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Antimicrobial Susceptibility of *Ehrlichia phagocytophila*

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Human granulocytic ehrlichiosis is a recently described disease caused by an obligate intracellular gram-negative organism recently named *Ehrlichia phagocytophila*. To expand our knowledge of the susceptibility of *E. phagocytophila*, we tested six New York State isolates for susceptibility to 12 antimicrobials using an HL-60 cell culture system. All of the isolates were susceptible to doxycycline (MIC, ≤0.125 μg/ml; minimum bactericidal concentration [MBC], 0.125 to 0.5 μg/ml), rifampin (MIC, ≤0.125 μg/ml; MBC, ≤0.125 μg/ml), ofloxacin (MIC, ≤2 μg/ml; MBC, ≤2 μg/ml), levofloxacin (MIC, ≤1 μg/ml; MBC, ≤1 μg/ml), and trovafloxacin (MIC, ≤0.032 μg/ml; MBC, ≤0.032 μg/ml). Isolates were uniformly resistant to amoxicillin, ceftriaxone, erythromycin, azithromycin, clarithromycin, and amikacin. For one strain, the MBC of chloramphenicol was ≤8 μg/ml. These data suggest that quinolone antibiotics and rifampin may be alternative agents for patients with intolerance to tetracyclines.

Human granulocytic ehrlichiosis was first described in 1994 (2) and has subsequently been reported from other regions of the United States and from Europe (1, 7, 11). The infection is caused by the obligate intracellular pathogen *Ehrlichia phagocytophila. E. phagocytophila* is transmitted by *Ixodes* sp. ticks (10). Patients acutely infected with this organism demonstrate a rapid clinical response to doxycycline therapy (1, 2). Successful outcomes have also been reported in two patients who were treated with rifampin (4) and one who received chloramphenicol (5). However, spontaneous resolution of illness may also occur without any antimicrobial therapy. To date, only three isolates, one of which was from New York State, have been tested for susceptibility to antimicrobials (6). Alternatives to doxycycline are needed for treatment of young children, pregnant women, and patients intolerant of tetracyclines. In order to expand upon the limited existing data, we tested six New York State isolates of *E. phagocytophila* for susceptibility to a panel of 12 antimicrobials.

MATERIALS AND METHODS

Isolation and culture of New York *E. phagocytophila* isolates. The New York *E. phagocytophila* isolates tested were recovered as described previously from patients suspected of being infected (1, 6). Infected cells were propagated in HL-60 cells using RPMI 1640 medium without antimicrobials and supplemented with 10% heat-inactivated fetal bovine serum (FBS). Culture positivity in HL-60 cells and percent infection were based on detection of morulae in cytospin slide preparations stained with Wright's stain.

Sources of antimicrobials. The following antimicrobials were purchased from Sigma Chemical Co. (St. Louis, Mo.): amikacin, amoxicillin, ceftriaxone, chloramphenicol, doxycycline (as the hydrochloride form), erythromycin (as ethyl succinate), ofloxacin, and rifampin. Trovafloxacin mesylate (CP-062, 993-03; lot 25381-087-02) and azithromycin hydrate (CP-009, 219-27; lot 25381-088-02) were obtained from Pfizer (Groton, Conn.). Levofloxacin (RWJ-25213-097-AX) was a gift from R. W. Johnson. Clarithromycin (A-56268.0; lot 456707-AX) was supplied by Abbott Laboratories (Chicago, Ill.).

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Preparation of antimicrobials. Stock solutions of amoxicillin, ceftriaxone, chloramphenicol, doxycycline, ofloxacin, levofloxacin, trovafloxacin, and amikacin were prepared in deionized distilled water. Erythromycin and azithromycin were dissolved in ethanol. We prepared rifampin by dissolving it in methanol. A stock solution of clarithromycin was prepared using acetone as the solvent. Antibiotics prepared in distilled water were filter sterilized by using 0.2 μm-pore-size filters (Nalge Company, Rochester, N.Y.). All stock solutions were serially diluted with the tissue culture medium RPMI 1640 prior to addition to cell cultures to yield the final concentrations that were tested.

Antimicrobials were tested at the following concentrations: doxycycline, 0.125, 0.5, 2, and 4 μg/ml; rifampin, 0.125 and 0.5 μg/ml; ofloxacin, 0.5 and 2 μg/ml; levofloxacin, 0.2, 1, 4, and 10 μg/ml; trovafloxacin, 0.032 and 0.125 μg/ml; amoxicillin, 32 μg/ml; ceftriaxone, 64 μg/ml; chloramphenicol, 8, 16, and 32 μg/ml; erythromycin, 8 μg/ml; azithromycin, 8 μg/ml; clarithromycin, 0.2, 1, 4, and 10 μg/ml; amikacin, 0.2, 1, 4, and 10, 16, and 32 μg/ml.

Testing of susceptibility of *E. phagocytophila* isolates to antimicrobials. Six New York State *E. phagocytophila* isolates (6008 and 6003, cultured in 1996; 7013, 7019, and NY13, cultured in 1997; and NY18, cultured in 1998) recovered from adult patients were tested for susceptibility to 12 antimicrobials in an in vitro HL-60 cell culture system. All strains were low passage (passaged less than six times). The New York State isolate previously tested for susceptibility (6) was not included in this study. When infection of HL-60 cells reached 25 to 30% (based upon the presence of morulae demonstrated by Wright staining), various concentrations of antimicrobials were added to infected cell cultures. Control cultures were performed without the addition of antimicrobial agents. For each antimicrobial concentration tested, 10-ml cultures in T-25 tissue culture flasks (Sarstedt, Inc., Newton, N.C.) containing 2×10^5 HL-60 cells/ml were used. The cells were cultured at 37°C in 5% CO_2 in medium containing RPMI 1640 medium supplemented with 10% heat-inactivated FBS. Two-milliliter aliquots of cells were removed from cultures on day 3 after the addition of antimicrobials (day 3 time point). The rest of the cultures were washed three times, each with 10 ml of antibiotic-free RPMI 1640 medium, and the cells were reseeded at 2×10^5 viable cells/ml in 5 ml of antimicrobial-free RPMI 1640 medium containing 10% FBS for an additional 5 days prior to harvesting (day 8 time point). For each time point, cytospin smear preparations were evaluated independently by four individuals. Infection was determined based on the presence of morulae by counting 200 cells after Wright staining. Tests were performed in duplicate with each antimicrobial for all experiments.

Definitions. The MIC of an antibiotic was defined as the lowest concentration of the antibiotic that reduced the percentage of *E. phagocytophila*-infected HL-60 cells by greater than 90% compared to control cultures at the day 3 time point. The minimal bactericidal concentration (MBC) of an antibiotic was defined as the lowest concentration of the antimicrobial that reduced the percentage of *E. phagocytophila*-infected HL-60 cells by greater than 90% compared to the control at the day 8 time point.

TABLE 1. Susceptibilities of six New York State isolates of *E. phagocytophila* to antibmicrobials

Antibiotic	MIC (μg/ml)	MBC (μg/ml)
Doxycycline	≤0.125	0.125–0.5
Rifampin	≤0.125	≤0.125
Ofloxacin	≤2	≤2
Levofloxacin	≤1	≤1
Trovafloxacin	≤0.032	≤0.032
Amoxicillin	≥32	≥32
Ceftriaxone	≥64	≥64
Chloramphenicol	>16	>8[b]
Erythromycin[a]	>8	>8
Azithromycin	>8	>8
Clarithromycin	>10	>10
Amikacin	>16	>16

[a] Erythromycin, erythromycin ethyl succinate.
[b] For a single isolate, the MBC was ≤8 μg/ml.

Viability of *E. phagocytophila*-infected HL-60 cells in the presence of the following concentrations of antibiotics was tested using the trypan blue exclusion test: doxycycline, 0.125 and 0.5 μg/ml; rifampin, 0.5 μg/ml; ofloxacin, 2 μg/ml; levofloxacin, 10 μg/ml; trovafloxacin, 0.125 μg/ml; amoxicillin, 32 μg/ml; ceftriaxone, 64 μg/ml; chloramphenicol, 32 μg/ml; erythromycin, 8 μg/ml; azithromycin, 8 μg/ml; clarithromycin, 10 μg/ml; amikacin, 10 μg/ml. Testing was performed with both 3-and 8-day specimens of all *E. phagocytophila* strains. Control samples without *E. phagocytophila* infection and *E. phagocytophila*-infected cells without antibiotics were also tested for viability.

RESULTS

All six isolates of *E. phagocytophila* were inhibited by doxycycline, rifampin, ofloxacin, levofloxacin, and trovofloxacin at concentrations lower than the National Committee for Clinical Laboratory Standards breakpoints for susceptibility to *Streptococcus pneumoniae, Haemophilus influenzae*, and enterobacteriaceae (except that no breakpoint has been established for trovofloxacin against enterobacteriaceae) (Table 1) (9). However, all strains were resistant to amoxicillin, ceftriaxone, erythromycin, azithromycin, clarithromycin, and amikacin using National Committee for Clinical Laboratary Standards guidelines for susceptibility of the aforementioned bacteria for which guidelines have been published. A single *E. phagocytophila* strain was susceptible to chloramphenicol at 8 μg/ml in the day 8 but not the day 3 cultures (Table 1). The MICs and MBCs were within 1 dilution of each other for each *E. phagocytophila* strain tested.

At the antibiotic concentrations tested, HL-60 cells had a mean day 3 viability of ≥85% with each antibiotic. On day 8, HL-60 cell viability was 95% with doxycycline, rifampin, ofloxacin, levofloxacin, and trovafloxacin. Viability was decreased in HL-60 cell cultures previously treated with amoxicillin (57%), ceftriaxone (43%), erythromycin (72%), azithromycin (77%), and clarithromycin (64%); as was that of control infected cells (32%).

DISCUSSION

Our data confirm and expand those of Klein et al. using the in vitro culture system developed by that group (6). In that study, only a single New York *E. phagocytophila* isolate was tested for antibiotic susceptibility (6). The six New York isolates of *E. phagocytophila* that we tested were susceptible in vitro to doxycycline, rifampin, levofloxacin, ofloxacin, and trovafloxacin. Klein et al. found that the three strains that they tested were susceptible to ciprofloxacin, ofloxacin, and trovafloxacin (6). We did not test for susceptibility to ciprofloxacin. Our data also demonstrate the lack of in vitro susceptibility of *E. phagocytophila* to macrolide antibiotics (e.g., clarithromycin) not tested previously. The six isolates of *E. phagocytophila* that we tested were uniformly resistant to amikacin. Klein et al. found variable susceptibility to gentamicin (6). In both studies, *E. phagocytophila* was not uniformly resistant to chloramphenicol. However, most strains were resistant to this agent.

It is of note that susceptibility testing for *E. phagocytophila* is a biologic assay and lacks standardization. Interobserver differences in interpretation can be expected. This was observed mainly with cultures demonstrating large numbers of infected cells when *E. phagocytophila* was resistant to the antimicrobial being tested. Variability was minimal in assays when *E. phagocytophila* was susceptible to the antimicrobial agent being tested. The lack of growth of *E. phagocytophila* in HL-60 cells exposed to antibiotics to which it appears susceptible does not seem to be due to antimicrobial toxicity to the cells because the cells were viable. However, by 8 days, *E. phagocytophila*-infected HL-60 cells with no antimicrobials, or with antimicrobials to which *E. phagocytophila* was not susceptible, were heavily infected and were frequently not viable. A caveat in interpreting the MBC data is that HL-60 cells with no visible morulae may have actually had viable *E. phagocytophila* so that the antimicrobial killing was not necessarily 100%. Klein et al. waited 11 days after removal of antibiotics before harvesting and did not get regrowth of *E. phagocytophila* (6). However, because *E. phagocytophila* replication usually occurs rapidly in HL-60 cells when no antibiotics are present, it is likely that growth would occur by 5 days if the cells were infected with viable organisms. In our culture system, HL-60 cell growth and viability were sacrificed by waiting 11 days. Therefore, we used the shorter postantibiotic wash culture duration of 5 days.

There are considerable clinical data indicating that doxycycline is successful for the treatment of *E. phagocytophila* even when patients are quite ill. Data on the use of rifampin in the clinical setting are limited to a few case reports (4). Because patients (particularly younger patients) may have self-limited disease (3), the role of antibiotics in effecting cure of *E. phagocytophila* infection must be evaluated critically. To date, large-scale, randomized studies of the treatment of *E. phagocytophila* infection have not been performed. Without such studies, and without in vivo animal model data, health care providers need some rationale for using antibiotics other than doxycycline in specific situations, such as those involving young children, pregnant women, or patients with intolerance to doxycycline. Given the labor and expense of the biologic assay used here, unless an automated system for discrimination of *E. phagocytophila*-infected from noninfected cells can be developed, it is unlikely that large numbers of isolates will be tested. However, based upon the in vitro data presented here and those of Klein et al. (6), rifampin and certain quinolones may be beneficial for the treatment of patients who are intolerant of doxycycline. Rifampin may be an alternative to doxycycline for children and pregnant women. Although trovafloxacin had the lowest MICs of any agent, due to the risk of hepatotoxicity and because alternatives exist, it should not be used for the treatment of *E. phagocytophila* infection. *E. phagocytophila* is resistant to

amoxicillin, azithromycin, and erythromycin, as well as the cephalosporin ceftriaxone, agents that may be used to treat *Borrelia burgdorferi* infection. Because of the potential for coinfection (8), doxycycline should remain the agent of choice for empiric treatment of patients with erythema migrans in areas where both *E. phagocytophila* and *B. burgdorferi* are endemic.

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