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Bromothymol blue Broth: Improved Medium for Detection of *Ureaplasma urealyticum* (T-Strain Mycoplasma)

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Bromothymol blue (B) broth for the cultivation, detection, and identification of *Ureaplasma urealyticum* is described. In this medium, strains Cook and 960 had shorter generation times (60 min or less) and reached higher populations (over 10^8) than have yet been reported for this species. Furthermore, the indicator changes color before the end of logarithmic growth, and the cultures retain viability for at least 1 day thereafter, greatly simplifying the handling of the organism. When the populations in cultures of these two strains and seven new isolates were determined, growth was detected earlier and proceeded to higher final titers in B broth than in urease test color medium (U-9 broth). The inclusion of antibiotics in B broth for use in clinical laboratories (B/NL broth) made the medium selective, specific, and more sensitive for the isolation of *U. urealyticum*. Comparison of B/NL broth with genital mycoplasma (GM) agar and U-9 broth for the primary isolation of *U. urealyticum* was made with 183 urethral swabs. All 70 isolates were detected on B/NL broth, but only 66 and 63 isolates were detected on GM agar and in U-9 broth, respectively. Moreover, the cultures in B/NL broth were pure and at titers that generally showed good correlation with colony counts on GM agar.

Since its discovery by Shepard in 1956 (17), the cultivation of *Ureaplasma urealyticum* (T-strain mycoplasma) has presented problems to laboratory workers. The difficulty in growth detection, resulting from the absence of turbidity, was lessened in 1970 when Shepard and Lunceford introduced U-9 broth (20). This broth has an indicator system based on the ability of *U. urealyticum*, unique among mycoplasmas, to degrade urea to CO_2 and NH_3 (ammonia) (19). The resultant accumulation of NH_4^+ (ammonium ion) increases the pH to change the color of the phenol red indicator from yellow to red (alkaline color) (20). This change is not evident, however, until stationary growth is reached, and the cultures lose viability soon thereafter. Although the rapid decline characteristic of *Ureaplasma* cultures has been overcome through use of continuous culture (4), a vacuum-flow system (8), dialysis culture (12), and an ion-exchange resin with pH adjustment (21), none of these methods is applicable to the routine handling of cultures. Low cell yields are characteristic of the fluid media presently available for the cultivation of most mycoplasma species; for *U. urealyticum* strains of human origin in batch cultures, maximum populations exceeding 10^7 per ml have been reported only occasionally (e.g., 1, 6, 8a, 10, 11, 13, 19).

The aim of this study was to develop a specific indicator medium for *U. urealyticum* that would allow detection during logarithmic growth, give relatively high cell yields, and prolong viability for periods that were convenient for the handling of the organism. An evaluation of bromothymol blue (B) broth is presented.

MATERIALS AND METHODS

Organisms. *U. urealyticum* strains 7, 23, 27, 58, 354, Pirillo, Cook, and 960 (serotypes I through VIII [2]) and the Boston T strain (9) were kindly supplied by D. K. Ford, Department of Medicine, University of British Columbia, Vancouver, British Columbia, and by R. B. Kundsin, Peter Bent Brigham Hospital, Boston, Mass., respectively. *Mycoplasma hominis* ATCC 14027, *M. pneumoniae* ATCC 15531, and *M. fermentans* ATCC 19989 came from the American Type Culture Collection, Rockville, Md. *M. salivarium* and all other strains of *U. urealyticum* were isolated by the Mycoplasma Laboratory, Department of Medical Bacteriology, University of Alberta, Edmonton, Alberta. *U. Urealyticum* and *Mycoplasma* species were identified by the urease spot test (18) and the growth inhibition test (4a), respectively.

Media. B broth medium (270 mosmol/kg) consisted of PPLO (pleuropneumonia-like organism) broth without crystal violet (Difco Laboratories, Detroit, Mich.), 2.1 g; yeast extract (Difco), 0.1 g; 0.4% bromothymol blue (Fisher Scientific Co., Fair Lawn, N.J.) solution, 1.0 ml; and water 90 ml. After sterilization, this basal medium was supplemented with 10 ml of pooled normal horse serum (Connaught Laboratories, Toronto, Ontario) and urea (Fisher), and glycyl-L-histidyl-L-lysine acetate salt (GHL) (Calbiochem, La

Jolla, Calif.) at concentrations of 0.025% (wt/vol) and 0.02 μg/ml, respectively. The serum, supplied sterile and found mycoplasma-free, was adjusted to pH 6.0. It contained 15 mg of urea per 100 ml, making the final concentration in the broth at least 0.0265% (wt/vol) or 4.45 mM. The urea and GHL solutions were sterilized by filtration. For the growth of pure cultures, the broth contained 1 mg of ampicillin sodium (Ayerst Laboratories, Montreal, Quebec) per ml; for the cultivation of clinical material, 50 U of nystatin (E. R. Squibb & Sons, New York, N. Y.) per ml and (for final experiments) 20 μg of lincomycin hydrochloride (The Upjohn Co. of Canada, Don Mills, Ontario) per ml were also incorporated into the broth to inhibit the growth of contaminating yeasts and large-colony *Mycoplasma* species, respectively. B broth with added nystatin is designated B/N, and that with both nystatin and lincomycin is designated B/NL. After all additions had been made, the hydrogen ion concentrations of the broths were adjusted to 6.0. U-9 broth medium (317 mosmol/kg) was made as described by Shepard and Lunceford (19). Components common to B, B/N, B/NL, and U-9 broths were from the same lots. Tryptic digest broth (Fields) (lot 908609, Baltimore Biological Laboratory, Division of Bioquest, Cockeysville, Md.), not available in Canada, was kindly supplied by M. C. Shepard, Camp Lejeune, N.C. For one experiment, broth was made in which the bromothymol blue of B broth was replaced with phenol red (B/PR broth) and the phenol red of U-9 broth was substituted by bromothymol blue (U-9/BTB broth). For the arginine broth, used to determine the titers of *M. hominis* in B broth, arginine and cresol red replaced urea and bromothymol blue in the B broth formulation, at final concentrations of 0.5% (wt/vol) and 0.002% (wt/vol), respectively. The pH was adjusted to 7.4; when growth increased the pH to about 8.0, the medium turned from yellow to purple. The solid medium for cultivating genital mycoplasmas, GM agar, had a basal medium consisting of PPLO broth without crystal violet, 2.1 g; Ionagar no. 2 (Oxoid Ltd., London, England), 0.75 g (no longer available; this may be replaced by agar no. 1 [Oxoid]); yeast extract, 0.1 g; *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (Calbiochem) 1.19 g; and water, 90 ml. After sterilization and cooling to 50°C, it received the same supplements as B broth and nystatin. The pH was then adjusted to 6.0. Five-milliliter volumes were dispensed into disposable plastic petri plates (60 by 15 mm). Deionized, glass-distilled water was used in all instances. Basal media were sterilized for 15 min at 121°C and 18 lb/in^2. Adjustments to pH were made with 1 N HCl. Osmolal concentrations were measured as previously described (16). The gel strength of GM agar, as determined by the method described by Furness (7), was 16%. Broth media were stored at −20°C, whereas GM agar (always used within 1 week of preparation) was kept at 4°C.

Quantitative cultures. Inocula for growth curves were prepared as follows: for *U. urealyticum*, 1 ml of an exponential-phase culture (indicated by the initial color change of the broth) was diluted 1/100 in B broth and sonically treated in an ultrasonic cleaner (Fisher) with the power supply set at 100/3, for 10 s, the period giving maximum dispersal of cell clumps. A 4-ml amount of this suspension was introduced into 36 ml of test broth. For *M. hominis*, 0.4 ml of sonically treated, undiluted, logarithmic-phase culture was inoculated into 40 ml of broth. Immediately after inoculation and at appropriate periods during incubation (based on changes in the pH or indicator color), samples were removed for color-change unit$_{50}$ (CCU_{50}) determinations. CCU_{50} determinations of the titers of stock strains and new isolates were made as follows: 0.2 ml of culture was added to 1.8 ml of indicator broth, and the dilution was sonically treated. One-in-ten dilutions in broth were carried to a maximum of 10^{-9} for *U. urealyticum* and 10^{-12} for *M. hominis*. The contents of each tube were then divided into nine 0.2-ml volumes in wells of 0.3-ml capacity in sterile, disposable plastic microtiter plates that were then sealed with cellophane tape. For *M. hominis* assays in arginine broth, the wells were vented by pricking the tape with a sterile needle. The 50% end points (15) were determined and expressed as CCU_{50} per milliliter of test culture. Pipetting precision after six serial 1:10 dilutions of radioactively labeled protein was 3.0%. Based on 10 CCU_{50} determinations on a culture that contained an average of 6.9×10^7 CCU_{50}/ml, the standard deviation was 0.906. Urethral swabs obtained from males seen by the University Health Service were placed in 1 ml of transport medium (910 mosmol/kg) consisting of 10× Earle basal salt solution, 10 ml; fetal calf serum, 10 ml; sorbitol, 10 g; $NaHCO_3$, 0.3 g; nystatin, 5,000 U; and water, 80 ml. The pH was adjusted to 7.0, and the solution was sterilized by filtration. Specimens were mixed for 1 min on a Vortex mixer, and the fluid was expressed from the swabs. Volumes (0.1 ml) of fluid from each specimen were placed on GM agar and in 0.9 ml of each broth medium under trial. These 1:10 dilutions in broth were carried to 10^{-6}. Colony-forming units (CFU) on agar and color-change units (CCU) in broth were expressed per milliliter of specimen (i.e., per swab). To ensure uniform volumes, all media used for quantitative studies were dispensed with a Cornwall syringe. Incubation was carried out at 35°C, with broth under atmospheric conditions and agar in 5% (vol/vol) carbon dioxide in nitrogen. Incubation of broth media was continued until 1 day had passed without end-point changes. After 3 days of incubation, colonies on agar were enumerated with an inverted microscope (Diavert model, Leitz, Wetzlar, Germany) at a minimum magnification of ×40.

RESULTS

The establishment of the minimal effective concentration of each medium component led to cumulative improvements in the growth of *U. urealyticum*. Although initial trials were made with stock cultures, no modification was adopted until proven beneficial for the cultivation of new and primary isolates. For instance, although the eight serotype strains reached equal or higher titers in broth with a 5 or 10% rather than a 20% serum supplement, 5% serum was insufficient for the primary isolation of certain strains. One

isolate, strain 411/76, produced a color change in only the 10^{-1} dilution in broth containing 5% serum, but up to 10^{-4} in double the serum concentration, whereas another, strain 399/76, produced no color change in 5% serum, but had a titer of 10^3 in a serum concentration of 10%. Since the higher titers gave closer correlation with the colony counts on GM agar (1.6×10^3 and 3.1×10^3 CFU/ml, respectively), 10% serum was adopted for the final formulation.

The peptide GHL was incorporated into the broth because, for some batches or for certain strains of *U. urealyticum*, growth was markedly improved in terms of an earlier initial change in the indicator and a higher final titer. The pH and urea concentrations chosen were those that allowed maximum growth with a clear-cut color change indicative of urease activity. Yeast extract seemed not to be required by stock strains but has been left in the medium until its effect on primary isolation is assessed.

Data from an experiment comparing the growth of the type strain *U. urealyticum* 960 (serotype VIII) in B and U-9 broths are presented in Table 1. In B broth replication proceeded with shorter lag and generation times than in U-9 broth, resulting in an earlier color change and a higher titer. The initial color change of the bromothymol blue indicator in B broth was from yellow to chartreuse at pH 6.8. By pH 7.2 this developed into a definite green. In U-9 broth the phenol red indicator turned from a straw color to pale pink at pH 7.0; by pH 8.2 the medium was deep pink. In B broth the color change occurred evenly throughout the tube, whereas in U-9 broth it first developed at the bottom and then spread upward (20). Maximum populations in B broth were almost 10-fold greater than in U-9 broth. Furthermore, 0.1-ml samples taken at intervals after the color changes began contained viable cells for at least 30 h in B broth but become sterile between 9 and 12 h in U-9 broth. In different batches of B broth, *U. urealyticum* strain 960 had generation times of between 44 and 60 min, with maximum populations of at least 1.0×10^8 CCU_{50}/ml. A second stock strain, Cook (serotype VII), doubled in number every 32 min to a maximum population of 1.3×10^8 CCU_{50}/ml. The poorest response of the three strains examined was obtained with the Boston T strain, which had a generation time of 65 min and a maximum titer of 3.4×10^7 CCU_{50}/ml. The growth of *M. hominis* ATCC 14027 in B broth was also followed; it showed a doubling time of 113 min and maximum titers of at least 10^{10} (see below). Incubation of this culture was continued, and 0.1-ml volumes removed at weekly intervals were examined for viability. After 8 weeks of incubation, the culture contained about 2.0×10^3 CFU/ml; samples inoculated to GM agar and B broth during week 10 were sterile.

The CCU_{50} of logarithmic-phase cultures of the two stock strains (960 and Cook) and seven new isolates of *U. urealyticum* was determined in each of four types of broth media: B, B/PR, U-9, and U-9/BTB. Initial color changes in these media were detected in the order in which the broths are listed above. After "overnight" incubation (14 to 20 h) the average CCU_{50} per milliliter in B broth was 1.8 times that in U-9 broth. The final titers shown by B and B/PR broths were similar or greater than those obtained in U-9 and U-9/BTB broths, with the averages in B and B/PR broths (both 2.6×10^7 CCU_{50}/ml) exceeding titers in U-9 and U-9/BTB broth by 49 and 73%, respectively. Both initial and final color changes were more distinct in B broth than in B/PR broth and in U-9 broth than in U-9/BTB broth.

For the above experiments, the stock strains had been cultivated in B broth for several transfers but the new strains had been cultivated only once since their isolation in that medium. Since it could be argued that the better growth in B broth resulted from their adaptation to this medium, the strains were passaged three times in U-9 broth, and the experiment was then repeated in B and U-9 broths. After 24 h of incubation, the average CCU_{50} per milliliter in B broth was again almost twice that in U-9 broth; as before, the final titers in B broth exceeded those in U-9 broth, although this time by only 23%. Although some adaptation of the strains to U-9 broth may have occurred, the general pattern of growth in the two media was unaltered. In terms of the time taken for initial color changes, growth rates, maximum titers, and cul-

TABLE 1. *Growth of U. urealyticum 960 in B and U-9 broths*

Broth medium	Length of lag phase (h)	Generation time (min) of logarithmic growth	Initial color change (CCU_{50}/ml) at:		Maximum population detected (CCU_{50})	Color change complete at ~pH	Half-life (min) in decline phase[a]
			pH 6.8	pH 7.0			
B	<1	60	2.3×10^7		1.3×10^8	7.4	64
U-9	>1; <2	68		3.2×10^6	1.0×10^7	8.4	<4.3

[a] For 12 h after maximum population was reached.

ture longevity, B broth was superior to U-9 broth for the cultivation of the stock cultures and the new isolates tested.

Neither *M. pneumoniae, M. fermentans,* nor *M. salivarium* altered the color of either B or U-9 broth. Although both *M. hominis* and *U. urealyticum* grew in U-9 broth, only the latter changed the color of the medium. The effects of these two organisms in B broth differ (Table 2). Whether alone in pure culture or together in mixed culture, characteristic changes in pH and medium color were produced. Although this information proved useful when culturing specimens from the genital tract, the presence of *M. hominis* interfered with the accurate determination of *U. urealyticum* titers and the isolation of this organism in pure culture. To inhibit the growth of *M. hominis* and other large-colony mycoplasmas that might be present in clinical material, lincomycin was incorporated into the medium. In trials with *M. hominis* ATCC 14027, the organism sometimes persisted in the presence of 10, but never 20, μg of the antibiotic per ml. The higher concentration of lincomycin was adopted for B/NL broth and eliminated *M. hominis* from the cultures. Table 3 summarizes a comparison of the titers of *U. urealyticum* on primary isolation in B/N and B/NL broths (CCU per milliliter) and on GM agar (CFU per milliliter). The two broths appeared to behave similarly, with overall correlation (i.e., the sum of categories 1 and 2) in 94 and 92% of all specimens and titers within a factor of 10 (i.e., category 2) in 84 and 80% of all positive cultures for B/N and B/NL broths, respectively. When there was a discrepancy between titers (i.e., categories 3 and 4), the populations in broth exceeded those on agar in 23 of 33 instances. Despite this similarity in behavior, the initial color changes occurred earlier in B/NL broth than in B/N broth and, when actual titers were compared for 28 consecutive specimens from which *U. urealyticum* was isolated on all three media, 7 or about 25% of the strains had titers of 10^2 to 10^4 times higher in B/NL than in B/N broth. Furthermore, the higher titers showed closer correlation with the CFU per milliliter on GM agar than did the titers in B/N broth. This phenomenon occurred when other microorganisms (*M. hominis* or *Neisseria gonorrhoeae*) were isolated from the specimens (2/7) but also when *U. urealyticum* was the only procaryote isolated (5/7). Because B/NL gave more accurate titers than did B/N broth and also provided for the isolation of *U. urealyticum* in pure culture, it was used instead of B or B/N broth for the final laboratory trial.

The main consideration in selecting an indicator broth was its effectiveness for the primary isolation of *U. urealyticum.* The performances of B/NL and U-9 broths and GM agar in this regard were compared by using urethral swabs from 183 males that were received from the University Health Service over a 6-month period. The results of the mycoplasma cultures are shown in Table 4. Pure cultures of *U. urealyticum* were obtained in B/NL broth from the 10 specimens that also contained *M. hominis.* For 8/10 of these specimens, the titers in B/NL broth correlated with the CFU of *U. urealyticum* on GM agar; for the remaining 2/10, the titers in broth exceeded those on agar by 10- to 100-fold. For the 60 specimens from which *U. urealyticum* was the only mycoplasma isolated, detection depended on the medium. Although all 60 strains were detected in B/NL broth, four produced no growth on GM agar; these four and three other strains (a total of seven or almost 12%) failed to cause a color change in U-9 broth.

TABLE 2. *Changes in B broth resulting from the growth of M. hominis and U. urealyticum*

Species	Final pH range	Color
M. hominis	6.7–7.0	Chartreuse
U. urealyticum	7.3–7.6	Green
M. hominis and *U. urealyticum*	7.7 or higher	Bluish green
Uninoculated control (pH 5.9–6.1)	6.0–6.2	Yellow

TABLE 3. *Correlation of CFU per milliliter on GM agar with CCU per milliliter in B/N and B/NL indicator broths*[a]

Broth medium	No. of specimens examined	No color change in broth or growth on agar (1)	CCU in broth and CFU on agar differ:		Growth apparent in broth or agar only (4)
			<10× (2)	>10× (3)	
B/N	246	145	85	10	6
B/NL	204	121	66	5	12

[a] Titers ranged from less than 10 to 10^6 or greater per ml. Numbers in parentheses indicate categories.

TABLE 4. *Isolation of mycoplasmas from urethral swabs*

Culture result	No. of specimens
U. urealyticum only	60
M. hominis only	0
U. urealyticum and *M. hominis*	10
Overgrown by contaminant[a]	1
No mycoplasmas isolated	112
Total	183

[a] Non-urea-hydrolyzing gram-negative bacillus.

The titers for these seven strains in B/NL broth correlated with those on GM agar. As noted in Table 4, only one culture was lost through contamination, and no false positives were obtained. The initial color changes in the 1:10 dilutions of the 70 isolations of *U. urealyticum* in B/NL broth occurred as follows: 43 by 5 p.m. on day 1 after incubation, 23 more by 5 p.m. on day 2 after incubation, and the remaining 4 by day 3. Of these four cultures, three grew in B/NL broth but not in U-9 broth. No further changes occurred on continued incubation. The results of this trial confirmed B/NL as our choice of broth for the primary isolation of *U. urealyticum*.

DISCUSSION

Although the "[U-9] urease color test medium was not designed as a growth medium per se" (20), it probably is the most widely used broth for the detection of *U. urealyticum* and, therefore, was selected as the basis of comparison for this study. Color changes resulting from the cultivation of *U. urealyticum* in B broth preceded those in U-9 broth because of better growth. In B broth the type strain 960 (serotype VIII) replicated faster, reached higher titers, and retained viability longer than it did in U-9 broth (Table 1). A second stock culture, Cook (serotype VII), had the shortest generation time and the highest titer (1.8×10^8 CCU_{50}) yet reported for a *U. urealyticum* strain of human origin. The Boston T strain, which is reputedly difficult to cultivate (9), multiplied more slowly than either of the above strains, yet reached populations over 10^7 CCU_{50}/ml. Overall better growth in terms of initial color changes and final titers was demonstrated in B broth by two stock cultures and seven new isolates, even after their adaptation to U-9 broth.

Although no single component was responsible for the improved growth of *U. urealyticum* in B broth, the serum concentration and peptide GHL were among the contributing factors. The peptide has been found in fresh normal human serum and, whether from this natural source or chemically synthesized, has been shown to enhance macromolecular synthesis in certain eucaryotic cell systems (14). The effect of GHL on the growth of *U. urealyticum* may be associated with serum deficient in nutrients or growth factors that are required by at least some of the strains. B broth contains less urea than does U-9 broth (about 4.5 versus 8.4 mM); this is a major cause of the lower final pH values obtained in the former and probably accounts for prolonged viability. As noted in Tables 1 and 2, the bromothymol blue indicator changes color at a pH value lower than that triggering the appearance of phenol red in U-9 broth. Neither GHL nor bromothymol blue indicator has been used in previously published formulations of media for *U. urealyticum*.

Although the generation time of *M. hominis* 14027 in B broth (113 min) was within the range obtained in a buffered broth designed for its propagation (73 to 120 min [16]), it reached titers 10- to 100-fold greater than in the latter medium. Although this is an important finding in regard to producing large crops of *M. hominis*, the organism was present in about 14% of the urethral swabs containing *U. urealyticum* (Table 4), and its high titers and longevity interfered with the quantitation of *U. urealyticum* and also made the rescue of the latter in pure culture extremely difficult. This problem was overcome by incorporating lincomycin into the medium. Lincomycin is known to inhibit large-colony mycoplasmas but not *U. urealyticum* (3, 5). In addition to making B/NL broth truly specific for *U. urealyticum*, lincomycin increased the titers of this organism isolated from certain urethral swabs, even in the absence of *M. hominis* and bacteria. This phenomenon suggests the presence in these specimens of factors bound by, or microorganisms inhibited by, the antibiotic. These specimens will be cultured for *Chlamydia trachomatis*.

In mycoplasmology, as in bacteriology, generally one expects a broth medium to be more sensitive for the growth of a given organism than an agar medium of the same basic composition (e.g., see reference 16), and this has been shown again with B/N and B/NL broths and GM agar (Tables 3 and 4). The difference in the isolation of *U. urealyticum* in B and U-9 broths points to a difference in strain requirements for primary isolation. We are investigating growth requirements for the primary isolation and subsequent in vitro propagation of *U. urealyticum* as one set of criteria that may be applied to their identification as to subspecies.

I have used B and B/NL broths for 2 years. In my research, B broth has greatly simplified the handling of *U. urealyticum*, whether it be for the preparation of logarithmic-phase inocula, for growth studies, or for a variety of assays based on growth. I anticipate its usefulness when large cell crops are required. In diagnostic laboratories, B/NL broth dilution series provide a reliable means for the quantitative isolation of *U. urealyticum* in pure culture for further identification and antimicrobial susceptibility testing. The longevity of cultures in B/NL broth allows them to be handled within the routine of hospital laboratories. Most significant, of course, is that B/NL broth allows for the increased

isolation of *U. urealyticum*. This will improve diagnosis and allow for more accurate assessment of these fascinating procaryotes in nongonococcal urethritis and other infections of the human genital tract.

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