modified mycoplasma phenol red (MPR) (20) and modified SP-4 (16, 17, 19) broths. The MPR broth was adapted from the standard CDC PPLO medium formulation traditionally used for the isolation of mycoplasmas (20). SP-4, a relatively new and enriched medium originally developed for cultivating two tick-derived spiroplasma strains (15), has been used primarily for the recovery of *Mycoplasma pneumoniae* from throat washings (16) and for the isolation of a new *Mycoplasma* sp. from the genital tract (17). Recently, Tully et al. reported the usefulness of SP-4 for improved broth isolation of *M. hominis* (18).

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MATERIALS AND METHODS

Specimens. A total of 484 frozen (−70°C) patient specimens from genital sources, primarily urine sediments and cervical swabs in transport media (20), were recultured to a solid agar medium and four broth media. All of these specimens were previously positive for *U. urealyticum* (476 isolates) or *Mycoplasma* spp. (76 isolates) or both.

Specimen inoculation. Samples were thawed at room temperature and vortexed, and 0.1 ml of specimen was inoculated to each of the five media.

Media preparation. Components common to all media were obtained from the same lot numbers. A7 agar plates were prepared at three different times (with the same component lot numbers) to assure fresh media (less than 1 week old) for subculture of the broths. All broth media were prepared simultaneously, stored at 4°C, and used within 1 week of preparation. These included the following: modified MPR broth supplemented with urea (MPRU) or arginine (MPRA), and modified SP-4 broth with either urea (SP-4U) or arginine (SP-4A). Broth media supplemented with arginine and urea were used for culture of *Mycoplasma* spp. and *U. urealyticum*, respectively. Each broth was dispensed in 2-ml volumes into sterile plastic screw-capped tubes (Falcon Plastics, Cockeysville, Md.). Agar medium was dispensed in 3-ml amounts into sterile plastic petri plates (Falcon).

Modified MPR broths. MPRA and MPRU were prepared as previously described (20), except that ampicillin sodium (Wyeth Laboratories, Inc., Philadelphia, Pa.) in a final concentration of 1 mg/ml replaced penicillin or erythromycin or both (Table 1).

Modified SP-4 broths. SP-4U and SP-4A broths were prepared by the formulation previously described for SP-4 (19), with the following exceptions. Ultra-pure urea

(Schwartz/Mann, Inc., Cambridge, Mass.) at a final concentration of 0.05% (wt/vol) replaced glucose in SP-4U, and L-arginine hydrochloride (GIBCO Laboratories, Grand Island, N.Y.) at a final concentration of 0.5% (wt/vol) replaced glucose in SP-4A. Ampicillin (1 mg/ml) was used in place of penicillin. SP-4U contained lincomycin hydrochloride (The Upjohn Co., Kalamazoo, Mich.) at a final concentration of 15 μg/ml (Table 1).

Modified Shepard A7 agar. A7 agar was prepared as previously described (12) with *Ureaplasma* differential agar medium (A7) (GIBCO Diagnostics, Madison, Wis.). Penicillin was replaced with ampicillin (1 mg/ml) and amphotericin B (5 μg/ml). The final pH of the agar was 6.0 (Table 1).

Incubation, observation, and identification. A7 plates were incubated at 36°C in anaerobic Gas Pak jars (BBL Microbiology Systems, Cockeysville, Md.) and examined daily for 8 days and then once more at 14 days. The agar surface was scanned with the use of a low-power objective (×100) from a standard light microscope. Positive cultures were identified by characteristic colony morphology on Shepard A7 differential agar; *U. urealyticum* appeared as typical, brown, manganese accretion colonies, whereas *Mycoplasma* spp. usually demonstrated a fried-egg appearance without the development of deep brown manganese product. *Mycoplasma* spp. colonies displaying atypical morphology were confirmed with Dienes stain (3, 6).

Broth media were incubated at 36°C under atmospheric conditions and examined twice daily for the first 2 days and then once per day until day 8. Broths were observed macroscopically against a white background to facilitate detection of minimal color changes. Positive broths were confirmed by subculturing 0.1-ml volumes to fresh A7 agar plates as soon as any color change was detected. These plates were handled and read by the procedures used for the primary A7 plates.

RESULTS

U. urealyticum was recovered from 351 of 476 (73.7%) frozen, previously positive specimens with A7 differential agar medium or some combination of the broths supplemented with urea (or both). Of the 351 ureaplasma isolates recovered, 321 (91.5%) grew on A7 agar and 234 (66.7%) were obtained from the combined broths, MPRU and SP-4U (Table 2). Thirty-three percent (117 of 351) of the isolates were recovered from A7 agar only, whereas 8.5% (30 of 351) of the strains were detected in the supplemental broths alone. The SP-4U and MPRU broths yielded 96.7% (29 of 30) and 93.3% (28 of 30), respectively, of the 30 isolates that were not recovered from the primary A7 agar medium (Table 2). The average time to detection of positive cultures of *U. urealyticum* was 1.6 days for the broths and 3.2 days on A7 agar (Table 3).

The number of *Mycoplasma* spp. (76) recovered with A7 agar and arginine supplemented broths was the same as originally isolated. The combined broths of MPRA and SP-4A yielded 96.1% (73 of 76) of *Mycoplasma* spp. isolates, whereas only 23.7% (18 of 76) of the strains were recovered on A7 agar (Table 2). Recovery only from arginine-supplemented broths accounted for 58 of 76 (76.3%) isolates; of these, 100% (58 of 58) were detected in SP-4A broth and 87.9% (51 of 58) were obtained from MPRA broth (Table 2). The average time to detection of positive cultures for large colony mycoplasma was 2.2 (85.9%) and 2.1 (89.1%) days after inoculation of MPRA and SP-4A broths, respectively. An average of 8.9 days elapsed before growth on the primary A7 agar was observed (Table 3).

DISCUSSION

The majority of *U. urealyticum* isolates were detected by growth on A7 agar (91.5%), with each of the broths yielding an additional 8.5% of the isolates. Moreover, SP-4U and MPRU broths demonstrated comparable recovery rates. Positive cultures were detected earlier in broth than on A7 agar, 1.6 days compared with 3.2 days, respectively. These observations are not substantially different from those made on cultures inoculated with fresh samples where the majority of isolates were detected in 1 to 3 days depending upon the number of organisms in the original specimen.

To determine an optimal agar medium for the simultaneous isolation of *U. urealyticum* and *Mycoplasma* spp., Yajko et al. (21) compared A7B with PPLO, NYC, and E agars, and used U9 urease color test medium as the supplemental broth. A7B agar was superior for the recovery of both genital mycoplasmas, and U9 broth recovered an additional 5.6% of *U. urealyticum* isolates not detected on A7B agar. This enhancement of isolate recovery by the supplemental broth is similar to our observations. Leland and co-workers (5) compared two media systems, S1 and S2, consisting of urea broth-urea agar and Boston broth-A7 agar, respectively, for recovery of *U. urealyticum*. With the S2 system, 92% of *U. urealyticum* isolates were identified on A7 agar, and an additional 6% of isolates were detected in the supplemental Boston broth; these results are consistent with our findings. The S2 system was superior to S1 in the numbers of positive specimens detected, time to detection, and colony counts, but within each system the number of positive cultures identified by broth and agar was similar, in contrast to our experience where A7 agar yielded more isolates than did either broth. On reculture, 33% of our *U. urealyticum* isolates were obtained on A7 agar alone, a result consistent with previous observations in our laboratory with fresh specimens, in which A7 agar yielded more isolates than urea broth. Perhaps some of the isolates recovered on A7 agar alone were the more fastidious strains of *U. urealyticum*

TABLE 1. Characteristics of modified media for isolation of genital mycoplasmas

Medium	Deleted component(s)	Added component(s)	pH	Initial color	Color when positive
MPRU	Penicillin	Ampicillin	6.0	Straw yellow	Pink-red
MPRA	Penicillin, erythromycin	Ampicillin	7.0	Pale pink	Red
SP-4U	Glucose, penicillin	Urea, ampicillin, amphotericin, lincomycin	6.0	Straw yellow	Pink-red
SP-4A	Glucose, penicillin	Arginine, ampicillin, amphotericin	7.0	Pale pink	Red-purple
A7 agar	Penicillin	Ampicillin, amphotericin	6.0		

TABLE 2. Recovery of *U. urealyticum* and genital *Mycoplasma* spp. from A7 agar and urea-supplemented (MPRU, SP-4U) or arginine-supplemented (MPRA, SP-4A) broth media

Organism (no. of isolates)	Number (%) of isolates recovered from:							
	A7 agar[a]	MPRU broth	SP-4U broth	MPRA broth	SP-4A broth	Broths combined	A7 agar only[b]	Broths only
U. urealyticum (351)	321 (91.5)	222[c] (63.2)	227[d] (64.7)	NA[e]	NA	234 (66.7)	117 (33.3)	30[c,d] (8.5)
Mycoplasma spp. (76)	18 (23.7)	NA	NA	64 (84.2)	73[f] (96.1)	73 (96.1)	3 (3.9)	58[f] (76.3)

[a] Total number of isolates recovered on A7 agar with or without corresponding recovery from broths.
[b] Total number of isolates recovered only on A7 agar (i.e., these strains were not recovered from any broth).
[c] MPRU single recovery source for one isolate.
[d] SP-4U single recovery source for two isolates.
[e] NA, Not applicable.
[f] SP-4A single recovery source for seven isolates.

that prefer A7 agar to broth (4) or that require an extended incubation time to produce the necessary color changes in broth (10). Either SP-4U or MPRU broth could be used as a useful adjunct to Shepard A7 agar for enhancing recovery of *U. urealyticum*.

A supplemental broth was essential for the isolation of *Mycoplasma* spp. from genital tract specimens. Without the use of broths, 76.3% of positive cultures would have been reported negative, and of these, 100% were detected in SP-4A broth and 87.9% were obtained from MPRA broth. Comparing Boston broth and A7 agar with biphasic arginine broth and arginine agar for the recovery of *Mycoplasma* spp., Leland and co-workers (5) obtained similar isolation rates from both systems and from the broth and agar within each system. They used fresh as well as frozen urine samples, whereas in our study we used specimens frozen for various periods of time. Although a decline in titer during storage may have resulted in insufficient organisms to produce colonies on agar (4, 8, 13, 14), the broths no doubt acted as enrichment media to provide enhanced growth of the organisms present in the inoculum and therefore greater sensitivity in the recovery of *Mycoplasma* spp. During our original testing of these specimens, *Mycoplasma* spp. grew readily on A7 agar; as was observed with *U. urealyticum*, 100% grew on A7 agar, whereas 85% of the arginine broths were positive. The average time to detection of positive cultures on reisolation was 2 days for the broths, an observation consistent with other studies (5), and 9 days for A7 agar. The average time to detection of growth in cultures inoculated with fresh specimens was 4 to 5 days on A7 agar, indicating a doubling of time for detection of positive specimens after frozen storage. The best overall isolation, yielding 96.1% (73 of 76) of the *Mycoplasma* spp. isolated, was with SP-4A.

Recently, Tully and co-workers compared SP-4 broth supplemented with 0.1% arginine to two conventional mycoplasma culture media for the recovery of *M. hominis* from the urogenital tract (18). Of the isolates obtained, 33% (18 of 55) were recovered from SP-4 alone, further demonstrating the usefulness of this media for the enhanced detection of *M. hominis*.

In addition to evaluating modified SP4, urea, and arginine broths, we also examined bromothymol blue broth incorporating urea for the isolation of *Ureaplasma* sp. as well as substituting arginine and cresol red for the isolation of *Mycoplasma* spp. These broths were prepared as previously described (8) and tested in parallel with the other media reported in this study (data not shown). Recovery of *U. urealyticum* and particularly *Mycoplasma* spp. was decreased significantly when compared with that of the other two broths studied; in retrospect, the discrepancy was probably due to the storage methods employed after preparation. For this study, all media were stored at 4°C and used within 1 week. Since storage of bromothymol blue broth at 4°C for more than 24 h leads to deleterious performance, these broths must be stored at −20°C immediately after their preparation until used (8). The importance of determining proper storage requirements for media and adhering to them cannot be overemphasized. Although none of the other media tested in this evaluation appeared to have as strict a storage requirement, it would be difficult during routine testing to determine which of the isolates remain undetected as a result of decreased media performance unless storage parameters had been strictly defined.

Broths were examined twice each day for the first few days and subcultured as soon as minimal color changes were first noted. Failure to do so exposes the organisms to detrimental changes in pH and may yield falsely negative subcultures (4, 5, 10, 13). During this study, very few instances of false-positive color changes were noted in reading the broths. Overgrowth by bacteria or fungi also was not a problem. Prior freezing of the specimens along with the use of high concentrations of antibiotics may have had some influence on causing very few false color changes in the

TABLE 3. Time to detection of *U. urealyticum* and *Mycoplasma* spp. with broth and agar media

Medium (no. positive)	No. (%) of positive cultures detected on day postinoculation:									Avg time (days) to detection
	1	2	3	4	5	6	7	8	14	
U. urealyticum										
MPRU (222)	98 (44.1)	114 (51.4)	8 (3.6)	2 (0.9)						1.6
SP-4U (227)	120 (52.9)	90 (39.6)	15 (6.6)	2 (0.9)						1.6
A7 agar (321)	61 (19.0)	109 (34.0)	63 (19.6)	30 (9.3)	13 (4.0)	7 (2.2)	7 (2.2)	23 (7.2)	8 (2.5)	3.2
Mycoplasma spp.										
MPRA (64)	3 (4.7)	52 (81.2)	3 (4.7)	3 (4.7)	3 (4.7)					2.2
SP-4A (73)	4 (5.5)	61 (83.6)	6 (8.2)	2 (2.7)						2.1
A7 agar (18)					1 (5.6)			12 (66.7)	5 (27.8)	8.9

broths as a result of depressing the metabolism of epithelial cells or contaminating organisms. Amphotericin B, at the concentration used (5 μg/ml), will reportedly inhibit some strains of *Ureaplasma* sp. (9). Since polymyxin B also acts on the cell membrane, it, too, may be inhibitory. The overall isolation rate of *Ureaplasma* and *Mycoplasma* spp. in this study, or when using formulations containing these antimicrobial agents, may be decreased and should therefore be noted. However, the inhibitory effect of amphotericin B or polymyxin B does not account for the number of *U. urealyticum* strains that were recovered on A7 agar as compared with the broths since amphotericin B at similar concentrations was contained in all media. Only MPRU (and MPRA) contained polymyxin B, but this broth did not perform differently than SP-4U for the recovery of *U. urealyticum*.

A variety of broth (2, 4, 5, 8, 11) and agar (4, 5, 8, 11, 12, 21) media have been used for the cultivation of genital mycoplasmas. Although a standardized system of culture is not common among laboratories, it is recognized that the use of agar and broth media results in optimal recovery of these organisms (4, 5, 10, 13, 20). Shepard A7 differential agar or one of its more recent modifications (A7B and A8) has been suggested as the solid medium of choice (4, 5, 10, 12), whereas the preferred liquid media have included U9 (11), standard mycoplasma (20), Boston (4, 5), and bromothymol blue (8) broths. Growth comparisons of genital mycoplasmas in broth and agar demonstrate that isolation rates vary with the media selected. Indeed, some investigations found broth media superior to agar media for the detection of genital mycoplasmas (2, 8, 14), whereas other studies reported similar recovery rates from both types of media (4, 5, 11). The results obtained from this study found A7 agar to be superior for the isolation of *U. urealyticum*, whereas broth media were optimal for the recovery of the *Mycoplasma* spp. However, to detect the maximum number of positive cultures, it is strongly recommended that a combination of broth and agar be used for the optimal recovery of both organisms.

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