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AMINOGLYCOSIDE RESISTANCE DETERMINANTS IN MULTIRESISTANT *ESCHERICHIA COLI* AND *KLEBSIELLA PNEUMONIAE* CLINICAL ISOLATES FROM TURKISH AND SYRIAN PATIENTS

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Escherichia coli and *Klebsiella pneumoniae* are frequently found resistance to aminoglycosides in Turkey. The aim of this study was to investigate aminoglycoside resistance in clinical isolates of *E. coli* and *K. pneumoniae* from Turkey using both phenotypic and genotypic methods and screening for the prevalence of gene coding for common aminoglycoside-modifying enzymes (AMEs) and 16S rRNA methylase genes. A total of 88 consecutive, non-duplicated *E. coli* ($n = 65$) and *K. pneumoniae* ($n = 23$) isolates showing resistance or intermediate resistance to amikacin and/or gentamicin were collected between October 2013 and May 2015 from clinical samples received at Gaziantep Dr. Ersin Arslan Training and Research Hospital. Seventeen isolates were obtained from Syrian patients. Isolates resistant to any of the two aminoglycosides were tested by PCR for seven AME genes, and 22 isolates with amikacin MIC ≥16 mg/L were also tested for 16S rRNA methylase genes. In *E. coli* isolates, the most frequent genes were *aac(6′)-Ib* (50 strains; 76.9%) and *aac(3)-IIa* (40 strains; 70.7%), followed by *aph(3′)-Ia* (5 strains; 7.6%) and *ant(2″)-Ia* (2 strains; 3.1%). Among the 23 resistant *K. pneumoniae* isolates, the most prevalent gene was *aac(3′)-IIa* (87.0%) followed by *aac(6′)-Ib* (73.9%) and *aph(3′)-Ia* (8.6%). The *rmtC* gene was detected in one *K. pneumoniae* isolate. Resistance to aminoglycosides in clinical isolates of *E. coli* and *K. pneumoniae* from our center is predominantly caused

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by AAC(6′)-Ib and AAC(3)-II enzymes, while the occurrence of 16S rRNA methylases is so far limited.

Keywords: aminoglycoside-modifying enzyme, 16S rRNA methyltransferase, *E. coli*, *K. pneumoniae*

Introduction

Aminoglycosides are a large family of drugs that act at the ribosome by inhibiting one or more of the biochemical steps involved in translation. They are extensively used in the treatment of serious bacterial infections, particularly in combination with β-lactams or glycopeptides [1]. The increasing problem of multiresistance in Gram-negative bacteria and the introduction of new aminoglycoside analogues (e.g., plazomicin) warrant new studies aimed at understanding aminoglycoside resistance [2].

According to the Central Asian and Eastern European Survillance of Antimicrobial Resistance (CAESAR) annual report 2016, *Escherichia coli* and *Klebsiella pneumoniae* had high resistance to the third-generation cephalosporins, aminoglycosides, and fluoroquinolones in Turkey [3].

In terms of frequency, the most important determinant of aminoglycoside resistance in *E. coli, K. pneumoniae*, and many other Gram-negative bacteria is aminoglycoside-modifying enzymes (AMEs), of which three classes are defined according to their modifying activities: acetyltransferases (AAC), nucleotidyltransferases (ANT), and phosphotransferases (APH) [4]. Other mechanisms conferring aminoglycoside resistance include active efflux of the antimicrobial and reduced intake into the bacterial cell, and production of several 16S rRNA methylases with ArmA, RmtB, and RmtC being the most widespread [2, 5].

Genes encoding AMEs and 16S rRNA methylases are located on mobile genetic elements along with other resistance determinants, such as extended-spectrum β-lactamases (ESBLs) and carbapenemases, contributing to explain multidrug resistance in clinical isolates [6].

The aim of this study was to investigate aminoglycoside resistance in *E. coli* and *K. pneumoniae* using both phenotypic and genotypic methods.

Materials and Methods

Bacterial isolates

A total of 88 consecutive, non-duplicated *E. coli* and *K. pneumoniae* isolates collected from clinical samples between October 2013 and May 2015 at Gaziantep

Dr. Ersin Arslan Training and Research Hospital and showing resistance or decreased susceptibiliy to amikacin and/or gentamicin were studied. Seventeen isolates were from Syrian patients (9 *K. pneumoniae* and 8 *E. coli*).

Antimicrobial susceptibility testing

The strains were identified by both conventional methods and Vitek 2 Compact system (BioMérieux, France). Antibiotic susceptibility (aminoglycosides, carbapenems, and ciprofloxacin) and ESBL production of isolates were tested by the Vitek 2 Compact system. The isolates were selected based on Vitek 2 results. In addition, the disk diffusion method was also performed for amikacin, gentamicin, and tobramycin. The results were interpreted using clinical breakpoints as defined by the Clinical Laboratory and Standards Institute [7]. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as quality control strains.

Molecular characterization of aminoglycoside resistance genes

Isolates resistant to any of the indicated aminoglycosides were tested by PCR for seven AME genes. Twenty-two isolates (19 *E. coli* and 3 *K. pneumoniae*) with a minimum inhibitory concentration (MIC) of amikacin ≥16 mg/L were also tested for 16S rRNA methylase genes. As a control, 10 isolates susceptible to the indicated aminoglycosides were also used in the PCR analysis. Sets of primers for the following genes were included in the PCR assay: *aac(3)-Ia, aac(3)-IIa, aac(6)-Ib, ant(2)-Ia, aph(3)-Ia, aph(3)-IIa, aph(3)-Via, armA, rmtB, rmtC, and rmtD*. The primers for AME and methyltransferase genes and their expected amplicon sizes are shown in Tables I and II.

Genomic DNA was extracted using an InstaGeneTM Matrix Kit (Bio-Rad, Madrid, Spain) according to the manufacturer's instructions. Then, 2 µl of DNA were added to a reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, and 1 U of Taq DNA Polymerase (Bioline, London, UK). Amplification conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s [60 °C for *aac(6)-Ib*] and 72 °C for 1 min, and a final elongation at 72 °C for 10 min. PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide.

Results

Thirty-three (50.7%) *E. coli* isolates were resistant to amikacin by Vitek 2 compared to 25 (38.4%) by disk diffusion. Eight (34.7%) *K. pneumoniae* isolates

Table I. Primers used in the detection of aminoglycoside-modifying enzyme (AME) and expected amplicon sizes

Gene		DNA sequence (5′–3′)	Product (bp)	Reference
aac(6′)-Ib	aac6′-Ib-F	TTGCGATGCTCTATGAGTGGCTA	482	[8]
	aac6′-Ib-R	CTCGAATGCCTGGCGTGTTT		
aac(3)-IIa	aac3-IIa-F	GGCAATAACGGAGGCGCTTCAAAA	563	[1]
	aac3-IIa-R	TTCCAGGCATCGGCATCTCATACG		
aac(3)-Ia	aac3-Ia-F	GCAGTCGCCCCTAAAACAAA	464	[1]
	aac3-Ia-R	CACTTCTTCCCGTATGCCCAACTT		
aph(3′)-VIa	aph3′-VIa-F	AAAGCGATCAATGCAAAACC	310	[1]
	aph3′-VIa-R	TATCCGTGATATCGCCATGA		
ant(2″)-Ia	ant2″-Ia-F	CGTCATGGAGGAGTTGGACT	303	[1]
	ant2″-Ia-R	CGCAAGACCTCAACCTTTTC		
aph(3′)-Ia	ant2″-Ia-F	CGAGCATCAAATGAAACTGC	624	[1]
	ant2″-Ia-R	GCGTTGCCAATGATGTTACAG		
aph(3′)-IIa	aph3′-Ia-F	GAACAAGATGGATTGCACGC	680	[1]
	aph3′-Ia-R	GCTCTTCAGCAATATCACGG		

Table II. Primers used in the detection of methyltransferase genes and expected amplicon sizes

Gene		DNA sequence (5′–3′)	Product (bp)	Reference
armA	armA-F	CAAATGGATAAGAATGATGTT	777	[9]
	armA-R	TTATTTCTGAAATCCACT		
rmtB	rmtB-F	TCAACGATGCCCTCACCTC	459	[10]
	rmtB-R	GCAGGGCAAAGGTAAAATCC		
rmtC	rmtC-F	CGAAGAAGTAACAGCCAAAG	711	[11]
	rmtC-R	ATCCCAACATCTCTCCCACT		
rmtD	rmtD-F	GAGCGAACTGAAGGAAAAAC	730	[12]
	rmtD-R	CAGCACGTAAAACAGCTC		

were resistant to amikacin by Vitek 2 compared to 13 (56.5%) by the disk diffusion. Resistance to gentamicin among *E. coli* and *K. pneumoniae* was similar by disk diffusion and Vitek 2 [47 isolates each (72.3%) and 21 isolates each (91.3%), respectively]. Resistance of *E. coli* and *K. pneumoniae* to tobramycin, as defined by disk diffusion, was 100% and 93.8%, respectively (Table III). Resistance rates of *E. coli* and *K. pneumoniae* to carbapenems and ciprofloxacin are also shown in Table IV. Fifty-six (86.1%) *E. coli* and 22 (95.6%) *K. pneumoniae* isolates were found positive for ESBL production.

Overall, the most frequent genes were *aac(6′)-Ib* (67 strains; 76.1%) and *aac(3)-IIa* (66 strains; 75.0%), followed by *aph(3′)-Ia* (7 strains; 8.0%) and *ant (2″)-Ia* (2 strains; 2.3%). Among the isolates from Syrian patients (17 strains), the

most prevalent gene was *aac(3)-IIa* (82.3%), followed by *aac(6′)-Ib* (64.7%) and *aph(3′)-Ia* (5.8%).

The most frequent AME gene in 65 resistant *E. coli* isolates was *aac(6′)-Ib*, identified in 50 (76.9%) isolates, followed by *aac(3)-IIa* in 46 (70.7%) isolates, *aph(3′)-Ia* in 5 (7.6%), and *ant(2″)-Ia* in 2 (3.1%) isolates. The genes *aac(3)-Ia*, *aph(3′)-VIa*, and *aph(3′)-IIa* were not found in *E. coli* isolates. Among the 23 resistant *K. pneumoniae* isolates, the prevalence of AME genes was as follows: *aac(3)-IIa* was the most frequent one, identified in 20 (87.0%) isolates, followed by *aac(6′)-Ib* in 17 (73.9%) isolates and *aph(3′)-Ia* in 2 (8.6%). The genes *aac(3)-Ia*, *aph(3′)-VIa*, *aph(3′)-IIa,* and *ant(2″)-Ia* were not found in *K. pneumoniae* isolates.

In three (one *K. pneumoniae* and two *E. coli*) isolates, none of the seven investigated AME genes was detected. One of those *E. coli* isolates had an intermediate category to gentamicin by Vitek 2 but was susceptible to that agent by disk diffusion. The other *E. coli* isolate was resistant to all tested aminoglycosides. We detected the *rmtC* gene in the *K. pneumoniae* isolate.

Fifty-five isolates were presented with more than one gene. The combination of *aac(6′)-Ib* and *aac(3)-IIa* was the most common one for both *E. coli* (33 isolates; 51%) and *K. pneumoniae* (14 isolates; 61%) isolates, followed by *aac(3)-IIa* and *aph(3′)-Ia* in *E. coli* (5 isolates; 6%) and *K. pneumoniae* (1 isolate; 4.3%). One *E. coli* isolate harbored four genes: *aac(6′)-Ib* + *aac(3)-IIa*+*aph(3′)-Ia*

Table III. The resistance rates of aminoglycosides in *E. coli* and *K. pneumoniae* isolates

	E. coli		*K. pneumoniae*	
	Disk diffusion	Vitek 2	Disk diffusion	Vitek 2
Amikacin	50.7	38.4	34.7	56.5
Gentamicin	72.3	72.3	91.3	91.3
Tobramycin	93.8	ND	100	ND

Note: ND: non-detected.

Table IV. The antimicrobial resistance rates of carbapenems, ciprofloxacin, and ESBL production of *E. coli* and *K. pneumoniae* isolates by Vitek 2 method

	E. coli	*K. pneumoniae*
ESBL	86.1	95.6
Imipenem	4.6	17.3
Meropenem	4.6	17.3
Ertapenem	9.2	26.0
Ciprofloxacin	96.9	69.5

Note: ESBL: extended-spectrum β-lactamase.

+*ant(2″)-Ia*. One *K. pneumoniae* isolate harbored three genes: *aac(6′)-Ib* + *aac(3)-IIa* + *aph(3′)-Ia*.

Discussion

According to the CAESAR annual report 2016, the percentage of aminoglycoside (amikacin, gentamicin, and tobramycin) resistance was 29% for *E. coli* and 45% for *K. pneumoniae* among blood and cerebrospinal fluid isolates in Turkey in 2014 [3].

In this study, a total of 72.3% and 50.7% of the *E. coli* isolates were resistant or intermediate to gentamicin and amikacin by Vitek 2. For *K. pneumonia*, the prevalence of resistance was 91.3% and 34.7% to gentamicin and amikacin, respectively. In both *E. coli* and *K. pneumoniae*, the prevalence of reduced susceptibility was higher for tobramycin.

The finding of this study that the most frequent AME genes were *aac(6′)-Ib* followed by *aac(3)-IIa*, *aph(3′)-Ia*, and *ant(2″)-Ia* is in line with a Spanish study, in which the most prevalent AME genes in 420 ESBL-positive *E. coli* and 139 ESBL-positive *K. pneumoniae* were *aac(6′)-Ib* (16.2% and 44.6%, respectively) and *aac(3)-IIa* (14.7% and 43.1%, respectively) [13]. In an another Spanish study, including 257 *E. coli* isolates resistant to amoxicillin/clavulanic acid, the most prevalent AME genes were *aac(6′)-Ib* followed by *aph(3′)-Ia*, *ant(2″)-Ia*, and *aac(3′)-IIa*(2). However, in a study from Norway, PCR screening for AME genes showed that the most prevalent AME gene in both *E. coli* and *K. pneumoniae* was *aac(3)-II*, followed by *aac(6′)-Ib*, whereas *ant(2″)-Ia* was only identified in three *E. coli* isolates [6].

The *aac(6′)-Ib* gene, which is probably the most clinically relevant AAC in *Enterobacteriaceae*, is responsible for resistance to amikacin and tobramycin, but not gentamicin [4]. In this study, 17 out of the 18 (94.4%) isolates that were only positive for *aac(6′)-Ib* expressed phenotypic resistance to amikacin. Among the isolates with the *aac(6′)-Ib* gene, the prevalence of ESBL production and ciprofloxacin resistance was 91% and 94%, respectively. This finding supports the fact that the *aac(6′)-Ib* gene is usually associated with quinolone resistance genes or β-lactamase genes [4]. In another study in Turkey, the authors indicated that an *aac(6′)-IV* enzyme, presumably related to AAC(6′)-Ib, was the most common AME in *Klebsiella* spp. (37.5%), whereas AAC(3)-II were the most common one (58%) in *E. coli* [14]. Resistance to aminoglycosides in 16 ESBL-producing *Enterobactericeae* isolated in a Turkish hospital was explained by the presence of the *aac(3)-II* and the *aac(6′)-Ib-cr* genes. Four of those isolates harbored an additional *aph(3′)-I* gene [15].

In this study, the second most common AME gene was *aac(3)-IIa*, which causes resistance to gentamicin, netilmicin, and tobramycin [4]. In the isolates containing this gene, resistance rates to gentamicin (95.4% with MICs ≥ 16 mg/L) and tobramycin (96.9%) are in agreement with the expected phenotype. In addition, eight isolates (9%) harboring *aph(3′)-Ia* co-harbored the *aac(3′)-IIa* gene, and were resistant to gentamicin. The APH(3′)-I subclass shows a resistance profile including kanamycin and neomycin and is widely distributed among Gram-negative bacteria containing wide host range plasmids and transposons [4].

ANT(2″)-Ia is also commonly encoded by plasmids and transposons and mediates resistance to gentamicin, tobramycin, and kanamycin [4]. We found the *ant(2″)-Ia* gene in only two *E. coli* isolates, which were resistant to both gentamicin and tobramycin.

Among the Syrian patients ($n = 17$), the most prevalent gene was *aac(3)-IIa* (82.3%), followed by *aac(6′)-Ib* (64.7%) and *aph(3′)-Ia* (5.8%). The most frequent association was *aac(3)-IIa* and *aac(6′)-Ib* (52.9%). To the best of our knowledge, this is the first report of AMEs resistance from Syrian patients in Turkey.

K. pneumoniae and *P. aeruginosa* showing high-level resistance to clinically useful aminoglycosides through the production of acquired methyltransferases were identified in France and Japan, respectively, in 2003 [9, 16]. These enzymes are mostly located on transferable plasmids, and could be easily transferred to other bacterial species [5]. In this study, the *rmtC* gene was identified in a single *K. pneumoniae* isolated from a blood culture, which does not harbor any of the studied AME genes, produce an ESBL, and was also carbapenem-resistant. MICs of amikacin and gentamicin for this organism are >64 and >16 mg/L, respectively. In Turkey, the *rmtC* gene has been previously detected in four *K. pneumoniae* isolates resistant to both amikacin (MIC >512 mg/L) and gentamicin (MIC >128 mg/L) and producing the NDM-1 carbapenemase [17]. The *rmtB* gene has also been identified in an aminoglycoside-resistant *K. pneumoniae* isolate in Turkey. This represented the first report in Turkey of a clinical isolated with a single plasmid containing the genes *rmtB*, *qepA*, and $bla_{\text{CTX-M-15}}$ [10]. On the other hand, resistance due to 16S rRNA methyltransferases was not found in any of 37 aminoglycoside-resistant Turkish clinical isolates with an amikacin MIC ≥128 [18].

In conclusion, resistance to aminoglycosides in clinical isolates of *E. coli* and *K. pneumoniae* isolated in Gaziantep is predominantly caused by the AAC(6′)-Ib and the AAC(3)-II enzymes, while the occurrence of 16S rRNA methylases is so far limited. Further studies are needed to determine the importance of AMEs and 16S rRNA methyltransferase as causes of aminoglycoside resistance in Turkey.

Conflict of Interest

The authors declare no conflict of interest.

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