Susceptibility of Campylobacter Species to Nalidixic Acid, Enoxacin, and Other DNA Gyrase Inhibitors

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Nalidixic acid-resistant mutants of Campylobacter jejuni and C. coli as well as “C. laridis” strains showed cross-resistance to another DNA gyrase subunit A inhibitor, enoxacin (MIC, 32 µg/ml), whereas C. fetus subsp. fetus, C. fetus subsp. venerealis, and “C. hyointestinalis” strains were all susceptible to enoxacin (MIC, ≤2 µg/ml). All Campylobacter strains were resistant to novobiocin (MIC, 32 to 512 µg/ml), but most strains were susceptible to the other DNA gyrase subunit B inhibitors coumermycin A1 and clorobiocin.

The catalase-producing campylobacters include Campylobacter jejuni (the major gastrointestinal pathogen), C. coli, “C. laridis,” C. fetus subsp. fetus, C. fetus subsp. venerealis, and “C. hyointestinalis” (12). Nalidixic acid resistance has been used extensively for the differentiation of the catalase-positive Campylobacter species (13, 14). C. jejuni and C. coli are generally susceptible to nalidixic acid (30-µg disk), whereas the other species are resistant to this drug. However, nalidixic acid-resistant variants of C. jejuni may occur (2, 13, 17) and have been used for the selection of transconjugants in our studies of tetracycline resistance plasmid transfer in Campylobacter spp. (16). Nalidixic acid inhibits the A subunit of DNA gyrase, an enzyme required for DNA replication in Escherichia coli (5). Recently, analogs of nalidixic acid, for example, enoxacin (CI-919) and other 4-quinolones, with much greater intrinsic antibacterial activity have been developed (10). Resistance to novobiocin has also been noted in C. jejuni strains (11) and has been incorporated into selective media for the isolation of C. jejuni from stool specimens (3). Novobiocin inhibits the B subunit of DNA gyrase in E. coli (6). Coumermycin A1 and clorobiocin are novobiocin analogs which also inhibit E. coli DNA gyrase and interfere with DNA replication (8).

During biotyping studies, nalidixic acid-resistant variants of C. jejuni were occasionally observed around a 30-µg disk of nalidixic acid during testing of susceptible strains (H. Lior, unpublished data). In this study, the frequency with which nalidixic acid-resistant mutants arise in C. jejuni and C. coli was determined. Cross-resistance to other DNA gyrase inhibitors was also investigated.

Campylobacter species were either from our own collections or were kindly supplied by John Bryner, National Animal Disease Center, Ames, Iowa. All strains were obtained from different sources (human or animal) to avoid duplication of the same strain. Differentiation of the Campylobacter species has been described previously (4, 14), and the tests used were as follows: oxidase; catalase; growth at 25, 37, or 43°C; 1% glycine; 0.04% triphenyltetrazolium chloride; 0.1% trimethylamine N-oxide dihydrate; susceptibility to nalidixic acid and cephalexin; H2S production in triple sugar iron agar and ferrous sulfate-metabisulfite medium; DNase; and hippurate hydrolysis. All strains were stored at −70°C in glycerol citrate. Before susceptibility testing, isolates were streaked on Mueller-Hinton agar (Oxoid Ltd., London, England) and incubated at 37°C in a CO2 incubator with 7% CO2 and 85% humidity (C. jejuni, C. coli, “C. laridis,” and C. fetus subsp. fetus) or in jars with 5% O2–10% CO2–85% N2 (C. fetus subsp. venerealis and “C. hyointestinalis”). Single colonies were picked from Mueller-Hinton agar plates after 48 h of incubation, inoculated into 2 ml of Mueller-Hinton broth (Oxoid Ltd.), and incubated at 37°C for 24 h. Three representative colonies were tested. After incubation, the broth cultures were diluted 10-fold and inoculated onto antibiotic plates with a Steers replicator (15). The antibiotics tested were novobiocin (purchased from Sigma Chemical Co., St. Louis, Mo.) and nalidixic acid (purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis.). Coumermycin A1 was kindly provided by Hoffmann-La Roche Inc., Nutley, N.J., clorobiocin was provided by May and Baker, Ltd., Dagenham, Essex, England, and enoxacin was provided by Parke-Davis Canada Inc., Toronto, Ontario, Canada. Ciprofloxacin was provided by Miles Pharmaceuticals, West Haven, Conn. The diluted Campylobacter cultures contained approximately 107 CFU/ml and, consequently, the replicator delivered 104 to 106 CFU per spot to the antibiotic plates. MICs were determined after 24 and 48 h of incubation.

Mutant strains of C. jejuni and C. coli resistant to nalidixic acid and enoxacin were selected from single colonies after growth in broth at 37°C in a CO2 incubator to 108 CFU/ml. The cultures were concentrated 10 times by centrifugation and resuspended in Mueller-Hinton broth so that 3 × 109 to 7 × 109 CFU/ml was obtained. Aliquots of 0.1 ml were plated on Mueller-Hinton agar containing doubling dilutions of nalidixic acid and enoxacin. Plates were incubated at 37°C in a CO2 incubator and examined after 48 and 96 h.

The MICs of various DNA gyrase inhibitors for C. jejuni and C. coli are shown in Table 1. Some of the strains were found to be resistant to nalidixic acid, with MICs of up to 256 µg/ml. However, our sample of C. jejuni strains was heavily biased towards nalidixic acid-resistant strains which we encountered during biotyping studies. The MIC for 90% of the strains, therefore, was higher than would normally be encountered in routine testing of C. jejuni isolates.

Spontaneous nalidixic acid-resistant mutants of C. jejuni UA535 and C. coli UA37 arose at frequencies of 2.5 × 10−8 and 7.8 × 10−9, respectively, per cell plated on selective media containing 32 µg of nalidixic acid per ml. As the

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concentration of nalidixic acid was increased fourfold, the frequency of mutation decreased about 10-fold (<10^{-9} per cell plated). It was, therefore, difficult to select mutants on media containing 64 or 128 μg of nalidixic acid per ml. Similarly, enoxacin-resistant mutants of C. jejuni UA535 and C. coli UA37 arose at frequencies of 2.9 x 10^{-8} on 4 μg of enoxacin per ml but of only <10^{-9} on 8 μg of enoxacin per ml. Resistant mutants were stable, as defined by continued resistance, after multiple passages on drug-free medium.

All nalidixic acid-resistant strains of C. jejuni and C. coli (MIC >64 μg/ml) were also enoxacin resistant (MIC, 32 μg/ml). This cross-resistance was observed with the mutants which we selected (Table 2) as well as with nalidixic acid-resistant clinical isolates of C. jejuni and C. coli. The direct correlation between nalidixic acid and enoxacin was also seen for four strains of “C. laridis,” including UA487. “C. laridis” is a Campylobacter species which is naturally resistant to nalidixic acid (2, 13). In contrast, the other Campylobacter species examined showed no cross-resistance (Table 2). Despite the fact that all strains of C. fetus subsp. fetus, C. fetus subsp. venerealis, and “C. hyointestinalis” were resistant to nalidixic acid (MIC, 128 or 256 μg/ml), none had an enoxacin MIC of greater than 2 μg/ml. Subsequently, ciprofloxacin, another nalidixic acid analog, was tested. Nalidixic acid-resistant strains of C. jejuni and C. coli and “C. laridis” strains showed cross-resistance with ciprofloxacin (MIC, 8 μg/ml), whereas nalidixic acid-resistant strains of C. jejuni and C. coli and other Campylobacter species were all susceptible (MIC, ≤1 μg/ml).

Novobiocin, at a concentration of 5 μg/ml, has been used to select C. jejuni and C. coli from stool specimens (3). The novobiocin MICs for all of the Campylobacter species tested were ≥32 μg/ml. However, the four “C. laridis” strains tested appeared to be relatively more resistant to novobiocin than the other species (MIC, >512 μg/ml). Novobiocin, coumermycin A1, and clorobiocin all inhibited the B subunit of E. coli DNA gyrase (6, 8). It was, therefore, of interest to determine whether any cross-resistance existed between novobiocin and its analogs. Most C. jejuni and C. coli strains were highly susceptible to both coumermycin A1 and clorobiocin (Table 1), with MICs of clorobiocin ranging from 1 to 2 μg/ml and those of coumermycin A1 ranging from 0.25 to 1 μg/ml. C. fetus subsp. fetus and C. fetus subsp. venerealis strains were also highly susceptible, “C. laridis” strains, which were highly resistant to novobiocin (MIC, >512 μg/ml), were resistant to clorobiocin (MIC, 16 μg/ml) but susceptible to coumermycin A1 (MIC, 0.5 μg/ml).

Resistance to nalidixic acid can rapidly be selected in gram-negative bacteria (1). Experiments with Klebsiella, Providencia, Enterobacter, Serratia, and E. coli strains have, in general, revealed MICs for resistant mutants to be 8- to 16-fold higher than those for parent strains with the 4-quinolones and 16- to 64-fold higher with nalidixic acid (7). Thus, nalidixic acid-resistant mutants of C. jejuni and C. coli showed cross-resistance to enoxacin similar to that observed in other bacterial genera. It is of interest that “C. laridis,” a species in which all strains are characteristically nalidixic acid resistant, also showed cross-resistance to enoxacin. In contrast, the MICs of enoxacin for the other nalidixic acid-resistant Campylobacter strains (C. fetus subsp. fetus, C. fetus subsp. venerealis, and “C. hyointestinalis”), which were highly resistant to nalidixic acid, did not exceed 2 μg/ml.

Previous studies with nalidixic acid have shown that mutations affect either the A subunit of DNA gyrase in E. coli (5) or drug permeability (9). DNA replication has not been studied in Campylobacter species, and no DNA gyrase has yet been isolated from Campylobacter species, although it is likely that the mechanisms of nalidixic acid resistance are similar to those in other gram-negative bacteria. It is tempting to speculate that nalidixic acid resistance in Campylobacter species which show no cross-resistance (C. fetus and “C. hyointestinalis”) is mediated by a different resistance mechanism.
ance mechanism than that seen in the cross-resistant Campylobacter species (C. jejuni, C. coli, and “C. laridis”).

Most Campylobacter strains are resistant to novobiocin. Novobiocin and its analogs coumermycin A1 and clorobiocin inhibit the B subunit of E. coli DNA gyrase (6, 8). All C. jejuni and C. coli strains were susceptible to coumermycin A1 and clorobiocin (MICs, 0.25 to 2 μg/ml). Similarly, C. fetus and “C. hyointestinalis” strains were also highly susceptible. Only “C. laridis” were susceptible to at least two inhibitors of the B subunit of DNA gyrase. Therefore, resistance to novobiocin may depend on the inability of the antibiotic to penetrate the cell rather than any intrinsic resistance of the DNA gyrase to novobiocin.

ACKNOWLEDGMENTS
We thank D. C. Hooper for his assistance in obtaining some of the antibiotics and for his comments on the manuscript. J. Hargreaves and C. Berkowitz for technical assistance, and J. Bryner for strains. D.E.T. received a scholarship and L.-K.N. received a studentship from the Alberta Heritage Foundation for Medical Research.

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