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FULL PAPER

Antimicrobial susceptibility and prevalence of extended-spectrum beta-lactamases in clinical strains of *Klebsiella pneumoniae* isolated from pediatric and adult patients of two Polish hospitals

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SUMMARY

Klebsiella pneumoniae due to the presence of multiple antibiotic resistance mechanisms is one of the most threatening human pathogens nowadays. The aim of the study was to characterize antimicrobial susceptibility, presence of resistance mechanisms and the prevalence of selected genes encoding ESBLs in 170 *K. pneumoniae* isolates recovered from children and adults hospitalized in two Polish medical centers from 2008 to 2015.

The phenotypic identification of strains was confirmed by amplification of *mdh* gene. ESBLs, metallo-beta-lactamases, *Klebsiella pneumoniae* carbapenemases and OXA-48 were detected using phenotypic tests. The $bla_{\text{CTX-M-1}}$, bla_{TEM} and bla_{SHV} ESBL genes were amplified by PCR.

Pediatric *K. pneumoniae* isolates displayed significantly higher resistance to piperacillin/tazobactam, cefoxitin, imipenem, amikacin and ciprofloxacin than strains obtained from adults ($P<0.05$). The presence of ESBLs, OXA-48, KPC and MBL was confirmed in 80.6%, 21.8%, 8.2% and 2.4%, respectively, of the tested strains. The CTX-M-1 enzymes were predominant (91.2%), followed by TEM (63.5%) and SHV (11.8%). The bla_{TEM} was significantly more common in adults than in children ($P<0.05$). Dual or triple *bla* genes were observed in 55.9% and 8.2% of *K. pneumoniae* isolates.

Further local epidemiological studies are required to monitor the dissemination of multidrug-resistant *K. pneumoniae* strains.

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INTRODUCTION

Klebsiella pneumoniae is classified as a Gram-negative, non-motile and capsulated bacillus which occurs naturally both in the environment and on human mucous membranes of the gastrointestinal tract and oropharynx microbiome. The bacteria can spread from these colonized areas to distinct tissues causing acute infections, including pneumonia, urinary tract infections, wound infections, bacteremia and liver abscesses. *Klebsiella pneumoniae* is a common opportunistic nosocomial pathogen, accounting for about one third of all Gram-negative infections overall (Navon-Venezia *et al.,* 2017). These infections occur mainly in patients with an impaired immune system, although it has recently been revealed that hypervirulent strains can invade immunocompetent individuals as well. Furthermore, *K. pneumoniae* is also responsible for serious community-onset infections. Along with its high prevalence and virulence, *K. pneumoniae* is a major source of antimicrobial resistance (Hennequin and Robin, 2016; Paczosa and Mecsas, 2016).

The most common resistance mechanisms observed in *K. pneumoniae* are beta-lactamases, including extended-spectrum enzymes (ESBL) and carbapenemases. ESBLs hydrolyze penicillin, first-, second- and third-generation cephalosporins and monobactams. Cephamycins and carbapenems remain active against ESBL-producing strains. Although the majority of ESBLs belong to class A of the Ambler scheme, they are divided into the following families: TEM, SHV, CTX-M, PER, VEB, GES, BES, OXA, TLA, SFO, IBC, BEL and PME (Vourli *et al.,* 2003; Poirel *et al.,* 2012; Bush, 2013; Hawkey, 2015).

ESBL originated from the broad-spectrum beta-lactamases, such as SHV-1, TEM-1 and TEM-2. The first enterobacterial strain harboring the extended-spectrum beta-lactamase SHV-2 (a mutant of SHV-1 enzyme) was isolated in Germany in 1983. Furthermore, TEM-type ESBLs, isolated in 1984 for the first time from *K. pneumoniae* in France, are derivatives of TEM-1 and TEM-2. Currently, TEM and SHV ESBLs occur in the most clinically relevant enterobacterial species, but they play an essential role in *K. pneumoniae* and *Escherichia coli* strains (Livermore *et al.,* 2007; Cantón *et al.,* 2008).

Key words:
Klebsiella pneumoniae, multidrug-resistant, beta-lactamases, CTX-M, TEM, SHV.

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Another significant family of ESBLs with strong cefotaxime-hydrolyzing activity are CTX-M enzymes. Currently more than 124 variants of CTX-M are recognized; they have been divided into five subgroups: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25. In Europe, the most common CTX-Ms are CTX-M-3 and CTX-M-15 (subgroup CTX-M-1) as well as CTX-M-9 and CTX-M-14 (subgroup CTX-M-9) (Coque *et al.,* 2008; Poirel *et al.,* 2012; D'Andrea *et al.,* 2013).

ESBL-producing strains are predominantly recovered from hospitalized patients; however, the most serious aspect is the growing rate of ESBL-producers among strains causing community-onset infections. In the late 1990s, the predominant ESBLs were TEM (TEM-4, TEM-24, TEM-52) and SHV (SHV-2, SHV-5, SHV-12), detected mainly among nosocomial strains of *K. pneumoniae* (Livermore *et al*., 2007). Unexpectedly, in the 2000s, the epidemiology of beta-lactamases has changed dramatically: the members of CTX-Ms have become the most common enzymes, widely disseminated among other than *K. pneumoniae* Enterobacteriaceae species and even non-fermenters (Livermore *et al.,* 2007; Cantón *et al.,* 2008; Coque *et al.,* 2008; Arhoune *et al.,* 2017).

The resistance rates in *K. pneumoniae* have steadily increased over the years. In Poland, the proportion of ESBL producers among nosocomial *K. pneumoniae* strains was 40.4% (Empel *et al.,* 2008). According to the Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net), the percentage of *K. pneumoniae* strains resistant to third-generation cephalosporins was 39.7%. in Poland in 2010. The proportion of ESBL-positive strains among these isolates was 98.9% (ECDC 2011). From 2014 to 2017, the rate of *K. pneumoniae* rods resistant to third-generation cephalosporins was 63.0% to 68.2%. A majority of the above-mentioned isolates was ESBL-positive (ECDC 2018). In European countries, the lowest occurrence of strains with resistance to third-generation cephalosporins was observed in Sweden (1.7%), Norway (2.1%) and Finland (4.0%), the highest in Romania (70.6%), Greece (74.6%) and Bulgaria (75.6%) (Coque *et al.,* 2008; ECDC 2011).

The horizontal transfer of highly mobile plasmids containing beta-lactamases-encoding genes determines the rapid spread of multidrug resistance among bacteria (Baraniak *et al.,* 2013). Moreover, genes conferring resistance to other antimicrobials (aminoglycosides, trimethoprim, sulphonamides, tetracyclines, chloramphenicol) could be localized on these mobile genetic elements (Arhoune *et al.,* 2017). The multidrug-resistant (MDR) strains, due to their high epidemic potential, have been responsible for many outbreaks of hospital-acquired infections. *Klebsiella pneumoniae* is one of the most worrisome and threatening MDR pathogens. The principal vehicle for the introduction and spread of MDR Gram-negative bacilli in humans is fecal carriage (Boo *et al.,* 2005; Hawkey, 2015). Therefore, to prevent dissemination of MDR organisms, every patient admitted to a hospital should be screened for rectal carriage of MDR *K. pneumoniae* (Landman *et al.,* 2005; Coque *et al.,* 2008; Sękowska *et al.,* 2014).

Outbreaks of infection caused by ESBL-producing *Klebsiella* strains have been reported both in adult and pediatric patients. Most available studies on the prevalence of ESBL-producing organisms are restricted to adults, so the role of these bacteria in the infection or colonization in children remains largely unknown. Detailed comparisons of resistance trends in children and adults are also limited (Chandramohan and Revell, 2012; Sękowska *et al.,* 2014). The present study therefore investigated the clinical isolates of *K. pneumoniae* among inpatients in pediatric and adult wards with respect to their antimicrobial susceptibility, presence of resistance mechanisms and the prevalence of genes encoding CTX-M-1, TEM and SHV extended-spectrum beta-lactamases.

MATERIALS AND METHODS

Patients and strains

The present study included 170 patient-unique strains of *K. pneumoniae*, 84 of which were isolated from patients at the University Children's Hospital of Kraków, Poland and 86 from adults hospitalized at the Rydygier Memorial Hospital in Kraków, Poland. The strains were collected from 2008 to 2015.

Children (47 male; 56.0% and 38 female; 45.2%) were admitted to different wards, such as cardiac surgery (35; 41.7%), intensive care (14; 16.7%), cardiology (11; 13.1%), general surgery (8; 9.5%) and newborn pathology (6; 7.1%). Clinical specimens included stool (69; 82.1%) and perianal swabs (12; 14.3%), both taken during screening for rectal carriage of MDR *K. pneumoniae* as part of routine clinical practice, as well as, less frequently, urine (2; 2.4%) and endotracheal aspirate (1; 1.2%). Mean patient age was 1 year 9 months (from 1 month to 17 years).

Most of the adult patients stayed in the following units: intensive care (33; 38.4%), neurology (15; 17.4%) and surgery (10; 11.6%). *Klebsiella pneumoniae* strains were mainly isolated from the respiratory system (39; 45.3%), urine (23; 26.7%) and blood (10; 11.6%). The average age of adult patients was 61 (from 19 to 90). Sixty-one males (70.9%) and 25 females (29.1%) were included in the analysis.

Chi-square test of independence or one-tailed Fisher exact probability test was used to assess the incidence of resistance to selected antimicrobials, to analyze resistance mechanisms and to investigate determinants of resistance among *K. pneumoniae* strains isolated from two age groups of patients. The significance level was assumed as $P<0.05$. All statistical analyses were performed using StatsDirect, version 2.7.9 (StatsDirect Ltd., Cheshire, UK). The study was approved by the Ethics Committee of the Jagiellonian University Medical College (KBET/129/B/2011).

Phenotypic characterization of Klebsiella pneumoniae strains

The initial species identification, performed by the Rydygier Memorial Hospital laboratory staff as part of routine practice, was based on the analysis of biochemical profiles of the isolates using the Vitek 2 Compact system with GN ID card (bioMérieux, Marcy l'Étoile, France). Bacterial strains isolated from the Children's Hospital patients were biochemically identified by the API 20E test (bioMérieux, Marcy l'Étoile, France), according to the manufacturer's instructions.

In vitro susceptibility of *K. pneumoniae* isolates, recovered from adults, to antibacterial agents (including amoxicillin with clavulanic acid, piperacillin with tazobactam, cefoxitin, imipenem, amikacin, gentamicin, norfloxacin, ciprofloxacin, tetracycline, and trimethoprim with sulfamethoxazole) was analyzed by the Vitek 2 Compact semiquantitative system, according to the guidelines of the Clinical and Laboratory Standards Institute criteria and interpret-

ed according to the CLSI breakpoints (CLSI 2008). The reference strain was *Escherichia coli* ATCC 25922.

The drug susceptibility of *K. pneumoniae* strains cultured from children was determined by the Kirby-Bauer technique. Discs containing the following antibacterial agents were used: piperacillin (30 µg), piperacillin/tazobactam (30/6 µg), cefotaxime (5 µg), ceftazidime (10 µg), ceftriaxone (30 µg), cefoxitin (30 µg), cefepime (30 µg), aztreonam (30 µg), gentamicin (10 µg), amikacin (30 µg), tobramycin (10 µg), norfloxacin (10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg) and trimethoprim with sulfamethoxazole (1.25/23.75 µg) (Oxoid, Basingstoke, United Kingdom). The MIC values for imipenem, meropenem, ertapenem, doripenem, colistin and tigecycline were assessed by the Etest method (bioMérieux, Marcy l'Étoile, France).

A total of 170 isolates were tested for ESBL using the double-disk synergy test (Drieux *et al.,* 2008). *Escherichia coli* ATCC 25922 served as a positive and *K. pneumoniae* ATCC 700603 as a negative control of ESBL production. The strains were also screened for the presence of metallo-beta-lactamases (MBL) by phenotypic EDTA double-disc synergy test (Lee *et al.,* 2003) and for KPC (*Klebsiella pneumoniae* carbapenemase) with 3-aminophenyl boronic acid as an inhibitor of KPC-type beta-lactamases (Doi *et al.,* 2008). The presence of OXA-48 carbapenemases was evaluated using the temocillin disc (10 µg) (Argenta, Poznań, Poland) (van Dijk *et al.,* 2014).

Molecular characterization of Klebsiella pneumoniae strains

Molecular tests were conducted at the Department of Pharmaceutical Microbiology of the Jagiellonian University Medical College. Bacterial DNA was extracted with the Genomic Mini isolation kit (A&A Biotechnology, Gdynia, Poland), according to the manufacturer's instructions.

Confirmation of species identification

The species identification of all 170 isolates was confirmed using the PCR molecular assay with Mdh-F (5'-GCGTGGCGGTAGATCTAAGTCATA-3') and Mdh-R (5'-TTCAGCTCCGCCACAAAGGTA-3') species-specific primers that target the *mdh* gene, as previously described (Thong *et al.,* 2011) (364 bp). The PCR conditions were as follows: initial denaturation at 95 C for 5 min, 30 cycles of denaturation at 96 C for 1 min, annealing at 52 C for 30 s, and extension at 72 C for 1 min, followed by a final extension at 72 C for 10 min. The reaction was performed in a volume of 25 µl, containing: 1×PCR buffer, 0.2 mM each of dATP, dGTP, dCTP, and dTTP (Promega, Madison, Wisconsin, USA), 0.4 µM of each of the primers (Genomed, Warszawa, Poland), 40 ng of genomic DNA, 2 mM $MgCl_2$, and 0.625 U of *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA).

Detection of selected ESBL-encoding genes

The genes encoding CTX-M-1-like, TEM-like and SHV-like ESBLs were detected in all of the analyzed *K. pneumoniae* bacilli. Molecular identification of $bla_{\text{CTX-M-1}}$ genes was performed by the monoplex PCR assay with P1C (5'-TTAATTCGTCTCTTCCAGA-3') and P2D (5'-CAGCGCTTTTGCCGTCTAAG-3') oligonucleotides. TEM-A (5'-ATAAAATTCTTGAAGAC-3') and TEM-B (5'-TTACCAATGCTTAATCA-3') primers were used for amplification of bla_{TEM} genes, while bla_{SHV} genes were detected with SHV-A (5'-ACTGAATGAGGCGCTTCC-3') and SHV-C (5'-CGCACCCCGCTTGCT-3') primers. Two different PCR conditions were used. The PCR assay for $bla_{\text{CTX-M-1}}$ and bla_{SHV} was conducted with the following cycle profile: initial denaturation at 95 C for 2 min, followed by 40 cycles at 94 C for 30 s, 55 C for 30 s, 72 C for 45 s, and a final extension at 72 C for 5 min. Amplification of bla_{TEM} genes was performed under the following conditions: denaturation at 95 C for 3 min, followed by 30 cycles consisting of denaturation at 94°C for 15 s, annealing at 42 C for 30 s, elongation at 72 C for 30 s and a final extension at 72 C for 7 min (Gniadkowski *et al.,* 1998a; Gniadkowski *et al.,* 1998b). The PCR products of the expected sizes of 970 bp, 1070 bp and 220 bp for $bla_{\text{CTX-M-1}}$, bla_{TEM} and bla_{SHV} genes, respectively, were obtained. All amplifications were carried out in a T personal thermocycler (Biometra, Göttingen, Germany). PCR amplicons were separated by 2% agarose gel electrophoresis and then visualized with ethidium bromide staining (Sigma-Aldrich Chemie, Munich, Germany).

RESULTS

Phenotypic characterization of Klebsiella pneumoniae strains

A total of 170 *K. pneumoniae* strains included in the study were isolated over several years (2008 - 2015); therefore, their susceptibility was tested against different antimicrobials and the isolates had different phenotypical characteristics (*Table 1*). The levels of resistance to the antimicrobials tested were compared in the groups of strains (isolated from adults and children) to determine the statistical significance of our results. In general, pediatric *K. pneumoniae* isolates showed a significantly higher resistance level to piperacillin/tazobactam, cefoxitin, imipenem, amikacin and ciprofloxacin than strains obtained from adults ($P<0.05$). Furthermore, there were no significant differences in the resistance rates to gentamicin, norfloxacin and trimethoprim/sulfamethoxazole between children and adults ($P>0.05$) (*Table 1*).

Beta-lactams, except for carbapenems, showed low activity against strains recovered from children. Out of the remaining antimicrobials tested, only colistin and tigecycline showed good *in vitro* activity against pediatric *K. pneumoniae* isolates. On the contrary, when considering *K. pneumoniae* bacilli isolates from Rydygier Memorial Hospital, both imipenem and amikacin might be considered treatment options (*Table 1*).

While we have confirmed phenotypically the presence of ESBLs in 80.6% (137/170) of the tested strains, OXA-48, KPC and MBL carbapenemases occurred in 21.8% (37/170), 8.2% (14/170) and 2.4% (4/170), respectively, of isolates. It is worth noting that, while ESBLs were present statistically significantly more often in *K. pneumoniae* from adults, OXA-48 and KPC enzymes occurred more frequently in *K. pneumoniae* from children ($P<0.05$) (*Table 2*). According to the definition proposed by a group of international experts headed by Magiorakos, all examined strains exhibited multidrug-resistant phenotypes, i.e., acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories (Magiorakos *et al.,* 2012).

Molecular characterization of Klebsiella pneumoniae strains

All isolates included in this study were confirmed as *K. pneumoniae* by amplification of the *mdh* gene. ESBL

genes were found in 94.7% (161/170) of the tested strains of MDR *K. pneumoniae*, while 5.3% (9/170) did not carry any of the amplified genes. In our study, the CTX-M-1 enzymes were predominant, followed by TEM and SHV detected in 91.2% (155/170), 63.5% (108/170) and 11.8% (20/170), respectively, of isolates. We have reported the differential occurrence of ESBL genes among *K. pneumoniae* strains recovered from patients of various ages, e.g., bla_{TEM} were statistically significantly more common in adults than in children ($P<0.05$). No significant differences were

Table 1 - *Phenotypic characterization of Klebsiella pneumoniae strains recovered from pediatric and adult patients of two Polish hospitals, including susceptibility to selected antimicrobials.*

Antibiotics / chemotherapeutic agents	*K. pneumoniae strains from children (n=84)*			*K. pneumoniae strains from adult (n=86)*			*P value*
	No. [%]			*No. [%]*			
	R	*I*	*S*	*R*	*I*	*S*	
Piperacillin	84 (100.0)	0 (0.0)	0 (0.0)	–	–	–	–
Amoxicillin with clavulanate	–	–	–	64 (75.3)	18 (21.2)	3 (3.5)	–
Piperacillin with tazobactam	67 (79.8)	15 (17.8)	2 (2.4)	46 (53.5)	21 (31.4)	13 (15.1)	0.0002
Cefoxitin	66 (78.6)	0 (0.0)	18 (21.4)	19 (22.1)	8 (9.3)	59 (68.6)	<.0001
Cefotaxime	84 (100.0)	0 (0.0)	0 (0.0)	–	–	–	–
Ceftazidime	84 (100.0)	0 (0.0)	0 (0.0)	–	–	–	–
Ceftriaxone	66 (95.6)	2 (2.9)	1 (1.5)	–	–	–	–
Cefepime	81 (96.4)	0 (0.0)	3 (3.6)	–	–	–	–
Meropenem	3 (5.6)	18 (33.3)	33 (61.1)	–	–	–	–
Imipenem	6 (11.1)	10 (18.5)	38 (70.4)	0 (0.0)	0 (0.0)	86 (100.0)	0.0027
Doripenem	10 (19.6)	11 (21.6)	30 (58.8)	–	–	–	–
Ertapenem	29 (53.7)	2 (3.7)	23 (42.6)	–	–	–	–
Aztreonam	83 (98.8)	1 (1.2)	0 (0.0)	–	–	–	–
Amikacin	23 (27.4)	31 (36.9)	30 (35.7)	9 (10.5)	1 (1.2)	76 (88.3)	0.0047
Gentamicin	60 (71.4)	2 (2.4)	22 (26.2)	52 (60.5)	1 (1.2)	33 (38.3)	0.1319 NS
Tobramycin	60 (87.0)	0 (0.0)	9 (13.0)	–	–	–	–
Ciprofloxacin	71 (84.5)	3 (3.6)	10 (11.9)	60 (69.8)	19 (22.1)	7 (8.1)	0.0222
Levofloxacin	64 (76.2)	6 (7.1)	14 (16.7)	–	–	–	–
Norfloxacin	73 (88.0)	5 (6.0)	5 (6.0)	70 (81.4)	0 (0.0)	16 (18.6)	0.2253 NS
Trimethoprim/sulfamethoxazole	73 (86.9)	4 (4.8)	7 (8.3)	78 (90.7)	0 (0.0)	8 (9.3)	0.4310 NS
Colistin	2 (8.7)	0 (0.0)	21 (91.3)	–	–	–	–
Tetracycline	–	–	–	12 (14.0)	10 (11.6)	64 (74.4)	–
Tigecycline	5 (19.2)	2 (7.7)	19 (73.1)	–	–	–	–

P value Chi-square test of Independence ($\chi 2$) or one-tailed Fisher exact probability test; P <0.05 was deemed statistically significant; NS - non significant; R - resistant; I - intermediate; S - susceptible.

Table 2 - *Phenotypic characterization of Klebsiella pneumoniae strains recovered from pediatric and adult patients of two Polish hospitals, including occurrence of ESBL, OXA-48, KPC, and MBL resistance mechanisms.*

Phenotypically confirmed resistance mechanism	*K. pneumoniae strains from children (n=84)*		*K. pneumoniae strains from adults (n=86)*		*P value*
	No.	*[%]*	*No.*	*[%]*	
ESBL	51	60.7	86	100.0	<.0001
OXA-48	30	35.7	7	8.1	<.0001
KPC	14	16.7	0	0.0	<.0001
MBL	4	4.8	0	0.0	0.0574 NS

P value Chi-square test of Independence ($\chi 2$) or one-tailed Fisher exact probability test; P <0.05 was deemed statistically significant; NS - non significant.

Table 3 - *Prevalence of genes encoding the most commonly revealed ESBL families in Poland: $bla_{CTX\text{-}M\text{-}1}$, bla_{TEM}, and bla_{SHV} in Klebsiella pneumoniae strains obtained from pediatric and adult patients of two Polish hospitals.*

Gene family	*K. pneumoniae strains from children (n=84)*		*K. pneumoniae strains from adults (n=86)*		*Summary*		*P value*
	No.	*[%]*	*No.*	*[%]*	*No.*	*[%]*	
$bla_{CTX\text{-}M1}$	77	58.8	78	51.3	155	54.8	0.8230 NS
bla_{TEM}	43	32.8	65	42.8	108	38.1	0.0009
bla_{SHV}	11	8.4	9	5.9	20	7.1	0.5967 NS
Total	131	100.0	152	100.0	283	100.0	–

P value Chi-square test of Independence ($\chi 2$); P < 0.05 was deemed statistically significant; NS - non significant.

Table 4 - *General comparison of the occurrence of single or multiple (encoding ESBLs of two or three different families) beta-lactamase genes among Klebsiella pneumoniae strains obtained from pediatric and adult patients of two Polish hospitals.*

Beta-lactamase genes	*K.pneumoniae strains from children (n=84)*		*K. pneumoniae strains from adults (n=86)*		*P value*
	No.	*[%]*	*No.*	*[%]*	
Single beta-lactamase gene	37	44.0	15	17.5	0.000167
Multiple beta-lactamase genes	43	51.2	66	76.7	0.000515
None of beta-lactamase genes	4	4.8	5	5.8	0.5148 NS
Total	84	100	86	100	-

P value Chi-square test of Independence ($\chi 2$) or one-tailed Fisher exact probability test; $P < 0.05$ was deemed statistically significant; NS - non significant.

Table 5 - *Detailed comparison of the occurrence of single or multiple (dual or triple) beta-lactamase genes among Klebsiella pneumoniae strains obtained from pediatric and adult patients of two Polish hospitals.*

Gene family/families	*K. pneumoniae strains from children (n=84)*		*K. pneumoniae strains from adults (n=86)*		*P value*
	No.	*[%]*	*No.*	*[%]*	
$bla_{CTX\text{-}M1}$	36	42.9	13	15.1	<.0001
$bla_{CTX\text{-}M1}$, bla_{TEM}	33	39.3	58	67.4	0.0002
$bla_{CTX\text{-}M1}$, bla_{TEM}, bla_{SHV}	8	9.5	6	7.0	0.5485 NS
absence of $bla_{CTX\text{-}M1}$, bla_{TEM}, bla_{SHV}	4	4.7	5	5.8	0.5148 NS
bla_{TEM}, bla_{SHV}	2	2.4	1	1.2	0.4911 NS
bla_{SHV}	1	1.2	2	2.3	0.5088 NS
$bla_{CTX\text{-}M1}$, bla_{SHV}	0	0.0	1	1.2	0.5058 NS
Total	84	100.0	86	100.0	-

P value Chi-square test of Independence ($\chi 2$) or one-tailed Fisher exact probability test; $P < 0.05$ was deemed statistically significant; NS - non significant.

observed in the occurrence of $bla_{\text{CTX-M-1}}$ or bla_{SHV}, either in the pediatric or in the adult population ($P>0.05$) (*Table 3*). The single ESBL gene occurred in 44.0% (37/84) and 17.5% (15/86), while multiple ESBL genes were harbored by 51.2% (43/84) and 76.7% (66/86), respectively, of *K. pneumoniae* strains taken from children and adults (*Table 4*). It is worth emphasizing that *K. pneumoniae* bacilli carrying only one of the detected ESBL genes occurred significantly more frequently in patients admitted to the University Children's Hospital ($P<0.05$), whereas strains producing two or three *bla* genes of different ESBL families were most often found in individuals from the Rydygier Memorial Hospital ($P<0.05$) (*Table 4*).

Our analysis of the co-occurrence of the detected ESBL-encoding genes among *K. pneumoniae* has revealed that the most common phenomenon is the co-existence of two genes, observed in 55.9% (95/170) of the isolates ($bla_{\text{CTX-M-1}}$ and bla_{TEM} in 53.5%; bla_{TEM} and bla_{SHV} in 1.8%; $bla_{\text{CTX-M-1}}$ and bla_{SHV} in 0.6%). Interestingly, the strains possessing both $bla_{\text{CTX-M-1}}$ and bla_{TEM} genes occurred significantly more often in adult individuals than in children ($P<0.05$). We have even observed that 8.2% (14/170) of strains harbored $bla_{\text{CTX-M-1}}$, bla_{TEM} and bla_{SHV} genes simultaneously. Nevertheless, considering isolates having only a single beta-lactamase gene, we noted a predominance of $bla_{\text{CTX-M-1}}$ (28.8%; 49/170), which could be detected more often in *K. pneumoniae* strains isolated from the pediatric population ($P<0.05$) (*Table 5*).

DISCUSSION

Gram-negative bacteria are currently considered the most critical therapeutic and epidemiological issue, responsible for growing levels of hospital-acquired infections. One of the most commonly isolated pathogens from this group is *K. pneumoniae*, which is also one of the species most often expressing an ESBL phenotype, usually accompanied by lack of activity of other classes of antimicrobials (Baroud *et al.,* 2013, Paczosa and Mecsas, 2016). In this work, we studied 170 clinical strains of MDR ESBL-producing *K. pneumoniae* collected from 2008 to 2015 from patients of two hospitals in Southern Poland. We determined their susceptibility to antimicrobials and identified the presence of resistance mechanisms. The occurrence of MDR *K. pneumoniae* was compared in two groups of patients: children and adults.

In our research, phenotypic testing of *K. pneumoniae* strains isolated from cases of gastrointestinal tract colonization and different sites of infection revealed that most of them were ESBL-producers (60.7% and 100.0%, respectively). In both groups of these isolates, we also detected OXA-48 (21.8%), KPC (8.2%), and MBL (2.4%) carbapenemases, although their production was not confirmed by molecular methods. Similar results were presented by Sękowska *et al.* (2014), who observed that 74.4% of *K. pneumoniae* strains isolated from rectal swabs produced ESBL. Furthermore, as in our study, co-occurrence of more than one beta-lactamase in a single isolate was confirmed, e.g., ESBL and MBL. Therefore, the monitoring of incidence of ESBL-positive *K. pneumoniae* strains seems to be crucial because colonization of the gastrointestinal tract is an important risk factor for the development of infections with strains with this phenotype. Pre-emptive screening for MDR Enterobacteriaceae, performed during admission to hospital, might become necessary to reduce the prevalence of these highly resistant bacteria in Polish hospitals (Sękowska *et al.,* 2012; Sękowska *et al.,* 2014).

An alarming level of drug resistant *K. pneumoniae* isolates, in which different resistance mechanisms have cumulated, has dramatically reduced the treatment options for nosocomial infections (Arhoune *et al.,* 2017). Abuse of carbapenems, effective against ESBL-producers, has re-

sulted in a shocking increase of resistance to this group of beta-lactams (Ruiz *et al.,* 2012; Marr and Russo, 2019). The data obtained in our investigations showed that carbapenems exhibited high activity against MDR *K. pneumoniae* strains, and thus remain the drugs of choice for the treatment of MDR ESBL-producing *K. pneumoniae* infections. Our observations are in line with a study by other investigators (Sękowska *et al.,* 2014; Mansury *et al.,* 2016). Furthermore, as long as tigecycline retains activity against most ESBL-producing Enterobacteriaceae (73.1% of susceptible MDR *K. pneumoniae* pediatric strains), it may be used as a possible treatment option for infections caused by ESBL-positive strains (Sekowska and Gospodarek, 2010).

In our analysis, including amplification of the most commonly reported ESBL genes ($bla_{\text{CTX-M-1}}$, bla_{SHV} and bla_{TEM}), the predominant enzymes were CTX-M-1 (91.2%). While SHV beta-lactamases are considered the least common (11.8%), the detection of TEM was confirmed in 63.5% of all isolates tested. A comparable prevalence of CTX-M-1 (58.8% and 51.3%) and SHV (8.4% and 5.9%) beta-lactamases was observed in *K. pneumoniae* strains taken from children and adults, respectively. However, the incidence of TEM enzymes was significantly higher in the adult population (42.8% versus 32.8% in pediatric patients).

Results of a national survey on the presence of ESBLs among the Enterobacteriaceae strains in 13 Polish hospitals showed that CTX-M-like ESBLs dominated (81.7%; CTX-M-3 and CTX-M-15), followed by SHV-like (17.5%; SHV-2, SHV-5 and SHV-12) and TEM-like enzymes (0.7%; TEM-19 and TEM-48) (Empel *et al.,* 2008). Likewise, the results of a survey conducted in Spain revealed a high rate of CTX-M-positive *K. pneumoniae* strains (75.9%) (Onnberg *et al.,* 2011).

Compared to our results, the prevalence of bla_{SHV}, $bla_{\text{CTX-M}}$ and bla_{TEM} genes in *K. pneumoniae* in Iran was lower (22.2%, 19.0% and 16.0%, respectively) (Mansury *et al.,* 2016). While ESBLs were detected phenotypically in 51.2% of 73 enterobacterial strains analyzed in India, they were confirmed by PCR technique only in 38.4% of them; 39.2% of the tested *K. pneumoniae* rods harbored $bla_{\text{CTX-M}}$, 25.0% bla_{SHV} and 17.9% bla_{TEM} genes. It is noteworthy that the frequency of occurrence of *bla* genes was higher in *K. pneumoniae* compared to those in *E. coli* (Shahid *et al.,* 2011). Our results also contrasted with the data reported previously by Liao *et al.* (2017), in which they found that the bla_{SHV} gene was the predominant ESBL genotype (74.5%) among 47 *K. pneumoniae* strains from China. CTX-M enzymes were detected more rarely (25.5%), while no TEM-type strain was detected (Liao *et al.,* 2017). Another study performed in Macedonia also disclosed the predominance of SHV (73.2%; 30/41) and CTX-M (65.9%; 27/41) ESBLs among *K. pneumoniae* isolates. Additionally, multiple ESBL genes occurred more frequently (58.5%) than a single ESBL gene (41.5%) in this group of strains (Kaftandzieva *et al.,* 2011).

Data regarding the occurrence and diversity of various types of ESBLs among Enterobacteriaceae isolated from pediatric patients are scarce (Chandramohan and Revell, 2012). Although ESBL producers cause neonatal infections less frequently than do Gram-positive cocci, they are responsible for a higher mortality rate among these patients (Wójkowska-Mach *et al.,* 2013). Another clinical trial, conducted in six Polish neonatal intensive care units, showed that the most common enterobacterial species isolated from neonates was *E. coli* (12.4%), followed by *Klebsiella* spp. (9.1%) and *Enterobacter* spp. (4.2%). The ESBL phenotype was found in 37% of isolates (mainly in *K. pneumoniae*), of which 89.3% produced CTX-M-type, 70.2% TEM-type and 8.5% SHV-type enzymes. Furthermore, the most common coexisting genes were $bla_{\text{CTX-M}}$ and bla_{TEM}, as in our analysis (Wójkowska-Mach *et al.,* 2013).

Our results were in line with a study conducted in Texas (USA), whose authors confirmed the predominance of CTX-M beta-lactamases in Enterobateriaceae isolated from children. While in a group of 94 strains, including *E. coli*, *Klebsiella* spp., *Enterobacter* spp. and others, the most commonly isolated ESBL-type was CTX-M (74%; 70/94), followed by TEM (27%; 26/94) and SHV (24%; 23/94); the SHV-type dominated in *Klebsiella* spp. (Chandramohan and Revell, 2012). Contrary to these findings, the predominant gene detected in 84.8% of the 138 ESBL-producing *K. pneumoniae* isolates obtained from pediatric inpatients in China was bla_{TEM}, followed by bla_{SHV} (50.7%) and $bla_{\text{CTX-M-1}}$ (31.9%) (Li *et al.,* 2014).

The present study proved a high prevalence (64.1%; 109/170) of dual or even triple beta-lactamase genes in single *K. pneumoniae* isolates. This is consistent with the results of other authors (Baraniak *et al.,* 2002; Dzierżanowska *et al.,* 2010; Shahid *et al.,* 2011; Chandramohan and Revell, 2012; Wójkowska-Mach *et al.,* 2013; Mansury *et al.,* 2016). However, some researchers did not find any strain with accumulation of all three beta-lactamase determinants (Baraniak *et al.,* 2002; Shahid *et al.,* 2011).

In another study conducted in Poland, among 110 ESBL-producing *K. pneumoniae* clinical isolates collected from three pediatric hospitals, 41.8% (46/110) produced a single ESBL, while 58.2% (64/110) strains harbored multiple ESBLs, including 53.7% (59/110) with *bla* genes for ESBLs of two families and 4.5% (5/110) with *bla* genes of three families (CTX-M, SHV and TEM) (Dzierżanowska *et al.*, 2010). Our report also revealed the co-existence of dual (55.9%) or triple (8.2%) beta-lactamase genes encoding ESBLs , which might suggest that in Poland the accumulation of *bla* genes for different ESBLs in *K. pneumoniae* has increased (Dzierżanowska *et al.,* 2010). These findings were also consistent with observations of *K. pneumoniae* strains obtained from pediatric patients in China (Li *et al.,* 2014).

Our study highlights the increasing problem of ESBL-producing Enterobacteriaceae that cause infections in both pediatric and adult patients. It is probable that the high resistance levels of isolates included in our study might be associated with the reference status of hospitals to which the most severely ill patients were admitted from the entire region of Southern Poland. In general, these patients had been previously hospitalized and underwent antibiotic therapy. We have demonstrated the predominance of CTX-M-type enzymes among tested *K. pneumoniae* isolates. The most alarming aspect is the detection of strains possessing dual or triple *bla* genes in the analyzed collection of isolates, which might be explained by the high mobility of plasmid-located genes that encode ESBLs and are easily transferred among clinical strains and between species in nature.

In conclusion, higher resistance rates to tested antimicrobials as well as more frequent occurrence of carbapenemases was demonstrated among *K. pneumoniae* strains isolated from children compared to strains recovered from adults, in whom the most common resistance mech-

anism was ESBL beta-lactamases. The differences in drug susceptibility revealed between the two groups of isolates may result from different times of isolation of the tested strains: from adults - 2008-2010 and from children - 2010-2015. Since then, antibiotics policies and the use of antimicrobials to treat infections have changed, which may also have influenced the phenotypic and genotypic characteristics of the analyzed strains. Differences in antimicrobial susceptibility and the prevalence of resistance mechanisms between the *K. pneumoniae* strains isolated from children and from adult patients observed in this report may also result from various sites of collection of clinical specimens (perianal swabs versus samples taken from site of infection) and not from the tested population. However, it should be emphasized that in the limited number of pediatric studies performed worldwide, our study provides important insights into the epidemiology and resistance of *K pneumoniae* strains isolated from children. Further epidemiological analysis and local studies are required to clarify the dissemination and circulation of different EBSLs, which are viewed as a global public health problem. Considering the dramatic growth in resistance among *K. pneumoniae* isolates, screening for gastrointestinal colonization with this pathogen should be a mandatory component of infection control strategies.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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