International Clones of *Klebsiella pneumoniae* and *Escherichia coli* with Extended-Spectrum β -Lactamases in a Czech Hospital[▽]

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A 2-month survey of extended-spectrum β -lactamase (ESBL) producers was performed in a Czech hospital. *Klebsiella pneumoniae* produced SHV-2, -5, or -12, *Escherichia coli* produced CTX-M-9 or -15, and other species produced TEM-92 or -132. All *K. pneumoniae* and *E. coli* isolates belonged to sequence types (STs) or clonal complexes (CCs) spread across the world (*K. pneumoniae* clonal complex 11 [CC11], CC14, and sequence type 101 [ST101] and *E. coli* CC31, CC73, CC131, and CC405) and carried various plasmids (mainly with A/C- and FII-type replicons).

Plasmid-encoded extended-spectrum β -lactamases (ESBLs) hydrolyzing penicillins, cephalosporins, and monobactams are a major reason behind the resistance of members of the family *Enterobacteriaceae* to antimicrobials (4, 21, 35). Organisms with these enzymes, especially *Klebsiella pneumoniae* and *Escherichia coli*, have been disseminating worldwide over the last two decades, reaching alarming prevalence rates in some countries or larger geographic areas (5, 43). From among several ESBL types identified, enzymes of the TEM, SHV, and more recently, CTX-M families have been playing prominent roles (30, 39). Growing evidence indicates that global spread of some ESBL variants greatly depends on particular clones of the producer species and on specific types of plasmids carrying their genes (11, 12, 24, 31, 33).

In the Czech Republic, the data on ESBLs have been scarce (25, 27). However, in 2007, the rates of ESBL-producing *K. pneumoniae* and *E. coli* nosocomial invasive isolates were as high as 30.8% and 6.2%, respectively (P. Urbášková, unpublished results). This work aimed to produce a detailed analysis of ESBL-producing organisms from a large Czech hospital.

In June and July 2006, a 2-month survey was performed in the University Hospital in Plzeň, Czech Republic (1,800 beds). All nonrepeated *Enterobacteriaceae* isolates identified as ESBL producers were collected. Species identification was carried out by Entero test 24 (Pliva Lachema Diagnostika, Brno, Czech Republic), and ESBLs were detected by the double-disk test with modifications for the species with natural AmpC β -lactamases (15). Twenty-four isolates were identified, including 10 *K. pneumoniae* isolates, 9 *E. coli* isolates, 3 *Providencia stuartii* isolates, 1 *Enterobacter cloacae* isolate, and 1 *Proteus mirabilis* isolate (Table 1). All these isolates represented nosocomial contamination. Several isolates had no or unclear clinical significance (four *K. pneumoniae* and *E. coli* isolates from

throat swabs that were surveillance cultures and the *K. pneumoniae* 1838/06 isolate from the sputum of a geriatric patient that represented colonization of the respiratory tract). Of the total number of 1,710 enterobacterial isolates recovered in the hospital over the study period (including surveillance cultures), ESBL producers represented 1.4% of the isolates, including 3.4% of *K. pneumoniae* isolates (293 isolates in total) and 1.2% of *E. coli* isolates (768 isolates in total).

The MICs of antimicrobials were determined by broth dilution as proposed by EUCAST (19). The isolates showed increased MICs of β -lactams and various patterns of resistance to other antimicrobials (Table 2). Some variations in the MICs of particular β -lactams between different strains, e.g., those of piperacillin with tazobactam for *K. pneumoniae*, might reflect the presence of additional resistance mechanisms in some clones of these organism. Two isolates, one *K. pneumoniae* isolate and one *E. coli* isolate, showed resistance to colistin (MIC, >64 μ g/ml). Transfer of the ESBL-associated resistance was performed with *E. coli* A15 resistant to rifampin (rifampicin) as a recipient, as described previously (22). Transconjugants, selected with 2 μ g/ml cefotaxime or ceftazidime and 128 μ g/ml rifampin, were obtained for five *K. pneumoniae* isolates, four *E. coli* isolates, and the *E. cloacae* isolates. Along with resistance to β -lactams, they showed various combinations of resistance to other compounds (Table 1).

β -Lactamases were visualized by isoelectric focusing, followed by a bioassay for enzymes with cefotaxime- or ceftazidime-hydrolyzing activity (2). Eight species-specific β -lactamase profiles were observed (Table 1). The putative ESBLs had pI values of 8.2 or 7.6 in *K. pneumoniae*, 8.9 (mostly) or 7.9 in *E. coli*, and 5.9 in *P. stuartii* and *P. mirabilis*. The *E. cloacae* isolate produced both the pI 8.2 and 5.9 enzymes. Additionally, almost all isolates expressed β -lactamases with a pI of 5.4 (probably TEM-1), and most of the *E. coli* isolates had enzymes with a pI of 7.4 (probably OXA-1). The transconjugants produced ESBLs like those in the corresponding isolates, together or not with the other β -lactamases (Table 1).

For all but one isolate, PCR detection and sequencing of *bla*_{SHV}, *bla*_{CTX-M}, and *bla*_{TEM} ESBL genes were performed as

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TABLE 1. Clinical data, typing, β -lactamase content, and plasmid characteristics of the study isolates

Isolate	Species	Ward ^a	Specimen	Phylogroup ^b	Typing ^c		pIs of β-lactamases found by IEF ^d	ESBL	Plasmid data ^e				
					PFGE type	ST			Replicon(s)	bla _{OXA-1}	bla _{TEM-1}	aac(6')- Ib-cr	Resistance in R ⁺ /f
1829/06	<i>K. pneumoniae</i>	NICU	Catheter		A	101	8.2 , 7.6, 5.4	SHV-12	FII				—
1830/06	<i>K. pneumoniae</i>	NICU	Throat swab		B	14	7.6, 5.4	SHV-2	A/C				
1833/06	<i>K. pneumoniae</i>	NICU	Sputum		B	ND	<u>7.6</u> , 5.4	SHV-2	A/C				
1835/06	<i>K. pneumoniae</i>	NICU	Throat swab		B	14	<u>7.6</u> , 5.4	SHV-2	A/C				
1831/06	<i>K. pneumoniae</i>	NICU	Sputum		C1	14	8.2 7.6, 5.4	SHV-5	A/C				CML, TET
1832/06	<i>K. pneumoniae</i>	NICU	Throat swab		C1	ND	8.2 , 7.6, 5.4	SHV-5	A/C				CML, TET
1834/06	<i>K. pneumoniae</i>	NICU	Wound swab		C1	ND	8.2 , 7.6, 5.4	SHV-5	A/C				CML, TET
1838/06	<i>K. pneumoniae</i>	Geriatrics	Sputum		C2	14	8.2 , 7.6, 5.4	SHV-5	A/C				CML, TET
1836/06	<i>K. pneumoniae</i>	Geriatrics	Urine		D1	11	8.2, 7.6, 5.4	SHV-5	A/C				
1837/06	<i>K. pneumoniae</i>	Geriatrics	Urine		D2	ND	<u>8.2</u> , 7.6, 5.4	SHV-5	A/C				
1841/06	<i>E. coli</i>	Urology	Urine	D	a	405	<u>7.9</u> , 5.4	CTX-M-9	II				—
1839/06	<i>E. coli</i>	Cardiosurgery	Urine	B2	b	638	<u>8.9</u> , 5.4	CTX-M-15	A/C				
1840/06	<i>E. coli</i>	Cardiosurgery	Urine	B2	b	ND	8.9, 5.4	CTX-M-15	A/C				
1842/06	<i>E. coli</i>	Geriatrics	Urine	D	c1	393	8.9 , 7.4 , 5.4	CTX-M-15	FII	+	+	+	GEN
1843/06	<i>E. coli</i>	Geriatrics	Urine	D	c2	393	8.9 , 7.4 , 5.4	CTX-M-15	FII	+	+	+	GEN
1844/06	<i>E. coli</i>	Oncology	Urine	B2	d	131	8.9 , 7.4 , 5.4	CTX-M-15	FII	+	+	+	GEN, SXT
1846/06	<i>E. coli</i>	IMICU	Urine	B2	e1	131	<u>8.9</u> , 7.4, 5.4	CTX-M-15	FII, FIA, Y	+	+	+	
1847/06	<i>E. coli</i>	IMICU	Urine	B2	e2	131	<u>8.9</u> , 7.4, 5.4	CTX-M-15	FII, FIA, Y	+	+	+	
1848/06	<i>E. coli</i>	IMICU	Throat swab	B2	e1	ND	8.9, 7.4, 5.4	CTX-M-15	FII, FIA, Y	+	+	+	
1849/06	<i>E. cloacae</i>	Hematology	Blood				8.4* , 8.2 , 5.9 , 5.4	SHV-12, TEM-132	FII, FIA				GEN, SXT
1850/06	<i>P. stuartii</i>	SICU	Wound swab		α		8.0*, <u>5.9</u>	TEM-92	A/C, W				
1851/06	<i>P. stuartii</i>	SICU	Urine		α		8.0*, <u>5.9</u>	TEM-92	A/C, W				
1852/06	<i>P. stuartii</i>	SICU	Urine		α		8.0*, <u>5.9</u>	TEM-92	A/C, W				
1853/06	<i>P. mirabilis</i>	SICU	Urine				5.9, 5.4	TEM-132	F1C, P				

^a NICU, neurosurgical intensive care unit (ICU); IMICU, internal medicine ICU; SICU, surgical ICU.^b Phylogrouping was performed only for *E. coli* isolates.^c PFGE typing was not performed for single *E. cloacae* and *P. mirabilis* isolates; MLST was carried out only for selected *K. pneumoniae* and *E. coli* isolates. ND, not determined.^d IEF, isoelectric focusing; pI values in boldface type refer to β -lactamases that were produced by transconjugants, while pI values that are underlined represent enzymes that hydrolyzed cefotaxime and/or ceftazidime in the bioassay. Asterisks indicate β -lactamases that were not stained by 0.5 mM nitrocefin with 0.3 mM cloxacillin (probably natural AmpC cephalosporinases of *E. cloacae* and *P. stuartii*).^e Detection of the *bla*_{OXA-1}, *bla*_{TEM-1}, and *aac*(6)-*Ib-cr* genes was performed only for the CTX-M-15-producing *E. coli* isolates with FII replicon plasmids.^f Non- β -lactam resistance markers in transconjugants (R⁺). Abbreviations: CML, chloramphenicol; GEN, gentamicin; SXT, co-trimoxazole; TET, tetracycline. Symbol: —, no transferable resistance to non- β -lactams was observed for these isolates.

TABLE 2. Antimicrobial susceptibility of study isolates

Isolate and/or PFGE type ^a	MIC(s) (μg/ml) of antimicrobial ^b													
	AMP	AMS	PIP	TZP	CTX	CAZ	FEP	ATM	MEM	GEN	CIP	TET	SXT	CML
<i>K. pneumoniae</i>														
Type A	>64	16	>64	4	8	>16	2	>32	0.125	16	1	4	>128	8
Type B	>64	>64	>64	>64	4, 8	4, 16	1, 4	2, 4	0.125	>32	0.25, 2	4, >64	>128	>64
Type C	>64	>64	>64	32, >64	4, 8	16, >16	0.5, 4	>32	0.125	>32	2, 8	>64	>128	>64
Type D	>64	>64	>64	>64	4, 8	>16	2, 4	>32	0.125	>32	>32	>64	>128	32, >64
<i>E. coli</i>														
Type a	>64	32	>64	4	16	4	>16	16	0.125	4	>32	>64	>128	>64
Type b	>64	32, >64	>64	8	>16	>16	>16	>32	0.125	0.5, 1	>32	1, >64	1, >128	32, 64
Type c	>64	16	>64	4, 8	>16	>16	16	>32	0.125	>32	>32	4, >64	2, >128	>64
Type d	>64	16	>64	32	>16	>16	>16	>32	0.125	>32	>32	2	>128	4
Type e	>64	8, 32	>64	8	>16	16, >16	16, >16	32, >32	0.125	32, >32	>32	1, 2, >64	1, >128	1, 4
<i>E. cloacae</i> 1849/06	>64	>64	>64	>64	>16	>16	>16	>32	0.25	16	2	16	>128	16
<i>P. stuartii</i> type α	>64	4, 8	>64	8	16	8, 16	4, 8	2, 4	0.125	1, 4	>32	>64	>128	16, 32
<i>P. mirabilis</i> 1853/06	>64	16	>64	2	>16	>16	16	>32	0.125	1	0.125	>64	>128	4

^a *K. pneumoniae*, *E. coli* and *P. stuartii* isolates are grouped according to PFGE types.

^b Abbreviations: AMP, ampicillin; AMS, ampicillin with sulbactam; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CML, chloramphenicol; CST, colistin; CTX, cefotaxime; FEP, cefepime; GEN, gentamicin; MEM, meropenem; PIP, piperacillin; SXT, co-trimoxazole; TET, tetracycline; TZP, piperacillin with tazobactam.

described previously (17). The *bla*_{CTX-M-9}-like gene encoding the β-lactamase with a pI of 7.9 in *E. coli* 1841/06 was identified first by multiplex PCR (45) and then amplified and sequenced as proposed by Eckert et al. (16). For *K. pneumoniae* isolates, the PCR of *bla*_{SHV} genes was performed with plasmid DNA, and for *E. cloacae* and *P. mirabilis* isolates, the *bla*_{TEM} ESBL genes were cloned first in *E. coli* DH5α (20) using vector pHSG298 (41). In *K. pneumoniae*, the ESBLs with a pI of 8.2 were SHV-5 (also in *E. cloacae*) or SHV-12, and the enzymes with a pI of 7.6 were SHV-2 (Table 1). The β-lactamases with pIs of 8.9 and 7.9 in *E. coli* were identified as CTX-M-15 and CTX-M-9, respectively. The ESBLs with a pI of 5.9 were either TEM-92 (in *P. stuartii*) or TEM-132 (in *E. cloacae* and *P. mirabilis*). Whereas the SHV and CTX-M enzymes belonged to “cosmopolitan” ESBL types (21, 30), the TEM variants have rarely been observed so far (13, 18, 46). The presence of the *ISEcp1* element in the vicinity of *bla*_{CTX-M} genes in *E. coli* isolates was analyzed as reported previously (1). It was located 49 bp and 42 bp upstream from *bla*_{CTX-M-15} and *bla*_{CTX-M-9}, respectively, demonstrating that these genes were present in widespread *ISEcp1* transposition modules (3, 9, 16, 30, 33, 38).

Pulsed-field gel electrophoresis (PFGE) was performed by the method of Struelens et al. (40); DNA banding patterns were interpreted by the method of Tenover et al. (42). Four and five PFGE types were discerned among *K. pneumoniae* and *E. coli* isolates, respectively, correlating with their β-lactamase profiles (Table 1). There were two PFGE types of SHV-5-producing *K. pneumoniae* (types C and D) and four types of CTX-M-15-producing *E. coli* (types b to e). Clusters of two to four related isolates were usually specific for particular wards, indicating parallel local dissemination of several enterobacterial strains.

Thirteen *K. pneumoniae* and *E. coli* isolates of all PFGE types were subjected to multilocus sequence typing (MLST) as described previously (14, 44). Databases available at www.pasteur.fr (*K. pneumoniae*) and www.mlst.net (*E. coli*) were used for assigning sequence types (STs) and clonal complexes (CCs). The *K. pneumoniae* isolates represented sequence type 11 (ST11) (clonal complex 11 [CC11]; PFGE type D), ST14 (CC14; PFGE types B and C), and ST101 (PFGE type A) (Table 1), which have been observed worldwide (12, 14, 36) (www.pasteur.fr). Within the ST14 clone, the similarity between PFGE patterns B and C1/C2 was around 58.0% (Dice coefficient), and between C1 and C2, it was 93.8%, which might reflect the more recent diversification of the clone. Isolates of all these STs have been mostly recovered from humans (www.pasteur.fr); recently, ST101 bovine isolates were reported in the United States (36). The data on ESBLs in these clones have often been partial (14), not available (36), or difficult to compare because of the use of another MLST scheme (26). Recently, two Hungarian epidemic *K. pneumoniae* clones with CTX-M-15 were assigned to ST11 and ST15 (12), the latter one was classified in CC14 together with ST14 (14). However, in this study, the ST11 and ST14 isolates produced either SHV-2 or SHV-5.

The *E. coli* isolates also represented STs belonging to international clones or CCs with pathogenic strains from humans, namely, ST131 (CC131; PFGE types d and e), ST393 (CC31; PFGE type c), ST405 (CC405; PFGE type a), and ST638 (CC73; PFGE type b) (Table 1) (44) (www.mlst.net). Within

ST131, the similarity between PFGE patterns d and e1/e2 was >60% (d and e1, 68.3%; d and e2, 63.0%), whereas between e1 and e2, it was 97.6%. ST131 and ST405 were recently described as *E. coli* clones disseminating with CTX-M-15 ESBL on a global scale (11, 29, 32, 37); however, in this study, while ST131 did indeed produce CTX-M-15, ST405 produced CTX-M-9. Phylogenetic grouping of *E. coli* isolates was performed by PCR as proposed by Clermont et al. (10). They all represented the more-virulent group B2 or D (Table 1).

PCR-based replicon typing of plasmids was performed by the method of Carattoli et al. (6), using total DNA from transconjugants or from clinical isolates when these were non-mating. Replicon profiles are shown in Table 1. Replicon A/C was found in all *K. pneumoniae* isolates except for the single SHV-12 producer with replicon FII. Different β -lactamase profiles or mating abilities suggested that the A/C-type plasmids with *bla*_{SHV-2/SHV-5} genes varied in *K. pneumoniae* isolates of different PFGE types (B, C, and D). Links between replicon A/C and *bla*_{SHV} genes and between replicon FII and *bla*_{SHV-12} were reported before (8, 31). The FII replicon was predominant in *E. coli* with CTX-M-15 ESBL and has been identified in isolates of three PFGE types (c, d, and e). The FII-type plasmids were probably specific for each of these PFGE types as shown by differences in their mating ability and transferable resistance markers. A number of reports demonstrated the strong link between *bla*_{CTX-M-15} and FII-type plasmids (7, 11, 23, 24, 33). These molecules often carry also *bla*_{OXA-1}, *bla*_{TEM-1}, and *aac*(6')-Ib-cr genes, which was confirmed in this work by specific PCRs (11) and sequencing (Table 1). The remaining *E. coli* with CTX-M-15 (PFGE type b) had only replicon A/C, which has been rarely observed (31). Similarly sporadic have been II-type plasmids with *bla*_{CTX-M-9}-like genes (24, 34), as found here for *E. coli* producing CTX-M-9. The isolates with TEM-type ESBLs had specific replicon profiles, including the *E. cloacae* and *P. mirabilis* isolates which both expressed TEM-132. All the above data showed a diversity of plasmids, even in isolates with the same ESBL variant (SHV-5, CTX-M-15, and TEM-132), correlating well with clonal diversity. They demonstrated that plasmid transfer was not an important factor of ESBL dissemination in the hospital during the study period.

This report demonstrates that nosocomial ESBL-producing subpopulations of *K. pneumoniae* and *E. coli* may entirely consist of clones with higher dissemination potential. It confirms further the particular role of *E. coli* ST131 with FII-type plasmids carrying the *bla*_{CTX-M-15} gene in current ESBL epidemiology (11, 29, 32). However, by showing that other clones identified produced different ESBLs than elsewhere (11, 12), this study contributes to the growing knowledge that spread of the international *K. pneumoniae* and *E. coli* clones is not associated strictly with specific β -lactamases but that these are being acquired independently by particular clone variants in different locales (28, 37). It is possible that the *E. coli* clones with CTX-M-15 ESBL had been more recent introductions to the hospital's environment, while the *K. pneumoniae* with SHV-2 or SHV-5 ESBL had been circulating there for a longer period. This hypothesis, however, requires a comparative analysis of ESBL producers from the Plzeň hospital and the Czech Republic from before 2006, and such a study is now being conducted.

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