ORIGINAL PAPERS

Adv Clin Exp Med 2007, **16**, 2, 239–247
ISSN 1230-025X

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Conjugative Transfer of Multiresistance Plasmids from ESBL-positive *Escherichia coli* and *Klebsiella* spp. Clinical Isolates to *Escherichia coli* Strain K12 C600

Transfer koniugacyjny plazmidów wieloporności z ESBL-dodatnich klinicznych szczepów *Escherichia coli* i *Klebsiella* spp. do szczepu *Escherichia coli* K12 C600

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Abstract

Objectives. The aim of this study was to evaluate the transfer frequency of plasmid-borne genes coding for extended-spectrum β-lactamases (ESBLs) from clinical isolates of *E. coli* and *Klebsiella* spp. to the *E. coli* K12 C600 recipient strain. Additionally, the antimicrobial susceptibility of the donor strains and transconjugants obtained in mating experiments were studied.

Material and Methods. A total of 51 ESBL-producing *E. coli* (n = 32) and *Klebsiella* spp. (n = 19) clinical strains isolated from children hospitalized in the Medical University Hospital in Wrocław, Poland, were used in this study. Transfer of plasmids carrying ESBL-encoding genes was performed using the conjugational broth method. ESBL production was detected by the double-disk synergy test (DDST). The minimal inhibitory concentrations (MICs) of antimicrobial drugs were determined by an agar dilution technique on Mueller-Hinton agar. The presence of the $bla_{\text{CTX-M}}$ gene in donor strains and transconjugants was determined by PCR.

Results. The majority of the isolates studied (92.2%) transferred ESBL-encoding plasmids to the *E. coli* K12 C600 recipient strain with a frequency of 10^{-5}–10^{-1} per donor strain. Donor strains and transconjugants displayed resistance patterns typical of ESBL producers. They were resistant to cefotaxime, cefrtiaxone, and aztreonam but susceptible to carbapenems and oxyimino-β-lactams (ceftazidime, cefotaxime, ceftriaxone, and aztreonam) in combination with clavulanic acid. Moreover, the majority of the strains exhibited a high level of resistance to non-β-lactam antimicrobials (gentamicin, amikacin, co-trimoxazole). The MIC values of cefotaxime and cefrtiaxone were significantly higher than those of ceftazidime, suggesting that this resistance may result from CTM-X-type ESBLs. PCR based on primers specific for CTX-M-type β-lactamases confirmed the presence of the $bla_{\text{CTX-M}}$ gene in 31 (66%) donor strains and 23 (48.9%) transconjugants.

Conclusions. The majority of the strains tested harbored conjugative plasmids coding for CTX-M-type ESBLs. Additionally, genes conferring resistance to antimicrobial agents other than β-lactams were often co-transferred to the recipient strain in the conjugation process, indicating that these determinants were carried by ESBL-encoding plasmids (**Adv Clin Exp Med 2007, 16, 2, 239–247**).

Key words: ESBL, CTX-M, plasmids, multiresistance.

Streszczenie

Cel pracy. Określenie częstości przekazywania genów plazmidowych kodujących β-laktamazy o rozszerzonym spektrum substratowym (ESBL) z klinicznych szczepów *Escherichia coli* i *Klebsiella* spp. do szczepu biorcy *E. coli* K12 C600. Oznaczono ponadto wrażliwość na leki przeciwbakteryjne szczepów dawców oraz uzyskanych w krzyżówkach transkoniugantów.

Materiał i metody. W badaniach zastosowano 51 szczepów klinicznych wytwarzających ESBL: *E. coli* (n = 32) oraz *Klebsiella* spp. (n = 19), wyizolowanych od dzieci hospitalizowanych w Akademickim Szpitalu Klinicznym we Wrocławiu (Polska). Przekazywanie plazmidów zawierających geny kodujące ESBL przeprowadzono za pomocą metody koniugacji w podłożu bulionowym. Wytwarzanie ESBL wykrywano testem synergizmu dwóch krążków (DDST). Minimalne stężenia hamujące (MIC) leków przeciwbakteryjnych oznaczono metodą seryjnych roz-

cieńczeń w podłożu agarowym Mueller-Hintona. Występowanie genu bla_{CTX-M} w szczepach dawców i transkoniugantach oznaczono metodą PCR.
Wyniki. Większość badanych szczepów (92,2%) przekazywała plazmidy kodujące ESBL do szczepu biorcy *E. coli* K12 C600 z częstością 10^{-5}–10^{-1} w przeliczeniu na komórkę dawcy. Szczepy dawców oraz transkoniuganty wykazywały typowe dla producentów ESBL wzorce oporności. Charakteryzowały się opornością na cefotaksym, ceftriakson, aztreonam oraz wrażliwością na karbapenemy i oksyimino-β-laktamy (ceftazydym, cefotaksym, ceftriakson i aztreonam) skojarzone z kwasem klawulanowym. Większość szczepów wykazywała ponadto wysoki poziom oporności na nie-β-laktamowe leki przeciwbakteryjne (gentamycynę, amikacynę, kotrimoksazol). Wartości MIC dla cefotaksymu i ceftriaksonu były znacznie wyższe w porównaniu z wartościami MIC dla ceftazydymu. Wyniki te mogą sugerować oporność wynikającą z wytwarzania ESBL typu CTX-M. Metoda PCR z wykorzystaniem sekwencji starterowych swoistych dla β-laktamaz typu CTX-M potwierdziła obecność genu bla_{CTX-M} u 31 (66%) szczepów dawców i 23 (48,9%) transkoniugantów.
Wnioski. Większość badanych szczepów zawierała plazmidy koniugacyjne kodujące β-laktamazy ESBL typu CTX-M. Geny warunkujące oporność na inne niż β-laktamy leki przeciwdrobnoustrojowe były ponadto często przekazywane w procesie koniugacji do szczepu biorcy, co wskazuje na ich umiejscowienie w obrębie plazmidów kodujących ESBL (**Adv Clin Exp Med 2007, 16, 2, 239–247**).

Słowa kluczowe: ESBL, CTX-M, plazmidy, wieloooporność.

The production of β-lactamases is considered the predominant mechanism of β-lactam resistance in Gram-negative bacteria of the *Enterobacteraiceae* family [1–3]. The extensive clinical utilization of the third-generation cephalosporins (3GC) ceftazidime, cefotaxime, and ceftriaxone at the beginning of the eighties has been responsible for the emergence of new variants of β-lactamases among Gram-negative enterobacteria causing hospital-acquired infections [4]. These enzymes were given the name extended-spectrum β-lactamases (ESBLs) to reflect their enhanced activity against oxyimino-β-lactams, including 3GC and monobactams. They effectively hydrolyze penicillins, cephalosporins, and monobactams; however, they are not active against carbapenems and cephamycins and remain susceptible to inhibition by β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam [1, 3].

ESBL-producing Gram-negative rods are undoubtedly one of the most important etiological agents of many severe and life-threatening nosocomial infections, such as bacteremia, pneumonia, and urinary tract infections, particularly in intensive care units and neonatal and surgical wards. The genes encoding ESBLs are usually localized on large, transferable plasmids that can easily become widespread in Gram-negative bacilli [2, 5]. Resistance to β-lactam antibiotics resulting from the synthesis of ESBLs may occur in all Gram-negative rods, but *Escherichia coli* and *Klebsiella* spp. strains are the predominant ESBL producers in many countries worldwide [6]. ESBL-producing strains often display high-level resistance to other classes of antibiotics and chemotherapeutics (e.g. aminoglycosides, co-trimoxazole, tetracycline). This results from the fact that genes coding for ESBLs and those conferring resistance to other antimicrobial drugs may reside within the same conjugative plasmids [7]. For this reason, the global spread of multiresistant ESBL-producing organisms poses a serious clinical problem limiting therapeutic options.

The present study aimed to evaluate the transfer frequency of ESBL-encoding plasmids from *Escherichia coli* and *Klebsiella* spp. clinical isolates to the *E. coli* K12 C600 recipient strain. In addition, the susceptibility to various antibiotics and chemotherapeutics and the presence of the bla_{CTX-M} gene in donor strains and their transconjugants obtained in mating experiments were determined.

Material and Methods

Bacterial Strains

Fifty-one ESBL-positive clinical isolates, including *Escherichia coli* (n = 32), *Klebsiella pneumoniae* (n = 17), and *Klebsiella oxytoca* (n = 2), were collected during a two-year period (2004-2005) from children hospitalized in pediatric wards of the Medical University Hospital in Wrocław, Poland. The isolates were recovered from various specimen types, mostly from urine, stool, pus, throat swabs, and blood samples. Identification of the strains was verified by the ATB automated identification system (bioMérieux, France), using the ID 32 E test according to the manufacturer's instructions.

Antibiotic Susceptibility Testing

The MIC values of the antibiotics and chemotherapeutics tested were determined by the agar dilution technique on Mueller-Hinton agar (Oxoid) according to the Clinical Laboratory Standards Institute (formerly NCCLS) guidelines [8]. MICs of

the oxyimino-β-lactams aztreonam, cefotaxime, ceftazidime, and ceftriaxone were determined alone and in a fixed concentration of clavulanic acid (2 mg/l). The inoculum was 10^4 colony forming units (cfu) per spot deposited on the Mueller-Hinton agar. MIC values were read after 18 h of incubation at 35°C. *E. coli* strains ATCC 25922 and ATCC 35218 were used as quality reference strains. Standard powders of antimicrobial drugs were obtained from the following suppliers: aztreonam (Bristol-Myers Squibb), ceftazidime (Glaxo Wellcome), ceftriaxone (Hoffmann-La Roche Inc.), amikacin, cefotaxime, gentamicin (Sigma Chemical Co.), imipenem (Merck Sharp & Dohme Research), meropenem (Zeneca), lithium clavulanate (GlaxoSmithKline Pharma), chloramphenicol, co-trimoxazole, norfloxacin, and tetracycline (Polfa Tarchomin).

ESBL Production

ESBL production was detected by the double-disk synergy test (DDST) according to Jarlier et al. [9]. This test was performed by placing disks of ceftazidime, cefotaxime, and aztreonam (30 μg each) at a distance of 20 cm (center to center) from a disk containing amoxicillin + clavulanic acid (20 and 10 μg, respectively). The strains that showed synergy between oxyimino-β-lactams and clavulanic acid were considered to produce ESBL enzymes.

Transfer of Plasmids Encoding ESBL

Conjugational transfer of plasmids encoding ESBL was performed with all ESBL-positive strains studied (resistant to ceftazidime or cefotaxime but susceptible to nalidixic acid) using the mixed broth method. *E. coli* K12 C600, which is resistant to nalidixic acid and susceptible to all β-lactams, was used as the recipient strain. Briefly, equal volumes (1 ml) of cultures of the donor and the recipient strains (10^9 cfu per ml), grown in nutrient broth (Difco), were mixed and incubated for 24 h at 37°C. Transconjugants were selected on MacConkey agar (Biomed) supplemented with nalidixic acid (64 mg/l) (Chinoin, Hungary) to inhibit the growth of the donor strains, and with ceftazidime or cefotaxime (4 mg/l) to inhibit the growth of the recipient strain. The transfer frequency of plasmid-mediated ESBL was expressed as the transconjugant cfu number relative to the donor cfu number after the mating period.

Plasmid DNA Preparation

Plasmid DNA was extracted from the donor strains and their transconjugants by the alkaline method using the Qiagen Plasmid Mini Kit (Qiagen, Germany) according to the manufacturer's procedure.

PCR Amplification of the $bla_{CTX\text{-}M\text{-}1}$ Gene

Plasmid DNA preparations from the donor strains and their transconjugants were used as templates for $bla_{CTX\text{-}M}$ gene amplification. The oligonucleotide primers used for the PCR assays were: P1C 5' – TCGTCTCTTCCAG – 3' and P2D 5' – –CAGCGCTTTTGCCGTCTAAG – 3' (Bionovo, Legnica, Poland). The PCR conditions were: 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and finally 3 min at 72°C [10]. The size of the PCR products was approximately 1 kb.

Results

All the 51 clinical isolates studied, comprising 32 *Escherichia coli* and 19 *Klebsiella* spp., were identified as ESBL producers on the basis of positive results by the DDST. To determine whether the ESBL phenotype was transferable, conjugation experiments were conducted with all these isolates as donors and the *Escherichia coli* K12 C600 strain as the recipient. Forty-seven (92.2%) of the strains analyzed transferred ESBL-encoding plasmids to the recipient strain (Tab. 1). The transfer frequency ranged from 1.5×10^{-5} to 4.5×10^{-1} per donor cell for *E. coli* and from 1.6×10^{-5} to 5.8×10^{-1} per donor cell for *Klebsiella* spp. strains.

The results of the susceptibility testing for the 47 donor strains and their transconjugants are shown in Table 2. All the donor strains were uniformly resistant to cefotaxime (MIC range: 256 to > 1024 mg/l), ceftriaxone (MIC range: 512 to > 1024 mg/l) and aztreonam (MIC range: 32 to > 1024 mg/l) but susceptible to imipenem, meropenem, and oxyimino-β-lactams (ceftazidime, cefotaxime, ceftriaxone, aztreonam) in combination with clavulanic acid (MIC: < 1 mg/l). Resistance to ceftazidime (MIC range: 32–256 mg/l) was demonstrated in 22 donor strains (46.8%).

In other classes of antimicrobial drugs, susceptibility testing gave the following results: all donor strains were fully resistant to co-trimoxazole (MIC: > 1024 mg/l) and 46 (97.9%) of them were resistant to gentamicin (MIC range: 256 to > 1024 mg/l) and amikacin (MIC range: 1024 to > 1024 mg/l). Resistance to tetracycline (MIC range: 16–512 mg/l) was demonstrated in 20 (42.6%) of the isolates and to chloramphenicol (MIC range: 32 to > 1024 mg/l) in 6 (12.8%) of the isolates.

Table 1. Transfer frequency of ESBL-encoding plasmids from strains tested (n = 51) to the *E. coli* K12 C600 recipient strain

Tabela 1. Częstość przekazywania plazmidów kodujących ESBL z badanych szczepów (n = 51) do szczepu biorcy *E. coli* K12 C600

ESBL-positive strains tested (Badane szczepy ESBL(+))	Transfer frequency (Częstość transferu)	ESBL-positive strains tested (Badane szczepy ESBL(+))	Transfer frequency (Częstość transferu)	ESBL-positive strains tested (Badane szczepy ESBL(+))	Transfer frequency (Częstość transferu)
Escherichia coli 1	2.0×10^{-5}	*Escherichia coli* 39	5.0×10^{-2}	*Klebsiella pneumoniae* 11	1.8×10^{-2}
Escherichia coli 5	2.1×10^{-1}	*Escherichia coli* 42	1.5×10^{-1}	*Klebsiella pneumoniae* 24	4.7×10^{-4}
Escherichia coli 6	6.3×10^{-4}	*Escherichia coli* 43	1.4×10^{-3}	*Klebsiella pneumoniae* 25	3.2×10^{-1}
Escherichia coli 9	1.7×10^{-5}	*Escherichia coli* 44	3.8×10^{-1}	*Klebsiella pneumoniae* 32	1.9×10^{-1}
Escherichia coli 10	2.9×10^{-5}	*Escherichia coli* 48	3.5×10^{-1}	*Klebsiella pneumoniae* 35	2.1×10^{-4}
Escherichia coli 12	–	*Escherichia coli* 49	4.5×10^{-1}	*Klebsiella pneumoniae* 36	5.8×10^{-1}
Escherichia coli 13	1.1×10^{-1}	*Escherichia coli* 51	2.9×10^{-1}	*Klebsiella pneumoniae* 40	1.6×10^{-2}
Escherichia coli 15	–	*Escherichia coli* 56	3.4×10^{-1}	*Klebsiella pneumoniae* 41	3.8×10^{-1}
Escherichia coli 16	1.1×10^{-3}	*Escherichia coli* 58	–	*Klebsiella pneumoniae* 47	1.3×10^{-2}
Escherichia coli 22	3.8×10^{-3}	*Escherichia coli* 64	1.2×10^{-3}	*Klebsiella pneumoniae* 52	7.4×10^{-2}
Escherichia coli 23	3.8×10^{-4}	*Escherichia coli* 86	3.0×10^{-1}	*Klebsiella pneumoniae* 61	–
Escherichia coli 26	1.5×10^{-1}	*Escherichia coli* 91	1.5×10^{-1}	*Klebsiella pneumoniae* 62	1.6×10^{-2}
Escherichia coli 27	3.2×10^{-1}	*Escherichia coli* 93	1.3×10^{-4}	*Klebsiella pneumoniae* 63	1.3×10^{-2}
Escherichia coli 30	6.1×10^{-5}	*Escherichia coli* 94	4.2×10^{-2}	*Klebsiella pneumoniae* 65	5.1×10^{-3}
Escherichia coli 33	1.3×10^{-2}	*Escherichia coli* 95	1.5×10^{-5}	*Klebsiella pneumoniae* 67	1.6×10^{-5}
Escherichia coli 34	9.4×10^{-3}	*Klebsiella pneumoniae* 7	1.1×10^{-2}	*Klebsiella oxytoca* 66	7.7×10^{-2}
Escherichia coli 38	1.0×10^{-1}	*Klebsiella pneumoniae* 8	4.0×10^{-4}	*Klebsiella oxytoca* 87	9.3×10^{-3}

On the other hand, all but one (*E. coli* 51) of the donor strains were susceptible to norfloxacin (MIC: 16 mg/l).

The MIC values of the antibiotics and chemotherapeutics tested for transconjugants were substantially lower but reflected well the data obtained for the respective donor strains (Tab. 2). All the transconjugants exhibited resistance to cefotaxime (MIC range: 128–1024 mg/l), ceftriaxone (MIC range: 64–1024 mg/l), aztreonam (MIC range: 32–256 mg/l), and co-trimoxazole (MIC range: 1024 to > 1024 mg/l), but were susceptible to imipenem, meropenem, and oxyimino-β-lactams in combination with clavulanic acid (MIC: < 1 mg/l). Compared with the donor strains however, the transconjugant strains displayed lower rates of resistance to gentamicin (85.1% vs. 97.9%) and to amikacin (87.2% vs. 97.9%). Resistance to chloramphenicol was found in one transconjugant only (MIC: 32 mg/l), but none of them were resistant to norfloxacin. Moreover, these strains demonstrated a high level of resistance to co-trimoxazole (MIC: 1024 to > 1024 mg/l) and aminoglycosides tested (in most cases, MICs from 256 to > 1024 mg/l).

PCR results based on P1C and P2D primers specific for the CTX-M family of ESBLs revealed the presence of the $bla_{\text{CTX-M}}$ gene in 31 (66%) of the 47 donor strains (19 *E. coli*, 10 *K. pneumoniae* and 2 *K. oxytoca*) (Fig. 1). Among the transconjugants obtained in the mating experiments, the $bla_{\text{CTX-M}}$ gene was detected in 23 (48.9%) strains (Fig. 2). PCR products were of the expected size of approximately 1 kb.

Resistance to non-β-lactam drugs was, in many cases, co-transferred from donors to the recipient strain with ESBL-encoding plasmids. As shown in Table 3, the most frequently observed resistance pattern among transconjugants (72.3%) was resistance to three non-β-lactam drugs: gentamicin, amikacin, and co-trimoxazole, followed by resistance to co-trimoxazole (12.8%), and then resistance to four antimicrobials: gentamicin, amikacin, co-trimoxazole, and tetracycline (10.6%). The remaining two resistance patterns: to five compounds (gentamicin, amikacin, co-trimoxazole, tetracycline, chloramphenicol) and to two compounds (amikacin, co-trimoxazole), were detected in individual strains only.

Discussion

The first clinical isolates of ESBL-producing species of the *Enterobacteriaceae* family (*K. pneumoniae*, *K. ozenae*, and *Serratia marcescens*) were reported in 1983 in Germany [4]. Hospital outbreaks caused by ESBL-producing bacteria in subsequent years were reported in France [11] and the USA [12]. Nowadays, ESBL production is considered one of the main β-lactam resistance mechanisms [3, 6]. ESBL-encoding genes are usually carried by large and transferable plasmids. Thus the plasmid localization of the genetic determinants facilitates their horizontal spread in bacterial populations, particularly by means of conjugation.

Table 2. Antimicrobial susceptibility of donor strains and their transconjugants

Tabela 2. Wrażliwość na leki przeciwbakteryjne szczepów dawców i ich transkoniugantów

Antibacterial drugs (Leki przeciwbakteryjne)	Donor strains (Szczepy dawców) n = 47		Transconjugants (Transkoniuganty) n = 47	
	no (%) of resistant strains range of MIC (mg/l)	no (%) of susceptible strains range of MIC (mg/l)	no (%) of resistant strains range of MIC (mg/l)	no (%) of susceptible strains range of MIC (mg/l)
Ceftazidime (Ceftazydym)	22 (46.8%) MIC 32–256	25 (53.2%) MIC 2–8	22 (46.8%) MIC 32–128	25 (53.2%) MIC 2–8
Ceftazidime + clavulanic acid (Ceftazydym + kwas klawulanowy)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Cefotaxime (Cefotaksym)	47 (100%) MIC 256–> 1024	0 (0%) –	47 (100%) MIC 128–1024	0 (0%) –
Cefotaxime + clavulanic acid (Cefotaksym + kwas klawulanowy)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Ceftriaxone (Ceftriakson)	47 (100%) MIC 512–> 1024	0 (0%) –	47 (100%) MIC 64–1024	0 (0%) –
Ceftriaxone + clavulanic acid (Ceftriakson + kwas klawulanowy)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Aztreonam (Aztreonam)	47 (100%) MIC 32–> 1024	0 (0%) –	47 (100%) MIC 32–256	0 (0%) –
Aztreonam + clavulanic acid (Aztreonam + kwas klawulanowy)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Imipenem (Imipenem)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Meropenem (Meropenem)	0 (0%) –	47 (100%) MIC < 1	0 (0%) –	47 (100%) MIC < 1
Gentamicin (Gentamycyna)	46 (97.9%) MIC 256–> 1024	1 (2.1%) MIC < 1	40 (85.1%) MIC 32–> 1024	7 (14.9%) MIC < 1
Amikacin (Amikacyna)	46 (97.9%) MIC 1024–> 1024	1 (2.1%) MIC < 1	41 (87.2%) MIC 64–> 1024	6 (12.8) MIC < 1–16
Tetracycline (Tetracyklina)	20 (42.6%) MIC 16–512	27 (57.4%) MIC < 1–4	6 (12.8%) MIC 16–32	41 (87.2%) MIC < 1–4
Norfloxacin (Norfloksacyna)	1 (2.1%) MIC 16	46 (97.9%) MIC < 1–2	0 (0%) –	47 (100%) MIC < 1
Co-trimoxazole (Kotrimoksazol)	47 (100%) MIC > 1024	0 (0%) –	47 (100%) MIC 1024–> 1024	0 (0%) –
Chloramphenicol (Chloramfenikol)	6 (12.8%) MIC 32–> 1024	41 (87.2%) MIC < 1–8	1 (2.1%) MIC 32	46 (97.9%) MIC < 1–8

In the present study, the majority of the ESBL-producing isolates (47/51) transferred plasmid-mediated ESBLs to the *E. coli* K12 C600 recipient strain. The transfer frequency varied from 10^{-5} to 10^{-1} per donor strain. It is worth noting that almost 60% of the donor strains (28/47) were found to transfer plasmid-mediated ESBLs with the very high frequency of 10^{-2}–10^{-1} per donor cell. These results confirm a common and very effective mechanism of ESBL dissemination among Gram-negative rods. The remaining four isolates tested (*E. coli* 12, *E. coli* 15, *E. coli* 58, and *K. pneumoniae* 61) gave negative results in the mating experiments, suggesting that the genes responsible for ESBL production were probably integrated with chromosome or nonconjugative plasmids. All transconjugants obtained in the mating experiments were found to express the ESBL phenotype, as confirmed by conventional DDST.

The donor strains and their transconjugants

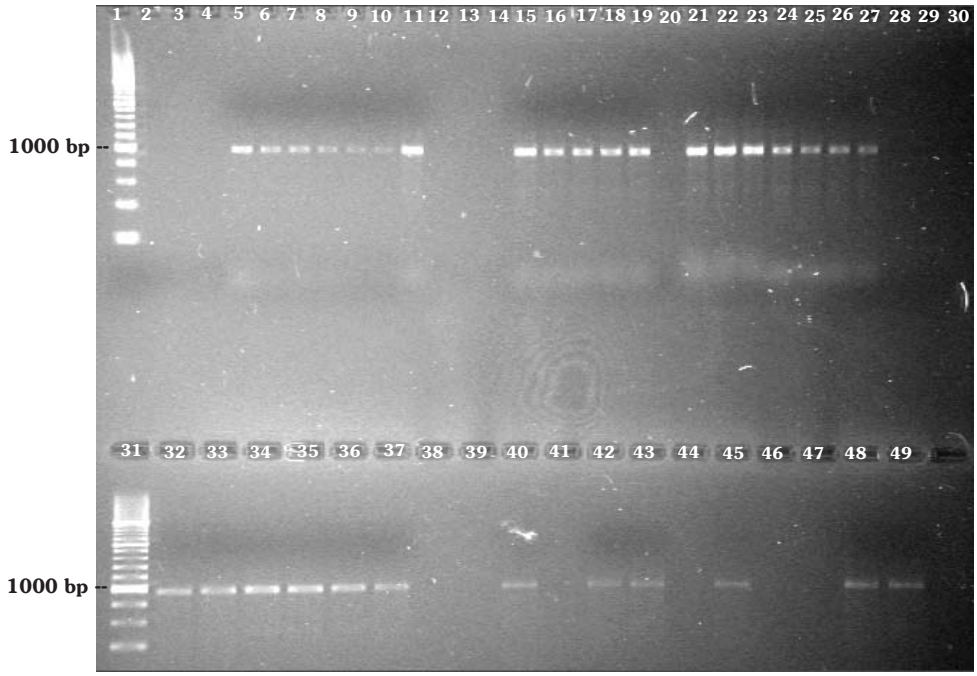

Fig. 1. Agarose gel electrophoresis of PCR products in donor strains. Lanes: 1 and 31 – molecular size of DNA markers. Positive results of PCR – lines: 5 (*E. coli* 9); 6 (*E. coli* 10); 7 (*E. coli* 13); 8 (*E. coli* 16); 9 (*E. coli* 22); 10 (*E. coli* 23); 11 (*E. coli* 26); 15 (*E. coli* 34); 16 (*E. coli* 38); 17 (*E. coli* 39); 18 (*E. coli* 42); 19 (*E. coli* 43); 21 (*E. coli* 48); 22 (*E. coli* 49); 23 (*E. coli* 51); 24 (*E. coli* 91); 25 (*E. coli* 93); 26 (*E. coli* 94); 27 (*E. coli* 95); 32 (*K. pneumoniae* 7); 33 (*K. pneumoniae* 8); 34 (*K. pneumoniae* 24); 35 (*K. pneumoniae* 35); 36 (*K. pneumoniae* 36); 37 (*K. pneumoniae* 40); 40 (*K. pneumoniae* 41); 42 (*K. pneumoniae* 47); 43 (*K. pneumoniae* 52); 45 (*K. pneumoniae* 65); 48 (*K. oxytoca* 66); 49 (*K. oxytoca* 87)

Ryc. 1. Elektroforeza w żelu agarozowym produktów PCR szczepów dawców. Ścieżki 1 i 31 – markery długości fragmentów DNA. Dodatnie wyniki PCR – ścieżki: 5 (*E. coli* 9); 6 (*E. coli* 10); 7 (*E. coli* 13); 8 (*E. coli* 16); 9 (*E. coli* 22); 10 (*E. coli* 23); 11 (*E. coli* 26); 15 (*E. coli* 34); 16 (*E. coli* 38); 17 (*E. coli* 39); 18 (*E. coli* 42); 19 (*E. coli* 43); 21 (*E. coli* 48); 22 (*E. coli* 49); 23 (*E. coli* 51); 24 (*E. coli* 91); 25 (*E. coli* 93); 26 (*E. coli* 94); 27 (*E. coli* 95); 32 (*K. pneumoniae* 7); 33 (*K. pneumoniae* 8); 34 (*K. pneumoniae* 24); 35 (*K. pneumoniae* 35); 36 (*K. pneumoniae* 36); 37 (*K. pneumoniae* 40); 40 (*K. pneumoniae* 41); 42 (*K. pneumoniae* 47); 43 (*K. pneumoniae* 52); 45 (*K. pneumoniae* 65); 48 (*K. oxytoca* 66); 49 (*K. oxytoca* 87)

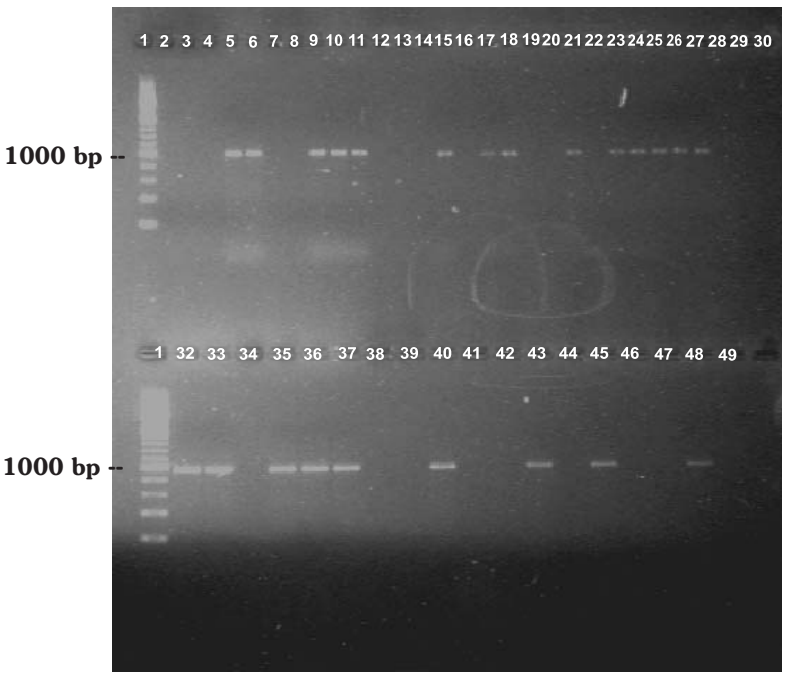

Fig. 2. Agarose gel electrophoresis of PCR products in transconjugants (T) Lanes: 1 and 31 – molecular size of DNA markers. Positive results of PCR – lines: 5 (T 9); 6 (T 10); 9 (T 22); 10 (T 23); 11 (T 26); 15 (T 34); 17 (T 39); 18 (T 42); 21 (T 48); 23 (T 51); 24 (T 91); 25 (T 93); 26 (T 94); 27 (T 95); 32 (T 8); 33 (T 24); 35 (T 35); 36 (T 36); 37 (T 41); 40 (T 47); 43 (T 52); 45 (T 65); 48 (T 66)

Ryc. 2. Elektroforeza w żelu agarozowym produktów PCR transkoniugantów (T) Ścieżki 1 i 31 – markery długości fragmentów DNA. Dodatnie wyniki PCR – ścieżki: 5 (T 9); 6 (T 10); 9 (T 22); 10 (T 23); 11 (T 26); 15 (T 34); 17 (T 39); 18 (T 42); 21 (T 48); 23 (T 51); 24 (T 91); 25 (T 93); 26 (T 94); 27 (T 95); 32 (T 8); 33 (T 24); 35 (T 35); 36 (T 36); 37 (T 41); 40 (T 47); 43 (T 52); 45 (T 65); 48 (T 66)

Table 3. Resistance patterns to non-β-lactam antibacterial drugs co-transferred with ESBL phenotype

Tabela 3. Wzory oporności na nie-β-laktamowe leki przeciwbakteryjne przekazywane wraz z fenotypem ESBL

Resistance patterns (Wzory oporności)	No. (%) of transconjugants (Liczba (%) transkoniugantów)
Gm, An, Sxt	34 (72.3)
Sxt	6 (12.8)
Gm, An, T, Sxt	5 (10.6)
Gm, An, T, Sxt, C	1 (2.1)
An, Sxt	1 (2.1)

Abbreviations: Gm – gentamicin, An – amikacin, T – tetracycline, Sxt – co-trimoxazole, C – chloramphenicol.

Skróty: Gm – gentamycyna, An – amikacyna, T – tetracyklina, Sxt – kotrimoksazol, C – chloramfenikol.

displayed susceptibility patterns typical of ESBL producers. All of them were uniformly resistant to cefotaxime, ceftriaxone, and aztreonam but susceptible to imipenem, meropenem, and oxyimino-β-lactams combined with clavulanic acid. These results support previous observations that carbapenems remain the antibiotics of choice for the treatment of infections caused by ESBL-producing strains [1, 13].

It should be emphasized that the donor strains displayed significantly higher MIC values of cefotaxime (256 to > 1024 mg/l) and cefrtiaxone (512 to > 1024 mg/l) than those of ceftazidime (2–256 mg/l). This may suggest that this resistance results from cefotaximase activity, e.g. CTX-M-type ESBLs. In order to check this hypothesis, PCR was performed with P1C and P2D primers specific for the CTX-M ESBLs. As expected, the presence of the $bla_{\text{CTX-M}}$ gene was detected in 31 (66%) of the donor strains, while the percentage of transconjugants harboring this gene was lower (48.9%).

CTX-M-type ESBLs display an enhanced activity against cefotaxime and ceftriaxone, but their activity against ceftazidime is significantly lower [14, 15]. The global expansion of CTX-M ESBLs was observed in the mid 1990s. The number of ESBLs representing the CTX-M family has been rapidly growing in recent years. Nowadays, these enzymes are considered to be one of the most common ESBLs worldwide [3]. The clinical isolates producing CTX-M-type ESBLs have been reported in different geographical areas, such as Europe [10, 16, 17], South America [18–20], Asia [21, 22], and North America [23].

ESBL-positive strains often display high levels of resistance to antibiotics and chemotherapeutics other than β-lactams, such as aminoglycosides, co-trimoxazole, and tetracycline, suggesting that the genes responsible for this resistance are carried by ESBL-encoding plasmids [13, 24, 25]. For this reason, such ESBL-producing multiresistant organisms pose a serious therapeutic problem in hospital settings. It is worth noting that ESBL-encoding genes and those conferring resistance to non-β-lactam drugs carried by the same conjugative plasmids may disseminate in bacterial populations even in the absence of β-lactams in the environment. Thus, selective pressure resulting from the clinical utilization of non-β-lactam antimicrobials contributes to the maintenance of plasmids coding for ESBLs.

In the present study, all the donor strains were resistant to co-trimoxazole. Additionally, resistance to gentamicin and amikacin was demonstrated in all but one donor strain (97.9%). These results are in agreement with those reported previously [24–26]. On the other hand, the percentages of donor strains exhibiting resistance to tetracycline and chloramphenicol were significantly lower (42.6% and 12.8%, respectively), while resistance to norfloxacin was demonstrated in one isolate only. In contrast, in the study by Puerto et al. [27], 62 (53.9%) out of 115 ESBL-producing *E. coli* clinical isolates were resistant to norfloxacin. The low norfloxacin resistance rate obtained in the present study may be explained by the fact that all the strains studied were recovered from hospitalized children. Thus, fluoroquinolones are not recommended for the treatment of infections in children because of their possible toxicity [28].

In summary, the results of this study demonstrate that multidrug resistance among enterobacteria is predominantly the result of the dissemination of transferable plasmids carrying resistance genes. Moreover, there is a very strong association between ESBL production and resistance to different antimicrobial agents, particularly to aminoglycosides and co-trimoxazole.

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Conflict of interest: None declared

Received: 24.01.2007
Revised: 8.03.2007
Accepted: 8.03.2007