

In Vitro Susceptibility of *Prototheca* spp. to Gentamicin

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One hundred strains of *Prototheca zopfii*, *Prototheca wickerhamii*, *Prototheca moriformis*, *Prototheca stagnora*, and *Prototheca ulmnea*; five strains of *Chlorella protothecoides*; and two strains of *Candida albicans* were obtained from a number of different clinical and environmental sources and were tested for their in vitro susceptibility to the antibacterial agent gentamicin. All *Prototheca* strains were susceptible to gentamicin at concentrations between 0.3 and 0.9 $\mu\text{g/ml}$. A modified macrobroth dilution MIC assay with a colorimeter and a microbroth dilution assay with a 96-well plate reader were the two methods used to determine the MICs.

Prototheca spp. are achlorophyllous, unicellular algae which are opportunistic pathogens for humans (3, 5, 13, 18) and animals (4, 6, 12, 16). Questions about appropriate therapy for protothecosis still exist. Antialgal agents have not been developed as a class, so other agents must be found. Antifungal agents such as amphotericin B and ketoconazole may work for the treatment of algal infections, because of the obvious similarities between fungi and algae and the modes of action of these two antifungal agents, but little documentation is found in the world literature. Conventional methods for predicting fungal susceptibilities are not reproducible and do not correlate clinically (7-9, 17). Also, there is no evidence that the recommended susceptibility testing methods for fungi will work with algae such as *Prototheca* spp. Previous investigators have shown that *Prototheca* spp. are susceptible to gentamicin by using a disk diffusion assay (2, 11), but there have been no reports of therapeutic use of gentamicin for protothecosis. This investigation was undertaken to confirm the patterns of susceptibility of *Prototheca* spp. to gentamicin and also to devise accurate, reproducible methodologies for predicting protothecal susceptibilities.

Susceptibility test medium as described by Pore et al. (16) was modified by increasing the morpholinepropanesulfonic acid (MOPS) concentration from 10 to 30 mM, increasing the dextrose concentration from 20 to 30 mM, eliminating the rose bengal, and autoclaving the ingredients at 17 lb/in² and 121°C for 15 min instead of using filter sterilization.

Prototheca spp. were cultured on Sabouraud dextrose agar medium (Becton Dickinson, Cockeysville, Md.) at 35 \pm 1°C for 48 h, harvested with an inoculating loop, and placed into 4 ml of susceptibility test medium in a Falcon 2057 tube (17 by 100 mm; Becton Dickinson, Lincoln Park, Nebr.) The inoculum was adjusted to 95% transmittance or about 4 \times 10⁴ cells per ml in 1.5 ml of susceptibility test medium in a Falcon 2054 tube (12 by 75 mm; Becton Dickinson) by using a Hach colorimeter (DR100) equipped with a 450-nm filter (Hach, Loveland, Colo.). The inoculum density determined by the colorimeter was verified by three different methods. First, serial dilutions of the inoculum were prepared and each was plated onto Sabouraud dextrose agar medium to obtain CFU. The second method used a hemocytometer (improved Neubauer Ultra plane; American Scientific Products, McGraw Park, Ill.), and the third method used a

Coulter counter (model ZM; Coulter Electronics, Inc., Hialeah, Fla.). Two-tenths milliliter of the adjusted inoculum was added to 0.8 ml of STM and 0.5 ml of a 3 \times gentamicin solution in a tube (12 by 75 mm). The tubes were placed on a rotary drum (New Brunswick Scientific, Edison, N.J.) at 0.2 rpm and incubated at 35 \pm 1°C for 48 h in the dark. After 48 h, the tubes were analyzed for turbidity by using the colorimeter. The MIC cutoff point was determined by the following criteria: the tube with the lowest concentration of antibiotic that clearly inhibited visible growth was determined to contain the MIC per the National Committee on Clinical Laboratory Standards (14). It was determined that the MIC would equal \geq 97% transmittance when read on a colorimeter.

For the microbroth dilution assay, the inoculum was prepared exactly as for the macrobroth dilution assay. A 96-well tissue culture plate (3595; Costar Corp., Cambridge, Mass.) was used, and all reagent and inoculum volumes were reduced exactly 10 \times . Instead of using a 1.5-ml total volume as in the macrobroth dilution assay, a 0.15-ml total volume was used; likewise, 0.05 ml of antibiotic preparation was used. The plates were incubated at 35 \pm 1°C for 48 h. After 48 h, the plates were agitated with a 96-well plate shaker (American Scientific Products) for 15 min to uniformly distribute the cells in each well. After mixing, absorbance was read with a 96-well plate reader equipped with a 450-nm filter (Titertek, Multiskan plus MK11; ICN biomedical, Costa Mesa, Calif.). To corroborate the automated reader values, turbidity was visually confirmed. The MIC absorbance cutoff point for the 96-well plate reader was set at 0.016 absorbance, which is equal to 97% transmittance.

All strains used in this study were chosen at random from the culture collection of R. S. Pore. Of 100 *Prototheca* sp. strains tested, 7% were from human isolates, 35% were from animal sources, and 58% were from environmental sources. All strains were tested twice.

All *Prototheca* sp. strains tested were susceptible to gentamicin, with MICs between 0.3 and 0.9 $\mu\text{g/ml}$. The variability between species was as follows. The macrobroth dilution MICs (macro-MICs) for all *Prototheca zopfii* strains were between 0.4 and 0.8 $\mu\text{g/ml}$, and the macro-MICs for 66% of the strains were 0.6 $\mu\text{g/ml}$. The macro-MICs for all *Prototheca wickerhamii* strains were between 0.3 and 0.9 $\mu\text{g/ml}$, and the macro-MICs for 75% of the strains were 0.6 $\mu\text{g/ml}$. The macro-MICs for the five *Prototheca moriformis* strains were between 0.4 and 0.6 $\mu\text{g/ml}$, with an average of 0.5 $\mu\text{g/ml}$. The macro-MICs for the six *Prototheca stagnora*

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TABLE 1. Gentamicin MIC₅₀^a test results with 100 *Prototheca* strains

| Microorganisms (no. tested) | MIC ₅₀ (range) |
|--|---------------------------|
| <i>P. zopfii</i> (43) | 0.6 (0.4–0.8) |
| <i>P. wickerhamii</i> (43) | 0.6 (0.3–0.9) |
| <i>P. moriformis</i> (5) | 0.5 (0.4–0.6) |
| <i>P. stagnora</i> (6) | 0.4 (0.3–0.7) |
| <i>P. ulmnea</i> (3) | 0.3 (0.2–0.7) |
| <i>C. protothecoides</i> ^b (5)..... | 0.7 |
| <i>C. albicans</i> (2)..... | >10.0 |

^a MIC₅₀, MIC for 50% of the isolates tested. MIC₅₀s are given in micrograms per milliliter.

^b *C. protothecoides*, *Chlorella protothecoides*.

strains were between 0.3 and 0.6 µg/ml, with an average of 0.4 µg/ml. The macro-MICs for three strains of *Prototheca ulmnea* were between 0.2 and 0.7 µg/ml, with an average of 0.3 µg/ml. The macro-MICs for the two *Candida albicans* strains were both >10 µg/ml (Table 1). Excellent agreement was demonstrated between the micro- and macrobroth dilution data; overall, there was an 87% correlation at ±0.02 µg/ml.

MIC has been defined by the National Committee for Clinical Laboratory Standards as the lowest concentration of antibiotic that inhibits clearly visible growth. To partially overcome the inherent bias in the conventional susceptibility tests, guidelines that would allow the use of a 96-well plate reader and a colorimeter to analyze results for the macro- and microbroth dilution experiments were developed. The macro-MIC guidelines called for the MIC cutoff point to be set at 97% transmittance when being read by a colorimeter. This value was chosen for the following reasons. The macro-MIC tubes were inoculated with 8.0×10^3 cells from an inoculum of 4.0×10^4 cells per ml, which produced a colorimeter reading of 99% transmittance. During preliminary studies concerning the macro-MIC cutoff point, it was observed that an increase in cell numbers up to 8.6×10^3 cells per ml could be observed in the tube exhibiting the macro-MIC and still meet the visual criteria for the National Committee for Clinical Laboratory Standards definition of MIC. It was also observed that there was a 2% variation in light transmittance in a broth-filled tube; inoculum and defects in the tubes (12 by 75 mm) caused transmittance errors equaling 3%.

Recently, a Belgian investigator demonstrated that in the absorbance range between 0.0 and 0.6, a straight-line relationship exists between the A_{450} and A_{595} and the dry weight of microplate cultures, suggesting that culture absorbance is an accurate indicator of fungal or algal biomass (1).

Gentamicin is a commonly prescribed antibacterial agent, but there are no known reports concerning the treatment of an algal or fungal infection with gentamicin. In two reports, in vitro susceptibility to gentamicin has been described for *Prototheca* spp. In 1974, McDonald, Richard, and Anderson tested 48 strains of *P. zopfii* with 20 antibacterial agents and 7 antifungal agents by using a modified disk diffusion technique. They found that 37.5% of the strains tested were susceptible to gentamicin, the only aminocyclitol studied (11). In 1975, Cassal and Gutierrez performed the same study with eight strains of *P. wickerhamii* by using a modified disk diffusion assay and concluded that 100% of the strains tested were susceptible to gentamicin (2). During

preliminary studies in our laboratory, it was noticed that *Prototheca* spp. were susceptible to all aminocyclitols tested by using a disk diffusion technique but that gentamicin caused a larger zone of inhibition than did amikacin, kanamycin, netilmicin, streptomycin, and tobramycin.

Since MICs for *Prototheca* sp. strains were well within the therapeutically achievable levels of gentamicin in human serum of 4 to 10 µg/ml (19), the possibility that gentamicin may be an effective treatment for protothecosis was suggested. The experimental use of gentamicin on protothecosis in animals should be tested.

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