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Extended-Spectrum-β-Lactamase-Producing *Enterobacteriaceae* in Yaounde, Cameroon

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Organisms producing extended-spectrum β-lactamases (ESBLs) have been reported in many countries, but there is no information on the prevalence of ESBL-producing members of the family *Enterobacteriaceae* in Cameroon. A total of 259 *Enterobacteriaceae* strains were isolated between 1995 and 1998 from patients at the Yaounde Central Hospital in Cameroon. Enterobacterial isolates resistant to extended-spectrum cephalosporin and monobactam were screened for ESBL production by the double-disk (DD) synergy test. Thirty-one (12%) of these *Enterobacteriaceae* strains were shown to be positive by the DD synergy test, suggesting the presence of ESBLs. Resistance to oxyimino-cephalosporins and monobactams of 12 (38.7%) of the 31 strains—i.e., 6 *Klebsiella pneumoniae*, 4 *Escherichia coli*, 1 *Citrobacter freundii*, and 1 *Enterobacter cloacae* strain—was transferred to *E. coli* HK-225 by conjugation. Resistance to gentamicin, gentamicin plus trimethoprim-sulfamethoxazole, or trimethoprim-sulfamethoxazole was cotransferred into 6, 2, and 1 of these transconjugants, respectively. All 12 transconjugants were resistant to amoxicillin, piperacillin, all of the cephalosporins, and aztreonam but remained susceptible to cefoxitin and imipenem. Crude extracts of β-lactamase-producing transconjugants were able to reduce the diameters of inhibition zones around disks containing penicillins, narrow- to expanded-spectrum cephalosporins or monobactams when tested against a fully susceptible *E. coli* strain but had no effect on such zones around cefoxitin, imipenem, and amoxicillin-clavulanate disks. The β-lactamases produced by the 12 tranconjugants turned out to be SHV-12 by DNA sequencing. Therefore, the ESBL SHV-12 is described for the first time in Cameroon.

Many extended-spectrum β-lactamases (ESBLs) are plasmid-mediated derivatives from TEM- and SHV-type enzymes and cause resistance to expanded-spectrum cephalosporins. They belong to Bush group 2be (6). Since their initial description in Germany in 1983 (13), ESBLs have diversified and spread worldwide. Several ESBLs appear to be particularly widely disseminated, being found in many countries, whereas others seem to occur more commonly in one or few countries (4). The various national patterns of antibiotic consumption in hospitals probably account for the differences in distribution of these enzymes. In an attempt to detect and study the dissemination of ESBLs in a central African country (Cameroon), we collected and characterized producers of such enzymes among clinical isolates of *Enterobacteriaceae* at Yaounde Central Hospital between 1995 and 1998. The ESBL SHV-12 was found in several species of *Enterobacteriaceae* for the first time in Cameroon.

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MATERIALS AND METHODS

Bacterial strains. A total of 259 isolates, members of the *Enterobacteriaceae* family were collected from patients in Yaounde Central Hospital (Table 1). Isolates were collected over 3-year period (April 1995 to March 1998) from urine, pus, and blood. The isolates were identified by conventional techniques (9) and were confirmed by the API 20 E (bioMérieux, France). There were no replicate strains isolated from any patient in the present study. *Escherichia coli* HK 225 (12), which is resistant to rifampin and streptomycin, was used as a recipient strain for transfer experiments by conjugation. *E. coli* ATCC 25922 was used as a control strain for antimicrobial susceptibility testing and as a negative control for PCR experiments. *E. coli* K12R111 (provided by Danielle Sirot) encoding TEM-1 and plasmid pMPA encoding SHV-2A were used as positive controls for bla_{TEM} and bla_{SHV} genes, respectively.

Antimicrobial susceptibility testing and detection of ESBL producers. Antimicrobial susceptibility was determined by disk diffusion tests according to the methods of the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards) (16) by using disks from BBL Microbiology Systems (Cockeysville, Md.). The tested antibiotics were amoxicillin, amoxicillin-clavulanate, piperacillin, cefazolin, cefoxitin, cefotaxime, ceftazidime, aztreonam, imipenem, gentamicin, ciprofloxacin, and trimethoprim-sulfamethoxazole.

The double-disk (DD) synergy test (11) was used for detection of ESBLs in clinical and transconjugant strains. A central disk of amoxicillin-clavulanate was surrounded by disks with cefotaxime, ceftriaxone, ceftazidime, and aztreonam at a distance of ca. 19 mm (center to center) on a Mueller-Hinton agar plate (Difco Laboratories, Detroit, MI) inoculated according to the standard procedures (16). Distortion of the peripheral inhibition zones of surrounding antibiotics toward the central disk with clavulanate was indicative for an ESBL. The tests were repeated with a disk spacing of 15 mm (center to center).

TABLE 1. Occurrence of ESBL-producing phenotypes among members of the family *Enterobacteriaceae*

Organism	Total isolates analyzed	No. of isolates with ESBL phenotypes (%)
Escherichia coli	91	13 (14.3)
Klebsiella spp.	64	12 (18.8)
Proteus spp.	56	1 (1.8)
Enterobacter spp.	31	2 (6.5)
Citrobacter spp.	17	3 (17.6)
Total	259	31 (12)

MICs for positive DD synergy test strains were determined by using the E-test (AB Biodisk, Solna, Sweden) according to the instructions of the manufacturer.

Conjugation experiments. Transfer of resistance phenotypes was performed by a liquid mating method (19) in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.). Culture mixtures were incubated overnight at 37°C with clinical isolates as donors and *E. coli* HK 225 as the recipient. After conjugation, bacterial suspensions were plated onto agar containing 50 μg of ampicillin, 10 μg of ceftazidime, 2 μg of cefotaxime, 100 μg of rifampin, and 2,000 μg of streptomycin per ml. The resulting transconjugants were purified and identified with API 20 E strips. The frequencies of transfer were determined per input donor cell.

Characterization of β-lactamases. Transconjugant strains were grown at 37°C with shaking in brain heart infusion broth (BBL Microbiology Systems, Cockeysville, Md.) containing ceftazidime (10 μg/ml), cefotaxime (2 μg/liter), and ampicillin (50 μg/liter). The cells were harvested during late log phase (8) by centrifugation at 3,000 × *g* at 4°C, washed twice in 0.1 M phosphate buffer (pH 7.0), and resuspended in 1/50 of the original volume. After cell rupture by sonication on ice, the cellular debris was removed by two centrifugations at 5,000 × *g*, each for 30 min at 4°C. Both times, the supernatant was carefully removed without solid particles. The crude extracts were stored at −20°C.

The extracts' ability to hydrolyze β-lactam antibiotics (amoxicillin, amoxicillin-clavulanate, piperacillin, cefazolin, cefoxitin, ceftriaxone, cefotaxin, ceftazidime, aztreonam, and imipenem) was evaluated by a microbiological method (15). Disk diffusion test were performed according to the methods of the CLSI (16), and antimicrobial disks were immediately impregnated with 10 μl of crude extract after their application. The inhibition zones produced against *E. coli* HK 225 were compared to those observed by untreated disks. A reduction of the inhibition zone diameter as a consequence of the applied extract was considered evidence for β-lactamase activity.

Molecular analysis techniques. DNA of transconjugants strains was extracted with phenol and chloroform, precipitated with isopropanol, and resuspended in TE buffer (3). *bla* genes were amplified by PCR with the following oligonucleotide primers: TEM OT-1-F (5′-TTGGGTGCACGAGTGGGTTA-3′) and OT-2-R (5′-TAATTGTTGCCGGGAAGCTA-3′), which amplified a 465-bp fragment of the bla_{TEM} gene (2), and SHV-F (5′-CGC CGG GTT ATT CTT ATT TGT CGC-3′) and SHV-R (5′-TCT TTC CGA TGC CGC CGC CAG TCA-3′), which amplified a 1,017-bp fragment of the bla_{SHV} gene (17). The cycle conditions were as follows: 30 cycles, with 1 cycle consisting of denaturation at 95°C for 30 s, annealing at 52°C (TEM) or 68°C (SHV) for 30 s, and extension at 72°C for 1 min. Each PCR program was preceded by a denaturation step of 94°C for 5 min and followed by a final denaturation at 72°C for 10 min. The SHV PCR products were digested with the restriction enzyme NheI (17).

Sequencing of the PCR products was performed by using the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, CA) according to the recommendations of the manufacturer in an ABI Prism 310 genetic analyzer. Sequence data were processed and analyzed with the version 9.0 GCG Sequence Analysis Software Package (Genetics Computer Group, Madison, WI).

Sequence accession number. The nucleotide sequence of bla_{SHV-12} from *C. freundii* 09 is filed in the GenBank database under the accession number AY940490.

RESULTS

Detection of ESBL-producing isolates. A total of 259 isolates (124 urine isolates, 120 isolates from pus, and 15 isolates from blood) belonging to the family *Enterobacteriaceae* were studied, among which 31 (12%) potential ESBL producers were identified by the DD synergy test. Characteristic clavulanate-induced distortions of inhibition zones that were indicative for ESBL production were found in 86, 90, 62, and 97% of the strains around the disks containing cefotaxime, ceftriaxone, ceftazidime, and aztreonam, respectively. The ESBL producer's isolates were investigated, and the ESBL-producing phenotype was found most frequently among *Klebsiella* species (18.8%; *K. pneumoniae* [$n = 11$] and *K. oxytoca* [$n = 1$]), followed by *Citrobacter* species (17.6%; *C. freundii* [$n = 3$]) and *E. coli* (14.3%) (Table 1). Of the ESBL-producing strains, 15 were from urine, 11 were from pus, and 5 were from blood; isolates were mainly from patients in the intensive care unit (ICU) (38.7%) and the surgical (45.1%) ward (Table 2).

MICs of extended-spectrum cephalosporins exhibited a notable variability among different isolates, but MICs of CAZ were always ≥2 μg/ml (Table 2). All of the isolates were resistant to amoxicillin, piperacillin, and cephalothin, and most of them were also resistant to amoxicillin-clavulanate, gentamicin, and trimethoprim-sulfamethoxazole. All of the isolates were susceptible to imipenem, and most of them were also susceptible to cefoxitin, ciprofloxacin, and amikacin (Table 2).

Transferability of ESBL genes. Among potential ESBL producers, 12 (38.7%) were transferred oxyimino-cephalosporin resistance to *E. coli* HK 225. The transfer frequencies were between 9×10^{-8} and 6.7×10^{-4} per input donor. The isolates included six of *K. pneumoniae*, four of *E. coli*, one of *E. cloacae*, and one of *C. freundii*. Resistance determinants against the following non-β-lactam antibiotics were cotransferred to *E. coli* HK 225: gentamicin (six cases), gentamicin and trimethoprim-sulfamethoxazole (two cases), and trimethoprim-sulfamethoxazole (one case). In contrast, resistance to cefoxitin or to other antibiotic classes was not transferred (Table 3).

β-Lactamase type. Supernatants containing crude β-lactamase extracts from transconjugants did not affect the inhibition zones of *E. coli* HK 225 around cefoxitin, imipenem, and amoxicillin-clavulanate disks. The supernatants did, however, significantly reduce the inhibition zones around disks containing amoxicillin, piperacillin, cefazolin, cefotaxime, ceftriaxone, ceftazidime, and aztreonam (data not shown). These results were consistent with the presence of the detected ESBLs.

All 12 transconjugants were subjected to molecular detection and characterization procedures aimed at bla_{TEM} and bla_{SHV} genes. From all 12 strains, no PCR product was obtained with TEM specific primers, whereas a characteristic 1,017-bp amplimer was synthesized, which proved to be degradable by the restriction enzyme NheI into two fragments of 770 and 247 bp in length. This positive PCR/NheI test indicated that an SHV-ESBL was produced by all transconjugants and hence by the 12 corresponding donor clinical isolates (Table 3).

Nucleotide sequencing revealed that all 12 amplimers were 100% homologous to each other along their entire length. This also confirmed the PCR/NheI results and showed that the deduced amino acid sequences differed in the following positions (Ambler numbering [1]) from the SHV-1 standard (4): leucine 35→glutamine, glycine 238→serine, and glutamic acid 240→lysine. Thus, SHV-12 (http://www.lahey.org/studies/webt.htm) was identified in all of the 12 strains (Table 3). More-

TABLE 2. Distribution, clinical features, antimicrobial susceptibility test, and resistance transfer of *Enterobateriaceae* with ESBL phenotype

Organism	Isolate	Ward	Specimen	MIC (μg/ml)[a]									DD synergy test	Other resistance marker(s)[b]	Transfer of ESBL gene
				Amx	Amc	Pip	Cfz	Fox	Ctx	Cro	Caz	Imp			
Escherichia coli	15/98	Surgery	Pus	>256	32	>256	>256	32	16	32	6	0.19	+	Sxt	−
	J51	Surgery	Pus	>256	>256	>256	>256	8	3	4	8	0.125	+		−
	J55	Surgery	Pus	>256	>256	>256	>256	>256	>256	>256	24	0.19	+	Gm, Sxt	+
	H302	ICU	Blood	>256	16	>256	>256	3	4	3	4	0.125	+	Gm, Cip, Sxt	−
	301/98	ICU	Pus	>256	12	>256	>256	12	12	16	12	0.25	+	Gm, Cip, Sxt	−
	424/97	Obstetrics/ Genecology	Urine	>256	>256	>256	>256	>256	>256	>256	>256	1.5	+	Gm, Amk, Sxt	−
	J6	Surgery	Urine	>256	16	>256	>256	3	2	3	32	0.125	+	Sxt	+
	268/98	ICU	Urine	>256	24	>256	>256	12	12	16	32	0.125	+	Gm, Cip, Sxt	+
	288/98	ICU	Urine	>256	8	>256	>256	6	8	12	64	0.125	+	Cip, Sxt	+
	H304	ICU	Blood	>256	24	>256	>256	4	4	4	32	0.19	+	Gm, Cip, Sxt, Amk	−
	U349	ICU	Urine	>256	12	>256	>256	3	48	48	96	0.125	+	Gm, Cip, Sxt	−
	508	Surgery	Pus	>256	8	>256	>256	3	4	2	24	0.19	+	Gm, Sxt	−
	H281	ICU	Blood	>256	8	>256	>256	1.5	3	2	16	0.19	+	Sxt	−
Klebsiella pneumoniae	U229	ICU	Urine	>256	16	>256	>256	3	48	32	>256	0.125	+	Gm, Sxt	+
	U271	Surgery	Urine	>256	32	>256	>256	2	256	256	96	0.19	+	Gm, Sxt	+
	U284	Surgery	Urine	>256	32	>256	>256	2	>256	>256	>256	0.19	+	Gm, Amk, Sxt	+
	J55	Surgery	Pus	>256	16	>256	>256	6	16	12	64	0.19	+	Gm	+
	J51	Surgery	Pus	>256	16	>256	>256	3	3	6	64	0.25	+	Gm, Sxt	−
	203cp	Urology	Urine	>256	24	>256	48	3	1	2	12	0.25	+	Gm, Sxt	−
	176cp	ICU	Blood	>256	24	>256	>256	4	2	3	12	0.125	+	Gm, Sxt	−
	J21	Urology	Urine	>256	3	8	12	3	1	1.5	4	0.19	+	Sxt	−
	083	ICU	Blood	>256	12	>256	>256	3	2	2	3	0.125	+	Gm, Sxt	−
	48/42	Surgery	Urine	>256	16	>256	>256	96	24	32	32	0.125	+	Sxt	+
	508	Surgery	Pus	>256	24	>256	>256	3	16	32	6	0.19	+	Gm, Sxt	+
Klebsiella oxytoca	103	Urology	Urine	>256	16	>256	>256	4	4	4	12	0.125	+	Gm, Sxt	−
Enterobacter aerogenes	1086	Surgery	Pus	>256	24	>256	>256	96	1.5	3	16	0.19	+	Gm, Sxt	−
Enterobacter cloacae	189	Surgery	Urine	>256	>256	>256	>256	>256	>256	>256	>256	0.25	+	Gm, Sxt	+
Citrobacter freundii	09/98	ICU	Pus	>256	48	>256	>256	>256	8	16	96	0.19	+	Gm, Sxt	+
	J13	Urology	Urine	>256	64	>256	>256	>256	12	16	32	0.19	+	Gm, Cip, Sxt	−
	U271	Surgery	Urine	>256	>256	>256	>256	93	96	96	64	0.125	+	Gm, Sxt	−
Proteus mirabilis	294	ICU	Pus	>256	8	>256	>256	1.5	4	8	8	0.125	+	Gm, Sxt	−

[a] Amx, amoxicillin; Amc, amoxicillin-clavulanic acid (2:1); Pip, piperacillin; Cfz, cefazolin; Fox, cefoxitin; Ctx, cefotaxime; Cro, ceftriaxone; Caz, ceftazidime; Atm, aztreonam; Imp, imipenem.
[b] Amk, amikacin; Gm, gentamicin; Cip, ciprofloxacin; Sxt, trimethoprim-sulfamethozaxole.

TABLE 3. Characteristics of transconjugant strains

Transconjugant *E. coli* strains	Antibiotic resistance pattern[a]	*bla* gene		β-Lactamase type
		TEM	SHV	
Hk-225-U288	ESBL	−	+	SHV-12
Hk-225-J55E	ESBL, Gm	−	+	SHV-12
Hk-225-J6	ESBL	−	+	SHV-12
Hk-225-U268	ESBL, Gm	−	+	SHV-12
Hk-225-508K	ESBL, Gm	−	+	SHV-12
Hk-225-U229	ESBL, Sxt	−	+	SHV-12
Hk-225-48/42	ESBL	−	+	SHV-12
Hk-225-U271	ESBL, Gm, Sxt	−	+	SHV-12
Hk-225-U284	ESBL, Gm, Sxt	−	+	SHV-12
Hk-225-J55K	ESBL, Gm	−	+	SHV-12
Hk-225-U189	ESBL, Gm	−	+	SHV-12
Hk-225-09	ESBL, Gm	−	+	SHV-12
Hk-225		−	−	−

[a] Gm, gentamicin; Sxt, trimethoprim-sulfamethozaxole.

over, two silent mutations at codons 138 and 268 were the same as those described in the original sequence of bla_{SHV-12} (EMBL accession no. X98105 [18]), namely, CTG and ACG, as opposed to CTA and ACC, respectively, in the standard bla_{SHV-1} sequence. However, one additional G-to-C transversion immediately after the TAA stop codon was detected (position 935 in X98105).

The bla_{SHV-12} sequence and flanking regions of *C. freundii* 09 were deposited in GenBank (accession no. AY940490) as the representative for the 12 isolates listed in Table 4.

The 12 SHV-12-producing isolates exhibited a relatively narrow clinical distribution. They were mainly obtained from the surgical ward between 1996 and 1998 (Table 4).

DISCUSSION

The aim of this study was to determine the prevalence and characterize ESBL producers among members of the family *Enterobacteriaceae* in Yaounde Central Hospital. We found isolates that expressed an ESBL producer phenotype among every genus that was tested. ESBLs were present in 31 (12%) among the 259 *Enterobacteriaceae* isolates evaluated here. The prevalence among genera is varied, with rates of 18.8% for *Klebsiella* spp. and 1.8 to 17.6% for all other genera. Of 31 ESBL producer isolates, 12 (38.7%) transferred the ESBL gene to *E. coli* HK 225 and were also found to produce SHV-12 ESBL. These data represent the first report of the prevalence of ESBL producers among *Enterobacteriaceae* in Cameroon.

TABLE 4. Distribution of SHV-12-producing clinical isolates of *Enterobacteriaceae*

Species	Isolate	Date (day/mo/yr)	Ward
Escherichia coli	J6	14/02/1997	Surgery
	J55E	19/08/1997	Surgery
	U268	13/03/1998	ICU
	U288	17/03/1998	ICU
Klebsiella pneumoniae	508K	26/07/1996	Surgery
	J55K	19/08/1997	Surgery
	U229	04/03/1998	ICU
	48/42	04/03/1998	Surgery
	U271	13/03/1998	Surgery
	U284	16/03/1998	Surgery
Enterobacter cloacae	U189	24/02/1998	Surgery
Citrobacter freundii	09	16/02/1998	ICU

In the present study, most ESBL producers were collected from patients in the surgical ward and the ICU. In these wards, isolates are exposed to great antibiotic pressure. Furthermore, many of these patients are particularly vulnerable to infection because they are immunocompromised or have an easy avenue of access for bacteria (23).

The susceptibility test data showed that the ESBL producers which were resistant to most β-lactams and non-β-lactams such as gentamicin and trimethoprim-sulfamethoxazole were multidrug-resistant strains. The ESBL producers usually carry a multiresistant plasmid, the genes conferring resistance to β-lactam and non-β-lactam antibiotics (10, 23). All of the ESBL producers were susceptible to imipenem, and most were also susceptible to cefoxitin, amikacin, and ciprofloxacin. If the patients are infected by ESBL producers, carbapenem may be used (5, 14).

The results of the present study are evidence of an ongoing outbreak during the sampling period from 1996 to 1998 at Yaoundé Central Hospital of ESBL-producing organisms attributable to at least four species of *Enterobacteriaceae*: *K. pneumoniae*, *E. coli*, *C. freundii*, and *E. cloacae*. Although the bla_{SHV-12} gene was cotransferable along with other resistance determinants upon conjugation and was found in several species, it seemed possible that the determinant is located on a large low-copy broad-host-range plasmid, as is usually the case (10, 21). In the present study, we focused on determinants that were easily transferable by conjugation in vitro (more than a third of the ESBL isolates from Yaounde Central Hospital) because these are the most clinically relevant factors with regard to the speed of dissemination.

Within the collection of 31 strains that yielded positive DD results, ceftazidime and aztreonam showed the lowest (62%) and highest (96%) rates of detection, respectively. These results indicate that at maximum sensitivity, when looking for ESBLs, several oxyimino-cephalosporins and aztreonam should be used simultaneously for DD testing. This is in agreement with the recommendations by Coudron et al. (7). Moreover, this finding underlines the fact that synergy tests and other physiological tests in general are of limited sensitivity in detecting ESBLs, a fact that has been stated already in 1995 (14) and confirmed by careful studies involving site-directed mutagenesis, as well as different copy number cloning vectors in isogenic systems (20).

ESBLs are now a problem for hospitalized patients worldwide. The rates of ESBL producers among *Klebsiella* sp. and *E. coli* at our center are 18.8 and 14.3%, respectively. This prevalence is lower for *Klebsiella* sp. and higher for *E. coli* than that reported by the SENTRY worldwide surveillance program, in which the ESBL prevalences in *K. pneumoniae* and *E. coli* were 45 and 8.5% (Latin America), 25 and 7.9% (Western Pacific), or 23 and 5.3% (Europe), respectively (22).

In conclusion, the present study emphasizes the importance of screening for ESBLs even in countries where such enzymes

have not been reported previously. The plasmid-mediated ESBLs have been already disseminated to four different species of *Enterobacteriaceae* and escalated into a multiclinic outbreak at Yaounde Central Hospital by the time they were discovered.

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