In Vitro Culture System To Determine MICs and MBCs of Antimicrobial Agents against *Treponema pallidum* subsp. *pallidum* (Nichols Strain)

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A new procedure for determining the susceptibility of *Treponema pallidum* subsp. *pallidum* to antimicrobial agents was developed, utilizing a tissue culture system which promotes the *in vitro* multiplication of this organism. In the absence of antibiotics, *T. pallidum* (Nichols virulent strain) multiplied an average of 10-fold when incubated for 7 days in the presence of *Ehrlichia* rabbit epithelial cell cultures. Varyed concentrations of penicillin G, tetracycline, erythromycin, and spectinomycin were added to triplicate cultures to determine their effects on treponemal multiplication, motility, and virulence. The MIC of each antibiotic was defined as the lowest concentration which prevented treponemal multiplication, whereas the MBC was defined as the lowest concentration which abrogated the ability of the cultured treponemes to multiply and cause lesions in rabbits. The in vitro culture technique provided highly reproducible MICs and (in parentheses) MBCs of each of the antibiotics tested: aqueous penicillin G, 0.0005 (0.0025) μg/ml; tetracycline, 0.2 (0.5) μg/ml; erythromycin, 0.005 (0.005) μg/ml; and spectinomycin, 0.5 (0.5) μg/ml. The significance of these results in light of the in vivo activities and the previous in vitro evaluations of these antibiotics is discussed. The *T. pallidum* in vitro cultivation system shows promise as a method for studying the interaction between *T. pallidum* and antimicrobial agents and for screening new antibiotics for syphilitic therapy.

Although benzathine benzylpenicillin is generally a very effective therapy for syphilis, several reasons exist for examining the susceptibility of *Treponema pallidum* to other antimicrobial agents (21, 37). First, only tetracycline and erythromycin are currently recommended by the Centers for Disease Control (6) and the World Health Organization (43) as alternate therapies for penicillin-sensitive individuals; there is evidence of treatment failure (14, 23, 39) and resistance (40) with erythromycin, and tetracycline is contraindicated for pregnant women and children (6, 43). Second, the use of benzathine penicillin for the treatment of neurosyphilis has been questioned due to its poor blood-brain barrier penetrability (21, 41), leading physicians to substitute multiple injections of intravenous administration of aqueous penicillin G (6, 41), procaine penicillin (6), or ceftriaxone (24) to ensure adequate treatment of these patients. Third, isolation of an erythromycin-resistant strain of *T. pallidum* from a syphilitic patient (40) and evidence for a plasmid in *T. pallidum* (32) indicate that future development of widespread antibiotic resistance in this organism is possible. Finally, it would be desirable to identify therapeutic regimens capable of treating gonorrhea, chlamydial infections, and syphilis concomitantly; this would require the identification of antimicrobial agents effective against each bacterium. The efficacies of a number of new agents, including cephalosporins and quinolones, in the treatment of gonorrhea and chlamydial infections are currently being investigated, and knowledge of their effects on syphilitic infection would also be of value.

Four assays have been used previously to examine the susceptibility of *T. pallidum* to antimicrobial agents (37): loss of motility or virulence during short-term incubation of static (nonmultiplying) suspensions of *T. pallidum* with the agents (7, 10–12, 30): inhibition of growth of easily cultivable, avirulent treponemal species (1–4, 13, 17, 20, 26, 42), such as *T. phagedenis*; inhibition of [35S]methionine incorporation into proteins by *T. pallidum* (40); L. V. Stamm, J. T. Stapleton, and P. J. Bassford, Jr., Antimicrob. Agents Chemother., in press); and in vivo studies with *T. pallidum*-infected rabbits (5, 12, 25, 27). Each of these methods has limitations. The first method was used because, until recently (15), in vitro multiplication of *T. pallidum* had not been obtained. Many antimicrobial agents are much more effective against actively multiplying organisms, so the results obtained with nonmultiplying suspensions may be inaccurate. The usefulness of results obtained with other species of *Treponema* is questionable, because these organisms appear to be distantly related to *T. pallidum*, based on DNA homology and GC content (29). Also, *T. pallidum* is microaerophilic (8, 16), whereas the other *Treponema* are anaerobic (38), indicating significant metabolic differences. Protein synthesis inhibition is a promising approach (40) but would be expected to be much more sensitive to protein synthesis inhibitors than to antibiotics with other modes of action. Animal studies are a necessary prerequisite to human trials but are expensive and cumbersome, particularly if more than one dose or treatment regimen is to be tested.

In 1981, Fieldsteel et al. (15) described a tissue culture system in which consistent *T. pallidum* multiplication can be obtained, as confirmed in our laboratory (33–35). *T. pallidum* is incubated in a modified tissue culture medium in the presence of SF1Ep cottontail rabbit epithelial cells under 1.5 to 3.0% oxygen at 33 to 34°C. Under these conditions, up to 100-fold multiplication of the treponemes with full retention of virulence is possible over a 12- to 15-day period of incubation, but continuous in vitro culture has not yet been achieved (35). The possibility of using the Fieldsteel et al. culture system...
to more accurately assess the antimicrobial susceptibility of *T. pallidum* was explored in the present study. Using this technique, we determined the MICs and MBCs of four antibiotics whose efficacy in the treatment of syphilis has been established previously. Our results indicate that the *T. pallidum* in vitro culture system will be useful for studying the effects of antimicrobial agents on *T. pallidum* and determining the potential efficacy of new agents in the treatment of syphilis.

MATERIALS AND METHODS

**Reagents.** The modified basal reduced minimal medium (BRMM) used for *T. pallidum* cultivation consisted of Eagle minimal essential medium (MEM) with Earle salts and 1× nonessential amino acids, 2.5 g of d-glucose per liter, 31.3 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.90 mM sodium pyruvate, 0.126 g of resazurin (Sigma Chemical Co., St. Louis, Mo.) per liter, and 0.63 mM dithiothreitol and was prepared as described previously (15, 33). Dithiothreitol is required for pre-reduction of the medium; however, most of it is oxidized at the time that the antibiotics are added (35), and it does not appear to affect drug activity. All tissue culture media reagents were purchased from Flow Laboratories (McLean, Va.), and dithiothreitol and HEPES were obtained from Calbiochem (La Jolla, Calif.). BRMM was supplemented with 20% fetal bovine serum from a single lot (Hyclone lot no. 100315; Sterile Systems, Logan, Utah) that was pretested for *T. pallidum* growth-supporting activity. The serum was heat-inactivated at 56°C for 30 min prior to use.

Penicillin G potassium for injection was obtained from Squibb (Princeton, N.J.), tetracycline hydrochloride was from Bristol Laboratories (Syracuse, N.Y.), and spectinomycin hydrochloride was from Upjohn (Kalamazoo, Mich.) under the brand name Trobicin. Erythromycin (base) was kindly provided by Upjohn Laboratories. Antibiotic stock solutions were prepared immediately prior to use. Penicillin, tetracycline, and spectinomycin were dissolved in distilled water without further treatment. Erythromycin was dissolved first in 1 ml of 95% ethanol and then diluted with distilled water; the amount of ethanol introduced into the cultures (<4% v/v) was not inhibitory to growth. Stock solutions were sterilized by filtration and serially diluted in sterile distilled water as necessary to provide the desired concentrations when added in quantities of 25 to 200 μl per 10 ml of culture.

**Tissue culture.** SFlEp cells (a cottontail rabbit epithelial cell line) were kindly provided by A. H. Fieldsteel (deceased) and D. L. Cox (Texas College of Osteopathic Medicine, Fort Worth, Tex.) or purchased from the American Type Culture Collection (Rockville, Md.). The cells were maintained in MEM-10% calf serum without antibiotics in a CO₂ incubator at 37°C. The cells used in these experiments were between passages 80 and 88.

**Bacteria.** The Nichols strain of *T. pallidum* subsp. *pallidum* was originally obtained from J. N. Miller, UCLA School of Medicine, Los Angeles, Calif. The bacterial strain was maintained by intratesticular injection of rabbits and prepared for inoculation into in vitro culture as described previously (15, 34).

*T. pallidum* cultivation and antimicrobial susceptibility testing. The *T. pallidum* cultivation system has been described in detail (15, 34) and is outlined in Fig. 1. Briefly, SFlEp cells were inoculated into 4-oz (ca. 125-ml) prescription bottles containing MEM-10% calf serum and allowed to grow for 2 days. Three to four hours prior to the introduction of *T. pallidum*, the MEM-10% calf serum medium was replaced with BRMM with 20% heat-inactivated fetal bovine serum. The antibiotic under study was then added to triplicate cultures for each concentration tested. In the initial experiments, a wide range of antibiotic concentrations were tested, varying from 10 to 10⁻⁷ μg/ml. Once the susceptibility range was determined, narrower concentration ranges (consisting of fivefold or twofold serial dilutions) were tested.

Following preequilibration in an atmosphere of 1.5% O₂-5% CO₂-93.5% N₂ (either in a microaerobic chamber [34] or in individually gassed flasks [15]), the cultures were infected with approximately 10⁷ *T. pallidum*. The cultures were incubated in the low-O₂ atmosphere at 34°C for 7 days. To assess the number and motility of *T. pallidum* after incubation, the cultures were trypsinized as previously described (15) to release the *T. pallidum* from the SFlEp cells. Samples from each culture were then examined by dark-field microscopy in Helber bacteria-counting chambers (Hawksley & Sons, Lancing, Sussex, England) (34), and *T. pallidum* concentration and motility were determined.

**Virulence.** To assess the virulence of the *T. pallidum* following incubation with antibiotics, samples of the 7-day cultures were used to infect New Zealand white rabbits as described previously (36). Each antibiotic was examined in a separate experiment. The antibiotic susceptibility testing procedure was carried out as described above, and pooled...
samples from the triplicate cultures for each antibiotic concentration were injected intradermally (0.1 ml per site) into the shaved backs of rabbits. Groups of three rabbits were inoculated at duplicate sites with each of the pooled culture samples, for a total of 6 sites per antibiotic concentration and 12 sites per animal. The inoculation sites were observed daily for 45 days for the emergence of raised, erythematous lesions. Needle aspirates of representative lesions were examined by dark-field microscopy for motile treponemes to verify the presence of an active treponemal infection.

**RESULTS**

**Determination of MICs.** In all experiments, the *T. pallidum* cultures were incubated for 7 days in the presence or absence of various concentrations of antibiotics. The 7-day time interval was chosen because it permitted sufficient multiplication in antibiotic-free control cultures (average, 9.9-fold; range, 3- to 17.5-fold) to allow unequivocal detection of growth inhibition. The observed multiplication rate (average generation time, 51 h) was similar to both in vivo measurements (28) and previous results in the Fieldsteel et al. system (15, 16, 33–35).

The effects of the antibiotics on the growth and motility of *T. pallidum* are shown in Fig. 2 and Tables 1 through 4. Each antibiotic exhibited a characteristic inhibition pattern which was consistent from experiment to experiment. The MIC of each antibiotic was defined as the lowest concentration which consistently inhibited growth (fold increase ≤ 1, Tables 1 through 4). In general, motility was greatly reduced at the MIC of each antibiotic but was retained to some degree at concentrations greater than that required to inhibit multiplication (Tables 1 to 4).

*T. pallidum* was susceptible to extremely low concentrations of aqueous penicillin G (Fig. 2A, Table 1), yielding an MIC of 0.0005 μg/ml. The MIC of 0.2 μg/ml obtained for tetracycline (Fig. 2B and Table 2) was much greater than that of penicillin; however, it was still within the effective concentration range for this antibiotic. Erythromycin inhibited the in vitro multiplication of *T. pallidum* at concentrations of 0.005 μg/ml or more (Fig. 2C and Table 3), and no multiplication was seen at spectinomycin concentrations of ≥ 0.5 μg/ml (Fig. 2D, Table 4).

**Determination of MBCs.** The MBC is generally defined as the concentration of antibiotic necessary to kill 99.9% of the organisms. To determine the MBCs of the antibiotics tested in this study, the virulence of the cultured *T. pallidum* was assessed by inoculating samples from cultures at each antibiotic concentration into the shaved backs of rabbits and monitoring for lesion development. Because dermal lesions are reported to be produced by as few as 20 to 50 *T. pallidum* (28) (and as few as 10 organisms in our unpublished experiments), infectivity is an extremely sensitive means of assessing treponemal viability. The time of lesion development is also related to the number of virulent organisms present. In previous studies with freshly extracted organisms (36), 10⁶, 10⁵, 10⁴, and 10³ *T. pallidum* resulted in lesion development at 4.1, 6.2, 8.9, 14.8, and 17.2 days postinoculation, respectively.

The MBC was defined as the lowest antibiotic concentration at which the cultured *T. pallidum* were incapable of multiplying in vivo and causing lesions. Samples (0.1 ml) from the MIC cultures indicated in Tables 1 through 4 were inoculated intradermally at duplicate sites in the shaved backs of three rabbits. For practical reasons, the number of organisms present in each inoculum could not be adjusted to the same concentration. Overall, our MBC procedure is analogous to the standard method of plating out a small volume of MIC broth cultures and observing for growth or lack of growth.

Table 5 summarizes the results of the virulence experiments. In most cases, increasing concentrations resulted

![FIG. 2. Effects of the presence of increasing concentrations of (A) aqueous penicillin G, (B) tetracycline, (C) erythromycin, and (D) spectinomycin on the numbers of *T. pallidum* harvested after 7 days of in vitro culture. Each curve represents a separate experiment, and the corresponding inocula are indicated on the right side of the graph. Each point indicates the mean of triplicate cultures.](http://aac.asm.org/)
first in an increase in the time to lesion appearance as the MIC was approached, followed by a loss of lesion development. The MBCs corresponding to the loss of virulence are given in Table 6. The MBCs correlated closely with the concentrations inhibiting multiplication and motility. At some of the antibiotic concentrations (e.g., 0.005 µg of erythromycin per ml and 0.5 µg of spectinomycin per ml), lesions failed to develop despite the presence of >10⁶ motile T. pallidum per inoculum, indicating that virulence may be more sensitive to the effects of antibiotics than is motility in this system.

**DISCUSSION**

In the present study, the practicality of using the Fieldsteel et al. culture system for assessing the antimicrobial susceptibility of T. pallidum was demonstrated. The results obtained with each of the antibiotics tested were highly reproducible and provided distinct, unambiguous MIC and MBC values, as summarized in Table 6. The MIC could be defined within a two- to fivefold concentration range; in cases of variation in MICs between experiments, the higher concentration was selected. Interpretation of the virulence results (which served as the basis for the MBC determinations) was complicated by the reduced number of organisms in samples containing drug concentrations below the MIC. However, sufficient numbers (>7 × 10⁵) were present to demonstrate that viability was greatly reduced in those samples.

The Nichols virulent strain used in this study has been used in nearly all of the previous antimicrobial susceptibility studies, as well as in other research involving T. pallidum subsp. pallidum. This strain, which was originally isolated from a patient with neurosyphilis in 1911 (31), is still pathogenic to humans (18) and by all measures appears to have properties very similar to those of more recent isolates. Other strains were not investigated because of the technical difficulty of maintaining a large number of T. pallidum strains in rabbits and in vitro culture. Cox et al. (9) recently demonstrated that multiplication of other venereal syphilis isolates can be obtained in the Fieldsteel et al. culture system. It would therefore be possible (albeit laborious) to use the in vitro culture system to test other venereal syphilis strains.

**TABLE 1. Effect of penicillin on multiplication and motility of T. pallidum**

<table>
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<tr>
<th>Penicillin concn (µg/ml)</th>
<th>Fold increase in treponemes ± SD</th>
<th>% Motile</th>
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<th>2</th>
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* Numbers 1 through 4 represent separate experiments. Experiment 4 was used in virulence determinations (Table 5).

**TABLE 2. Effect of tetracycline on the multiplication and motility of T. pallidum**

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<th>Tetracycline concn (µg/ml)</th>
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* See Table 1, footnote a. Experiment 3 was used in virulence determinations (Table 5).

a The MIC for treponemes.
isolates and determine whether significant variation in susceptibility occurs. Although penicillin treatment failures have been known to occur (19, 22), it was not possible in these cases to document that the failures were due to antimicrobial agent resistance. However, the isolation of an erythromycin-resistant strain (40) (Street strain 14) and the occurrence of erythromycin treatment failures indicate that variations in *T. pallidum* antimicrobial susceptibility could potentially become a significant health care problem.

Comparison of the results obtained in the Fieldsteel et al. culture system with previous antibiotic susceptibility determinations is difficult because of the wide variation in the results obtained with other systems (37). In general, the in vitro culture technique described here appears to be more sensitive than the other in vitro procedures. The quantity of penicillin required to reduce *T. pallidum* motility to 50% after 18 h of in vitro incubation was reported by Nell to be 0.001 to 0.003 μg/ml (30), somewhat higher than the MIC of 0.0005 μg/ml obtained in the present study. Both of these values are within the expected range of the in vivo clearance level of 0.005 to 0.01 μg/ml of serum reported by Eagle et al. (12) for *T. pallidum* infection in rabbits. The values obtained with easily cultivable species of *Treponema* are highly variable and are in general much greater than those obtained for *T. pallidum*. Penicillin MICs for *T. phagedenis* biotypes Kazan and Reiter, *T. vincentii*, and *T. refringens* ranged from 0.005 to 1.0 μg/ml, and the reported MBCs extended from 0.02 to >1,000 μg/ml (1-4, 13, 26, 42). This variability, which was also evident in studies examining tetracycline and erythromycin susceptibilities (1, 2, 20, 26), is apparently due both to differences in experimental procedures and to true variations in susceptibility among the tested strains.

As with other bacteria, in vitro activity of an antimicrobial agent against *T. pallidum* may not always correlate well with in vivo efficacy due to the pharmacodynamics of the drug. The correlation was good in the case of aqueous penicillin G, which is very effective in vivo and which also exhibited an extremely low MIC in our system (0.0005 μg/ml). The correlation was poor, however, for spectinomycin, which provided an MIC of 0.5 μg/ml and yet is ineffective in the treatment of syphilis both in humans and in experimental animals (37: B. T. Craig, R. J. Rice, R. J. Arko, J. A. Crawford, E. F. Hunter, and S. A. Larsen, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, A-139, p. 24). The inefficacy of spectinomycin is apparently due to poor tissue penetrability rather than lack of bactericidal activity. Concentrations of spectinomycin in tissue fluid are very low even in the presence of high levels in serum, as shown recently in the implanted-chamber model in rabbits (R. J. Rice, S. E. Thompson, R. J. Arko, E. F. Hunter, P. M. Burleigh, B. P. Craig, and S. A. Larsen, submitted for publication). Since *T. pallidum* infiltrates the interstitial spaces in tissue, spectinomycin may not reach the organisms in sufficient concentrations to eradicate infection. This inter-

### TABLE 3. Effect of erythromycin on the multiplication and motility of *T. pallidum*

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<thead>
<tr>
<th>Erythromycin concn (μg/ml)</th>
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*See Table 1, footnote a. Experiment 2 was used in virulence determinations (Table 5).

† The MIC for treponemes.

### TABLE 4. Effect of spectinomycin on the multiplication and motility of *T. pallidum*

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</table>

*See Table 1, footnote a. Experiment 4 was used in virulence determinations (Table 5).

† The MIC for treponemes.
TABLE 5. Effect of penicillin G, tetracycline, erythromycin, and spectinomycin on the retention of virulence by *T. pallidum*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>T. pallidum inoculum (organisms)</th>
<th>% Motile</th>
<th>No. of lesions/6 inoculated</th>
<th>Mean day of lesion development ± SD</th>
</tr>
</thead>
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<tr>
<td>Penicillin G</td>
<td></td>
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<td>96</td>
<td>6</td>
<td>8.3 ± 0.5</td>
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<tr>
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<tr>
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In summary, we believe that this system represents the first in vitro method of obtaining reliable MICs of antimicrobial agents against *T. pallidum*. The complexity of the in vitro culture technique precludes its use as a standard laboratory procedure. However, this approach will be useful in the selection of new treatment regimens for further evaluation and in the study of the interaction of antimicrobial agents with *T. pallidum*.

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LITERATURE CITED


