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Distribution of 16S rRNA Methylases Among Different Species of Aminoglycoside-Resistant *Enterobacteriaceae* in a Tertiary Care Hospital in Poland

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Abstract

Background. Aminoglycosides are a group of antimicrobial agents still the most commonly used in the treatment of life-threatening bacterial infections in human and animals. The emergence and spread of 16S rRNA methylases, which confer high-level resistance to the majority of clinically relevant aminoglycosides, constitute a major public health concern.

Objectives. Our goal was to evaluate the distribution of 16S rRNA methylases among different species of *Enterobacteriaceae* during a five month-long survey in a tertiary hospital in Warszawa, Poland.

Material and Methods. In the survey, a total of 1770 non-duplicate clinical isolates were collected from all hospital wards in a tertiary hospital in Warszawa, Poland. The survey was conducted between 19 April and 19 September 2010. The ability to produce 16S rRNA methylase was examined by determining MICs for gentamicin, kanamycin, amikacin by means of the agar dilution method. The isolates resistant to high concentration of aminoglycosides were PCR tested for genes: *armA*, *rmtA*, *rmtB* and *rmtC*. PCR products were subjected to DNA sequencing by the Sanger method. The genetic similarity of the ArmA-producing isolates was analysed by pulsed-field gel electrophoresis (PFGE).

Results. ArmA was the only 16S rRNA methylase detected in 20 of 1770 tested isolates. The overall prevalence rate of ArmA was 1.13%. In *K. pneumoniae* (n = 742), *P. mirabilis* (n = 130), and *E. cloacae* (n = 253) collected in the survey, the prevalence of ArmA was 0.4%, 0.8% and 5.9%, respectively. The PFGE revealed both horizontal and clonal spread of the *armA* gene in the hospital.

Conclusions. The prevalence of 16S rRNA methylase ArmA reported in this study is significantly higher than observed in other countries in Europe (Adv Clin Exp Med 2016, 25, 3, 539–544).

Key words: *Enterobacteriaceae*, 16S rRNA methylases, ArmA.

Aminoglycosides are still the most commonly used in the treatment of life-threatening infections caused by Gram-negative and Gram-positive bacteria in both human and animals. In clinical settings, aminoglycosides are often administered together with β -lactams and fluoroquinolones. Aminoglycoside-modifying enzymes: aminoglycoside phosphotransferases (APHs), aminoglycoside nucleotidyltransferases (ANTs) or aminoglycoside acetyltransferases (AACs) are the most common

mechanisms of resistance to aminoglycosides reported in Gram-negative bacteria.

In the past decade, a distinct type of aminoglycoside resistance mechanism emerged globally, one that confers high-level resistance to the majority of clinically relevant aminoglycosides [1–6]. This mechanism provides resistance to 4, 6-disubstituted deoxystreptamines (kanamycin, amikacin, gentamicin, netilmicin, tobramycin) by post-transcriptional methylation of the A site of 16S

ribosomal RNA. Methylation is carried out by enzymes known as 16S rRNA methylases. To date, eight distinct plasmid-mediated 16S rRNA methylases (ArmA, RmtA, RmtB, RmtC, NpmA, RmtD, RmtE, RmtD2) have been identified in human and animal *Enterobacteriaceae* from various geographic areas, including Eastern Asia, Europe, and the Americas [7]. Among them, ArmA and RmtB are predominant [7]. ArmA (aminoglycoside resistance methyltransferase A) is the most common methylase identified in clinical *Enterobacteriaceae* strains in Europe [8]. ArmA was reported first in 2003 by Gallimand and colleagues [9] in *K. pneumoniae* BM4536 isolated from a French patient. However, the nucleotide sequence encoding ArmA was found as a part of pCTX-M-3 plasmid (GenBank accession no. AF550415) of *C. freundii* from Poland deposited in GenBank in 2002. ArmA is encoded by the *armA* gene which was found in a variety of plasmids ranging from 57 to 196 kb, often within transposon (Tn1548) [7].

We previously reported a *K. pneumoniae* strain coproducing ArmA and *Klebsiella pneumoniae* carbapenemase (KPC-2) [10]. Considering the data from other countries [2, 4, 9, 11], as well as a relatively high incidence of CTX-M-3 in Poland [12], little is known about the prevalence of 16S rRNA methylases in clinical strains of *Enterobacteriaceae*. Therefore, the aim of this prospective study was to assess the prevalence of 16S rRNA methylase producers among aminoglycoside resistant clinical isolates of *Enterobacteriaceae* collected in a tertiary hospital in Warszawa, Poland.

Material and Methods

Bacterial Isolates and Antimicrobial Susceptibility

16S rRNA methylase survey was conducted during a five-month period in a 1030-bed tertiary care hospital in Warszawa, Poland. There was no preference for a specific hospital ward, clinical symptoms, patients group or sample type. From 19 April to 19 September 2010, a total of 1770 non-duplicate isolates of *Enterobacteriaceae* were isolated from clinical samples. The isolates belonged to: *E. coli* (n = 742), *K. pneumoniae* (n = 451), *E. cloacae* (n = 253), *P. mirabilis* (n = 130), *M. Morganii* (n = 41), *K. oxytoca* (n = 40), other (n = 113). Species identification and antimicrobial susceptibility testing were performed by Vitek 2 system (bioMérieux, France). To isolate 16S rRNA methylase producers, a stepwise selection was performed. The first selection step was to

select bacterial-strains that are resistant to at least two aminoglycosides. All of the 1770 isolates collected in the survey were tested against gentamicin and tobramycin or gentamicin and amikacin using Vitek 2 – cards. The latter set of aminoglycosides was exclusively used for isolates from urine samples. In total, 113 isolates were found to be simultaneously resistant to two aminoglycosides. These isolates were subjected to further examinations.

The ability to produce 16S rRNA methylase was examined by determining a minimum inhibitory concentration (MICs) for gentamicin, kanamycin, amikacin (Sigma-Aldrich, USA) using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Among the 113 preselected *Enterobacteriaceae* isolates, 20 were resistant to high concentration (MIC range from 128 to > 1024 mg/L) of gentamicin, kanamycin, and amikacin. All the 20 isolates resistant to the high concentration of 4,6-disubstituted deoxystreptamines tested were thoroughly investigated as probable 16S rRNA methylase producers. The susceptibility results were interpreted in accordance with European Committee on Antimicrobial Susceptibility Testing (EUCAST) interpretive standards [14]. In addition, neomycin MICs were determined by the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines [13].

PCR and DNA Sequencing

Genes encoding the most common 16S rRNA methylases, including ArmA, RmtA, RmtB and RmtC, were detected by PCR amplification using primers and conditions described previously by Fritsche et al. [8]. All the DNA sequences were queried against the National Center for Biotechnology Information (NCBI) nucleotide database using the BLASTn algorithm.

PFGE Typing

The genetic similarity of the 16S rRNA-producing isolates was analysed by pulsed-field gel electrophoresis (PFGE) as described previously [14] using the CHEF-DR II system (Bio-Rad Laboratories, USA) and endonuclease *XbaI* (Thermo Scientific, Lithuania) with switching time of 3–30 s for 24 h at 14°C and voltage gradient of 6.0 V cm⁻¹. PFGE patterns were analysed using the BioNumerics software v. 6.6 (Applied Maths, Belgium). Similarity clustering analyses were performed using UPGMA and Dice correlation coefficient with a tolerance of 1.2%. The PFGE-typing was performed twice.

Results

Amongst the total of 1770 *Enterobacteriaceae* isolates collected during the survey, 113 isolates were found to be resistant to at least two aminoglycosides, posing 16S rRNA methylase activity. This group consisted of *K. pneumoniae* (n = 66), *E. coli* (n = 20), *E. cloacae* (n = 20), *Proteus mirabilis* (n = 4), *Citrobacter freundii* (n = 1), *Morganella morganii* (n = 1) and *Serratia marcescens* (n = 1). Among them, only 20 isolates showed high-level (MIC $\geq$ 128 mg/L) resistance to gentamicin, kanamycin, amikacin. All these isolates except the one presented low MICs value to neomycin (with MIC range of 1–8 mg/L) (Table 1). This subgroup of potential 16S rRNA methylase-producers (n = 20) encompassed: *E. cloacae* (n = 15), *K. pneumoniae* (n = 3), *C. freundii* (n = 1) and *P. mirabilis* (n = 1).

Antimicrobial susceptibility of the tested isolates resistant to the high concentration of aminoglycosides (n = 20) are shown in Table 1.

The *armA* gene was found in all of the 20 *Enterobacteriaceae* isolates revealing high-level resistance to aminoglycosides. The DNA sequencing of *armA* PCR products of the tested isolates revealed 100% nucleotide identity to the *armA* reference sequence (GenBank accession no. AY220558). No PCR product for *rmtA*, *rmtB* and *rmtC* genes was detected in any tested isolate.

*Xba*I-PFGE patterns and the dendrogram of the pattern similarity for all *ArmA*-producing isolates tested are shown in Fig. 1. The PFGE typing was successfully performed in 17 isolates including *E. cloacae* (n = 14) and *K. pneumoniae* (n = 3). *E. cloacae* isolate no. 205 was non-typeable in repeated experiments. Single isolates of

Table 1. Aminoglycoside resistance patterns of 20 *Enterobacteriaceae* isolates producing 16S rRNA methylase *ArmA*

No of isolate	Bacterial species	Specimen	MIC (mg/L) of:			
			GEN	AMK	KAN	NEO
30	<i>E. cloacae</i>	urine	256	1024	> 1024	2
31	<i>E. cloacae</i>	urine	256	1024	> 1024	2
226	<i>E. cloacae</i>	urine	512	> 1024	> 1024	2
63	<i>E. cloacae</i>	urine	> 1024	1024	> 1024	2
51	<i>E. cloacae</i>	urine	128	512	> 1024	1
216	<i>E. cloacae</i>	urine	256	1024	> 1024	2
187	<i>E. cloacae</i>	urine	512	> 1024	> 1024	2
90	<i>E. cloacae</i>	urine	512	> 1024	> 1024	2
117	<i>E. cloacae</i>	urine	1024	> 1024	> 1024	2
228	<i>E. cloacae</i>	urine	> 1024	> 1024	> 1024	2
186	<i>E. cloacae</i>	urine	256	1024	> 1024	2
40	<i>E. cloacae</i>	wound	512	> 1024	> 1024	2
176	<i>E. cloacae</i>	wound	512	> 1024	> 1024	4
205	<i>E. cloacae</i>	catheter	512	> 1024	> 1024	2
229	<i>E. cloacae</i>	bronchial washings	> 1024	> 1024	> 1024	8
65	<i>K. pneumoniae</i>	urine	> 1024	1024	1024	2
227	<i>K. pneumoniae</i>	urine	> 1024	> 1024	> 1024	2
114	<i>K. pneumoniae</i>	wound	> 1024	> 1024	> 1024	> 128
68	<i>C. freundii</i>	urine	> 1024	> 1024	> 1024	2
32	<i>P. mirabilis</i>	urine	256	> 1024	> 1024	4
–	<i>E. coli</i> DH5 α	–	0.125	0.25	1	0.5

AMK – amikacin; GEN – gentamicin; KAN – kanamycin; NEO – neomycin.

Fig. 1. Dendrogram of patterns similarity of ArmaA-producing clinical *Enterobacteriaceae* isolates. (a.) – *E. cloacae*; (b.) – *K. pneumoniae*

C. freundii and *P. mirabilis* were excluded from PFGE-typing.

Twelve PFGE-XbaI patterns were distinguished among the 14 *E. cloacae* isolates genotyped by PFGE. Two pairs of indistinguishable *E. cloacae* isolates (No. 63 and 117) and (No. 187 and 40) were detected. Three clonal groups (A, B, and C) were found among the genotyped *E. cloacae* isolates. All the three *K. pneumoniae* isolates typed by the PFGE were apparently distinct.

ArmA was the only 16S rRNA methylase detected among 1770 clinical isolates of *Enterobacteriaceae* collected in the tertiary hospital during the survey period. The methylase was identified in 20 isolates of four *Enterobacteriaceae* species (Table 1). The overall prevalence rate of ArmA was 1.13%. Among 742 *K. pneumoniae*, 130 *P. mirabilis* and 253 *E. cloacae* isolates collected in the survey, the respective prevalence rates of ArmA were 0.4%, 0.8% and 5.9%.

Discussion

In recent years, the role of the *Enterobacteriaceae* as opportunist pathogens has become increasingly important. They are the most significant

agents of urinary tract infections, pneumonia and bacteraemia, especially in patients who are hospitalized for an extended period of time or are living in a nursing home. The therapy of aminoglycosides in combination with broad-spectrum β -lactams has been widely used to treat severe bacterial infections. Consequently, antimicrobial resistance including multi-drug resistance (MDR) is increasingly reported among *Enterobacteriaceae* worldwide [15].

In Poland, 16S rRNA methylase ArmA have been also reported. Little is, however, known about the prevalence of 16S rRNA methylases in the country. We have previously reported the occurrence of 16S rRNA methylase ArmA in clinical isolates of *K. pneumoniae* in hospitals located in two major cities in Poland [14]. These findings have prompted us to investigate the dissemination of 16S rRNA methylases in a tertiary hospital in Warszawa. We performed a prospective study for five months to determine the incidence of the methylase producing *Enterobacteriaceae*. Since 16S rRNA methylases confer resistance to a variety of aminoglycosides, bacterial isolate resistance to at least two aminoglycoside agents was the primary selection condition to fish out the probable methylase producers.

To the best of our knowledge, the presented study is the first prospective study on the prevalence of 16S rRNA methylases in clinical isolates of *Enterobacteriaceae* in Poland. Despite a number of reports on 16S rRNA methylase producing *Enterobacteriaceae* published to date [2, 3, 5, 10, 11], the prospective studies devoted to the assessment of the methylase producers prevalence are relatively rare in Europe.

With value of 1.13% for all *Enterobacteriaceae* the 16S rRNA methylase-producers prevalence determined herein is significantly higher than in other European countries, e.g. Greece (0.3%) or Belgium (0.12%) [2, 3]. The prevalence determined in our study is, however, lower than reported from Korea – 2.8% or China – 3.8% [16, 17]. It is noteworthy that all the 16S rRNA methyltransferase producing isolates expressed ArmA exclusively. This finding may suggest that ArmA predominates in Poland. However, in Europe RmtB and RmtC enzymes were also reported [18]. Although we screened all the isolates with high-level resistance to aminoglycosides against *rmtB* and *rmtC*, neither gene was detected. In contrast, RmtB was reported to predominate in Greece [3]. Our data is, therefore, consistent with studies indicating that ArmA may be more frequent than RmtB among clinical isolates of *Enterobacteriaceae* in Europe [2, 11].

Neomycin resistant 16S rRNA methylase producing isolates have been reported relatively rarely.

NpmA is the only methylase conferring resistance to neomycin [19] reported to date. The neomycin-resistant ArmA producing isolate No. 114 collected during our survey was, however, negative in PCR for the *npmA* gene. This may suggest that the neomycin resistance in isolate No. 114 is most likely conferred by an aminoglycoside modifying enzyme that was co-produced with ArmA.

ArmA was reported to occur worldwide in *Enterobacteriaceae* with the most common *K. pneumoniae* and *E. coli* [7, 20]. In our study, *Enterobacter cloacae* was the most prevailing *Enterobacteriaceae* species producing ArmA. The overall prevalence rates of *armA* were 0.4% (3/742) for *K. pneumoniae*, and 5.9% (15/253) for *E. cloacae*. What is noteworthy is that the prevalence rate of ArmA in *E. cloacae* was the highest in Europe when compared with reports published elsewhere [2, 11]. This rate (5.9%) was also higher than 3.9% reported in South Korea but lower than in China, where the rate was 10% [16, 17].

Among less commonly reported [2, 11] *Enterobacteriaceae* species producing ArmA found in our study there were single isolates of *Citrobacter freundii* and *Proteus mirabilis*. In line with other reports, our data shows that *armA* is widely spread among the various species of *Enterobacteriaceae* and may argue for horizontal dissemination of 16S rRNA methylases. Furthermore, the PFGE-typing of *E. cloacae* and *K. pneumoniae* indicated horizontal spread of ArmA had likely occurred.

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References

- [1] Yamane K, Wachino J, Suzuki S, Shibata N, Kato H, Shibayama K, Kimura K, Kai K, Ishikawa S, Ozawa Y, Konda T, Arakawa Y: 16S rRNA methylase-producing, gram-negative pathogens, Japan. *Emerging Inf Dis* 2007, 13, 642–646.
- [2] Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, de Mendonca R, Rodriguez-Villalobos H, Struelens M, Glupczynski Y: Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. *J Antimicrob Chemother* 2007, 59, 459–464.
- [3] Galani I, Souli M, Panagea T, Poulakou G, Kanellakopoulou K, Giamarellou H: Prevalence of 16S rRNA methylase genes in *Enterobacteriaceae* isolates from a Greek University Hospital. *Clin Microbiol Infect* 2012, 18, E52–E54.
- [4] Yan JJ, Wu JJ, Ko WCh, Tsai SH, Chuang ChL, Wu HM, Lu YJ, Li JD: Plasmid-mediated 16S rRNA methylases conferring high-level aminoglycoside resistance in *Escherichia coli* and *Klebsiella pneumoniae* isolates from two Taiwanese hospitals. *J Antimicrob Chemother* 2004, 54, 1007–1012.
- [5] Berçot B, Poirel L, Nordmann P: Plasmid-mediated 16S rRNA methylases among extended-spectrum β -lactamase-producing *Enterobacteriaceae* isolates. *Antimicrob Agents Chemother* 2008, 52, 4526–4527.
- [6] Yang J, Ye L, Wang W, Luo Y, Zhang Y, Han L: Diverse prevalence of 16S rRNA methylase genes *armA* and *rmtB* amongst clinical multidrug-resistant *Escherichia coli* and *Klebsiella pneumoniae* isolates. *Int J Antimicrob Agents* 2011, 38, 348–351.
- [7] Wachino J, Arakawa Y: Exogenously acquired 16S rRNA methyltransferases found in aminoglycoside-resistant pathogenic Gram-negative bacteria: An update. *Drug Resistance Updates* 2012, 15, 133–148.
- [8] Fritsche TR, Castanheira M, Miller GH, Jones RN, Armstrong ES: Detection of methyltransferases conferring high-level resistance to aminoglycosides in *Enterobacteriaceae* from Europe, North America, and Latin America. *Antimicrob Agents Chemother* 2008, 52, 1843–1845.
- [9] Galimand M, Courvalin P, Lambert T: Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. *Antimicrob Agents Chemother* 2003, 47, 2565–2571.

- [10] Zacharczuk K, Piekarska K, Szych J, Zawidzka E, Sulikowska A, Wardak S, Jagielski M, Gierczyński R: Emergence of *Klebsiella pneumoniae* coproducing KPC-2 and 16S rRNA methylase ArmA in Poland. *Antimicrob Agents Chemother* 2011, 55, 443–446.
- [11] Sabtcheva S, Saga T, Kantardjiev T, Ivanova M, Ishii Y, Kaku M: Nosocomial spread of *armA*-mediated high-level aminoglycoside resistance in *Enterobacteriaceae* isolates producing CTX-M-3 beta-lactamase in cancer hospital in Bulgaria. *J Chemother* 2008, 20, 593–599.
- [12] Gołębiewski M, Kern-Zdanowicz I, Zienkiewicz M, Adamczyk M, Zylńska J, Baraniak A, Gniadkowski M, Bardowski J, Cegłowski P: Complete nucleotide sequence of the pCTX-M3 plasmid and its involvement in spread of extended-spectrum β -lactamase gene *bla*_{CTX-M-3}. *Antimicrob Agents Chemother* 2007, 51, 3789–3795.
- [13] Clinical and Laboratory Standards Institute (CLSI). Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard, 8th ed. Document M07-A8. CLSI, Wayne, PA, USA 2009.
- [14] Zacharczuk K, Piekarska K, Szych J, Jagielski M, Hidalgo L, Millán ÁS, Gutiérrez B, Rastawicki W, González-Zorn B, Gierczyński R: Plasmid-borne 16S rRNA methylase ArmA in aminoglycoside – resistant *Klebsiella pneumoniae* in Poland. *J Med Microbiol* 2011, 60, 1306–1311.
- [15] EARS-Net Annual Reports. Antimicrobial resistance surveillance in Europe 2010. Annual report of the European Antimicrobial Resistance Surveillance Network (www.ecdc.europa.eu).
- [16] Wu Q, Zhang Y, Han L, Sun J, Ni Y: Plasmid-mediated 16S rRNA methylases in aminoglycoside-resistant *Enterobacteriaceae* isolates in Shinghai, China. *Antimicrob Agents Chemother* 2009, 53, 271–272.
- [17] Kang HY, Kim KY, Kim J, Lee JCh, Lee YCh, Cho DCh, Seol SY: Distribution of conjugative-plasmid-mediated 16S rRNA methylase genes among amikacin-resistant *Enterobacteriaceae* isolates collected in 1995 to 1998 and 2001 to 2006 at a University Hospital in South Korea and identification of conjugative plasmids mediating dissemination of 16S rRNA methylase. *J Clin Microbiol* 2008, 46, 700–706.
- [18] Hopkins KL, Escudero JA, Hidalgo L, Gonzalez-Zorn B: 16S rRNA methyltransferase RmtC in *Salmonella enterica* serovar Virchow. *Emerg Infect Dis* 2010, 16, 712–715.
- [19] Wachino J, Shibayama K, Kurokawa H, Kimura K, Yamane K, Suzuki S, Shibata N, Ike Y, Arakawa Y: Novel plasmid-mediated 16S rRNA m1A1408 methyltransferase, NpmA, found in a clinically isolated *Escherichia coli* strain resistant to structurally diverse aminoglycosides. *Antimicrob Agents Chemother* 2007, 51, 4401–4409.
- [20] Galimand M, Sabtcheva S, Courvalin P, Lambert T: Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. *Antimicrob Agents Chemother* 2005, 49, 2949–2953.

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