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Comparative Activities of Clavulanic Acid, Sulbactam, and Tazobactam against Clinically Important β-Lactamases

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Clavulanic acid, sulbactam, and tazobactam are inhibitors of a variety of plasmid-mediated β-lactamases. However, inhibition data for these three inhibitors with a wide range of different plasmid-mediated β-lactamases have not yet been compared under the same experimental conditions. A number of groups have inferred that clavulanic acid inhibits extended-spectrum TEM and SHV β-lactamases, but inhibition data have rarely been published. In this study, the 50% inhibitory concentrations of these three β-lactamase inhibitors for 35 plasmid-mediated β-lactamases have been determined. Of these 35 β-lactamases, 20 were extended-spectrum TEM- or SHV-derived β-lactamases. The other 15 enzymes were conventional-spectrum β-lactamases such as TEM-1 and SHV-1. Clavulanic acid was a more potent inhibitor than sulbactam for 32 of the 35 plasmid-mediated β-lactamases tested. In particular, clavulanic acid was 60 and 580 times more potent than sulbactam against TEM-1 and SHV-1, respectively, currently the two most clinically prevalent gram-negative plasmid-mediated β-lactamases. Statistical analysis of the data of the 50% inhibitory concentrations showed that clavulanic acid was 20 times more active overall than sulbactam against the conventional-spectrum enzymes. In addition, clavulanic acid was 14 times more potent than sulbactam at inhibiting the extended-spectrum enzymes. Tazobactam also showed significantly greater activity than sulbactam against the two groups of β-lactamases. There were no significant differences between the overall activities of tazobactam and clavulanic acid against the extended-spectrum TEM and SHV enzymes and conventional-spectrum enzymes, although differences in their inhibition profiles were observed.

β-lactamases are plasmid-encoded or chromosomally encoded bacterial enzymes which hydrolyze β-lactam antibiotics. Plasmid-mediated β-lactamases can transfer rapidly between bacterial genera and consequently pose a major threat to the successful use of β-lactam agents. More than 60 different types of plasmid-encoded β-lactamases have been characterized, and for the purpose of this work, the enzymes have been classified as either conventional-spectrum or TEM- and SHV-derived extended-spectrum enzymes. The conventional-spectrum β-lactamases are from Bush groups 2a, 2b, 2c, and 2d, and extended-spectrum TEM and SHV enzymes are all from group 2b′ (4).

The conventional-spectrum enzymes include enzymes such as TEM-1 and SHV-1, which do not confer resistance to cephalosporins such as ceftazidime and cefotaxime. A recent survey of 802 gram-negative clinical isolates showed that TEM-1 and SHV-1 were responsible for mediating β-lactam resistance in 17% of clinical isolates (37). Other conventional-spectrum enzymes which are often found in clinical isolates include the OXA and PSE β-lactamases (1). The most common conventional-spectrum plasmid-mediated β-lactamase found in gram-positive bacteria is the penicillinase produced by the majority of *Staphylococcus aureus* clinical isolates (26).

Most of the extended-spectrum plasmid-mediated β-lactamases are derived from the TEM and SHV β-lactamase genes and confer transferable resistance to the newer broad-spectrum cephalosporins such as cefotaxime and ceftazidime. So far, at least 25 to 30 different extended-spectrum β-lactamases have been reported, and none of the enzymes appear to dominate the clinical situation worldwide. Therefore, a broad selection of extended-spectrum β-lactamases must be examined when assessing the activities of antimicrobial agents against such enzymes. The clinical prevalence of extended-spectrum β-lactamases in certain areas is increasing (35) and consequently compromises the effectiveness of the newer cephalosporins (17).

Combinations of β-lactam antibiotics and β-lactamase inhibitors have proved successful at treating infections caused by bacteria producing β-lactamases (22). The potency of such combinations continues to be largely assessed by susceptibility testing (2). Such studies have suggested that clavulanic acid and other β-lactamase inhibitors differentially inhibit various types of plasmid-mediated β-lactamases (15, 16, 19). However, the activity of a particular combination against clinical isolates is affected by the interplay of many different factors which are discussed in detail by Thomson et al. (36). One major component which affects the overall antibacterial activity of a β-lactam–β-lactamase inhibitor combination is the susceptibility of the β-lactamase to the inhibitor, and it is this component which has been addressed in our study. As there are now many different types of plasmid-mediated β-lactamase, any assessment of the potency of different β-lactamase inhibitors must be performed on a broad range of β-lactamases. Therefore, this paper reports the results of a comprehensive study which has examined the β-lactamase inhibitory activities of clavulanic acid, sulbactam, and tazobactam against 35 isolated plasmid-mediated β-lactamases. Twenty of the enzymes were extended-spectrum β-lactamases, and the remainder were conventional-spectrum plasmid-mediated β-lactamases.

MATERIALS AND METHODS

Antibiotics. Sulbactam was supplied by Pfizer Central Research, and tazobactam was obtained from Lederle Laboratories. Clavulanic acid was prepared in our laboratories.

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TABLE 1. Strains and plasmids used in study

Bush group and strain	Plasmid[a]	β-Lactamase	Reference
Group 2a β-lactamases			
S. aureus Russell		Staphylococcal penicillinase	14
Group 2b β-lactamases			
E. coli K-12 J53-2 Pro$^-$ Met$^-$ Rf^r	R6K	TEM-1	13
	RP4	TEM-2	13
	R1010	SHV-1	30
Group 2b′ β-lactamases			
E. coli C600 Nal^r The$^-$ Leu$^-$ Thi$^-$	pCF04	TEM-3	33
	pCFF14	TEM-5	29
E. coli K-12 J53-2 Pro$^-$ Met$^-$ Rf^r	pMG226	TEM-6	3
E. coli C600 Nal^r The$^-$ Leu$^-$ Thi$^-$	pLF100	TEM-7	12
E. coli K-12 J53-2 Pro$^-$ Met$^-$ Rf^r	pMG228	TEM-9	34
	pJPQ100	TEM-10	31
	pUD17	SHV-2	20
	pUD18	SHV-3	18
E. coli BM 694 Nal^r, Cla derived	pAFF1	SHV-5	11
E. coli K-12 J53-2 Pro$^-$ Met$^-$ Rf^r	100Kb	Enzyme A	27[b]
	100Kb	Enzyme B	27[b]
	RP4C	Enzyme C	27[b]
	RP4D	Enzyme D	27[b]
	R1818E	Enzyme E	27[b]
	pUK700	TEM-E1	27[b]
	pUK721	TEM-E2	27[b]
	pUK723	TEM-E3	27[b]
	pUK724	TEM-E4	27[b]
	pCFF44	CAZ-3	32
	pUK730	DJP-1	27[b]
	pMG204b	TLE-1	25
Klebsiella oxytoca		MJ-1	8
Group 2c β-lactamases			
Pseudomonas aeruginosa PU21 Ilv$^-$ Leu$^-$ Str^r Rf^r		PSE-4	10
Branhamella catarrhalis		BRO-1	9
Group 2d β-lactamases			
E. coli K-12 J53-2 Pro$^-$ Met$^-$ Rf^r	R455	OXA-1	7
	R46	OXA-2	7
	pMG203	OXA-4	25
	pMG54	OXA-5	25
	pMG204b	OXA-7	25
P. aeruginosa PA038 Leu$^-$ Rf^r Nal^r	pMG39	OXA-6	25
P. aeruginosa PU21 Ilv$^-$ Leu$^-$ Str^r Rf^r	R151	PSE-2	23

[a] The plasmid is identified if it was named in the original publication.
[b] Including references cited within.

Strains and plasmids. The *Escherichia coli* transconjugant strains used in this study are listed in Table 1. These strains were selected because they produced high levels of plasmid-mediated β-lactamase. Enzymes A and B were obtained from TEM-1 by in vitro spontaneous mutation; in the same way, enzymes C and D were derived from TEM-2 and enzyme E was derived from PSE-4 (27). TEM-E2, TEM-E1, CAZ-lo, and TEM-E4 have pIs similar to those of enzymes A, B, C, and D, respectively (27). However, isoelectric focusing data in isolation are not sufficient to assess whether two TEM β-lactamases with similar pIs are identical (17). The *S. aureus* Russell β-lactamase was representative of a typical staphylococcal penicillinase.

Preparation of β-lactamases. A 1-liter culture (nutrient broth no. 2) of each strain listed in Table 1 was shaken overnight at 37°C. The cells were harvested by centrifugation for 15 min at 6,000 × *g*. The bacterial pellets were washed in 25 mM sodium phosphate buffer (pH 7), and the centrifugation was repeated. One milliliter of 25 mM sodium phosphate buffer was then added to the final pellet, and the cells were resuspended to give 3 ml of cell suspension, which was disrupted by ultrasound. The cell lysate was cleared by centrifugation for 1 h at 32,000 × *g*.

β-Lactamase identification. Each β-lactamase was checked by analytical isoelectric focusing (24). The enzymes were examined on gels containing a 1:1 ratio of pH 3.5 to pH 10 ampholines and either pH 4 to pH 6 or pH 9 to pH 11 ampholines, depending on the expected pI of the β-lactamase. The prepared enzymes were focused alongside known β-lactamase preparations. Each preparation from the *E. coli* transconjugants was shown to produce only one plasmid-mediated enzyme with a trace amount of the *E. coli* K-12 chromosomal enzyme. The other strains all produced the single plasmid-mediated enzyme.

TABLE 2. Geometric means and 95% confidence intervals for IC_{50}s of clavulanic acid and tazobactam for OXA-1 and SHV-5

β-lactamase and inhibitor	No. of determinations	IC_{50} (μM)		
		Range	Geometric mean	95% CI[a]
OXA-1				
Clavulanic acid	8	1.19–2.11	1.58	1.34–1.84
Tazobactam	8	1.00–1.98	1.45	1.21–1.75
SHV-5				
Clavulanic acid	9	0.01–0.02	0.013	0.009–0.016
Tazobactam	9	0.06–0.10	0.076	0.066–0.086

[a] 95% CI, 95% confidence interval.

Assay conditions for the determination of IC_{50}s. The activities of β-lactamase inhibitors were assessed by determining the concentration of inhibitor which inhibits by 50% the hydrolysis of nitrocefin by a particular β-lactamase (IC_{50}).

The IC_{50}s were determined by an automated microtiter assay system as described previously (28). The amount of enzyme was normalized to give approximately 70 μM nitrocefin hydrolyzed per min.

Inhibitors were assayed at eight different concentrations, ranging from 0.005 to 100 μM. The dilution of each inhibitor and its subsequent transfer into microtiter plates were performed automatically with a Hamilton Micro Lab AT Plus. The β-lactamase and inhibitor were preincubated for 5 min at 37°C, and the assay was initiated by the rapid addition of nitrocefin to create a final concentration of 0.2 mM.

Calculation of IC_{50}s. The KinetiCalc software provided with the Biotek reader allowed the plots of absorbance against time for all 96 wells to be simultaneously displayed in real time. The initial rates of hydrolysis at each inhibitor concentration were calculated, and the IC_{50}s (μM) were determined by plotting percentage inhibition against inhibitor concentration (28).

Statistical analysis of IC_{50} data. The reproducibility of the methodology was examined by measuring the IC_{50}s of clavulanic acid and tazobactam for SHV-5 and OXA-1 on at least eight separate occasions. Analyses were performed on the IC_{50}s after $\log_{10}$ transformation, and geometric means with 95% confidence intervals were calculated for each inhibitor with each enzyme. The reproducibility was assessed by expressing the range of the confidence interval as a percentage of the mean IC_{50}.

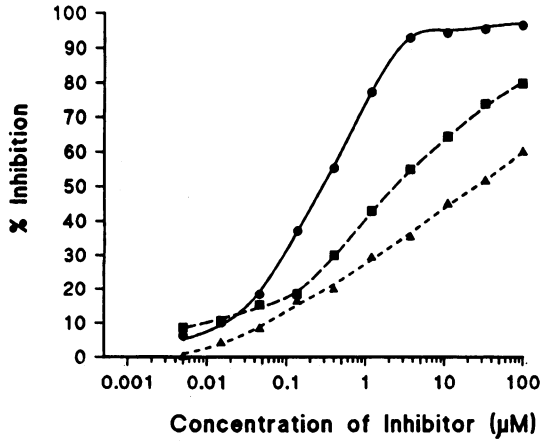

FIG. 1. Inhibition of *S. aureus* (Russell) penicillinase by clavulanic acid (●), sulbactam (▲), and tazobactam (■).

TABLE 3. IC_{50}s of β-lactamase inhibitors for 35 plasmid-mediated β-lactamases

β-lactamase	IC_{50} (μM)		
	Clavulanic acid	Sulbactam	Tazobactam
S. aureus Russell	0.28	26	2.3
TEM-1	0.09	6.1	0.04
TEM-2	0.18	8.7	0.05
SHV-1	0.03	17	0.14
TEM-3	0.03	0.03	0.01
TEM-5	0.03	1.2	0.28
TEM-6	0.12	0.45	0.17
TEM-7	0.10	0.62	0.18
TEM-9	0.29	0.90	0.34
TEM-10	0.03	0.34	0.08
SHV-2	0.05	2.8	0.13
SHV-3	0.04	2.7	0.10
SHV-5	0.01	0.63	0.08
Enzyme A	0.09	0.48	0.07
Enzyme B	0.01	0.12	0.01
Enzyme C	0.33	10	0.09
Enzyme D	0.04	0.57	0.01
Enzyme E	0.09	3.6	0.11
TEM-E1	0.05	0.64	0.02
TEM-E2	0.09	1.6	0.05
TEM-E3	0.02	0.20	0.06
TEM-E4	0.06	0.79	0.04
CAZ-3	0.13	2.5	0.06
DJP-1	0.01	0.21	0.02
TLE-1	0.11	5.5	0.05
MJ-1	0.09	40	0.43
PSE-4	0.15	3.7	0.10
BRO-1	0.02	0.02	0.02
OXA-1	1.8	4.7	1.4
OXA-2	1.4	0.14	0.01
OXA-4	8.4	16	5.6
OXA-5	3.1	18	0.25
OXA-6	1.6	51	1.7
OXA-7	0.36	40	0.61
PSE-2	0.81	37	0.94

Statistical analysis of the IC_{50}s after $\log_{10}$ transformation was performed by analysis of variance, taking into account the inhibitor, the enzyme, and the two groups of enzymes (conventional-spectrum and extended-spectrum TEM and SHV). Differences in the geometric means were back-transformed to ratios, i.e., relative potencies, of the inhibitors for the different groups of enzymes. The corresponding confidence limits were also determined.

RESULTS

Table 2 shows the statistical analysis of at least eight IC_{50}s for four different enzyme-inhibitor interactions. This illustrates that the semiautomated technique described in this paper was acceptably reproducible, with 95% confidence intervals less than 40% of the geometric mean in three of the four enzyme-inhibitor interactions. The 95% confidence interval for the fourth combination (clavulanic acid with SHV-5) was within 60% of the geometric mean (Table 2).

Figure 1 shows the inhibition profiles from which the IC_{50}s for *S. aureus* Russell β-lactamase were calculated. This illustrates that clavulanic acid was 93 times more active than sulbactam and 8 times more active than tazobactam against the *S. aureus* enzyme. The graphs for other IC_{50}s are not shown,

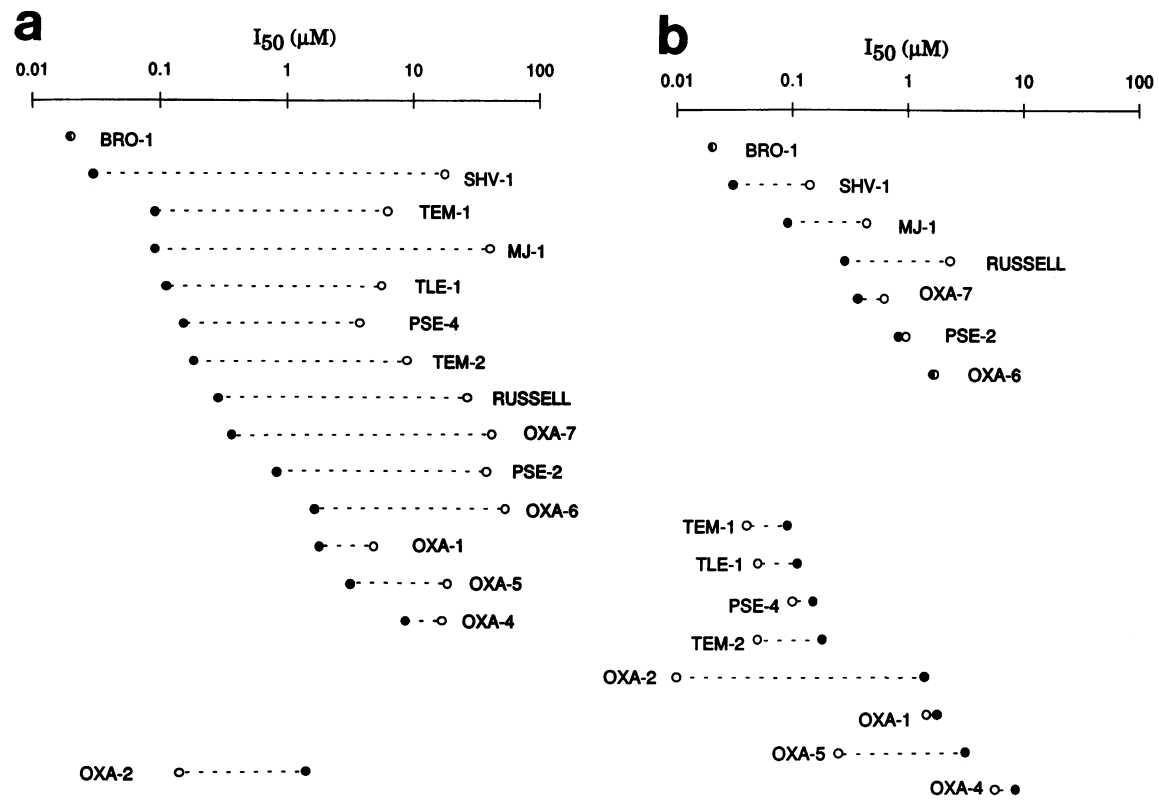

FIG. 2. Comparison of the IC_{50}s of β-lactamase inhibitors against conventional-spectrum β-lactamases. (a) IC_{50}s of clavulanic acid (●) and sulbactam (○); (b) IC_{50}s of clavulanic acid (●) and tazobactam (○).

and subsequent discussion of inhibition data refers directly to IC_{50}s (these are tabulated in Table 3 for reference). Figures 2 and 3 summarize these data, and for simplicity, the enzymes were ranked according to their susceptibilities to clavulanic acid.

Figure 2 compares the IC_{50}s of clavulanic acid with those of tazobactam and sulbactam for the conventional-spectrum plasmid-mediated β-lactamases. Figure 2a shows that clavulanic acid was more potent than sulbactam against almost all of the enzymes in this group. OXA-2 was the only enzyme against which sulbactam demonstrated better activity than clavulanic acid. Statistical analysis of the IC_{50}s demonstrates that, overall, clavulanic acid was 20 times more potent than sulbactam against these 15 enzymes (Table 4).

Figure 2b shows that tazobactam and clavulanic acid had similar activities against the 15 conventional-spectrum enzymes. Analysis of the IC_{50}s failed to reveal a significant difference in overall potency between the two inhibitors and this group of enzymes (Table 4). The comparison of panels a and b of Fig. 2 clearly demonstrates that tazobactam was more potent than sulbactam. In fact, overall, tazobactam was significantly more potent than sulbactam against this group of enzymes (Table 4).

Clavulanic acid was also a more potent inhibitor than sulbactam for 19 of the 20 extended-spectrum plasmid-mediated β-lactamases (Fig. 3a). TEM-3 was the only extended-spectrum enzyme against which the activity of sulbactam was comparable to that of clavulanic acid. Statistical analysis of the IC_{50}s shows that, for the 20 enzymes studied, clavulanic acid was 14 times more potent overall than sulbactam (Table 4).

Figure 3b compares the β-lactamase inhibitory activities of clavulanic acid and tazobactam against 20 TEM- and SHV-derived extended-spectrum β-lactamases. Both inhibitors were highly active, with IC_{50}s of less than 0.5 μM. In terms of overall potency, the difference between them was not statistically significant.

DISCUSSION

Detailed kinetic studies have been essential in elucidating the mode of action of mechanism-based β-lactamase inhibitors (5, 21). However, because of the complexities of such interactions, these studies have been confined to a limited number of representative β-lactamases. The proportion of clinical isolates producing plasmid-mediated β-lactamases is high (37), and the diversity of these enzymes is also expanding as an inevitable consequence of microbial adaptation (27). Consequently, from a clinical perspective, it is also essential to investigate the activities of the commercially available β-lactamase inhibitors against a broad variety of clinically derived β-lactamases. Therefore, we have determined the IC_{50}s of the three commercially available β-lactamase inhibitors for 35 plasmid-mediated β-lactamases. IC_{50}s measure only the activity of a β-lactamase inhibitor at a fixed time interval after incubation with an enzyme (5 min in this study). The values obtained are dependent on various kinetic parameters associated with the interactions of both inhibitor and substrate with β-lactamase. Nevertheless, IC_{50}s do provide the most practical way of evaluating the relative activities of β-lactamase inhibitors for such a large number of enzymes. For these reasons, many other groups have adopted IC_{50} assays as a means of characterizing β-lactamases (5, 19, 31).

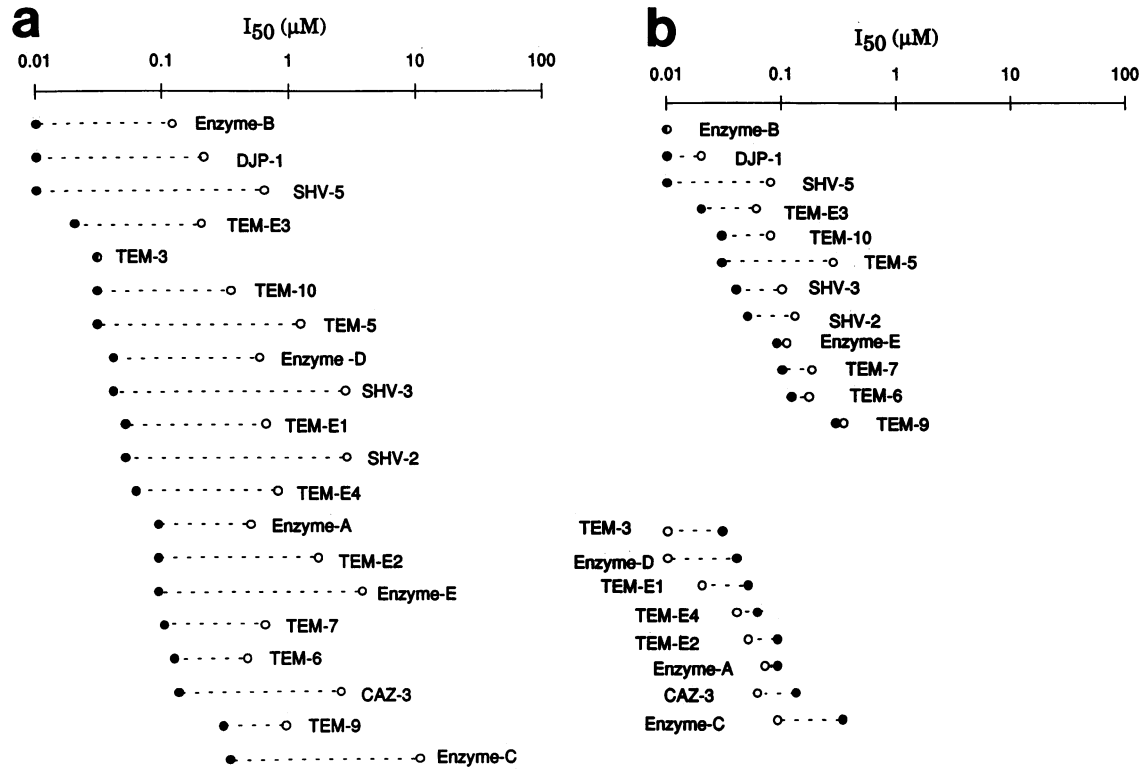

FIG. 3. Comparison of the IC_{50}s of β-lactamase inhibitors against TEM- and SHV-derived extended-spectrum β-lactamases. (a) IC_{50}s of clavulanic acid (●) and sulbactam (○); (b) IC_{50}s of clavulanic acid (●) and tazobactam (○).

The semiautomated method for the determination of IC_{50}s proved to be a rapid method for comparing the activities of β-lactamase inhibitors with an acceptable level of reproducibility.

These results show that clavulanic acid was a more potent inhibitor than sulbactam of both conventional-spectrum and extended-spectrum TEM and SHV enzymes. Jacobs et al. (15) determined the MICs of ampicillin in combination with clavulanic acid and sulbactam for 12 strains of *E. coli* producing conventional-spectrum β-lactamases, and although IC_{50}s and MICs cannot be directly correlated, the MICs do suggest that clavulanic acid had greater activity than did sulbactam against conventional-spectrum enzymes. The only enzyme for which the IC_{50} of sulbactam was lower than that of clavulanic acid was OXA-2. However, OXA-2 is of minor clinical significance, being produced by <1% of ampicillin-resistant enterobacterial strains (1). Previously published IC_{50}s (6) also showed that clavulanic acid was a more potent inhibitor than sulbactam of the *S. aureus* penicillinase, TEM-1, SHV-1, PSE-4, and four extended-spectrum TEM and SHV β-lactamases (5).

Overall, clavulanic acid and tazobactam were equipotent against both sets of enzymes. However, each inhibitor had a distinct inhibition profile. The IC_{50}s of clavulanic acid for *S. aureus* Russell, SHV-1, SHV-5, MJ-1, and TEM-5 were 8.1-, 4.7-, 5.9-, 4.8-, and 9.3-fold lower, respectively, than those of tazobactam. In contrast, tazobactam was more active than clavulanic acid against OXA-2 and OXA-5 (137- and 12.3-fold, respectively). Given the reproducibility of the assay and the location of these values at the extremes of the distribution of relative potencies, these differences may be considered significant.

In conclusion, we have reported the use of automated instrumentation to determine the activities of clavulanic acid, sulbactam, and tazobactam against a substantial number of previously reported plasmid-mediated β-lactamases. Overall, these IC_{50}s for 35 β-lactamases showed that clavulanic acid and tazobactam were more effective β-lactamase inhibitors than sulbactam and that clavulanic acid had activities equivalent to those of tazobactam. Although the inhibitory activity of the β-lactamase inhibitor is only one of the many components which contribute to the overall MIC of a β-lactam–β-lactamase inhibitor combination, this ranking of the activities of β-lacta-

TABLE 4. Relative potencies for pairs of inhibitors with 95% confidence intervals

Inhibitor pair	Relative potency (95% confidence interval) for:		
	All β-lactamases (n = 35)	Conventional-spectrum β-lactamases (n = 15)	Extended-spectrum TEM and SHV β-lactamases (n = 20)
Clavulanic acid-sulbactam	16.6 (10.1–27.4)	20.5 (9.6–43.9)	14.2 (7.3–27.4)
Clavulanic acid-tazobactam	0.95 (0.58–1.56)	0.70 (0.33–1.49)	1.19 (0.62–2.31)
Tazobactam-sulbactam	17.54 (10.7–28.9)	29.4 (13.8–63.0)	11.9 (6.1–22.9)

mase inhibitors has also been reported with MIC determinations conducted on 184 ampicillin-resistant clinical isolates (2). This demonstrates that clavulanic acid and tazobactam are potent inhibitors not only of the conventional-spectrum β-lactamases but also of the newer enzymes.

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