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Lack of Correlation Between β-Lactamase Production and Susceptibility to Cefamandole or Cefoxitin Among Spontaneous Mutants of *Enterobacteriaceae*

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A large number of cultures of gram-negative bacteria were examined for their susceptibility to various concentrations of cefamandole, cefoxitin, carbenicillin, and nalidixic acid. Heterogeneity of susceptibility was demonstrated in individual cultures to all of these antibiotics. Resistant clones isolated from cefamandole or cefoxitin plates were examined for β-lactamase production. Approximately 13% of 262 resistant clones acquired the ability to produce a β-lactamase. Examination of the substrate profile of the β-lactamases from some of these clones revealed no change in the specific activity of these enzymes for cefamandole, cephaloridine, or compound 87/312 as compared with their parental enzymes. This study clearly shows that some resistant clones do not produce β-lactamases, whereas some susceptible strains produced significant amounts of these enzymes. We conclude from these findings that little correlation exists between β-lactamase production and decreased susceptibility to cefamandole or cefoxitin. The results suggest the possibility that characteristics other than β-lactamase production may be responsible for resistance in *Enterobacteriaceae*.

A new semisynthetic cephalosporin, cefamandole, exhibits a wide spectrum of antibacterial activity (2, 4, 5, 7, 12, 13, 18, 19). Extension of the spectrum of cephalosporin activity by this compound is particularly noteworthy in that it includes many *Enterobacter* species and *Haemophilus influenzae* (8). It has been suggested that the ability of cefamandole, as well as cefoxitin, to inhibit growth of *Enterobacter* spp. is in part due to the stability of these compounds to hydrolysis by bacterial β-lactamases (15, 19). However, evidence that β-lactamase is a limiting factor in bacterial susceptibility has been obtained in only a few instances, specifically, resistance to some penicillins by *Staphylococcus aureus* (3), *H. influenzae* (9), and *Neisseria gonorrhoeae* (1, 16). Among the majority of gram-negative bacteria, this relationship is somewhat less certain.

Recently, a paper by Findell and Sherris reported data suggesting that the discrepancy between broth and agar dilution minimal inhibitory concentration (MIC) measurements of cefamandole toward *Enterobacter* could be explained by a high spontaneous mutation rate to resistance (6). In that study, two types of resistant isolates were found, one type which produced a β-lactamase and one in which no β-lactamase could be detected. These findings raise some interesting questions about the relationship of spontaneous resistance and β-lactamase production to the clinical efficacy of cefamandole.

We examined a large number of gram-negative bacteria, including laboratory strains and isolates from a clinical trial for efficacy of cefamandole. In addition, resistant derivatives from a representative group of these strains were isolated and examined. We quantitated the spontaneous appearance of resistant progeny, production of β-lactamase, and susceptibility of these bacterial strains and isolates to cefamandole, cefoxitin, carbenicillin, and nalidixic acid.

MATERIALS AND METHODS

Bacterial strains. Cultures of *Enterobacteriaceae* used were laboratory strains from our culture collection or recent clinical isolates (designated by the prefix C) received from a number of laboratories involved in clinical trials of cefamandole. All cultures were identified by the API-20 system (Analytab Products, Inc., Plainview, N.Y.).

Susceptibility testing. Antibiotic susceptibility was measured by the agar dilution procedure with $\log_2$ dilutions of the antibiotics added to a Penassay-type agar. Cultures were grown overnight in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and diluted 1:100 with sterile water and then 1:10 in 0.25% agar. A 19-prong metal replicator which delivers

approximately 10^4 bacteria per spot was used to inoculate the plates. The MIC was determined as the lowest concentration showing no growth (or less than four isolated colonies) after overnight incubation at 37°C. Antibiotic solutions were freshly prepared in sterile water from standard powders obtained from the following sources: cefamandole lithium salt and cephaloridine, Eli Lilly & Co., Indianapolis, Ind.; cefoxitin sodium salt, Merck & Co., Inc., Rahway, N.J.; nalidixic acid, Calbiochem, San Diego, Calif.; and carbenicillin disodium salt, Roerig Pharmaceuticals, New York, N.Y.

β-Lactamase determination. (i) Spot plate assay. β-Lactamase activity of whole cultures was determined by a spot plate assay as described previously (8) by using a chromogenic cephalosporin substrate, compound 87/312, obtained from Glaxo Research Ltd., Greenford, Middlesex, England. A color change from yellow to red within 10 min was used as the end point.

(ii) Spectrophotometric assay. Quantitation of β-lactamase in crude extracts was determined spectrophotometrically with compound 87/312 (14). Specific activities of the β-lactamase using cefamandole and cephaloridine as substrates were determined as previously described (11). Specific activity was calculated as micromoles of substrate hydrolyzed per hour per milligram of protein.

Crude cell-free extracts were prepared from late-log-phase (6-h) cultures. The cells were collected by centrifugation at 20,000 × *g* for 10 min, washed once with 0.05 M phosphate buffer, pH 7.0, and subjected to sonic disruption with the microprobe of a sonic oscillator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). Cell debris was removed by centrifugation at 20,000 × *g* for 10 min. Protein content of the crude extracts was determined by the method of Lowry et al. (10).

Induction of β-lactamase. Induction of β-lactamase was determined in a number of strains by using cefamandole as the inducer at one-fourth of its MIC. Cefamandole was added to a 4-h culture in brain heart infusion broth, and incubation was continued for another 2 h. Noninduced cultures were prepared simultaneously by adding a corresponding amount of water at 4 h to duplicate cultures.

Determination of resistance frequency and selection of resistant mutants. The number of resistant cells in a 6-h culture was determined by spreading 0.1 ml of $\log_{10}$ dilutions on plates containing increasing concentrations of antibiotic. The frequency of resistant mutants at a particular antibiotic concentration was calculated by dividing the number of cells per milliliter growing at that antibiotic concentration by the number of cells per milliliter in the absence of antibiotic. Putative resistant clones (9 to 15) were selected from colonies growing on the antibiotic-containing plates. These isolates were inoculated into brain heart infusion broth and incubated overnight at 37°C. The agar dilution MIC and spot plate β-lactamase activity of these isolates was determined with these subcultures.

RESULTS

The heterogeneity of susceptibility in a culture of *Enterobacter aerogenes* EB-1 to cefamandole is shown in Table 1. The agar dilution MIC for this culture was 8 μg/ml at an inoculum of 10^4 cells per spot. However, at higher cell densities, it was apparent that the culture contained resistant cells. As the antibiotic concentration was increased, the number of resistant clones recoverable from the culture decreased. At 80 μg/ml (10 times the MIC) the number of resistant cells per ml appeared to become constant. At this concentration, the ratio of resistant cells to total cells was 3×10^{-6}, or 3 resistant clones in 10^6 cells. Thus, not only are there cells in the culture with MICs above 8 μg/ml, but there also is a heterogeneity among these resistant clones.

Seven strains of *E. aerogenes*, seven strains of *Enterobacter cloacae*, three strains of *Proteus*, two strains of *Klebsiella pneumoniae*, and one strain of *Escherichia coli* were examined, and the frequency of occurrence of resistance to cefamandole, cefoxitin, carbenicillin, and nalidixic acid was determined. For cultures subjected to the selective pressure of cefamandole and cefoxitin, the resistant isolates were examined for the presence of β-lactamase by a semiquantitative spot plate procedure (8).

The frequency of isolation of clones resistant to these four antibiotics varied from a high of 1.5×10^{-4} to 3.3×10^{-9}. None of these four antibiotics exhibited any special propensity to select for resistant clones among the four gram-negative genera examined, nor was the frequency of resistance found for any given antibiotic significantly different from the other three antibiotics. It is apparent, therefore, that the ability of these cultures to spawn resistant derivatives is not restricted to β-lactams and certainly not restricted to cefamandole.

Among the 182 resistant clones isolated in the presence of cefamandole, 38 were found to produce β-lactamase. However, 13 of these 38 were isolated from a parent that produced β-lacta-

TABLE 1. *Growth of E. aerogenes strain EB-1 on plates containing cefamandole*[a]

Concn of cefamandole (μg/ml)	Viable cells per ml
0	1.2×10^9
5	5.3×10^6
10	1.5×10^5
20	2.1×10^4
40	3.7×10^3
80	3.5×10^3

[a] The agar dilution MIC was 8 μg/ml. The frequency of resistant cells at 10 times the MIC was $(3.5 \times 10^3)/(1.2 \times 10^9) = 3.0 \times 10^{-6}$.

mase. Thus, 25 (14.8%) isolates had acquired the ability to produce β-lactamase. Similarly, 80 strains were selected from cefoxitin-containing plates, of which 8 (11%) acquired the ability to produce β-lactamase.

Isolates from plates containing either cefamandole or cefoxitin were examined in detail for MICs to cefamandole and cefoxitin. Inducible and constitutive production of β-lactamase was also quantified by use of compound 87/312 as described above. A tabulation of the MICs and specific activity of β-lactamase in crude extracts from strain C32 and its derivatives is shown in Table 2. Culture C32 and 11 isolates from it exhibited an MIC range of from 3.1 to >100 μg/ml against cefamandole, and all were resistant to cefoxitin (MIC, >100 μg/ml).

When the spot plate (compound 87/312) assay was used, only isolates C32-175 and C32-171 were judged capable of producing β-lactamase. However, when β-lactamase was quantified in crude extracts of induced cultures of the remaining isolates, low levels of β-lactamase were found in the original culture, C32, and four of the resistant clones, C32-166, C32-172, C32-176, and C32-173. Among those cultures that produced low levels of enzymes after induction, the MIC toward cefamandole ranged from 3.1 to >100 μg/ml, thus indicating no clear correlation between MIC and β-lactamase. Only two strains, C32-175 and C32-171, produced large quantities of β-lactamase and had high (>100 μg/ml) MICs toward cefamandole.

Table 2 also shows similar data for strains C24 and C20. Strain C24 and all of the resistant clones isolated from it produced relatively large amounts of β-lactamase, but the group had a varied range of MICs toward cefamandole and cefoxitin. Strain C20 and its resistant clones also showed a varied MIC pattern, but none of these cultures were found to produce β-lactamase. Ability to produce β-lactamase or the lack thereof appears, therefore, to bear no direct relationship to susceptibility toward either cefamandole or cefoxitin.

Comparison of the specific activity, either in-

TABLE 2. *β-lactamase activity compared with agar dilution MIC values of resistant clones*

Strain	Agar dilution MIC (μg/ml) vs		β-Lactamase sp act[a]	
	Cefamandole	Cefoxitin	Noninduced	Induced
E. aerogenes				
C32	3.1	>100	1	2
C32-166	6.2	>100	1	2
C32-174	6.2	>100	1	1
C32-167	12.5	>100	1	1
C32-168	12.5	>100	1	1
C32-169	25	>100	1	1
C32-170	25	>100	1	1
C32-172	>100	>100	1	3
C32-176	>100	>100	1	6
C32-173	>100	>100	1	8
C32-175	>100	>100	304	291
C32-171	>100	>100	1,030	1,049
Proteus rettgeri				
C24	1.6	0.8	226	226
C24-194	1.6	0.8	201	187
C24-190	3.1	0.8	186	199
C24-191	3.1	0.8	179	202
C24-187	25	12.5	175	188
C24-188	25	12.5	159	173
C24-189	25	6.2	173	189
E. cloacae				
C20	3.1	25	ND[b]	ND
C20-130[c]	6.2	50	ND	ND
C20-129[c]	6.2	100	ND	ND
C20-123[c]	50	100	ND	ND
C20-125[c]	50	>100	ND	ND
C20-104	50	>100	ND	ND
C20-106	50	>100	ND	ND

[a] Specific activity expressed as micromoles of compound 87/312 hydrolyzed per hour per milligram of protein.
[b] ND, Not detected.
[c] Isolates selected from cefoxitin plates.

duced or uninduced, of β-lactamase in clinical isolates with their MICs to cefamandole and cefoxitin (Table 3) again shows the lack of agreement between MIC and enzyme production. An eightfold difference in MIC was found for cultures C43 and C9, although the β-lactamase activity was identical. Culture C14 showed a relatively low MIC and a fairly high level of induced β-lactamase. When cultures C41 and C5 are compared, it is clear that the culture with the lower MIC produced considerably more β-lactamase. Cultures C49 and C24 again show no agreement between β-lactamase and MIC. In this group of 11 cultures from two genera and four species, only two (C13 and C33) could be considered resistant by virtue of β-lactamase production on the basis of this type of analysis.

The possibility exists and has been suggested that spontaneous mutants recovered from experiments similar to those presented here or recovered without the selective pressure of cefamandole might owe their resistance to a qualitative change in the β-lactamase such that it had a higher specific activity against cefamandole than the wild-type enzyme (6). Table 4 shows the specific activity of β-lactamase in

TABLE 3. *β-Lactamase activity compared with agar dilution MIC values of clinical isolates*

Strain	Agar dilution MIC (μg/ml) vs		β-Lactamase sp act[a]	
	Cefamandole	Cefoxitin	Noninduced	Induced
E. cloacae				
C43	3.1	>100	1	2
C9	25	>100	1	1
C41	25	>100	56	55
C5	>100	>100	1	9
E. aerogenes				
C32	3.1	>100	1	2
C36	12.5	>100	1	4
C13	>100	>100	1	327
Proteus morganii				
C14	6.2	6.2	1	58
C33	25	3.1	140	382
Proteus rettgeri				
C49	0.8	6.2	0.3	3
C24	1.6	0.8	208	190

[a] Specific activity expressed as micromoles of compound 87/312 hydrolyzed per hour per milligram of protein.

TABLE 4. *Substrate profile of selected β-lactamases*

Strain	Agar dilution MIC (μg/ml) vs		Induced β-lactamase sp act[a]		
	Cefamandole	Cefoxitin	Compound 87/312	Cephaloridine	Cefamandole
E. colacae					
C20	3.1	25	0	0	0
C20-123	50	100	0	0	0
C41	25	100	55	28	12
C5	>100	>100	9	3	0
E. aerogenes					
C32	3.1	>100	2	0.6	0
C32-173	>100	>100	8	6	0
C32-175	>100	>100	291	227	1
C32-171	>100	>100	1,049	832	7
C13	>100	>100	327	212	0.5
Proteus morganii					
C14	6.2	6.2	58	21	0.3
C33	25	3.1	382	121	2
Proteus rettgeri					
C49	0.8	12.5	3	0.9	0
C24	1.6	0.8	226	106	15
C24-187	25	12.5	188	92	9

[a] Specific activity expressed as micromoles of substrate hydrolyzed per hour per milligram of protein.

crude extracts from selected cultures assayed against compound 87/312, cephaloridine, and cefamandole, along with the MICs for cefoxitin and cefamandole. No increase in β-lactamase activity was found between culture C20 and its more resistant derivative C20-123, even though the MIC of C20-123 for cefamandole is 16 times higher than that of C20. Culture C24-187 produced an enzyme with a slightly lower specific activity against all three substrates than its more susceptible parent C24, even though the MICs of C24-187 are 16-fold higher than those of C24.

Assay of the β-lactamases from resistant derivatives of culture C32 provides examples of the association of both high and low enzyme production with high MIC values. The enzyme from culture C32-173 had no detectable activity against cefamandole; however, the overall enzymatic activity of this culture was quite low. Cultures C32-175 and C32-171 produced large quantities of β-lactamase, although the specific activity of the enzymes against cefamandole was still low. Comparison of the ratios of specific activity of the β-lactamases from these cultures toward cephaloridine and compound 87/312 indicates that they have essentially the same substrate profile. These data indicate that resistant mutants selected in the presence of cefamandole may or may not produce large amounts of β-lactamase and that, of those cultures which produce increased amounts of enzyme, this enzyme does not appear to be qualitatively different from the parental enzymes.

DISCUSSION

Antibiotic-resistant clones were easily isolated from cultures of various *Enterobacteriaceae* in this study. This heterogeneity of susceptibility was, however, not limited to cefamandole but was also demonstrated with cefoxitin, carbenicillin, and nalidixic acid. Among 23 clones isolated in the presence of cefamandole or cefoxitin which exhibited a decreased susceptibility to the antibiotics, only 2 produced significantly larger amounts of β-lactamase than did their parental strains. Examination of the substrate profile of the β-lactamases from these isolates revealed no change in the specific activity of the enzymes toward cefamandole, cephaloridine, or compound 87/312, as compared with the parental enzymes. In a broader survey of resistance and β-lactamase production, about 13% of the isolates examined in a semiquantitative manner for enzymes were capable of producing β-lactamase. A similar percentage of β-lactamase producers was found with either cefamandole or cefoxitin as the selective agent. These results agree reasonably well with those reported in other studies in that two types of resistant clones are recoverable from *Enterobacteriaceae,* one which produces a β-lactamase and one which does not produce a β-lactamase (6). However, we found only about 13% of these clones to be β-lactamase producers, whereas Findell and Sherris (6) reported about 50% producers among resistant clones.

The most straightforward and popular explanation for resistance of bacteria to β-lactam antibiotics in organisms which produce a β-lactamase involves enzymatic destruction of the antibiotic. Certain cases of penicillin resistance, as mentioned above, with *Staphylococcus, Neisseria,* and *Haemophilus* appear unequivocal. The certainty of this relationship is not so apparent when most gram-negative bacteria are examined. The results of this study and others clearly show that some resistant clones do not produce β-lactamase and that some susceptible strains often produce significant amounts of this enzyme (6, 17). The incongruity of these findings suggests the possibility of other genetic determinants in *Enterobacteriaceae,* segregating independently from β-lactamase, which may be responsible for resistance more often than is β-lactamase production. Two mechanisms which are frequently proposed to explain non-β-lactamase-mediated resistance are decreased cell permeability and increased intrinsic resistance. An example of the difficulty of trying to explain resistance in bacteria to β-lactams by the change in a single characteristic is a recent finding (D. A. Preston, personal communication) that one of the well-studied culture pairs (*E. cloacae* P99 and P99N), presumably isogenic except for β-lactamase production and often cited as a clear demonstration of the role of β-lactamase in resistance, is in fact not isogenic. The β-lactamase-producing strain, P99, was found to be a poor pathogen for mice, whereas infection with strain P99N, the β-lactamase-negative strain, was easily established. Thus, because the isogenic relationship of this pair cannot be assured, one cannot be certain that the presence or absence of β-lactamase is responsible for the resistance pattern of these two strains.

Regardless of the in vitro behavior of an antibiotic, the most critical examination of the effectiveness of an antibiotic lies in its ability to treat clinical infections. Many of the strains used in this study were derived from successfully treated infections during the initial clinical trial of cefamandole. Therefore, although it is possible to isolate resistant clones from these bacterial cultures in the presence of cefamandole, conditions which exist in clinical infections apparently are not conducive to elaboration of the resistant organisms, and successful therapy can be achieved (L. R. Levine and E. McCain, Program

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