JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 1982, p. 709–714
0095-1137/82/100709-06$02.00/0

Vol. 16, No. 4

Comparative Evaluation of Media for Isolation of *Ureaplasma urealyticum* and Genital *Mycoplasma* Species

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Received 26 April 1982/Accepted 14 June 1982

Two media systems were compared for isolation of *Ureaplasma urealyticum* and genital *Mycoplasma* sp. System 1 (S-1) consisted of arginine agar and an arginine biphasic medium for isolation of *Mycoplasma* sp. and urea agar and urea broth for isolation of *U. urealyticum*. System 2 (S-2) utilized Boston broth, which is a urea-containing broth, and A7 agar, both of which support the growth of both species. Urine samples, some freshly collected and some known-positive frozen samples, were used as inocula for the two media systems. With S-1, *U. urealyticum* was recovered in 68% of *U. urealyticum*-positive cultures; 58% were detected in urea broth, and 60% were identified on urea agar. When the S-2 system was used for culture of the same samples, *U. urealyticum* was recovered in 98% of the cultures, with 94% detected in Boston broth and 92% identified on A7 agar. *Mycoplasma* sp. was recovered in S-1 and S-2 in 97 and 100% of *Mycoplasma* sp.-positive cultures, respectively. The S-1 arginine broth gave positive results for 89% of the cultures, and the arginine agar was positive for 97% of the cultures. The S-2 Boston broth and A7 agar gave positive results for 92 and 97% of the cultures, respectively. For the isolation of *U. urealyticum*, colony counts were higher, growth was seen sooner, and colonies were larger when the S-2 media system was used. Overall, the cost per test of the S-2 system was less both in technologist time and in reagent costs.

At present there is evidence that *Ureaplasma urealyticum* and genital *Mycoplasma* sp. are involved in neonatal infections (5, 10, 11) and that they play a role in genital diseases such as nonspecific urethritis (7) and reproductive failure in both men and women (3, 4). To further clarify the importance of these organisms in human pathology, techniques must be available for their accurate isolation and identification in the clinical laboratory. Many types of media, including various broths, agars, and biphasic systems, have been employed. In our laboratory a traditional media system (12) was utilized. This system, consisting of an arginine agar plate, an arginine biphasic medium, a urea agar plate, and urea broth medium, provided a satisfactory means of isolating both *U. urealyticum* and *Mycoplasma* sp. However, observed recovery rates of *U. urealyticum* were lower than those reported by Kundsin et al. (6), who used an A7 differential agar plate (9). Kundsin has also used Boston broth, a modified urea-containing medium, for isolation of *U. urealyticum* (R. B. Kundsin, personal communication). The present investigation was undertaken to compare the traditional system (S-1) with the system (S-2) of Kundsin et al. in terms of recovery rates, time required for positive reactions to occur, colony size, and relative cost.

MATERIALS AND METHODS

Media system 1 (S-1). Arginine biphasic medium, arginine agar, urea broth, and urea agar were prepared as described by Velleca et al. (12). Agar media were dispensed into sterile petri plates (60 by 15 mm), at 5 ml of agar per plate. Urea broth was dispensed in 2.5-ml volumes into screw-capped tubes (16 by 125 mm). Tubes of arginine biphasic medium were prepared by placing 1 ml of agar medium and 1.5 ml of broth medium in screw-capped tubes (16 by 125 mm). All S-1 media were used within 2 weeks of preparation as recommended (12).

Media system 2 (S-2). A7 agar was prepared as described by Shephard and Lunceford (9) with the following modification: 8 g of powdered plain agar (Difco Laboratories, Detroit, Mich.) was added per liter of medium. The agar was dispensed into sterile petri plates (60 by 15 mm) at 5 ml of agar per plate. The S-2 Boston broth, a modification of Ford broth (2), was developed by R. B. Kundsin (personal communication), Peter Bent Brigham Hospital, Boston, Mass. It was prepared as follows: 4.2 g of PPLO broth without CV (Difco) was mixed with 200 ml of distilled water, and the pH was adjusted to 5.5 with 1 N HCl. This mixture was autoclaved at 121°C for 15 min and cooled to 56°C in a water bath. The following components were added aseptically: 2 ml of CVA enrichment

(GIBCO Laboratories, Grand Island, N.Y.), 25 ml of horse serum, 25 ml of 25% yeast extract (Flow Laboratories, McLean, Va.), 25 ml of 10% urea, 1.2 ml of 0.4% phenol red, 2.5 ml of penicillin (100,000 U/ml), and 2 ml of 2% L-cysteine hydrochloride. The Boston broth was dispensed in 2.5-ml volumes screw-capped tubes (16 by 125 mm). The S-2 media were used within 1 month of preparation.

Fresh urine samples. Forty freshly voided urine specimens were collected and transported to the laboratory within 1 h after collection. Samples were from male and female patients attending a community sexually-transmitted diseases clinic and from hospital laboratory employees. Urine was plated in quantities of 0.1, 0.01, and 0.001 ml on arginine, urea, and A7 agar plates. A 10-ml aliquot of the remaining urine was then centrifuged at 1,500 × *g* for 10 min in a Sorvall GLC-2 desk-top centrifuge, 9 ml of the supernatant fluid was discarded, and 0.1 ml of resuspended sediment was used to inoculate each of the following media: arginine agar, urea agar, A7 agar, arginine biphasic broth, urea broth, and Boston broth.

Frozen samples. Sediments of 43 known positive samples were used. *U. urealyticum*, *Mycoplasma* sp., or both had been recovered from these urine specimens upon previous routine culturing. The samples were retrieved from −70°C freezer storage and thawed at room temperature. Each was diluted to 10 ml in Eagle minimum essential medium modified with Earle salts (Flow Laboratories) and mixed thoroughly. These samples were then cultured as described above for freshly voided urine samples.

Incubation and observation of media. All plates were incubated at 36°C in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) with $H_2 + CO_2$ generator packages. Broth and biphasic media were incubated at 36°C in an ambient air incubator. All media were examined daily during a 7-day period, and the time, in days, of the first observed growth or color change was recorded. Agar plates were examined with the low-power (×10) microscope objective of a standard light microscope, and an ocular micrometer was used to measure colony diameters. Broths were examined macroscopically for color change. For the urea broth medium, a color change from yellow to bright pink was considered positive. In the arginine biphasic medium, a positive reaction was observed as a color change from pale orange to pink. Boston broth, which supports the growth of both *Mycoplasma* sp. and *U. urealyticum*, showed two types of color change. One type involved an initial change from yellow to pale salmon-pink. This was observed when *Mycoplasma* sp. alone was present. This color change sometimes progressed to bright pink upon prolonged incubation. A second type of color change appeared as an initial and rapid change from yellow to bright pink. This was observed when *U. urealyticum*, alone or in combination with *Mycoplasma* sp., was present.

Cultures were never reported as positive solely on the basis of color change in a tube medium. Observation of colonies with characteristic morphology on agar plates was required for a positive report. Broths showing color change in agar-negative cultures were subcultured to appropriate media to observe growth. If no growth was observed on agar, the color change in broth was considered as a false-positive result. Note that subculture of the broth must be attempted before the color change is complete; recovery of viable organisms from tubes showing complete color change is seldom successful (6).

Identification of organisms. Identification of *U. urealyticum* on the S-2 A7 agar was made by observation of characteristic brown colonies. Identification of colonies on the S-1 urea agar also relied upon observation of characteristic morphology. A direct urease test (8) was performed in cases in which colony morphology was atypical. Identification of *Mycoplasma* sp. on agar media was made by observation of the typical fried-egg colonies. No further confirmatory tests were performed. Artifacts were differentiated from colonies by using the modified Dienes stain (12).

Analysis of data. Because no statistically significant differences were found between results on fresh and frozen urine samples, data from all samples were pooled and analyzed together. Evaluation of isolation rates obtained with various media was performed by using the chi-square test. The average time of the first positive reading was calculated as a mean value, using the number of days elapsed between culture inoculation and observation of a positive result. The standard deviation was calculated, and means were compared by using Student's *t* test. Any medium showing gross contamination, false-positive results, or negative results was not included in these calculations. This calculation for Boston broth readings was performed on cultures with only *U. urealyticum* or only *Mycoplasma* sp. to eliminate interference due to color change produced by the other organism in mixed culture. Comparison of colony counts reflects values from only those cultures which showed growth of organisms on both types of agar being compared; cultures in which either the S-1 or S-2 plates showed no growth of the organisms or were overgrown with contaminants were not included in colony count comparisons.

Calculation of costs. The cost of reagents and supplies for each type of medium was determined on the basis of current catalog prices. A charge for technologist time for preparation of media was included in the final cost per tube or plate of each medium. Technologist time for examination of cultures was calculated based on the assumption that each culture was examined daily for 7 days and that the technologist was paid $0.12 per min. The final cost of technologist time per culture was determined by multiplying 7 days × $0.12/min × number of minutes per day per culture.

RESULTS

Of 83 urine samples cultured, *U. urealyticum* was recovered from 50. With S-1, this strain was isolated from 68% of the *U. urealyticum*-positive samples, with the initial urea broth giving positive results in 58% of the cases and the initial urea agar showing growth in 60% of the cultures (Table 1). Overgrowth of urinary tract organisms on the S-1 urea agar plate was seen in 25% of the cultures in which recovery of *U. urealyticum* was unsuccessful. With the S-2 media, *U. urealyticum* was recovered in 98% of the positive cultures, with the initial Boston broth giving positive results in 94% of the cultures and the initial A7 agar showing growth in 92% of the

cultures. S-2 provided significantly ($P < 0.001$) better isolation rates of *U. urealyticum* than did S-1. Statistically significant differences ($P < 0.001$) in the recovery rate of this strain were also determined when urea broth and Boston broth were compared and when urea agar and A7 agar were compared. Positive results were evident earlier with the S-2 media (Table 1). The average time of the first positive reading obtained with the S-2 Boston broth was 1.69 ± 0.97 days, compared to 2.97 ± 1.50 days ($P < 0.001$) obtained with the S-1 urea broth. The average time of the first positive reading for the S-2 A7 agar was 2.02 ± 1.13 days, compared to 4.00 ± 1.70 days ($P < 0.001$) for the S-1 urea agar. Representative *U. urealyticum* colonies ranged from 30 to 160 μm on S-2 A7 agar (Fig. 1A) compared with 10 to 30 μm on the S-1 urea agar (Fig. 1B). Colony counts were much higher on S-2 A7 agar (Table 2). In 46% of the *U. urealyticum*-positive cultures, the A7 agar plate had 10 or more times as many colonies as did the urea agar; in 12% there were 5 to 9 times as many; and in 42%, the A7 and urea agar had counts which were approximately equal (± fourfold difference).

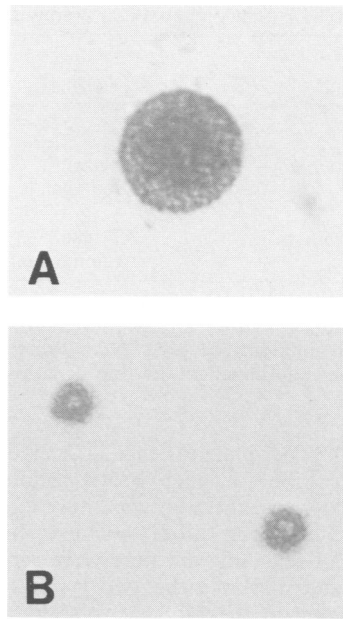

FIG. 1. *U. urealyticum* (A) on A7 agar and (B) on urea agar. Magnification, ×100.

Of the 83 urine samples cultured, *Mycoplasma* sp. was recovered from 37. With S-1, this species was isolated from 97% of *Mycoplasma*-positive samples, with the arginine biphasic medium giving positive reactions in 89% of the cultures and the arginine agar plate showing growth in 97% of the cultures. With S-2, *Mycoplasma* sp. was recovered from 100% of the positive cultures, with the Boston broth giving positive reactions in 92% of the cultures and the A7 agar plate showing growth in 97% of the cultures (Table 3). There was no statistically significant difference in *Mycoplasma* sp. isolation rates between S-1 and S-2, between arginine biphasic medium and Boston broth, or between arginine agar and A7 agar. The average time of the first positive reading in *Mycoplasma*-positive cultures was 1.73 ± 0.46 days for S-2 Boston broth, 2.09 ± 0.63 days for the S-1 arginine biphasic medium, 2.58 ± 0.89 days for S-1 arginine agar, and 2.39 ± 1.13 days for S-2 A7 agar. Differences in the average time of the first positive reading were not statistically significant. *Mycoplasma* colony counts on A7 agar and arginine agar were approximately equal (± fourfold difference) in 68% of the cultures (Table 2). Colony counts on A7 agar were greater by fourfold or more in 9% of the cultures, whereas colony counts on arginine agar were greater by fourfold or more in 23% of the cultures. The *Mycoplasma* colonies were of comparable size on both A7 and arginine agar.

The reagent and preparation cost was approxi-

TABLE 1. Identification of 50 *U. urealyticum*-positive urine samples

Media system	Total no. of positive cultures identified (%)	Medium	No. of positive cultures identified (%)	Time of first positive reading (days ± SD)
S-1	34 (68)	Urea broth	29 (58)	2.97 ± 1.50
		Urea agar	30 (60)	4.00 ± 1.7
S-2	49 (98)[a]	Boston broth	47 (94)[b]	1.69 ± 0.97[b,c]
		A7 agar	46 (92)[d]	2.02 ± 1.13[d]

[a] Difference was statistically significant ($P < 0.001$) compared to S-1.
[b] Difference was statistically significant ($P < 0.001$) compared to urea broth.
[c] Calculated on pure cultures of *U. urealyticum* only, to eliminate color change interference when *Mycoplasma* sp. is present.
[d] Difference was statistically significant ($P < 0.001$) compared to urea agar.

TABLE 2. Comparison of colony counts in recovery of *Mycoplasma* sp. and *U. urealyticum*

Strain	Medium	% of colony counts that were:		
		Equal (± fourfold difference)	Five- to ninefold higher	≥10-fold higher
U. urealyticum[a]	A7 agar	42	12	46
	Urea agar	42	0	0
Mycoplasma sp.[b]	A7 agar	68	6	3
	Arginine agar	68	10	13

[a] In 20 additional cultures, *U. urealyticum* was recovered on A7 agar; no colonies were seen on urea agar. In four additional cultures, the strain was recovered on urea agar; no colonies were seen on A7 agar.

[b] In one additional culture, *Mycoplasma* sp. was recovered on A7 agar; no colonies were seen on arginine agar. In one additional culture, the strain was recovered on arginine agar; no colonies were seen on A7 agar.

mately $0.30 per plate for each agar medium and $0.15 per tube for each broth medium. The arginine biphasic medium was more expensive, costing $0.30 per tube, because increased amounts of reagents and time were required to prepare and dispense the two types of media which make up the biphasic system. The S-1 media (two agar plates, one broth tube, one biphasic culture) cost $1.05 per culture, and the S-2 media (one agar plate, one broth tube) cost $0.45 per culture.

Technologist time for daily examination of cultures involved approximately 10 s per broth or biphasic medium and 1 min for each agar medium with the exception of the urea agar; urea agar required a longer examination time of 1.5 min. Daily reading time was 2.84 min for S-1 and 1.17 min for S-2. During a 7-day incubation period, the cost for technologist time for reading of a culture was $2.39 for S-1 and $0.98 for S-2. Total cost per culture (technologist time and reagent cost) was $3.44 for S-1 and $1.43 for S-2.

DISCUSSION

Two media systems were compared for isolation of *U. urealyticum* and *Mycoplasma* sp. from urine samples. Concerning isolation of *U. urealyticum*, S-2 had many advantages. This system successfully identified a significantly larger percentage of *U. urealyticum*-positive cultures, and evidence of growth of organisms was observed sooner. Higher colony counts and larger colonies were found in the S-2 A7 agar than on the S-1 urea agar.

Overgrowth of common urinary tract organisms was responsible for 25% of the false-negative results on the S-1 urea agar. This contamination, although present in some cases on S-2 A7 agar, was not as troublesome because the *U. urealyticum* colonies, which appeared sooner and were larger on A7 agar, were visible before the contamination could overgrow. The S-1 urea broth frequently demonstrated a color change from yellow to orange during days 5, 6, and 7 of incubation. *U. urealyticum* could not be isolated from these cultures, so the color change was considered to be a false-positive reaction. Such false-positive reactions were not observed with the S-2 Boston broth.

With the S-1 system, positive reactions were often seen in the urea broth before colonies were visible on the urea agar; the urea broth, therefore, required immediate subculturing to an ad-

TABLE 3. Identification of 37 *Mycoplasma* sp.-positive urine samples

Media system	Total no. of positive cultures identified (%)	Medium	No. of positive cultures identified (%)	Time of first positive reading (days ± SD)
S-1	36 (97)	Arginine biphasic	33 (89)	2.09 ± 0.63
		Arginine agar	36 (97)	2.58 ± 0.89
S-2	37 (100)[a]	Boston broth	34 (92)[b]	1.73 ± 0.46[b,c]
		A7 agar	36 (97)[d]	2.39 ± 1.13[d]

[a] Difference was not statistically significant compared to S-1.

[b] Difference was not statistically significant compared to arginine broth.

[c] Calculated on pure cultures of *Mycoplasma* sp. only, to eliminate color change interference when *U. urealyticum* is present.

[d] Difference was not statistically significant compared to arginine agar.

ditional agar plate. With the S-2 system, subculturing of this nature was seldom required because colonies were usually visible on the A7 agar at the time that a positive reaction was observed in the Boston broth.

The overall recovery rate of *Mycoplasma* sp. from urine samples was comparable for the S-1 and S-2 systems, which successfully identified 97 and 100% of the *Mycoplasma*-positive cultures, respectively. *Mycoplasma* colony sizes were equal on the S-2 A7 and the S-1 arginine agar, and the average times of the first positive readings were very similar for both systems. Colony counts were equal (within ± fourfold) in 68% of cultures; 9% had larger counts on S-2 A7 agar and 23% had larger counts on S-1 arginine. Contamination was not a problem with either type of agar or tube medium.

Analysis of results here confirms the conclusion of Kundsin et al. (6) regarding the need for utilization of both broth and agar media for initial specimen inoculation. None of the individual media types used in this study was sufficient to isolate either *Mycoplasma* sp. or *U. urealyticum* in 100% of the positive cultures. Results here likewise confirm the conclusions of Bredt and Bink (1), who found that strains of *U. urealyticum* differ in their ability to grow in broth. Yaoko et al. (D. Yaoko, T. Balston, D. Wood, and W. K. Hadley, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, G8, p. 80) compared recovery rates of genital *Mycoplasmataceae* on four agar media and found superior recovery rates of *U. urealyticum* with A7 agar. Results from this study as well as the work of Fiacco et al. (V. Fiacco, M. J. Miller, E. Carney, and W. J. Martin, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, C34, p. 277) indicate that recovery rates of genital *Mycoplasma* sp. and *U. urealyticum* can be increased by the use of A7 agar with a supplemental broth medium for initial isolation.

The urea concentration in Boston broth is 0.88%. Most urease color test broths for ureaplasma isolation utilize lower urea concentrations ranging from 0.03 to 0.1% (2, 12). Ford and MacDonald (2) demonstrated that a urea concentration of 1.0% enhanced growth of ureaplasma, although, after a 6- to 8-h period of rapid growth, resulting alkalinity inhibited growth of organisms and caused an increased death rate. We feel that the increased urea concentration of nearly 1% in Boston broth enhances early growth of ureaplasmas and makes this broth a valuable diagnostic aid by providing an early indication of ureaplasma growth. Death of organisms due to increasing alkalinity must be avoided by rapid subculture of organisms to another medium if growth of organisms is to be maintained for a prolonged period.

The ease of handling and preparation of the S-2 media is notable. Because fewer types of media are required, the cost of the S-2 system is less for reagents, glassware, and petri dishes, and less time is required for media preparation. This system eliminates the need for preparation of the more expensive and time-consuming biphasic medium. The S-2 media have a shelf-life of at least 1 month (6), whereas the S-1 media must be used within 2 weeks of preparation (12).

The S-2 system offers advantages in terms of technologist time. Each culture has only one plate and one tube which must be examined, compared with two plates and two tubes for the S-1 system. Colonies on the S-2 A7 agar are larger and easier to see, which decreases the time required for examination of each agar plate. Because growth is visible on the S-2 A7 agar very early in the course of culture, the need for subculture of positive broths is infrequent.

Both S-1 and S-2 systems have been recommended for isolation of *U. urealyticum* and *Mycoplasma* sp. from genital swab samples. For S-1, it is suggested that a transport medium be utilized for transport of genital swab samples to the laboratory (12). Transport medium is not recommended for genital swab transport when the S-2 system is used (6).

During a 2-year period we have cultured genital swab samples from a population of patients attending a community hospital obstetrics clinic. Initial studies using the S-1 media with transport medium yielded *U. urealyticum* in 60% of the cultures. In the same population, the recovery rate of *U. urealyticum* increased to 93% when we started using the S-2 system. In the same population, the recovery rate of *Mycoplasma* sp. was 40% with the S-1 system. This rate did not change when the S-2 system was used.

Our findings indicate that enhanced recovery rates of *U. urealyticum* and lower laboratory costs can be obtained by using the S-2 system. Furthermore, this increase in recovery is sufficient to raise questions about previously reported prevalence ratios of *U. urealyticum* in a variety of diseases in which S-1 or a similar system was used for primary isolation.

ACKNOWLEDGMENTS

We thank R. B. Kundsin, Angeles Parrino, and the staff of the Specialty Microbiology Laboratory, Peter Bent Brigham Hospital, Boston, Mass., for technical advice and encouragement.

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