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Research Article

The Occurrence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ Genes in Extended-Spectrum β-Lactamase-Positive Strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* in Poland

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Bacteria belonging to the *Enterobacteriaceae* family that produce extended-spectrum β-lactamase (ESBL) enzymes are important pathogens of infections. Increasing numbers of ESBL-producing bacterial strains exhibiting multidrug resistance have been observed. The aim of the study was to evaluate the prevalence of *bla*$_{\text{CTX-M}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{TEM}}$ genes among ESBL-producing *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis* strains and to examine susceptibility to antibiotics of tested strains. In our study, thirty-six of the tested strains exhibited *bla*$_{\text{CTX-M}}$ genes (*bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{CTX-M-91}}$, and *bla*$_{\text{CTX-M-89}}$). Moreover, twelve ESBL-positive strains harbored *bla*$_{\text{SHV}}$ genes (*bla*$_{\text{SHV-18}}$, *bla*$_{\text{SHV-7}}$, *bla*$_{\text{SHV-2}}$, and *bla*$_{\text{SHV-5}}$), and the presence of a *bla*$_{\text{TEM}}$ gene (*bla*$_{\text{TEM-1}}$) in twenty-five ESBL-positive strains was revealed. Among *K. pneumoniae* the multiple ESBL genotype composed of *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{CTX-M-3}}$, *bla*$_{\text{SHV-18}}$, *bla*$_{\text{SHV-7}}$, *bla*$_{\text{SHV-2}}$, and *bla*$_{\text{SHV-5}}$ genes encoding particular ESBL variants was observed. Analysis of bacterial susceptibility to antibiotics revealed that, among β-lactam antibiotics, the most effective against *E. coli* strains was meropenem (100%), whereas *K. pneumoniae* were completely susceptible to ertapenem and meropenem (100%), and *P. mirabilis* strains were susceptible to ertapenem (91.7%). Moreover, among non-β-lactam antibiotics, gentamicin showed the highest activity to *E. coli* (91.7%) and ciprofloxacin the highest to *K. pneumoniae* (83.3%). *P. mirabilis* revealed the highest susceptibility to amikacin (66.7%).

1. Introduction

Bacteria belonging to the *Enterobacteriaceae* family have been reported worldwide as etiologic factors of many nosocomial infections [1]. Infections caused by *Enterobacteriaceae* rods are difficult to manage because of the reduction of therapeutic possibilities, resulting from constantly increasing resistance of these organisms to antibiotics [2]. Production of ESBLs is one of the most prevalent resistance mechanisms in Gram-negative bacilli. Initially, ESBLs were predominantly described in *K. pneumoniae* and *E. coli* strains, but recently the enzymes were found in other genera of the *Enterobacteriaceae* family [3]. ESBL-producing bacteria exhibit effective hydrolyzation of β-lactam antibiotics, including broad-spectrum β-lactam drugs and monobactams, except cefamycins and β-lactam inhibitors [4]. The resistance usually depends on expression of *bla* genes belonging to the inter alia *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, and *bla*$_{\text{CTX-M}}$ genes family. The *bla*$_{\text{TEM}}$,

bla_{SHV}, and $bla_{CTX\text{-}M}$ genes are responsible for production of, respectively, TEM β-lactamases, SHV β-lactamases, and CTX-M β-lactamases, large families of enzymes with evolutionary affinity. Since the first TEM-1 β-lactamase was discovered, one hundred eighty-five new β-lactamases of the TEM family have been reported worldwide, whereas ninety-three variants are responsible for production of ESBLs. Among one hundred seventy-two enzyme types of the SHV family, forty-five have been reported as extended-spectrum β-lactamases. The CTX-M family comprises more than sixty enzymes (http://www.eucast.org/clinicalbreakpoints, [5, 6]).

It is known that *bla* genes encoding antibiotic resistance may be placed on transferable elements such as plasmids or transposons. This localization of *bla* genes can facilitate a horizontal spreading of antibiotic resistance among bacterial strains [7]. Due to the noticeable geographical differentiation of *bla* genes among ESBL-producers here we examined the prevalence of bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes among ESBL-producing strains of *Klebsiella pneumoniae*, *Escherichia coli*, and *Proteus mirabilis*, with the specification of their variants. The study was focused on the searching for genes bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ based on reports in the literature describing them as the most commonly occurring among *Enterobacteriaceae* family [8, 9]. Moreover, due to the alarming reports in the literature about the emergence of *Enterobacteriaceae* ESBL-positive strains exhibiting multiresistance phenotype, the next aim of our study was to evaluate the sensitivity of tested strains to antibiotics, other groups than β-lactam antibiotics, and to indicate the antibiotic with the highest activity.

2. Material and Methods

Tests were carried out on thirty-six ESBL-positive isolates including twelve strains of *K. pneumoniae*, twelve strains of *P. mirabilis*, and twelve strains of *E. coli*. All strains for the study were chosen on the basis of the screening test for the detection of ESBL-type enzymes. The isolates were collected from clinical specimens of patients hospitalized at the University Hospital in Bialystok (Poland) from the period of the first quarter of year 2013. The isolates were recovered from various clinical specimens mostly urine, tracheal secretions, throat swabs, rectal swabs, and wound swabs. The majority of collected strains originated from the intensive care unit, the hematology clinic, and the urology clinic. The identification of the strains was performed by the VITEK 2 GN cards and the automated identification system (bioMérieux, France) according to the procedure and following the manufacturer's instructions. Control strains used in this study included *K. pneumoniae* ATCC 700603, *E. coli* ATCC 35218, and *E. coli* ATCC 25922.

2.1. Antibiotic Susceptibility Testing and Determination of ESBL. Susceptibility of clinical isolates to β-lactams (amoxicillin, ampicillin, aztreonam, cefepime, ceftriaxone, ertapenem, and meropenem) and to ciprofloxacin, amikacin, gentamicin, levofloxacin, tobramycin, and trimethoprim/sulfamethoxazole was tested by using AST-N093 cards and the automated VITEK 2 system. Susceptibility was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org/clinicalbreakpoints). The thirty-six *Enterobacteriaceae* strains including twelve strains of *K. pneumoniae*, twelve strains of *P. mirabilis*, and twelve strains of *E. coli* from hospitalized patients were found to be ESBL-producers. The presence of ESBL phenotype was confirmed both by the double-disk-synergy test (DDST) [10] and VITEK 2 automated system.

2.2. Plasmid DNA Preparation. The *Enterobacteriaceae* strains were cultured overnight on Trypticase Soy Broth, (Emapol, Poland) at 37°C. Plasmid DNA was extracted from *Enterobacteriaceae* strains by the alkaline method with the Plasmid Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol.

2.3. PCR Amplification of bla Genes. Prepared plasmid DNA was used as templates for bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ gene amplification. Molecular detection of the *bla* genes was carried out with the Cyclone 96 (PEQLAB Biotechnology, GmbH, Germany) thermal cycler. The primers used for the polymerase chain reaction (PCR) assays were forward primer *bla*TEM-F (5′-GCTCACCCAGAAACGCTGGT-3′), reverse primer *bla*TEM-R (5′-CCATCTGGCCCCAGTGCTGC-3′), forward primer *bla*SHV-F (5′-CCCGCAGCCGCTTGAGCAAA-3′), reverse primer *bla*SHV-R (5′-CATGCTCGCCGGCGTATCCC-3′), forward primer *bla*CTX-M-F (5′-SCSATGTGCAGYACCAGTAA-3′), and reverse primer *bla*CTX-M-R (5′-ACCAGAAYVAGCGGBGC-3′). The oligonucleotide primers *bla*SHV and *bla*TEM were designed on the basis of the nucleotide sequence of bla_{TEM} and bla_{SHV} genes reported in the National Center for Biotechnology Information (NCBI) Gen Bank database, while *bla*CTX-M primers were synthesized according to a previously published protocol [11]. The PCR mixture, in a final volume of 25 μL, contained 10 pmol/μL of each primer (1 μL), 12.5 μL of 2x PCR RED Master Mix (DNA-Gdansk, Poland), 3 μL of template DNA, and 7.5 μL of ultra pure H_2O. PCR conditions for the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes were selected based on the properties of the primers and were, respectively, 5 min at 95°C, thirty-five cycles (1 min at 94°C, 1 min at 58°C, and 1 min at 72°C), and finally 10 min at 72°C; 5 min at 95°C, thirty-five cycles (1 min at 94°C, 1 min at 58.5°C, and 1 min at 72°C), and finally 10 min at 72°C; and 3 min at 94°C, thirty-five cycles (30 s at 94°C, 30 s at 55°C, and 45 s at 72°C), and finally 10 min at 72°C.

2.4. Detection of PCR Products and Sequence Analysis. PCR amplicons were separated electrophoretically in Mini-Sub Cell GT (BIO-RAD, USA) at 5 V/cm for 90 min in 1.5% agarose gel (Basica LE GQT Agarose, PRONA Marine Research Institute, Spain) containing 0.5 μg/mL of ethidium bromide (MP Biomedicals, Poland) in borate buffer (TBE, Tris-Borate-EDTA) and photographed using the ChemiDoc XRS imaging system (BIO-RAD) and Quantity One 1-D Analysis Software (Bio-Rad, USA). Then, the positions of

amplification products were estimated with the position of the molecular weight marker. PCR products with a length of 686 bp (bla_{TEM}), 733 bp (bla_{SHV}), and 585 bp (bla_{CTX-M}) were purified from the agarose gel using Gel-Out Kit (A&A Biotechnology) and then sequenced using 3130xls Genetic Analyser (Applied Biosystems, USA). Nucleotide sequences of bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes were analyzed and compared with sequences available in the NCBI database using Basic Local Alignment Search Tool (BLAST) algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3. Results and Discussion

The analysis of selected strains was concerned with the searching for bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes in plasmid materials of tested strains, which were confirmed by phenotypic tests as producers of extended-spectrum β-lactamases. Regarding the literature reports describing a substantial number of genes encoding β-lactamases inter alia ESBLs, we focused our research on the families of *bla* genes which are presented in literature as the most common among *Enterobacteriaceae* strains [12]. Moreover, limited number of reports regarding the prevalence of *bla* genes among *Enterobacteriaceae* strains in Poland meant that this topic has been the subject of research.

Performed analysis with use of PCR reaction and specific primers for the bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes revealed variation in occurrence of *bla* genes among tested strains (Table 1). Studies have shown that the genes responsible for the production of CTX-M β-lactamases were more prevalent among tested strains in comparison to the genes encoding SHV-type or TEM-type β-lactamases. The presence of bla_{CTX-M} genes was observed among all tested *Enterobacteriaceae* strains, whereas, the presence of the bla_{SHV} genes was limited to all tested strains of *K. pneumoniae*. Moreover, PCR results based on primers specific for the *bla*TEM-type β-lactamases revealed the presence of bla_{TEM} genes among twenty-five of thirty-six tested strains, including two strains of *E. coli*, twelve strains of *K. pneumoniae*, and eleven strains of *P. mirabilis*.

In the next stage of the study, the PCR products of the expected size, which were observed in electrophoresis, were sequenced. The sequencing process led to the determination of the nucleotide sequences of the obtained products of PCR reactions and the identification of different types of genes whose presence was detected. Sequences of bla_{CTX-M} genes detected by electrophoretic analysis were identified as $bla_{CTX-M-15}$ in all tested strains of *E. coli* and among 91.7% of *K. pneumoniae* strains. In 8.3% of *K. pneumonia* strains the presence of $bla_{CTX-M-3}$ genes responsible for production of extended-spectrum β-lactamase CTX-M-3 was noticed. Moreover, DNA sequencing revealed the prevalence of $bla_{CTX-M-91}$ genes encoding CTX-M-91 extended-spectrum β-lactamase that were detected in 66.7% of *P. mirabilis*. Among the remaining 33.3% of *P. mirabilis* the presence of $bla_{CTX-M-89}$ encoding CTX-M-89 extended-spectrum β-lactamase was observed. Additionally, DNA sequencing revealed that 16.7% of *E. coli*, 100% of *K. pneumoniae*, and 91.7% of *P. mirabilis* strains harbored bla_{TEM-1} genes encoding TEM-1 enzyme with activity of broad-spectrum β-lactamase. Furthermore, *K. pneumoniae* strains were found to carry the following bla_{SHV} genes: bla_{SHV-18} (41.7%), bla_{SHV-7} (8.3%), bla_{SHV-2} (58.3%), and bla_{SHV-5} (25%), encoding different extended-spectrum β-lactamses. The results provide information about the diversity of *bla* genes presence among *K. pneumoniae*, *P. mirabilis*, and *E. coli* strains, in the North-Eastern Polish, that were harboring mainly $bla_{CTX-M-15}$ genes. As reported, world literature, both $bla_{CTX-M-15}$ and $bla_{CTX-M-3}$ genes, detected among tested strains, belong to CTX-M-1 group, which is often described as *Enterobacteriaceae*. Global reports describe, in addition to the CTX-M-15 and CTX-M-3 detected in our study, CTX-M-9 and CTX-M-14 as the dominant variants among the CTX-M family and the most widespread enzymes among non-TEM and non-SHV plasmid mediated ESBLs [13]. These results are consistent with reports describing CTX-M-family enzymes as the group that, during the last few years, has become predominant [14]. Furthermore, in the present study $bla_{CTX-M-91}$ and $bla_{CTX-M-89}$ among *P. mirabilis* were identified. These genes exhibit genetic similarity with $bla_{CTX-M-25}$ encode enzymes belonging to the sub-CTX-M-25 with extended-spectrum β-lactamase activity [15, 16]. Genes $bla_{CTX-M-91}$ and $bla_{CTX-M-89}$ are uncommon but their occurrence in *P. mirabilis* and *Enterobacter cloacae* was reported [17, 18]. Moreover, our results are in agreement with the literature that bla_{TEM-1} genes and TEM-1 β-lactamase are a prevalent plasmid-mediated β-lactamase in Gram-negative bacteria [19]. As reported in the literature, the occurrence of bla_{TEM} genes in *Enterobacteriaceae* can be as high as 61% [20]. Strains of *Enterobacteriaceae* showing the presence of bla_{TEM} genes responsible for the production of particular ESBL enzymes such as TEM-47, TEM-4, TEM-29, TEM-85, TEM-93, and TEM-94 have also been described [21]. Moreover, the presence of the bla_{SHV} genes was observed only in tested strains of *K. pneumoniae*, which confirms the position of *K. pneumoniae* bacteria species as organisms commonly harboring genes encoding enzymes of the SHV family. The results of our study showed a lack of bla_{SHV} genes in strains of *E. coli* and *P. mirabilis*, which does not exclude the possibility of the occurrence of these genes among species of these bacteria, which is supported by the published literature describing the strains of *E. coli* showing the presence of genes bla_{SHV-5} and bla_{SHV-12} [22]. The SHV-family enzyme variants of bla_{SHV-18}, bla_{SHV-7}, bla_{SHV-2}, and bla_{SHV-5} detected in our study are also revealed among ESBL-producing *Enterobacteriaceae* strains in Europe [23]. The presence of bla_{SHV}, bla_{CTX-M}, and bla_{TEM} genes among tested rods of the *Enterobacteriaceae* family is presented in Table 1.

It should be noted that tested strains of *K. pneumoniae* have the genes responsible for the production of two ESBLs. They had the following genotypes: $bla_{CTX-M-15}$ and bla_{SHV-18}; $bla_{CTX-M-15}$ and bla_{SHV-7}; $bla_{CTX-M-3}$ and bla_{SHV-2}; $bla_{CTX-M-15}$ and bla_{SHV-5}; $bla_{CTX-M-15}$ and bla_{SHV-2}. The phenomenon of multiple ESBL-production is becoming more common. It has been reported that, among strains of ESBL-positive *Enterobacteriaceae*, 44% harbored *bla* genes for multiple ESBLs [24].

Table 1: Overview over all strains, identified *bla* genes, and resistance phenotypes.

Name of strain	$bla_{\text{CTX-M}}$ genes	bla_{TEM} genes	bla_{SHV} genes	Resistance phenotype to non-β-lactam antibiotics						Active β-lactam antibiotics
1 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R	AN^R	GM^R	LEV^R	TM^R	SXT^R	MEM
2 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ETP, FEP, MEM
3 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ATM, FEP, ETP, MEM
4 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ATM, FEP, ETP, MEM
5 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ATM, FEP, ETP, MEM
6 E	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	—			GM^R		TM^R	SXT^R	FEP, MEM
7 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ATM, FEP, ETP, MEM
8 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R	AN^R		LEV^R	TM^R	SXT^R	FEP, ETP, MEM
9 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	ETP, MEM
10 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	FEP, ETP, MEM
11 E	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	—	CIP^R			LEV^R		SXT^R	ETP, MEM
12 E	$bla_{\text{CTX-M-15}}$	—	—	CIP^R			LEV^R	TM^R	SXT^R	FEP, ETP, MEM
1 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-18}}$		AN^R	GM^R			SXT^R	ETP, MEM
2 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-7}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
3 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-18}}$			GM^R	LEV^R		SXT^R	ETP, MEM
4 K	$bla_{\text{CTX-M-3}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-2}}$	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
5 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-5}}$	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
6 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-18}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
7 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-5}}$			GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
8 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-5}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
9 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-2}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
10 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-2}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
11 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-18}}$		AN^R	GM^R	LEV^R	TM^R	SXT^R	ETP, MEM
12 K	$bla_{\text{CTX-M-15}}$	$bla_{\text{TEM-1}}$	$bla_{\text{SHV-18}}$			GM^R	LEV^R		SXT^R	ETP, MEM
1 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
2 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R	AN^R	GM^R	LEV^R	TM^R	SXT^R	MEM
3 P	$bla_{\text{CTX-M-89}}$	$bla_{\text{TEM-1}}$	—	CIP^R	AN^R	GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
4 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, FEP, ETP, MEM
5 P	$bla_{\text{CTX-M-89}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
6 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
7 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
8 P	$bla_{\text{CTX-M-89}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
9 P	$bla_{\text{CTX-M-89}}$	—	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
10 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R		GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
11 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R	AN^R	GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM
12 P	$bla_{\text{CTX-M-91}}$	$bla_{\text{TEM-1}}$	—	CIP^R	AN^R	GM^R	LEV^R	TM^R	SXT^R	ATM, ETP, MEM

AN: amikacin; ATM: aztreonam; FEP: cefepime; ETP: ertapenem; MEM: meropenem; CIP: ciprofloxacin; GM: gentamicin; LEV: levofloxacin; TM: tobramycin; SXT: trimethoprim/sulfamethoxazole; R: resistant; E: *E. coli*; P: *P. mirabilis*; K: *K. pneumoniae*.

Table 2: Susceptibility of *Enterobacteriaceae* ESBL-positive strains ($n = 36$).

Antibiotic	*E. coli*			*K. pneumoniae*			*P. mirabilis*		
	R	I	S	R	I	S	R	I	S
AMC	100%			100%			100%		
AM	100%			100%*			100%		
CRO	100%			100%			100%		
ATM	16.7%	25%	33.3%	100%			8.3%		91.7%
FEP	8.3%	16.7%	75%	83.3%	16.7%		16.7%	83.3%	
ETP	16.7%		83.3%			100%	8.3%		91.7%
MEM			100%			100%			100%
CIP	91.7%		8.3%	16.7%		83.3%	100%		
LEV	91.7%		8.3%	75%		25%	100%		
AN	16.7%	58.3%	25%	58.3%	25%	16.7%	33.3%		66.7%
GM	8.3%		91.7%	100%			100%		
TM	75%	8.3%	16.7%	75%		25%	100%		
SXT	100%			100%			100%		

AN: amikacin; AMC: amoxicillin/clavulanic acid; AM: ampicillin; ATM: aztreonam; FEP: cefepime; CRO: ceftriaxone; ETP: ertapenem; MEM: meropenem; CIP: ciprofloxacin; GM: gentamicin; LEV: levofloxacin; TM: tobramycin; SXT: trimethoprim/sulfamethoxazole; R: resistant; I: intermediate; S: susceptible; *intrinsic resistance.

To summarize, DNA sequencing has allowed us to identify specific variants of the *bla* genes among ESBL-positive *Enterobacteriaceae*. The majority of the *bla* genes detected in our study was encoding enzyme variants that are commonly found in Europe. This may indicate the possibility of the dissemination of *bla* genes among *Enterobacteriaceae*, which may be associated with the occurrence of *bla* genes on the mobile genetic elements.

With the ability of bacteria to produce ESBL enzymes, the phenomenon of broad spectrum resistance to β-lactam antibiotics is observed and confirmed by numerous reports [25]. That relationship was also shown in our analysis. The antibiotic susceptibilities of ESBL-producers are presented in Table 2. Among *E. coli* strains expressing ESBL activity, high levels of resistance to ampicillin (100%), amoxicillin/clavulanic acid (100%), ceftriaxone (100%), and aztreonam (16.7%) were observed. Tested strains of *E. coli* were susceptible to cefepime (75%), ertapenem (83.3%), and meropenem (100%). All *K. pneumoniae* ESBL-positive strains were resistant to ampicillin (intrinsic resistance), amoxicillin/clavulanic acid, ceftriaxone, and aztreonam. Furthermore, ESBL-positive *K. pneumoniae* strains were fully susceptible to meropenem (100%) and ertapenem (100%). Additionally, *P. mirabilis* ESBL-positive strains were completely resistant to ampicillin, amoxicillin/clavulanic acid, and ceftriaxone. High activity to *P. mirabilis* revealed aztreonam (91.7%), ertapenem (91.7%), and meropenem (100%) (Table 2).

Epidemiological data indicate that, over the past few years, antimicrobial resistance has dramatically increased. Additionally, more and more studies present data about the constantly increasing resistance to both β-lactams and other groups of antibiotics in the bacteria of the *Enterobacteriaceae* family [26, 27]. The constant increase of simultaneous resistance to various classes of antibiotics significantly reduces the possibility of therapeutic treatment of infections caused by ESBL-producers [28–30]. That prompted us to analyze the level of resistance among ESBL-positive tested strains.

ESBL-producers revealed high resistance to antibiotic groups other than β-lactams. The results of our study are in line with global reports [28, 31–33]. Our analysis showed a significant degree of ESBL-positive *E. coli* resistance to such antibiotics as ciprofloxacin, levofloxacin, and tobramycin. The percentages of resistant strains were, respectively, 91.7%, 91.7%, and 75%. *K. pneumoniae* ESBL-positive strains appeared to be resistant to such antibiotics as levofloxacin (75%), amikacin (58.3%), gentamicin (100%), and tobramycin (75%). *P. mirabilis* strains revealed resistance towards ciprofloxacin (100%), levofloxacin (100%), gentamicin (100%), and tobramycin (100%). Moreover, all tested strains were fully resistant to trimethoprim/sulfamethoxazole (100%) (Table 2).

ESBL-positive strains showing simultaneous resistance to both β-lactams and antibiotics of other groups are defined as multidrug-resistant strains [30]. Frequent occurrence of multidrug-resistant strains as an etiological factor of infections is described in numerous reports [34, 35]. Characteristic location of genes responsible for resistance is considered to be the reason for the prevalence of this phenomenon. Resistant genes for β-lactamases are often located in mobile genetic elements such as plasmids and integrons, whereby the horizontal transfer of these genes is possible not only in bacteria of the same species but also between bacteria of *Enterobacteriaceae* species and nonfermenting rods [36, 37]. In *Enterobacteriaceae*, resistant genes are located on plasmids, where genes responsible for resistance to different groups of antibiotics may be located in a close neighborhood and thus may be transmitted at the same time to other bacteria [38, 39].

The majority of tested clinical isolates possessing particular *bla* genes were resistant to at least one antibiotic from three different classes of antibiotics, which classifies them as multidrug-resistant bacteria. The occurrence of ESBL-positive strains expressing multidrug resistance to antibiotics has remained the dominant problem in the therapy of

infections caused by Gram-negative bacilli [40]. Bacterial resistant phenotypes and the presence of *bla* genes among tested strains are summarized in Table 1. The analysis revealed that among *E. coli* strains carrying the $bla_{\text{CTX-M-15}}$ gene, the most prevalent phenotype of resistance included resistance towards ciprofloxacin, levofloxacin, tobramycin, and trimethoprim/sulfamethoxazole. Moreover, all strains of *P. mirabilis* carrying $bla_{\text{CTX-M-91}}$ and $bla_{\text{TEM-1}}$ genes appeared to be resistant to ciprofloxacin, gentamicin, levofloxacin, tobramycin and trimethoprim/sulfamethoxazole. *K. pneumoniae* strains with simultaneous presence of the *bla* genes of the SHV ($bla_{\text{SHV-18}}$, $bla_{\text{SHV-7}}$, $bla_{\text{SHV-5}}$, and $bla_{\text{SHV-2}}$), TEM ($bla_{\text{TEM-1}}$), and CTX-M ($bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-3}}$) families presented a resistance profile including resistance against amikacin, gentamicin, levofloxacin, tobramycin, and trimethoprim/sulfamethoxazole (Table 1).

Observations of a high level of antibiotic resistance among strains analyzed in the present study are disturbing but in agreement with previously published results that showed susceptibility of multiple ESBL *K. pneumoniae* strains to meropenem, ertapenem, imipenem, and ciprofloxacin [41].

Our data demonstrated the prevalence of particular *bla* genes responsible for production appropriate β-lactamases in tested ESBL-positive *Enterobacteriaceae* strains. The dominance of the $bla_{\text{CTX-M-15}}$ genes among *E. coli* and *K. pneumoniae* and $bla_{\text{CTX-M-91}}$ genes among *P. mirabilis* was revealed. Both $bla_{\text{CTX-M-15}}$ genes and $bla_{\text{CTX-M-91}}$ genes are responsible for production of extended-spectrum β-lactamases. Moreover, the prevalence of $bla_{\text{TEM-1}}$ genes responsible for the production of broad-spectrum β-lactamases among *K. pneumoniae* and *P. mirabilis* has been shown, and *bla* genes of the SHV family ($bla_{\text{SHV-18}}$, $bla_{\text{SHV-7}}$, $bla_{\text{SHV-2}}$, and $bla_{\text{SHV-5}}$) responsible for the production of ESBL enzymes have been detected in strains of *K. pneumoniae*. Among tested rods, only *K. pneumoniae* strains revealed the simultaneous presence of $bla_{\text{CTX-M-15}}$ and $bla_{\text{CTX-M-3}}$ genes in combination with $bla_{\text{TEM-1}}$ and particular types of bla_{SHV} ($bla_{\text{SHV-18}}$, $bla_{\text{SHV-7}}$, $bla_{\text{SHV-2}}$, and $bla_{\text{SHV-5}}$) genes. It is noteworthy that among *K. pneumoniae* the multiple-ESBL genotype composed of $bla_{\text{CTX-M-15}}$, $bla_{\text{CTX-M-3}}$, $bla_{\text{SHV-18}}$, $bla_{\text{SHV-7}}$, $bla_{\text{SHV-2}}$, and $bla_{\text{SHV-5}}$ genes encoding particular ESBL variants was observed. Moreover, our study revealed a high level of resistance to antibiotics and the prevalence of multidrug-resistant bacteria among ESBL producers.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Paweł Sacha and Piotr Wieczorek contributed equally to this work.

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References

[1] R. Gaynes and J. R. Edwards, "Overview of nosocomial infections caused by gram-negative bacilli," *Clinical Infectious Diseases*, vol. 41, no. 6, pp. 848–854, 2005.

[2] J. D. D. Pitout, "Multiresistant *Enterobacteriaceae*: new threat of an old problem," *Expert Review of Anti-Infective Therapy*, vol. 6, no. 5, pp. 657–669, 2008.

[3] P. Mathur, A. Kapil, B. Das, and B. Dhawan, "Prevalence of extended spectrum β lactamase producing Gram negative bacteria in a tertiary care hospital," *Indian Journal of Medical Research*, vol. 115, pp. 153–157, 2002.

[4] K. Bush and G. A. Jacoby, "Updated functional classification of β-lactamases," *Antimicrobial Agents and Chemotherapy*, vol. 54, no. 3, pp. 969–976, 2010.

[5] A. M. Al-Jassera, "Extended-spectrum beta-lactamases (ESBLs): a global problem," *Kuwait Medical Journal*, vol. 38, no. 3, pp. 171–185, 2006.

[6] A. A. Shah, F. Hasan, S. Ahmed, and A. Hameed, "Characteristics, epidemiology and clinical importance of emerging strains of Gram-negative bacilli producing extended-spectrum β-lactamases," *Research in Microbiology*, vol. 155, no. 6, pp. 409–421, 2004.

[7] A. El salabi, T. R. Walsh, and C. Choichanic, "Extended spectrum β-lactamases, carbapenemases and mobile genetic elements responsible for antibiotics resistance in Gram-negative bacteria," *Critical Reviews in Microbiology*, vol. 39, no. 2, pp. 113–122, 2012.

[8] K. Bush, "Extended-spectrum β-lactamases in North America, 1987–2006," *Clinical Microbiology and Infection*, vol. 14, no. 1, pp. 134–143, 2008.

[9] R. Cantón, A. Novais, A. Valverde et al., "Prevalence and spread of extended-spectrum β-lactamase-producing *Enterobacteriaceae* in Europe," *Clinical Microbiology and Infection*, vol. 14, no. 1, pp. 144–153, 2008.

[10] V. Jarlier, M. H. Nicolas, G. Fournier, and A. Philippon, "Extend-ed broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns," *Reviews of Infectious Diseases*, vol. 10, no. 4, pp. 867–878, 1988.

[11] A. Sundsfjord, G. S. Simonsen, B. C. Haldorsen et al., "Genetic methods for detection of antimicrobial resistance," *Acta Pathologica et Immunologica Scandinavica*, vol. 112, no. 11-12, pp. 815–837, 2004.

[12] K. Bush, "Bench-to-bedside review: the role of β-lactamases in antibiotic-resistant Gram-negative infections," *Critical Care*, vol. 14, no. 3, pp. 224–232, 2010.

[13] U. Govinden, C. Mocktar, P. Moodley, A. W. Sturm, and S. Y. Essack, "Geographical evolution of the CTX-M β-lactamase: an update," *African Journal of Biotechnology*, vol. 6, no. 7, pp. 831–839, 2007.

[14] D. M. Livermore, R. Canton, M. Gniadkowski et al., "CTX-M: changing the face of ESBLs in Europe," *Journal of Antimicrobial Chemotherapy*, vol. 59, no. 2, pp. 165–174, 2007.

[15] S. E. McGettigan, B. Hu, K. Andreacchio, I. Nachamkin, and P. H. Edelstein, "Prevalence of CTX-M β-lactamases in Philadelphia, Pennsylvania," *Journal of Clinical Microbiology*, vol. 47, no. 9, pp. 2970–2974, 2009.

[16] J. Vervoort, "Characterization of two new CTX-M-25-Group extended-spectrum beta-lactamase variants identified in *Escherichiacoli* isolates from Israel," *PLoS ONE*, vol. 7, no. 9, Article ID e46329, 2012.

[17] K. Kasahara and P. H. Edelstein, "Novel *Proteus mirabilis* CTX-M," http://www.uniprot.org/uniprot/D0ES28.

[18] I. Park and K. H. Kim, "Novel plasmid-encoded cefotaxime-hydrolyzing CTX-M-89 extended-spectrum beta-lactamase from clinical isolates of Enterobacter cloacae," http://www.uniprot.org/uniprot/C5IUN2.

[19] T. Palzkill and D. Botstein, "Identification of amino acid substitutions that alter the substrate specificity of TEM-1 β-lactamase," *Journal of Bacteriology*, vol. 174, no. 16, pp. 5237–5243, 1992.

[20] L. G. Mesa, A. R. Morí, L. N. Becerra et al., "Phenotypic and molecular identification of extended-spectrum β-lactamase (ESBL) TEM and SHV produced by clinical isolates *Escherichia coli* and *Klebsiella* spp. in hospitals," *Revista Cubana de Medicina Tropical*, vol. 59, no. 1, pp. 52–58, 2007.

[21] A. Baraniak, J. Fiett, A. Mrówka, J. Walory, W. Hryniewicz, and M. Gniadkowski, "Evolution of TEM-type extended-spectrum β-lactamases in clinical *Enterobacteriaceae* strains in Poland," *Antimicrobial Agents and Chemotherapy*, vol. 49, no. 5, pp. 1872–1880, 2005.

[22] T. M. Coque, F. Baquero, and R. Canton, "Increasing prevalence of ESBL-producing *Enterobacteriaceae* in Europe," *Euro Surveillance*, vol. 13, no. 47, pp. 1–11, 2008.

[23] P. Sacha, D. Ojdana, P. Wieczorek et al., "Profiles of phenotype resistance to antibiotic other than β-lactams in *Klebsiella pneumoniae* ESBLs-producers, carrying blaSHV genes," *Folia Histochemica et Cytobiologica*, vol. 48, no. 4, pp. 663–666, 2010.

[24] C. H. Jones, M. Tuckman, D. Keeney, A. Ruzin, and P. A. Bradford, "Characterization and sequence analysis of extended-spectrum-β-lactamase-encoding genes from *Escherichia coli, Klebsiella pneumoniae*, and *Proteus mirabilis* isolates collected during tigecycline phase 3 clinical trials," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 2, pp. 465–475, 2009.

[25] S. Nijssen, A. Florijn, M. J. M. Bonten, F. J. Schmitz, J. Verhoef, and A. C. Fluit, "Beta-lactam susceptibilities and prevalence of ESBL-producing isolates among more than 5000 European *Enterobacteriaceae* isolates," *International Journal of Antimicrobial Agents*, vol. 24, no. 6, pp. 585–591, 2004.

[26] C. Schultsz and S. Geerlings, "Plasmid-mediated resistance in *Enterobacteriaceae*: changing landscape and implications for therapy," *Drugs*, vol. 72, no. 1, pp. 1–16, 2012.

[27] J. P. Lynch, N. M. Clark, and G. G. Zhanerl, "Evolution of antimicrobial resistance among *Enterobacteriaceae* (focus on extended spectrum beta-lactamases and carbapenemases)," *Expert Opinion on Pharmacotherapy*, vol. 14, no. 2, pp. 199–210, 2013.

[28] H. Giamarellou and G. Poulakou, "Multidrug-resistant gram-negative infections: what are the treatment options?" *Drugs*, vol. 69, no. 14, pp. 1879–1901, 2009.

[29] H. Giamarellou, "Multidrug-resistant Gram-negative bacteria: how to treat and for how long," *International Journal of Antimicrobial Agents*, vol. 36, no. 2, pp. S50–S54, 2010.

[30] N. H. Chan-Tompkins, "Multidrug-resistant gram-negative infections bringing back the old," *Critical Care Nursing Quarterly*, vol. 34, no. 2, pp. 87–100, 2011.

[31] D. L. Paterson, "Recommendation for treatment of severe infections caused by *Enterobacteriaceae* producing extended-spectrum β-lactamases (ESBLs)," *Clinical Microbiology and Infection*, vol. 6, no. 9, pp. 460–463, 2000.

[32] S. Babypadmini and B. Appalaraju, "Extended spectrum β-lactamases in urinary isolates of *Escherichia coli* and *Klebsiella pneumoniae*: prevalence and susceptibility pattern in a tertiary care hospital," *Indian Journal of Medical Microbiology*, vol. 22, no. 3, pp. 172–174, 2004.

[33] S. Jaiswal, N. Kastury, A. K. Kapoor, A. R. Tiwari, and A. Bhargava, "A study of antibiotic sensitivity pattern in gram-negative urinary isolates with special reference to extended spectrum beta lactamase producers," *Biomedicine*, vol. 27, no. 4, pp. 162–167, 2007.

[34] G. M. Rossolini, E. Mantengoli, J.-D. Docquier, R. A. Musmanno, and G. Coratza, "Epidemiology of infections caused by multiresistant Gram-negatives: ESBLs, MBLs, panresistant strains," *New Microbiologica*, vol. 30, no. 3, pp. 332–339, 2007.

[35] A. M. Nicasio, J. L. Kuti, and D. P. Nicolau, "The current state of multidrug-resistant gram-negative bacilli in North America: insights from the society of infectious diseases pharmacists," *Pharmacotherapy*, vol. 28, no. 2, pp. 235–249, 2008.

[36] K. Bush, "Alarming β-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*," *Current Opinion in Microbiology*, vol. 13, no. 5, pp. 558–564, 2010.

[37] P. Sacha, P. Wieczorek, D. Ojdana et al., "Antibiotic susceptibility and the presence of presence of bla_{IMP} and bla_{VIM} genes among *Pseudomonas aeruginosa* strains resistant or susceptible to imipenem," *Progress in Health Sciences*, vol. 2, no. 1, pp. 12–18, 2012.

[38] M. A. Leverstein-van Hall, A. T. A. Box, H. E. M. Blok, A. Paauw, A. C. Fluit, and J. Verhoef, "Evidence of extensive interspecies transfer of integron-mediated antimicrobial resistance genes among multidrug-resistant *Enterobacteriaceae* in a clinical setting," *Journal of Infectious Diseases*, vol. 186, no. 1, pp. 49–56, 2002.

[39] A. Carattoli, "Resistance plasmid families in *Enterobacteriaceae*," *Antimicrobial Agents and Chemotherapy*, vol. 53, no. 6, pp. 2227–2238, 2009.

[40] R. Colodner, "Extended-spectrum β-lactamases: a challenge for clinical microbiologists and infection control specialists," *The American Journal of Infection Control*, vol. 33, no. 2, pp. 104–107, 2005.

[41] D. Dierżanowska, W. Kamińska, K. Semczuk et al., "Carriage of genes for various extended spectrum beta-lactamases: a novel resistance strategy of *Klebsiella pneumoniae* in Poland," *International Journal of Antimicrobial Agents*, vol. 35, no. 4, pp. 392–395, 2010.