High prevalence of stably derepressed class-I β -lactamase expression in multiresistant clinical isolates of *Enterobacter cloacae* from Greek hospitals

E. TZELEPI, L. S. TZOUVELEKIS, A. C. VATOPOULOS*†, A. F. MENTIS, A. TSAKRIS*
and N. J. LEGAKIS‡

Department of Bacteriology, Hellenic Pasteur Institute, * Department of Microbiology, Hippokration General Hospital, † Department of Hygiene and Epidemiology and ‡ Department of Microbiology, School of Health Sciences, National University of Athens, 11521 Athens, Greece

Summary. Susceptibilities to cefotaxime (Ctx) and ceftazidime (Caz) were examined for 90 recent clinical isolates of *Enterobacter cloacae* from Greek hospitals. Most (68%) of the isolates were resistant to both drugs, and all were resistant to ceftazidime. β -Lactamase activities against cephaloridine in crude extracts from Ctx-Caz-resistant isolates were high, irrespective of whether or not the cells were grown with ceftazidime as an inducer of the chromosomal β -lactamase, indicating stable derepression of the gene for the enzyme. On the other hand, double disk antagonism tests showed that all the Ctx-Caz-sensitive isolates possessed inducible expression of this β -lactamase. Iso-electric focusing revealed the presence of five forms of the chromosomal β -lactamase, randomly distributed amongst the Ctx-Caz-resistant and -sensitive isolates. Plasmid-mediated β -lactamases of TEM and PSE types also were found in many isolates. These data indicate that the extremely high prevalence of Ctx-Caz-resistant *E. cloacae* isolates in Greek hospitals is attributed to the dissemination of mutants which constitutively overproduce the class-I chromosomal β -lactamase. Over 90% of these isolates exhibited cross-resistance to aminoglycosides, suggesting the accumulation of unrelated antibiotic resistance mechanisms.

Introduction

The introduction of newer cephalosporins such as cefotaxime and ceftazidime has improved the potential for effective treatment in serious infections caused by gram-negative pathogens. However, the clinical use of these antimicrobial agents was followed rapidly by the emergence of resistance in several species, including *Enterobacter cloacae*. Several studies have shown that cephalosporin resistance in *E. cloacae* is due mainly to the overproduction ("stable derepression") of the class-I β -lactamase encoded by the chromosomal *ampC* gene.^{1,2} Stably derepressed mutants are segregated from inducible strains at relatively high frequencies.³ Therapeutic failures with cephalosporin treatment have been associated with the selection and ultimate dominance of these variants.^{4,5} Combination therapy with a cephalosporin plus an aminoglycoside is often employed to prevent development of resistance.⁶

Hospitals in the Athens area have recorded an alarming frequency of isolation of *E. cloacae* strains highly resistant to aminoglycosides and to cefotaxime and ceftazidime (unpublished data). The present study

was undertaken to obtain further information on the extent and the nature of this phenomenon.

Materials and methods

Bacterial isolates

A total of 90 randomly selected, non-replicate clinical isolates of *E. cloacae* from separate patients was examined. They were collected from various clinical specimens, mainly of urine and sputum, from 12 hospitals in the Greater Athens area; 60 isolates were from four major hospitals (11–20 isolates per hospital) and 30 isolates were from eight minor units (1–7 isolates per hospital). All were from patients with active infection. The bacteria were identified by the API 20E System (API BioMérieux, La Balme Les Grottes, France).

Antibiotic susceptibility tests

Antibiotic susceptibilities of the isolates were determined in Mueller-Hinton Broth (Oxoid) by a microdilution technique. An inoculum of 10^4 cfu/ml was used and the isolates were considered to be resistant or sensitive according to the recom-

recommendations of the Working Party of the British Society for Antimicrobial Chemotherapy.⁷ On this basis, cefotaxime and ceftazidime resistances were defined as MIC > 1 and > 2 mg/L, respectively.

Detection of inducible β -lactamases

Screening for inducible β -lactamase production was by the cefoxitin diffusion method.⁸ Inducer disks containing 10 μ g of cefoxitin (AB Biodisc, Solna, Sweden) were used in combination with disks containing 30 μ g of cefotaxime and 30 μ g of ceftazidime (Oxoid).

Extraction of β -lactamases

Sonic extracts of cultures grown for 6 h in Trypticase Soya Broth (Oxoid) were used for the detection of β -lactamases and for hydrolysis studies. The protein content of the extracts was measured by the BioRad Protein Assay kit (BioRad Laboratories, Richmond, CA, USA).

Induction of β -lactamases

Induction of β -lactamases was investigated as follows: the organisms were incubated overnight at 37°C in Trypticase Soya Broth, and 1 ml of each culture was transferred into 9 ml of the same broth. After 90 min, cefoxitin was added to a final concentration of 50 mg/L. Incubation was continued for 4 h, after which the β -lactamase was released by sonic treatment.

Hydrolytic studies

β -Lactamase hydrolysis rates were studied by a UV spectrophotometric method, as previously described,⁹ with cephaloridine (Glaxo, Greenford) as the substrate. The cephaloridine solution was prepared in 0.1 M phosphate buffer (pH 7.0) at a final concentration of 1 mM, and hydrolysis was measured at 37°C in 3-ml cuvettes, at a wavelength of 295 nm. Results were expressed as units of hydrolytic activity (1 unit = 1 nmol of antibiotic hydrolysed/min/mg of protein).

Analytical iso-electric focusing (IEF)

IEF was performed on polyacrylamide gels as described elsewhere.¹⁰ β -Lactamases were visualised by overlying gels with filter paper moistened with a solution of nitrocefin (Oxoid) 0.5 mg/ml in 0.1 M sodium phosphate buffer (pH 7.0) containing dimethylsulphoxide 1% v/v. In some experiments, the focused gels were flooded with a 1 mM potassium clavulanate solution before the application of nitrocefin. This allowed the in-situ distinction between class I and plasmid-mediated enzymes, as the latter, but not the former, are inhibited by clavulanate.¹¹

Table I. Activities of 10 antimicrobial agents against 90 *E. cloacae* isolates *in vitro*

Antimicrobial agent	MICs (mg/L)		
	MIC50	MIC90	Range
Carbenicillin	1024	1024	4–1024
Cefoxitin	256	1024	32–1024
Cefotaxime (Ctx)	128	512	1–512
Ceftazidime (Caz)	128	256	1–512
Imipenem	0.5	1	< 0.06–4
Gentamicin	4	256	< 1–1024
Netilmicin	128	1024	< 1–1024
Tobramycin	64	256	< 1–1024
Amikacin	16	128	< 1–1024
Norfloxacin	0.125	0.5	< 0.06–4

Results

The in-vitro activities of the 10 antimicrobial agents tested against the 90 *E. cloacae* isolates are presented, in terms of MIC50 and MIC90 values, in table I. Most of the isolates were highly resistant to carbenicillin, cefoxitin, cefotaxime (Ctx), and ceftazidime (Caz). Imipenem and norfloxacin remained highly active. Amongst the aminoglycosides, gentamicin was active against the greatest proportion of strains; netilmicin, tobramycin and amikacin were less active.

Of the 90 isolates, 61 (68%) were resistant to both cefotaxime and ceftazidime (MIC > 1 and > 2 mg/L, respectively) (table II). Many were highly resistant to these two drugs, for which the MIC50 values were 128 mg/L. Also, 65 isolates (72%) were resistant to one or more aminoglycosides (MIC > 1 mg/L for gentamicin, netilmicin or tobramycin, and > 4 mg/L for amikacin). Interestingly, 57 (93%) of the 61 Ctx-Caz-resistant isolates were cross-resistant to one or more aminoglycosides; resistance to aminoglycosides was much less frequent (28%) amongst the Ctx-Caz-sensitive isolates (χ^2 test, $p < 0.001$) (table II).

Cefoxitin disk diffusion tests revealed that all the Ctx-Caz-sensitive isolates possessed inducible β -lactamases. The test was not applicable to the Ctx-Caz resistant isolates, because none of them gave a substantial inhibition zone around a disk containing one of the third generation cephalosporins. In quantitative assays, it was found that growth in the presence of cefoxitin strongly induced cephaloridine-hydrolysing activity in all the Ctx-Caz-sensitive isolates. Uninduced β -lactamase activities ranged from 10 to 80 units, but in all cases exceeded the 3000 units after induction with cefoxitin. High levels of β -lactamase activity against cephaloridine (> 1000 units) were found in crude extracts of all the Ctx-Caz-resistant isolates, even when the cultures were grown without induction with cefoxitin. This finding, taken together with their Ctx-Caz-resistance phenotype, indicates that these isolates were hyperproducers of chromosomal β -lactamases. Interestingly, substantial differences in inducibility were observed amongst these

Table II. Resistance phenotypes of 90 *E. cloacae* isolates, and relationship between susceptibility to third generation cephalosporins (3GC) and aminoglycosides (AMG)

Resistance phenotypes*	No. of isolates that were			
	3GC ^r -AMG ^r	3GC ^r -AMG ^s	3GC ^s -AMG ^r	3GC ^s -AMG ^s
Fox Cb Ctx Caz Gm Tm An Net	33			
Fox Cb Ctx Caz — Tm An Net	19			
Fox Cb Ctx Caz Gm Tm — Net	5			
Fox Cb Ctx Caz — — — —		4		
Fox Cb — — Gm Tm An Net			3	
Fox Cb — — — Tm An Net			5	
Fox Cb — — — — — —				4
Fox — — — — — —				17
Totals	57	4	8	21

* Fox, cefoxitin; Cb, carbenicillin; Ctx, cefotaxime; Caz, ceftazidime; Gm, gentamicin; Tm, tobramycin; An, amikacin; Net, netilmicin.

Table III. Iso-electric points (pIs) of β -lactamases produced by *E. cloacae* isolates in relation to resistance and sensitivity to third generation cephalosporins (3GC^r and 3GC^s)

β -Lactamases (pIs)	Number of isolates (90)	Number of isolates	
		3GC ^r (61)	3GC ^s (29)
8.0	8	3	5
8.0+5.4	24	15	9
8.0+6.1	1	1	—
8.4	20	13	7
8.4+5.4	6	4	2
8.4+5.4+5.6	4	4	—
8.7	5	3	2
8.7+5.4	4	2	2
8.7+5.4+5.6	2	2	—
9.0+5.4	2	1	1
9.0+6.1	2	2	—
9.2+5.4	4	3	1
9.2+6.9	6	6	—
9.2+5.4+6.1	1	1	—
9.2+5.4+6.9	1	1	—

suggests that, in some cases, derepression was not total. Iso-electric focusing revealed the production of five forms of the chromosomal β -lactamase, with iso-electric points (pIs) ranging from 8.0 to 9.2 (table III, figure).

Fifty-six (62 %) of the isolates also possessed one or more secondary β -lactamases, with pIs of 5.4 (53 % of the isolates), 5.6 (7 %), 6.1 (4 %) and 6.9 (8 %). These secondary enzymes were presumably plasmid-mediated, and were inhibited *in situ* by flooding the focused gels with clavulanate solution. The occurrence of these β -lactamases did not correlate with resistance to Ctx and Caz. Also, hydrolysis rates from crude extracts of isolates possessing such enzymes did not differ significantly from the rates observed with isolates that had chromosomal enzymes only. Thus, it is suggested that the distortion in the measurements of β -lactamase activity caused by the presence of secondary enzymes was negligible.

organisms. In 46 (75 %) cases, induction by cefoxitin resulted in < 1.5-fold increase in β -lactamase activity; in the remaining 15 (25 %) the degree of induction varied from two-fold up to 10-fold. This observation

Discussion

Colonisation of patients with *Enterobacter* species may be enhanced by the use of β -lactam antibiotics,¹² and an association between intensive use of these

Figure. Analytical iso-electric focusing (pH range 3.5–9.5) of β -lactamases in crude extracts of *E. cloacae* clinical isolates, and reference standards. All the pI variants of chromosomal β -lactamase types found among the 90 isolates studied are represented in lanes 3–19. Standard pIs of the β -lactamases TEM-1, PSE-2 and SHV-1 (lane 1) are indicated on the left. In lane 2, an additional marker (OXA-3, pI 7.1) is present.

drugs and the development of clinically important *E. cloacae* infections has also been reported.^{4,5,13}

It has been reported that most (70–80%) clinical isolates of *E. cloacae* possess inducible class-I chromosomal β -lactamases.^{8,14,15} These isolates are resistant to cefoxitin but susceptible to third-generation cephalosporins such as cefotaxime and ceftazidime. The remaining isolates are either stably derepressed producers of the class-I enzymes and are resistant to virtually all β -lactams, or are basal producers, susceptible to most β -lactam drugs including cefoxitin.¹⁶

Third-generation cephalosporins are poor inducers of the class-I enzymes but remain labile to their hydrolytic action. Moreover, these β -lactamases exhibit high affinity for the newer cephalosporins, a fact which facilitates their hydrolytic activity in the periplasmic space.¹⁷ Therefore, derepressed but not inducible cells express resistance and the compounds can select such variants more readily than other β -lactam drugs.¹⁸ Large bacterial numbers at the site of infection, low concentrations of the drug, and prior therapy with β -lactam antibiotics have been proposed as important factors for the selection of resistant variants *in vivo*.^{19,20}

In contrast with the above reports, we found that most of the isolates tested in the present study (68%) were stably derepressed and so were resistant to both cefotaxime and ceftazidime. These data clearly indicate an unusually high incidence of stably derepressed variants of *E. cloacae* in Greek hospitals. On the other hand, basal producers of class-I enzymes were not detected. It should be mentioned that decreased outer-membrane permeability in *E. cloacae* isolates may play an important synergic role in the expression of high levels of resistance to cephalosporins²¹ and we cannot discount its possible contribution in some of the isolates.

The diversity of different pI forms of class-I β -lactamase is of academic rather than of clinical interest, since the isolates show a similar spectrum of resistance irrespective of which enzyme-subtype they produce.¹⁶ However, the high incidence of secondary β -lactamases observed is of interest. Enzymes with pIs of 5.4 and 5.6 were found most commonly. These almost certainly correspond to TEM-1 and TEM-2 β -lacta-

mases, respectively. These enzymes can confer resistance to carboxy- and ureido-penicillins, expanding the resistance of chromosomal β -lactamase inducible *E. cloacae* strains. More rarely, enzymes with pIs of 6.1 and 6.9 were found; these may have been PSE-2 and PSE-3, respectively.

As well as a high frequency of Ctx-Caz resistance among the isolates tested, a similarly high frequency of aminoglycoside resistance was also evident. Moreover, aminoglycoside resistant isolates were significantly more often resistant to cefotaxime and ceftazidime than were aminoglycoside susceptible ones, indicating a linkage of the two resistance phenotypes. In further studies on aminoglycoside resistance in the same *E. cloacae* collection, various transferable plasmids carrying genes for more than one aminoglycoside-modifying enzyme were found. Detailed typing showed that most of the isolates were epidemiologically distinct (unpublished data).

The high frequency of resistant gram-negative bacilli in Greek hospitals is documented,²² but the degree of cross-resistance and the mechanisms involved have not yet been fully assessed. In previous studies, it was shown that the high frequency of isolation of multi-resistant *Pseudomonas aeruginosa* in Greek hospitals was due to the dissemination of a single strain,^{23,24} whereas resistance of *Klebsiella pneumoniae* and *E. coli* to newer cephalosporins and aminoglycosides resulted from the spread of plasmids.²⁵ In contrast to these reports, it seems that simultaneous resistance to newer cephalosporins and aminoglycosides in the *E. cloacae* strains isolated is due to the occurrence of two genetically unrelated mechanisms, one chromosomal and the other plasmid-mediated.

Infections due to multiresistant *E. cloacae* are difficult to treat. Nevertheless, imipenem and quinolones retained their activity and could serve as alternatives. However, the accumulation of unrelated resistance mechanisms within the same bacterial population is of special interest and poses a serious problem which is difficult to confront.²⁶ The data presented here clearly suggest the need for a continuous and detailed surveillance on susceptibility trends in order to formulate effective antibiotic policies in Greek hospitals.

References

1. Lindberg F, Normark S. Common mechanism of AmpC β -lactamase induction in enterobacteria: regulation of the cloned *Enterobacter cloacae* P99 β -lactamase gene. *J Bacteriol* 1987; **169**: 758–763.
2. Nicolas M-H, Honore N, Jarlier V, Philippon A, Cole ST. Molecular genetic analysis of cephalosporinase production and its role in β -lactam resistance in clinical isolates of *Enterobacter cloacae*. *Antimicrob Agents Chemother* 1987; **31**: 295–299.
3. Wiedemann B. Genetic and biochemical basis of resistance of Enterobacteriaceae to β -lactam antibiotics. *J Antimicrob Chemother* 1986; **18**: Suppl B: 31–38.
4. Dworzack DL, Pugsley MP, Sanders CC, Horowitz EA. Emergence of resistance in gram-negative bacteria during therapy with expanded-spectrum cephalosporins. *Eur J Clin Microbiol* 1987; **6**: 456–459.
5. Johnson MP, Ramphal R. β -Lactam-resistant *Enterobacter* bacteremia in febrile neutropenic patients receiving monotherapy. *J Infect Dis* 1990; **162**: 981–983.
6. Giamerellou H. Aminoglycosides plus beta-lactams against gram-negative organism. *Am J Med* 1986; **80** Suppl 6 B: 126–137.
7. Working Party of the British Society for Antimicrobial Chemotherapy. Breakpoints in in-vitro antibiotic sensitivity testing. *J Antimicrob Chemother* 1988; **21**: 701–710.
8. Anonymous. Incidence of inducible beta-lactamases in gram-negative septicemia isolates from twenty-nine European laboratories. *Eur J Clin Microbiol* 1987; **6**: 460–466.

9. O'Callaghan CH, Muggleton PW, Ross GW. Effects of β -lactamase from gram-negative organisms on cephalosporins and penicillin. In: Hobby GL (ed) *Antimicrobial agents and chemotherapy*. Bethesda, MD. 1969: 57-63.
10. Matthew M, Harris AM, Marshall MJ, Ross GW. The use of analytical isoelectric focusing for detection and identification of β -lactamases. *J Gen Microbiol* 1975; **88**: 169-178.
11. Sanders CC, Sanders WE, Moland ES. Characterization of β -lactamases in situ on polyacrylamide gels. *Antimicrob Agents Chemother* 1986; **30**: 951-952.
12. Flynn DM, Weinstein RA, Nathan C, Gaston MA, Kabins SA. Patients' endogenous flora as the source of "nosocomial" *Enterobacter* in cardiac surgery. *J Infect Dis* 1987; **156**: 363-368.
13. Mortimer J, Miller S, Black D, Kwok K, Kirby WM. Comparison of cefoperazone and mezlocillin with imipenem as empiric therapy in febrile neutropenic cancer patients. *Am J Med* 1988; **85**: 17-20.
14. Moritz VA, Carson PBD. Cefoxitin sensitivity as a marker for inducible beta-lactamases. *J Med Microbiol* 1986; **21**: 203-207.
15. Lindh E, Dornbusch K, Jalakas K, Forsgren A. Antibiotic susceptibility and β -lactamase production in clinical isolates of *Enterobacter* spp. *APMIS* 1990; **98**: 462-470.
16. Yang Y, Livermore DM, Williams RJ. Chromosomal β -lactamase expression and antibiotic resistance in *Enterobacter cloacae*. *J Med Microbiol* 1988; **25**: 227-233.
17. Livermore DM. Do β -lactamases 'trap' cephalosporins? *J Antimicrob Chemother* 1985; **15**: 511-514.
18. Normark S. Mechanisms of β -lactamase induction in enterobacteria. *Med Malad Infect* 1989; **19**: 13-15.
19. Sanders CC, Sanders WE. Type I β -lactamases of gram-negative bacteria: interactions with β -lactam antibiotics. *J Infect Dis* 1986; **154**: 792-800.
20. Weinstein RA. Endemic emergence of cephalosporin resistant *Enterobacter*: relation to prior therapy. *Infect Control Hosp Epidemiol* 1986; **7**: 120-123.
21. Bush K, Tanaka SK, Bonner DP, Sykes RB. Resistance by decreased penetration of β -lactam antibiotics into *Enterobacter cloacae*. *Antimicrob Agents Chemother* 1985; **27**: 555-560.
22. Greek Society for Microbiology. Antibiotic resistance among gram-negative bacilli in 19 Greek hospitals. *J Hosp Infect* 1989; **14**: 177-181.
23. Legakis NJ, Koukoubanis N, Malliara K, Michalitsianos D, Papavassiliou J. Importance of carbenicillin and gentamicin cross-resistant serotype O:12 *Pseudomonas aeruginosa* in six Athens hospitals. *Eur J Clin Microbiol* 1987; **6**: 300-303.
24. Pitt TL, Livermore DM, Pitcher D, Vatopoulos AC, Legakis NJ. Multi-resistant serotype O:12 *Pseudomonas aeruginosa*: evidence for a common strain in Europe. *Epidemiol Infect* 1989; **103**: 565-576.
25. Vatopoulos AC, Philippon A, Tzouveleakis LS, Komninou Z, Legakis NJ. Prevalence of a transferable SHV-5 type β -lactamase in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Greece. *J Antimicrob Chemother* 1990; **26**: 635-648.
26. Vatopoulos AC, Tzouveleakis LS, Tzelepi E, Legakis NJ. Cross-resistance to norfloxacin and other antimicrobial agents among clinical isolates of gram-negative bacteria. *Int J Exp Clin Chemother* 1990; **3**: 201-205.