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Profiles of phenotype resistance to antibiotic other than β-lactams in *Klebsiella pneumoniae* ESBLs-producers, carrying *bla*$_{SHV}$ genes

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Abstract: Extended spectrum β-lactamases production is one of the most common mechanism of resistance to extended spectrum β-lactam antibiotics is increasing worldwide. Twenty five strains of *Klebsiella pneumoniae* isolated from clinical specimens were tested. Based on the phenotypic confirmatory test all these strains were defined as ESBL producers named ESBL(+). The plasmid DNA from each strains was used to investigate the presence of *bla*$_{SHV}$ genes responsible for extended spectrum β-lactamases production. Moreover, susceptibility of these strains to antibiotic other than β-lactams in was tested.

Keywords: *Klebsiella pneumoniae*; *bla*$_{SHV}$ genes; extended spectrum β-lactamases; plasmid DNA.

Introduction

Extended spectrum β-lactamases (ESBLs) belong to a wide group of enzymes named β-lactamases. A large number of these enzymes and a need for their understanding caused that tries of their classification have been undertaken. At present, two systems of β-lactamases classification are commonly used. One based on the molecular structure of the enzyme and the second on their functional characteristics [1]. The presence of serine at the active site in ESBLs classify them into A-molecular class of Ambler scheme. Whereas, characteristic activity properties caused classification of the majority of ESBLs into 2be group whereby the Bush-Jacoby-Medeiros scheme. Generally β-lactam enzymes hydrolyze antibiotics which include β-lactam ring [2]. ESBLs are able to hydrolyze penicillins, cephalosporins of the first, second, third and fourth generation. In addition, they are capable of hydrolyzing monobactam, but not cephamycins or carbapenems. Moreover, ESBLs are easily inhibited by the β-lactamase inhibitors such as clavulanic acid or sulbactam [3].

As extended spectrum β-lactamases constitute a wide group of enzymes likewise genes number which expression is necessary for synthesis of appropriate enzymes. A range group of genes responsible for ESBLs synthesis belong mainly into *bla*$_{SHV}$ and *bla*TEM families [4]. ESBLs are common identified among clinical *Enterobacteriaceae*, frequently in *Klebsiella pneumoniae*. *Klebsiella pneumoniae* resistance to β-lactam antibiotics caused by ESBLs production is important problem in treatment of infections [5]. β-lactam antibiotics because of high efficacy and low toxicity were frequently used in therapy. Nowa-

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days in connection with the fact that penicillins, cephalosporines and carbapenems are included in substrate spectrum of ESBLs a significant restriction of therapeutic possibilities for use of these antibiotics has been observed [6].

The aim of this study was to analyze the presence of bla_{SHV} genes in *Klebsiella pneumoniae* strains using genetically methods. An attempt to define the most frequent profiles of phenotype resistance to antibiotics other than β-lactams in *Klebsiella pneumoniae* ESBLs producers has been undertaken. Moreover, we try to establish a relationship between antibiotic susceptibility patterns and presence of particular bla_{SHV} genes in *Klebsiella pneumoniae* strains.

Materials and methods

Bacterial isolates. Twenty five strains of *Klebsiela pneumoniae* isolated from clinical specimens of patients hospitalized at the University Hospital in Bialystok (Poland) were included in this study. Identification of the strains was performed with using rapid ID 32 E strips for identification in ATB automated system (BioMerieux). Control strains used in this study included *Klebsiella pneumoniae* ATCC 700603, *Escherichia coli* ATCC 35218 and *Escherichia coli* ATCC 25922.

Antibiotic susceptibility. The minimal inhibitory concentrations (MICs) of antibiotics were determined in Mueller-Hinton broth by a microdilution method according to the recommendations of CLSI (Clinical and Laboratory Standards Institute) [7]. MICs of the following antibiotics were tested: gentamicin, (Gentamycin TZF, Polfa-Tarchomin), ciprofloxacin (Ciprinol, Krka), tetracycline (Sigma), tigecycline (Tygacil, Wyeth Pharmaceuticals). The range of antibiotics concentrations used in the study were from 0.032 μg/ml to 512 μg/ml.

Screening of ESBL-producing isolates. ESBLs production was screened by the DDST method (double-disc diffusion test) according with CLSI (Clinical and Laboratory Standards Institute) recommendations [7]. Tests were performed on Mueller-Hinton agar (Oxoid) with using 30μg antibiotic discs of ceftazidime, cefotaxime, cefepime and aztreonam placed 20 mm from discs containing 30μg of amoxicillin/clavulanic acid (Becton, Dickinson and Company). A positive result of test was indicated by changes of the inhibition zones.

Plasmid DNA isolation. *Klebsiella pneumoniae* strains were cultured overnight on TSB (*Trypticase Soy Broth*, Emapol) at 37°C and plasmids DNA extraction from *Klebsiella pneumoniae* isolates was performed with the Plasmid Mini (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's instruction.

Molecular detection of the bla_{SHV} genes. PCR assays were carried out with the Cyclone 96 (PEQLAB Biotechnology, GmbH) thermal cycler. PCR amplification of the bla_{SHV} genes was performed using the oligonucleotide primers designed on the basis of the nucleotide sequence reported in the National Center for Biotechnology Information (NCBI) GenBank database. Primers used in PCR amplification: forward primer: bla_{SHV}f 5'-CCCGCAGCCGCTTGAGCAAA-3' and reverse primer: bla_{SHV}r 5'-CATGCTCGCCGGCGTATCCC-3'. The PCR mixture, in a final volume of 25 μl, contained: 10 pmol/μl of each primer (1μl), 12.5 μl of 2x PCR RED Master Mix (DNA-Gdansk), 3 μl of template DNA and 7.5 μl of ultra pure H_2O. The following conditions for PCR amplification were used: denaturation at 94°C for 60 s, annealing at 57°C for 60 s and extension at 72°C for 60 s repeated for 35 cycles and final extension at 72°C for 10 min.

Detection of PCR products and sequence analysis. PCR amplicons were separated electrophoretically at 5 V/cm for 90 min in 1.5% agarose gel containing 0.5 μg/ml of ethidium bromide in TBE buffer and photographed using the Bio Rad ChemiDoc XRS imaging system. Then, the positions of amplification products were estimated with the position of the molecular weight marker.

PCR products with a length of 733 bp were purified from the agarose gel using Gel-Out kit (A&A Biotechnology, Gdynia, Poland) and then sequenced using an Applied Biosystems model 3130xls Genetic Analyser. A nucleotide sequences from bla_{SHV} gene were analyzed and compared with sequences available in the National Center for Biotechnology Information (NCBI) GenBank database.

Statistical analysis. Statistical analysis was performed with using Statistica 8.0 (StatSoft) software. Values of $p<0.05$ were considered as statistically significant.

Results and discussion

The aim of this study was to investigate an antibiotic susceptibility of twenty five *Klebsiella pneumoniae* strains which were defined as ESBL-producers based on phenotypic test with using the double disc synergy test. Minimal inhibitory concentrations of gentamicin, ciprofloxacin, tetracycline and tigecycline were determined for all these strains.

Among ESBL-producing isolates we observed 80% isolates were susceptible to ciprofloxacin. Furthermore, all tested isolates showed a high rate of resistance to gentamicin. Other investigators showed similar susceptibility of *Klebsiella pneumoniae* EBLs-producers to ciprofloxacin and relationship between production of ESBLs and resistance to aminoglycosides [8 9]. Moreover, 68% of tested strains were susceptibility to tetracycline. Additionally, in this study we obtained high susceptibility of ESBLs-producers to tigecycline. 88% of the isolates were susceptibility to tigecycline. Other investigators describe such high activity of tigecycline to *Klebsiella pneumoniae* ESBLs-producers [10]. Statistical analysis reveal statistically significant difference in the distribution of MICs between follows antibiotics: tetracycline and ciprofloxacin, tetracycline and gentamicin, ciprofloxacin and gentamicin, tigecycline and gentamicin. It is important to note that no statistically significant difference was observed between distribution of tetracycline and tigecycline MIC values, but low MIC_{50} and MIC_{90} values of tigecycline may indicate on high activity of this antibiotic on ESBLs-producers.

Because of high variety of bla_{SHV} genes family it was interesting to determine what variants of these genes were carried by tested *Klebsiella pneumoniae* strains [11]. Therefore, in the next part of this study a sequencing of amplicons was carried out.

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Table 1. Distribution of minimal inhibitory concentrations of tetracycline, ciprofloxacin, gentamicin and tigecycline in *Klebsiella pneumoniae* ESBL (+) strains. The percentage of ESBLs-producing strains which were susceptible, intermediate and resistant to tested antibiotics.

Antibiotic	Distribution of the MIC (μg/mL)															MIC_{50}	MIC_{90}	Range of MIC	S	I	R
	0.032	0.064	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	512						
Tetracycline	-	-	-	-	-	8	5	4		3	-	3	-	2	-	2	64	1 – 256	68%	-	32%
Ciprofloxacin	3	-	-	3	7	7	-	1	-	2	-	1	-	1	-	0.5	16	0.032 – 256	80%	-	20%
Gentamicin	-	-	-	-	-	-	-	-	-	-	4	3	2	2	14	512	512	32 – 512	-	-	100%
Tigecycline	-	-	-	-	-	3	19	-	1	2	-	-	-	-	-	2	8	1 – 16	88%	-	12%

Abbreviations: MIC – minimal inhibitory concentration; MIC_{50} – minimal inhibitory concentration required to inhibit the growth of 50% of organisms; MIC_{90} – minimal inhibitory concentration required to inhibit the growth of 90% of organisms; S – susceptible; I – intermediate; R – resistant.

Table 2. The presence of bla_{SHV} genes responsible for production of particular extended spectrum β-lactamases from SHV family in strains of *Klebsiella pneumoniae* ESBL-producers.

SHV types	n	Antibiotic susceptibility [%]											
		CIP			GM			TC			TG		
		R	I	S	R	I	S	R	I	S	R	I	S
SHV-2	7	14%	-	86%	100%	-	-	57%	-	43%	14%	-	86%
SHV-5	8	25%	-	75%	100%	-	-	12.5%	-	87.5%	12.5%	-	87.5%
SHV-7	2	100%	-	-	100%	-	-	-	-	100%	-	-	100%
SHV-12	2	-	-	100%	100%	-	-	-	-	100%	-	-	100%
SHV-18	6	-	-	100%	100%	-	-	17%	-	83%	83%	-	17%

Abbreviations: n – number of strains; TG – tigecycline; CIP – ciprofloxacin; TC – tetracycline; GM – gentamicin. S – susceptible; I – intermediate; R – resistant.

Genetic study revealed, that among strains of *Klebsiella pneumoniae* with phenotypic confirmatory production of ESBLs, extended spectrum β-lactamases-encoding bla_{SHV} genes were present. The sequencing showed that the majority of DNA isolates from ESBLs producers carried bla_{SHV-5} genes (eight strains), seven isolates contained bla_{SHV-2} genes, six strains carried bla_{SHV-18} genes. Moreover, two isolates contained genes for SHV-7 and two others for SHV-12. Epidemiological survey in Europe show that the most frequent bla_{SHV} genes responsible for extended spectrum β-lactamases production are bla_{SHV-5}, bla_{SHV-12}, bla_{SHV-2} [12,13].

We investigate relationship between antibiotic susceptibility and the presence of SHV types. In the case of SHV-2 ESBLs isolates, resistance rates were as follows: tigecycline 14%, cirofloxacin 14%, gentamicin 100% and tetracycline 57%. All the SHV-5 ESBLs-producing *Klebsiella pneumoniae* strains were susceptible to tigecycline (87.5%), ciprofloxacin (75%), and tetracycline (87.5%). All strains carrying bla_{SHV-7} gene were susceptible to tigecycline and tetracycline and resistant to ciprofloxacin and gentamicin. Reported isolates possessing the ESBLs SHV-12 were susceptible to tigecycline, ciprofloxacin and tetracycline and resistant to gentamicinan. Additionally, isolates carrying bla_{SHV-18} were susceptible to ciprofloxacin (100%), tetracycline (83%), tigecycline (83%) and resistance to gentamicin (100%).

It is well known that SHV enzymes are distributed worldwide. Moreover, genes encoded ESBLs are mainly carried by easily transferable plasmids, which allows for quick spreading of ESBLs among strains of Gram-negative rods. bla_{SHV} genes encoding for ESBLs predominantly have been found in *Klebsiella pneumoniae* but in case of its presence on plasmids there is a very high risk of its transmission [14]. They were detected in *Escherichia coli*, *Salmonella ssp*, and *S. enterica*. ESBLs also have been reported in *S. marcescens* and *E. cloacae* [15].

Numerous infection caused by organisms producing ESBLs have been observed worldwide. The presence of resistance connected with of extended spectrum β-lactamases production in bacteria may lead to serious therapeutic implications in infections caused by these organisms. As mentioned above, ESBLs are responsible for higher levels of resistance to extended spectrum cephalosporins. Results of our study reveal

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that tigecycline seems to be an opportunity in antibiotic choices for infections caused by ESBLs-producing gram-negative rods.

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