Molecular characterization of extended-spectrum beta-lactamases in *Enterobacteriaceae* from patients of two hospitals in Saxony, Germany

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Between January and September 2003, 39 isolates of the family *Enterobacteriaceae* with phenotypically positive Vitek 1 extended-spectrum beta-lactamase (ESBL) test results were collected, originating from patients of two hospitals in Saxony, Germany. Plasmid DNA was isolated and screened by PCR for the presence of genes encoding beta-lactamases of SHV, TEM and CTX-M types. To differentiate ESBL and non-ESBL among SHV and TEM genes, detailed analysis of PCR products was performed. Twenty-four strains carried SHV-2, SHV-5 or SHV-12 genes. In a further 11 strains a CTX-M gene was detected. The CTX-M genes could be affiliated to the CTX-M-1 and CTX-M-9 cluster by RFLP analysis. In the case of four *Klebsiella oxytoca* isolates, hyperproduction of the chromosomal beta-lactamase K1 was inferred, because genes of the above-mentioned types were not detected. The strains contained plasmid DNA between 45 and 160 kb in size. Common plasmid restriction patterns among SHV-5 producers provided evidence of horizontal spread. Twenty strains had a MIC for cefotaxime of ≤ 4 mg l⁻¹, 18 strains had the same MIC for ceftazidime, and nine strains had this MIC of > 4 mg l⁻¹ for both antibiotics. The ESBL phenotypes often coincided with ciprofloxacin or gentamicin resistance.

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INTRODUCTION

Enterobacterial resistance against oxyimino-cephalosporins such as ceftriaxone and ceftazidime (CAZ) is a growing problem in the treatment of nosocomial infections, and is often caused by the production of extended-spectrum beta-lactamases (ESBLs) (Paterson *et al.*, 2003). ESBLs are frequently identified in *Klebsiella pneumoniae* and *Escherichia coli* (Gniadkowski *et al.*, 1998a), but also in other species, such as *Citrobacter* spp., *Enterobacter* spp. and *Pseudomonas aeruginosa*. ESBL producers have been found worldwide (Jacoby & Medeiros, 1991). The general substrate profile of ESBLs includes penicillins, cephalosporins and monobactams. More than 100 ESBL variants from different types are known. The most abundant types are represented by SHV, TEM, OXA and CTX-M (Gniadkowski, 2001). All types except OXA belong to Ambler class A or Bush group 2be. OXA enzymes belong to class D or group 2d (Bush, 2001). While TEM- and SHV-type ESBLs have developed from their ancestors SHV-1 and TEM-1/-2 by point

mutations, the origin of the other enzymes is less clear. The plasmid-encoded TEM-1/-2 enzymes are widespread and are the cause of resistance to broad-spectrum penicillins in many Gram-negative rods, such as *E. coli*, *Neisseria gonorrhoeae* and *Haemophilus influenzae* (Livermore, 1995). SHV-1 is the chromosomally encoded beta-lactamase K2 from *K. pneumoniae*, but has also been found to be plasmid encoded in other species (Chang *et al.*, 2001). For both TEM-1 and SHV-1, an association with transposons is recognized (Heritage *et al.*, 1999). Some enzymes of the CTX-M type are identical or similar to species-specific beta-lactamases from *Kluyvera* spp. (Bonnet, 2004). The detection of ESBL production in microbial diagnostics is especially problematic, since such micro-organisms often seem to be susceptible to broad-spectrum cephalosporins *in vitro* (Gniadkowski, 2001). A failure to detect ESBLs and subsequent treatment with oxyimino-cephalosporins are associated with a higher risk of therapy failure (Paterson *et al.*, 2001). Higher mortality rates have also been described (Kim *et al.*, 2002). Although the first ESBLs were discovered in Germany (Kliebe *et al.*, 1985), investigations of ESBLs in Germany have not yielded consistent results (Bauernfeind *et al.*, 1993; Babini & Livermore, 2000). In order to get information on the regional spread of ESBL, we characterized 39 strains that were isolated from patients in two local hospitals and that tested ESBL positive with a Vitek 1 ESBL test (bioMérieux).

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Abbreviations: CAZ, ceftazidime; CFP, cefepime; CIPR, ciprofloxacin; CTX, cefotaxime; ESBL, extended-spectrum beta-lactamase; GENT, gentamicin; ICU, intensive care unit; IMIP, imipenem; PIP, piperacillin; PITA, piperacillin/tazobactam.

METHODS

Bacterial strains. The strains included in this study were isolated between January and September 2003 from specimens of patients hospitalized at two local hospitals in the region of Dresden, designated hospital A and hospital B. The isolates showed decreased susceptibility to oxyimino-cephalosporins and were phenotypically tested for ESBL production by the Vitek 1 ESBL test (bioMérieux). ESBL test-positive strains were collected and stored in glycerol cultures. In total, from 37 patients, 21 *K. pneumoniae* strains, 10 *E. coli* strains, six *K. oxytoca* strains and one strain each of *Citrobacter freundii* and *Enterobacter aerogenes* were isolated.

Transfer of resistance determinants. In order to investigate the conjugative transfer of resistance determinants, broth mating experiments were performed. The plasmid-free, rifampicin-resistant *E. coli* strain A15 R⁻ was used as an acceptor (Elwell & Falkow, 1986). This strain was kindly provided by Branka Bedenić, 'A. Štampar' Medical School, University of Zagreb, Croatia. Overnight Luria-Bertani (LB) broth cultures of the acceptor and the respective donor strain were mixed in a ratio of 1:2 and incubated for 18 h at 37 °C without shaking. The selection of transconjugants was performed by spreading 100 µl of the mixture onto MacConkey agar plates containing 200 mg rifampicin l⁻¹ as well as 2 mg CAZ l⁻¹ or cefotaxime (CTX). As some *K. pneumoniae* strains developed rifampicin resistance during mating, colonies were subcultured on citrate agar (Simmons citrate agar, Oxoid) to detect citrate-positive, rifampicin-resistant *K. pneumoniae* donor strains.

Preparation of plasmid DNA. Plasmid DNA was obtained using a modified alkaline-lysis method (Sambrook *et al.*, 1989), with slight modifications. Briefly, a further purification step with phenol/chloroform was added before DNA precipitation in 2-propanol. In some strains, the quality of this preparation was not sufficient for the subsequent restriction analysis, probably because of the presence of capsular polysaccharides. In these cases, bismuth (III) pentahydrate (Sigma-Aldrich) and sodium salicylate were added to the LB medium before incubation in final concentrations of 0.5 and 2.5 mM, respectively, as described by Domenico *et al.* (1992). After incubation and before alkaline lysis, EDTA (pH 10) was added to a final concentration of 5 mM to bind bismuth ions. Unlike the method of Domenico *et al.* (1992), the treatment with a detergent was omitted. Plasmids were separated on a 0.8% (w/v) agarose gel in Tris/borate/EDTA buffer for 1.5 h at 120 V.

Restriction of plasmid DNA. Aliquots of 10 µl of the plasmid DNA preparation were subjected to restriction with 1 µl *EcoRI* (10 U µl⁻¹; Fermentas), 3 µl Buffer *EcoRI*⁺ (Fermentas), and 16 µl nuclease-free deionized water for 2 h at 37 °C. DNA was separated on a 1% (w/v) agarose gel for 17 h at 30 V together with the molecular mass standard peqGOLD DNA-Sizer IX (Peqlab Biotechnology).

PCR. In order to detect genes encoding Ambler class A beta-lactamases (*bla*), a standard PCR was performed with plasmid DNA as a template. For amplification of genes encoding SHV beta-lactamases (*bla_{SHV}*), primers MN I (5'-CGC CGG GTT ATT CTT ATT TGT CGC-3') and MN II (5'-TCT TTC CGA TGC CGC CGC CAG TCA-3') were used (Nüesch-Inderbinen *et al.*, 1996). The thermal cycling conditions used for the PCR with these primers included 30 cycles at 94 °C for 30 s, 68 °C for 60 s, and 72 °C for 60 s, with a final extension of 72 °C for 300 s. The 1016 bp PCR product contained the entire ORF. For amplification of genes encoding TEM beta-lactamases (*bla_{TEM}*), primers TEM-F (5'-ATA AAA TTC TTG AAG ACG AAA-3') and TEM-R (5'-GAC AGT TAC CAA TGC TTA ATC A-3') were used with thermal cycling conditions as described elsewhere (Wu *et al.*, 2001). The 1080 bp amplicon reached from 214 bp upstream of the start codon to the stop codon. In order to detect

genes for CTX-M beta-lactamases (*bla_{CTX-M}*), a PCR was performed with primers and conditions described elsewhere (Edelstein *et al.*, 2003). The amplicon is a 544 bp intragenic fragment. Ten microlitres of PCR product was electrophoresed in a 1% (w/v) agarose gel for 1 h at 140 V together with a Smartladder DNA standard (Eurogentec).

DNA sequencing. Since the above-mentioned MN and TEM primers are specific for all *bla_{SHV}* and *bla_{TEM}*, including the non-ESBL variants, it was necessary to sequence the PCR products. For this purpose, PCR products were purified with a QIAquick PCR purification kit (Qiagen). Sequencing reactions were performed with the primers used for PCR detection using a BigDye Terminator Cycle Sequencing Ready Reaction Premix (Applied Biosystems). After purification with AutoSeq-G50 columns (Amersham Biosciences), products of the sequencing reaction were subjected to direct sequencing on an ABI Prism 377 automated sequencer (Applied Biosystems). The sequences were analysed using the program Bioedit (Hall, 1999). Chromatograms were visually inspected for double peaks as signs of the presence of different *bla* genes from the same type.

Differentiation between *bla_{ESBL-SHV}* and *bla_{non-ESBL-SHV}* in a PCR product. In the case of double peaks within the chromatograms of *bla_{SHV}* sequences, a 40 µl aliquot of the corresponding PCR product was digested with 5 µl *NheI* (10 U µl⁻¹; Fermentas) and 5 µl 10× Buffer Y⁺/Tango (Fermentas) with BSA for 3 h at 37 °C. Digested DNA was separated on 1.5% (w/v) agarose gels for 2 h at 140 V. As described elsewhere (Nüesch-Inderbinen *et al.*, 1997), most *bla_{ESBL-SHV}* genes contain the *NheI* restriction site, in contrast to *bla_{non-ESBL-SHV}* genes. Thus, both fragments could be separated from each other. The *bla_{non-ESBL-SHV}* fragment was extracted from the gel under visualization with UV light. The extraction was performed using a QIAquick Gel Extraction kit (Qiagen), according to the guidelines of the manufacturer. The product was directly subjected to sequencing, as described above, without any further purification. In this way, mixed sequence signals at characteristic positions inside ORFs of some SHV sequences could be assigned to ESBL and non-ESBL enzymes, respectively.

RFLP analysis of *bla_{CTX-M}*. A further subgrouping of *bla_{CTX-M}* was performed according to Edelstein *et al.* (2003). For this purpose, 10 µl of the products of CTX-M-specific PCR were digested with 0.9 µl *PstI* (10 U µl⁻¹), 0.2 µl *PvuII* (20 U µl⁻¹), 2 µl 10-fold NEBuffer 3 (enzymes and buffer, New England BioLabs) and 6.9 µl nuclease-free distilled water at 37 °C for 3 h. Restriction fragments were electrophoresed in a 3% (w/v) agarose gel for 2 h at 140 V together with the DNA standard Smartladder SF (Eurogentec).

Determination of MICs. In order to phenotypically characterize the resistance properties of the strains, MICs were determined for CTX (Aventis), CAZ (GlaxoSmithKline), cefepime (CFP; Bristol-Myers Squibb), piperacillin (PIP; Ratiopharm) in combination with tazobactam (Wyeth Pharma), imipenem (IMIP; Merck, Sharp and Dohme), gentamicin (GENT; Merck) and ciprofloxacin (CIPR; Bayer). MICs were determined by the agar dilution method on Mueller-Hinton agar plates containing antibiotics in concentrations of a geometric series according to the DIN 58940-6 guidelines of the German Institute for Standardization (Deutsches Institut für Normung, 1989). MIC determination for the PIP/tazobactam (PITA) combination was tested using a fixed concentration of 4 mg tazobactam l⁻¹ and varying concentrations of PIP.

RESULTS

The Department of Medical Microbiology and Hygiene is responsible for clinical microbiological diagnostics of patients from all wards of two hospitals in Dresden and

its vicinity. The origins of the individual strains are listed in Table 2. Most strains (19) were isolated from clinical specimens originating from patients' airways, followed by urine (12), skin (three), body cavities (three) and blood (two). All 39 strains belonged to the family *Enterobacteriaceae*.

SHV-specific PCR and sequencing

SHV-specific PCR revealed *bla*_{SHV} genes in all 21 *K. pneumoniae*, in two *E. coli* and one each in *K. oxytoca*, *Ent. aerogenes* and *C. freundii* isolates. The DNA sequences of the PCR products showed seven alleles encoding SHV-2, nine encoding SHV-5, four encoding SHV-12 and one each encoding SHV-1 and SHV-28. The latter two enzymes are not known to mediate extended-spectrum resistance. However, SHV-28 has been described in another ESBL-conferring strain (Ndugulile *et al.*, 2005), and has been suggested to confer ESBL-mediated resistance.

A further four raw DNA sequences contained mixed signals in the respective electropherograms at characteristic positions, all at amino acid position 238 according to the numbering provided by Ambler (Ambler *et al.*, 1991). This problem was resolved by *NheI* restriction endonuclease digestion, as described above. It resulted in the detection of two SHV alleles in a single strain, as follows: *bla*_{SHV-2} paired with *bla*_{SHV-1} twice; *bla*_{SHV-2} paired with *bla*_{SHV-28} once; *bla*_{SHV-12} paired with *bla*_{SHV-1} once. The sequence of *K. pneumoniae* KP01 revealed a non-functional start codon, damaged by a T to G mutation; apart from that it was identical to the deduced SHV-1 sequence. In conclusion, the ESBL phenotype could be explained by SHV ESBL production in 24 strains only, in spite of a positive SHV PCR result in 26 cases. *Ent. aerogenes* EA05 (single *bla*_{SHV-1}),

K. pneumoniae KP12 (single *bla*_{SHV-28}) and KP01 (defective start codon) putatively express other resistance mechanisms in addition to the production of SHV-type enzymes. Coding and non-coding genetic polymorphisms are summarized in Table 1. Since all ESBL genes from the same SHV type were identical among themselves, only one representative example is shown for all strains of the same ESBL type.

TEM-specific PCR

As mentioned above, only in 24 strains could the ESBL characteristic be explained by the presence of SHV ESBL genes. The genetic origin of ESBL production in the remaining 15 strains was therefore located in genes of other types. Accordingly, all strains were screened for further genes. A TEM-specific primer set was used to detect *bla*_{TEM} genes. In 12 strains, *bla*_{TEM} genes could be detected in this way. Sequencing of the PCR products with the same primers used for PCR revealed reliable chromatograms up to Ambler position 285. All 12 PCR products consisted of *bla*_{TEM} sequences identical to published *bla*_{TEM-1} sequences (Backman *et al.*, 2000). They originated from four SHV-5 producers (KP09, KP13, KP19, KP22), three SHV-12 producers (KP03, KP10, EC29), and two *K. oxytoca* and four *E. coli* not producing SHV enzymes. The ESBL phenotypes could not be explained by the presence of *bla*_{TEM} genes in this survey.

CTX-M-specific PCR and RFLP analysis of PCR products

As ESBLs from type CTX-M have been increasing in frequency in Western and Central Europe for some years (Alobwede *et al.*, 2003), further screening for this type was performed. With respect to the genetic heterogeneity within

Table 1. Coding and silent mutations in ORFs of sequenced *bla*_{SHV} genes compared with SHV-1 (GenBank accession no. AF124984)

Isolate*	Enzyme	Codon†								
		Tyr/Phe-7	Leu/Gln-35	His-112	Leu-138	Arg-215	Gly/Ser-238	Glu/Lys-240	Ile-260	Thr-268
		TAT	CTA	CAC	CTA	CGG	GGC	GAG‡	ATT	ACC
KP02, KP03	SHV-1	—	—	CAT	CTG	CGA	—	GAA	ATC	—
KP23	SHV-1	—	—	—	—	—	—	—	—	—
EA05	SHV-1	—	—	CAT	CTG	CGA	—	GAA	ATC	ACG
KP12, KP33	SHV-28	TTT	—	CAT	CTG	CGA	—	GAA	ATC	—
KP37	SHV-2	—	—	—	—	—	AGC	—	—	—
KP34	SHV-5	—	—	—	—	—	AGC	AAG	—	—
KO26	SHV-12	—	CAA	—	CTG	—	AGC	AAG	—	ACG

*Isolates are abbreviated as follows: KP, *K. pneumoniae*; EA, *Ent. aerogenes*; KO, *K. oxytoca*.

†The amino acid residue (in the case of a coding mutation, both amino acids are shown), followed by the position according to the Ambler numbering scheme (Ambler *et al.*, 1991). The sequence of a prototype SHV-1 sequenced by Rice *et al.* (1999) is shown above each column, and identity to that sequence is marked by '—'. Mutated nucleotides are shown in bold type.

‡At this codon, two possible mutations occur: a coding mutation at the first position and a non-coding one at the third position.

Table 2. Overview over all strains, identified enzyme(s), ward of isolation, MICs and assigned resistance phenotype

Abbreviations: anaesthes., anaesthesiology; HA, hospital A; HB, hospital B.

Strain*	Enzyme(s)	Ward	Plasmid restriction pattern (kb)	MIC (mg l ⁻¹)							Resistance-type
				PITA	CTX	CAZ	CFP	GENT	CIPR	IMIP	
KP01	CTX-M-1-Gr.	Surgical ICU, HA		8	64	4	8	≤1	≤1	≤2	CTX
R + 01				4	32	4	8	≤1	≤1	≤2	CTX
KP12	CTX-M-1-Gr., SHV-28	HB		2	8	≤2	≤2	≤1	≤1	≤2	CTX
R + 12				4	64	≤2	8	≤1	≤1	≤2	CTX
EA05	CTX-M-1-Gr., SHV-1	Urological, HA		16	64	32	8	≤1	4	≤2	CTX
EC07	CTX-M-1-Gr.	Medical, HA		8	16	≤2	8	≤1	4	≤2	CTX
EC16	CTX-M-1-Gr.	Medical, HA		64	128	8	32	≤1	>4	≤2	CTX
EC17	CTX-M-1-Gr.	Medical, HA		64	128	8	32	≤1	4	≤2	CTX
EC30	CTX-M-1-Gr.	Surgical ICU, HA		2	64	4	16	≤1	≤1	≤2	CTX
R + 30				> 128	> 1024	8	> 64	≤1	≤1	≤2	CTX
EC31	CTX-M-1-Gr., TEM-1	Medical, HA		4	4	≤2	≤2	≤1	>4	≤2	CTX
EC40	CTX-M-1-Gr., TEM-1	Paediatric, HA		2	32	≤2	4	≤1	≤1	≤2	CTX
R + 40				4	8	≤2	≤2	≤1	≤1	≤2	CTX
EC25	CTX-M-9-Gr., TEM-1	Anaesthes. ICU, HA	G (140)	2	32	≤2	8	≤1	≤1	≤2	CTX
R + 25			G (140)	≤1	64	≤2	32	≤1	≤1	≤2	CTX
EC27	CTX-M-9-Gr., TEM-1	Surgical ICU, HA	G (140)	4	32	≤2	16	≤1	≤1	≤2	CTX
R + 27			G (140)	2	64	≤2	32	≤1	≤1	≤2	CTX
KP02	SHV-1, SHV-2	HB		8	4	4	≤2	≤1	≤1	≤2	CTX
KP06	SHV-2	HB	A (70)	8	4	4	≤2	>4	≤1	≤2	CTX
KP14	SHV-2	Anaesthes. ICU, HA	A (70)	16	4	4	≤2	>4	≤1	≤2	CTX
KP21	SHV-2	Paediatric ICU, HA	B (90)	> 128	64	16	4	≤1	≤1	≤2	CTX
KP24	SHV-2	Paediatric ICU, HA	B (90)	> 128	32	256	8	≤1	≤1	≤2	CAZ
KP23	SHV-1, SHV-2	Surgical ICU, HA	C (90)	> 128	8	16	4	≤1	4	≤2	CTX
KP32	SHV-2	HB	C (90)	32	≤2	4	≤2	≤1	2	≤2	CTX
KP33	SHV-28, SHV-2	Urology, HA	D (110)	8	≤2	4	≤2	>4	≤1	≤2	CTX
KP37	SHV-2	Urology, HA	D (110)	16	≤2	≤2	≤2	>4	≤1	≤2	CTX
KP11	SHV-2	HB		> 128	8	16	4	>4	≤1	≤2	CTX
KP04	SHV-5	HB		16	8	128	≤2	≤1	2	≤2	CAZ
EC20	SHV-5	Paediatric, HA		2	≤2	32	≤2	≤1	≤1	≤2	CAZ
R + 20				4	64	4	≤2	≤1	≤1	≤2	CTX
KP28	SHV-5	HB	F' (160)	16	4	128	≤2	4	2	≤2	CAZ
R + 28			F' (160)	> 128	16	1024	≤2	>4	2	≤2	CAZ
KP34	SHV-5	Anaesthes. ICU, HA	F' (160)	> 128	4	128	≤2	4	2	≤2	CAZ
KP39	SHV-5	Surgical, HA	F (90)	8	≤2	128	≤2	≤1	≤1	≤2	CAZ
KP09	SHV-5, TEM-1	HB	E (200)	> 128	≤2	128	≤2	2	≤1	≤2	CAZ
KP13	SHV-5, TEM-1	Medical ICU, HA		16	≤2	128	≤2	4	≤1	≤2	CAZ
KP19	SHV-5, TEM-1	HB	E' (215)	> 128	4	128	≤2	4	≤1	≤2	CAZ
KP22	SHV-5, TEM-1	Surgical ICU, HA		> 128	4	128	≤2	2	≤1	≤2	CAZ
KP03	SHV-12, TEM-1, SHV-1	HB		> 128	≤2	128	≤2	>4	>4	≤2	CAZ
KP10	SHV-12, TEM-1	HB		> 128	4	32	≤2	>4	≤1	≤2	CAZ
R + 10				4	≤2	8	≤2	≤1	≤1	≤2	CAZ
EC29	SHV-12, TEM-1	Medical ICU, HA		> 128	16	128	8	≤1	≤1	≤2	CAZ
R + 29				> 128	64	512	8	≤1	≤1	≤2	CAZ
CF18	SHV-12	Surgical ICU, HA		8	4	128	≤2	≤1	≤1	≤2	CAZ
R + 18				4	8	64	≤2	≤1	≤1	≤2	CAZ
KO26	SHV-12, K1	Surgical ICU, HA		> 128	64	> 1024	8	4	≤1	≤2	CAZ
KO08	K1	HB		> 128	32	≤2	≤2	≤1	4	≤2	CTX
KO15	K1, TEM-1	HB		> 128	64	≤2	≤2	≤1	≤1	≤2	CTX
KO36	K1	HB		> 128	≤2	≤2	≤2	≤1	2	≤2	CTX
KO38	K1	Surgical ICU, HA		> 128	4	≤2	≤2	≤1	≤1	≤2	CTX
ECA15				4	≤2	≤2	≤2	≤1	≤1	≤2	

*Transconjugant strains are named R+.

the group of *bla*_{CTX-M}, a subgrouping into five clusters (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and recently discovered CTX-M-25) was performed using sequence homology criteria (Bonnet, 2004). To detect the presence of CTX-M ESBL genes in the strains used in this study, a primer pair sensitive to all *bla*_{CTX-M} genes independent of cluster affiliation was chosen. However, this primer pair produces false-positive results for the species-specific chromosomally encoded beta-lactamase (*bla*) genes of some Gram-negative strains (Edelstein *et al.*, 2003). Among the species used in this work, only the *K. oxytoca* strains with their chromosomal beta-lactamase K1 encoded by *bla*_{OXY} were affected. In fact, an amplicon could be obtained by this PCR analysis from DNA preparations from all these strains. In addition, a positive result could be obtained from eight *E. coli*, two *K. pneumoniae* and the *Ent. aerogenes* strains. For all non-*K. oxytoca* strains, RFLP analysis of PCR products was performed in order to obtain information about the cluster affiliation of the genes found. Restriction patterns were compared with the expected ones described in the original publication (Edelstein *et al.*, 2003). For the new cluster of CTX-M-25-like enzymes (CTX-M-25 and CTX-M-26), which was not discovered at the date of development of the method, a restriction map was created with Bioedit. The digestion of such a CTX-M-25-like PCR product would result in fragments of 422 and 120 bp in length, which can be distinguished from the restriction patterns of the other clusters. The PCR products of all strains, except two *E. coli* strains, displayed the fragment of a *bla*_{CTX-M-1}-like gene. Two *E. coli* isolates, EC25 and EC27, carried a gene belonging to the CTX-M-9 cluster. The PCR products of *K. oxytoca* strains were sequenced using the same primer pair as for PCR detection. This procedure was performed to identify mixed signals in sequence chromatograms that would have occurred in the case of the simultaneous presence of a *bla*_{OXY} encoding K1 and a *bla*_{CTX-M}. All sequences of *K. oxytoca* strains were clear cut in encoding K1. Table 2 shows the detected enzyme genes, the plasmid restriction patterns and the MIC values for all strains.

Mating experiments

Transconjugants could be obtained from 11 strains only. If a mating experiment was unsuccessful, it was repeated with CTX or CAZ as selective agent. Transconjugant plasmid DNA was obtained and PCRs were performed in the same way as in the case of donor strains. Two SHV-5 producers, three SHV-12 producers, four of the strains producing a CTX-M-1-like enzyme and both strains with the *bla*_{CTX-M-9}-like gene transferred their ESBL gene to *E. coli* A15 R⁻. Five of the 11 donors carried an additional TEM-1, but only one of them co-transferred it to the acceptor strain. SHV PCR of plasmid DNA from transconjugants of two CTX-M producers with an additional *bla*_{non-ESBL-SHV} was negative.

Analysis of plasmids

In order to detect the presence of common restriction bands among plasmids of strains with the same ESBL type and

among their transconjugants, plasmid DNA was isolated and digested with *Eco*RI. Five different restriction patterns were found among SHV-2 producers, the band sizes of which ranged from approximately 70 to 130 kb. Four of the restriction patterns appeared to be paired. Two of the pairs with identical plasmid restriction patterns were isolated from the same departments within hospital A. In the case of the other two paired restriction patterns, one pair of strains was isolated at hospital A and one at hospital B. Analysis of unrestricted plasmid DNA of SHV-5 producers revealed at least two single bands, which allowed us to infer the presence of at least two independent plasmids. All restriction patterns of SHV-5-producing strains plasmid DNA had common elements, which can be inferred from Fig. 1. Strains EC20 and KP28 were two SHV-5 producers from which transconjugants could be obtained. All restriction bands of the donor pattern were present in transconjugants in both cases (110 and 160 kb). SHV-5-producing strains were isolated at both hospitals. Among SHV-12-producing strains, no common plasmid DNA restriction pattern could be observed, other than a common 6 kb band in KP10, CF18 and KO26. Plasmid sizes ranged from 45 to 160 bp. The CTX-M producers EC07, EC16 and EC17 had a common restriction pattern, probably because of three common bands in electrophoresis of their undigested plasmid DNA. In addition to these three bands there were a further one at EC07, two at EC16 and four at EC17. All three strains were isolated from the same patient of hospital A on two different dates. The two strains EC25 and EC27 producing a CTX-M-9-like beta-lactamase had an identical plasmid restriction profile (~140 kb). Both were isolated from intensive care units (ICUs) of hospital A. As was

Fig. 1. Restriction patterns of plasmid DNA of SHV-5-carrying strains after *Eco*RI digestion show partly common bands. Lanes: A and M, DNA-Sizer Smartladder (10, 8, 6, 5, 4, 3 kb); B, R+28; C, KP28; D, KP34; E, KP39; F, KP22; G, KP04; H, KP09; I, KP13; J, KP19; K, EC20; L, R+20; N, DNA-Sizer IX (48, 38, 33, 29, 24, 23, 19, 17, 15, 12, 10, 8 kb).

the case with the SHV-12 producers, there was no common restriction pattern observable upon gel electrophoresis of digested plasmid DNA of the other CTX-M-producing strains. Total plasmid DNA of the strains ranged from approximately 40 to 90 kb in size.

Determination of MICs

The MICs for selected antibiotics are summarized in Table 2. Depending on their MICs, a resistance type was assigned to CTX (MIC for CAZ greater than twofold the MIC for CTX) and CAZ (MIC for CAZ greater than fourfold the MIC for CTX) (Bedenič *et al.*, 2001). The resistance type correlated with ESBL genotype as follows. All strains producing SHV-2 and CTX-M beta-lactamases were assigned to a CTX resistance type, while SHV-5- and SHV-12-producing strains displayed a CAZ type. KP24 and the transconjugant from EC20, R+20, were exceptions. Surprisingly 10 strains had a MIC ≤ 2 mg l⁻¹ for CTX and 12 had the same for CAZ. The SHV-2 producer KP37 and the K1 carrier KO36 did not grow at this concentration, either on CTX or CAZ. In summary, 20 strains appeared to be susceptible to CTX (MIC ≤ 4 mg l⁻¹) and 19 to CAZ (MIC ≤ 4 mg l⁻¹). Nine strains were susceptible to both antibiotics according to the German standard tests. CFP was revealed to be more effective against ESBL producers; only four strains were resistant. The MICs towards PITA were distributed bimodally: 20 strains had a MIC between 2+4 and 16+4 mg l⁻¹, while 17 strains still grew at concentrations of 512+4 mg l⁻¹. Twelve strains were resistant towards GENT (MIC ≥ 4 mg GENT l⁻¹). KP28 transferred this resistance together with an ESBL to the donor strain as well as reduced susceptibility towards CIPR (MIC ≥ 2 mg CIPR l⁻¹), in common with 12 other strains. All strains were completely susceptible to IMIP (MIC ≤ 2 mg IMIP l⁻¹).

DISCUSSION

SHV-2- and SHV-5-type ESBLs are spread worldwide, and their occurrence in Germany has already been known for some time (Bauernfeind *et al.*, 1993). The SHV-12 allele has usually been found in Asia (Kim *et al.*, 2005; Liu *et al.*, 2004). It has also been detected in the other four continents (De Champs *et al.*, 2004; Nüesch-Inderbinen *et al.*, 1996; Mulvey *et al.*, 2004; Howard *et al.*, 2002). All *bla*_{SHV-12} genes found here carried the same characteristic silent mutations (Table 1) as the one first described (Nüesch-Inderbinen *et al.*, 1996) and all the other sequenced genes of this type published in the NCBI database. Amongst the SHV-12 producers analysed in this study we found no common plasmid restriction patterns. Probably, *bla*_{SHV-12} is part of a worldwide-disseminating mobile element. Besides *K. pneumoniae*, *C. freundii* (CF18), *E. coli* (EC29) and *K. oxytoca* (KO26) also contain SHV-12 genes. Moreover, the SHV-12 gene has been found in *Serratia liquefaciens* (GenBank accession no. AY273807), *Enterobacter cloacae* (AF462395) and *Acinetobacter baumannii* (AY259163). Although

first identifications of CTX-M-type ESBLs occurred about 15 years ago (Bauernfeind *et al.*, 1990), for years, dissemination has only been known in endemic areas of Asia, Southern America and Eastern Europe (Bonnet, 2004). Recently, dissemination of this ESBL type has been observed (Coque *et al.*, 2002; Alobwede *et al.*, 2003). Detection of CTX-M-type enzymes in Dresden underlines this trend. Most of the strains carried genes for a CTX-M-1-like enzyme. For CTX-M-3, the most important representative of this group, epidemic occurrence in Poland (Gniadkowski *et al.*, 1998b) and Russia (Edelstein *et al.*, 2003) is known. In those two studies, the two CTX-M-9-like carriers EC25 and EC27 had identical plasmid restriction profiles, in contrast to the CTX-M-1-like enzyme carriers found in this study. Both were isolated from ICUs of hospital A, implicating cross-infection. Their 160 kb plasmid could be transferred *in vitro*, suggesting further dissemination of this enzyme type. The most disseminated representative of this enzyme type, CTX-M-14, has already been found in Spain (Coque *et al.*, 2002), France and the UK (Alobwede *et al.*, 2003).

Most of the strains able to transfer their resistance *in vitro* were SHV-12 or CTX-M producers, implicating the role of transconjugation in dissemination, while transconjugants could not be obtained from SHV-2 producers. A similarly low transconjugation success was reported from a molecular survey of SHV-producing *Klebsiella* strains in Switzerland (Nüesch-Inderbinen *et al.*, 1996). In that study, 26 of 37 *bla*_{SHV}-carrying strains were unable to transfer ESBL genes. In our survey, 19 of 24 strains did not transfer the ESBLs. In contrast, six of 11 CTX-M carriers were shown to transconjugate the CTX-M genes. Probably, clonal expansion is a more important mechanism in the dissemination of SHV-2 and SHV-5 genes.

All but one of the non-ESBL SHV sequences (*bla*_{SHV-1} in KP23) carried silent mutations that were different to those found in ESBL genes, making co-evolution improbable (Table 1). SHV-28 had already been identified from China (GenBank accession nos AF538324 and AY299299). This allele differs from SHV-1 by an amino acid substitution at position 7 according to Ambler's standard numbering scheme for class A beta-lactamases (Ambler *et al.*, 1991). Since the mature protein starts with Ambler position 26 compared to SHV-1 (Péduzzi *et al.*, 1989), there should not be any difference either in biochemical properties or in isoelectric point. The detected SHV-1 from *Ent. aerogenes* EA05 must have been plasmid encoded, because from this species only a chromosomally encoded Ambler class C beta-lactamase (AmpC) is known, which is produced only in small amounts, but is inducible. The same applies to *C. freundii*. The beta-lactamase K1 is the chromosomally encoded class A beta-lactamase of *K. oxytoca*. It is encoded by two genes, *bla*_{OXY-1} and *bla*_{OXY-2} (Mammeri *et al.*, 2001). Intrinsically, K1 mediates only moderate resistance towards penicillins. However, at present, up to 20% of isolates hyperproduce this enzyme in Europe. The

consequence is a significant resistance towards ceftriaxone, CTX and aztreonam. This must be the case with the *K. oxytoca* strains in this study, as one can observe typical signs of K1 hyperproduction in the resistance patterns, such as resistance to PITA and at least reduced susceptibility to CTX (Table 2) (Livermore, 1995). Hyperproduction is more usual with *bla*_{OXY-2}-encoded enzymes, and can be caused by point mutations within the promoter sequence (Jeong *et al.*, 2001; Fournier *et al.*, 1995). In the case of *K. oxytoca* KO26, in which an SHV-12 beta-lactamase had already been identified, K1 was encoded by *bla*_{OXY-1}. In the case of the other four *K. oxytoca* strains, K1 was encoded by *bla*_{OXY-2}. With respect to the Vitek 1 ESBL test as performed in our department, it is known that it cannot distinguish between a K1 hyperproducer and a 'classical' ESBL carrier (Leverstein-van Hall *et al.*, 2002). Although K1 was assigned to group 2be (ESBL) within the functional beta-lactamase classification scheme of Bush–Jacoby–Medeiros (Bush *et al.*, 1995), in fact, ESBL detection in K1 hyperproducers represents a false-positive result, because it is not necessary to regard such strains as resistant to all cephalosporins independently of their resistance patterns, as is the case with ESBL carriers. Moreover, these strains cannot transfer their resistance horizontally. KO26 demonstrates that *K. oxytoca* strains are potential ESBL producers, and false-negative ESBL test results should be regarded as more dangerous than false-positive results in that regard. Paterson *et al.* (2001) have described the higher risk of therapy failure in the treatment by oxyimino-cephalosporins of infections with ESBL-producing strains, even if the strains appear to be susceptible *in vitro*. The number of strains having a MIC defined as susceptible to CAZ or CTX underlines the necessity to test with both antibiotics and cut-offs below 4 mg l⁻¹. Experts suggest 2 mg l⁻¹ (Witte & Mielke, 2003; Geiss *et al.*, 2003). CFP seems to be more effective than CTX and CAZ. One should consider the possibility of the decreasing efficacy of this beta-lactam due to inoculum effects and changes in the outer-membrane protein profile during therapy, particularly in the case of infections that are connected with high bacterial inocula *in vivo*, such as endocarditis, meningitis, septic arthritis, osteomyelitis, abscesses and other deep-seated infections (Thomson & Moland, 2001). The bimodal distribution of MICs towards PITA has recently been observed and investigated (Babini *et al.*, 2003). It is caused by titration effects when testing with fixed concentrations of tazobactam. The distribution becomes unimodal if the ratio of piperacillin to tazobactam is kept constant at 1 : 8. Babini *et al.* (2003) emphasize the general susceptibility of ESBLs towards inhibition with tazobactam. If strains are susceptible to PITA *in vitro*, it should be considered as a therapeutic option. The prevalence of ESBL-producing strains decreases if hospitals reduce their use of oxyimino-cephalosporins in favour of beta-lactam inhibitor plus piperacillin (Gniadkowski, 2001; Bradford, 2001). In our survey ESBL production coincided with fluoroquinolone (28 % reduced susceptibility to CIPR) and aminoglycoside resistance (30 % GENT resistance in this survey), similar to results seen elsewhere. Thus, fewer therapeutic options

remain for treatment, and that is one reason why the emergence of ESBLs should be controlled in the future. In this context, the importance of molecular diagnostics will increase, as they are a more reliable method than phenotypic testing.

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