

Antibiotic Susceptibilities of *Anaplasma (Ehrlichia) phagocytophilum* Strains from Various Geographic Areas in the United States

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We tested the antibiotic susceptibilities of eight strains of *Anaplasma phagocytophilum* (the agent of human granulocytic ehrlichiosis) collected in various geographic areas of the United States, including Minnesota, Wisconsin, California, and New York. The results are homogeneous and show that doxycycline, rifampin, and levofloxacin are the most active antibiotics against these strains in vitro.

The agent of human granulocytic ehrlichiosis (HGE) was first described in 1994 in the upper Midwest of the United States (3, 4, 11) and then in other regions of this country (1, 19, 34) as well as in Europe (5, 23, 31, 32). The HGE agent, *Ehrlichia phagocytophila* (the agent of tick-borne fever of sheep and cattle in Europe), and *Ehrlichia equi* (the agent of equine and canine granulocytic ehrlichiosis) have recently been moved into the genus *Anaplasma* in the reorganized family *Anaplasmataceae* and unified in a single species, *Anaplasma phagocytophilum* (10, 12, 14). Wild rodents, deer, sheep, cattle, and horses are probable reservoirs of these bacteria (28), whereas ticks, including *Ixodes scapularis* and *Ixodes pacificus* in the United States and *Ixodes ricinus* in Europe, are considered major vectors for human transmission (9, 28). HGE is most often an asymptomatic to mild disease, but more-severe and even fatal cases have been described (4, 28). Doxycycline is the drug of choice for treating patients with HGE (1, 3, 4, 12, 13). However, safe alternatives to tetracyclines may be needed or desired for children younger than 8 years old because of the potential for tooth discoloration, for pregnant women because of the danger of bone toxicity for the fetus, for allergic patients, and for patients with gastric intolerance to these compounds (30). Failures in chloramphenicol treatment of patients with HGE have been reported (12), whereas the clinical usefulness of rifampin for treatment of pregnant women with HGE has been suggested (8). Only a few in vitro studies have assessed the antibiotic susceptibilities of this species (21, 22), and recent investigations have clearly delineated the diversity and heterogeneity of *A. phagocytophilum* strains of different geographic origins (2, 24). Here, we report the antibiotic susceptibilities of eight strains collected from human or animal sources in very different geographic areas of the United States.

A. phagocytophilum strains (Table 1) were grown in the human promyelocytic cell line HL-60 at 37°C in an atmosphere of 5% CO₂ with RPMI 1640 (18) supplemented with 1% fetal bovine serum and 2 mM L-glutamine as the culture medium.

Three times per week, HL-60 cells were counted to maintain a concentration between 2×10^5 and 10^6 cells/ml, while the percentage of infected cells was monitored by detection of intracellular morulae in cytospin slides stained with LeukoStat (Fisher, Pittsburgh, Pa.). On the first day of antibiotic susceptibility testing, infected cells were centrifuged ($400 \times g$ for 5 min) and the supernatant was replaced by fresh medium to allow removal of extracellular bacteria. Infected and uninfected HL-60 cells were mixed to obtain 3.0×10^5 cells/ml, of which 5% were infected as determined by LeukoStat staining. This cell suspension was dispensed into each well of 96-well microtiter plates (180 μ l per well).

Antibiotics were added at concentrations 10-fold higher than the desired final concentrations (20 μ l per well, with three wells for each of the eight antibiotic concentrations tested for each strain). Three wells receiving 20 μ l of drug-free RPMI 1640 served as growth controls. Plates were incubated at 37°C in 5% CO₂ for 3 days. Then, 100 μ l of incubation medium in each well was replaced by fresh RPMI 1640 (with or without the final antibiotic concentrations tested), and all plates were reincubated for an additional 3 days. The infection rate in each well was determined on the sixth day of incubation of cultures by preparing cytospin slides stained with LeukoStat as described above. At that time, at least 50% of the cells in all growth control wells were infected. The lowest antibiotic concentration resulting in $\leq 5\%$ infected cells was considered the MIC. This corresponded to significant reduction in bacterial growth compared with controls at the 95% confidence interval, as determined by Student's *t* test. The absence of antibiotic-induced cell toxicity was verified at the time of MIC determination. Viable cell counts were determined by trypan blue staining in the three wells corresponding to the MICs, and cell counts were compared with those in uninfected HL-60 controls at the 95% confidence interval by using Student's *t* test. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used to control the accuracy of the antibiotic concentrations used in previous experiments. MICs were determined by using Mueller-Hinton broth, with an inoculum of 10^5 bacteria/ml and an 18-h incubation at 37°C, according to NCCLS guidelines (27).

MICs determined for the *S. aureus* and *E. faecalis* control strains were in the ranges expected for all antibiotics. The

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TABLE 1. *A. phagocytophila* strains

Strain ^b	Source	Geographical origin	Isolation date (mo/day/yr)	No. of passages ^a
Webster	Human	Wisconsin	6/7/96	4 + 2
MRK ^c	Horse	California	7/25/97	15 + 2
Spooner	Human	Wisconsin	2/10/97	8 + 2
NY8	Human	New York state	3/25/97	4 + 1
97E13	Dog	Minnesota	10/30/97	2 + 2
Sawyer	Human	Wisconsin	3/3/97	2 + 2
98E4	Dog	Minnesota	6/16/98	3 + 4
Bayfield	Human	Wisconsin	8/30/99	5 + 5

^a The number of culture passages after primary isolation plus the number of additional passages needed for antibiotic susceptibility testing.

^b All strains except MRK are HGE agents.

^c MRK was formerly designated *E. equi*.

results for the tested *A. phagocytophilum* strains are summarized in Table 2. All strains were resistant to the beta-lactam compounds ampicillin and ceftriaxone, to the aminoglycoside amikacin, to the macrolide compound erythromycin, and to the azalide compound azithromycin. The combination of sulfamethoxazole and trimethoprim was effective only at high concentrations, and a few morulae were still visible at the highest concentration tested (i.e., 100 µg of sulfamethoxazole/ml and 20 µg of trimethoprim/ml) for all the strains. Compounds with a bacteriostatic activity included doxycycline (MICs, ≤0.03 µg/ml), rifampin (MICs, ≤0.03 µg/ml), levofloxacin (MICs ranging from 0.06 to 0.5 µg/ml), and chloramphenicol (MICs ranging from 2 to 8 µg/ml). Cell counts in infected HL-60 cultures with the various antibiotic concentrations were not statistically different from those in the respective drug-free controls, except for amikacin, which was toxic to HL-60 cells at a concentration of 128 µg/ml.

Our study confirms and expands the results of previous reports of the in vitro antibiotic susceptibilities of *A. phagocytophilum* (21, 22) and suggests that diversity in susceptibility to antimicrobial agents is infrequent despite the antigenic diversity of strains. Our method for determination of MICs for *Ehrlichia* spp. was original and different from previously described methods (6, 7, 21, 22). In these experiments, the point of inhibition of ehrlichial growth, rather than the reduction in the percentage of infected cells (21, 22), was used to determine MIC. This principle fits better with traditional standardized test methods recommended by the NCCLS (27). The method

was highly reproducible, and MICs were well defined. In contrast, some variability in results and difficulty in their interpretation were previously reported by Horowitz et al. (21), who used methods based on the reduction in the percentage of infected cells. Furthermore, our tests were made in triplicate and were repeated to allow statistical evaluation of data.

Doxycycline and rifampin were the most active drugs in vitro. Tetracycline compounds are considered the first-line antibiotics for treatment of ehrlichial diseases (1, 3, 4, 12, 13). These drugs also have the advantage of showing additional activity against *Borrelia burgdorferi*, the agent of Lyme disease, which is transmitted by the same tick vector (9). Ampicillin, ceftriaxone, and amikacin were not active in vitro. These compounds are ineffective in treatment of infections caused by obligately intracellular pathogens, including rickettsioses, Q fever (caused by *Coxiella burnetii*), and ehrlichioses (26). Chloramphenicol shows poor in vitro activity, with MICs very close to the peak concentrations achievable in human serum (17), and failures in the treatment of HGE patients with this antibiotic have been reported (12). The combination of sulfamethoxazole and trimethoprim also demonstrated poor in vitro activity. More surprisingly, erythromycin and azithromycin were not active, confirming the results of previous experiments by Klein et al. (22) and Horowitz et al. (21). *A. phagocytophilum* has also previously been shown to be resistant to clarithromycin (21). Natural resistance to macrolide and azalide compounds is most often associated with methylation or point mutation of specific nucleotides (most often adenosine

TABLE 2. MICs for *A. phagocytophila* strains

Antibiotic ^a	MIC (µg/ml) for strain:							
	Webster	MRK	Spooner	NY8	97E13	Sawyer	98E4	Bayfield
Ampicillin	>128	>128	>128	>128	>128	>128	>128	>128
Ceftriaxone	>128	>128	>128	>128	>128	>128	>128	>128
Amikacin	>64	>64	>64	>64	>64	>64	>64	>64
Chloramphenicol	4	4	2	2	8	2	4	2
Erythromycin	>16	>16	>16	>16	>16	>16	>16	>16
Azithromycin	>16	>16	>16	>16	>16	>16	>16	>16
Doxycycline	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03
Rifampin	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03	≤0.03
Levofloxacin	0.5	0.25	0.06	0.125	0.5	0.5	0.5	0.5
Sulfamethoxazole-trimethoprim ^b	50/10	25/5	100/20	25/5	25/5	50/10	100/20	100/20

^a Ampicillin, ceftriaxone, amikacin, erythromycin, rifampin, and sulfamethoxazole-trimethoprim were obtained from Sigma-Aldrich Chemical Co. (St. Louis, Mo.); azithromycin and doxycycline were obtained from Pfizer (Brooklyn, N.Y.); chloramphenicol was obtained from Parke-Davis (Morris Plains, N.J.); and levofloxacin was obtained from Ortho-McNeil (Spring House, Pa.).

^b Sulfamethoxazole-trimethoprim was used at a 5:1 (wt/wt) ratio.

2058) of 23S rRNA (33). These specific antibiotic resistance mechanisms in *A. phagocytophila* should be investigated.

Rifampin has been used successfully in a limited number of patients, mainly pregnant women with HGE (8). However, the use of rifampin as an alternative to tetracyclines should be considered cautiously because of the possibility of rapid selection of resistant ehrlichial populations, as has been demonstrated for many bacterial species (15, 20). Levofloxacin was active against all strains tested, confirming the findings of previous in vitro experiments (21, 22). Thus, fluoroquinolones might represent potential therapeutic alternatives to tetracyclines, but they have not received FDA approval for use in children and pregnant women (29). However, a *gyrA*-mediated resistance in the related species *Ehrlichia canis* and *Ehrlichia chaffeensis* that corresponds to a single amino acid difference in the GyrA protein in *A. phagocytophilum* has recently been described (25). Thus, acquired resistance in *A. phagocytophilum* due to a comparable mechanism should be expected, especially since MICs of levofloxacin, ranging from 0.06 to 0.5 µg/ml, are actually very close to the levels achievable in human serum (16). More clinical data are needed to define a safe alternative to tetracyclines for patients who have HGE. As discussed by Horowitz et al. (21), the choice of a therapeutic alternative should take into account the possibility of coinfection with *Borrelia burgdorferi*, since both bacteria are transmitted by the same tick vector (9, 28).

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