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## Carriage of genes for various extended-spectrum $\beta$ -lactamases: a novel resistance strategy of *Klebsiella pneumoniae* in Poland

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## ABSTRACT

Among 110 randomly sampled strains from a collection of 247 extended-spectrum  $\beta$ -lactamase (ESBL)-producing clinical isolates of *Klebsiella pneumoniae* collected from hospitalised children in three paediatric hospitals in Poland, 64 strains (58.2%) with multiple ESBLs were found, including five non-clonal strains (4.5%) harbouring *bla* genes for ESBLs of three families (CTX-M, SHV and TEM). This is the first report of the emergence of triple ESBL-producing *K. pneumoniae* in Poland. In addition, *K. pneumoniae* strains harbouring *bla* genes for TEM-130 and TEM-132 ESBLs were detected in Poland for the first time. Epidemiological analysis of the multiple ESBL-producing *K. pneumoniae* isolates by pulsed-field gel electrophoresis (PFGE) revealed a relatively high genetic diversity between isolates producing the same combination of enzymes. Clonally related strains were uncommon.

## 1. Introduction

Extended-spectrum  $\beta$ -lactamases (ESBLs) are the predominant resistance agents to oxyimino- $\beta$ -lactams in clinical isolates of Gram-negative bacteria. In general, ESBLs are capable of hydrolysing penicillins, broad-spectrum cephalosporins and aztreonam. Owing to their rapid dissemination and diversity, ESBLs are an increasing problem worldwide [1]. Genes encoding ESBLs usually map on mobile genetic elements within large plasmids, which often spread horizontally. Initially, resistance to oxyimino-cephalosporins in Gram-negative rods was mainly due to dissemination of TEM- and SHV-type ESBLs. During the last 10 years, CTX-M-type ESBLs have become predominant [2]. ESBLs are frequently reported to be co-produced with penicillinases in clinical isolates of Enterobacteriaceae. *Klebsiella pneumoniae* strains producing multiple ESBLs were isolated for the first time in Chicago in 1990 and 1991. The strains were found to produce two different ESBLs of the same family (TEM-12 and TEM-10) in a single isolate [3]. Later, *K. pneumoniae* isolates producing multiple ESBLs of different families (TEM and SHV) were collected in Durban, South Africa [4]. Strains harbouring *bla* genes encoding for ESBLs of two or three different families emerged soon after [5]. In Poland, *K. pneumoniae* isolates simultaneously expressing CTX-M-3 and SHV- or TEM-type ESBLs were reported in 2002 [6]. The aim of this study was to determine the prevalence of multiple ESBL-producing *K. pneumoniae* isolates in three selected paediatric hospitals in Poland. Here we report 64 *K. pneumoniae* clinical isolates carrying *bla* genes for simultaneous expression of two or three different ESBLs.

## 2. Materials and methods

### 2.1. Bacterial strains

A total of 110 strains was randomly selected from a collection of 247 ESBL-producing *K. pneumoniae* isolates recovered from various clinical specimens from children hospitalised during 2000–2007 in the reference hospital Children's Memorial Health Institute (CMHI) in Warsaw ( $n = 137$ ), the neonatology ward in Poznań Medical University Hospital (PMUH) ( $n = 61$ ) and the Clinic of Paediatrics, Haematology and Oncology in the Warsaw Medical University Hospital (WMUH) ( $n = 49$ ). All selected strains were confirmed as ESBL-producers by the double-disk synergy test using a combination of cefotaxime, ceftazidime and amoxicillin/clavulanic acid [7].

### 2.2. Polymerase chain reaction (PCR) screening for extended-spectrum $\beta$ -lactamase genes and DNA sequencing

The conserved core regions of the *bla*<sub>CTX-M</sub>, *bla*<sub>SHV</sub> and *bla*<sub>TEM</sub> genes, which represent the most commonly reported ESBL families in Poland, were amplified by PCR [8] to identify isolates genetically competent to produce  $\beta$ -lactamases of different families simultaneously. Further, the exact ESBL genotypes were determined by DNA sequencing. Briefly, the core regions of the *bla* genes and the overlapping upstream and downstream gene fragments were amplified by PCR using the primers listed in Table 1. Both DNA strands of the PCR amplicons were sequenced using an automated fluorescent DNA sequencer ABI PRISM 310 (Applied Biosystems, Foster City, CA) and a BigDye<sup>®</sup> Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) in accordance with the manufacturer's

instructions. Primers for PCR and DNA sequencing were the same. FinchTV V 1.4 and CLC Free Workbench V 6.0 software were used for processing of the sequencing data and to assemble the complete gene sequences.

### 2.3. Antimicrobial susceptibility testing

Minimum inhibitory concentrations of cefotaxime, ceftazidime, cefepime, piperacillin/tazobactam, ciprofloxacin, imipenem, meropenem and ertapenem were determined for each multiple ESBL-producing isolate by the agar dilution method on Iso-Sensitest agar [9]. Quality control was performed using strains *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC 70060.

### 2.4. Pulsed-field gel electrophoresis (PFGE)

Isolates revealing a multiple ESBL genotype were subjected to PFGE typing with a CHEF DR II System (Bio-Rad, Hercules, CA). Briefly, agarose-embedded genomic DNA was digested with *Xba*I (Fermentas, Vilnius, Lithuania) and digestion products were separated in 1% w/v agarose gel (Pulsed Field Certified Agarose; Bio-Rad) in TBE buffer [45 mM Tris-base, 45 mM boric acid, 1 mM ethylene diamine tetra-acetic acid (EDTA), pH 8.0]. Electrophoresis was carried out at 6 V/cm at 14 °C for 10 h with a switch time of 5 s to 15 s, followed by 10 h with a switch time of 15 s to 45 s using a CHEF DR II System.

A dendrogram illustrating the genetic similarity of the multiple ESBL-producing isolates was generated using the Molecular Analyst Program (Bio-Rad).



### 3. Results and discussion

Among the 110 tested strains of *K. pneumoniae*, 46 carried a single *bla* gene encoding for ESBL, whilst 64 strains harboured *bla* genes for at least two ESBLs of different families (Table 2). The majority of isolates producing a single ESBL harboured the *bla*<sub>CTX-M-3</sub> gene (39/46) and the remaining isolates carried the following genes: *bla*<sub>SHV-5</sub> ( $n = 3$ ); *bla*<sub>CTX-M-15</sub> ( $n = 2$ ); *bla*<sub>TEM-47</sub> ( $n = 1$ ); and *bla*<sub>TEM-48</sub> ( $n = 1$ ). Fifty-nine isolates and five isolates, respectively, were found to carry two or three *bla* genes for different ESBL families.

The combination of *bla*<sub>CTX-M-3</sub> with *bla*<sub>TEM-48</sub> or *bla*<sub>SHV-5</sub> predominated in isolates producing two ESBLs (dual ESBLs). The less common *bla*<sub>CTX-M-15</sub> gene was found in combination with *bla*<sub>TEM-48</sub> or *bla*<sub>TEM-47</sub>, found in strains isolated from CMHI only. The *bla*<sub>TEM</sub> genes for TEM-130 and TEM-132 were detected in *K. pneumoniae* strains producing two ESBLs. Interestingly, these two TEM variants have not been reported in Poland. TEM-130 has not yet been fully described. Its amino acid sequence differs from TEM-129 by a single substitution (D35P) (<http://www.lahey.org/Studies/temtable.asp>). Therefore, in this study TEM-130 was considered as the ESBL. TEM-132, first reported in 2005, was originally found in neighbouring Slovakia [10]. Notably, 36 of the dual ESBL-producing *K. pneumoniae* isolates tested in this study were collected in CMHI, whereas only 23 were isolated from patients in the two regional hospitals PMUH and WMUH. All the isolates genetically competent to produce three ESBLs (triple ESBL genotype) were collected exclusively from CMHI patients. They had the following four genotypes: (i) *bla*<sub>CTX-M-3</sub>, *bla*<sub>TEM-47</sub> and *bla*<sub>SHV-5</sub>; (ii) *bla*<sub>CTX-M-3</sub>, *bla*<sub>TEM-48</sub> and *bla*<sub>SHV-5</sub>; (iii) *bla*<sub>CTX-M-3</sub>, *bla*<sub>TEM-86</sub> and *bla*<sub>SHV-5</sub>; and (iv) *bla*<sub>CTX-M-15</sub>, *bla*<sub>TEM-48</sub> and *bla*<sub>SHV-5</sub>. The prevalence of isolates

showing a multiple ESBL genotype in CMHI was higher (67.2%) than in PMUH (57.9%) and WMUH (40.0%). In contrast to the two regional hospitals, CMHI is the reference paediatric hospital to which serious cases from the whole of Poland are admitted. Before admission to CMHI, many patients had prolonged or multiple hospitalisation events in a primary-care hospital, often connected with one or two antibiotic therapies.

All the multiple ESBL-producing *K. pneumoniae* strains were susceptible to imipenem, meropenem and ertapenem, and some were susceptible to piperacillin/tazobactam (Table 2). These strains were also susceptible to ciprofloxacin, which is in contrast to reports by Paterson et al. [11] and Damjanova et al. [12] who found a close relationship between ESBL production and ciprofloxacin resistance in *K. pneumoniae*. This may be due to the strongly limited consumption of fluoroquinolones in paediatric hospitals.

It is noteworthy that the majority of the multiple ESBL genotypes observed in this study were composed of genes encoding for ESBL variants that are common in Poland [13,14]. This finding may argue for self-dependent acquisition of the most common genes encoding ESBL by *K. pneumoniae* clinical isolates. It was particularly interesting to determine whether *K. pneumoniae* isolates sharing the same multiple ESBL genotype are clonal. Therefore, all 110 isolates were subjected to PFGE. The majority of the multiple ESBL-producing isolates from CMHI were non-clonal with a genetic similarity <70% (Fig. 1). In contrast, the multiple ESBL-producing isolates from both local hospitals (PMUH and WMUH) were mostly clonal. Two clones covering six and three isolates and only two non-clonal ones were found in PMUH.

Similarly, five clones of two isolates each, together with only two non-clonal isolates, were detected among the multiple ESBL-producing isolates from WMUH. Findings from these two local hospitals may argue for an enhanced epidemic potential of *K. pneumoniae* harbouring multiple *bla* genes for ESBLs.

Multiple ESBL-producing *K. pneumoniae* appear to occur worldwide. Among bloodstream *K. pneumoniae* isolates collected in 12 hospitals in Argentina, Australia, Belgium, South Africa, Taiwan, Turkey and the USA, up to 19.2% were found to produce multiple ESBLs [5]. Isolates of such a phenotype may be even more common. Recently, it has been reported that 44% of ESBL-producing isolates of *E. coli*, *K. pneumoniae* and *Proteus mirabilis* harboured *bla* genes for multiple ESBLs [15].

In Poland, the last country-wide survey on ESBL-producing Enterobacteriaceae was conducted between November 2003 and January 2004 [13]. This survey showed that 1.5% of 264 isolates collected in regional secondary-care hospitals co-produced two different ESBLs (CTX-M-15 and SHV-5). No isolates capable of producing three different ESBLs were found. In contrast, the results of this study showed that 58.2% of ESBL-producing *K. pneumoniae* isolates revealed a multiple ESBLs genotype. This could be explained by differences in studied patients, bacterial species and the study period. The current study was focused on *K. pneumoniae*, which is known to develop novel resistance traits rapidly. The tested isolates were collected from patients hospitalised between 2000 and 2007, mostly from the reference hospital (CMHI). It is noteworthy that the majority of the multiple ESBL-producing isolates (82.8%) were collected after 2003. All the triple ESBL-producing isolates emerged in

CMHI after 2004. In light of this, it may be supposed that the accumulation of multiple *bla* genes for different ESBLs in *K. pneumoniae* is tending to increase in Poland. Further studies are therefore required to deepen our knowledge of mechanisms leading to the selection and dissemination of multiple ESBL-expressing isolates.

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### **Competing interests**

None declared.

### **Ethical approval**

Not required.

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**Fig. 1.** Dendrogram of the clonal relationship of multiple extended-spectrum  $\beta$ -lactamase (ESBL)-producing *Klebsiella pneumoniae* isolates from the reference hospital Children's Memorial Health Institute (CMHI) in Warsaw. The dendrogram was generated from pulsed-field gel electrophoresis (PFGE) patterns of *Xba*I-digested genomic DNA. Each isolate is characterised by a number (culture identifier) and the multiple ESBL genotype.

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**Table 1**

Nucleotide sequences of polymerase chain reaction (PCR) primers used in the study

Gene	Fragment	Forward (5'→3')	Reverse (5'→3')	Size (bp)	Reference
<i>bla</i> <sub>CTX-M</sub>	Upstream	CTTTCGGAAGCATAAAATCGG	CTCAGCCAGTGACATCGTCC	472	This study
<i>bla</i> <sub>CTX-M</sub>	Core	TGCAGTACCAGTAAGGTGATGG	CCAGATAACCGCGATATCGTTG	546	[8]
<i>bla</i> <sub>CTX-M</sub>	Downstream	TGGTGACATGGATGAAAGGC	GCACTTTTGCCGTCTAAGGC	311	This study
<i>bla</i> <sub>TEM</sub>	Upstream	CGCTCATGAGACAATAACC	AAGGATCTTCACCTAGATCC	379	This study
<i>bla</i> <sub>TEM</sub>	Core	AGTTCTGCTATGTGGTGCGG	GCTAGAGTAAGTAGTTCGCC	387	[8]
<i>bla</i> <sub>TEM</sub>	Downstream	AGCCATACCAAACGACGAGC	AAGGATCTTCACCTAGATCC	439	This study
<i>bla</i> <sub>SHV</sub>	Upstream	AAGAGGAATTGTGAATCAGC	GATCCTGCTGGCGATAGTGG	387	This study
<i>bla</i> <sub>SHV</sub>	Core	GAGCGAAAGATCCACTATCG	CCTCATTCAGTTCGGTTTCC	241	[8]
<i>bla</i> <sub>SHV</sub>	Downstream	GATCGGCGACAACGTCACC	TTAGCGTTGCCAGTGCTCGATC	412	This study

**Table 2**

Molecular characterisation of the *bla* genes among multiple extended spectrum  $\beta$ -lactamase (ESBL)-producing *Klebsiella pneumoniae* ( $n = 64$ ) and activity of selected antibiotics against tested isolates

Genotype (no. of isolates)	No. of isolates in hospital			MIC (mg/L)							
	CMHI	PMUH	WMUH	CTX	CAZ	FEP	PIP/TAZ	CIP	ETP	IPM	MEM
CTX-M-3, TEM-47 ( $n = 8$ )	5	3	0	32–256	2–256	4–128	4–128	0.015–0.5	0.015–0.25	0.12	0.03–0.06
CTX-M-3, TEM-48 ( $n = 22$ )	12	5	5	32–256	2–256	2–128	1–64	0.015–0.12	0.015–0.5	0.12	0.06
CTX-M-3, TEM-86 ( $n = 3$ )	3	0	0	16–256	2–128	2–16	1–32	0.5	0.12	0.12	0.06
CTX-M-3, TEM-130 ( $n = 2$ )	2	0	0	4–32	16–32	2–32	4–16	0.015–0.06	0.015–0.06	0.12	0.06
CTX-M-3, TEM-132 ( $n = 2$ )	2	0	0	8–32	4–16	4–8	4–128	0.03	0.06–0.25	0.06	0.03
CTX-M-15, TEM-47 ( $n = 3$ )	3	0	0	64–128	4–64	8–16	8–128	0.015–0.03	0.06–0.25	0.06–0.12	0.03–0.06
CTX-M-15, TEM-48 ( $n = 5$ )	5	0	0	128	32–128	16–32	32–64	0.06–0.12	0.25–0.5	0.12	0.03–0.06

CTX-M-3, SHV-5 ( <i>n</i> = 12)	4	3	5	16– 256	2–16	4–64	1–32	0.015– 0.25	0.015– 0.25	0.12	0.06
SHV-5 + TEM-48 ( <i>n</i> = 2)	0	0	2	32–64	2	2	0.5	0.06	0.015	0.12	0.06
CTX-M-3, TEM-47, SHV- 5 ( <i>n</i> = 1)	1	0	0	256	16	16	32	0.03	0.5	0.12	0.06
CTX-M-3, TEM-48, SHV- 5 ( <i>n</i> = 2)	2	0	0	64– 256	4–8	16	4–32	0.015– 0.06	0.03–0.5	0.12	0.06
CTX-M-3, TEM-86, SHV- 5 ( <i>n</i> = 1)	1	0	0	32	128	16	8	0.12	0.06	0.12	0.06
CTX-M-15, TEM-48, SHV-5 ( <i>n</i> = 1)	1	0	0	128	4	16	8	0.03	0.06	0.12	0.06

CMHI, Children's Memorial Health Institute; PMUH, Poznań Medical University Hospital; WMUH, Warsaw Medical University

Hospital; MIC, minimum inhibitory concentration; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; PIP/TAZ,

piperacillin/tazobactam; CIP, ciprofloxacin; ETM, ertapenem; IPM, imipenem; MEM, meropenem.

