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Significance of Appropriate Techniques and Media for Isolation and Identification of *Ureaplasma urealyticum* from Clinical Specimens

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Controversy over the association of *Ureaplasma urealyticum* with reproductive failure may be due to methods used to isolate the microorganism. *U. urealyticum* isolations from clinical material should be done simultaneously in broth and on Shepard's differential agar medium (A7) containing manganese sulfate. Urine sediments result in a 9% ($P = 0.0002$) higher rate of isolation than cervical and urethral swabs. Primary isolations may not display standard textbook morphology. Isolated colonies may be present, but brown streaks in cervical mucus or a coalescent haze around epithelial cells in urine sediment may also be seen in areas of concentrated growth. The broth and agar media used, method of incubation, type of specimen, and method of storing specimens before culture are all factors which influence the recovery of *U. urealyticum*.

The association of *Ureaplasma urealyticum* (25) with genitourinary tract infections and reproductive failure ranging from infertility to spontaneous abortion, premature births, and low birth weight is fraught with controversy. Responsible investigators can be found, some implicating the ureaplasmas in infertility (7, 8, 11, 12), others insisting that they are mere commensals and not associated with infertility (4, 9, 20), some implicating them in spontaneous abortion (3, 13, 19), others exonerating them from etiological relationship with spontaneous abortion (14, 26). Investigators on both sides of the controversy are convinced of the validity of their data and totally honest in their evaluations. The controversy over *U. urealyticum* as an etiological agent of nongonococcal urethritis has been resolved by the recent publication of the results of a human experiment in which two volunteers were inoculated with *U. urealyticum* and both developed urethritis (29).

An explanation for these differences of opinion can be related to the specimens used for isolation, the methods used for transport and storage, and media used for primary isolation of ureaplasmas. Many strains exist which do not grow on the media routinely used for their isolation in some laboratories. If a high percentage of fastidious strains fails to be isolated, conclusions reached on the basis of strains actually isolated are not accurate.

The ultimate criterion for ureaplasma and mycoplasma identification is characteristic growth on agar, acceptance of the Dienes stain (15) by the mycoplasmas, and, in the case of the ureaplasmas, demonstration of urease.

MATERIALS AND METHODS

The clinical specimens reported on were obtained during January through December 1976 from patients sent to the Peter Bent Brigham Hospital's Surgical Bacteriology Laboratory by local gynecologists and obstetricians because of reproductive failure or genitourinary tract infection. The cervical swab of the female was taken by the physician in the office and brought to the laboratory by the patient. The urethral swab of the male patient was taken by the patient himself, using a Calgiswab, in our laboratory. Urine specimens of both males and females were obtained in the laboratory and were processed within 30 min.

The urine was freshly voided with no prior preparation of the perineum. The first part of the stream was optimal since it contained the urethral washings. A moist cervical, urethral, or vaginal swab with no transport medium was taken to the laboratory within the hour for culture. If specimens were sent distances, they were shipped frozen in dry ice. Storage at −70°C was preferable, but even at this low temperature, losses in titer occurred after freezing.

All swabs were carefully rolled on the agar medium and then dropped into broth. The change of pH in broth after incubation suggested the presence of ureaplasmas and was used as a signal indicating the possible presence of ureaplasmas. Agar blocks were studied for actual visualization of colonies before any culture was considered positive.

The broth used was Ford's pH 6.0 (6), or Shepard's M10, also pH 6.0 (22). Both broths supported the growth of more fastidious ureaplasmas. The previous U9 broth (23) in our hands did not support all strains, and in those instances the broth was unchanged while

ureaplasma colonies were visualized on agar.

The agar medium originally used in our earlier studies for isolating ureaplasmas and mycoplasmas was Dienes soft horse serum agar (15). The A7 differential agar medium of Shepard and Lunceford, pH 6.0 (24), is now used. This medium, which supports growth of more fastidious strains, was used in this study.

All broth and agar media were refrigerated at 4°C and used within the month. Due to the volume of work, media have never lasted longer than a month. In-use quality control testing has shown the media to be satisfactory for this period of time.

Methods of incubation. The broth was incubated by placing the inoculated tube into a 36°C incubator. Ureaplasma growth characteristically showed a pH change starting at the bottom of the tube, with the change in color gradually rising to the top. The best time for subculture was when the medium was just starting to change at the bottom of the tube. When the color change was complete to the top of the tube, ureaplasmas were no longer viable and could not be subcultured.

Agar plates could be placed in a GasPak jar for incubation, using the generator package producing H_2 + CO_2. Agar plates in this study were set up for Fortner (J. Fortner, Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. **108**:155, 1928) incubation. The Fortner method has been shown to support the growth of fastidious anaerobes (16). This method consisted of placing a triangle of a nutrient medium containing dextrose cut from a poured agar plate so that it adhered to the cover of the ureaplasma culture plate. Blood agar base agar supplemented with 1% dextrose was satisfactory. The agar triangle was then streaked with *Serratia marcescens*. The petri plate was then put together; the cover with the adherent *S. marcescens*-streaked agar was placed over the base of the plate which had been inoculated with the specimen. Paraffin softened by the addition of white petrolatum was pipetted into the space between the cover and bottom of the petri dish, using a Pasteur pipette with a bulb. The paraffin solidified rapidly, and the plate was put into the 36°C incubator in the customary inverted position for incubation.

Plates could be either scanned unopened under low power or opened with a forceps. Agar blocks, approximately 1 cm^2, were cut with a flamed surgical scalpel and placed on glass slides, colony side uppermost. Dienes-stained (15) cover slips were superimposed on the agar block, and the block was completely scanned, using low power (×160) first and then oil immersion (×900). The micrometer in the eyepiece was calibrated for use with a Zeiss microscope to measure the diameters of the colonies at 72-h incubation.

Water used in the preparation of media was single distilled daily, using a Barnstead still. It was tested monthly for chemical properties, pH, conductivity, and microorganisms per milliliter. Toxicity for microorganisms was also tested. These quality control procedures are done to comply with the requirements of the Commonwealth of Massachusetts Department of Environmental Quality Engineering.

RESULTS

The most common problems in the isolation of ureaplasmas from clinical specimens involve types of specimens, method of collection, transport and/or storage of specimens, media used, conditions of incubation, and experience in identifying isolates from clinical material.

Types of specimens. An analysis of data from patient cultures for 1976 are shown in Tables 1 and 2. Only data from patients having both a urine and genital culture were used. Totally negative primary cultures and cultures after antibiotic treatment were not included. Only one set of cultures per patient was included. Of the 659 women and 66 men whose cultures were tabulated, one of the four media used was positive. A broth and an agar were inoculated simultaneously for each specimen. The most commonly positive specimen was the urine in both men and women. In women, the urine sediment resulted in 9% more isolations than the cervix. In men, the urine sediment resulted in 13% more isolations than the urethra. Agreement between urine and cervix was 88% in females, and agreement between urine and urethra was 86% in males.

Because mycoplasmas and ureaplasmas are found intimately associated with and adherent to cells, it is better to spin down body fluids such as urine, spinal fluid, and amniotic fluid and use the sediments for culture. Some researchers have stated that since centrifugation of the urine before culture is "a laborious and time-consuming procedure," this was eliminated and genital cultures were used alone (14). In our experience, centrifuged urine sediments yield more positive cultures than the cervix in women and more positive cultures than the urethra in men. Table 2 shows culture results over a 1-year period. Thus, an overall 9% of mycoplasma cultures would be considered negative if centrifuged ur-

TABLE 1. *Comparison of isolation of U. urealyticum*

Patients	Urine[a]	Cervix	Urethra	Total
Females	485 (74)	428 (65)		659
Males	43 (65)		34 (52)	66
All	528 (73)[b]	462 (64)		725

[a] Numbers in parentheses are percentages.
[b] $\chi^2 = 13.8$; $P = 0.0002$.

TABLE 2. *Comparison of isolation of U. urealyticum from urine and cervix (females) and urine and urethra (males)*

Correlation	659 Females[a]	66 Males
Agreement		
Positive or negative	582 (88)	57 (86)
Nonagreement		
Urine positive, cervix/urethra negative	67 (10)	9 (14)
Urine negative, cervix/urethra positive	10 (2)	0 (0)

[a] Numbers in parentheses are percentages.

ine cultures were not done in conjunction with genital cultures.

Conditions of incubation. Table 3 shows the colony size of six strains of ureaplasmas grown under different conditions of incubation. The laboratory strain, Boston T, did not grow under aerobic conditions or in the candle jar. Colonies were very small, somewhat larger on the Fortner plate than in the GasPak. Other laboratory strains did grow under all tested conditions of incubation as did the two strains on primary isolation. Size of colonies differed with strains, indicating that some strains could be easily missed since colonies were small (16 to 18 μm) and, in the case of one primary isolate, extremely small under all conditions of growth.

Freezing. Twelve fresh urine sediments ranging in titer from 10^1 to 10^4 color-changing units were frozen and stored at −70°C. All strains showed a drop in titer. One strain dropped from 10^4 to 10^0 after 3 days in the freezer. Strains varied in their survival independent of original titer, and survival could not be predicted. Clearly, fresh urines should be used for isolations. Most researchers freeze urine specimens before culture, which could lead to many false negatives among infected patients harboring low numbers of ureaplasmas.

Media. Boston T strain, a laboratory strain originally isolated from the placenta of a midtrimester spontaneous abortion, did not grow on New York City (NYC) medium (5) or in U9 broth. It did grow on differential A7 medium, Dienes soft horse agar, Ford's broth, and M10.

A comparison NYC medium with A7 was done by using a patient's urine sediment as the inoculum. Both plates were incubated in a candle jar for 4 days at 37°C. NYC medium yielded ureaplasma colonies which measured 21 ± 6 μm, whereas A7 yielded colonies measuring 35 ± 10 μm, a difference of 14 μm ($P < 0.001$). These measurements were made on the NYC medium by using a direct stain consisting of 10% urea followed by 0.8% manganese sulfate (21). Without the use of this stain, colonies on NYC medium were difficult to visualize and measure and could easily be missed.

Ureaplasmas did not increase the turbidity of broth with increasing titers. Broth cultures were clear, with no developed sediment at the time pH changes were noticed. Changes at the bottom of the tube were seen within a few hours if the specimen had a high titer of ureaplasmas. The change in pH could not, however, be considered as absolute evidence of the presence of ureaplasmas. One must always suspect that the broth used may not support the growth of fastidious strains of ureaplasmas. False positives as well as false negatives do occur. Shepard's earlier broth, U9, in our hands did not support the growth of the Boston T strain isolated from the placenta of a spontaneous midtrimester abortion. The newer M10 broth does support its growth. Alkaline pH changes did occur in broth because of the presence of penicillin-resistant, urease-positive microorganisms, and the broth was found to contain *Rhodotorula* or *Proteus* species. When other microorganisms were present, the broth became cloudy and a sediment could usually be seen in the bottom of the tube. This was not true when ureaplasmas alone had changed the medium; then the broth was clear and there was no sediment. It is also conceivable that bacteria as well as ureaplasmas may be present, and in that case the broth may be cloudy and yet have a characteristic pH change. The agar plate must then be carefully examined for ureaplasma colonies.

Agar plates can also be suspected of not supporting a particular ureaplasma isolate when broth changes occur but no colonies are seen. There may be too few ureaplasmas to visualize on the agar plate but enough in the specimen inoculated with broth to multiply and alter the pH. A repeat specimen can be requested from the patient. The other alternative is to attempt subculture of the broth before the color change is complete in the hope of securing a successful agar subculture with colonies confirming the presence of ureaplasmas.

Negative broth cultures have been observed with many colonies on the agar plate inoculated simultaneously. The converse also has occurred where a positive broth and negative agar plate

TABLE 3. *Average[a] colony size in μm of U. urealyticum under different conditions of incubation*

Medium	Method of incubation	Avg colony size (μm)					
		Laboratory strain				Primary isolation	
		Boston T	Ault	Royston	Brown	Bair	Culver
A7	Aerobic	NG[b]	137	59	180	67	18
A7	CO_2	NG	149	NG	210	49	18
A7	Fortner	18	148	121	165	52	21
A7	GasPak	16	152	130	186	48	30

[a] Average of 10 to 20 colonies.
[b] NG, No growth.

result from simultaneous inoculation of the same specimen. Fortunately, agreement does usually occur, and in those cases the presence of ureaplasma is readily confirmed.

When U9, as originally formulated without L-cystein-HCl supplement, and M10 broth media were inoculated simultaneously, approximately 10% of the ureaplasmas did not grow in U9 but did grow in M10. Consequently, 10% of isolates would have been missed by using one particular broth alone as an indication of the presence of ureaplasmas.

Morphology on primary isolation. A source of difficulty in mycoplasma and ureaplasma cultures is that clinical isolates do not look like textbook mycoplasmas. Uniform, circular fried eggs on agar are more characteristic of laboratory-cultivated strains than of actual isolates in the diagnostic laboratory. Figure 1 shows the standard textbook appearance of mycoplasmas and ureaplasmas under low power (× 160). Figures 2, 3, and 4 show *Mycoplasma hominis* and *U. urealyticum* on primary isolation from a placenta, a cervix, and an oropharyngeal aspirate from a newborn.

DISCUSSION

Many strains of ureaplasmas have fastidious growth requirements. Hayflick and Stanbridge estimate that 70% of all mycoplasmas cannot be

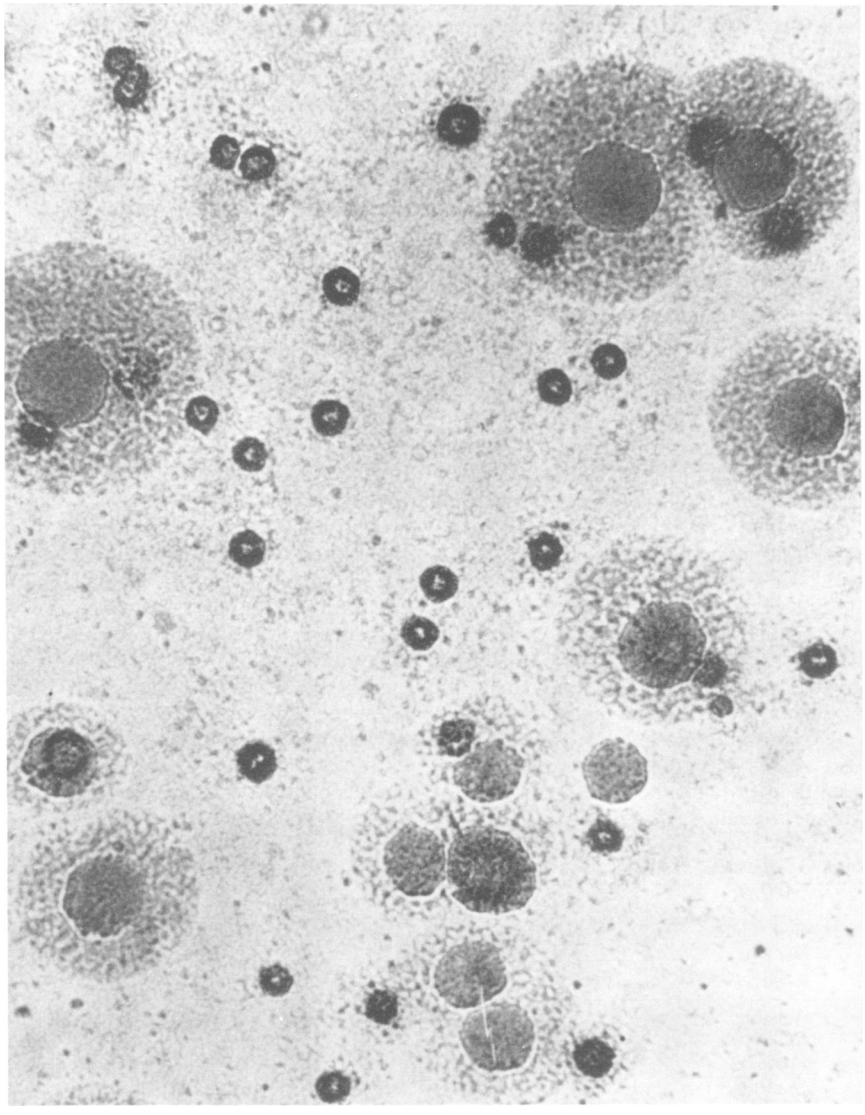

FIG. 1. *M. hominis and U. urealyticum on A7 agar (×160) (Courtesy of M. C. Shepard).*

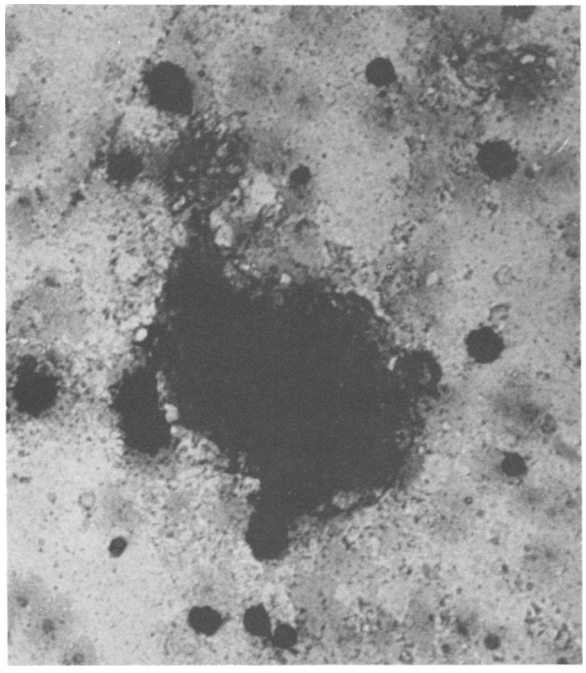

FIG. 2. *M. hominis and U. urealyticum from oropharyngeal aspirate of a newborn (×160).*

subcultured (10). Relying, therefore, on subculture for identification eliminates a large number of fastidious strains from the study and leaves the investigator with only the readily growing strains. The error introduced by such techniques is obvious. If 10% of strains are not isolated, a 10% error is introduced when doing test and control group investigation. It is possible that the test group is predominantly infected with the fastidious strains, thus grossly skewing the conclusions reached.

Specimens routinely done for genitourinary tract infections are urine and cervix in women and urine and urethra in men. Nasopharyngeal swabs on a wire holder (Calgiswab) are used for the male urethra. Shepard has found scrapings taken with a wire loop to give the most satisfactory male specimens. The logistics of taking such a culture in a busy microbiology laboratory are insurmountable. In the laboratory, the male patient is briefly instructed, handed the sterile nasopharyngeal swab in a paper packet, a sterile test tube to receive the swab, and a urine container, and asked to produce a urine specimen after taking the urethral swab himself.

Cultures of the cervical os are taken by the patient's gynecologist in the office. The tenacity of the cervical mucus at different stages of the menstrual cycle may result in poor specimens. Vaginal cultures could probably be substituted or done in addition to cultures of the cervix and urine. Also to be considered is the possibility that endometrium or Fallopian tube specimens may be the best specimens to study and that, in fact, cultures of urine, cervix, and vagina are but poor approximations of the true site of colonization and infection.

Transport media are not recommended for use with specimens brought to the laboratory. Transport media designed specifically for mycoplasmas and ureaplasmas contain constituents inhibitory to other microorganisms. Thus, a high penicillin level would prevent recovery of gonococci and other penicillin-susceptible microorganisms. Because our specimens are frequently studied for the isolation of a wide range of microorganisms including chlamydia, the effect of constituents of the transport medium upon their recovery is not known. Another factor to consider is that metabolites essential for mycoplasma growth and carried over from their natural environment may be diluted out by the transport medium. A comparison of transport medium (Culturette) with a plain swab for ureaplasma isolation definitively shows 1 h to be the maximum holding time for specimens at room temperature (S. Poulin and R. B. Kundsin, manuscript in preparation).

The NYC medium originally devised for the isolation of gonococci was shown to also support the growth of some, but not all, ureaplasma strains. Thayer-Martin (30) medium in our

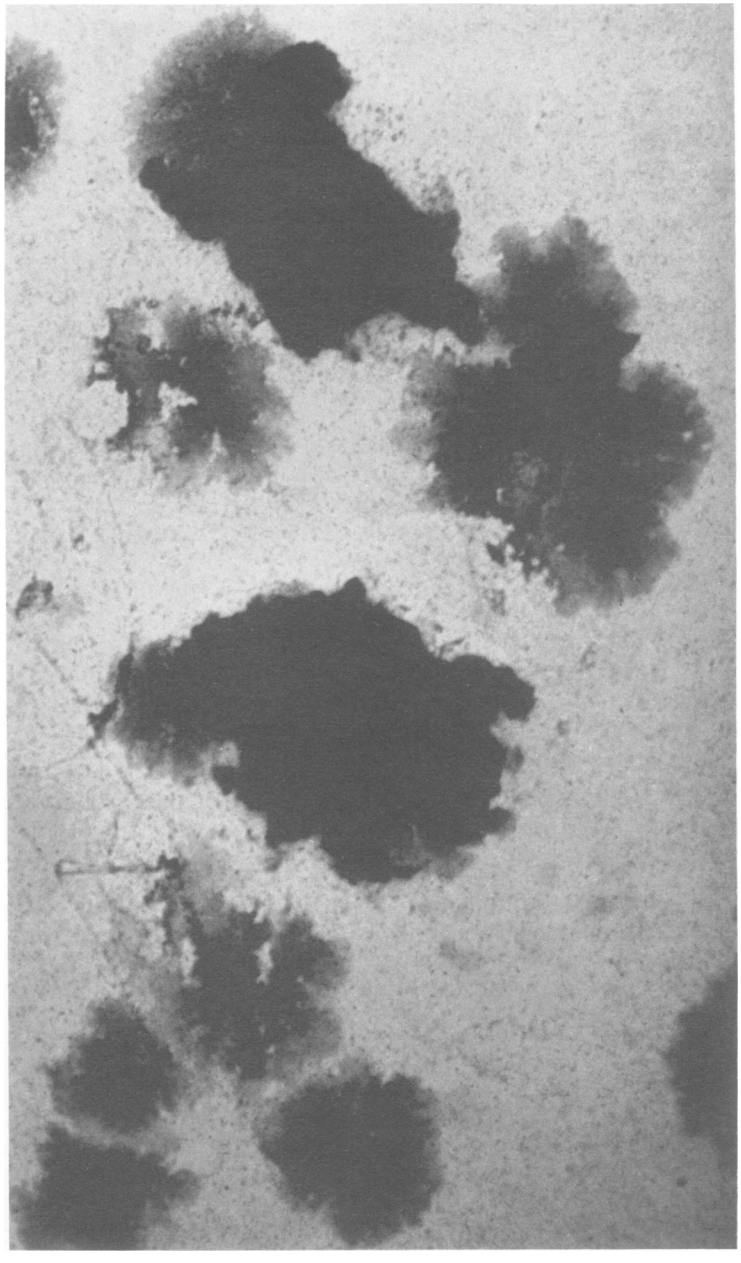

FIG. 3. *M. hominis and U. urealyticum from placenta (×160).*

hands also supports the growth of some strains of ureaplasmas; subcultures can also be made successfully from Thayer-Martin. But most important, the investigator needs assurance that fastidious strains can be isolated and identified. It is also probable that presently noncultivable ureaplasmas exist. Media and techniques of incubation may have to be changed in the future when the requirements of more fastidious strains are known. We are currently relying on metabolites brought over from their natural environment to initiate growth on primary isolation, and subcultures may not be successful for characterization of strains.

Robertson (18) has described a bromothymol blue broth in which ureaplasmas grow to attain higher titers than in other previously used broths, and after a color change, ureaplasmas

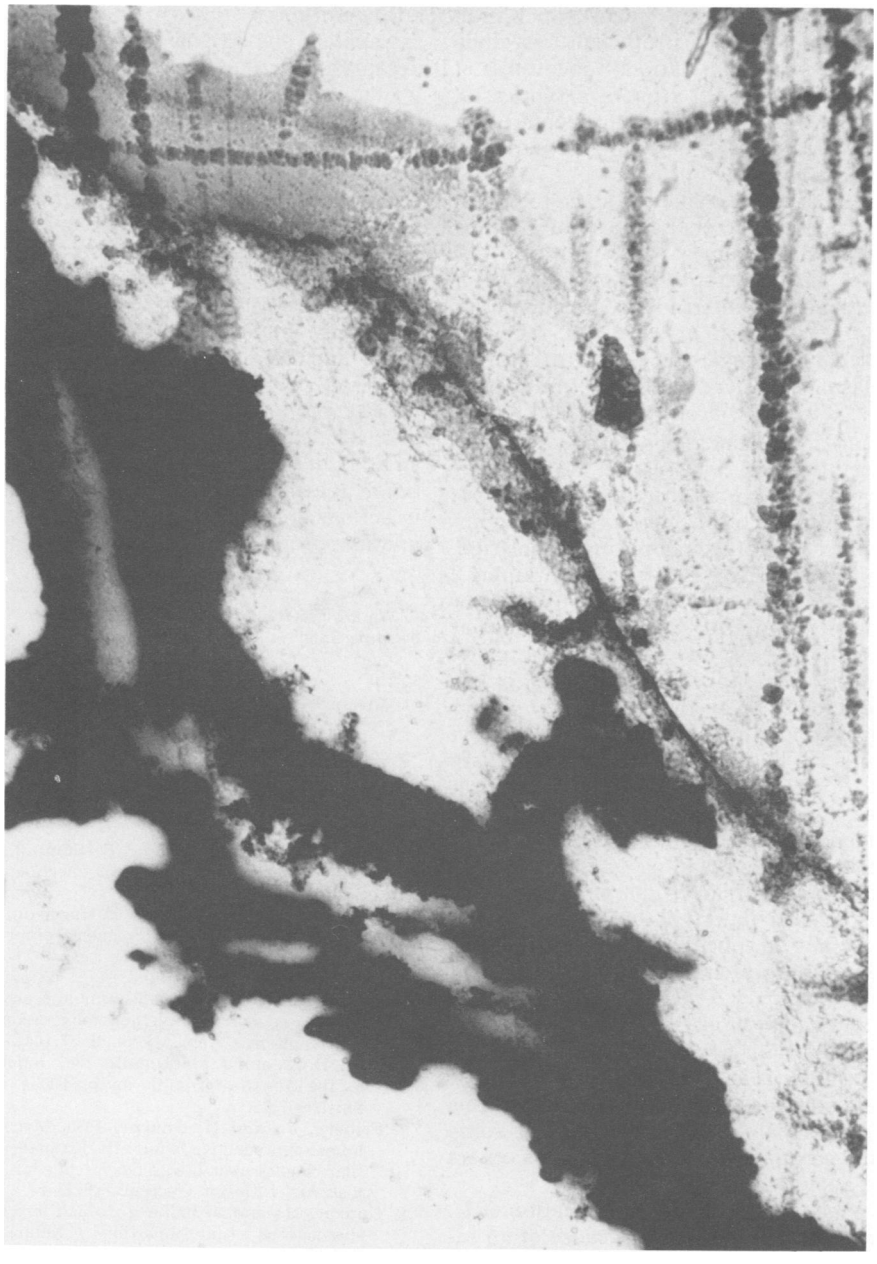

FIG. 4. *M. hominis and U. urealyticum in streaks of mucus from cervix (×160).*

remain viable for longer periods. A higher isolation rate of ureaplasmas from clinical specimens was also reported. Of 70 strains isolated in bromothymol blue broth, 7 (10%) could not be isolated in U9 broth. Bromothymol blue broth may turn out to be superior to broths currently used, but further work needs to be done.

Harrison et al. (9) in a study of doxycycline treatment and infertility, concluded that mycoplasmas were not associated with primary infertility. Their specimens were frozen at −20°C and then inoculated into indicator broth for subculture. These techniques would result in a substantial number of negative cultures from specimens which are, in fact, positive for ureaplasmas. Freezing results in a 2-logarithm loss. Ur-

ines were not utilized as specimens, which is an additional 9 to 13% loss. Broth cultures alone were used for primary isolation. Subcultures of positive broths would not grow in 70% of cases. Consequently, their data represent technical errors, with substantial loss of isolates invalidating their conclusions.

DeLouvois et al. (4) make the same technical errors as Harrison et al. because they report using the same techniques.

Shoub et al. (20), reporting on cultures of seminal fluid from infertile males, state that specimens were placed in U9 broth, then observed for color change, and subcultured at 48 h. Ureaplasmas are dead and cannot be subcultured at 48 h from U9 broth.

Braun et al. (1), in a paper discussing the prevalence of genital mycoplasma in pregnancy and their relationship to prematurity and postpartum fever, used broth media containing lincomycin and erythromycin for primary isolation. Subcultures were made, and a disk containing 2 or 15 μg of erythromycin for *U. urealyticum* or 2 μg of lincomycin for *M. hominis* was placed at the end of the streak on the subculture plate. Identification of ureaplasmas was based on inhibition of growth by erythromycin. *M. hominis* was identified by growth inhibition by lincomycin. Strains of both microorganisms that are resistant to these antibiotics at the concentrations used are known (27), and therefore their use for identification is not valid. Growth not inhibited by erythromycin could have been *U. urealyticum* as well as *M. hominis*. The same investigators also reported that 10% of specimens could not be subcultured for ureaplasmas from urea broths which did exhibit a pH rise of 0.8 units or more.

Bredt and Bink (2) observed that some media such as differential agar with manganese sulfate resulted in more isolations than media without manganese sulfate. They also found that strains differed in their ability to grow in broth: some preferred a serum-rich medium, whereas others preferred a low-serum broth.

Razin et al. (17) recommend incubation of *U. urealyticum* under 100% CO_2 because of an increase in the size of most colonies of the one laboratory strain they tested. They suggest that incubation in 100% CO_2 would also facilitate isolation and identification of ureaplasmas from clinical material. Our results (Table 3) indicate that of five strains tested (three laboratory strains and two strains isolated from urine sediment), two strains did not grow under 100% CO_2, and of the remaining three that did grow only one strain averaged a larger colony size under CO_2 than under other conditions of incubation. Different ureaplasmas apparently prefer different conditions of growth, and it is not valid to extrapolate one strain's preferences to all other ureaplasmas.

Stalheim et al. (28), using ureaplasmas from human and bovine sources, was able to demonstrate cilia-stopping activity and histological lesions in organ cultures of the bovine oviduct. These investigators commented that other researchers found no effect of ureaplasmas in bovine organ cultures, probably because of the use of inappropriate methods.

At the present state of the art, all ureaplasma isolations should be done simultaneously in broth and on Shepard's differential agar medium, A7, containing manganese sulfate. Mycoplasma and ureaplasma cultures require highly specialized techniques, and such isolations should not be casually assumed. It is even more unfortunate when conclusions regarding patient infections are reached on the basis of insensitive and inappropriate techniques.

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