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Antimicrobial Susceptibility of *Ureaplasma urealyticum*

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An antimicrobial susceptibility test, a two-tube broth dilution and disk elution method for *Ureaplasma urealyticum*, was modified to incorporate some of the standard procedures followed in traditional antimicrobial testing. The susceptibility pattern of the species was reevaluated by determining the effect of various antimicrobial agents on 21 vaginal isolates. All isolates were inhibited by tetracycline congeners (1 to 6 μg/ml) and killed by methenamine mandelate (0.6 mg/ml). All but one isolate were inhibited by erythromycin (0.4 to 3 μg/ml). Only eight isolates were inhibited by nalidixic acid (1 to 6 μg/ml), and seven were inhibited by nitrofurantoin (20 to 60 μg/ml), whereas all isolates were resistant to rifampin (1 μg/ml) and trimethoprim-sulfamethoxazole (5 μg/ml). The in vitro technique described can readily be performed on individual patient isolates before the initiation of antimicrobial therapy.

During the last decade, *Ureaplasma urealyticum* was evaluated extensively as a possible pathogen of humans (2, 6, 16-19). The questionable pathogenicity of the organism caused a demand for cultivation and susceptibility testing of the species in clinical microbiology. Successful laboratory cultivation and antibiotic susceptibility testing of *U. urealyticum* have been greatly aided with the introduction of appropriate media (9, 12, 13) and careful attention to isolation and culture techniques (8, 9, 12, 13). The susceptibility tests, however, have been performed by using diverse methodologies resulting in interpretations and conclusions which have been difficult to compare (1, 3, 5-7, 15-17).

The initial objective of the present study was to modify the antimicrobial susceptibility test reported by Spaepen and Kundsin (16) and to incorporate some of the standard procedures followed in traditional antimicrobial susceptibility testing, that is, use of pure cultures of test organisms, standardization of the inoculum size, use of culture medium free of antibiotics, and more sensitive observation of results within 24 h of inoculation. Once the technique was standardized, there developed a second objective, which was to reevaluate the susceptibility patterns previously reported for *U. urealyticum*.

The susceptibility of 21 clinical isolates was determined to the natural and semisynthetic tetracyclines, erythromycin, and other antimicrobial agents, particularly those used in the treatment of common urinary tract infections.

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MATERIALS AND METHODS

Specimens. Vaginal swabs taken during routine physical examinations by private gynecologists were inoculated immediately to modified agar medium A7 (MA7; see below) and incubated in either 5% CO_2 or a candle jar at 37°C. None of the patients had a history of tetracycline treatment failure for genitourinary tract infection.

MA7 was prepared by dissolving 24 g of Trypticase soy broth (111768; BBL Microbiology Systems, Cockeysville, Md.) and 0.15 g of manganous sulfate monohydrate in 825 ml of distilled water and adjusting to pH 5.5 with 1 N HCl. Eleven grams of agar (49-180-03; Inolex, Glenwood, Ill.) was added, and the mixture was autoclaved at 121°C for 15 min. After cooling to 50°C, the following were added: horse serum (4-012; Flow Laboratories, Rockville, Md.), 200 ml; 100× MEM (Modified) Vitamins (6-224D; Flow Laboratories), 5 ml; yeast extract (30-003-49; Flow Laboratories), 10 ml; 10% urea, 10 ml; 4% L-cysteine, 2.5 ml; penicillin G, 1,000,000 U; and 0.1% amphotericin B (Fungizone), 1.5 ml.

Isolation. Plates with *U. urealyticum* growth, as observed under ×100 magnification, were subcultured by removing a 1.0- to 1.5-cm^2 rectangle of agar containing colonies and placing it into 2 ml of modified broth medium A7 (BA7).

BA7, an antibiotic-free medium, was prepared by dissolving 24 g of Trypticase soy broth in 940 ml of distilled water, adjusting the pH to 5.5 with 1 N HCl, and autoclaving at 121°C for 15 min. After cooling, the following were added: horse serum, 100 ml; 4% L-cysteine, 2.5 ml; 10% urea, 10 ml; and 1% phenol red (Na^+), 1.0 ml. At the first indication of *U. urealyticum* growth (18 to 24 h), as evidenced by a color change from yellow to orange (pH 6.8), positive broths were

subcultured to MA7 by utilizing the Fortner technique for 24- to 48-h incubation (8). Organisms were stored at −70°C until used in antimicrobial susceptibility studies. Isolates remained viable at this temperature for at least 3 months. An isolation technique similar to this has recently been described (14).

Method for testing antimicrobial susceptibility. Modifications of the broth dilution technique of Schneierson and Amsterdam (10) and the disk elution technique of Spaepen and Kundsin (16) were used to determine the antimicrobial susceptibilities of the *U. urealyticum* isolates.

Stored isolates were thawed at room temperature, and a rectangular piece of agar was cut out, placed into 2 ml of BA7, and incubated for 18 to 24 h. Positive broths (pure cultures) were then shaken by using a Vortex mixer, 0.15 ml was inoculated into 5 ml of BA7, and the subculture was incubated for 18 to 24 h. The percent transmittance at 580 nm was then measured. Growth was detected by a decrease in transmittance compared with that of an uninoculated tube of BA7. A 0.1-ml inoculum containing 10^4 to 10^5 colony-forming units of *U. urealyticum*, presumably in log phase, was added to 5-ml volumes of BA7 containing different antimicrobial agents. These mixtures were shaken and incubated at 37°C. Growth controls and uninoculated medium controls containing antimicrobial agents were simultaneously incubated. Each of these controls plus selected test cultures was subcultured to MA7 daily for 5 days to ascertain the viability of the organisms.

Immediately before inoculation of the medium, antimicrobial agents were added to BA7 by placing prepared disks (BBL) into the tubes of medium. The antibiotics and final concentrations tested are shown in Table 1. The one exception was minocycline (Lederle Laboratories, Pearl River, N.Y.), which was prepared in aqueous solution and added in a 0.1-ml volume containing either 5 or 30 μg of the drug. Control tests of disk content were performed by using the appropriate antibiotic disks, Mueller-Hinton agar medium, and cultures of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. None of the antimicrobial agents caused significant color change of BA7 during elution in uninoculated medium.

TABLE 1. *Susceptibility of 21 clinical isolates of U. urealyticum to tetracycline congeners, and other antimicrobial agents used to treat common genitourinary tract infections*[a]

Antibiotic	Concn (μg/ml of medium)	No. of susceptible isolates
Chlortetracycline	1.0	21
Demeclocycline	1.0	21
Oxytetracycline	1.0	19
	6.0	2
Tetracycline	1.0	21
Doxycycline	1.0	21
Methacycline	1.0	21
Minocycline	1.0	21
Erythromycin	0.4	2
	3.0	18
Methenamine mandelate	600	21
Nalidixic acid	1.0	1
	6.0	7
Nitrofurantoin	20	4
	60	3
Rifampin	1.0	0
Trimethoprim-sulfamethoxazole	5.0	0

[a] An isolate was defined as susceptible if when cultured for 24 h in BA7 with the antimicrobial agent it did not have a transmittance (580 nm) within 2 positive standard deviations from the mean transmittance of 21 growth controls.

After 24 h of incubation of *U. urealyticum* in the presence of antimicrobial agents, transmittance at 580 nm was measured. Antimicrobial activity was determined by comparing the change in the transmittance in BA7 growth controls with changes occurring in inoculated BA7 containing antimicrobial agents. Since the change in transmittance of growth controls was so consistent after 24 h of incubation, interpretation of susceptibility was based on the mean transmittance of the growth controls for the 21 different isolates (see Fig. 1). After 24-h readings, a *U. urealyticum* isolate was considered susceptible to a specific concentration of antimicrobial agent if the transmittance of the culture with that antimicrobial agent was above 2 positive standard deviations from the mean of the growth controls.

When evaluating the tetracyclines, erythromycin, nalidixic acid, and nitrofurantoin, organisms susceptible to the lower concentration tested (Table 1) were regarded as very susceptible, whereas organisms susceptible to only the higher concentration were regarded as fairly susceptible. Ureaplasmas not sensitive to the higher concentration were categorized as resistant to the antimicrobial agent. For rifampin, methenamine mandelate, and trimethoprim-sulfamethoxazole those organisms susceptible to the single concentration tested (Table 1) were regarded as susceptible to that antimicrobial agent.

Stability of antimicrobial agents in BA7. Since there existed a potential for the deterioration of antimicrobial agents in the BA7 test system, the breakdown of these substances was assessed by assaying for the antimicrobial agent that was eluted from commercially prepared disks placed in small volumes of BA7 at 37°C for 1 to 5 days. Samples (25 ml) of eluted antimicrobial agent in BA7 were added to sterile filter paper disks on plates of Mueller-Hinton agar medium seeded with a common standardized suspension of *S. aureus* ATCC 25923 of *E. coli* ATCC 25922. The calculated potency of the new disks, assuming no antimicrobial agent breakdown during the incubation period, is shown in Table 2.

Plates were prepared in triplicate and incubated overnight, and the zones of inhibition were measured with a micrometer. A decrease in the average zone diameter, which occurred with increasing incubation time of the antimicrobial agent in BA7, was accepted as an indication of antimicrobial agent degradation.

RESULTS

Isolation. Isolated ureaplasmas were easily subcultured, provided this was done when an indication of growth first appeared. *U. urealyticum* growth is characterized by golden-brown colonies on MA7 (13) and as a gradual color

TABLE 2. *Zone of inhibition diameters obtained with disks prepared with antimicrobial agents eluted from commercial disks in sterile BA7 at 37°C*[a]

Antibiotic	Concn (μg/disk)[b]	Mean zone diameters (mm)[c] for elution time of:			
		2 h	24 h	48 h	120 h
Chlortetracycline	4.6	19.7 ± 0.3	17.3 ± 0.5	15.0 ± 0.4	12.6 ± 0.8
Demeclocycline	4.6	21.7 ± 0.4	21.6 ± 0.7	21.0 ± 0.5	19.7 ± 0.6
Oxytetracycline	4.6	18.7 ± 0.6	18.4 ± 0.1	17.7 ± 0.7	13.1 ± 0.2
Tetracycline	4.6	20.0 ± 0.3	19.6 ± 0.4	19.0 ± 0.5	18.2 ± 0.7
Doxycycline	4.6	22.8 ± 0.7	22.7 ± 0.3	22.9 ± 0.7	20.4 ± 0.8
Methacycline	4.6	21.9 ± 0.4	21.7 ± 0.8	19.6 ± 0.1	15.4 ± 0.1
Minocycline	3.8	21.7 ± 0.5	21.5 ± 0.8	21.3 ± 0.9	19.6 ± 0.9
Erythromycin	2.3	19.2 ± 0.6	18.6 ± 0.4	17.5 ± 0.3	16.3 ± 0.2
Methenamine mandelate	500	NZ	NZ	NZ	NZ
Nalidixic acid	4.6	15.7 ± 0.3	14.2 ± 0.7	14.2 ± 0.1	14.8 ± 0.5
Nitrofurantoin	46.0	NZ	NZ	NZ	NZ
Rifampin	0.8	25.6 ± 0.3	24.7 ± 0.3	23.4 ± 0.7	23.6 ± 0.8
Trimethoprim-sulfamethoxazole	3.8	19.7 ± 0.4	21.7 ± 1.1	20.6 ± 0.4	19.1 ± 0.6
Control, sterile BA7 (25 μl)		NZ	NZ	NZ	NZ

[a] *S. aureus* ATCC 25923 was the test organism for all substances except nalidixic acid, which was tested against *E. coli* ATCC 25922.

[b] Approximate concentration, assuming no antimicrobial agent breakdown during the elution period.

[c] Zone diameters given ± standard deviation. NZ, Unmeasurable or no zone.

change from yellow to orange-red in BA7. In an attempt to determine whether the uniformity of growth had a consistent influence on the indicator system, the color change of BA7 was measured as a change in transmittance at 580 nm. From the growth controls from 21 clinical isolates in pure culture, 0.1-ml samples of 24-h cultures with 65 to 70% transmittance were used to inoculate 5 ml of freshly prepared BA7. Whereas uninoculated BA7 remained unchanged, the transmittance of inoculated BA7 routinely decreased to 66% (standard deviation, ±4%) in 24 h. In Fig. 1 the mean transmittance obtained with the growth controls during a 5-day period is illustrated. Transferring 0.1 ml after 24 h of incubation consistently resulted in a positive subculture in 18 to 24 h. After 48 h, the transmittance decreased to 58% (standard deviation, ±10%), but subculturing at this time or later rarely resulted in growth.

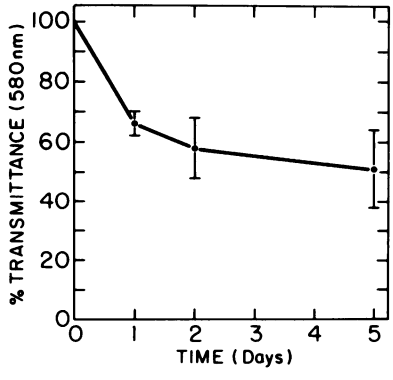

FIG. 1. *Transmittance (580 nm) of BA7 medium during 5 days of incubation at 37°C. BA7 was inoculated with 10^4 to 10^5 colony-forming units of U. urealyticum. Each point and vertical bar represents the mean ± standard deviation for 21 different isolates.*

Stability of antimicrobial agents in BA7. As shown in Table 2, most, but not all, of the antimicrobial agents gave zones of inhibition of decreased size with increased elution time, suggesting breakdown of some of the substances. Since little change in zone size was apparent after 24 h of incubation, the antimicrobial susceptibility tests were interpreted after 18 to 24 h of incubation. The amount of antimicrobial agent remaining after this time appears questionable, and continued measurement of transmittance may be an unreliable indication of antimicrobial susceptibility.

Antimicrobial susceptibility patterns of *U. urealyticum*. From the data in Table 1, it appears that after 24 h of incubation all *U. urealyticum* isolates tested were very susceptible to tetracycline, chlortetracycline, demeclocycline, doxycycline, methacycline, and minocycline. Two isolates (9.5%) were only fairly susceptible to oxytetracycline. All of the isolates had extended viability in the presence of tetracycline at 1 μg/ml, for at any time during 5 days incubation the ureaplasmas grown in the presence of these antibiotics could be subcultured, provided the transmittance had not decreased to 65% or below. The viability of organisms in cultures containing tetracyclines at 6 μg/ml was usually prolonged for only 2 to 3 days, in the

absence of a color change.

The anti-ureaplasmal effectiveness of other antimicrobial agents used to treat common urinary tract pathogens is also shown in Table 1. The one isolate (4.8%) resistant to erythromycin was very susceptible to each tetracycline tested. Of the two isolates which were only fairly susceptible to oxytetracycline, both were susceptible to erythromycin. Of the 14 isolates which were resistant to nitrofurantoin, 11 were also resistant to nalidixic acid, whereas of 13 resistant to nalidixic acid, 11 were also resistant to nitrofurantoin. The one isolate resistant to erythromycin was very susceptible to both nalidixic acid and nitrofurantoin. As with the growth controls, ureaplasmas cultured in the presence of these antimicrobial agents remained viable and could be subcultured until the transmittance decreased to approximately 65%.

It is interesting that another urinary tract antiseptic, methenamine mandelate, proved to be a very effective ureaplasmacidal agent, as all isolates were sensitive to 0.6 mg/ml in this in vitro system (Table 1). Methenamine mandelate was the only antimicrobial agent to alter the pH of the culture medium in the absence of organisms. The pH of the medium decreased slightly to approximately pH 5, presumably due to the mandelic acid portion of this agent.

Rifampin and trimethoprim-sulfamethoxazole were ineffective against the ureaplasmas tested (Table 1). When subcultures were made of ureaplasmas grown in the presence of these antimicrobial agents, the extent of growth resembled that of the growth controls.

DISCUSSION

The special enriched media required for the growth and identification of *U. urealyticum* (8, 9, 12), plus the cultural characteristics of the organism (13), make it difficult to use the traditional paper disk-agar diffusion method of antimicrobial susceptibility testing with the species. Antimicrobial testing has been performed, however, by using broth media and antibiotic eluted from commercial disks (16). The testing procedure presently reported further standardizes the above technique by using pure cultures of test organisms, standardizing inoculum size, using antibiotic-free culture medium, and permitting more sensitive observation of results within 24 h of inoculation. Standardization similar to that found in traditional bacteriological testing was incorporated to reduce the possibility of error in interpretation of results.

The test system was constructed to provide for the elution of antimicrobial agents from commercially prepared disks placed in 5 ml of BA7 (initially antibiotic free) which had just been inoculated with 10^4 to 10^5 colony-forming units of *U. urealyticum* in pure culture. Basically, the system was used as a two-tube dilution procedure employing a high and low concentration of antimicrobial agent.

When interpreting antimicrobial susceptibility tests with *U. urealyticum* one must consider the growth kinetics of the microorganism as well as the in vitro stability of the antimicrobial agents in the system. Since the reported technique resulted in substantial growth of *U. urealyticum* at 24 h, the endpoint for judging susceptibility was recorded at that time to preclude significant antibiotic decay.

To provide a high confidence limit to the interpretation of susceptibility, an organism was considered susceptible to an antimicrobial agent if after 24 h of incubation in the presence of the antimicrobial agent, the transmittance measured was not within 2 standard deviations of the mean transmittance observed with the 21 growth controls.

Each of the *U. urealyticum* isolates examined was considered susceptible to the tetracyclines (1 to 6 μg/ml) tested (Table 1). Ford and Smith (7) reported that five of their stock strains of *U. urealyticum* were susceptible to tetracycline in vitro at a concentration of 0.75 μg/ml, whereas one resistant isolate was not susceptible until a concentration of 15 μg/ml was achieved. Braun et al. (3) reported the median initial minimal inhibitory concentration for 11 strains of *U. urealyticum* as 0.4 μg/ml. Spaepen and Kundsin (16) found that 13% of their isolates were resistant to 1 μg of the tetracyclines tested per ml; specifically, 85.2, 83.3, 72.2, and 33.3% were inhibited by minocycline, doxycycline, demeclocycline, and tetracycline, respectively. Many of their isolates were resistant to both a tetracycline and erythromycin. The disparity between their data and ours may be a result of differences in the serotypes isolated, the source and number of isolates tested, and the difference in incubation time. The same study (16) implies that doxycycline and minocycline might have greater anti-ureaplasmal activity than the class representative, tetracycline, suggesting the "class concept" of disk testing cannot be applied to *U. urealyticum*. This, however, is not apparent from the data presented in Table 1.

The macrolide antibiotic erythromycin (3 μg/ml) has recently been reported as an ineffective anti-ureaplasmal agent (16), inhibiting only 3.7% of the isolates studied. This is in contrast to the present study (Table 1) and the studies by Ford and Smith (7) who reported that all four of the isolates they tested were susceptible to 1 μg/ml,

Braun et al. (3) who reported a median initial minimal inhibitory concentration of 1.6 μg/ml for 11 strains, and Shepard et al. (15) who reported a minimal inhibitory concentration of 3.12 μg/ml. The contrasting data may again be a reflection of the different sources and serotypes of *U. urealyticum* isolates, as well as methodology.

Chlamydiae and ureaplasmas are apparently both colonizers and opportunistic pathogens of the urethra, each possibly associated with nongonococcal urethritis (2). Recent clinical studies have indicated that rifampin, an antichlamydial agent, is ineffective against *U. urealyticum* (5), and results obtained with the present in vitro system support that study.

As a result of the activity of rifampin, Coufalik et al. (5) have suggested that this antimicrobial agent may be helpful in defining the role of *U. urealyticum* in nongonococcal urethritis. In an analagous way, the results we obtained with the urinary tract antiseptic methenamine mandelate suggest that it too may be used to distinguish between chlamydia- or ureaplasma-associated urethritis. Although methenamine mandelate is not recommended for in vitro tests in routine bacteriology, the pH of our test system (pH 5.5) seemed appropriate for its use. The antiseptic was routinely ureaplasmacidal. When tested, the acidic character of methenamine mandelate slightly lowered the already acid pH of the BA7. It was not determined whether the slight alteration in pH or the presence of methenamine was responsible for the activity.

Nalidixic acid and nitrofurantoin are also well known for their antibacterial activity and use as urinary tract antiseptics. Very limited in vitro activity of nitrofurantoin (20 to 60 μg/ml) against ureaplasmas has been reported here, as could be expected after Braun et al. (3) found the initial minimal inhibitory concentration range to be 12.5 to 1,000 μg/ml. It also appears from our studies that nalidixic acid (6 μg/ml) is not sufficiently active against *U. urealyticum* in vitro, similar to the interpretation by Braun et al. (3) when they tested one strain.

Trimethoprim-sulfamethoxazole is an antimicrobial agent commonly used to treat urinary tract infection. Sulfonamides reportedly have little effect on mycoplasmas (3), and their effect on *U. urealyticum* in clinical and in vitro studies was limited (1). With our system, trimethoprim-sulfamethoxazole (5 μg/ml) appears to be ineffective in inhibiting these organisms. Shepard (11) found a similar lack of activity with trimethoprim lactate. It should be noted, however, that thymidine is a likely component of BA7, and its presence in the culture medium may be responsible for blocking the activity of trimethoprim-sulfamethoxazole (4), resulting in its ineffectiveness in our study.

In conclusion, antimicrobial susceptibility testing with *U. urealyticum* may be more time-consuming than routine bacteriological testing, but it can be performed reliably and in a standardized manner. When performed as such, tetracycline and erythromycin still appeared as active agents in vitro. In addition, methenamine mandelate was also active, whereas trimethoprim-sulfamethoxazole, nalidixic acid, nitrofurantoin, and rifampin were all ineffective in vitro.

As has been previously suggested, in vitro antimicrobial susceptibilities should be determined on isolates from patients and cohorts before the initiation of therapy (17). Since resistant strains of *U. urealyticum* may appear, repeat cultures may be necessary 1 to 2 weeks after treatment (17).

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