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Susceptibility of *Enterobacter* to Cefamandole: Evidence for a High Mutation Rate to Resistance

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Cefamandole minimum inhibitory concentrations (MICs) of 10 strains of *Enterobacter* were determined by the ICS agar dilution and broth dilution procedures. Agar dilution MICs ranged from 1 to 8 μg/ml, with an inoculum of 10^4 organisms/spot. Broth dilution MICs were consistently higher, with an inoculum of approximately 7×10^5 organisms/ml. Seven strains showed MICs of ≥64 μg/ml. There was a marked inoculum effect in broth, and skipped tubes were often observed. Variants resistant to 32 μg/ml or more were isolated by direct selection and were shown to occur at a frequency of approximately 10^{-6} to 10^{-7}. A mutant showing a 16-fold increase in agar dilution MIC was also isolated by indirect selection. These variants and others isolated from broth in the presence of cefamandole were tested for ability to inactivate the antibiotic, using both a biological and a chemical procedure. Two distinct classes of variants were seen. Twelve of 28 were shown by both methods to inactivate the antibiotic, whereas the others, including the indirectly selected mutant, did not. The wild types were also negative by both tests. The higher cefamandole MICs of *Enterobacter* in broth, thus, appeared to reflect a high frequency of resistant variants that were not detected with the inoculum and end point criteria usually used in agar dilution methods. The ability of some variants to inactivate cefamandole may have resulted from a mutation that extended the activity of *Enterobacter* cephalosporinase to include this antibiotic.

Cefamandole, a semisynthetic cephalosporin, has a wide spectrum of antibacterial activity which has been shown to include *Enterobacter* species (3, 13, 18). However, discrepancies exist between the degree of susceptibility of *Enterobacter* found by different investigators. Wick and Preston (18), using an agar dilution technique, reported minimum inhibitory concentrations (MICs) of 8 μg/ml or less. Others have found *Enterobacter* to be less susceptible (13). It appeared that the discrepancies were due, at least in part, to higher MICs with broth dilution as compared with agar dilution methods (R. B. Kammer, personal communication).

Discrepancies between the results of dilution procedures can be due to several different factors. Spontaneous inactivation of the antibiotic may be more rapid in some broth media than in others (15). Antibiotic inactivation by the inoculum may reduce its concentration to subinhibitory levels in broth, but the phenomenon may be obscured in an agar dilution test by diffusion of more antibiotic to the site of inoculation from the surrounding agar. Finally, a high mutation rate to resistance may lead to full overnight growth in a tube of broth containing a concentration of antibiotic inhibitory to all but the mutant, a situation that may yield only a single colony in an agar dilution test. A high mutation rate may also be manifested by the phenomenon of "skipped" tubes in the broth dilution series (1). These phenomena reflect the genetic heterogeneity of the inoculum and the larger number of cells usually used in broth dilution procedures.

The purposes of our studies were to confirm the differences in the results of broth and agar dilution cefamandole susceptibilities with *Enterobacter* and to distinguish between the possible explanations.

MATERIALS AND METHODS

Bacterial strains. Six strains of *E. cloacae*, three of *E. aerogenes*, and one of *E. liquefaciens* (syn. *Serratia liquefaciens*) were obtained from the clinical microbiology laboratories at the University of Washington Hospital, Seattle. All were clinical isolates.

Susceptibility test methods. Cefamandole lithium was kindly supplied by Eli Lilly & Co. Stock

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solutions of 4,000 and 1,280 μg of active cefamandole base per ml were prepared in distilled water and stored at −20 C. Sodium cephalothin was obtained from Ames Co. and similar stock solutions were prepared.

Agar dilution procedures followed the protocol of a previous study (2). Mueller-Hinton agar (Difco) and $\log_2$ dilutions of antibiotic were used. Inocula were prepared by adjusting young broth cultures to the density of the barium sulfate standard used for the diffusion test (5). This was taken to equate to approximately 1.5×10^8 bacteria/ml. Further dilutions were prepared in Mueller-Hinton broth to yield the inoculum desired on the plate surface. Plates were seeded with a Steers replicator head (17) delivering approximately 0.002 ml/spot and incubated overnight at 35 C. The MIC was read as the lowest concentration of antibiotic that completely prevented visible growth, except that one colony was ignored with an inoculum of 10^4 organisms/spot, and three colonies were ignored with an inoculum of 10^5/spot.

Broth dilution MICs were determined in Mueller-Hinton broth according to the ICS protocol (2). The inocula were adjusted to equate to the barium sulfate turbidity standard and were then diluted in broth to yield the final concentrations required. Incubation conditions were those of the agar dilution test. The MIC was taken as the lowest concentration of antibiotic that inhibited growth, as judged by the production of visible turbidity. In several instances, the skipped-tube phenomenon was observed. This was manifested by one or occasionally two tubes failing to grow when growth occurred at the next highest concentration. Skipped tubes were ignored in reading the MIC.

Diffusion susceptibilities were determined by the standardized single disk procedure (5). Cefamandole disks contained 30 μg of antibiotic.

Procedures for detecting antibiotic destruction. A diffusion assay method based on that of Sabath (16) was used to determine the amount of cefamandole activity remaining after exposure to bacterial suspensions. The sample was centrifuged and then sterilized by filtration through a membrane filter (0.45 μm Millipore) before performing the assay. Filter paper disks (6-mm diameter) were loaded with 0.02 ml of the sample and of appropriate standards and placed on agar plates seeded with *Bacillus subtilis* spore suspension (Difco). Zones of inhibition were measured after 18 h of incubation at 35 C.

A modification (H. A. Ericsson, personal communication) of the method of Kjellander and Myrbäck (10) was used to test isolates for qualitative evidence of inactivation of cefamandole. A swab was used to inoculate an agar plate with an 18-h broth culture of a cefamandole-susceptible strain of *Sarcina lutea*. Colonies of *Enterobacter* were picked from agar plates and heavily streaked to the inoculated plate in the form of a cross. A cefamandole disk (30 μg) was placed in the center of the cross streak, and the plate was incubated overnight. Destruction of antibiotic diffusing from the disk by the *Enterobacter* was manifested by a clover leaf pattern of growth inhibition of the *Sarcina*, with growth adjacent to the *Enterobacter* streaks but inhibition between them. Strains of *Enterobacter* that did not inactivate the antibiotic had essentially no influence on the large circular zone of inhibition of the *Sarcina*. This procedure provides excellent conditions for expressing enzyme induction because the test strain is exposed to gradually increasing concentrations of antibiotic diffusing from the disk.

A modification (6) of the membrane technique of Knox and Smith (11) was also used to detect cephalosporinase activity. Membrane filters (Millipore) were laid on Mueller-Hinton agar plates and spot inoculated with the colonies to be tested and with *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922) as negative controls. After incubation for 6 h, the membrane was removed and placed on filter paper that had been moistened with a solution of 25 mg of cefamandole in 2% Andrade indicator per ml. Colonies were designated positive when they developed a deepening red color as compared with the background pink of the filter paper and control colonies. Final readings were made after 6 h.

Selection of resistant variants. The Lederberg replica plating technique was used to demonstrate the occurrence of spontaneous mutants in a bacterial population (12). Inhibitory plates contained 32 μg of cefamandole per ml. When the number of resistant bacteria in the inoculum had been increased sufficiently with replica plating, individual colonies were sampled. Each colony was touched with a sterile toothpick and transferred first to a control plate and then to an inhibitory plate.

Resistant variants were also selected directly by adding known numbers of organisms to melted agar at 45 C that contained inhibitory concentrations of cefamandole. Plates were poured and incubated overnight. Any colonies developing were scored, and their resistance was confirmed by lightly touching their surface with an inoculating needle and streaking over an octant of an antibiotic-containing agar plate.

RESULTS

Comparison of broth and agar dilution results. The results of agar dilution, broth dilution, and agar diffusion cefamandole susceptibility tests on the 10 strains of *Enterobacter* are shown in Table 1. Agar dilution MICs ranged from 1 to 8 μg of cefamandole per ml, with an inoculum of 10^4 organisms/spot. With an inoculum 10-fold higher, the MICs of five strains increased two- to fourfold. In several instances, one to three colonies grew in concentrations above the arbitrarily defined MIC for this inoculum. For example, two colonies of *E. cloacae* 9 were found on the 4 μg/ml plate and one each on the 8 and 16 μg/ml plates. Agar diffusion tests all yielded large zones, with diameters of 20 to 27 mm.

Duplicate broth dilution tests were made

TABLE 1. *Influence of method and inocula on Enterobacter susceptibility test results with cefamandole*

Strain	Agar dilution MIC (μg/ml) at inoculum size of (per spot):		Broth dilution MIC (μg/ml) at inoculum size of (per ml)[a]:			Agar diffusion zone diam (mm)
	10^4	10^5	7×10^4	7×10^5	7×10^6	
E. cloacae 2	1	2	16	64 (8)	64	24
			8	16		
E. cloacae 6	1	2	8	64 (8)	>64	27
			8	64		
E. cloacae 9	4	4	32	>64	>64	24
			16	>64		
E. cloacae 12	2	8	8	64 (16)	>64	24
			16	>64 (32)		
E. cloacae 16	2	2	4	16	32	25
			8	>64 (8, 32)		
E. cloacae 17	8	8	>64	>64	>64	20
			>64	>64		
E. aerogenes 4	2	4	2	16 (4)	64 (8, 16)	24
			2	64 (2, 8, 16)		
E. aerogenes 8	1	1	4	32 (1, 4, 8)	64	24
			2	4		
E. aerogenes 14	1	1	2	4	64	25
			2	8		
E. liquefaciens 11	4	16	16	64		25
			8	64 (16)	>64	

[a] Figures in parentheses show the antibiotic concentrations in "skipped" tubes that failed to grow in the broth dilution series.

with inocula of approximately 7×10^4 and 7×10^5/ml and single tests at 7×10^6/ml. Results were generally higher than agar dilution MICs, even with the lowest inoculum concentration tested. There was a marked inoculum effect with all strains except *E. cloacae* 17, and skipped tubes were frequently seen when an inoculum of 7×10^5 organisms/ml was used. These results strongly suggested a nonhomogeneous inoculum with respect to cefamandole susceptibility.

All 10 strains were resistant to cephalothin, as determined by the agar dilution procedure, with an inoculum of 10^4 organisms/spot. The MICs for strains 2 and 8 were 128 μg/ml. The cephalothin MICs for the other eight strains were >128 μg/ml.

Evidence for cefamandole-resistant mutants in the inoculum of broth dilution tests. The skipped-tube phenomenon was studied with *E. cloacae* strains 2 and 6, *E. aerogenes* strains 4 and 8, and *E. liquefaciens* strain 11. The broth cultures that grew in the next highest concentration of cefamandole after the skipped tubes were streaked to nutrient agar plates without antibiotic. The corresponding wild-type strains were also streaked to these plates. Single colonies were touched with an inoculating wire and spread on octants of agar plates containing 32 μg of cefamandole per ml. All isolates from the antibiotic-containing tubes grew, whereas those from the wild-type strains did not. Resistance was stable on subculture. Broth dilution tests seeded with 7×10^5 *E. aerogenes* 4 per ml, both from the tube containing the highest concentration in which growth had occurred and after overnight growth in the absence of antibiotic, each showed MICs of ≥400 μg/ml.

A mutant with increased resistance to cefamandole was obtained from *E. aerogenes* 4 by indirect selection using the Lederberg technique. This clone showed a 16-fold increase in agar dilution MIC over the wild type but otherwise behaved identically when tested by a battery of 20 biochemical tests.

The proportions of resistant variants in broth cultures of five strains were determined by incorporating 10^7 organisms in agar plates containing 32 μg of cefamandole per ml. The numbers of colonies growing are shown in Table 2. The frequencies of resistant cells were approximately 10^{-6} to 10^{-7}, which is compatible with a relatively high mutation rate to resistance. Repeated subculture of *E. aerogenes* 8 at intervals of 6 h resulted in no obvious change in the proportion of cells resistant to 32 μg/ml, which implies either a high back mutation rate or some selective disadvantage for these mutants.

A kinetic growth study of *E. cloacae* 9 was made to demonstrate the increase in the pro-

TABLE 2. *Number of resistant colonies found in plates containing 32 μg of cefamandole per ml and inoculated with 10^7 organisms*

Strain	No. of resistant colonies per plate containing 10^7 organisms[a]
E. cloacae 9	7, 2
E. cloacae 12	4, 2
E. cloacae 16	2, 1
E. aerogenes 8	6, 10
E. liquefaciens 11	3, 3

[a] Each frequency determination was performed twice for each strain.

portions of resistant variants with time in broth containing 16 μg of cefamandole per ml. Conditions in the experiment were designed to simulate those in a tube of a broth dilution susceptibility test. A total of 10 ml of broth containing 7×10^5 organisms/ml was added to an equal volume of broth containing cefamandole. Samples were removed during incubation, and viable counts were determined in the absence of antibiotic and in the presence of 16 μg/ml. The results are summarized in Table 3 and are fully compatible with the selection of a resistant mutant cell type by cefamandole, whereas most of the wild-type cells were killed by the antibiotic. The somewhat higher count at 18 h in the absence of the antibiotic may reflect growth of surviving wild-type cells after destruction of antibiotic by the mutant.

Mechanisms of resistance to cefamandole. Twenty-two variants resistant to 32 μg of cefamandole per ml were isolated from five strains of *Enterobacter* using the poured plate method described above. The resistant colonies were streaked to agar plates in the absence of antibiotic and incubated overnight at 35 C. Single colonies were picked to agar slants, and their resistance was checked by streaking to plates containing 32 μg of the antibiotic per ml. Tests for antibiotic inactivation by these clones, by the wild types, and by the colony selected by the Lederberg procedure were made using both the "clover leaf" procedure and the membrane filter technique. The results of the two methods were identical and are shown in Table 4. Ten of 22 resistant variants inactivated the antibiotic. The other 12, the wild types, and the indirectly selected mutant did not. Similar findings were obtained with the six resistant variants isolated from the broth dilution tests. Those derived from *E. aerogenes* 4 and 8 inactivated the antibiotic in contrast to the other four. All wild-type strains and variants showed marked inactivation of cephalothin by this procedure.

TABLE 3. *Kinetic growth study of E. cloacae 9 in broth containing 16 μg of cefamandole per ml*

Length of incubation (h)	Viable count per ml	No. of resistant variants per ml
0	7×10^5	<10
6	7×10^4	4×10^2
18	8×10^8	5×10^8

TABLE 4. *Cefamandole-inactivating ability of resistant variants*

Parent strain	No. of resistant variants tested	No. of variants inactivating cefamandole
E. cloacae 9	5	2
E. cloacae 12	4	2
E. cloacae 16	3	3
E. aerogenes 8	5	3
E. liquefaciens 11	5	0

The finding that two different types of resistant variants were being selected, one that inactivated cefamandole and one that did not, was confirmed in washed cell suspension experiments using the wild-type *E. aerogenes* 4 and the two types of variants derived from it. Equal volumes of 10^9 cells in 0.1 M phosphate buffer (pH 7.4) were added to 32 μg of cefamandole per ml and incubated at 37 C. A control tube containing only 16 μg of cefamandole per ml was similarly incubated. The residual antibiotic was bioassayed after 18 h of incubation. The percentages of the initial concentrations of cefamandole remaining were 75% in the control, 66% with the wild type, 60% with the mutant selected by the Lederberg procedure, and below the levels of detection with the variant that inactivated antibiotic in the qualitative tests. Essentially identical results were obtained with final concentrations of 50 μg of cefamandole per ml.

DISCUSSION

These studies have confirmed the discrepancies between broth and agar dilution cefamandole susceptibility tests with *Enterobacter*. Cefamandole agar dilution MICs with the standard inoculum of 10^4 organisms indicate inhibition by readily achieved blood levels. However, broth dilution MICs were substantially higher, there was a marked inoculum effect, and skipped tubes were common. These discrepancies appear to be due to a relatively high mutation rate to resistance. The higher MICs in broth dilution tests were a result of the greater chance of a resistant variant being

present in the larger numbers of cells used as inocula and the fact that a single cell would yield overnight growth in broth. The skipped tubes are a statistical phenomenon and reflect the absence of resistant variants in the inoculum for that particular tube.

Enterobacter species are highly resistant to most cephalosporin antibiotics, including cephalothin and cephaloridine. They produce β-lactamase cephalosporinases (6, 7, 9, 14), which are capable of destroying the antibiotics. These enzymes appear to be an important determinant of the resistance of *Enterobacter*, in that loss through mutation may be associated with regaining antibiotic susceptibility (8). Other factors may be involved in resistance (4), including relative lack of permeability to the antibiotics. The activity of cefamandole against *Enterobacter* has been correlated with stability of the antibiotic to cephalosporinase (18). We were unable to detect inactivation of cefamandole by any of the wild types that we studied.

Two classes of cefamandole-resistant variants were isolated in this study. Half of the variants gained the ability to inactivate cefamandole, whereas the other half did not. Mutation in the structural gene for the cephalosporinase may have extended its range of activity to include cefamandole and thus account for the resistance of the first class of variant. The second class, which included a mutant obtained by indirect selection, appears to owe its resistance to some other mechanism because inactivation of cefamandole could not be detected, even with the clover leaf procedure which provides good conditions for enzyme induction. However, the presence of low levels of intracellular cephalosporinases cannot be excluded.

The clinical significance of the relatively high mutation rate to cefamandole resistance remains to be determined. In studies on the in vitro effect of cephalothin and cephaloridine, Benner et al. (1) demonstrated a marked inoculum effect, found skipped tubes in broth dilution tests, and isolated resistant variants of *Klebsiella*. Despite this, these agents have proved useful chemotherapeutics in *Klebsiella* infections.

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