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Occurrence of Extended-Spectrum β-Lactamases among *Escherichia coli* Isolates from Hospitalized and Healthy Children

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ABSTRACT. The prevalence of extended-spectrum β-lactamases (ESBL) was determined among isolates of *Escherichia coli* (n = 63) isolated from hospitalized (43) and healthy (20) children. Ten isolates (21 %) were ESBL-positive for two screening tests, the double disk-synergy test and the *Oxoid* Combination Disk method. One ESBL-positive isolate came from a healthy child. The transfer frequency of oxyimino-β-lactam resistance from ESBL-producing isolates to *E. coli* K12 C600 recipient strain ranged from 10^{-8} to 10^{-5} per donor cell. Donor strains and transconjugants displayed susceptibility patterns typical of ESBL producers. They were resistant to oxyimino-β-lactams but susceptible to clavulanic acid and carbapenems. Seven out of the 10 ESBL-positive isolates were found to produce MR/MS fimbria, which may play an important role in the colonization of the human intestinal mucosa.

Abbreviations

	CFU	colony forming unit				MS	mannose-sensitive (fimbria)					
	ESBL	extended-spectrum β-lactamases				OBLR	oxyimino-β-lactam resistance					
	DDST	double disk-synergy test				OCDM	*Oxoid* Combination Disk method					
	MR	mannose-resistant (fimbria)										
Antibiotics	Amc	amoxicillin	Azt	aztreonam	Cef	cefotaxime		Cfz	ceftazidime	Cpd	cefpodoxime	
	Ctx	ceftriaxone	Imi	imipenem	Mer	meropenem		Pip	piperacillin	Nal	nalidixic acid	
	Cla	clavulanate										

Pathogenic strains of *Escherichia coli* remain a significant cause of diarrhea. Adhesion to the intestinal mucosa as well as the production of hemolysins and toxins by *E. coli* strains are an important factor in their pathogenesis (*see*, *e.g.*, Liptáková *et al.* 2002). Genes encoding many of these virulence factors and antibiotic resistance are very often localized on plasmids and transposons which can be horizontally transferred from one species to another, facilitating spreading of virulent strains among hospitalized patients (Hacker 1992; Nataro *et al.* 1998; Bogyiová *et al.* 2002).

The extensive clinical utilization of oxyimino-β-lactams (ceftazidime, cefotaxime, aztreonam) at the beginning of the eighties has been responsible for the appearance of an increasing number of resistant Gram-negative bacilli (Jacoby *et al*. 1991; Tejedor-Junco *et al*. 1998; Bujdáková *et al.* 2001). The resistance is usually due to the expression of plasmid-born extended-spectrum β-lactamases. The first ESBL-producing isolates were discovered in Germany in 1983 (Knothe *et al*. 1983). Since then, they have been reported worldwide as etiologic factors of many severe and life-threading infections. The enzymes efficiently hydrolyze broad-spectrum β-lactam drugs, including the third-generation cephalosporins and monobactams, but are not active against cefamycins and β-lactam inhibitors (Livermore 1995; Medeiros 1997). ESBL predominantly occur in *Klebsiella* spp. and *Escherichia coli* strains but recently the enzymes were found in other genera of the family *Enterobacteriaceae* as well as in nonfermentative Gram-negative bacteria (Vahaboglu *et al*. 2001).

ESBL-producing bacterial strains exhibiting a wide multidrug resistance are considered as alert pathogens, particularly as very important agents responsible for nosocomial infections. The prevalence of OBLR due to ESBL production varies significantly with different geographical areas as well as in a particular hospital. The investigations carried out in Western Europe showed that 14–16 % of clinical isolates of *K. pneumoniae* expressed ESBL phenotype (Livermore *et al*. 1996). On the other hand, avery low occur-

rence of ESBL positive *E. coli* and *K. pneumoniae* strains was reported in Dutch hospitals (less than 1 %) (Stobberingh *et al*. 1999).

Here we determined the occurrence of ESBL-producing *E. coli* among strains isolated from hospitalized children with diarrhea and from healthy children. Additionally, antibiotic susceptibility, transfer of OBLR and MS and MR fimbria production were studied.

MATERIAL AND METHODS

Bacterial strains. A total of 63 isolates of *Escherichia coli* were examined; 43 were isolated from children with diarrhea hospitalized in the *Pediatric Medical University Hospital* (Wrocław, Poland) and 20 came from healthy children at the age from 2 weeks to 3 years as a control group. The isolates were recovered from stool samples and were nonrepetetive (one strain from one child). Species identification of the strains was done by the ATB automated identification system (*bioMérieux*, France). All examined strains were serotyped by agglutination test with antisera to enteropathogenic *E. coli*.

Antibiotic susceptibility testing. The MIC of β-lactam antibiotics were determined by an agar dilution technique on Mueller–Hinton agar (*Oxoid*) according to the *NCCLS* (2001) recommendations. MIC of β-lactams were determined alone or in a fixed concentration of clavulanic acid (2 mg/L). The inoculum was 10^4 CFU per spot deposited on the Mueller–Hinton agar. MIC were read after a 18-h incubation at 35 °C; *E. coli* strains ATCC 25922 and ATCC 35218 were used as the quality reference strains. Standard powders of β-lactam antibiotics were obtained from the following suppliers: aztreonam (*Bristol-Myers Squibb*); ceftazidime (*Glaxo Wellcome*); ceftriaxone (*Hoffmann-La Roche Inc*.); cefotaxime (*Sigma Chemical Co.*); imipenem (*Merck Sharp & Dohme Research*); meropenem (*Zeneca*); lithium clavulanate (*GlaxoSmithKline Pharma*); piperacillin (*Polfa Tarchomin*).

ESBL production was detected by two methods – the double disk-synergy test according to Jarlier *et al*. (1987) and the *Oxoid* Combination Disk method. DDST was performed by placing disks of Cfz, Cef and Azt (30 μg each) at distances of 25 and 30 mm (center to center) from a disk containing Amc + Cla (20 and 10 μg, respectively). The strains that showed synergy between oxyimino-β-lactams and Cla were considered to produce ESBL enzymes. The OCDM depends on comparing the zones given by Cpd (10 μg) and Cpd + Cla (10 and 1 μg, respectively) disks. ESBL production is inferred if the zones given by the disks with Cla are 5 mm larger than those without the inhibitor (Carter *et al*. 2000).

Transfer of oxyimino-β-lactam resistance. Conjugational transfer of OBLR was performed using the mixed broth method. *E. coli* K12 C600, which is resistant to Nal and susceptible to all β-lactam antibiotics, was used as recipient strain. Equal volumes (1 mL) of cultures of the donor and the recipient strains (1/pL, *i.e*. 10^9 CFU per mL) grown in nutrient broth (*Difco*) were mixed and incubated for 1 d at 37 °C. Transconjugants were selected on MacConkey agar (*Biomed*) supplemented with Nal (64 mg/L) (*Chinoin*, Hungary), to inhibit the growth of donor strains, and Cfz (4 mg/L), to inhibit the growth of recipient strain. Transfer frequency of OBLR was expressed as a number of transconjugants relative to the number of donor CFU after the mating period.

Hemagglutination test. MS and MR fimbria were detected in a hemagglutination test according to Duguid *et al*. (1987) with 3 % erythrocytes: human group A, bovine, horse, sheep, goat and rabbit in the presence of 2 % methyl β-D-mannopyranoside (*Sigma*). The presence of MR hemagglutination was confirmed by heating tested strains at 65 °C for ½ h. MR hemagglutination disappeared after heating.

Table I. Transfer frequency (TF) of oxyimino-β-lactam resistance from ESBL producers to *E. coli* K12 C600 recipient strain

EC[a]	TF, × 10^{-8}
6/1	180
7/1	7400
9/1	1000
12/1	32
25/2	310
33/1	8
36/1	12
48/2	8
60/1	–

[a]Numbering of donor strains.

RESULTS AND DISCUSSION

Ten out of the 63 *E. coli* isolates from hospitalized and healthy children were found to be ESBL-positive, resulting in an overall prevalence of 15.9 %. Among 43 clinical isolates 9 (21 %) were ESBL-positive. It is surprising that one strain expressing ESBL activity (*E. coli* 60/1) was isolated from a healthy child. We also demonstrated that the results of the OCDM were in agreement with those obtained by DDST.

Nine out of the 10 ESBL-positive isolates were subjected to conjugation experiments. The remaining strain (*E coli* 17/1) was excluded because of its resistance to Nal. All ESBL-positive *E. coli* strains

except one (strain 60/1) used as donors in mating experiments transferred OBLR to *E. coli* K12 C600 with a frequency of 10^{-8}–10^{-5} per donor cell (Table I). All transconjugants displayed ESBL expression which was confirmed by the conventional DDST as well as the OCDM.

All of the non-ESBL-producing isolates (n = 53) were significantly susceptible to all antibiotics used (MIC < 250 μg/L; *data not shown*). The antibiotic susceptibilities of ten ESBL-producers are presented in Table II. The ESBL-positive strains were resistant to at least one of the oxyimino-β-lactam antibiotics tested. Five of ten ESBL-positive isolates displayed resistance to all third-generation cephalosporins and Azt used. MIC values of Cef and Ctx (in most cases 256–1024 mg/L) were higher than those of Cfz (2–32 mg/L) and Azt (16–128 mg/L). The activity of oxyimino-β-lactams was efficiently restored by Cla (MIC < 250–500 μg/L) suggesting ESBL production. ESBL-expressing isolates were uniformly resistant to Pip (MIC from 256 to >1024 mg/L) but susceptible to Imi and Mer (MIC < 250 μg/L). Our findings support previous suggestions that carbapenems are stable in use against ESBL (Livermore 1995). The antimicrobial susceptibility patterns for transconjugants were similar to those obtained for donor strains (Table III).

Table II. MIC (mg/L) of β-lactam antibiotics alone and in combination with clavulanic acid for ESBL-positive *E. coli* isolates (donors)

Antibiotic(s)[a]	6/1[b]	7/1[b]	9/1[b]	12/1[b]	17/1[b]	25/2[b]	33/1[b]	36/1[b]	48/2[b]	60/1[b]
Cfz	32	32	32	2	16	32	8	8	8	8
Cfz + Cla	0.5	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Cef	512	512	1024	512	128	2	256	64	1	256
Cef + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Ctx	512	1024	1024	1024	512	4	512	128	1	512
Ctx + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Azt	128	128	128	128	16	128	64	32	32	32
Azt + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Pip	1024	1024	1024	1024	1024	1024	>1024	1024	256	1024
Mer	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Imi	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

[a]Azt – aztreonam, Cef – cefotaxime, Cfz – ceftazidime, Ctx – ceftriaxone; Imi – imipenem, Mer – meropenem, Pip – piperacillin; Cla – clavulanic acid at a fixed concentration of 2 mg/L.
[b]Numbering of *E. coli* donor strains.

Table III. MIC (mg/L) of β-lactam antibiotics alone and in combination with Cla for transconjugants[a]

Antibiotic(s)[b]	T 6/1	T 7/1	T 9/1	T 12/1	T 25/2	T 33/1	T 36/1	T 48/2
Cfz	16	16	32	32	128	32	32	32
Cfz + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	0.5	0.5	<0.25
Cef	512	256	256	256	16	256	256	2
Cef + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Ctx	512	256	256	512	8	256	512	2
Ctx + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Azt	128	64	128	64	256	128	1024	64
Azt + Cla	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	0.5	<0.25
Pip	1024	512	1024	1024	256	>1024	256	64
Mer	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25
Imi	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25	<0.25

[a]Obtained in mating between *E. coli* 6/1 donor strain and recipient; numbering *see* Table II. [b]*See* footnote *a* in Table II.

The bacterial adherence to epithelial cell surfaces due to MR/MS fimbria is an important step in the colonization process (Hacker 1992; Majtán and Majtánová 2001; Waldon *et al.* 2002). The majority of ESBL-positive isolates (7 out of 10) showed the presence of MR/MS fimbria agglutinating at least one of the erythrocyte types tested (Table IV). These fimbria may be important virulence determinants involved in the colonization of human intestinal mucosa by resistant strains of *E. coli*. Three ESBL-positive strains – *E. coli*

12/1, 33/1 and 36/1 – belonged to enteropathogenic *E. coli* ("EPEC" strains) and represented O25, O26 and O127 serogroups, respectively.

Table IV. The presence of mannose-sensitive (MS) and mannose-resistant (MR) fimbria among ESBL-producing strains of *E. coli*

Group of children	Numbering of strains	Serogroup[a]	Erythrocytes[b]					
			human	bovine	horse	goat	sheep	rabbit
With diarrhea	6/1	np	MS	–	MS	–	MS	MS
	7/1	np	–	–	–	–	–	–
	9/1	np	MS	–	MS	-	–	MS
	12/1	O127	–	–	MS	MS, MR	MR	MS, MR
	17/1	np	–	–	–	–	–	–
	25/2	np	–	–	–	–	–	–
	33/1	O25	MS, MR	–	–	–	–	MS
	36/1	O26	MS, MR	–	MS	–	MS, MR	MS, MR
	48/2	np	MS, MR	MS, MR	MS, MR	MS, MR	MR	MS
Healthy	60/1	np	MS, MR	–	MS	MR	MR	MS

[a]np – nonpathogenic strain. [b]*See Materials and Methods.*

It is remarkable that *E. coli* strain isolated from a healthy child (isolate 60/1), apart from ESBL production, was demonstrated to be also MR-fimbriated. Probably the child carrying this strain, without any symptoms of infection, may be regarded as a potential source of this virulent *E. coli* strain. We found that there is a probability of transmission of this pathogen in child's environment. The linkage between ESBL production and colonization factor can also contribute to the persistence of resistant *E. coli* strains *in vivo*, even in the absence of antibiotic therapy selection pressure.

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