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Incidence and Mechanisms of Resistance to the Combination of Amoxicillin and Clavulanic Acid in *Escherichia coli*

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Among *Escherichia coli* organisms isolated at St. Thomas's Hospital during the years 1990 to 1994, the frequency of resistance to amoxicillin-clavulanic acid (tested by disk diffusion in a ratio of 2:1) remained constant at about 5% of patient isolates (10 to 15% of the 41 to 45% that were amoxicillin resistant). Mechanisms of increased resistance were determined for 72 consecutively collected such amoxicillin-clavulanic acid-resistant isolates. MICs of the combination were 16-8 μg/ml for 51 (71%) of these and ≥32-16 μg/ml for the remainder. The predominant mechanism was hyperproduction of enzymes isoelectrically cofocusing with TEM-1 (β-lactamase activities, >200 nmol of nitrocefin hydrolyzed per min per mg of protein) which was found in 44 isolates (61%); two isolates produced smaller amounts (approximately 150 nmol/min/mg) of such enzymes, and two isolates hyperproduced enzymes cofocusing with TEM-2. Eleven isolates produced enzymes cofocusing with OXA-1 β-lactamase, which has previously been associated with resistance to amoxicillin-clavulanic acid. Ten isolates produced increased amounts of chromosomal β-lactamase, and four of these additionally produced TEM-1 or TEM-2. Three isolates produced inhibitor-resistant TEM-group enzymes. In one of the enzymes (pI, 5.4), the amino acid sequence change was Met-67→Val, and thus the enzyme is identical to TEM-34. Another (pI, 5.4) had the substitution Met-67→Ile and is identical to IRT-I67, which we propose now be given the designation TEM-40. The third (pI, 5.2) had the substitution Arg-241→Thr; this enzyme has not been reported previously and should be called TEM-41. The rarity and diversity of inhibitor-resistant TEM-group enzymes suggest that they are the result of spontaneous mutations that have not yet spread.

Resistance to ampicillin and amoxicillin in *Escherichia coli* is predominantly caused by the plasmid-encoded β-lactamase TEM-1 (reviewed by Wiedemann et al. [34]), which is sensitive to β-lactamase inhibitors such as clavulanic acid. It has become apparent that there are several mechanisms by which *E. coli* can be resistant to β-lactam–β-lactamase inhibitor combinations such as amoxicillin plus clavulanic acid. Since chromosomally encoded Bush group 1 β-lactamases are less sensitive than group 2 enzymes to inhibitors (6), overproduction of the *E. coli* chromosomal β-lactamase is one cause of this resistance (3, 15). Some plasmid-encoded β-lactamases such as OXA-1 are less sensitive than TEM-1 to inhibition by clavulanic acid, so organisms that produce these enzymes are more frequently resistant to amoxicillin-clavulanic acid (15, 39). Overproduction of TEM-1 also results in resistance (7, 17, 21, 24, 25, 27, 31, 37), as does deficiency in the OmpF and/or OmpC porins in conjunction with TEM-1 production (22).

The most recently discovered mechanism of resistance to amoxicillin-clavulanic acid is production of β-lactamases derived from TEM-1 but with substantially reduced sensitivity to clavulanic acid and other β-lactamase inhibitors. This was reported initially for laboratory mutants generated by degenerate oligonucleotide mutagenesis (19). Substitution of the methionine residue at position 69 in the Ambler numbering system (1) (position 67 in the actual amino acid sequence of TEM-1 [28]) by one of the aliphatic hydrophobic amino acids—leucine, isoleucine, or valine—resulted in resistance to inhibition by clavulanic acid, as to a lesser extent did substitution by threonine. There have subsequently been several reports of such enzymes in clinical isolates (4, 5, 9, 10, 13, 29, 30, 32, 39). In general, these enzymes occur in *E. coli*, but one has been found in a strain of *Citrobacter freundii* from a calf (13). The amino acid sequences of some inhibitor-resistant enzymes have been deduced from DNA sequences (Table 1); they have an amino acid substitution at position 69 or 244, sometimes accompanied by changes at other positions.

In this paper we report recent results for resistance in *E. coli* to amoxicillin and amoxicillin-clavulanic acid from our hospital and from studies of the types and quantities of β-lactamases produced by strains collected during a 3-month period in 1993.

MATERIALS AND METHODS

Organisms and susceptibility tests. The study of incidence of resistance was conducted with isolates from all specimens other than feces submitted to the Microbiology Department, St. Thomas's Hospital, during the years 1990 to 1994, inclusive. *E. coli* was identified on the basis of its Gram-stained appearance and β-glucuronidase activity. The biochemical tests in API 20E strips (Analytab Products, La Balmes les Grottes, Montalier Vercier, France) were used for confirmation when necessary. Tests for antibiotic susceptibility were performed routinely by the disk diffusion comparative method (35). Antibiotic-containing discs were purchased from Unipath (Basingstoke, Hampshire, United Kingdom). Repeat isolates (within 30 days) from the same patient were excluded from analysis, unless susceptibility patterns were significantly different. All isolates of *E. coli* from specimens other than feces collected during October to December 1993, from both inpatients and outpatients, that were resistant to amoxicillin-clavulanic acid by this method were saved for further study.

MICs were determined by agar dilution on Diagnostic Sensitivity Test Agar (Oxoid CM261) with an inoculum of approximately 10^4 organisms per spot as described previously (37). Antibiotic powders of known potency were obtained from SmithKline Beecham, Betchworth, Surrey, United Kingdom (amoxicillin and clavulanic acid); Bayer UK, Newbury, Berkshire, United Kingdom (mezlocillin); Glaxo Group Research, Greenford, Middlesex, United Kingdom (cephaloridine and cefuroxime); Merck, Sharp and Dohme, Hoddesdon, Hertfordshire, United Kingdom (cefoxitin); and Bristol-Myers Squibb, Hounslow, Middlesex, United Kingdom (cefadroxil). The combination of amoxicillin and clavulanic acid was tested at a fixed ratio of 2:1, and results are expressed as amoxicillin

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TABLE 1. Amino acid sequence differences of inhibitor-resistant TEM β-lactamases

Enzyme	pI	Amino acid at position[a]:						Reference(s)
		69	165	182	244	275	276	
TEM-1	5.4	Met	Trp	Met	Arg	Arg	Asn	28
TEM-30 (TRI-2, IRT-2)	5.2				Ser			4, 32, 39
TEM-31 (TRI-1, IRT-1)	5.2				Cys			4, 32, 39
IRT-3 (TEM-32)	5.4	Ile		Thr				4
TEM-33 (IRT-5)	5.4	Leu						39
TEM-34 (IRT-6)	5.4	Val						39
TEM-35 (IRT-4)	5.2	Leu					Asp	5, 39
TEM-36 (IRT-7)	5.2	Val					Asp	39
IRT-8 (TEM-37)	5.2	Ile					Asp	9
IRT-9 (TEM-38)	5.2	Val				Leu		9
IRT-10 (TEM-39)	5.4	Leu	Arg				Asp	9
TEM-40 (IRT-I67)		Ile						4, 9; this article

[a] In the Ambler et al. (1) numbering scheme for class A β-lactamases.

concentrations. National Committee for Clinical Laboratory Standards criteria for susceptibility and resistance were applied when available (18) and were as follows: amoxicillin (without or with clavulanic acid), cefoxitin, and cefuroxime, ≤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).

Confidence intervals for resistance frequencies were calculated by the binomial method (2). Confidence intervals of the MIC at which 50% of the isolates are inhibited were calculated as described by Martin and his colleagues (16).

β-Lactamase studies. Cells were harvested from 20-h brain heart infusion broth (Oxoid CM225) cultures by centrifugation and resuspended in 0.5 ml of phosphate buffer (0.1 M, pH 7), and β-lactamase was released by sonication. Enzymes were identified by isoelectric focusing in Agarose-IEF (Pharmacia) gels containing Pharmalyte (pH range, 3 to 10; Pharmacia) and subsequent staining with nitrocefin (100 μg/ml). Preparations of strains known to produce TEM-1, TEM-2, OXA-1, SHV-1, or PSE-4 (kindly provided by R. B. Sykes and M. Matthew, Glaxo Group Research) were used as standards. The presence of chromosomal β-lactamase was recorded only when its band developed rapidly (within 2 min).

β-Lactamase activity was measured by monitoring the rate of hydrolysis of nitrocefin by ultrasonically disrupted cell suspensions as described previously (37). Enzyme activity was standardized against the total protein concentration in the enzyme preparation, as estimated by the biuret method (11). Inhibition of the hydrolysis of nitrocefin by clavulanic acid was measured after 10 min of preincubation. Enzymes were screened at a single concentration of clavulanic acid (0.1 μg/ml); a wider range of concentrations (0.02 to 20 μg/ml) was tested on enzymes which were not at least 90% inhibited.

Hyperproduction of TEM β-lactamase was arbitrarily defined as an activity of >200 nmol of nitrocefin hydrolyzed per min per mg of protein, since in a previous study (37), approximately 20% of TEM-1-producing clinical isolates of *E. coli* were found to have such activity.

PCR and DNA sequencing. Isolates of *E. coli* were inoculated in 3 ml of brain heart infusion broth and incubated for 20 h at 37°C with shaking. Cells from 1.5 ml of overnight culture were harvested by centrifugation in an Eppendorf centrifuge for 5 min. After the supernatant was decanted, the pellet was resuspended in 0.5 ml of sterile distilled water. The cells were lysed by heating at 95°C for 10 min.

The amplification primers A [5′-d(GTATGGATCCTCAACATTTCCGT GTCG)-3′ starting at position 205] and B [5′-d(ACCAAAGCTTAATCAGT GAGGCA)-3′ starting at position 1067] used for PCR have been described previously (39). The composition of the reaction mix was as follows: reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100), 0.2 mM (each) deoxynucleotide triphosphate (ATP, CTP, GTP, TTP), 5 μM (each) the two primers, and 1.5 mM $MgCl_2$ in a total volume of 48 μl. Sample lysate (1 μl) and 1 U of *Taq* polymerase were added to the reaction mixture, and the samples were mixed by vortexing and centrifuged briefly before 50 μl of mineral oil was layered onto the surface. Thermal cycling was performed in a Hybaid (Teddington, Middlesex, United Kingdom) Omnigene Thermal Reactor under the following conditions: 35 cycles of 25 s at 92°C, 2 min at 65°C, and 3 min at 75°C and a final extension step of 5 min at 75°C.

The PCR products were purified by ammonium acetate (4 M, 1 volume) precipitation in the presence of 2 volumes of isopropanol at room temperature for 30 min. DNA was recovered by centrifugation at top speed in a microcentrifuge for 30 min; this was followed by a 70% ethanol wash. After drying, the pellet was resuspended in sterile distilled water.

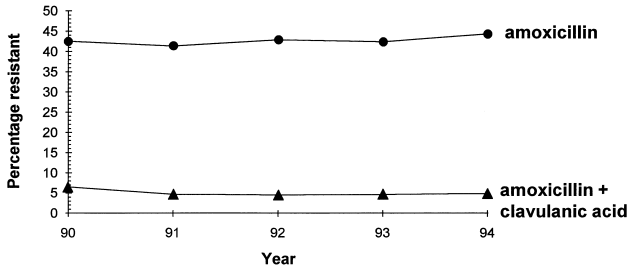

FIG. 1. Resistance in *E. coli* to amoxicillin and amoxicillin-clavulanic acid (2:1) at St. Thomas's Hospital. There were 4,520 isolates in 1990, 4,967 in 1991, 5,559 in 1992, 6,317 in 1993, and 7,017 in 1994.

The sequencing reactions were performed directly on the purified PCR product by use of the quick annealing method, as described in the instructions for the AutoRead Sequencing Kit (Pharmacia Biotech, St. Albans, Hertfordshire, United Kingdom). Template DNA (10 μg) and unlabelled primers (25 μM) were first denatured in the presence of NaOH (1 M) at 80°C for 3 min, placed on ice, and then neutralized with HCl (1 M). Primers A and B (see above) were usually used for sequencing. Labelling of the primer was performed by the addition of 4 U of T7 DNA polymerase and fluorescein-15-dATP labelling mix, with subsequent incubation at 37°C for 10 min. Sometimes a fluorescently labelled primer B (see above) synthesized by Pharmacia Biotech was used. Dideoxy termination reactions were carried out by the addition of the template DNA-labelled primer to each of four termination mixes and incubation at 37°C for 5 min. The reactions were stopped by the addition of formamide containing Dextran Blue (5 mg/ml). The DNA sequences of the samples were determined with an Automated Laser Fluorescent DNA sequencer (Pharmacia Biotech). The samples were heat denatured at 80°C for 3 min before loading on a 6% Hydrolink Long Ranger gel containing 7 M urea and 1.2× Tris-borate-EDTA. The samples were run at 37 W (1,800 V, 60 mA, 44°C) in 0.6× Tris-borate-EDTA, with a sampling time of 2 s (8-h running time) and a laser power of 3 mW.

Reagents. *Taq* DNA polymerase, 10× *Taq* polymerase buffer, and magnesium chloride were supplied by Promega (Promega Corporation, Southampton, United Kingdom). Nucleotides were obtained from Sigma (Poole, Dorset, United Kingdom). Sterile distilled water was molecular biology grade (Bio-Rad, Hemel-Hempstead, Hertfordshire, United Kingdom). Synthetic oligonucleotide primers were obtained from Pharmacia Biotech. The AutoRead Sequencing Kit and Automated Laser Fluorescent grade urea were supplied by Pharmacia Biotech. Hydrolink Long Ranger gel was obtained from Hoefer (Newcastle-under-Lyme, Staffordshire, United Kingdom). All other reagents were ANALAR grade, obtained from BDH (Lutterworth, Leicestershire, United Kingdom).

RESULTS

The frequency of resistance to amoxicillin and amoxicillin-clavulanic acid, as assessed by disk diffusion tests, for *E. coli* at St. Thomas's Hospital from 1990 to 1994 is shown in Fig. 1. There was little change in the resistance frequencies during this period. Amoxicillin resistance remained at about 42 to 44%, and resistance to amoxicillin-clavulanic acid remained at about 5%. Not surprisingly, resistance to amoxicillin-clavulanic acid was encountered only in amoxicillin-resistant isolates, 11% of which were resistant in 1994.

Table 2 shows the β-lactamases tentatively identified by iso-

TABLE 2. β-Lactamases produced by 72 amoxicillin-clavulanic acid-resistant isolates of *E. coli*

Enzyme characteristic(s)	No. of isolates
pI, 5.4 (cofocusing with TEM-1)	48
pI, 5.6 (cofocusing with TEM-2)	2
pI, 5.2	1
pI, 7.4 (cofocusing with OXA-1)	11
Chromosomal	6
Chromosomal; pI, 5.4	3
Chromosomal; pI, 5.6	1

TABLE 3. MICs for isolates with different mechanisms of reduced susceptibility to amoxicillin-clavulanic acid

Characteristic(s) of β-lactamase producers (no. of isolates)	Compound	MIC (μg/ml)[a]		
		Range	50%	90%
pI, 5.4; activity, >150 nmol/min/mg (46)	Amoxicillin	2,048–>4,096	>4,096	>4,096
	Amoxicillin-clavulanic acid	16–32	16	32
	Mezlocillin	128–>1,024	256	1,024
	Cephaloridine	4–128	32	128
	Cefadroxil	8–32	8	16
	Cefuroxime	2–8	4	8
	Cefoxitin	2–16	8	8
pI, 5.2 or 5.4; activity, 49–103 nmol/min/mg (3)[b]	Amoxicillin	2,048–>4,096	4,096	>4,096
	Amoxicillin-clavulanic acid	32	32	32
	Mezlocillin	32–64	64	64
	Cephaloridine	4–16	4	16
	Cefadroxil	8	8	8
	Cefuroxime	4–8	4	8
	Cefoxitin	8	8	8
pI, 7.4 (11)	Amoxicillin	512–>4,096	1,024	4,096
	Amoxicillin-clavulanic acid	16–64	16	32
	Mezlocillin	16–256	32	128
	Cephaloridine	2–8	2	4
	Cefadroxil	4–8	8	8
	Cefuroxime	2–16	4	8
	Cefoxitin	4–32	8	8
Chromosomal enzyme (6)	Amoxicillin	256–>4,096	512	>4,096
	Amoxicillin-clavulanic acid	16–64	32	64
	Mezlocillin	4–64	16	64
	Cephaloridine	16–>128	16	>128
	Cefadroxil	64–>64	>64	>64
	Cefuroxime	8–64	16	64
	Cefoxitin	16–>64	32	>64

[a] 50% and 90%, MICs at which 50 and 90% of the isolates are inhibited, respectively.
[b] Two isolates cofocused with TEM-1, and one had a pI of 5.2.

electric focusing in the 72 consecutive nonduplicate isolates, collected in October to December 1993, that were resistant or only moderately susceptible to amoxicillin-clavulanic acid. The majority (48 of 72; 67%) produced an enzyme that cofocused with a standard TEM-1 enzyme (pI, 5.4). The second most common pI was 7.4 (i.e., enzymes cofocusing with OXA-1), which was detected in 14% of the isolates. Ten isolates were identified as producing increased amounts of the chromosomal β-lactamase (pI, >8), mainly on the basis of their high degree of resistance to cefadroxil (MICs, ≥64 μg/ml) and at least reduced susceptibility to cefoxitin (MICs, ≥16 μg/ml) and, usually, cefuroxime (MICs, ≥16 μg/ml); four of these isolates additionally produced TEM-group enzymes (pI, 5.4 or 5.6). Only one isolate produced a β-lactamase with an isolectric point of 5.2.

The in vitro activities of β-lactams against isolates that produced different β-lactamases are summarized in Table 3. Producers of β-lactamases with a pI of 7.4 were less resistant to amoxicillin than were pI 5.4 β-lactamase producers with high β-lactamase activities. These pI 5.4 β-lactamase producers were also more resistant to mezlocillin than members of the other groups and more resistant to cephaloridine than were pI 5.2 or 5.4 β-lactamase producers with relatively low β-lactamase activities or pI 7.4 β-lactamase producers. In contrast, the chromosomal β-lactamase producers were more resistant to cefadroxil than members of the other groups and were also more resistant to cefuroxime and cefoxitin than were pI 5.4 β-lactamase producers with high activities of pI 7.4 β-lactamase producers.

Of the 72 isolates, 51 (71%) were moderately susceptible to amoxicillin-clavulanic acid, while the remainder were resistant to ≥32 μg/ml. pI 5.4 β-lactamase producers with high β-lactamase activities were generally moderately susceptible to amoxicillin-clavulanic acid, with only 5 of the 46 isolates (11%) being resistant, as were 4 of the 11 (36%) pI 7.4 β-lactamase producers. In contrast, all three pI 5.2 or pI 5.4 β-lactamase producers with relatively low β-lactamase activities were resistant, as were five of the six (83%) chromosomal β-lactamase producers.

β-Lactamase activities for isolates that produce enzymes cofocusing with TEM-1 are shown in Fig. 2. Two isolates had activities in the range of 49 to 84 nmol of nitrocefin hydrolyzed

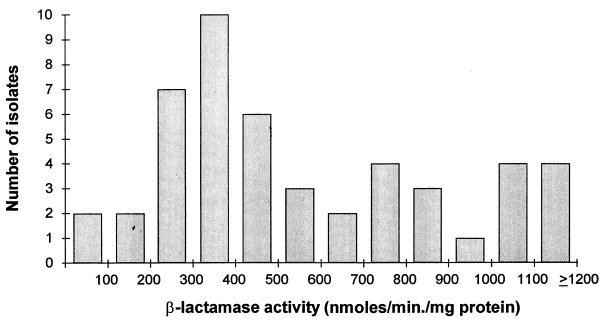

FIG. 2. β-Lactamase activities for isolates that produce enzymes that cofocus with TEM-1.

TABLE 4. Comparison of clavulanate-sensitive and clavulanate-resistant TEM enzymes

Isolate	β-Lactamase		IC_{50} of clavulanate (μg/ml)[b]	MIC (μg/ml) of:		Amino acid at position:			Enzyme
	pI	Activity[a]		Amoxicillin	Amoxicillin-clavulanic acid	69	244	276	
TEM-1	5.4		≤0.01			Met	Arg	Asn	TEM-1
55446	5.4	158	≤0.01	>4,096	16				TEM-1
46503	5.4	159	≤0.01	>4,096	16				TEM-1
53951	5.4	311	≤0.01	>4,096	16				TEM-1
57641	5.4	84	0.33	4,096	32	Val			TEM-34 (IRT-6)
52017	5.4	49	5.2	4,096	32	Ile			TEM-40 (IRT-I67)
51864	5.2	103	0.44	>4,096	32		Thr		TEM-41

[a] Nanomoles of nitrocefin hydrolyzed per min per mg of protein.
[b] IC_{50}, 50% inhibitory concentration.

per min per mg of protein; another two had activities in the range of 101 to 200 nmol/min/mg of protein but 44 (92%) had activities of >200 nmol/min/mg of protein and thus were classified as hyperproducers. The isolate with a β-lactamase with an isoelectric point of 5.2 also had relatively low β-lactamase activity (103 nmol/min/mg of protein). The two isolates with pI 5.6 β-lactamases had high activities (550 to 1849 nmol/min/mg of protein).

The β-lactamases from isolates that produced only an enzyme with a pI of 5.2, 5.4, or 5.6 were tested for inhibition by clavulanic acid (0.1 μg/ml); all were at least 90% inhibited, apart from the enzymes from the three isolates (57641, 51864, and 52017) with low activities (49 to 103 nmol/min/mg of protein). These three β-lactamases were tested at a wider range of concentrations in comparison with clavulanate-sensitive enzymes from isolates with slightly higher activities (158 to 159 nmol/min/mg of protein) and a single isolate with high β-lactamase activity (Table 4). The enzymes from isolates 55446, 46503, and 53951 with high, or relatively high, activities of clavulanic acid-sensitive TEM enzymes showed no sequence differences from the standard TEM-1. In contrast, the enzymes from isolates 57641, 51864, and 52017, which were at least 44- to 520-fold less sensitive than TEM-1 to inhibition by clavulanic acid, had various changes in the amino acid sequences (Table 4).

DISCUSSION

The frequency of resistance to amoxicillin (40 to 45%) that we found for the years 1990 to 1994 was similar to that found for 1982 to 1991 (26) and indicates that amoxicillin resistance in *E. coli* in our hospital has reached a plateau after increases in earlier years. The amoxicillin resistance frequency was higher than the 30 to 37% reported for the United States (12, 33) but similar to the 41% found by Henquell et al. (10) for French isolates in 1993 and to the frequencies reported for several European countries (33) and lower than the 58 to 68% reported for Spain and Israel (8, 33).

It has been noted that difficulties encountered in measuring susceptibility to amoxicillin-clavulanic acid can cause disagreements between laboratories and that evaluation of increased resistance is specific for each laboratory (38). This probably explains the wide range of frequencies of resistance to amoxicillin-clavulanic acid that have been reported for amoxicillin-resistant *E. coli*, including 3% for Spanish isolates collected from 1983 to 1988 (23), 10% for isolates collected in Belgium (38), 28% for isolates collected in Spain (8), 33% for strains collected in 1993 in Clermont-Ferrand, France (10), 35% for American strains collected in 1992 (12), and 41% for strains collected in Hong Kong from 1984 to 1988 (14). In the present study, we found about 11% of amoxicillin-resistant strains to be resistant to the combination of amoxicillin and clavulanic acid by disk diffusion tests, but 71% of these were subsequently shown to be moderately susceptible rather than resistant on the basis of MICs. Previously, in a study of blood culture isolates in which all isolates were tested by MIC determination, we found 10% to be resistant (26) and another 30% to be moderately susceptible, with amoxicillin-clavulanic acid MICs of 16 μg/ml. The discrepancy suggests that the disc test, which does not have the "moderately susceptible" category, may underestimate the numbers of moderately susceptible or resistant isolates. The lack of change in the proportion of amoxicillin-resistant strains that were resistant to the combination during the years 1990 to 1994 confirms previous reports (25, 26).

All isolates were inhibited by 64 μg of amoxicillin per ml plus 32 μg of clavulanic acid per ml. However, this does not necessarily reflect inhibition of the β-lactamase reducing the amoxicillin MIC to this level, since MICs of clavulanic acid alone for *E. coli*, including a strain that produced large amounts of the chromosomal β-lactamase, were reported to be about 32 μg/ml (20). Because of the relatively low level of MICs for resistant strains of *E. coli*, it is possible that simple urinary tract infections caused by even such resistant strains may respond to the high concentrations of the drugs in urine.

The existence of clavulanate-resistant TEM derivatives that cofocus with TEM-1 means that isoelectric focusing is less reliable for the identification of plasmid-determined β-lactamases than was once thought. Nevertheless, the proportion of isolates that produced pI 5.4 β-lactamases tentatively identified as TEM-1 (67%) is fairly close to the figure (78%) we found for a collection of amoxicillin-resistant isolates (36) and is close to the range of values expected for *E. coli* (34). Enzymes cofocusing with OXA-1 (pI, 7.4) occurred more frequently (15% compared with 4.9%); since numbers were small this may be the result of chance, but it is relevant that OXA-1 production has previously been associated with reduced susceptibility to amoxicillin-clavulanic acid (15, 39).

The predominant mechanism of reduced susceptibility to amoxicillin-clavulanic acid in our isolates was hyperproduction of TEM-1 β-lactamase, which occurred in 76% of the strains. In a further two strains, relatively high TEM-1 production (approximately 150 nmol/min/mg of protein) was probably the cause of the resistance. Henquell et al. (10) ascribed such resistance to hyperproduction of TEM-1 in 41% of isolates but thought that increased production contributed to intermediate resistance in 93% of isolates.

Henquell et al. (10) detected clavulanate-resistant TEM β-lactamases in 153 of 417 (37%) amoxicillin-clavulanic acid-

resistant *E. coli* isolates from urinary tract infections in France but in none of the 290 strains with intermediate resistance. The enzymes were principally TEM-30 (found in 22 of 107 isolates tested), TEM-37 (20 isolates), TEM-33 (17 isolates), TEM-34 (14 isolates), and TEM-36 (12 isolates) (9). In the present study, clavulanate-resistant TEM derivatives were produced by only three of 21 (14%) isolates resistant to ≥32 μg of amoxicillin-clavulanic acid per ml but by none of the moderately susceptible isolates. All three of these enzymes were different. This, combined with their rarity in our collection, suggests that they are the result of spontaneous mutations and that the mutant genes may not have the capacity to spread. One of the clavulanate-resistant enzymes (TEM-34) has been reported previously (39). The change from methionine to isoleucine at position 69 has been reported previously for a laboratory strain and the β-lactamase called IRT-I67 (4); it was also reported by Henquell et al. (9) but not clearly distinguished from TEM-32, since they omitted changes at position 182 from their Table 2. We believe that this enzyme should be distinguished from TEM-32, which has an additional Met-182→Thr change, and brought unambiguously into the TEM series as TEM-40. The Arg-to-Thr change at position 241 has not been previously reported, so we propose that this enzyme be called TEM-41.

In summary, there has been little change over the last five years in the incidence of resistance to amoxicillin and amoxicillin-clavulanic acid in *E. coli* isolates from our hospital. Both clavulanate-resistant enzymes and hyperproduction of TEM-1 β-lactamase contribute to the resistance to amoxicillin-clavulanic acid, but hyperproduction is by far the most frequent mechanism. In contrast to the situation in France, production of inhibitor-resistant TEM derivatives is uncommon at the present time and so far does not appear to have spread among clinical isolates of *E. coli* in London.

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