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β-Lactamases Responsible for Resistance to Expanded-Spectrum Cephalosporins in *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* Isolates Recovered in South Africa

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Although resistance to the expanded-spectrum cephalosporins among members of the family *Enterobacteriaceae* lacking inducible β-lactamases occurs virtually worldwide, little is known about this problem among isolates recovered in South Africa. Isolates of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* resistant to expanded-spectrum cephalosporins recovered from patients in various parts of South Africa over a 3-month period were investigated for extended-spectrum β-lactamase production. Antibiotic susceptibility was determined by standard disk diffusion and agar dilution procedures. Production of extended-spectrum β-lactamases was evaluated by using the double-disk test, and the β-lactamases were characterized by spectrophotometric hydrolysis assays and an isoelectric focusing overlay technique which simultaneously determined isoelectric points and general substrate or inhibitor characteristics. DNA amplification and sequencing were performed to confirm the identities of these enzymes. The *P. mirabilis* and *E. coli* isolates were found to produce TEM-26-type, SHV-2, and SHV-5 extended-spectrum β-lactamases. An AmpC-related enzyme which had a pI of 8.0 and which conferred resistance to cefoxitin as well as the expanded-spectrum cephalosporins was found in a strain of *K. pneumoniae*. This is the first study which has identified organisms producing different extended-spectrum β-lactamases from South Africa and the first report describing strains of *P. mirabilis* producing a TEM-26-type enzyme. The variety of extended-spectrum β-lactamases found among members of the family *Enterobacteriaceae* isolated from major medical centers in South Africa is troubling and adds to the growing list of countries where these enzymes pose a serious problem for antimicrobial therapy.

The production of β-lactamases is an important mechanism of resistance to β-lactam antibiotics among gram-negative bacteria. Expanded-spectrum cephalosporins have been specifically designed to resist degradation by the older broad-spectrum β-lactamases such as TEM-1, TEM-2, and SHV-1. The response to the expanded-spectrum cephalosporins among members of the family *Enterobacteriaceae* lacking inducible β-lactamases has been the production of mutant forms of the older β-lactamases called extended-spectrum β-lactamases (ESBLs). These enzymes are capable of hydrolyzing the newer cephalosporins and aztreonam (11). Studies by biochemical and molecular techniques indicate that many ESBLs are derivatives of older TEM-1, TEM-2, or SHV-1 β-lactamases, some of which differ from the parent enzyme by only one or two amino acids (11). In addition, resistance to the expanded-spectrum cephalosporins has also arisen in *Klebsiella pneumoniae* and *Escherichia coli* via the acquisition of plasmids containing the chromosomally encoded AmpC β-lactamase found in *Enterobacter* spp., *Pseudomonas aeruginosa*, and *Citrobacter* spp. (3, 25, 26).

Although ESBL-producing members of the family *Enterobacteriaceae* were first reported in Europe in 1983 and 1984, ESBLs have now been found in organisms recovered from patients on all continents except Antarctica (14, 27). The occurrence of organisms producing ESBLs varies widely, with some types more prevalent in Europe (TEM-3) and others more prevalent in the United States (TEM-10, TEM-12 and TEM-26), while others appear worldwide (SHV-2 and SHV-5) (30).

Reports concerning the existence of members of the family *Enterobacteriaceae* producing ESBLs in Africa have been limited to Saharan countries, and information from sub-Saharan Africa is scarce. Members of the family *Enterobacteriaceae* producing SHV-2 have been isolated from three different African countries, namely, Tunisia (6), Senegal (29), and Egypt (29), while TEM-3, TEM-20, and TEM-21 have also been recovered from Tunisia (6). *K. pneumoniae* strains resistant to the expanded-spectrum cephalosporins were the cause of a nosocomial outbreak in South Africa in the late 1980s, but the mechanism of resistance was not described (9). Resistance to the expanded-spectrum cephalosporins has also been observed in other species of the family *Enterobacteriaceae* such as *E. coli* and *Proteus mirabilis*. Therefore, a study was designed to characterize and describe the mechanisms responsible for resistance to the expanded-spectrum cephalosporins among clinical isolates of *K. pneumoniae*, *E. coli*, and *P. mirabilis* recovered from various medical centers in South Africa.

MATERIALS AND METHODS

Bacterial strains. During a period of 3 months in 1993, 37 strains of *K. pneumoniae* (13 blood, 5 burn, 7 wound, and 11 tracheal isolates), 4 strains of *P. mirabilis* (all wound isolates), and 4 strains of *E. coli* (1 blood, 1 burn, and 2 wound isolates) were collected from patients at the following medical centers in South Africa: Tygerberg Hospital near Cape Town, King Edward VIII Hospital in Durban, Chris Hani Baragwanath Hospital in Soweto, and Pretoria Academic Hospital in Pretoria. The strains were provided in response to a request for all strains of the family *Enterobacteriaceae* that lacked inducible β-lactamases and that were intermediate or resistant to cefotaxime or ceftazidime. The total

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TABLE 1. MICs for the different resistance phenotypes observed in *K. pneumoniae*, *E. coli*, and *P. mirabilis*

Species and resistance phenotype	No. of strains	MIC range (μg/ml)[a]							
		pip	tzp	ctx	caz	atm	fox	fep	imi
K. pneumoniae									
Kpn1	8	>128	2–4	0.25–1	>128	16–64	2–4	0.12	0.12
Kpn2	28	>128	2–>128	4–64	4–128	1–128	2–8	0.5–4	0.12–1
Kpn3	1	64	16	4	4	2	>128	0.12	0.12
E. coli									
Ec1	3	>128	1	1	>128	16	4	0.12	0.12
Ec2	1	>128	32	16	4	2	8	2	0.12
P. mirabilis	4	128	0.25–0.5	0.25–0.5	16–64	0.5–2	2–4	2	0.5–1

[a] pip, piperacillin; tzp, piperacillin-tazobactam (4 μg/ml); ctx, cefotaxime; caz, ceftazidime; atm, aztreonam; fox, cefoxitin; fep, cefepime; imi, imipenem.

number of strains screened is unknown, and at this time the referring hospitals did not perform more sensitive screening tests for ESBL detection. Therefore, accurate prevalence data were not obtained.

Thirty-four of the 43 patients involved (including all from whom isolates from blood were obtained) had received an expanded-spectrum cephalosporin during the 4 weeks prior to isolation of the organisms described above. Fifteen patients (including eight patients from whose blood isolates were obtained) were receiving either cefotaxime or ceftazidime at the time that the isolates were cultured and were considered not to be responding to these agents.

Susceptibility testing and antibiotics. Antibiotic susceptibility was determined by standard disk diffusion (21) and agar dilution (20) procedures. Standard powders of the following antimicrobial agents were kindly provided by the indicated companies: piperacillin and tazobactam, Lederle Laboratories (Wayne, N.J.); cefoxitin and imipenem, Merck (Rathway, N.J.); cefotaxime, Hoechst-Roussel Pharmaceuticals Inc. (Somerville, N.J.); ceftazidime, Glaxo Group Research Ltd. (Greenford, England); and aztreonam and cefepime, Bristol-Myers Squibb (Princeton, N.J.). Disks for the agar diffusion procedures were obtained from Becton Dickinson Microbiology Systems (Cockeysville, Md.). For quality control purposes, the following quality control strains were run simultaneously with the test organisms: *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *E. coli* ATCC 35218, and *Staphylococcus aureus* ATCC 29213. Throughout this study, results were interpreted by using the criteria of the National Committee for Clinical Laboratory Standards for disk diffusion (21) and broth dilution (20) procedures.

Double-disk test. All the strains were screened for the production of ESBLs by using the double-disk test as described by Jarlier et al. (15). A potentiation of the zones of cefotaxime, ceftriaxone, ceftazidime, or aztreonam by clavulanic acid represented a positive test result and was indicative of the possible presence of an ESBL.

β-Lactamase characterization. Overnight cultures in 5 ml of Trypticase soy broth were diluted with 45 ml of fresh broth and were incubated with shaking for 4 h at 37°C. The cells were harvested by centrifugation at 4°C, washed with 1 M potassium-phosphate buffer (pH 7.0), suspended, and sonicated. After sonication, crude extracts were obtained by centrifugation at 5,858 × *g* for 1 h. One strain, *K. pneumoniae* Pit 68, with a suspected AmpC β-lactamase, was induced with cefoxitin as described previously (28). The rates of hydrolysis of 100 μM solutions of nitrocephin, cephalothin, cefotaxime, ceftazidime, and aztreonam were determined by spectrophotometric assays with crude β-lactamase extracts (23).

The β-lactamases in the sonic extracts were assessed for pIs and general substrate and inhibitor characteristics in polyacrylamide gels (4, 18, 31). As controls, crude β-lactamase preparations from the following organisms possessing different TEM and SHV enzymes were examined simultaneously with the *K. pneumoniae*, *E. coli*, and *P. mirabilis* strains: TEM-1 [from *E. coli* RTEM(R6K)], TEM-2 [from *E. coli* 1752E(RP1)], TEM-10 [from *E. coli* C600(pK2)], TEM-26 [from *E. coli* HB101(pJPQ101), SHV-1 [from *E. coli* J53(R1010)], SHV-2 (from *Klebsiella ozaenae* 2180), SHV-3 [from *E. coli* J53(pUD18)], SHV-4 [from *E. coli* J53-2(pUD21)], and SHV-5 [from *E. coli* ClaNal(pAFF2)].

DNA amplification by PCR. The organisms were inoculated into 5 ml of Luria-Bertani broth (Difco, Detroit, Mich.) and incubated for 20 h at 37°C with shaking. Cells from 1.5 ml of an overnight culture were harvested by centrifugation at 17,310 × *g* in a Hermle centrifuge for 5 min. After the supernatant was decanted, the pellet was resuspended in 500 μl of distilled water. The cells were lysed by heating at 95°C for 10 min, and cellular debris was removed by centrifugation at 17,310 × *g* for 5 min. The supernatant was used as a source of template for amplification.

The following oligonucleotide primers specific for the SHV and TEM genes were designed by using MacVector, version 4.5 (Kodak/IBI): for SHV genes, A [5′-(CACTCAAGGATGTATTGTG)-3′] and B [5′-(TTAGCGTTGCCAGTGC TCG)-3′] corresponding to nucleotide numbers 103 to 121 and 988 to 970, respectively, of Mercier and Levesque (19); for TEM genes, C [5′-(TCGGGGAA ATGTGCGCG)-3′] and D [5′-(TGCTTAATCAGTGAGGCACC)-3′] corresponding to nucleotide numbers 90 to 105 and 1062 to 1042, respectively, of Sutcliffe (34). Primers A and B amplified a 885-bp fragment, while primers C and D amplified a 971-bp fragment. The specificities of the SHV and TEM primers for amplification of SHV and TEM genes, respectively, were tested by using the following β-lactamase controls; TEM-1 (pACYC177), MIR-1 (from *K. pneumoniae* 96D), and SHV-7 (pCLL3410).

PCR amplifications were carried out on a DNA Thermal Cycler 480 instrument (Perkin-Elmer, Cetus, Norwalk, Conn.) with the Gene Amp DNA amplification kit containing AmpliTaq polymerase (Perkin-Elmer, Roche Molecular Systems, Inc., Branchburg, N.J.). The composition of the reaction mixture was as follows: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, each of the four deoxynucleoside triphosphates at a concentration of 0.2 mM, and 1.2 U of AmpliTaq in a total volume of 49 μl. A total of 1 μl of sample lysate was added to the reaction mixture, and the mixture was centrifuged briefly before 50 μl of mineral oil was layered onto the surface. The PCR program consisted of an initial denaturation step at 96°C for 15 s, followed by 24 cycles of DNA denaturation at 96°C for 15 s, primer annealing at 50°C for 15 s, and primer extension at 72°C for 2 min. After the last cycle the products were stored at 4°C. The PCR products (1/10 volume) were analyzed by electrophoresis with 1.4% agarose gels in TAE buffer (0.04 M Tris-acetate, 0.002 M EDTA [pH 8.5]). The gels were stained with ethidium bromide, and the PCR products were visualized with UV light. A single band was observed for TEM amplified products with a single primer set. Two amplified products were observed with the SHV primer set. The larger product, which corresponded to the expected size of the SHV-specific product, was gel purified with a 1.4% agarose gel in TAE buffer, and the purified PCR product was used for sequence analysis.

PCR products were sequenced by automated PCR cycle sequencing with dye-terminator chemistry by using a DNA stretch sequencer from Applied Biosystems.

RESULTS

Resistance phenotypes. All the strains except *K. pneumoniae* Pit 68 gave a positive disk potentiation when cefotaxime, ceftriaxone, aztreonam, and/or ceftazidime disks were used. The MICs of piperacillin, piperacillin-tazobactam, cefotaxime, ceftazidime, aztreonam, and cefoxitin revealed three different resistance phenotypes (Kpn1, Kpn2, and Kpn3) in the *K. pneumoniae* strains and two resistance phenotypes (Ec1 and Ec2) in *E. coli* strains (Table 1). The phenotypes Kpn1 and Ec1 involved high-level resistance to ceftazidime (MICs, >128 μg/ml) but susceptibility to cefotaxime (MIC range, 0.25 to 1 μg/ml), while Kpn2 and Ec2 involved decreased susceptibility to both cefotaxime (MIC range, 4 to 64 μg/ml) and ceftazidime (MIC range, 4 to 128 μg/ml). Kpn3, represented by *K. pneumoniae* Pit 68, involved resistance to cefoxitin (MIC, >128 μg/ml) and decreased susceptibility to cefotaxime, ceftazidime, and aztreonam (MICs ≥2 μg/ml) (Table 1). The *P. mirabilis* isolates showed decreased susceptibility to ceftazidime (MIC range, 16 to 64 μg/ml) and susceptibility to cefotaxime (MIC range, 0.25 to 0.5 μg/ml).

β-Lactamases. Strains representing the Kpn1 and Ec1 phenotypes produced β-lactamases with pI values of 5.6 and 7.6

TABLE 2. Characteristics of β-lactamases produced by different resistance phenotypes

Species and resistance phenotype	No. of strains	pI	ctx hydrolysis[a]	Inhibition[b] by the following:		Most similar β-lactamases
				clox	clav	
K. pneumoniae						
Kpn1	8	5.6	Yes	No	Yes	TEM-10 or -26
		7.6	No	No	Yes	SHV-1
Kpn2	28	5.4	No	No	Yes	TEM-1
		7.6	Yes	No	Yes	SHV-2 or -8
		8.2	Yes	No	Yes	SHV-5
Kpn3	1	5.4	No	No	Yes	TEM-1
		8.0	Yes	Yes	No	AmpC
E. coli						
Ec1	3	5.4	No	No	Yes	TEM-1
		5.6	Yes	No	Yes	TEM-10 or -26
Ec2	1	5.4	No	No	Yes	TEM-1
		7.6	Yes	No	Yes	SHV-2 or -8
P. mirabilis	4	5.6	Yes	No	Yes	TEM-10 or -26

[a] Hydrolysis of 0.75 μg of cefotaxime (ctx) per ml; cefotaxime was used in the substrate-based isoelectric focusing overlay technique (12).
[b] The inhibitors used in the isoelectric focusing overlay technique were clavulanic acid (clav) and cloxacillin (clox) (13).

respectively, while phenotype Kpn2 and Ec2 involved enzymes with pIs of 5.4, 7.6, and 8.2 (Table 2). *K. pneumoniae* Pit 68, representing phenotype Kpn3, produced two β-lactamases with pIs of 5.4 and 8.0, respectively. The *P. mirabilis* strains showed a single enzyme with a pI of 5.6 (Table 2). The enzymes with pIs of 5.4, 5.6, 7.6, and 8.2 aligned with TEM-1 (pI 5.4), TEM-10 or TEM-26 (pI 5.57), SHV-1, SHV-2, or SHV-8 (pI 7.6), and SHV-5 (pI 8.2) (Table 2). It was therefore necessary to investigate these enzymes further. On isoelectric focusing gels, all of the β-lactamases except for the enzyme with a pI of 8.0 were inhibited by clavulanate, a characteristic of Bush group 2 enzymes (8). The enzyme with a pI of 8.0 was inhibited by cloxacillin, which correlates with Bush group 1 cephalosporinases (8). The substrate-based technique showed hydrolysis of 0.75 μg cefotaxime per ml at the bands focusing at pIs of 5.6, 8.0, and 8.2 and for some enzymes at the bands focusing at a pI of 7.6 (Table 2). Control enzymes of TEM-10, TEM-26, SHV-2, and SHV-5 showed hydrolysis of cefotaxime in this assay (Table 2).

Hydrolysis assays with nitrocefin, cefotaxime, ceftazidime, and aztreonam were performed with strains possessing single β-lactamases. All the strains assayed hydrolyzed cefotaxime, ceftazidime, and aztreonam to some extent (Table 3).

DNA amplification and sequencing. The DNAs from organisms producing single β-lactamases were amplified and sequenced. Strains producing ESBLs with pIs of 5.6, which aligned with TEM-10 and TEM-26, were amplified with the TEM primers (Table 4). The amino acids at positions 104, 164, and 240 (numbering of Ambler et al. [1]) were used to determine that this enzyme was more similar to TEM-26 (32). Amino acids deduced from amplicon sequences included lysine at position 104, serine at position 164, and glutamine at position 240 (Table 4). Strains producing ESBLs with pI values of 7.6 and 8.2, which aligned with SHV-2 and SHV-5, respectively, were amplified with SHV primers (Table 4). The amino acids at positions 205, 238, and 240 (numbering of Barthélémy et al. [2]) were used to identify the ESBL involved. The arginine at position 205, the serine at position 238, and the glutamic acid at position 240 of the deduced amino acid sequence of strains producing an ESBL with a pI of 7.6 indicated the presence of SHV-2 (13) (Table 4). *K. pneumoniae* Pit 82, producing an ESBL with a pI of 8.2, had a lysine at position 240, indicating the presence of SHV-5 (7) (Table 4).

DISCUSSION

Although members of the family *Enterobacteriaceae* producing ESBLs have been recovered from medical centers in South Africa since the late 1980s, little is known about the various types present or their prevalence (10). ESBL-producing *K. pneumoniae* strains have been involved in nosocomial outbreaks in intensive care units at several academic centers, leading to the temporary closure of one of the units. Furthermore, in Durban, a strain of *K. pneumoniae* producing an ESBL has been responsible for the failure of therapy with an expanded-spectrum cephalosporin (16).

This is the first report to identify definitively the different types of ESBLs produced by members of the family *Enterobacteriaceae* isolated from major medical centers in South Africa. The following ESBLs, produced by *K. pneumoniae*, *E. coli*, and *P. mirabilis*, were identified: a TEM-26-type β-lactamase, SHV-2, SHV-5, and an AmpC β-lactamase with a pI of 8.0. This represents the major types of β-lactamases found worldwide. *K. pneumoniae* and *E. coli* producing TEM-26 were first isolated from cancer patients in a children's hospital in Stanford, Calif. (22), and have subsequently been described in England (12) and France (33). This is the first report of *K. pneumoniae* and *E. coli* producing a TEM-26-type β-lactamase outside the United States, Europe, and the United Kingdom. Members of the family *Enterobacteriaceae* producing SHV-2 and SHV-5 have been described worldwide (30), and South Africa has now been added to the list of countries where these enzymes are present.

The occurrence of an AmpC β-lactamase encoded on a plasmid recovered from strains of *K. pneumoniae* resistant to cefoxitin, as well as the expanded-spectrum cephalosporins, was first reported from Providence, R.I. (25). These strains were involved in a nosocomial outbreak, and imipenem was the only β-lactam antibiotic active against the strains involved. Since that initial report, *E. coli* and *K. pneumoniae* strains producing plasmid-mediated AmpC β-lactamases have been isolated worldwide (35). These Bush group 1 β-lactamases appear to originate from chromosomal genes of *Enterobacter*, *Citrobacter freundii*, and *P. aeruginosa* (5). This report of a South African strain adds another isolate of *K. pneumoniae* which appears to produce this type of β-lactamase. The DNA responsible for encoding

TABLE 3. Hydrolysis profiles of cell extracts containing a single β-lactamase

Strain	β-Lactamase pI	Hydrolysis (nmol of substrate[a] hydrolyzed/min/mg of protein)			
		Nitrocefin	Cefotaxime	Ceftazidime	Aztreonam
K. pneumoniae					
Pit 16	5.6	114	4	3	0.6
Pit 100	7.6	159	11	0.1	0.9
Pit 82	8.2	136	11	0.2	0.9
E. coli					
Pit 64	5.6	275	1	2	0.4
Pit 56	7.6	143	9	0.1	0.8
P. mirabilis Pit 85	5.6	138	5	1	0.5

[a] A 100 μM solution of substrate was used.

TABLE 4. Identification of extended-spectrum β-lactamases occurring in South Africa

Strain[a]	pI	Amplification with the following:		Amino acid at the indicated position						β-Lactamase
				TEM[b]			SHV[c]			
		TEM primers	SHV primers	104	164	240	205	238	240	
K. pneumoniae										
Pit 16	5.6	Yes	No	Lys	Ser	Glu				TEM-26 type
Pit 100	7.6	No	Yes				Arg	Ser	Glu	SHV-2
Pit 82	8.2	No	Yes				Arg	Ser	Lys	SHV-5
E. coli										
Pit 64	5.6	Yes	No	Lys	Ser	Glu				TEM-26 type
Pit 56	7.6	No	Yes				Arg	Ser	Glu	SHV-2
P. mirabilis Pit 85	5.6	Yes	No	Lys	Ser	Glu				TEM-26 type

[a] Strains with single β-lactamases were used for sequencing.
[b] Numbering according to Sutcliffe (34).
[c] Numbering according to Mercier and Levesque (19).

this enzyme needs to be cloned and sequenced to determine if this is a new type of plasmid-mediated AmpC β-lactamase.

β-Lactamases with extended-spectrum activities were first isolated from *P. mirabilis* in 1991 (36). This enzyme, FPM-1, was very similar to the chromosomal cephalosporinase of *Proteus vulgaris*. Subsequently, *P. mirabilis* strains producing TEM-3 have been isolated in France (17), and *P. mirabilis* producing TEM-10 have been isolated in the United States (24). This is the first report describing clinical isolates of *P. mirabilis* that produce TEM-26.

The variety of β-lactamases present in South Africa is disconcerting and probably reflects the overuse of the newer expanded-spectrum cephalosporins by the medical community at large. Although this investigation did not address the issue of prevalence, there is not doubt that the widespread dissemination of organisms producing ESBLs and plasmid-mediated AmpC enzymes will severely limit the therapeutic options of physicians facing these organisms, because the carbapenems are the only β-lactam drugs uniformly active against organisms producing these β-lactamases. Organisms producing these enzymes pose a serious threat for the future treatment of infections at large, and if these problems are to be minimized, it is important that these newer antimicrobial agents be used sparingly and with discretion.

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