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

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

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
ribsominal 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 RNA 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 (bioMerieux, 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 RNA 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 morgani (n = 1) and Serratia marcescens (n = 1). Among them, only 20 isolates showed high-level (MIC ≥ 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.

XbaI-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. Aminoglicoside resistance patterns of 20 Enterobacteriaceae isolates producing 16S rRNA methylase ArmA

<table>
<thead>
<tr>
<th>No of isolate</th>
<th>Bacterial species</th>
<th>Specimen</th>
<th>MIC (mg/L) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>GEN</td>
</tr>
<tr>
<td>30</td>
<td>E. cloacae</td>
<td>urine</td>
<td>256</td>
</tr>
<tr>
<td>31</td>
<td>E. cloacae</td>
<td>urine</td>
<td>256</td>
</tr>
<tr>
<td>226</td>
<td>E. cloacae</td>
<td>urine</td>
<td>512</td>
</tr>
<tr>
<td>63</td>
<td>E. cloacae</td>
<td>urine</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>51</td>
<td>E. cloacae</td>
<td>urine</td>
<td>128</td>
</tr>
<tr>
<td>216</td>
<td>E. cloacae</td>
<td>urine</td>
<td>256</td>
</tr>
<tr>
<td>187</td>
<td>E. cloacae</td>
<td>urine</td>
<td>512</td>
</tr>
<tr>
<td>90</td>
<td>E. cloacae</td>
<td>urine</td>
<td>512</td>
</tr>
<tr>
<td>117</td>
<td>E. cloacae</td>
<td>urine</td>
<td>1024</td>
</tr>
<tr>
<td>228</td>
<td>E. cloacae</td>
<td>urine</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>186</td>
<td>E. cloacae</td>
<td>urine</td>
<td>256</td>
</tr>
<tr>
<td>40</td>
<td>E. cloacae</td>
<td>wound</td>
<td>512</td>
</tr>
<tr>
<td>176</td>
<td>E. cloacae</td>
<td>wound</td>
<td>512</td>
</tr>
<tr>
<td>205</td>
<td>E. cloacae</td>
<td>catheter</td>
<td>512</td>
</tr>
<tr>
<td>229</td>
<td>E. cloacae</td>
<td>bronchial washings</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>65</td>
<td>K. pneumoniae</td>
<td>urine</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>227</td>
<td>K. pneumoniae</td>
<td>urine</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>114</td>
<td>K. pneumoniae</td>
<td>wound</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>68</td>
<td>C. freundii</td>
<td>urine</td>
<td>&gt; 1024</td>
</tr>
<tr>
<td>32</td>
<td>P. mirabilis</td>
<td>urine</td>
<td>256</td>
</tr>
<tr>
<td>-</td>
<td>E. coli DH5α</td>
<td>-</td>
<td>0.125</td>
</tr>
</tbody>
</table>

AMK – amikacin; GEN – gentamicin; KAN – kanamycin; NEO – neomycin.
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-lactamas 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. 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 pmrA 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


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