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ESBL-Producing *Escherichia coli* Isolated from Children with Acute Diarrhea – Antimicrobial Susceptibility, Adherence Patterns and Phylogenetic Background*

Szczepy *Escherichia coli* wytwarzające β-laktamazy ESBL izolowane od dzieci z ciężką biegunką – wrażliwość na leki, typy adhezji i pochodzenie filogenetyczne

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Abstract

Background. *Escherichia coli* remains the principal bacterial pathogen in childhood diarrhea and constitutes an important public health problem, especially in developing countries. Diarrheagenic *E. coli* strains often display resistance to β-lactams due to the production of extended-spectrum β-lactamases (ESBLs).
Objectives. A total of thirty ESBL-producing *E. coli* strains colonizing the gastrointestinal tracts of children with acute diarrhea were studied in order to determine their antimicrobial susceptibility, adherence patterns to the HEp-2 cell line and phylogenetic background.
Material and Methods. ESBL production was detected by the double disk synergy test (DDST). The minimal inhibitory concentrations (MICs) of antibacterial drugs were determined by an agar dilution technique on Mueller-Hinton agar. The presence of bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ determinants in the strains studied was ascertained by polymerase chain reaction (PCR).
Results. The strains displayed the resistance pattern typical of ESBL producers. The majority of them (23 out of 30) were found to produce CTX-M-type ESBLs conferring a high level of resistance to oxyimino-β-lactams, especially to cefotaxime and ceftriaxone. In many cases, the strains exhibited resistance to non-β-lactam antimicrobials, such as gentamicin, amikacin, co-trimoxazole and tetracycline. On the other hand, these strains were uniformly susceptible to carbapenems, to oxyimino-β-lactams combined with clavulanic acid and to tigecycline. The *E. coli* strains were distributed among the four main phylogenetic groups: A, B1, B2 and D. The *in vitro* adhesion assay revealed that all but two of the strains adhered to the HEp-2 epithelial cell line. Aggregative and diffuse adherence patterns were found to be the most prevalent.
Conclusions. CTX-M-type enzymes were the most prevalent ESBLs among the strains studied. As many as 40% of the diarrheagenic *E. coli* isolates were found to belong to phylogenetic group D, which usually comprises *E. coli* strains associated with extraintestinal infections. The effectiveness of tigecycline against ESBL-producing *E. coli* strains was similar to that of imipenem and meropenem (**Adv Clin Exp Med 2012, 21, 2, 187–192**).

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

Streszczenie

Wprowadzenie. *Escherichia coli* pozostaje nadal głównym bakteryjnym patogenem biegunek u dzieci, stanowiąc istotny problem zdrowotny, zwłaszcza w krajach rozwijających się. Szczepy *E. coli* wywołujące biegunki często wykazują oporność na większość β-laktamów, wynikającą z wytwarzania β-laktamaz o rozszerzonym spektrum substratowym (ESBL).

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Cel pracy. Przedmiotem badań było 30 szczepów *E. coli* wytwarzających ESBL, kolonizujących przewód pokarmowy dzieci z ostrą biegunką. Celem badań było oznaczenie wrażliwości na leki przeciwbakteryjne, typu adhezji do linii komórkowej HEp-2 oraz pochodzenia filogenetycznego.
Materiał i metody. Wytwarzanie ESBL wykrywano testem synergizmu dwóch krążków (DDST). Minimalne stężenia hamujące (MIC) leków przeciwbakteryjnych oznaczono metodą seryjnych rozcieńczeń w podłożu agarowym Mueller-Hintona. Występowanie genów bla_{TEM}, bla_{SHV} i bla_{CTX-M} w badanych szczepach oznaczono metodą PCR.
Wyniki. Badane szczepy odznaczały się typowymi dla producentów ESBL wzorcami oporności. Większość z nich (20 spośród 30) wytwarzała enzymy ESBL typu CTX-M, warunkujące wysoki poziom oporności na oksyimino-β--laktamy, a zwłaszcza cefotaksym i ceftriakson. W wielu przypadkach badane szczepy wykazywały oporność na inne niż β-laktamy leki przeciwbakteryjne, takie jak: gentamycyna, amikacyna, kotrimoksazol i tetracyklina. Ponadto wszystkie szczepy cechowały się wrażliwością na karbapenemy, oksyimino-β-laktamy skojarzone z kwasem klawulanowym oraz tigecyklinę. Badane szczepy *E. coli* należały do 4 grup filogenetycznych: A, B1, B2 i D. Przeprowadzone *in vitro* badania nad adhezją wykazały, że wszystkie szczepy *E. coli*, z wyjątkiem dwóch, adherowały do nabłonkowej linii komórkowej Hep-2. Dominującymi wzorami adhezji były adhezja typu agregacyjnego i rozproszonego.
Wnioski. Wśród badanych szczepów enzymy CTX-M stanowiły najczęstszy typ ESBL. Aż 40% biegunkowych izolatów *E. coli* należało do filogenetycznej grupy D zawierającej zazwyczaj szczepy *E. coli* związane z zakażeniami pozajelitowymi. Skuteczność tigecykliny wobec szczepów *E. coli* wytwarzających ESBL była zbliżona do aktywności imipenemu i meropenemu (**Adv Clin Exp Med 2012, 21, 2, 187–192**).

Słowa kluczowe: *E. coli*, ESBL, oporność na leki przeciwbakteryjne, adhezja, grupy filogenetyczne.

Escherichia coli remains the leading bacterial cause of diarrhea in children, particularly in developing countries [1–4]. In contrast to their commensal counterparts, diarrheagenic *E. coli* (DEC) strains possess an arsenal of distinct virulence factors, such as adhesins, enterotoxins and hemagglutinins, which specifically contribute to their ability to cause diarrheal disease. Bacterial adherence to intestinal mucosa due to fimbrial and non-fimbrial adhesins is considered the first step in the development of infection caused by many DEC pathotypes [5–7]. Moreover, multidrug-resistant DEC strains pose a serious clinical challenge in many hospital settings. The predominant mechanism of resistance to β-lactam antibiotics in *E. coli* is the production of plasmid-borne extended-spectrum β-lactamases (ESBLs). Since the first report at the beginning of the 1980s [8], ESBL-producing organisms have become widespread throughout the world. ESBLs are known to confer resistance to most β-lactams, with the exception of cephamycin and carbapenems, but remain susceptible to β-lactam inhibitors [9]. Additionally, ESBLs exhibit a high degree of diversity and are classified into several families, of which CTX-M enzymes (so-called cefotaximases) are now the most prevalent ESBLs in many parts of the world [10, 11]. ESBL-producing *E. coli* often display resistance to non-β-lactam antibiotics and chemotherapeutics. This results from the fact that genes coding for ESBLs and those conferring resistance to other antimicrobial drugs often reside within the same conjugative plasmids. As the authors noted in a previous publication, the conjugational transfer of plasmid-mediated ESBLs occurs efficiently in the intestinal tract, where enteric rods, in particular *E. coli*, often act as a reservoir of self transmissible resistance markers that can be exchanged between species of the *Enterobacteriaceae* family [12].

The aim of the present study was to determine the antimicrobial susceptibilities of ESBL-producing *E. coli* strains colonizing the gastrointestinal tracts of children with acute diarrhea. In addition, the adherence patterns to the Hep-2 cell line and the phylogenetic background of the strains were investigated.

Material and Methods

Bacterial Strains

Thirty ESBL-producing *Escherichia coli* strains isolated from children (age range 1–5 years, mean age 2.2 years) with acute diarrhea, hospitalized in the Wroclaw Medical University Pediatric Hospital (Wrocław, Poland) were included in the study. The isolates were collected from stool samples during a two-year period (2008–2009) and were nonrepetetive. Species identification of the strains was done by the ATB automated identification system (bioMérieux, France) using the ID 32 E test according to the manufacturer's instructions.

Susceptibility Testing and Detection of ESBLs

The minimal inhibitory concentration (MIC) values of the antimicrobial agents selected for the study was determined by an agar dilution technique on Mueller-Hinton agar (Oxoid) according to the Clinical and Laboratory Standards Institute recommendations [13]. The MICs of oxyimino-β-lactams (aztreonam, cefotaxime, ceftazidime and ceftriaxone) were determined alone and in clavu-

lanic acid, at a fixed concentration of 2 mg/l. The inoculum was 10^4 colony-forming units (CFU) per spot deposited on the Mueller-Hinton agar. *E. coli* strains ATCC 25922 and ATCC 35218 were used as the quality reference strains. The standard antimicrobial powders tested were obtained from the following suppliers: aztreonam (Bristol-Myers Squibb), ceftazidime (Glaxo Wellcome), ceftriaxone (Hoffmann-La Roche Inc.), amikacin, cefotaxime, gentamicin, tetracycline (Sigma Chemical Co.), imipenem (Merck Sharp & Dohme Research), meropenem (Zeneca), clavulanic acid (GlaxoSmithKline Pharma), co-trimoxazole (trimethoprim/sulfamethoxazole, Polfa Tarchomin), tigecycline (Wyeth). ESBL production was determined by the double disk synergy test (DDST) according to Jarlier et al. [14]. The test was performed by placing disks of ceftazidime, cefotaxime and aztreonam (30 μg each) at a distance of 20 mm (from center to center) from a disk containing amoxicillin with 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. *Klebsiella pneumoniae* ATCC 700603 was used as the positive control, and *E. coli* ATCC 25922 as the negative control.

Plasmid DNA Preparation

Plasmid DNA was extracted from the strains studied by the alkaline method, using the Qiagen Plasmid Mini Kit (Qiagen) according to the manufacturer's instructions.

PCR Amplification of bla_{TEM}, bla_{SHV} and bla_{CTX-M} Determinants

Plasmid preparations from the isolates tested were used as templates for bla_{TEM}, bla_{SHV}, and bla_{CTX-M} gene amplification. The oligonucleotide primers used for the polymerase chain reaction (PCR) assays were as follows: TEM-A and TEM-B, specific for bla_{TEM} were 5'–ATAAAATTCTTGAAGACGAAA–3' and 5'–GACAGTTACCAATGCTTAATCA–3', respectively [15]. SHV-A and SHV-B, specific for bla_{SHV} were 5'–ACTGAATGAGGCGCTTCC–3' and 5'–ATCCCGCAGATAAATCACC–3', respectively [16]. P1C and P2D, specific for bla_{CTX-M}, were 5'–TCGTCTCTTCCAG–3' and 5'–CAGCGCTTTTGCCGTCTAAG–3', respectively [17]. PCR conditions for bla_{TEM} gene amplification were: 3 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 42°C, 1 min at 72°C, and finally 3 min at 72°C. PCR conditions for bla_{SHV}, and bla_{CTX-M} gene amplification were: 3 min at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, 30 s at 72°C, and finally 3 min at 72°C. PCR reactions were carried out in a T3 thermocycler (Biometra GmbH, Gottingen, Germany).

Adherence Assay

Adherence to the HEp-2 cell line was measured as described by Cravioto et al. [18]. Cells were grown to a nearly confluent monolayer in a chamber slide at 37°C in a 5% CO_2 atmosphere in minimal essential medium supplemented with fetal bovine serum (10% v/v) and antibiotic-antimycotic solution (penicillin 0.1 U/ml, streptomycin 0.1 U/ml, amphotericin B 0.25 mg/l). For the adherence assay the culture medium was replaced with minimum essential medium supplemented with fetal bovine serum (1% v/v) and methyl α-D-mannopyranoside (0.5% v/v) without antibiotics. Cultures of the strains tested (25 μl), grown overnight in Luria broth, were added to the cells and then incubated for 6 hours at 37°C in a 5% CO_2 atmosphere.

After 3 hours of incubation the cells were washed, fresh medium was added and the cells were incubated for a further 3 hours. The slides were then washed three times with phosphate buffered saline, fixed with methanol (70% v/v), and stained with Giemsa (10% v/v). The assay was repeated twice or three times for each strain.

Phylogenetic Group Distribution

The *E. coli* isolates were assigned to one of the four main phylogenetic groups: A, B1, B2 and D, according to the combination of three genetic determinants: *chuA*, *yjaA*, and the DNA fragment TSPE4.C2 as described by Clermont et al. [19]. The amplification conditions were as follows: Each step consisted of 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, and there was a final extension step of 7 min at 72°C. Thirty cycles were performed for the amplification.

Results and Discussion

Susceptibility of ESBL-Producing *E. coli* to Antimicrobial Agents

The *E. coli* isolates studied displayed resistance patterns typical of ESBL producers. As the authors previously reported [12, 20, 21] the majority of the isolates studied (24 out of 30) were resistant to cefotaxime (MIC range: 512 to 1024 mg/l), ceftriaxone (25 out of 30, MIC range: 512 to 1024 mg/l) and aztreonam (21 out of 30, MIC

range: 32 to 1024 mg/l). On the other hand, resistance to ceftazidime (MIC range: 32 to 256 mg/l) was observed in 13 out of 30 isolates. Moreover, all the strains were susceptible to imipenem, meropenem (MIC: < 1 mg/l) and oxyimino-β-lactams (ceftazidime, cefotaxime, ceftriaxone, and aztreonam) in combination with clavulanic acid (MIC range: < 1 to 4 mg/l). It is worth noting that the majority of the isolates were also resistant to non-β-lactam antimicrobial agents. All but three of the strains showed resistance to co-trimoxazole (MIC: > 1024 mg/l). In addition, resistance to tetracycline (MIC range: 128 to 512 mg/l), amikacin (MIC range: 1024 to > 1024 mg/l) and gentamicin (MIC range: 32 to > 1024 mg/l) was observed in 17, 15 and 22 strains, respectively. In contrast, the strains were uniformly susceptible to tigecycline (MIC: < 1 mg/l). These results confirm the high activity of tigecycline against ESBL-producing isolates and are in accordance with those previously reported by other authors [22, 23] As previously reported [12, 24], this new semisynthetic antimicrobial, belonging to the glycylcyclines, demonstrates excellent activity against a wide variety of Gram-positive and Gram-negative bacteria, including ESBL-producing organisms. For this reason, tigecycline could be considered an encouraging antimicrobial for the treatment of infections involving these microorganisms. However, this antibiotic is not recommended in adolescents under 18 years of age due to lack data on its safety, and should not be used in children under 8 years of age because of tooth discoloration.

Detection of the *bla*$_{\text{CTX-M}}$ Gene

As the authors noted in earlier articles [12, 20], the ESBL-producing *E. coli* strains studied demonstrated significantly higher MIC values of cefotaxime and ceftriaxone (MIC range: 512 to1024 mg/l) than those of ceftazidime (MIC range: 32 to 256 mg/l). These findings indicate that this resistance may result from CTX-M-type ESBL activity. To investigate this possibility, PCR was performed with P1C and P2D primers specific for CTX-M enzymes. As expected, the *bla*$_{\text{CTX-M}}$ determinant was detected in 23 of 30 ESBL-positive isolates. The remaining strains were found to possess *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ determinants (4 isolates and 3 isolates, respectively). As the authors previously pointed out [12], CTX-M-type β-lactamases emerged in the late 1980s, shortly after the introduction of cefotaxime in clinical practice. Global expansion of the enzymes, however, was observed in the mid 1990s. CTX-M β-lactamases have been derived from the chromosomally encoded enzymes of *Kluyvera* spp. [25]. In general, these enzymes preferentially hydrolyze cefotaxime and ceftriaxone but their activity against ceftazidime and aztreonam is usually lower. Nowadays, CTX-M-type β-lactamases are the most prevalent ESBLs in *Enterobacteriaceae* in many geographical areas [10, 11, 26]. As previously noted [12, 20, 21], the studied strains' resistance to gentamicin, amikacin, tetracycline and co-trimoxazole may be determined by genes localized within the same multi-drug resistance plasmids that can be horizontally transferred from one species to another. Therefore, such multiresistant ESBL-producing organisms constitute a serious therapeutic problem and might be selected by various non-β-lactam drugs. Further conjugational experiments should be performed to confirm plasmid-borne resistance to non-β-lactam antimicrobials.

Phylogenetic Group Distribution

As mentioned above, *E. coli* strains can be assigned to one of the four main phylogenetic groups (A, B1, B2 or D) based on the combination of three genetic markers: *chuA*, *yjaA* and the DNA fragment TSPE4.C2 [19]. The strains derived from the B2 and D phylogroups have been shown to express more virulence factors than those from the phylogroups A and B1 [27]. In addition, pathogenic *E. coli* isolates assigned to B2 and D groups are usually associated with extraintestinal infections, whereas the majority of commensal strains belong to A and B1 phylogenetic groups [19, 28]. Previous studies have shown that diarrheagenic *E. coli* isolates may be distributed among all four of these phylogenetic groups, but the strains from group A are the most commonly found [3, 4]. In the present study, however, the largest number of *E. coli* strains (12 out of 30) were assigned to phylogroup D, followed by groups B1 (8 isolates), B2 (7 isolates) and A (3 isolates) (Table 1). Furthermore, the majority of the CTX-M-producing strains studied (16 out of 23) were found to belong to groups D and B2 (9 and 7 strains, respectively). These results appear to be in agreement with those reported by Pitout et al. [29].

Adherence Patterns

Bacterial adhesion to intestinal mucosa is considered the first step in the development of infections caused by many *E. coli* strains leading to diarrhea. Diarrheagenic *E. coli* strains are known to display three distinct patterns of adherence to epithelial cell lines, namely: localized adherence (LA), diffuse adherence (DA) and aggregative adherence (AA) [30]. In contrast to adhering isolates, so-called cell-detaching (CD) *E. coli* strains have been found to be capable of detaching epithelial cells from glass surfaces in adherence assays [31].

Table 1. ESBL-positive *Escherichia coli* strains' (n = 30) phylogenetic background and adherence patterns to the HEp-2 cell line

Tabela 1. Pochodzenie filogenetyczne i typy adhezji do linii komórkowej HEp-2 ESBL-dodatnich szczepów *Escherichia coli* (n = 30)

Type of ESBLs (Typ ESBL)	Number of isolates belonging to phylogenetic groups (Liczba izolatów należących do grup filogenetycznych)				Number of isolates displaying adherence patterns (Liczba izolatów wykazujących typy adhezji)				
	A	B1	B2	D	AA*	DA	CD	UD	NA
CTX-M (n = 23)	1	6	7	9	5	8	2	6	2
TEM (n = 4)	2	–	–	2	3		–	–	–
SHV (n = 3)	–	2	–	1	2	1	–	1	–
Total n = 30 (Ogółem n = 30)	3	8	7	12	10	9	2	7	2

* Adherence patterns: AA – aggregative, DA – diffuse, UD – undefined, CD – cell-detaching, NA – non-adherent.

* Typy adhezji: AA – agregacyjny, DA – rozsiany, UD – nieokreślony, CD – odrywający komórki, NA – niezdolny do adhezji.

The *in vitro* adhesion assay revealed that all but two of *E. coli* strains adhered to the HEp-2 epithelial cell line (Table 1). Aggregative adherence was the most prevalent adherence pattern, observed in 10 out of 30 isolates, followed by diffuse adherence (9 strains). The cell-detaching adherence (CD) pattern was observed in two strains only. On the other hand, out of the 30 isolates studied, 7 exhibited undefined adherence (UD), in which adhering bacterial cells do not form any characteristic pattern.

In conclusion, as most *E. coli* isolates examined demonstrated the ability to adhere to epithelial cells, there is a probability that these strains can also colonize the intestinal epithelium. For that reason, colonized children may become a potential source of multidrug ESBL-producing *E. coli* strains in the hospital environment.

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