Tetracycline-Resistant T-Mycoplasmas (*Ureaplasma urealyticum*) from Patients with a History of Reproductive Failure

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The susceptibilities of T-mycoplasmas (*Ureaplasma urealyticum*) to minocycline, demeclocycline, doxycycline, tetracycline, and erythromycin were determined by a direct tube dilution test. T-mycoplasma-positive urine sediments of 105 patients with a history of reproductive failure were used as inocula. Minocycline was found to be the most active of the group of antibiotics commonly used to eradicate T-mycoplasma infection. Based on the median initial minimum inhibitory concentration, minocycline was the lowest with 0.03 µg/ml, followed by demeclocycline and doxycycline with 0.125 µg/ml, tetracycline with 0.25 µg/ml, and erythromycin with 2.0 µg/ml. Six T-mycoplasma isolates which had been cloned three times were also tested for susceptibility to the same five antibiotics. The same susceptibility pattern was found. Strains resistant to high concentrations of all antibiotics occurred. Strong positive correlation was seen in 21 patients between in vitro highly resistant strains and positive posttreatment cultures. These results indicate that empirical treatment of genital mycoplasma infections is not justified. Cultures should be taken pretreatment, susceptibility testing performed prior to treatment, and follow-up cultures done posttreatment.

T-mycoplasmas have been implicated in nongonococcal urethritis by Shepard (26, 27) and Bennett et al. (4) and in human reproductive failure by Kundsin et al. (20, 21), Gnarpe and Friberg (14), and Horne et al. (17). Furthermore, Horne et al. (16) have described an endometrial lesion associated with the isolation of T strain mycoplasmas which may explain patient infertility. Determination of the antibiotic susceptibility of T-mycoplasmas recovered from patients who were treatment failures suggested that a possible cause for this failure was the existence of strains highly resistant to tetracycline, tetracycline analogues, and erythromycin. These antibiotics, as currently prescribed for T-mycoplasma infections, are shown in Table 1.

In the present study, a direct drug susceptibility test was performed based on a similar method described for mycobacteria (2). Urine sediment containing T-mycoplasmas was used as the inoculum instead of cloned T-mycoplasmas for two reasons. First, subculturing is frequently impossible. Hayflick (15) found that almost 70% of mycoplasmas isolated from human clinical materials could not be subcultured after initial isolation. Endogenous nutrients carried over from growth in the natural environment undoubtedly accounted for the initial isolation of this group of very fastidious mycoplasmas. Secondly, the composition of the growth medium has been shown to significantly influence certain properties. Because of their propensity to bind components from the medium, great caution must be exercised in describing characteristics of mycoplasma species. Their physical properties, antigenicity, antibiotic susceptibility, and even their reaction in certain diagnostic tests, have all been demonstrated to be related to the growth medium (6, 24). A number of studies have indicated that the mycoplasma cell membrane influences antibiotic susceptibility. For example, resistance to tetracycline has been shown to involve a reduced permeability of the membrane to the antibiotic, rather than an alteration in a ribosomal protein synthesis, in several mycoplasmas (9, 25).

MATERIALS AND METHODS

One hundred and five urine sediments from 105 patients found to be positive for T-mycoplasmas in the Surgical Bacteriology Laboratory, Peter Bent Brigham Hospital, Boston, Mass., were used in this study. These patients had a history of reproductive failure. Their specimens (urine and vaginal, and

TABLE 1. *Antibiotics which have been reported as successfully eradicating T-mycoplasmas from the genitourinary tract*

Antibiotic	Dose	Duration	Investigator
Minocycline	200 mg 100 mg	Day 1 7 more days	Hodgson (personal communication)
Doxycycline	200 mg 100 mg	Day 1 9 more days	Gnarpe & Friberg (14)
Demeclocycline	1 filmtab (300 mg)	10 days	Horne et al. (17)
Tetracycline	2 g	10 days	Shepard (27), Bennett et al. (4)
Erythromycin	2 g	10 days	Shepard (27)

cervical or urethral swab) were sent to the laboratory by private gynecologists and infertility clinics. The study was conducted over a 1-year period between August 1974 and August 1975. The T strains were identified by the simultaneous use of liquid and solid media as described by Kundsin (19).

Preparation of the antibiotic solutions. Standard minocycline, demeclocycline, and tetracycline powders of known potency were provided by the Lederle Laboratories (Pearl River, N.Y.). Erythromycin was purchased through the hospital pharmacy as sterile erythromycin gluceptate powder (Ilotycin, Dista Products Co., Eli Lilly and Co., Indianapolis, Ind.). Doxycycline was donated by the Pfizer Laboratories Division of Chas. Pfizer & Co., Inc. (New York, N.Y.). Minocycline hydrochloride had an activity of 876 μg/ml. The product was used without drying, because the potency factor corrects for moisture content, hydrochloride, and inactive materials. Demeclocycline hydrochloride and tetracycline hydrochloride were 100% active and needed no corrections.

The initial solutions (stock solutions) of minocycline, demeclocycline, and tetracycline were made up in 0.01 N hydrochloric acid, giving an antibiotic concentration of 1,000 μg/ml. These stock solutions were each sterilized by passage through a membrane filter (0.22-μm pore size, Millipore Corp., Bedford, Mass.). The erythromycin gluceptate and doxycycline hyclate were dissolved in Sterile Water for Injection (Eli Lilly and Co.) giving an antibiotic concentration of 1,000 μg/ml. Under aseptic conditions, using ultraviolet irradiation, each stock solution was dispensed in 1.8-ml aliquots into sterile polysterene tubes with pop-off caps (Falcon Products, Cockeysville, Md.) and frozen (−75 F [ca. −59.5 C]). Each tube was thawed only once prior to susceptibility testing and was used immediately to prepare broth dilutions.

Susceptibility test procedure. The minimal inhibitory concentration (MIC) was determined by using a direct drug susceptibility test as described for mycobacteria (2). Only urine sediments in which T-mycoplasmas had been visualized were used as inocula. The urine was dispensed in clear, sterile plastic tubes in aliquots of 12 ml. Each urine specimen was centrifuged in a clinical centrifuge (1,100 × g). The supernatant was discarded, and the remaining 1 ml of sediment was frozen at −75 F until the original sediment had been found positive for T-mycoplasmas.

Tubes containing 1 ml of Ford medium (7) with 200, 40, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06, and 0.03 μg of

antibiotic per ml were made up on the day each antibiotic was tested. To each tube 0.025 ml of thawed, well-mixed urine sediment containing T-mycoplasmas was added, using a calibrated microtiter pipette. The tubes were closed with tight-fitting plastic caps. For each isolate a control tube containing 1 ml of Ford medium and 0.025 ml of urine sediment with T-mycoplasmas was inoculated simultaneously. All tubes were incubated at 36 C for 10 days and observed daily for color change.

Of 105 specimens from different patients, 102 T-mycoplasma isolates were tested for susceptibility to minocycline, 97 to demeclocycline, 58 to doxycycline, 18 to tetracycline, and 35 to erythromycin.

The MIC was defined as the lowest concentration of antibiotic inhibiting color change in broth by the T-mycoplasma isolate tested. The initial MIC was determined as the lowest concentration of antibiotic inhibiting color change caused by a given T-mycoplasma at the time the control tube containing the same strain changed. The lowest concentration of antibiotic that completely inhibited color change after 6 days of incubation was also noted and will be referred to as the "final MIC." It is included because color changes were slower to develop in the presence of antibiotic than in the controls without antibiotic. Therefore incubation must be prolonged until no further color changes occur. Incubation beyond 6 days did not result in any further color change. The final MIC represents only an upper limit of the true MIC at 6 days because of possible deterioration of the antibiotic.

To establish that the presence of 0.01 N hydrochloric acid used as solvent for minocycline, demeclocycline, and tetracycline had no effect on the growth of T-mycoplasmas, several experiments were performed similar to the tube dilution method, using a stock solution of 0.01 N hydrochloric acid without antibiotic.

The T-mycoplasmas were not isolated from their original environment (urine) prior to testing. Table 2 shows the relative occurrence of other bacteria present in the 105 urine specimens used in this study.

To establish that cloned strains showed the same antibiotic susceptibilities, 6 isolates of T-mycoplasma which had been cloned three times were assayed, and the results were compared with those obtained from the clinical isolates. These strains were representative of isolates from different sources. Strains *960* and *K-12* were isolates from patients with nongonococcal urethritis (obtained

TABLE 2. Occurrence of microorganisms in the 105 urine cultures

Organisms[a]	No. of urine cultures (%)
T-mycoplasmas	63 (60.0%)
T-mycoplasmas and Mycoplasma hominis	19 (18.0%)
T-mycoplasmas and Candida albicans	10 (9.5%)
T-mycoplasmas and Proteus mirabilis	4 (3.8%)
T-mycoplasmas and Torulopsis glabrata	3 (2.8%)
T-mycoplasmas, Mycoplasma hominis, and Candida albicans	2 (1.9%)
T-mycoplasmas, Mycoplasma hominis, and Proteus vulgaris	2 (1.9%)
T-mycoplasmas, Proteus mirabilis, and Candida albicans	1 (0.9%)
T-mycoplasmas, Proteus mirabilis, and Torulopsis glabrata	1 (0.9%).

[a] Presence of few gram-positive cocci and/or lactobacilli possible.

from M. C. Shepard); NUN was from the urine of an asymptomatic nun; BROWN was from a symptomatic patient with genitoinfectious disease; BARBARA was from the cervix of a patient with carcinoma in situ; and BOSTON T was from the fetal membranes of a middle trimester spontaneous abortion.

Titration of the urine specimens. The titer of each specimen was determined by adding 0.2 ml of urine sediment to 1.8 ml of Ford medium (7). This was carefully mixed (Vortex-Genie) to break up clumps and serial 10-fold dilutions were made in the same medium using 0.2 ml to 1.8 ml, contained in clear plastic screw-cap tubes, up to 10^{-5}. The tubes were incubated at 36 C for seven days. The highest dilution in which a color change occurred was considered the end point. The results were read visually and expressed as titers of color-changing units (CCU). One hundred and twenty-five isolates of T-mycoplasmas in urine sediments were titrated.

Correlation between susceptibility test results and posttreatment urine cultures. Fifty of the 105 patients were recultured for the presence of T-mycoplasmas within a period of 4 to 8 weeks after completion of their antibiotic therapy. The posttreatment urine culture results were correlated with the final MIC of the prescribed antibiotic for each patient. Of these 50 patients, 2 were treated with minocycline, 25 with demeclocycline, 18 with doxycycline, and 4 with tetracycline. None was treated with erythromycin.

RESULTS

Of the 125 T-mycoplasma isolates titrated, 20 specimens with a titer of 10^1 CCU were not included in this study because they gave inconsistent results due to the low numbers of CCU. The distribution of the end points of the 105 remaining specimens is presented in Table 3. Most specimens had a titer of 10^3 CCU or greater. Regardless of the titer of organisms in

the urine sediments, the controls containing T strains without antibiotic showed color change within 48 h for all specimens. At a titer of 10^2 CCU, 20% of the controls changed within 24 h; at a titer 10^3 CCU, 75% of the controls changed within 24 h; at a titer of 10^4 CCU, 81% changed within 24 h; and at a titer of 10^5 CCU or greater, all controls changed within 24 h.

Figures 1 and 2 summarize the initial MIC and the final MIC of four tetracycline components and erythromycin against T-mycoplasmas present in urine sediments. The results show that minocycline was clearly the most active compound at the lowest concentrations, followed by demeclocycline, doxycycline, and tetracycline. Erythromycin was considerably less active than the tetracyclines with a clustering of values in the 4.0- to 200-μg/ml range (final MIC).

At an initial MIC of 0.03 μg/ml, 50% of the T-mycoplasmas were inhibited by minocycline, whereas only 25.8% were inhibited by demeclocycline, 13.8% by doxycycline, 11.1% by tetracycline, and 2.6% by erythromycin. At the final MIC, 2% of the T-mycoplasmas were still inhibited by 0.03 μg of minocycline per ml, whereas all the other antibiotics did not retain

TABLE 3. Distribution of urine titers of the 105 T-mycoplasmas tested

Titer (CCU)	No. of strains (%)
10^2	10 (9.5%)
10^3	24 (22.9%)
10^4	47 (44.8%)
$\geq 10^5$	24 (22.9%)

FIG. 1. Initial MICs for T-mycoplasmas in urine sediment of minocycline (102), demeclocycline (97), doxycycline (58), tetracycline (18), and erythromycin (35). Numbers in parentheses indicate the number of specimens tested to each antibiotic.
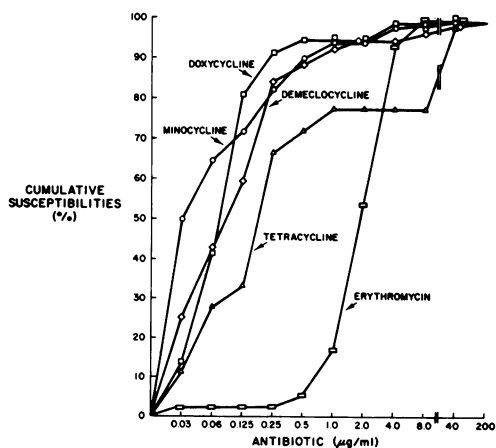
FIG. 2. *Final MICs for T-mycoplasmas in urine sediment of minocycline (102), demeclocycline (97), doxycycline (58), tetracycline (18), and erythromycin (35). Numbers in parentheses indicate the number of specimens tested to each antibiotic.*
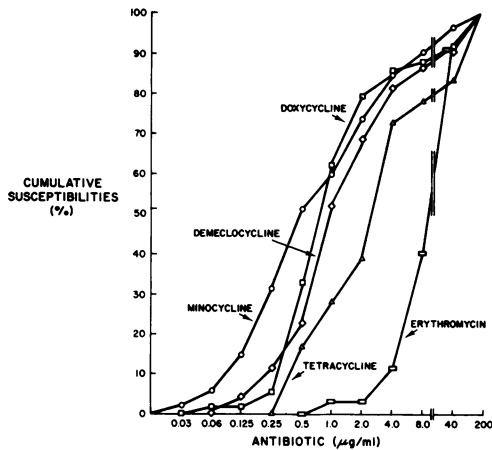
their activity. The median values for the initial MIC and the final MIC of minocycline (102 T strains), demeclocycline (97 T strains), doxycycline (58 T strains), tetracycline (18 T strains), and erythromycin (35 T strains) are presented in Table 4. Minocycline showed the lowest median values for both the initial MIC and the final MIC.

T-mycoplasmas were susceptible over a broad range of concentrations to each tetracycline. Several isolates deviated widely from the median for each drug. Strains resistant to 40 μg/ml for each antibiotic were found. Based on the final MIC, comparable percentages of T-mycoplasmas were inhibited by achievable blood levels (10, 29) of minocycline (73.5% inhibited by 2 μg/ml), demeclocycline (68.0% inhibited by 2 μg/ml), doxycycline (79.2% inhibited by 2 μg/ml) and tetracycline (72.2% inhibited by 4 μg/ml). However, only 11.4% of the T-mycoplasmas were inhibited by 4 μg of erythromycin per ml. Table 5 outlines the in vitro susceptibility results of six cloned T-mycoplasma strains tested to the same five antibiotics. The titer of each inoculum is presented in the same table. The results were consistent with those obtained from the urine sediments. The same susceptibility pattern was followed. Minocycline again was the most active antibiotic, followed by doxycyline, demeclocycline, tetracycline, and erythromycin. One strain (*NUN*) showed a lower final MIC for doxycycline. The *BOSTON T* strain was assayed twice, using inocula with different titers (once with 10^3 CCU, once with 10^4 CCU). Both tests gave the same MIC values. The *BOSTON T* strain was

the most susceptible strain, and *960* was the least susceptible. As was observed for the clinical isolates, the susceptibility appeared to vary with the isolate tested.

The presence of 0.01 N hydrochloric acid, used as a solvent for some of the antibiotics, appeared to have no effect on the susceptibility test. The color change occurred in all the tubes containing 0.01 N HCl in broth (similarly diluted as in the actual test) and T strains at the same time a color change was observed in the control tubes containing broth and T strains.

Table 6 outlines the data obtained by correlating the posttreatment urine culture results of 50 patients with the final MIC of the antibiotic used for treatment of their T-mycoplasma infection. Of these 50 patients, 29 had a negative urine specimen posttreatment and 21 had a positive urine specimen posttreatment. It was found that patients who carried T-mycoplasmas in the urine, requiring a final MIC of 4.0 μg or more of a tetracycline per ml in vitro, were still positive posttreatment. A final MIC of 1.0 μg/ml or less resulted in a negative urine specimen posttreatment for all but one patient who was still positive after treatment with doxycycline. A final MIC of 2.0 μg/ml in vitro gave mixed posttreatment results. Four patients were negative and four patients were still positive for T-mycoplasma posttreatment. Since 2.0 μg/ml appears to be a borderline concentration, the posttreatment outcome probably depends upon the antibiotic concentration attained during treatment, which is variable from patient to patient. Based on these data, T-mycoplasmas requiring a final MIC of 1.0 μg of the tetracyclines per ml could be considered as "susceptible," a final MIC of 2.0 μg/ml as "intermediate," and a final MIC of 4.0 μg/ml as "resistant." More data are needed to attest to the validity of this classification.

DISCUSSION

The results of this study indicate that minocycline is an active antibiotic against T-mycoplasmas with a median MIC well below reported blood serum concentrations achievable

TABLE 4. *Median MICs of five antimicrobial agents to T-mycoplasma isolates*

Antibiotic	No. of strains tested	Median (μg/ml)	
		Initial MIC	Final MIC
Minocycline	102	0.03	0.5
Demeclocycline	97	0.125	1.0
Doxycycline	58	0.125	1.0
Tetracycline	18	0.25	4.0
Erythromycin	35	2.0	40

TABLE 5. *Titer and susceptibilities of six cloned T-mycoplasmas to five antibiotics*

Antigen	Titer (CCU)	Initial MIC (final MIC) of antibiotics (µg/ml)				
		Minocycline	Doxycycline	Demeclocycline	Tetracycline	Erythromycin
BOSTON T	10^3, 10^4	0.06 (0.25)	0.125 (0.5)	0.25 (1.0)	0.5 (2.0)	2.0 (40)
960	$\geq 10^5$	0.25 (200)	0.5 (200)	1.0 (200)	4.0 (>200)	8.0 (>200)
NUN	$\geq 10^5$	0.5 (8.0)	0.5 (2.0)	1.0 (4.0)	1.0 (200)	8.0 (>200)
K-12	10^4	0.06 (1.0)	0.125 (2.0)	0.25 (4.0)	0.25 (8.0)	4.0 (40)
BROWN	$\geq 10^5$	0.125 (2.0)	0.25 (2.0)	0.5 (2.0)	1.0 (8.0)	4.0 (>200)
BARBARA	$\geq 10^5$	0.25 (2.0)	0.5 (2.0)	0.5 (2.0)	2.0 (8.0)	8.0 (40)

TABLE 6. *Correlation between the in vitro susceptibility test results and posttreatment urine specimen results for 50 patients*

Posttreatment culture results	Antibiotic used for treatment	No. of patients treated	No. of T-mycoplasmas with final MIC (µg/ml) of										
			0.03	0.06	0.125	0.25	0.5	1.0	2.0	4.0	8.0	40	200
Negative (29 patients)	Minocycline	1						1					
	Demeclocycline	16			2	2	2	6	3				
	Doxycycline	11				1	2	7	1				
	Tetracycline	1						1					
Positive (21 patients)	Minocycline	1								1			
	Demeclocycline	10							2	1	1	2	4
	Doxycycline	7						1	2	1	1	1	1
	Tetracycline	3									1	1	1

after therapeutic doses (10, 29). Doxycycline and demeclocycline, although not as active in vitro as minocycline against the majority of the T-mycoplasmas tested, still gave low median MIC values. Tetracycline was less active than its analogues. Erythromycin required the highest concentrations to inhibit the T strains. Braun et al. (5), using a different method, showed lower initial and final median MIC values for erythromycin than reported in this study. For tetracycline, however, they found a higher initial and lower final median MIC.

Unlike earlier investigations (11, 12, 13, 30) which showed that all T-mycoplasmas were susceptible to the tetracyclines, in this study many T-mycoplasma isolates were found to be resistant to a concentration of 8 µg/ml and higher. Recently Ford and Smith (8) have described the isolation of a tetracycline-resistant, erythromycin-sensitive T-mycoplasma from a patient with nonspecific urethritis.

Although little is known about attainable levels for these antimicrobial agents in those parts of the human body where these microorganisms are most frequently harbored, good correlation was noticed between T strains highly resistant in vitro and repeated positive cultures posttreatment. It is doubtful that reinfection would be responsible for consistent isolation of T-mycoplasmas for several reasons. Both husband and wife are treated simultaneously with the same antibiotic. Furthermore,

this study was done on a patient population with a history of infertility and a strong motivation to initiate a successful pregnancy. And finally, excellent correlation between the in vitro resistant T strains and positive posttreatment cultures was seen in 21 patients.

The results also indicate that the tetracyclines should not be recommended for the empirical treatment of T-mycoplasma infections because of the high frequency of resistant strains. Moreover, the different T-mycoplasmas showed a variability in their susceptibility pattern which cannot be ignored. Some strains may be eradicated with minocycline when treatment with demeclocycline has failed. No one tetracycline analogue can be proposed as the drug of choice because resistance to all four was found. The data obtained in this study illustrated the importance of susceptibility testing of T-mycoplasmas as a guide to rational antimicrobial therapy. It is suggested that all patients be recultured at a minimum of 4 weeks after completion of therapy to assure eradication of the T-mycoplasmas.

As mentioned earlier, an inoculum in which the T strains are left in their original environment was preferred because of the complex nutritional requirements of mycoplasmas. Isolation and cloning is in many cases impossible because many cannot be recultured after initial isolation from human clinical sources (15). The most fastidious T-mycoplasmas often appeared

to be the most resistant strains. Furthermore, cell membrane constituents have been known to change depending on the medium in which growth occurred (28). With this method the in vitro susceptibility testing emulates the environment in which the organisms have to be inhibited in vivo. As suggested by Shepard (see ref. 3), T strain mycoplasmas may occur inside cells, can get into cells and become protected from the action of antibiotics. The use of the sediment with epithelial cells is therefore a more realistic approach to antibiotic susceptibility in vivo than the use of T-mycoplasma cultures which have been subcultured.

Six cloned T-mycoplasma isolates were tested similarly for susceptibility to the five antibiotics and used as a comparison. The MIC values obtained for these cloned isolates fitted very well into the susceptibility pattern observed for the clinical isolates. One cloned strain and 10 clinical specimens were tested twice, and the results were reproducible.

The presence of bacteria and fungi had little effect on the observations in vitro because the test is based on a colorimetric reaction specific for T-mycoplasmas in a specific medium. Some penicillin-resistant microorganisms which give a positive urease reaction, such as *Proteus* species, are occasionally able to raise the pH of the broth and cause a color change. However, bacterial and fungal growth is indicated by obvious turbidity. Growth of the T strains produces no visible turbidity, and the color change generally commences at the bottom of the tube. On occasions where cloudy tubes were noticed, the results were not included in this study.

Earlier studies (1, 18, 22, 23) on the viability of mycoplasmas indicate that these organisms survive for relatively long periods when frozen in broth and stored at -20 to -70 C. In our laboratory, it was found that freezing and thawing of the urine sediments had no effect on the titer of the T-mycoplasmas if frozen at -75 F for up to 2 weeks. A change in titer by a factor of 10^{-1} to 10^{-2} was noticed for some isolates if stored longer than 2 weeks. The urine sediments used in this study were all tested for susceptibility within a week after freezing, and each specimen was titrated on the day the susceptibility test was performed. All specimens were frozen and thawed only once.

Taylor-Robinson (30) reported that a titer of 10^2 or 10^5 T-mycoplasmas gave the same minimum inhibitory concentration of an antibiotic. Braun et al. (5) have reported similar findings for T-mycoplasmas. Our findings are in agreement with those. The susceptibility results were not affected by the titer of the T strains in the urine sediments. The same MIC levels were found, regardless of the titer, for T-mycoplasmas in different urine sediments from the same patient. The titer served only as an indication as to whether the specimen contained sufficient organisms to produce a color change when diluted in the actual test procedure.

Both the initial MIC and final MIC are necessary for evaluating the susceptibility test results. The initial MIC serves mainly as a preliminary indicator, useful for the physician to initiate treatment. The final MIC is particularly valuable in detecting resistant strains, since not all of them have a high initial MIC reading.

Although this direct in vitro susceptibility method may not be an ideal method, it is a practical method for testing drug susceptibilities of T strains in a relatively short period of time so that the physician can use the information for treatment of T-mycoplasma infections. Cloning of T-mycoplasmas is a lengthy and time-consuming procedure and consequently is not feasible when the clinician is anxious to start therapy. Furthermore, the patients' results attest to the validity of this method. The performance of such in vitro susceptibility determinations uncovered drug-resistant strains and is necessary for the intelligent management of retreatment cases.

In conclusion, tetracyclines are still the drugs of choice for T-mycoplasma infections. They should not, however, be given empirically. A pretreatment susceptibility test and a posttreatment culture are essential. This study also suggests the need for a new drug effective against the tetracycline-resistant, erythromycin-resistant T-mycoplasmas.

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