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Genetic basis of enzymatic resistance of *E. coli* to aminoglycosides

Dominika Ojdana*, Anna Sieńko, Paweł Sacha, Piotr Majewski, Piotr Wieczorek, Anna Wieczorek, Elżbieta Tryniszewska

Department of Microbiological Diagnostics and Infectious Immunology, Medical University of Bialystok, Bialystok, Poland

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ABSTRACT

Purpose: Over the past years, an increase in resistance to aminoglycosides has been observed among *Enterobacteriaceae* rods. This resistance development reduces therapeutic options for infections caused by multidrug-resistance organisms. Because of the changing epidemiology of extended-spectrum β-lactamases (ESBLs) and resistance to aminoglycosides, we investigated the prevalence of the *aac(3)-Ia*, *aac(6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib* genes encoding aminoglycoside-modifying enzymes (AMEs) in ESBL-producing *Escherichia coli* as well as ESBL-non-producing isolates. To understand bacterial resistance to aminoglycoside antibiotics, we estimated resistance phenotypes and the presence of genes responsible for this resistance.

Materials and methods: The study was conducted on 44 *E.coli* strains originated from patients hospitalized at University Hospital of Bialystok. MIC values were obtained for gentamicin, amikacin, netilmicin, and tobramycin. Isolates were tested for the presence of the *aac(3)-Ia*, *aac(6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib* genes with the use of the PCR technique.

Results: Resistance to aminoglycosides was found in 79.5% of the isolates. The highest percentages of resistance were observed for tobramycin (70,5%) and gentamicin (59%), followed by netilmicin (43.2%) and amikacin (11.4%). PCR assays revealed the presence of *aac(6′)-Ib* among 26 (59.2%) strains, *aph(3")-Ib* among 16 (36.2%), *aac(3)-Ia* among 7 (15.9%), and *ant(2")-Ia* among 2 (4.6%) strains.

Conclusions: The enzymatic resistance against aminoglycosides in northeastern Poland among clinical isolates of *E. coli* is predominantly caused by *aac(6′)-Ib* and *aph(3")-Ib*. Amikacin may be used for therapy of infections caused by ESBL-producing *E. coli*, because of the low rates of resistance.

1. Introduction

Escherichia coli is an important bacterial species associated with nosocomial infections involving urinary tract infections, pneumoniae, bloodstream infections, and surgical site infections [1,2,3]. In recent years the increase of *E. coli* resistance to third-generation cephalosporin caused by producing extended-spectrum β-lactamases (ESBLs) was observed [4,5,6]. Aminoglycosides are an important group of antibiotics used in the treatment of serious infections by Gram-negative bacteria, including *E. coli* [7,8]. Over the last years there has been an increase in aminoglycoside resistance among ESBL-producing *E. coli* [9,10]. Bacterial resistance to aminoglycosides may be a result of chromosomal mutation as well as acquisition of mobile genetic elements (plasmids, integrons, transposons) harboring resistance genes [11,12]. Bacterial resistance to aminoglycosides is mediated by three different mechanisms, including enzymatic drug modification, decreased intracellular antibiotic accumulation, and the substitution of ribosomal proteins or mutation of rRNA [13]. Production of aminoglycoside-modifying enzymes (AMEs) is one of the most frequently occurring mechanisms of resistance to the aminoglycoside among *E. coli*. Enzymes belonging to N-acetyltransferases (ACC) inactivate aminoglycoside antibiotics by acetylation, O-nucleotidyltransferases (ANT) cause adenylation of aminoglycosides, and O-phosphotransferases (APH) can phosphorylate aminoglycosides [14,15]. The genes encoding AMEs often are located on plasmids carrying genes for ESBLs. Therefore the simultaneous resistance of bacteria to aminoglycosides and extended-spectrum cephalosporins remains a considerable challenge. Because the epidemiology of ESBLs and resistance to aminoglycosides is changing, we decided to investigate the prevalence of the *aac (3)-Ia*, *aac(6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib* genes in ESBL-producing *E. coli* as well as *E. coli* ESBL-non-producing isolates from the University Hospital of Bialystok. Moreover, we estimated the relationship between bacterial phenotypes of

* Corresponding author at: Department of Microbiological Diagnostics and Infectious Immunology Medical University of Bialystok, Waszyngtona Street 15a, 15-269 Bialystok, Poland.
E-mail address: dominika.ojdana@umb.edu.pl (D. Ojdana).

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aminoglycoside resistance and the prevalence of genes responsible for this resistance.

2. Matherial and methods

2.1. Strains

The study was conducted on 44 *E.coli* strains isolated during the period from the eighth of March 2013 to the twenty-eighth of May 2015 from various clinical materials, including urine (27.3%), blood (11.4%), pus (13.6%), tracheal secretions (18.2%), rectal swabs (15.9%), and wound swabs (13.6%). The collected strains were taken from patients hospitalized at intensive care units (45.4%), gastroenterology (18.2%), surgery (15.9%), hematology (9.1%), endocrinology (6.8%), cardiology (2.3%), and rheumatology (2.3%) clinics of University Hospital of Bialystok. All strains were identified using GN cards and the automated VITEK 2 GN card system and the automated identification system (bioMérieux, France), according to the manufacturer's instructions. Control strains included *Klebsiella pneumoniae* ATCC 700603, *E. coli* ATCC 35218, and *E. coli* ATCC 25922.

2.2. Determination of ESBL and antibiotic susceptibility testing

The presence of the ESBL phenotype was detected by the double-disk synergy test (DDST) [10]. Susceptibility of tested isolates to aminoglycoside antibiotics including gentamicin, amikacin, netilmicin, and tobramycin was prepared by using E-tests. The test was prepared on Mueller-Hinton agar in accordance with the manufacturer's instructions. Susceptibility was interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org/clinicalbreakpoints).

2.3. Plasmid DNA preparation

The *E. coli* strains were cultured overnight on Trypticase soy broth (Emapol, Poland) at 37 °C. Plasmid DNA was extracted from *E. coli* strains by the alkaline method with the Plasmid Mini Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol.

2.4. PCR amplification

Prepared plasmid DNA was used as templates for the amplification of aminoglycoside resistance genes *aac(3)-Ia, aac (6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib* using the LabCycler Gradient (SensoQuest GmbH, Goettingen, Germany). The primers used for the PCR assays are presented in Table 1. The PCRs for various AME genes were performed as previously described [16,17,18].

3. Results

3.1. ESBL detection and susceptibility testing

All the isolates of *E. coli* were screened for ESBL production. Among all 44 tested strains, 29 (65.9%) were found to be ESBL producers, whereas 15 (34.1%) were defined as ESBL non-producers.

Antibiotic susceptibility testing revealed differentiation among percentages of strains resistant to particular aminoglycosides, as shown in Table 2. Thirty-five (79.5%) tested strains presented resistance and/or intermediate susceptibility against tested aminoglycosides. Among these strains, 29 (65.9%) were ESBL producers and 6 (13.6%) were ESBL non-producers. Nine (20.5%) other strains that belonged to the ESBL non-producer group presented susceptibility to all tested aminoglycosides. The 26 (59%) tested strains were noticed to be resistant against gentamicin, whereas 20 (45.5%) of these strains were ESBL producers and 6 (13.5%) strains were ESBL non-producers. Moreover, a high resistance rate (31 (70.4%)) was observed for tobramycin. The resistance to tobramycin was presented in 25 (56.8%) strains from the group of ESBL producers and in 6 (13.6%) strains from the group of ESBL non-producers. Also, high rates of resistance in 19 tested strains (43.2%) were observed against netilmicin. Among these strains, 17 (38.6%) belonged to ESBL producers and 2 (4.6%) were ESBL non-producers. The highest percentage of susceptibility among tested strains (33 (75%)) was observed for amikacin. Among ESBL producers, 18 (41%) were susceptible to amikacin; among ESBL non-producers, susceptibility to amikacin was presented by 15 (34%) strains. Comparison of the aminoglycoside activity against ESBL producers and ESBL non-producers is presented in Table 2. The Wilcoxon rank-sum test revealed that there was a statistically significant ($p < 0.001$) difference between MIC values for amikacin, netilmicin, and tobramycin observed for *E. coli* strains from the group of ESBL producers and ESBL non-producers. For gentamicin there was no statistically significant difference ($p = 0.163$) between MIC values presented by *E. coli* strains from these two groups (Fig. 1).

The analysis of antimicrobial susceptibility results for tested strains revealed eight different resistance phenotypes against aminoglycosides. Resistance phenotypes involved simultaneous resistance of tested strains to GN, AM, NET, TOB (GN, gentamicin; AM, amikacin; NET, netilmicin; TOB, tobramycin), GN, NET, TOB; AM, NET, TOB; NET, TOB; GN, TOB; GN, NET; GN and TOB. The resistance phenotype involving GN, TOB was observed among 9 (20.4%) tested strains. Eight (18.2%) tested strains presented resistance to GN, NET, TOB. Resistance to all tested aminoglycosides was observed among 5 (11.4%) tested strains. Simultaneous resistance to TOB or to NET, TOB was observed among 4 (9.1%) tested strains. The remaining tested strains presented resistance phenotypes including GN (3 (6.8%)), AM, NET, TOB (1 (2.3%)), or GN, NET (1 (2.3%)).

Table 1
Names, sequences, and references of primers used for PCR reactions in the study.

Gene target	Names and sequences of primers	Amplicon size	Reference
aac(6′)-Ib	aacA4F 5′ GCTCTTGGAAGCGGGGACGG 3′ aacA4R 5′ TCGCTCGAATGCCTGGCGTG 3′	300 bp	[16]
aac(3)-Ia	aac3F 5′ GGCTCAAGTATGGGCATCAT 3′ aac3R 5′ TCACCGTAATCTGCTTGCAC 3′	389 bp	[17]
aph(3′′)-Ib	aph(3′′)F 5′ CCTTGGTGATAACGGCAATTC 3′ aph(3′′)R 5′ CCAATCGCAGATAGAAGGC 3′	548 bp	[18]
ant(4′)-IIa	ant(4′)F 5′ ATCGTCTGCGAGAAGCGTAT 3′ ant(4′)R 5′ TAAAACGCCTATCCGTCACC 3′	839 bp	[17]
ant(2′′)-Ia	ant(2′′)F 5′ GACACAACGCAGGTCACATT 3′ ant(2′′)R 5′ CGCAAGACCTCAACCTTTTC 3′	500 bp	[17]

Table 2
Antimicrobial susceptibility profiles of tested *E. coli* strains.

Group (n)	Antibiotics	Percentage (number) of strains with MIC values (μg/ml) of													
		0.25	0.5	1	2	4	8	16	32	64	128	256	512	1024	S	R
ESBL producers (29)	GN	–	–	17.3% (5)	6.9% (2)	6.9% (2)	–	10.3% (3)	9.1% (4)	24.2% (7)	10.3% (3)	3.3% (1)	–	6.9% (2)	≤2	>4
	AM	–	–	–	31% (9)	24.2% (7)	6.9% (2)	20.7% (6)	10.3% (3)	6.9% (2)	–	–	–	–	≤8	>16
	NET	–	6.9% (2)	–	–	24.2% (7)	3.3% (1)	–	–	3.3% (1)	13.8% (4)	48.5% (14)	–	–	≤2	>4
	TOB	–	–	–	–	10.3% (3)	13.8% (4)	17.3% (5)	13.8% (4)	–	23.2% (7)	17.3% (5)	–	3.3% (1)	≤2	>4
ESBL non-producers (15)	GN	–	13.3% (2)	46.7% (7)	–	–	–	–	–	6.7% (1)	26.6% (4)	–	6.7% (1)	–	≤2	>4
	AM	–	–	40% (6)	46.7% (7)	13.3% (2)	–	–	–	–	–	–	–	–	≤8	>16
	NET	–	53.2% (8)	6.7% (1)	–	26.7% (4)	6.7% (1)	6.7% (1)	–	–	–	–	–	–	≤2	>4
	TOB	–	33.2% (5)	20% (3)	6.7% (1)	–	6.7% (1)	20% (3)	–	6.7% (1)	–	6.7% (1)	–	–	≤2	>4

Abbreviations: S: susceptible; R: resistant; GN: gentamicin; AM: amikacin; NET: netilmicin; TOB: tobramycin; n: number of strains.

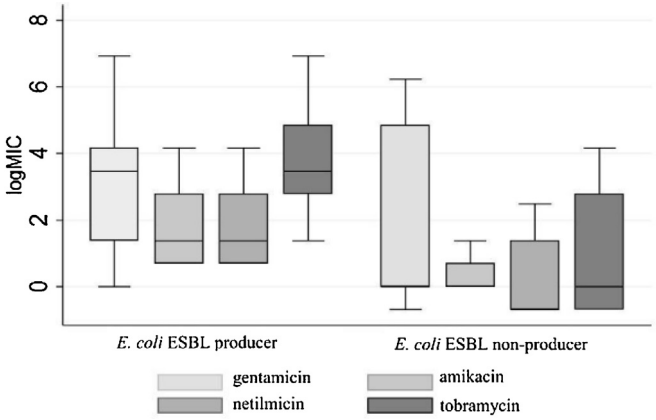

Fig. 1. Comparison of the MIC value median of aminoglycoside antibiotics for the tested *E. coli* strains of ESBL producers and ESBL non-producers.

3.2. Analysis of AMEs genes

The occurrence of genes encoding AMEs was identified in plasmid material from 35 strains (79.5%) that presented resistance or intermediate susceptibility against at least one of the tested aminoglycosides. Among five of the tested genes responsible for bacterial resistance to aminoglycosides, the presence of four genes was detected among all tested strains. PCR assays revealed the presence of *aac(6′)-Ib* among 26 (59.2%) strains, *aph(3″)-Ib* among 16 (36.2%), *aac(3-)Ia* among 7 (15.9%), and *ant(2″)-Ia* among 2

Table 3
Genes encoding AMEs, ESBLs and aminoglycoside resistance phenotypes among tested *E. coli* strains.

Genes encoding AMEs	Observed resistance phenotypes	Number of strains	Genes encoding ESBLs
aph(3″)-Ib, *aac(3)-Ia*, *aac(6′)-Ib*	GM^R TOB^R	1	(−)
ant(2″)-Ia, *aac(6′)-Ib*	TOB^R	1	bla_{CTX-M}
aac(3)-Ia, *aac(6′)-Ib*	GM^R AM^R NET^R TOB^R	2	bla_{SHV}; bla_{SHV}
	TOB^R	1	bla_{TEM}, bla_{CTX-M}
	GM^R NET^R TOB^R	2	bla_{SHV}; bla_{SHV}
	GM^R TOB^R	1	(−)
aac(6′)-Ib, *aph(3″)-Ib*	GM^R AM^R NET^R TOB^R	3	bla_{TEM}; bla_{TEM}, bla_{CTX-M}; bla_{TEM}, bla_{CTX-M}
	GM^R NET^R TOB^R	1	bla_{TEM}
	GM^R	2	bla_{TEM}, bla_{CTX-M}; bla_{CTX-M}
	NET^R TOB^R	1	bla_{CTX-M}
aac(6′)-Ib	AM^R NET^R TOB^R	1	bla_{CTX-M}
	NET^R TOB^R	3	bla_{CTX-M}; bla_{CTX-M}; bla_{CTX-M}
	GM^R NET^R TOB^R	2	bla_{CTX-M}; (−)
	GM^R TOB^R	3	bla_{CTX-M}; bla_{CTX-M}, bla_{TEM}; (−)
	TOB^R	2	bla_{CTX-M}; bla_{CTX-M}
aph(3″)-Ib	GM^R	1	bla_{SHV}, bla_{TEM}
	GM^R TOB^R	3	bla_{TEM}, bla_{CTX-M}; bla_{TEM}, bla_{CTX-M}; (−)
	GM^R NET^R TOB^R	3	bla_{TEM}, bla_{CTX-M}; bla_{TEM}, bla_{CTX-M}; (−)
	GM^R NET^R	1	bla_{TEM}, bla_{CTX-M}

Abbreviations: GN: gentamicin; AM: amikacin; NET: netilmicin; TOB: tobramycin; R: resistant; AMEs: aminoglycoside-modifying enzymes(−)no bla genes were detected.

(4.6%) strains. Further analysis revealed that *aac(6′)-Ib* and *aph(3")-Ib* genes were common among ESBL non-producers, and were detected among 4 (26,7%) and 3 (20%) strains, respectively; whereas, among ESBL producers, the most frequently detected genes encoding AMEs were *aac(6′)-Ib* and *aph(3")-Ib*, observed in 22 (75.9%) and 13 (44.8%) of isolates, respectively. In addition, few ESBL-producing strains presented *aac(3)-Ia* and *ant(2')-Ia* genes. Additionally, we noticed that one isolates harbored three genes encoding AMEs: *aph(3")-Ib*, *aac(3)-Ia*, *aac(6′)-Ib.* Moreover, simultaneous presence of two genes was detected among 14 (31.2%) tested strains. The *ant(2")-Ia* and *aac(6′)-Ib* genes were detected among 1 (2.3%) strain, *aac(3)-Ia* and *aac(6′)-Ib* genes were detected in 6 (13.6%) tested strains, and *aac(6′)-Ib* with *aph(3")-Ib* genes were seen in 7 (15.9%) strains. The presence of only one gene was observed among 19 (43.2%) tested strains. Eleven strains harbored only the *aac(6′)-Ib* gene, 8 (18.2%) strains harbored only the *aph (3")-Ib* gene, and 1 (2.3%) strain harbored only the *ant(2")-Ia* gene. Correlations between the presence of aminoglycoside resistance genes among tested strains and their aminoglycoside resistance profiles are shown in Table 3.

4. Discussion

In recent years, more and more reports about the occurrence of infections caused by multidrug-resistant *E. coli* have been reported [19]. Moreover, over the past years there has been an increase in aminoglycoside resistance among *Enterobacteriaceae* isolates [20,21]. The development of multidrug resistance has reduced therapeutic options for infections because of the growing number of organisms that are not susceptible to treatment [22]. This study focused on the evaluation of resistance to aminoglycoside antibiotics such as gentamicin, amikacin, netilmicin, and tobramycin among ESBL-producing as well as ESBL-non-producing *E. coli* strains. Additionally, this study evaluated the prevalence of *aac(3)-Ia*, *aac(6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib* genes and to estimate the relationship between bacterial phenotypes of aminoglycoside resistance and the prevalence of genes responsible for this resistance.

The results of DDST showed that the prevalence of ESBL is common among *E. coli* isolated from different clinical materials derived from hospital patients. These results are in accordance with literature reports showing 82% of isolates as ESBL producers among hospital isolates of *E. coli* [23]. Additionally, in our study *E. coli* isolates showed variable degrees of resistance against tested aminoglycosides as shown in Table 2. The highest percentages of resistance were observed for tobramycin (70.5%) and gentamicin (59%) followed by netilmicin (43.2%) and amikacin (11.4%). Among *E. coli* ESBL producers, the highest rates of resistance were observed for tobramycin (86.2%) and gentamicin (69%) followed by netilmicin (58%) and amikacin (24.2%). Among *E. coli* ESBL non-producers, the highest rates of resistance were observed for tobramycin (40%) and gentamicin (40%) followed by netilmicin (13.3%). Summarizing the results of susceptibility tests, amikacin was the aminoglycoside with the highest activity against *E. coli* ESBL producers as well as to *E. coli* ESBL non-producers with the percentages of susceptibility at 62.1% and 100%, respectively. Resistance to aminoglycosides was also the subject of research performed by *Lindemann* et al. who tested ESBL-producing E. coli clinical isolates and revealed high rates of their resistance to gentamicin (80.6%), netilmicin (89.4%), and tobramycin (94%). Moreover, this work is in accordance with our study in the aspect of high activity of amikacin against ESBL producers (6% resistance) [24]. The low rates of reduced susceptibility against amikacin was also observed by *Haldorsen* et al. and reached the level of 0.4%, whereas for gentamicin and tobramycin these rates were 3.2% and 3.4%, respectively [25].

All strains showing resistance or intermediate susceptibility to aminoglycoside antibiotics underwent molecular tests for detection genes: *aac(3)-Ia*, *aac(6′)-Ib*, *ant(4′)-IIa*, *ant(2")-Ia*, and *aph(3")-Ib*, encoding particular enzymes modifying aminoglycosides. PCR assays revealed that the AME genes were present in all of these isolates. Among these genes, *aac(6′)-Ib* and *aph(3")-Ib* were the most common, followed by *aac(3)-Ia*, and *ant(2")-Ia*, with rates of 59.2%, 36.2%, 15.9%, and 4.6%, respectively. Our results are in accordance with data from Spain where *Fernandez-Martinez* et al. presented *aac(6′)-Ib* and *ant(2")-Ia* as the one of the most frequently detected genes encoding AMEs in clinical isolates of *E. coli.* The AME genes found were 34.3% and 27.6% respectively [26]. *Xiao* et al. presented a study in which the *aac(3)-II* and *aac(6′)-Ib* genes were reported as dominant AME genes among Chinese clinical isolates of *E. coli.* In this study the *aac(3)-II* was reported among 162 strains while *aac(6′)-Ib* was reported among 50 strains. Additionally, 20 strains harbored the *ant(2")-I* gene, and *ant(3′)-I* with *aph(3)-II* genes were observed among 28 and 20 strains, respectively [27]. High prevalence of the *aph(3″)-Ib* gene was reported by *Miro* et al. [28]. PCR screening for AME genes performed by *Haldorsen* et al. also showed that the *aac(6′)-Ib* gene was the most prevalent among *E. coli* strains Moreover, in this study, the presence of *aac(3)-II* and *ant(2")-Ia* was also detected among tested *E. coli* strains [25]. French research conducted by *Fihman* et al. also observed the *aac(6′)-Ib* gene as common among *E. coli* [29]. Moreover, the coexistence of AME genes among particular strains was noticed. In our study three isolates harbored three genes encoding AMEs: *aph(3")-Ib*, *aac(3)-Ia*, and *aac(6′)-Ib.* The simultaneous presence of two genes (*ant(2")-Ia* and *aac(6′)-Ib, aac (3)-Ia* and *aac(6′)-Ib,* and *aac(6′)-Ib* with *aph(3")-Ib*)was detected among 14 (31.2%) tested strains. Strains carrying multiple aminoglycoside resistance genes were also detected by *Fernandez-Martinez* et al. during molecular analysis of AMEs in clinical isolates of *E. coli*. In this research the coexistence of *aac(6′)-Ib* and *aac(3)-IIa* was noticed in 7.6% of isolates, *aac(3)-IIa* and *aph(3′)-Ia* in 3.8%, *ant (2′)-Ia* and *aph(3′)-Ia* in 2.9%, and *aac(6′)-Ib* and *aph(3′)-Ia* in 1% of tested strains [29]. The presented data may indicate the geographic differentiation in the occurrence of AME genes. Also, significant differentiation may be observed in the phenotypes of resistance to aminoglycosides among *E. coli* as shown in Table 3. Differences in the phenotypes of resistance to aminoglycosides presented in this study, which did not always correspond with the known spectrum of enzymes action, may be explained by the possible presence of genes encoding other AMEs that we did not include in our study. Therefore, further studies are necessary to fully explain this phenomenon.

5. Conclusions

In conclusion, the enzymatic resistance against aminoglycosides in northeast Poland among clinical isolates of *E. coli* is predominantly caused by *aac(6′)-Ib* and *aph(3")-Ib*, while the occurrence of *aac(3)-Ia*, *ant(2")-Ia* is limited. The low rates of resistance against amikacin may suggest that this antibiotic can be used for the therapy of infections caused by ESBL-producing *E. coli.*

Conflict of interest

The authors declare no conflict of interest.

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