DISSEMINATION OF ST274 *KLEBSIELLA PNEUMONIAE* EPIDEMIC CLONE IN NEWBORN AND ADULT HOSPITAL SETTINGS HARBOURING SHV-2A OR CTX-M-15 TYPE EXTENDED SPECTRUM β -LACTAMASES-PRODUCING KNOWN PLASMIDS

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Molecular epidemiology and genetic features of an extended-spectrum β -lactamase (ESBL) producing *Klebsiella pneumoniae* epidemic clone (KP-EC) with elevated ciprofloxacin MIC (minimum inhibitory concentration) values from multiple nosocomial outbreaks and sporadic cases between 2006 and 2008 in Hungary were investigated.

As a result of continuous monitoring of ESBL-producing KP-ECs, 27 isolates collected from five healthcare facilities were selected for macrorestriction profile analysis by PFGE (pulsed field gel electrophoresis). Of these, 12 strains were isolated from adult inpatients, while 15 strains were from newborns. The MIC values for several antibiotics were determined by agar dilution technique. Molecular typing was further performed by PCR (polymerase chain reaction) and sequencing of several antibiotic resistance genes, plasmid profile analysis, transfer of resistance determinants and multilocus sequence typing (MLST).

All isolates showed moderate resistance to ciprofloxacin (MICs ranged from 0.5 to 8 mg L⁻¹). PFGE revealed the existence of only one genetic cluster defined as EC IV. *Pst*I digestion of plasmid DNA revealed two highly diverse restriction patterns in “adult” and “newborn” isolates corresponding to plasmids from the Hungarian Epidemic Clone and plasmids isolated from a neonatal nosocomial outbreak in 1998, respectively. Sequence analysis of β -lactamase genes from plasmids of 14 selected isolates detected *bla*_{SHV-2a} in strains isolated exclusively from newborns and *bla*_{CTX-M-15} in strains isolated exclusively from adult inpatients. MLST established that strains of the PFGE cluster belonged to a novel sequence type ST274.

ESBL-producing *K. pneumoniae* isolates belonging to the novel sequence type ST274 appeared in the newborn and adult hospital settings in Hungary and acquired SHV-2a or CTX-M-15 type enzymes, respectively. Thus, a new antimicrobial resistance strategy for successful conformation to distinct hospital settings was found.

Keywords: *Klebsiella pneumoniae*, allodemic plasmid, clonal spread, MLST

Introduction

One of the major factors involved in the current prevalence of extended-spectrum β -lactamase (ESBL)-producing *Enterobacteriaceae* is clonal spread. Nationwide dissemination of particular multidrug-producing *K. pneumoniae* clones has been observed in several countries [1].

We have previously shown that the high number of nosocomial outbreaks caused by SHV-type ESBL-producing *K. pneumoniae* seen in several Hungarian neonatal intensive care units (NICUs) in 2002 and 2003 was the consequence of extensive and effective dissemination of identical allodemic R-plasmids [2]. Conversely, the nationwide dissemination of CTX-M-15-type ESBLs among

K. pneumoniae in 2005 could have been explained by the rapid and efficient expansion of three large ciprofloxacin-resistant CTX-M-15-producing KP-ECs (HEC, EC II and EC III) exclusively in adult inpatients [3].

Since the dissemination of CTX-M-15-producing *K. pneumoniae* in Hungary proved to be clonal, we are performing continuous monitoring of ESBL-producing KP-ECs in Hungary.

Our study was conducted to investigate the molecular epidemiology and genetic features of an ESBL-producing KP-EC with elevated ciprofloxacin MIC values, which was isolated from multiple nosocomial outbreaks and sporadic cases between 2006 and 2008 in Hungary.

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Materials and methods

As a result of continuous monitoring of ESBL-producing KP-ECs from January 2006 to December 2008, 27 isolates collected from five healthcare facilities and submitted to the National Center for Epidemiology were selected for macrorestriction profile analysis by PFGE. Of these, 12 strains were isolated from adult inpatients (including five invasive samples from one nosocomial outbreak) in two healthcare facilities and 15 strains were isolated from newborns (including 12 invasive samples from two nosocomial outbreaks) in four healthcare facilities.

The MIC values were determined by agar dilution technique according to the CLSI guidelines [4] for the following antibiotics: ceftazidime, cefotaxime, cefepime, gentamicin, amikacin and ciprofloxacin. For efflux pump inhibitor test, the MIC value of ciprofloxacin was determined by using broth microdilution test in accordance with the guidelines established by the CLSI [4], in either the presence or absence of Phe-Arg- β -naphthylamide (Pa β N) at a concentration of 20 mg L⁻¹. Interpretation of the results with Pa β N was done in accordance with recent publications [5, 6].

The PFGE method was performed on 27 isolates in line with the standardized CDC protocol. Gels were interpreted with Fingerprinting II Informatix Software (Bio-Rad). Levels of similarity were calculated with the Dice coefficient, and UPGMA was used for the cluster analysis of the PFGE patterns. Pulsotypes (PTs) were defined at 85% similarity level between macrorestriction patterns and marked by letters according to the criteria established by Tenover [7]. Clonally related isolates were supposed if they belonged to the same PT.

Furthermore, molecular typing was performed by PCR and sequencing of several antibiotic resistance genes (β -lactamase genes (*bla*_{SHV}, *bla*_{CTX-M}, *bla*_{TEM}) [2, 8], genes of aminoglycosides-modifying enzymes (*aac*(6')-Ib, *aac*(3)-II) [3], mobile quinolone resistance genes (*qnrA*, *qnrB* and *qnrS*) [9, 10] and quinolone resistance determining regions (QRDRs) of *gyrA* and *parC*) [3], by plasmid profile analysis and plasmid RFLP (restriction fragment length polymorphism), and by transfer of resistance determinants to *Escherichia coli* K12 J5-3^{Rif} by conjugation and to *E. coli* DH5a by electroporation [3].

Multilocus sequence typing (MLST) of selected isolates was performed according to Diancourt et al. [11], as previously described.

Results and discussion

All isolates showed moderate resistance to ciprofloxacin (MICs ranged from 0.5 to 8 mg L⁻¹) (Table 1). The isolates from adult patients showed slightly elevated ciprofloxacin MIC values of 2 mg L⁻¹ uniformly, except isolate K53/08 (MIC value 8 mg L⁻¹). Among isolates from newborns, there is one isolate also representing moderate resistance to ciprofloxacin of 8 mg L⁻¹. The MICs for ceftazidim proved 64–128 mg L⁻¹ in the “adult isolates” and 8 mg L⁻¹ in the “newborn” isolates.

Macrorestriction profile analysis of 27 isolates revealed the existence of only one genetic cluster at 90% similarity level defined as L PT and EC IV (Fig. 1).

The QRDRs of *gyrA* and *parC* genes were analyzed in five isolates selected among the donor strains used in the conjugation and electroporation experiments. On the basis of the alterations of nucleotide sequences in the QRDRs of *GyrA* and *ParC* in the EC IV isolates, one amino acid change (Ser-83 Tyr) was detected in *gyrA* (Table 1). PCR screening for *qnrA*, *qnrB* and *qnrS* was also performed and showed negative results.

Conjugation studies were performed on three isolates from adult patients and on five isolates from newborn patients. A plasmid of ~90 kb from three “adult” isolates and another plasmid of ~90 kb from two “newborn” isolates were transferred to the *E. coli* J5-3^{Rif} recipient strain, whereas a plasmid of ~90 kb from three further “newborn” isolates could be transformed to the *E. coli* DH5a recipient strain.

Transconjugants and transformants were screened by PCR for *bla*_{CTX-M}, *bla*_{TEM} and *bla*_{SHV} genes and also for *aac*(3)-II and *aac*(6')-Ib genes, respectively (data not shown). Sequencing revealed that plasmids from “adult” isolates harboured *bla*_{CTX-M-15}, *aac*(6')-Ib-cr and *aac*(3)-II genes, while on plasmids from transconjugants and transformants of “newborn” isolates, *bla*_{SHV-2a} and *aac*(3)-II genes were found.

The MICs of ciprofloxacin showed slightly elevated values in the *aac*(6')-Ib-cr-harboursing “adult” isolates compared with the majority of the “newborn” isolates (Table 1). One of the “newborn” isolates (K61/08) showed 8 mg L⁻¹ ciprofloxacin MIC. According to the efflux pump inhibitor test, the elevation in this isolate can be explained with overexpression of efflux pump (Table 1). Interestingly, despite the presence of *aac*(6')-Ib-cr gene on plasmids from “adult” isolates, the ciprofloxacin MIC values of their transconjugants were not influenced. Thus, three different mechanisms used by ST274 EC for developing resistance to ciprofloxacin were supposed: the basic *gyrA* mutation, an efflux pump and the presence of *aac*(6')-Ib-cr gene. Cumulative mutations in QRDRs of *gyrA* and *parC* are responsible for the high-level resistance to ciprofloxacin in *K. pneumoniae*. Development of resistance to fluoroquinolones is a stepwise process [12], whereas higher fluoroquinolone MICs in Gram-negative bacteria need *gyrA* and *parC* mutations simultaneously [13, 14]. However, only the “first-step” mutation in *gyrA* conferring low-level ciprofloxacin resistance was found in *K. pneumoniae* ST274 clone. Accessory resistance mechanisms such efflux pump [15] and *aac*(6')-Ib-cr [16, 17] responsible for low-level quinolone resistance facilitate the further selection of subsequent higher level resistance mutations [14].

*Pst*I digestion of plasmid DNA from transconjugants or transformants revealed two highly diverse restriction patterns corresponding to “adult” and “newborn” isolates, respectively (Fig. 2). However, plasmids from “adult” EC IV isolates and those from the Hungarian Epidemic Clone isolates from 2005 [3] showed identical or very similar re-

Table 1. Features of extended-spectrum β -lactamase-producing *Klebsiella pneumoniae* representative outbreak isolates and their transconjugants (TCs) and transformants (Ts)*

ID of strains	Age of patient (years)	Sample	Centre of isolation; hospital ward	Year of isolation	Pulsotype	MLST	ESBL types	Aminoglycoside resistance genes		MIC (mg L ⁻¹)						CIP+ PA β N
								<i>aac(6')-Ib</i>	<i>aac(3)-II</i>	CAZ	CTX	FEP	GEN	AMK	CIP	
K130/06	34	CSF	BP1;Neurosurgery	2006	L	ND	CTX-M-15	<i>aac(6')-Ib-cr</i>	+	128	256	64	128	32	2	ND
K131/06	39	blood	BP1;Neurosurgery	2006	L	ST274	CTX-M-15	<i>aac(6')-Ib-cr</i>	+	128	256	64	128	32	2	1
K133/06	52	blood	BP1;Neurosurgery	2006	L	ND	CTX-M-15	<i>aac(6')-Ib-cr</i>	+	128	128	64	128	32	2	ND
K24/07	73	blood	BP1;ICU	2007	L	ND	CTX-M-15	<i>aac(6')-Ib-cr</i>	+	128	256	64	128	32	2	ND
K80/08	0	blood	BP2;NICU	2007	L	ND	SHV-2a	–	+	8	8	2	128	2	0.5	ND
K1/08	0	stool	BP2;NICU	2008	L	ND	SHV-2a	–	+	8	8	4	128	1	0.5	ND
K4/08	0	blood	P1;NICU	2008	L	ND	SHV-2a	–	+	8	8	2	128	2	0.5	0.25
K5/08	0	blood	P1;NICU	2008	L	ST274	SHV-2a	–	+	8	8	2	128	2	0.5	ND
K6/08	0	blood	P1;NICU	2008	L	ND	SHV-2a	–	+	8	8	4	128	4	0.5	ND
K35/08	0	throat	F1;NICU	2008	L	ND	SHV-2a	–	+	8	8	2	128	2	0.5	ND
K53/08	73	LRT	D1;Neurosurgery	2008	L	ND	CTX-M-15	<i>aac(6')-Ib-cr</i>	+	64	128	32	128	8	8	8
K61/08	0	blood	BP2;NICU	2008	L	ST274	SHV-2a	–	+	8	8	4	128	1	8	0.5
K62/08	0	blood	BP2;Cardiology	2008	L	ND	SHV-2a	–	+	64	128	128	128	2	0.5	0.5
K63/08	0	blood	BP2;NICU	2008	L	ND	SHV-2a	–	+	8	8	2	64	2	0.5	ND
TC130/06							CTX-M-15	+	+	4	64	4	16	0.5	≤ 0.064	ND
TC131/06							CTX-M-15	+	+	2	16	1	16	1	≤ 0.064	ND
TC133/06							CTX-M-15	+	+	8	64	2	32	1	≤ 0.064	ND
TC62/08							SHV-2a	–	+	2	8	1	16	0.5	≤ 0.064	ND
TC63/08							SHV-2a	–	+	2	8	1	16	0.5	≤ 0.064	ND
T4/8							SHV-2a	–	+	≤ 0.5	1	≤ 0.5	32	0.5	≤ 0.064	ND
T5/8							SHV-2a	–	+	≤ 0.5	1	≤ 0.5	32	0.5	≤ 0.064	ND
T61/8							SHV-2a	–	+	1	1	≤ 0.5	32	0.5	≤ 0.064	ND
J5–3								ND	ND	≤ 0.5	<0.5	≤ 0.5	≤ 0.25	≤ 0.5	≤ 0.064	ND
DH5 α								ND	ND	0.5	<0.5	≤ 0.5	≤ 0.25	≤ 0.5	≤ 0.064	ND

*CSF: cerebrospinal fluid, LRT: lower respiratory tract, ICU: intensive care unit, NICU: neonatal intensive care unit, CAZ: ceftazidime, CTX: cefotaxime, FEP: cefepime, GEN: gentamicin, AMK: amikacin, CIP: Ciprofloxacin; ND: not determined.

Dice (Opt: 1.00%) (Tol. 1.0%–2.0%)(H > 0.0%, S > 0.0%)(0.0%–100.0%)

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Fig. 1. Cluster analysis of *K. pneumoniae* macrorestriction profiles of different epidemic and outbreak clones (1998–2008)

Fig. 2. *Pst*I fingerprints of plasmids isolated from transconjugants (TCs) and transformants (Ts) of different epidemic clones.

Lane M, molecular weight marker; lane 1, TC130/06 (CTX-M-15); lane 2, TC131/06 (CTX-M-15); lane 3, TC133/06 (CTX-M-15); lane 4, T4/08 (SHV-2a); lane 5, T5/08 (SHV-2a); lane 6, T61/08 (SHV-2a); lane 7, TC62/08 (SHV-2a); lane 8, TC105/98 (SHV-2a); lane 9, TC280/05 (HEC-Ia; CTX-M-15); lane 10, TC79/05 (HEC-Ib; CTX-M-15)

striction patterns. Surprisingly, restriction patterns of plasmids from “newborn” EC IV isolates were indistinguishable from those of *bla*_{SHV-2a}-harbouring plasmids isolated from nosocomial outbreaks in two geographically distant NICUs in 1998 and 2002 [2]. It seems that some successful plasmids, harbouring a specific combination of resist-

ance genes, circulate for at least 10 years in different hospital settings across Hungary.

Sequence analysis of β -lactamase genes from plasmids of eight selected isolates detected *bla*_{SHV-2a} in strains isolated exclusively from newborns and *bla*_{CTX-M-15} in strains isolated exclusively from adult inpatients. MLST estab-

lished that strains of the L PFGE cluster belonged to a novel sequence type ST274 (Table 1).

We have shown that there has been a particular antimicrobial resistance strategy used by ST274 KP-EC for successful dissemination in quite diverse newborn and adult hospital settings. Such fusions of epidemic clones and long-standing epidemic plasmids at the stringent evolutionary pressure of hospital environment probably lead to the evolution and spread of highly resistant clones.

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