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BRIEF REPORT

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Nationwide spread of clonally related CTX-M-15-producing multidrug-resistant *Klebsiella pneumoniae* strains in Hungary

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Since the 1990s the CTX-M type beta-lactamases have comprised the most rapidly growing group of extended-spectrum beta-lactamases (ESBL). They have been detected increasingly in Europe, Asia, America and Africa, and CTX-M-15 has been particularly prevalent [1–3]. In Hungary, only the CTX-M-4 beta-lactamase has been found in one *Salmonella* serovar Typhimurium strain [4], and no more data on the occurrence and dissemination of CTX-M β-lactamases is currently available. In order to close this knowledge gap, the study presented here was initiated to characterize the nosocomial isolates of CTX-M-producing *Klebsiella* spp. submitted to the National Center for Epidemiology in Budapest in 2003.

The National ESBL Survey was initiated by the National Center for Epidemiology and started in June of 2002. In 2003, a total of 5,865 *K. pneumoniae* strains were isolated from patients attending participating Hungarian hospitals, and after preliminary antimicrobial susceptibility tests were performed, 158 presumably ESBL-producing isolates were submitted to the National Center for Epidemiology for confirmation. Seventeen of these 158 strains showed higher resistance to cefotaxime than to ceftazidime. The first strain was isolated in January 2003 at an intensive care unit in Pest County (Table 1). By the end of March, four strains with a similar resistance pattern had been isolated from patients attending the surgical ward of the same hospital. During the same year, 13 further strains were isolated from patients in surgery, urology, medical and nephrology wards from eight different hospitals across Hungary, predominantly from surgical wounds (10/17) and urine (3/17) (Table 1).

The isolates were identified using ATB ID 32 E (bioMérieux, Marcy l' Étoile, France). The putative production of an ESBL was tested using the combined disk method ESBL SET (Mast Diagnostics, Merseyside, UK). The ESBL-producing strain *K. pneumoniae* ATCC 700603 was used as a control strain. The MICs were determined using the E-test according to the recommendations of the Clinical and Laboratory Standards Institute (formerly the National Committee for Clinical Laboratory Standards).

The mating experiments were carried out with *E. coli* K12 J53-2 Rif^R as the recipient strain. The transconjugants were selected on Mueller–Hinton agar supplemented with cefotaxime (4 mg/l) and rifampicin (300 mg/l). For fingerprinting analysis, plasmid DNA from transconjugants was obtained using a QIAprep Spin Miniprep kit (QIAGEN, Hilden, Germany) and digested with *Pst*I and *Pvu*II (Biolabs, Ipswich, New England).

Amplification of the $bla_{CTX\text{-}M}$ gene was carried out with all isolates and their transconjugants with primers specific for all of the known $bla_{CTX\text{-}M}$ genes: forward 5′-TTT GCG ATG TGC AGT ACC AGT AA-3′ and reverse 5′-CGA TAT CGT TGG TGG TGC CAT A-3′. The amplified $bla_{CTX\text{-}M}$ gene fragments were subsequently digested by *Pst*I and *Pvu*II restriction endonucleases to separate the five CTX-M groups [5]. PCR to detect the presence of *aac(3)-II* in transconjugants was carried out using the primers and conditions described previously [6].

DNA sequencing was performed on transconjugants using ABI prism Big Dye Terminator Cycle Sequencing Ready Reaction kit with Ampli Taq^R DNA Polymerase FS

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Table 1 Clinical and in vitro characterization of multidrug-resistant CTX-M-15-producing *K. pneumoniae* isolates (K) and *E. coli* J 53 transconjugants (TK)

Isolate	Hospital location	Date of collection	Hospital ward	Specimen	Phage type	Pulsotype	MIC (μg/ml)									
							CTX	CAZ	AMC	FEP	FOX	IMP	GEN	AMK	TET	CIP
K34/03	Pest county	06.01.2003	ICU	Intra-abdominal	XIA3	N1	256	64	48	32	4	0.25	128	8	256	32
TK34/03							256	1.5	16	1	2	0.125	6	0.75	64	0.016
K46/03	Pest county	10.02.2003	Surgical	Wound	XIA1	N1										
K54/03	Pest county	11.02.2003	Surgical	Wound	XIA1	N1	256	96	256	32	16	0.19	32	3	256	32
TK54/03							24	3	3	2	1.5	0.125	3	1	64	0.032
K55/03	Pest county	28.02.2003	Surgical	Wound	XIA1	N1										
K83/03	Pest county	31.03.2003	Surgical	Wound	IA26	N1										
K85/03	Nograd county	31.03.2003	Surgical	Wound	XIA1	N1										
K86/03	Nograd county	08.04.2003	ICU	Intra-abdominal	XIA1	N1	256	64	48	32	4	0.25	128	8	256	32
TK86/03							256	2	32	0.5	1	0.125	4	0.5	96	0.016
K168/03	Nograd county	10.06.2003	Surgical	Wound	IA26	N1										
K171/03	Budapest I	11.06.2003	Urology	Urine	XIA1	N2	256	96	48	32	8	0.19	24	2	256	32
TK171/03							16	3	8	2	1.5	0.125	4	1	96	0.023
K153/03	Budapest I	11.06.2003	Traumatology	Nasal secretion	XIA1	N2										
K154/04	Budapest I	12.06.2003	Nephrology	Urine	XIA1	N2	256	96	96	32	16	0.19	32	3	256	32
TK154/03							24	3	3	2	1.5	0.125	3	0.75	64	0.032
K158/03	Budapest II	16.06.2003	Surgical	Wound	IA26	N2	256	48	48	32	32	0.25	128	6	256	32
TK158/03							256	3	32	1.5	1.5	0.125	4	0.5	96	0.023
K233/03	Tolna county I	16.06.2003	Medical	Urine	IA26	N1	256	64	48	32	16	0.19	96	6	256	32
TK233/03							128	4	24	2	0.75	0.125	4	0.75	96	0.016
K152/03	Pest county	16.07.2003	Surgical	Wound	XIA1	N1										
K150/03	Budapest III	16.07.2003	Medical	Blood	XIA1	N1	256	48	16	32	4	0.25	48	3	256	24
TK150/03							256	4	3	1	1	0.125	4	0.25	96	0.006
K165/03	Baranya county	16.09.2003	Surgical	Wound	XIA1	N1	256	64	24	24		0.25	0.5	3	256	32
TK165/03							256	4	6	0.75	0.75	0.125	0.125	1	96	0.016
K262/03	Tolna county II	03.10.2003	Surgical	Wound	XIA1	N1	256	64	48	32	16	0.19	96	8	256	32
TK262/03							256	2	24	6	1.5	0.125	6	0.5	64	0.023
J 53	–	–	–	–	–	–	0.03	0.09	1.5	0.023	1.5	0.09	0.06	0.19	ND	0.006
ATCC700603							4	32	2	1	32	0.19	6	2	ND	ND

MIC minimum inhibitory concentration, *CTX* cefotaxime, *CAZ* ceftazidime, *AMC* amoxicillin-clavulanic acid, *FEP* cefepime, *FOX* cefoxitine, *IMP* imipenem, *GEN* gentamicin, *AMK* amikacin, *TET* tetracycline, *CIP* ciprofloxacin

(Perkin–Elmer, Branchburg, NJ, USA) and analyzed in an ABI Prism 310 Automated Sequencer (Perkin Elmer Biosystems, Foster City, CA, USA) using the primer pair specific for $bla_{\text{CTX-M-1}}$: forward 5′-ATG GTT AAA AAA TCA CTG CG-3′ [1] and reverse 5′-CAG CGC TTT TGC CGT CTA AG-3′ [7]. Plasmid DNA from 17 *K. pneumoniae* isolates and from transconjugants was obtained using the alkaline lysis method [8], and agarose gel electrophoresis was performed in vertical agarose gels.

Phage typing with 15 phages [9] and PFGE analysis with *Xba*I were used to ascertain the epidemiological relationship among 17 ESBL-KP strains. Clustering of PFGE profiles was performed using UPGAMA and Dice coefficient with a 1% track-length tolerance level. Pulsotypes (up to six-band differences) and subtypes (up to three-band differences) were defined following the criteria established by Tenover et al. [10]; clonal relationships were supposed if the isolates belonged to the same pulsotype.

Antimicrobial susceptibility testing (Table 1) revealed a high level of resistance to cefotaxime, gentamicin, tetracycline and ciprofloxacin and moderate resistance to ceftazidime, amoxicillin/clavulanic acid, cefepime, and netilmicin in all strains but one. The latter, strain K165/03 from Baranya county, was susceptible to gentamicin, and the *aac(3)-II* gene was not detected in its transconjugant.

The 544 bp fragments specific for $bla_{\text{CTX-M}}$ were amplified in all strains. Further digestion of PCR products with *Pst*I and *Pvu*II showed that all $bla_{\text{CTX-M}}$ genes belonged to the CTX-M-1 ESBL-group. Plasmids from CTX-M-producing strains could be divided into three profiles: 137/2.7 KB (12 isolates), 137/38/2.7 KB (1 isolate) and 137/100/38/2.7 KB (4 isolates). Only the large plasmid of 137 KB was transferred to all transconjugants, and it harbored the $bla_{\text{CTX-M-15}}$, as demonstrated by sequencing, and the *aac(3)-II* genes (except TK165/03), as demonstrated by PCR (data not shown). Plasmid DNA from transconjugants was compared after digestion with *Pst*I and *Pvu*II restriction endonucleases (data not shown) and very similar restriction patterns were obtained; the gene encoding CTX-M-15 resided in the same plasmid of 137 KB in all 17 strains.

The 17 strains belong to three closely related phage types: IA26, XIA1 and XIA3 (Table 1). On the basis of these preliminary results, all strains were subjected to macrorestriction profile analysis by PFGE together with eight different SHV-producing outbreak strains previously isolated in different Hungarian neonatal intensive care units. According to cluster analysis, all CTX-M-15-producing *K. pneumoniae* isolates were clonally related at an 89% similarity level and were clearly different from the SHV-outbreak strains. The CTX-M-15-producing isolates were grouped into one clone represented by two subtypes with a four-band difference: N1 and N2 (Table 1). The results obtained with phage typing and PFGE were concordant and proved the existence of a strong correlation among the CTX-M-15-producing isolates.

The emergence of CTX-M-15 in *K. pneumoniae* in Hungary is not surprising considering its extended dissemination among different species of the family *Enterobacteriaceae* in Eastern Europe [1, 5]. This report represents the first description of countrywide spread of a CTX-M-15-producing KP clone in Hungary, and we propose calling it the Hungarian epidemic clone (HEC). This clone was isolated from patients at eight geographically distant hospitals in five Hungarian counties and in the city of Budapest, mainly from surgical care units and from postoperative wound infections. Interestingly, in different healthcare centers the strains were isolated at different time periods, month-by-month, during the year 2003.

In HEC, the $bla_{\text{CTX-M-15}}$ gene was located on the 137 KB self-transmissible plasmid, which confers co-resistance to aminoglycosides and tetracycline. In *E. coli* isolates from a French geriatric hospital, the $bla_{\text{CTX-M-15}}$ was located on the 120 KB conjugative plasmid [6]. In general, the CTX-M-encoding genes have been located on plasmids of different sizes between 7 and 160 KB and they are often transmissible by conjugation in vitro. These plasmids frequently carry genes for resistance to aminoglycosides, chloramphenicol, sulfonamide and tetracycline [2]. The epidemic spread of CTX-M-15-producing clones has only been reported for *E. coli* in the UK and France [3, 6]. The rapid dissemination of this Hungarian multidrug-resistant epidemic clone and particularly of its conjugative plasmid seriously endangers Hungarian healthcare institutions. Immediate intervention is needed for efficient eradication of this clone and continuous epidemiological monitoring is required to control its spread.

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