ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Apr. 1990, p. 622–627
0066-4804/90/040622-06$02.00/0

Vol. 34, No. 4

β-Lactamase Production in Members of the Family *Enterobacteriaceae* and Resistance to β-Lactam–Enzyme Inhibitor Combinations

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Received 12 June 1989/Accepted 26 December 1989

Recent reports that members of the family *Enterobacteriaceae* that produce high levels of certain β-lactamases are often resistant to ticarcillin-clavulanate prompted this study to assess the relationship between type and amount of enzyme produced and susceptibility to ticarcillin-clavulanate, piperacillin-tazobactam, and cefoperazone-sulbactam. Agar dilution MICs were determined by using 73 strains of *Enterobacteriaceae* that produced a single β-lactamase that had been characterized and quantified and a β-lactamase-negative control strain of *Escherichia coli*. For *E. coli* and *Klebsiella pneumoniae*, MICs of each combination increased as levels of TEM, SHV-1, or class IV enzymes increased. However, the percentage of strains that were resistant was highest for ticarcillin-clavulanate (32%), with only 18 and 6% resistant to piperacillin-tazobactam and cefoperazone-sulbactam, respectively. Strains producing PSE-1, regardless of level, were resistant or moderately susceptible to ticarcillin-clavulanate but were susceptible to piperacillin-tazobactam and cefoperazone-sulbactam. HMS-1 and OHIO-1 β-lactamases were associated with resistance to ticarcillin-clavulanate and piperacillin-tazobactam, respectively. High levels of class IV enzymes in *Klebsiella oxytoca* were associated with resistance to all three combinations. These results indicate that the level and type of β-lactamase produced by members of the family *Enterobacteriaceae* are important determinants of susceptibility to β-lactam–inhibitor combinations, especially ticarcillin-clavulanate.

Recent reports indicate that the β-lactamase inhibitor potassium clavulanate provides only partial protection from the hydrolytic action of TEM-1, SHV-1, and PSE-1 β-lactamases of some strains in the family *Enterobacteriaceae* (9, 14; J. L. Martinez, E. Cercenado, M. Rodriguez-Creixems, A. Delgado-Iribarren, and F. Baquero, Letter, Lancet ii:1437, 1987; H. Williams, A. King, K. Shannon, and I. Phillips, Letter, Lancet i:304–305, 1988; A. A. Medeiros, J. Martinez-Beltran, E. F. Papa, and C. O. O'Gara, Program Abstr. 28th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 491, 1988; M. Rodriguez-Creixems, E. Cercenado, P. Nieto, J. Garcia, M. Rivera, and E. Bouza, 28th ICAAC, abstr. no. 489, 1988). Failure to effectively inhibit these β-lactamases has been attributed to both the type of β-lactamase involved and the level at which it is produced (14; Medeiros et al., 28th ICAAC). Other studies (2, 5, 8), although not characterizing and quantifying β-lactamases, report resistance of *Enterobacteriaceae* to various combinations of β-lactam antibiotics and β-lactam inhibitors. These reports indicate a need to reexamine β-lactam–clavulanate combinations and also to investigate other β-lactam–inhibitor combinations to better understand their limitations. Therefore, a study was designed to assess the relationships between amount and type of β-lactamase and in vitro susceptibility to ticarcillin-clavulanate, piperacillin-tazobactam, and cefoperazone-sulbactam. To expand previous observations, a broader range of β-lactamases was included in this study. Also, since the purpose of the study was to compare the ability of each inhibitor to protect its companion drug, a single inhibitor concentration of 2 μg/ml was used. This allowed direct comparisons to be made.

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MATERIALS AND METHODS

Strains. Seventy-three strains of *Enterobacteriaceae* that produced a single β-lactamase and one fully susceptible, β-lactamase-negative strain of *Escherichia coli* (ATCC 25922) were included in the study. The β-lactamase-producing strains included clinical isolates which had been recovered from patients at the University of Texas M.D. Anderson Cancer Center, Houston (47 strains), and from patients at St. Joseph's Hospital, Omaha, Nebr. (13 strains) and 13 reference strains producing well-characterized β-lactamases. The latter included the following plasmid-mediated β-lactamases (kindly provided by A. A. Medeiros of Brown University, Providence, R.I.): TEM-1 [*E. coli* RTEM(R6K)], TEM-2 [*E. coli* 1725E(RP1)], OXA-1 [*E. coli* 1527(RGN238)], OXA-2 [*E. coli* 1573(R46)], OXA-3 [*E. coli* 1894E(R57b)], OXA-4 (*E. coli* 7259), OXA-5 [*E. coli* J53(pMG54)], OXA-7 (*E. coli* 7181), OHIO-1 (*E. coli* C600:075), SHV-1 [*E. coli* J53 (R1010)], and HMS-1 [*E. coli* J53.2(R997)]. L. Gutmann, University of Paris, Paris, France, provided TEM-7 (*E. coli*). The collection of isolates comprised 59 strains of *E. coli*, 12 strains of *Klebsiella pneumoniae*, and 3 strains of *Klebsiella oxytoca* and were chosen for the study because of their resistance to β-lactam antibiotics. They were not randomly chosen isolates.

Susceptibility tests. Susceptibility tests were performed by an agar dilution procedure (12) with the antibiotics (kindly provided by their respective manufacturers) prepared on the day of use. For tests involving β-lactamase inhibitors, the inhibitor was incorporated into the Mueller-Hinton agar at a constant concentration of 2 μg/ml. An inoculum of 10^4 CFU per spot was applied with a Steers inoculator (17) to the Mueller-Hinton agar plates.

MICs were interpreted by the National Committee for Clinical Laboratory Standards (NCCLS) guidelines. MICs of

TABLE 1. Comparative susceptibilities[a] of 74 study isolates to individual β-lactam antibiotics and β-lactam–inhibitor combinations

Agent	No. of strains (%)		
	Susceptible	Moderately susceptible	Resistant
Ticarcillin	3 (4)	2 (3)	69 (93)
Ticarcillin-clavulanate	28 (38)	22 (30)	24 (32)
Piperacillin	15 (20)	5 (7)	54 (73)
Piperacillin-tazobactam	55 (74)	6 (8)	13 (18)
Cefoperazone	54 (73)	6 (8)	14 (19)
Cefoperazone-sulbactam	65 (88)	3 (4)	6 (8)

[a] NCCLS interpretive criteria (see Materials and Methods).

TABLE 2. Comparative susceptibilities[a] of 42 TEM-1-producing *E. coli* to individual β-lactam antibiotics and β-lactam–inhibitor combinations

Agent	No. of strains (%)		
	Susceptible	Moderately susceptible	Resistant
Ticarcillin	1 (2)	0 (0)	41 (98)
Ticarcillin-clavulanate	14 (33)	17 (41)	11 (26)
Piperacillin	7 (17)	2 (5)	33 (78)
Piperacillin-tazobactam	34 (80)	4 (10)	4 (10)
Cefoperazone	32 (76)	3 (7)	7 (17)
Cefoperazone-sulbactam	41 (98)	1 (2)	0 (0)

[a] NCCLS interpretive criteria (see Materials and Methods).

≥128 μg of ticarcillin or piperacillin per ml and ≥64 μg of cefoperazone per ml indicated resistance, 32 to 64 μg of ticarcillin or piperacillin per ml and 32 μg of cefoperazone per ml indicated moderate susceptibility, and ≤16 μg of each drug per ml indicated susceptibility. These interpretive criteria applied to the drugs when they were tested alone and also in combination with β-lactamase inhibitors. The recommended NCCLS *E. coli* strains, ATCC 25922 and ATCC 35218, were included in the 74 strains because they constitute β-lactamase-negative and TEM-1-producing control strains for use in susceptibility tests of β-lactam antibiotics and β-lactam–clavulanate combinations.

Characterization of β-lactamases. Sonic extracts were prepared from 4-h cultures in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.). Washed, centrifuged cell pellets were subjected to 10 cycles of 15-s sonication treatments at 4°C, and all sonic extracts were frozen at −70°C until tested. β-Lactamases were characterized initially by determining their pIs in polyacrylamide gels (16), their susceptibilities to inhibition by cloxacillin and potassium clavulanate (16), and their abilities to hydrolyze penicillin G, cephalothin, and nitrocefin. These characteristics were then compared with those obtained with enzyme standards prepared from the reference strains. Additional substrates were examined as necessary whenever these initial characteristics did not provide definitive identification.

β-Lactamase assays. Substrate hydrolysis assays were determined in UV spectrophotometric assays monitoring the λ_{max} associated with the β-lactam ring (15). The amount of β-lactamase in sonic extracts was quantified by using nitrocefin as a substrate. One unit of activity was defined as the amount of enzyme hydrolyzing 1 nmol of nitrocefin per min per mg of protein in sonic extracts at pH 7.0 and 37°C. Relative hydrolysis rates were calculated by arbitrarily assigning a hydrolysis rate of 100 to penicillin to reflect relative activities of the enzymes in the sonic extracts.

RESULTS

Susceptibility profiles. The in vitro activities of the β-lactam–inhibitor combinations evaluated in this study differed considerably when the combinations were tested against strains which included a wide variety of types and levels of β-lactamases (Table 1). Among the 74 strains examined, the percentages of strains resistant to each drug alone were 93% for ticarcillin, 73% for piperacillin, and 19% for cefoperazone. When the inhibitors were added at 2 μg/ml, the percentages of strains resistant to each combination were 32% for ticarcillin-clavulanate, 18% for piperacillin-tazobactam, and 8% for cefoperazone-sulbactam. The percentages of susceptible isolates were as follows: cefoperazone-sulbactam, 88%; piperacillin-tazobactam, 74%; and ticarcillin-clavulanate, 38%. Thus, ticarcillin-clavulanate was considerably less active against this collection of *Enterobacteriaceae*.

These differences were further accentuated when results obtained with strains of *E. coli* which produced TEM-1 were considered. The percentages of strains resistant to each combination were 26% for ticarcillin-clavulanate, 10% for piperacillin-tazobactam, and 0% for cefoperazone-sulbactam, whereas the percentages of strains susceptible to each combination were 33% for ticarcillin-clavulanate, 80% for piperacillin-tazobactam, and 98% for cefoperazone-sulbactam (Table 2).

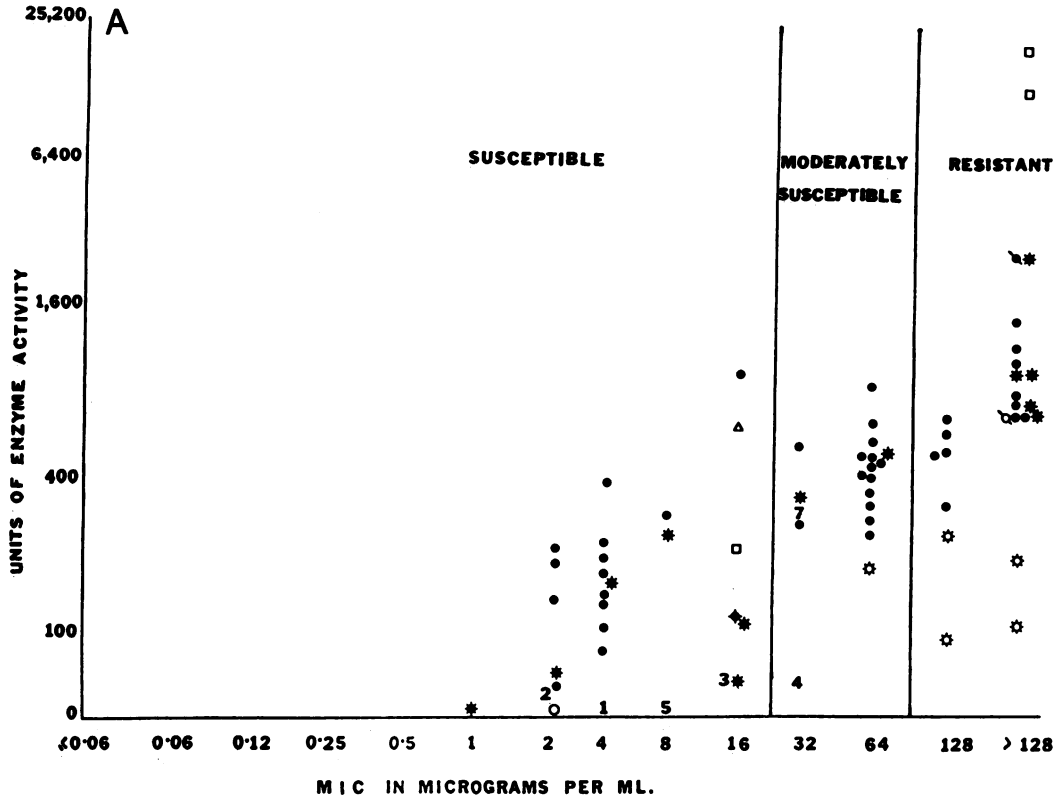
All combinations were generally less active against strains of *E. coli* ($n = 3$) and *K. pneumoniae* ($n = 10$) that produced SHV-1. The percentages of these strains that were resistant were 77% for ticarcillin-clavulanate, 46% for piperacillin-tazobactam, and 31% for cefoperazone-sulbactam (Table 3).

Correlation of susceptibility with enzyme type and level. With the exception of strains producing PSE-1, the susceptibility of the strains to each β-lactam–inhibitor combination was largely determined by the amount of β-lactamase produced (Fig. 1). This suggested that the protection provided by the inhibitors was limited and at times inadequate against strains of *Enterobacteriaceae* that produced high levels of certain β-lactamases. This problem affected ticarcillin-clavulanate more than piperacillin-tazobactam or cefoperazone-sulbactam. Production of high levels of TEM β-lactamases was associated with higher MICs of ticarcillin-clavulanate than of cefoperazone-sulbactam and piperacillin-tazobactam (Fig. 1). Strains of *K. pneumoniae* and *K. oxytoca* which produced high levels of SHV-1 and class IV β-lactamases,

TABLE 3. Comparative susceptibilities[a] of 13 SHV-1-producing isolates to individual β-lactam antibiotics and β-lactam–inhibitor combinations

Agent	No. of strains (%)		
	Susceptible	Moderately susceptible	Resistant
Ticarcillin	0 (0)	0 (0)	13 (100)
Ticarcillin-clavulanate	1 (8)	2 (15)	10 (77)
Piperacillin	1 (8)	2 (15)	10 (77)
Piperacillin-tazobactam	7 (54)	0 (0)	6 (46)
Cefoperazone	7 (54)	1 (8)	5 (38)
Cefoperazone-sulbactam	8 (61)	1 (8)	4 (31)

[a] NCCLS interpretive criteria (see Materials and Methods).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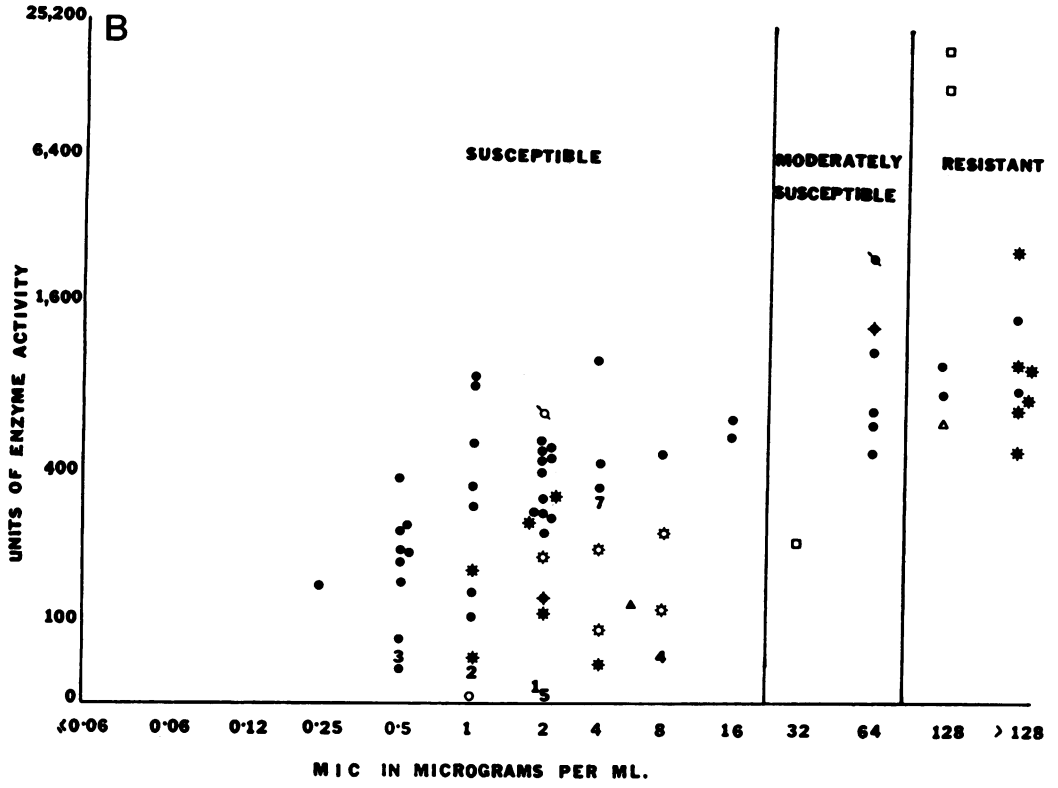
MIC IN MICROGRAMS PER ML.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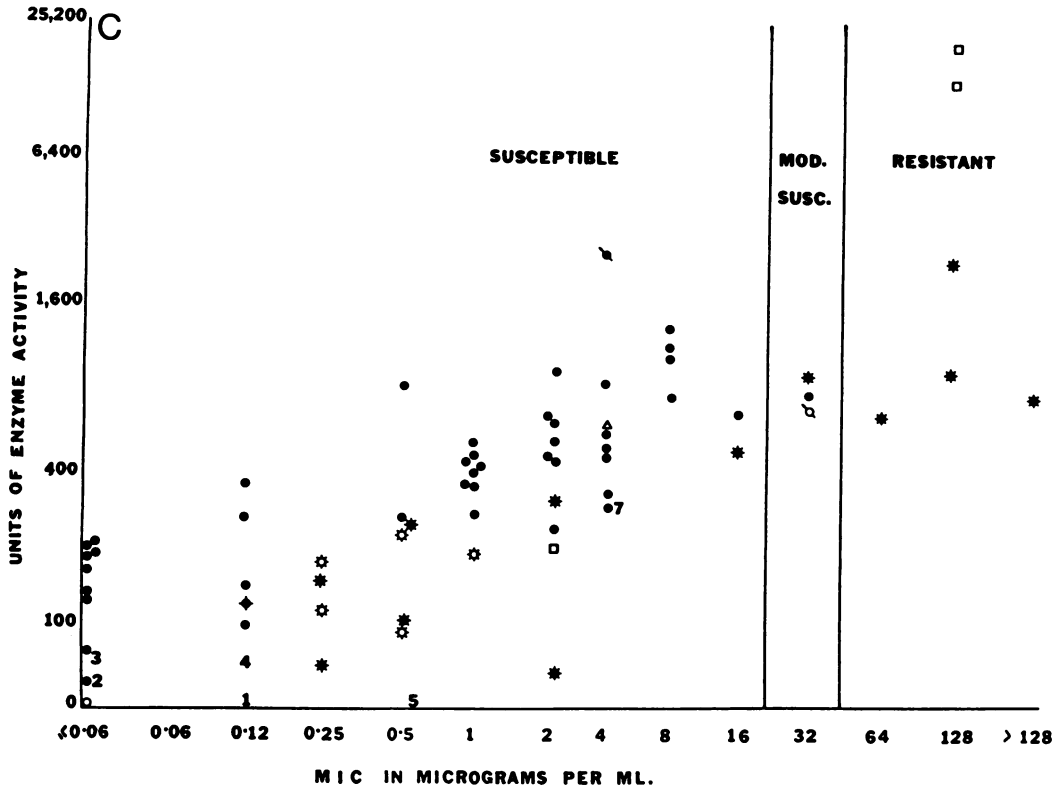

FIG. 1. Relationship between type and amount of enzyme produced and MICs for ticarcillin-clavulanate (A), piperacillin-tazobactam (B), and cefoperazone-sulbactam (C). Symbols: ●, TEM-1; ◔, TEM-2; ✦, TEM-7; ✸, SHV-1; ✿, PSE-1; □, class IV; △, OHIO-1; ◔, HMS-1; 1 through 7, OXA-1 through OXA-7; ○, ATCC 25922 (β-lactamase negative).

respectively, were resistant to all of the β-lactam–inhibitor combinations. However, strains producing lower levels of SHV-1 and class IV β-lactamases were susceptible to cefoperazone-sulbactam and, to a lesser degree, to piperacillin-tazobactam and to ticarcillin-clavulanate (Fig. 1).

In strains producing PSE-1, even low-level enzyme production was associated with resistance or moderate susceptibility to ticarcillin-clavulanate (Fig. 1A). In contrast, all strains producing PSE-1 were susceptible to piperacillin-tazobactam and cefoperazone-sulbactam regardless of the level of enzyme produced (Fig. 1B and C). The resistance to ticarcillin-clavulanate was probably due to the more rapid hydrolysis of ticarcillin by PSE-1 in comparison with the hydrolysis of piperacillin or cefoperazone by PSE-1 (Table 4).

Other β-lactamases which were associated with diminished susceptibility to the β-lactam–inhibitor combinations were OHIO-1, which was responsible for resistance to piperacillin-tazobactam; HMS-1, which was responsible for resistance to ticarcillin-clavulanate and moderate susceptibility to cefoperazone-sulbactam; and OXA-4 and OXA-7, which were responsible for only moderate susceptibility to ticarcillin-clavulanate (Fig. 1). The absence of a number of strains producing various levels of these enzymes precluded analysis of the role of amount of enzyme in susceptibility.

TABLE 4. Relative hydrolysis rates

Class IV	Relative rate of hydrolysis[a] in the following substrate:			
	Cephalothin (100 μM)	Ticarcillin (250 μM)	Piperacillin (250 μM)	Cefoperazone (100 μM)
TEM-1	4	2	18	6
Class IV	9	0.6	0.2	10.3
PSE-1	0.8	25	4	0.5
SHV-1	3	2	9	8

[a] Hydrolysis rate of 100 was arbitrarily assigned to penicillin, the standard.

DISCUSSION

Recent studies have reported the occurrence of resistance to amoxicillin-clavulanate and ticarcillin-clavulanate in clinical isolates of *Enterobacteriaceae* possessing β-lactamases that were previously thought to be adequately inhibited by clavulanate (9, 14; Martinez et al., Letter, Lancet; Williams et al., Letter, Lancet; Medeiros et al., 28th ICAAC; Rodriguez-Creixems et al., 28th ICAAC). In all studies there was a correlation between increasing resistance and increasing levels of TEM-1 production in *E. coli*.

The current study was more comprehensive than previous studies (7, 9, 14; Martinez, et al., Letter, Lancet; Williams et al., Letter, Lancet; Medeiros et al., 28th ICAAC; Rodriguez-Creixems et al., 28th ICAAC), and investigated a wider range of β-lactamases in *Enterobacteriaceae* and more diverse drug-inhibitor combinations. A major finding was that piperacillin-tazobactam and cefoperazone-sulbactam were usually much more active than ticarcillin-clavulanate against strains producing elevated levels of non-class I β-lactamases. However, increased resistance to all three combinations was associated with increased production of certain β-lactamases. This finding is consistent with other reports (9, 14; Martinez et al., Letter, Lancet; Williams et al., Letter, Lancet; Medeiros et al., 28th ICAAC; Rod-

riguez-Creixems et al., 28th ICAAC), in that resistance to some penicillins or cephalothin in combination with β-lactamase inhibitors increases with increasing levels of TEM-1 in *E. coli*. Some investigators, however, were unable to find good correlation between resistance and enzyme level for *E. coli* that produced OXA-1 (9; Medeiros et al., 28th ICAAC). In this study it was not possible, however, to evaluate the effect of different levels of OXA-1 and other OXA enzymes because only single strains that produced each type of OXA enzyme were included. It should also be noted that for many isolates, drug ability to cross the cell membrane and the susceptibility of penicillin-binding proteins (PBPs) help determine overall susceptibility to β-lactam antibiotics. Thus, it is not surprising that for some strains, high enzyme levels were not always associated with high MICs and vice versa (Fig. 1A and B).

In the case of TEM-1-producing strains of *E. coli*, the previously reported inability of clavulanate to overcome resistance due to high enzyme levels (9, 14; Martinez et al., Letter, Lancet; Williams et al., Letter, Lancet; Medeiros et al., 28th ICAAC; Rodriguez-Creixems et al., 28th ICAAC) was confirmed (Fig. 1A and Table 2). A total of 26% of strains producing this β-lactamase were resistant to ticarcillin-clavulanate, whereas the newer combinations, piperacillin-tazobactam and cefoperazone-sulbactam, were much more active, with only 10% of strains resistant to piperacillin-tazobactam and no strains resistant to cefoperazone-sulbactam. A further 41% of strains had diminished (moderate) susceptibility to ticarcillin-clavulanate, whereas only 10 and 2% of strains, respectively, had diminished susceptibility to piperacillin-tazobactam and cefoperazone-sulbactam. These findings delineated ticarcillin-clavulanate as a distinctly less active combination than piperacillin-tazobactam or cefoperazone-sulbactam against strains of *E. coli* which produced elevated levels of TEM-1.

Analysis of TEM-1 levels did not explain the order of potency against strains of *E. coli* that produced this enzyme (i.e., cefoperazone-sulbactam > piperacillin-tazobactam > ticarcillin-clavulanate). Clavulanate and tazobactam are far more potent inhibitors of TEM-1 (and most of the other enzymes in this study) than sulbactam (1, 3, 4, 7; F. Moosdeen, S. Yamabe, and J. Williams, Program Abstr. 1st Eur. Congr. Clin. Microbiol., abstr. no. 423, 1983), and ticarcillin is no more susceptible than either piperacillin or cefoperazone to hydrolysis by TEM-1 (Table 4). Therefore, all other factors being equal, ticarcillin-clavulanate would be expected to be the most effective combination against TEM-1-producing strains. Thus, the relatively low potency of ticarcillin-clavulanate must be due to other factors, such as outer membrane permeability and affinity for PBPs. Yoshimura and Nikaido (18) reported that cefoperazone penetrates the outer membrane of *E. coli* faster than piperacillin and carbenicillin. If ticarcillin has a penetration rate similar to that of carbenicillin, then the greater potency of cefoperazone-sulbactam could be a consequence of faster penetration by cefoperazone. This, however, does not explain the greater potency of piperacillin-tazobactam over that of ticarcillin-clavulanate. The difference between these combinations may be due to the greater affinity of piperacillin for PBP 3, the principal target of both drugs. This is suggested by a 20-fold-greater affinity of the ureidopenicillins—piperacillin, mezlocillin, and azlocillin—for PBP 3 of *E. coli* compared with the carboxypenicillin carbenicillin (6). If ticarcillin and carbenicillin have similar affinities for PBPs, the greater potency of piperacillin-tazobactam may be due to the 20-fold-greater affinity of piperacillin for PBP 3.

Resistance to all drug combinations in strains of *K. pneumoniae* and *K. oxytoca* which produced high levels of SHV-1 and class IV enzymes, respectively, indicated that high-level producers of these enzymes constitute a gap in the spectrum of all of these combinations when only 2 μg of inhibitor per ml is present. Sanders et al. (14) reported that by increasing the clavulanate concentration from 2 to 5 μg/ml, it was possible to reduce ticarcillin-clavulanate MICs four- to sixfold on average for strains producing high levels of SHV-1. Although 2 μg/ml is the clinically appropriate clavulanate concentration for MIC testing (12), it has not yet been established what concentrations of tazobactam and sulbactam are clinically relevant for MIC tests. Thus, the therapeutic implications of high levels of SHV-1 and class IV β-lactamases for piperacillin-tazobactam and cefoperazone-sulbactam are unknown. As with TEM-1, SHV-1 was associated with greater resistance to ticarcillin-clavulanate than to cefoperazone-sulbactam and piperacillin-tazobactam.

Resistance to ticarcillin-clavulanate in strains of *E. coli* which produced PSE-1 was probably due to the more rapid hydrolysis of ticarcillin by PSE-1. In this study, although PSE-1 showed preferential hydrolytic activity against ticarcillin compared with piperacillin and cefoperazone, the level of hydrolysis (25%) was lower than might have been predicted from the literature. Medeiros et al. (11) reported relative hydrolysis rates for carbenicillin of 81 to 114% for PSE-1 using a modified microiodometric assay technique, a technique which is more accurate for assaying penicillinase activity than cephalosporinase activity. In this study, a spectrophotometric assay (13) was used, since both penicillin and cephalosporin substrates were examined. The spectrophotometric technique is a satisfactory assay of cephalosporinase activity but is relatively insensitive for assaying penicillinase activity. Thus, the lower sensitivity of our method may explain the difference in carboxypenicillin hydrolysis rates for the two studies. At present, PSE-1 is only rarely found in *E. coli* (10). However, in institutions where PSE-1 occurs, it is possible that widespread use of ticarcillin-clavulanate may select for the increased incidence of this enzyme.

At present there are still many factors to be investigated with these and other β-lactam–inhibitor combinations. Clinically relevant in vitro susceptibility tests are needed to provide more meaningful data for piperacillin-tazobactam and cefoperazone-sulbactam. It should be stressed that in this study a concentration of 2 μg/ml was used for all inhibitors, since this is the clinically relevant concentration for clavulanate and direct comparisons were being made. It is possible that with the establishment of pharmacokinetic parameters for sulbactam and tazobactam, a higher inhibitor concentration would be indicated. Were this the case, then differences between β-lactam–inhibitor combinations seen in this study could become even larger. It is clear that there is considerable need for future studies with β-lactamase inhibitors so that we can better understand their proper uses and their limitations as therapeutic agents.

ACKNOWLEDGMENTS

This study was supported by grants from Lederle Laboratories, Pearl River, N.Y.; Roerig Division, Pfizer Pharmaceuticals, New York, N.Y.; and the Health Future Foundation, Omaha, Nebr.

We thank Ellen S. Moland and Stephanie Armor for their excellent technical assistance and Stephen Cavalieri and Charles New for providing additional clinical strains.

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