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ESBL-Producing *Escherichia coli* Isolated from Bloodstream Infections – Antimicrobial Susceptibility, Conjugative Transfer of Resistance Genes and Phylogenetic Origin

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Background. The prevalence of bloodstream infections (BSIs) due to ESBL-producing *Escherichia coli* (ESBL-EC) strains has increased dramatically over the past years.
Objectives. Characterization of ESBL-EC isolates collected from BSIs with regard to their antimicrobial susceptibility and phylogenetic background. The conjugative transfer of resistance determinants to the *E. coli* reference strain K12 C600 was also investigated.
Material and Methods. A collection of forty-eight ESBL-EC strains recovered from BSIs was subjected to the study. These strains were obtained from the ICU (intensive care unit) of the Medical University Hospital, Wrocław, Poland, during a four-year period (2009–2012). All the isolates were screened for ESBL production by the double disk synergy test (DDST). Transferability of plasmid-mediated resistance genes was performed by the conjugational broth method. Susceptibility to antibiotics and chemotherapeutics of clinical isolates and transconjugants was determined by the agar dilution method. PCR assay was used to detect the $bla_{\text{CTX-M}}$ gene in ESBL-EC tested and transconjugants. Affiliation to phylogenetic groups was done by the triplex PCR method.
Results. Conjugational transfer of plasmids responsible for ESBL to a recipient strain was successful for all the ESBL-EC analyzed (donors). The conjugation frequencies ranging from 2.3×10^{-7} to 5.2×10^{-1} per donor. *In vitro* susceptibility testing revealed that all the ESBL-EC isolates and their transconjugants were resistant to most of the antimicrobial agents tested with the exception of carbapenems, tigecycline, and β-lactam-clavulanate combinations. Moreover, all the donor strains and their transconjugants were found to contain the $bla_{\text{CTX-M}}$ gene. The majority of the isolates analyzed belonged to phylogroups B2 (62.5%) and D (25%), whereas groups B1 and A were less frequently represented (8.3% and 4.2%, respectively).
Conclusions. The results of the study confirm the need of antibiotic policies and effective infection control measures in hospital settings to minimize BSIs caused by multi-resistant ESBL-producing pathogens (**Adv Clin Exp Med 2014, 23, 6, 865–870**).

Key words: *E. coli*, ESBL, conjugational transfer, antimicrobial resistance, phylogenetic groups.

Plasmid-encoded extended-spectrum β-lactamases (ESBLs) are considered one of the most common mechanisms of resistance to β-lactam antibiotics in Gram-negative rods. Since the first description at the beginning of the 1980s, the number of ESBL types identified has been constantly growing. Currently, more than 700 distinct ESBL variants have been described worldwide. These enzymes are usually classified based on their molecular and biochemical properties as well as substrate preferences [1]. ESBLs constitute the major source of resistance to almost all penicillins, third-generation cephalosporins (3GC) and aztreonam with the exception of cephamycins and carbapenems. Moreover, their hydrolyzing activity is effectively blocked by β-lactamase inhibitors, such as clavulanate, sulbactam or tazobactam [2]. ESBLs are most commonly detected in a wide variety of enterobacterial species, predominantly in *Escherichia coli* and *Klebsiella pneumoniae* isolates responsible

for community- and hospital-acquired infections. As the authors reported previously, ESBL-expressing enterobacteria usually exhibit co-resistance to a wide range of non-β-lactam antibiotics and chemotherapeutics, including aminoglycosides, trimethoprim-sulfamethoxazole, tetracycline and chloramphenicol [3, 4]. This reduces significantly the number of antimicrobials to which the infecting microorganism is susceptible. Thus, treatment of severe infections caused by multiresistant *Enterobacteriaceae* expressing ESBLs is often limited to carbapenems [5]. ESBL-producing *Escherichia coli* (ESBL-EC) is considered the causative agent of many severe and life-threading infections, such as meningitis and bloodstream infections (BSIs). The prevalence of BSIs caused by ESBL-EC has increased dramatically over the past few years [6–8]. Moreover, hospital-acquired BSIs are frequently associated with many adverse outcomes, including increased rates of treatment failure, prolonged hospital stays, increased morbidity and mortality, and higher economic costs [9, 10]. This study was designed to characterize ESBL-EC clinical isolates collected from BSIs. The primary objectives of the study were to investigate: 1) the conjugative transfer of resistance determinants, 2) the antimicrobial susceptibility patterns, and 3) the phylogenetic group affiliation.

Material and Methods

Bacterial Strains

Between January 2009 and December 2012, a total of 48 non-duplicated ESBL-EC strains were isolated from blood samples from 32 women and 16 men (mean age 59.2 years) from the ICU of the Medical University Hospital, Wrocław, Poland. Identification to species level was carried out using the ATB ID32GN test (bioMérieux) according to the recommendations of the manufacturer.

Phenotypic ESBL Detection and Antimicrobial Susceptibility Testing

The ESBL status of each isolate tested was confirmed by standard double disk synergy test (DDST) using ceftazidime, cefotaxime, cefepime and amoxicillin/clavulanate disks [11]. *Klebsiella pneumoniae* ATCC 700603 was included as an ESBL-positive control. The minimal inhibitory concentrations (MICs) of selected antimicrobial agents were determined by the dilution method on Mueller-Hinton agar (bioMérieux) in accordance with the Clinical Laboratory Standards Institute (CLSI) guidelines [12]. MICs of the oxyimino-β-lactams, namely aztreonam, ceftriaxone, ceftazidime and cefotaxime, were also determined in the presence of clavulanate (2 mg/L). Control strains, including *E. coli* ATCC 25922 and *E. coli* ATCC 35218, were included in each susceptibility test. The antibiotics and chemotherapeutics tested were provided as follows: cefotaxime, gentamicin, amikacin, clavulanate lithium (Sigma Chemical Co., St Louis, MO, USA), aztreonam (Bristol-Myers Squibb, New Brunswick, NJ, USA), ceftriaxone (Roche, Basel, Switzerland), ceftazidime (Glaxo Wellcome, Stevenage, UK), meropenem (Zeneca, Macclesfield, UK), imipenem (Merck Sharp & Dohme Research), chloramphenicol, co-trimoxazole, tetracycline (Polfa, Tarchomin, Poland), and tigecycline (Wyeth).

Transferability of Plasmid-Mediated ESBLs

Conjugation experiments were carried out between ESBL-EC clinical isolates (donors) and *E. coli* K12 C600 (recipient), as described previously [4]. Transconjugants were selected on MacConkey agar plates containing nalidixic acid (64 mg/L) and cefotaxime (4 mg/L). Nalidixic acid inhibited the growth of the donors but did not affect that of the recipient. On the other hand, cefotaxime allowed the growth of the donors but inhibited that of the recipient. The combination of these two antimicrobials permitted the growth of the transconjugants. Conjugation frequencies were expressed as the number of transconjugants per donor.

Plasmid DNA Isolation and PCR Assay

Plasmid DNA was isolated from ESBL-EC donor strains and transconjugants using the Plasmid Mini Kit (Qiagen, Germany) in accordance with the manufacturer's methodology. The prepared plasmids were subjected to PCR for the presence of the $bla_{\text{CTX-M}}$ gene in donors and their transconjugants. The primer pairs and PCR conditions were as described previously [4].

Determination of Phylogenetic Groups

Affiliation of the ESBL-EC strains studied to phylogenetic groups (A, B1, B2, D) was done using the triplex PCR method initially developed by Clermont et al. [13]. This method applied primers targeting genes: *chuA*, *yjaA*, and a noncoding region, TSPE4.C2. The PCR assay consisted of initial

denaturation at 94°C for 5 min, followed by 30 cycles at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s and a terminal extension at 72°C for 7 min.

Results and Discussion

Conjugation Experiments

All of the 48 *E. coli* bloodstream isolates tested were found to express ESBL phenotype based on the positive results of the DDST. Overall, a total of 48 conjugation crossings between ESBL-EC clinical isolates (donors) and *E. coli* strain K12 C600 (recipient) were carried out. As shown in Table 1, transconjugants were obtained for all the analyzed isolates at frequencies of 2.3×10^{-7} to 5.2×10^{-1} per donor. Remarkably, a very high frequency of conjugation (10^{-2}–10^{-1} per donor) was demonstrated with thirty (62.5%) ESBL-EC isolates.

The transconjugants obtained in transferability assays were positive for ESBL production according to conventional DDST. The current study showed an extremely effective mechanism of ESBL dissemination in Gram-negative bacilli by means of conjugation is consistent with our previous observations [3, 4].

Table 1. Frequency of conjugative transfer of plasmid-encoded ESBL from ESBL-EC* clinical isolates (n = 48) to *E. coli* K12 C600 recipient

ESBL-EC (donors)	Transfer frequency	ESBL-EC (donors)	Transfer frequency
8	2.8×10^{-5}	74	2.0×10^{-2}
15	2.4×10^{-1}	76	8.5×10^{-1}
16	6.2×10^{-3}	77	1.8×10^{-3}
18	2.7×10^{-2}	81	3.8×10^{-1}
23	2.8×10^{-2}	82	3.7×10^{-1}
28	3.4×10^{-2}	85	4.0×10^{-1}
29	5.2×10^{-1}	88	2.9×10^{-1}
30	3.3×10^{-1}	89	2.4×10^{-1}
34	1.5×10^{-2}	90	1.4×10^{-1}
36	3.8×10^{-1}	92	5.2×10^{-3}
37	3.8×10^{-4}	94	3.3×10^{-1}
38	1.7×10^{-2}	96	2.3×10^{-7}
41	3.1×10^{-1}	98	1.8×10^{-4}
47	6.8×10^{-5}	100	4.2×10^{-2}
48	1.9×10^{-2}	102	4.5×10^{-3}
53	9.6×10^{-2}	103	3.1×10^{-2}
55	1.4×10^{-1}	104	4.0×10^{-4}
57	2.4×10^{-6}	105	1.3×10^{-4}
58	8.0×10^{-6}	106	5.1×10^{-2}
59	5.2×10^{-5}	107	2.1×10^{-1}
61	3.0×10^{-4}	109	8.8×10^{-3}
65	2.5×10^{-2}	111	3.2×10^{-2}
66	4.5×10^{-7}	115	2.3×10^{-1}
67	1.1×10^{-1}	119	9.1×10^{-6}

*ESBL-EC – clinical isolates of *Escherichia coli*, ESBL+ phenotype.

Susceptibility Testing

Table 2 summarizes the results of *in vitro* susceptibilities obtained for ESBL-EC clinical isolates (donors) and their transconjugants. Generally, the susceptibility of donor strains reflected well antibiotic-resistance patterns specific to ESBL-producing microorganisms. All of the ESBL-EC isolates were highly resistant to aztreonam, cefotaxime and ceftriaxone. On the other hand, 12 out of the 48 isolates were susceptible to ceftazidime. The MICs of third-generation cephalosporins and aztreonam were strongly reduced in the presence of β-lactamase inhibitor (clavulanate) at a fixed concentration of 2 mg/L. Additionally, ESBL-EC isolates remained susceptible to the carbapenems tested (meropenem and imipenem). These findings confirm that carbapenems are first line therapy in patients infected with ESBL-producing bacteria and are in agreement with previous reports [5, 14]. Apart from β-lactam drugs, the ESBL-EC isolates studied were uniformly resistant to co-trimoxazole and most of them (42/48) also displayed resistance to amikacin and gentamicin. Moreover, resistance to tetracycline and chloramphenicol was demonstrated in 18 and 6 strains, respectively. However, none of the ESBL-EC isolates was resistant to tigecycline. Our results demonstrate that tigecycline appears to be efficacious against multidrug resistant pathogens, including ESBL-producing organisms, and are consistent with data of previous studies [15, 16]. Almost all transconjugants obtained in the mating experiments showed similar antibiotic resistance profile as donor strains (Table 2). All of them were resistant to aztreonam, cefotaxime, and ceftriaxone, but susceptible to meropenem, imipenem and oxyimino-β-lactam-clavulanate combinations. Ceftazidime resistance was observed in 12 (25%) transconjugants. The data concerning the susceptibility to non-β-lactam antibiotics showed

Table 2. Resistance phenotypes of ESBL-EC donors (n = 48) and their transconjugants

Antibacterial agents	ESBL-EC donor strains		Transconjugants	
	no. (%) of resistant donors MICs (mg/L)	**no. (%) of susceptible donors MICs (mg/L)**	**no. (%) of resistant transconjugants MICs (mg/L)**	**no. (%) of susceptible transconjugants MICs (mg/L)**
CAZ	12 (25.0%) MIC 64–128	36 (75.0%) MIC 2–8	12 (25.0%) MIC 64–128	36 (75.0%) MIC 2–4
CAZ + CLA	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
CTX	48 (100%) MIC 512– > 1024	0 (0%) –	48 (100%) MIC 128–1024	0 (0%) –
CTX + CLA	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
CRO	48 (100%) MIC 256– > 1024	0 (0%) –	48 (100%) MIC 128–1024	0 (0%) –
CRO + CLA	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
ATM	48 (100%) MIC 128–512	0 (0%) –	48 (100%) MIC 128–256	0 (0%) –
ATM + CLA	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
IPM	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
MEM	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1
GM	42 (87.5%) MIC 256–1024	6 (12.5%) MIC < 1	36 (75%) MIC 128–1024	12 (25%) MIC < 1–2
AN	42 (87.5%) MIC 1024– > 1024	6 (12.5%) MIC < 1	35 (72.9%) MIC 512–1024	13 (27.1%) MIC < 1–2
T	18 (37.5%) MIC 16–512	30 (62.5%) MIC < 1–4	1 (2.1%) MIC 32	47 (97.9%) MIC < 1–2
SXT	48 (100%) MIC > 1024	0 (0%) –	36 (75%) MIC 1024	12 (25%) MIC 1–4
C	6 (12.5%) MIC 32– > 1024	42 (87.5%) MIC < 1–8	1 (2.1%) MIC 64	47 (97.9%) MIC < 1–4
TIG	0 (0%) –	48 (100%) MIC < 1	0 (0%) –	48 (100%) MIC < 1

CAZ – ceftazidime, CAZ + CLA – ceftazidime + clavulanate, CTX – cefotaxime, CTX + CLA – cefotaxime + clavulanate, CRO – ceftriaxone, CRO + CLA – ceftriaxone + clavulanate, ATM – aztreonam, ATM + CLA – aztreonam + clavulanate, IPM – imipenem, MEM – meropenem, GM – gentamicin, AN – amikacin, T – tetracycline, SXT – co-trimoxazole, C – chloramphenicol, TIG – tigecycline.

that 36 (75%) out of the 48 transconjugants showed resistance to co-trimoxazole and gentamicin. In addition, 35 (72.9%) of them were resistant to amikacin. Moreover, resistance to chloramphenicol and tetracycline was demonstrated in one transconjugant only, whereas all the transconjugants were uniformly susceptible to tigecycline. The results of the present study indicate that transferability of ESBL-mediated resistance to oxyimino-β-lactams is directly associated with the resistance to non-β-lactam antibacterial drugs. On the other hand, further studies should be carried out to demonstrate the genetic location of antibiotic resistance markers in the same ESBL-encoding plasmids.

PCR-Based Amplification of the bla_{CTX-M}

Cefotaximases (CTX-M-ases) constitute the predominant family among globally distributed ESBLs. These enzymes are not closely related to others families of ESBLs (TEM and SHV), but share a high level of identity with chromosomal β-lactamases derived from *Kluyvera* spp. [17]. CTX-M-type ESBLs confer higher levels of resistance to cefotaxime than to ceftazidime. The analysis of susceptibility patterns obtained for the ESBL-EC strains tested and their transconjugants revealed that MICs of cefotaxime were significantly higher than those of ceftazidime. Thus, these results may suggest the synthesis of ESBLs belonging to the CTX-M family. PCR-based assays revealed the presence of the bla_{CTX-M} gene in all ESBL-EC clinical isolates as well as their transconjugants. These findings demonstrate the predominance of CTX-M enzymes among ESBL producers and correlate well with our previous observations [4] as well as with data reported by other researchers [18, 19].

Phylogenetic Group Distribution Among ESBL-EC

Phylogenetic analyses, based on the combination of 3 DNA markers (chuA, yjaA, and TSPE4. C2) have classified *E. coli* strains into 4 main phylogroups, namely A, B1, B2 and D. It is noteworthy that the phylogenetic group affiliation of *E. coli* strains is closely associated with their pathogenic potential. Generally, commensal isolates belong mainly to A and B1 groups whereas pathogenic *E. coli* strains responsible for extraintestinal infections, particularly in hospital settings, are essentially link to the B2 and D phylogroups [13, 20]. The present study showed that the majority of ESBL-EC isolates belonged to phylogroups B2 (62.5%) and D (25%), whereas phylogenetic groups B1 and A were less frequently represented (8.3% and 4.2%, respectively). These findings are generally consistent with those obtained by Sannes et al. [21] and Bukh et al. [22], who demonstrated that bloodstream *E. coli* isolates belonged mostly to B2 and D phylogroups.

In conclusion, our study emphasizes the need to apply appropriate infection control measures to reduce severe hospital-acquired infections due to multiresistant ESBL-producing bacteria.

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