Effect of Hyperproduction of TEM-1 β-Lactamase on In Vitro Susceptibility of Escherichia coli to β-Lactam Antibiotics

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The susceptibility of 173 TEM-1-producing isolates of Escherichia coli was assessed by determination of MICs by the agar dilution method. MICs of amoxicillin, mezlocillin, cephaloridine, and, to a smaller extent, amoxicillin-clavulanic acid (but not cephalexin, cefuroxime, ceftaxime, cefazidime, or imipenem) were higher for isolates that produced large amounts of β-lactamase than for isolates that produced smaller amounts. The effect of fixed concentrations of clavulanic acid on resistance to amoxicillin was assessed for 34 selected TEM-1-producing isolates. Low concentrations of the inhibitor (0.5 to 1 μg/ml) reduced the amoxicillin MICs substantially for almost all the isolates, although the reductions were not sufficient to render any of the isolates amoxicillin susceptible. Higher concentrations of clavulanic acid had progressively greater effects on amoxicillin MICs, but even at 8 μg/ml some of the isolates with high β-lactamase activities remained resistant or only moderately susceptible to amoxicillin. All the isolates were inhibited by clavulanic acid (in the absence of amoxicillin) at concentrations of 16 to 32 μg/ml. TEM-1 β-lactamase activity was inhibited in vitro by clavulanic acid, but not totally, with approximately 2% of the initial activity remaining at 2 μg/ml and 0.4% remaining at 8 μg/ml. These findings suggest that the amount of β-lactamase activity is a major determinant of the degree of resistance to several β-lactam antibiotics and can make the difference between susceptibility and resistance to some compounds, notably the combination of amoxicillin with clavulanic acid.

Clavulanic acid [Z-(3R,5R)-2-β-hydroxyethylidene clavam-3-carboxylic acid] is an inhibitor of many β-lactamases, particularly the plasmid-determined enzymes (11, 12). One of these enzymes, TEM-1, is responsible for 80% or more of resistance to amoxicillin and other β-lactamase-sensitive antibiotics in Escherichia coli (reviewed by Wiedemann et al. [24]).

It has become apparent that the amount of β-lactamase produced by the bacterium affects the resistance profile, since there have been a number of reports of E. coli resistant to β-lactam agents expected to have activity against strains that produce TEM-1 or related β-lactamases. Although some have involved extended-spectrum enzymes that confer resistance to cephalexinlike enzymes and cefotaxime (see the work of Jacoby and Medeiros [5] for a review) or β-lactamase inhibitor-resistant enzymes (1, 22), there have also been reports of production of large amounts of TEM-1, TEM-2, or SHV-1 conferring resistance to ureidopenicillins and to combinations of a β-lactamase inhibitor with a β-lactamase-sensitive antibiotic (2, 8, 13, 16, 17, 21, 23, 25). The existence of such strains is not a new phenomenon; Page et al. (10) demonstrated β-lactamase hyperproduction and resistance to amoxicillin-clavulanic acid by TEM-1-producing strains isolated in 1967 to 1974 but gave no indication of how abundant such strains were in those days. There is no evidence that hyperproducers have increased as a proportion of β-lactamase-producing E. coli in recent years since Searlsingh et al. (19) examined amoxicillin-resistant, TEM-1-producing strains of E. coli and reported that there was no significant difference in the distribution of β-lactamase activities between 50 isolates collected in 1982 and 46 collected in 1989. Nevertheless, there is clear evidence that the proportion of isolates of E. coli that are

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resistant to amoxicillin has increased during the past 20 years (18, 20).

This study was undertaken to examine in more detail the effect of production of different amounts of TEM-1 β-lactamase on the susceptibility of E. coli to a range of β-lactam antibiotics and also to study the effects of different concentrations of clavulanic acid on the degrees of resistance to amoxicillin.

MATERIALS AND METHODS

Collection and identification of organisms. Consecutive isolates of E. coli were collected from urine specimens submitted to the Department of Microbiology, St. Thomas’ Hospital, between March and May 1991. The organisms were identified with API 20E strips, and antibiotic susceptibility was tested by the comparative disc method (26) in the clinical laboratory. Organisms that appeared resistant to amoxicillin were saved for further study on nutrient agar slants.

Determination of MICs. MICs were determined by inoculation, with a multiple inoculator (Denley, Billingshurst, Sussex, United Kingdom), of about 10⁶ CFU per spot suspended in brain heart infusion broth (Oxoid CM 225) on diagnostic sensitivity test agar (Oxoid CM 261) containing serial doubling concentrations of the antibiotic. E. coli NCTC 10418 was used as the control strain. The MIC was defined as the lowest concentration on which there was no visible growth after overnight incubation at 37°C. Antibiotics were kindly given, as powders of known potency, by the following companies: amoxicillin and clavulanic acid by SmithKline Beecham, Betchworth, Surrey, United Kingdom; cephaloridine, cephalexin, cefuroxime, and ceftazidime by Glaxo Group Research, Greenford, Middlesex, United Kingdom; cefotaxime by Roussel, Uxbridge, Middlesex, United Kingdom; and imipenem by Merck, Sharp and Dohme, Hoddesdon, Hertfordshire, United King-
dom. Except where explicitly stated otherwise, amoxicillin was combined with clavulanic acid in the ratio 2:1 by weight.

The MIC for 10% of strains tested (MIC<sub>M'C75</sub>, MIC<sub>M'C50</sub>, MIC<sub>M'C20</sub>, and MIC<sub>M'C10</sub>) were calculated as described by Hamilton-Miller (3). The MIC<sub>M'C</sub> was defined as the concentration immediately below the concentration at which at least one isolate was inhibited. Confidence intervals of the MIC<sub>M'C</sub> were calculated by the method described by Martin and his colleagues (7).

National Committee for Clinical Laboratory Standards criteria for susceptibility and resistance were applied (9) and were as follows: amoxicillin (without or with clavulanic acid), cefuroxime, and cephalaxin, ≤8 μg/ml (susceptible), 16 μg/ml (moderately susceptible), and ≥32 μg/ml (resistant); mezlocillin, ≤16 μg/ml (susceptible), 32 to 64 μg/ml (moderately susceptible), and ≥128 μg/ml (resistant); cefotaxime and ceftazidime, ≤8 μg/ml (susceptible), 16 to 32 μg/ml (moderately susceptible), and ≥64 μg/ml (resistant); and imipenem, ≤4 μg/ml (susceptible), 8 μg/ml (moderately susceptible), and ≥16 μg/ml (resistant).

Identification of β-lactamases and measurement of β-lactamase activity. Cells were harvested from overnight brain heart infusion broth cultures by centrifugation and resuspended in 0.5 ml of sodium phosphate buffer (0.1 M, pH 7), and β-lactamase was released by sonication (two bursts of 30 s in ice at an amplitude of 12 μm in an MSE Ultrasonic Disintegrator Mark II). Enzymes were identified by isoelectric focusing in Agarose-IEF (Pharmacia) gels containing Pharmalyte pH range 3 to 10; Pharmacia). Preparations of strains known to produce TEM-1, TEM-2, OXA-1, SHV-1, or PSE-4 were used as standards. After the focusing, β-lactamases were detected by spreading a solution of nitrocefin (100 μg/ml) on the surface of the gel. Isolates that produced TEM-1 as the sole detectable β-lactamase were included in the collection of TEM-1 producers. Strains that produced TEM-1 and also apparently overproduced the chromosomal β-lactamase (i.e., showed a significant band of activity at alkaline pH on isoelectric focusing) were excluded from the collection.

β-Lactamase activity was measured spectrophotometrically against 50 μM nitrocefin at 482 nm. The buffer used was 0.1 M sodium phosphate (pH 7), the light path was 1 cm, and all assays were performed at room temperature. Enzyme activity was standardized against the total protein concentration in the enzyme preparation, as estimated by the biuret method (4).

The inhibition of TEM-1 β-lactamase activity was measured against 20 μM nitrocefin after preincubation of the enzyme with clavulanic acid (0.5 to 16 μg/ml) for 10 min at room temperature.

RESULTS

The distribution of β-lactamase activities for the 173 isolates that produced TEM-1 alone (apart from the very small amounts of the chromosomally determined β-lactamase that is normally produced even by fully β-lactam-susceptible isolates of <i>E. coli</i>) is shown in Fig. 1. The activity was between 51 and 100 nmol of nitrocefin hydrolyzed per min per mg of protein for approximately one-third of the isolates; the next most common levels of activity were 1 to 50 (in fact, the lowest activity measured was 9 nmol/min/mg) and 101 to 150 nmol/min/mg, which when added to the 51 to 100-nmol/min/mg band accounted for 71% of the strains. However, 22% of the strains produced more than 200 nmol/min/mg, with 10% producing more than 300 nmol/min/mg and 5% producing more than 400 nmol/min/mg.

The susceptibility patterns of groups of isolates of <i>E. coli</i> that produced increasing amounts of TEM-1 β-lactamase are shown in Fig. 2 and 3. All the isolates were resistant to amoxicillin, but there was an increase in the degree of resistance as the β-lactamase activity increased, with the 95% confidence intervals of the MIC<sub>M'C</sub> (786 to 1,192 μg/ml) for the group with the lowest activity being significantly lower than the values for the group with the highest activity (3,419 to >4,096 μg/ml). MICs of mezlocillin and cephalexin were lower, but again there was an increase as the β-lactamase activity increased. In the case of mezlocillin the 95% confidence intervals of the MIC<sub>M'C</sub> for the group with the lowest β-lactamase activity was 19.1 to 28.9 μg/ml compared with 99.2 to 151 μg/ml for the group with highest activity; for cephalexin the ranges were 5.49 to 8.33 compared with 19.8 to 30.2 μg/ml for these two groups. The changes were less marked for the combination of amoxicillin with clavulanic acid; however, there was a significant difference between the group with the lowest β-lactamase activity (95% confidence interval of MIC<sub>M'C</sub> 8.29 to 12.6 μg/ml) and the group with the highest activity (95% confidence interval of MIC<sub>M'C</sub> 14.2 to 21.7 μg/ml). In contrast, there was no clear relationship between β-lactamase activity and degree of susceptibility to cephalaxin, cefuroxime, cefotaxime, ceftazidime or imipenem (Fig. 3).

Figure 4 shows the effect of clavulanic acid on amoxicillin MICs for 34 isolates of <i>E. coli</i> (selected to include a wide range of β-lactamase activities); at 0.5 μg/ml (data not shown) or 1 μg/ml the inhibitor reduced the amoxicillin MICs substantially for almost all the isolates, although the reductions were not sufficient to render any of the isolates amoxicillin susceptible. Higher concentrations of clavulanic acid had progressively greater effects on amoxicillin MICs, but even at 8 μg of clavulanic acid per ml some of the isolates with high β-lactamase activities remained resistant or only moderately susceptible to amoxicillin. All the isolates were inhibited by clavulanic acid (in the absence of amoxicillin) at concentrations of 16 to 32 μg/ml.

Figure 5 shows the inhibition of TEM-1 β-lactamase activity by clavulanic acid. Although there was considerable inhibition by concentrations as low as 0.5 μg/ml, activity on the order of 1% remained in the presence of 1 to 16 μg of the inhibitor per ml.
DISCUSSION

Our results confirm previous reports (13, 16–19) that MICs of amoxicillin and other β-lactam-sensitive β-lactam antibiotics are affected by the amount of TEM-1 β-lactamase synthesized by the isolate of E. coli. The effects on the combination of amoxicillin with clavulanic acid are noticeable though less marked than those on amoxicillin alone, mezlocillin, or cephalexin. Although TEM-1 β-lactamases activities of >200 nmol of nitrocefin hydrolyzed per min per mg protein were almost always associated with resistance to amoxicillin-clavulanic acid (MICs ≥ 16:8 μg/ml), the converse was not always true and other factors also probably contributed to resistance as discussed below. There were no significant effects of high β-lactamase production on the degree of susceptibility to cephalexin, cefuroxime, cefotaxime, ceftazidime, or imipenem; this is not surprising, since these compounds are relatively resistant to hydrolysis by TEM-1 in vitro (6, 15).

Although the amount of TEM-1 β-lactamase synthesized affects the susceptibility of E. coli to β-lactams, it does not appear to be the only factor, since some isolates are considerably more or less resistant than other isolates with similar β-lactamase activities and some isolates remained amoxicillin resistant in the presence of 8 μg of clavulanic acid per ml. This may well be the result of differences in permeability, as would be expected in a collection of clinical isolates, since effects of changes in porin content in conjunction with different amounts of TEM-1 activity on susceptibility have been reported by Reguera and his colleagues (14). Other possible explanations include differences in affinities to the essential penicillin-binding proteins, slight overproduction of the chromosomal enzyme (though not sufficient to be noticeable on the isoelectric focusing gel), or differences in the 50% inhibitory concentration of clavulanic acid for the β-lactamase. However, for the last explanation to be true, the alteration to the TEM-1 must have been such that the isoelectric point was not distinguishable from the normal value for the enzyme, since the strain would otherwise have been excluded from the collection.

The production of TEM-1 β-lactamase by a strain of E. coli is a very efficient mechanism for protecting the strain from amoxicillin, since the production of even relatively small amounts results in MICs much higher than the upper limit of susceptibility. Consequently, for a β-lactamase inhibitor to

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Susceptibility of TEM-1-producing isolates of E. coli to amoxicillin, mezlocillin, cephalexin, and amoxicillin plus clavulanic acid. The isolates were divided into three groups with increasing β-lactamase activities (expressed as nanomoles of nitrocefin hydrolyzed per minute per milligram of protein). The groups had the stated ranges of β-lactamase activity and contained the following numbers of isolates: 1 to 74 nmol/min/mg, 67 isolates; 75 to 120 nmol/min/mg, 41 isolates; and >120 nmol/min/mg, 65 isolates.

![Fig. 3](https://example.com/fig3.png)

**Fig. 3.** Susceptibility of TEM-1-producing isolates of E. coli to cephalexin, cefuroxime, cefotaxime, ceftazidime, and imipenem. See the legend to Fig. 2 for an explanation.
provide total protection to amoxicillin, the inhibition of the β-lactamase must be virtually complete. We found that β-lactamase activity of the order of 1% of the original activity remained after preincubation of TEM-1 with clavulanic acid (1 to 16 μg/ml). Reading's (11) results also indicate that the inhibition of TEM enzymes is not complete; furthermore, it was found that the concentration of clavulanic acid required to reduce the hydrolysis of cefaloridine by 50% for TEM-1 was 10-fold higher for whole cells than for cell-free β-lactamase. Consequently, we believe that the absence of complete inhibition of the β-lactamase is a major reason for the failure of the inhibitor to render all isolates amoxicillin susceptible, although other factors such as differences in permeability cannot be ruled out. In view of the high sensitivity of amoxicillin to TEM-β-lactamase, we remain to be convinced that any β-lactamase inhibitor can provide total protection for it in the clinical setting.

Strains of E. coli that hyperproduce TEM-1 were isolated long before clavulanic acid was used (10), so it would be very unwise to attribute the emergence of hyperproducers of TEM β-lactamase as a clinical problem solely to the use of β-lactam-β-lactamase inhibitor combinations, since the hyperproduction also confers a selective advantage in the presence of several other β-lactam compounds. Consequently, the clinical use of ureidopenicillins or TEM-1-labile cephalosporins is also likely to have contributed to the problem.

In summary, our findings confirm that the amount of β-lactamase activity is a major determinant of the degree of resistance to several β-lactam antibiotics and can be responsible for the difference between susceptibility and resistance for some compounds, notably the combination of amoxicillin with clavulanic acid.

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REFERENCES


