



BRIEF COMMUNICATION

Prevalence of aminoglycoside modifying enzyme and 16S ribosomal RNA methylase genes among aminoglycoside-resistant *Escherichia coli* isolates



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Aminoglycoside resistance determinants among 188 aminoglycoside-resistant blood culture *Escherichia coli* isolates from a tertiary hospital in Hong Kong, from 2004 to 2010 were investigated. Overall, 91% had *aac(3)-II*, 12.2% had *aac(6')-Ib/Ib-cr*, and 5.4% had the methylase genes (*rmtB*, *armA*). Aminoglycoside-resistant isolates with *aac(6')-Ib/Ib-cr*, *rmtB*, and *armA* often had coresistance to multiple other antibiotics.

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Introduction

Escherichia coli is the most common cause of Gram-negative bacteremia. For many years, a β -lactam in combination with

an aminoglycoside has been the standard therapy for empirical therapy suspected or confirmed Gram-negative bacteremia.^{1,2} Depending on the prevalence of resistance, the preferred aminoglycoside in most geographic areas is either gentamicin or amikacin.³ Genes encoding aminoglycoside-modifying enzymes (AME) and 16S ribosomal RNA (rRNA) methylases are the main causes of aminoglycoside resistance.^{3,4} In Gram-negative bacteria, the main gentamicin-resistant, AME genes are *aac(3)-I*, *aac(3)-II*, *aac(3)-III*, *aac(4)-IV*, and *ant(2'')-I*.³ Early epidemiological studies have

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shown that amikacin resistance often involved *aac(6′)-Ib* and occasionally *aph(4′)-II* and *aph(3′)-VI*.⁵ Because not all the substrates for a given AME may be tested resistant at the currently adopted breakpoints, interpretive reporting of susceptibility results is widely practiced according to the resistance profile of different aminoglycosides.⁶ Recent studies have also highlighted the increasing occurrence of 16S rRNA methylases, which cause high-level resistance [minimum inhibitory concentration (MIC) \geq 512 $\mu\text{g}/\text{mL}$] to all the 4-,6-aminoglycosides.⁴ Therefore, there is a need to regularly update the prevalent aminoglycoside resistance mechanisms. Here, we investigated the occurrence and distribution of the major AME and 16S rRNA methylases in aminoglycoside-resistant *E. coli*.

Materials and methods

Bacterial isolates

The isolates were recovered from blood cultures of patients who were treated in a regional hospital in Hong Kong, People's Republic of China. The hospital has 1400 acute care beds and provides service for all the clinical disciplines. All the isolates were stored in MicroBank (Pro-Lab Diagnostics, Cheshire, United Kingdom) at -80°C until testing. Bacteria were identified by the VITEK GNI system (bioMérieux, Hong Kong). All the blood culture isolates that were viable during 2004–2010 were included. Only one isolate per patient was included in this study. For those patients with more than one isolate, only the first isolate was tested. A total of 188 isolates of *E. coli* including 29 isolates from 2004 to 2008 and 159 isolates from 2009 to 2010 were included.

Antimicrobial susceptibility testing

The Clinical Laboratory Standards Institute's disc diffusion method was used for susceptibility testing: gentamicin \geq 15 mm (MIC \leq 4 $\mu\text{g}/\text{mL}$) sensitive, 13–14 mm (MIC 8 $\mu\text{g}/\text{mL}$) intermediate, \leq 12 mm (MIC \geq 16 $\mu\text{g}/\text{mL}$); and amikacin \geq 17 mm (MIC \leq 16 $\mu\text{g}/\text{mL}$) sensitive, 15–16 mm (MIC 32 $\mu\text{g}/\text{mL}$) intermediate, \leq 14 mm (MIC \geq 64 $\mu\text{g}/\text{mL}$).² The double-disk approximation method was used for detection of extended-spectrum β -lactamases (ESBL) as previously described.⁷

Microbiological methods

Genes encoding AME [*aac(3)-II*, *aac(6′)-Ib/Ib-cr*, *ant(2′′)-Ia*, *ant(4′)-IIa*, and *aph(3′)-VIa*] and 16S rRNA methylases (*rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *armA*) were sought by polymerase chain reaction (PCR) using specific primers^{1,2,8} and representative amplicons were sequenced for confirmation. Samples positive for *aac(6′)-Ib* were further analyzed by digestion with BstF5I (Takara Bio Inc., Otsu, Japan) to identify the *aac(6′)-Ib-cr* variant, which lacks the BstF5I restriction site present in the wild-type gene.⁹ Among the ESBL producers, genes related to the CTX-M subgroups (1, 2, 8, 9, and 25) were identified by PCR.¹⁰ Multilocus sequence typing (MLST) was carried out by the University of Warwick scheme (<http://mlst.warwick.ac.uk/mlst/>).

Statistical analysis

The Chi-square or Fisher's exact test were used for statistical analysis. A two-tailed $p < 0.05$ was considered significant. All analyses were performed using statistical software (SPSS, version 16.0; SPSS Inc., Chicago, IL, USA).

Results

All 188 isolates were gentamicin-resistant (Gen-R), of which 19.7% (37/188) and 32.4% (61/188) had coresistance to amikacin (Ak-R) and netilmicin, respectively. High-level resistance, as indicated by the absence of an inhibition zone around the discs, to gentamicin, amikacin, and netilmicin were detected in 98.4% (185/188), 19.7% (37/188), and 8.0% (15/188) of the isolates, respectively. Eleven isolates (5.9%) exhibited high-level resistance for all three aminoglycosides. The phenotypic and genotypic characteristics of the isolates stratified by gentamicin and amikacin resistance patterns are summarized in Table 1. The comparison showed that Gen-R/Ak-R isolates were more likely than Gen-R/Ak-S isolates to be ESBL positive, ciprofloxacin resistant, chloramphenicol resistant, and multidrug resistant.

PCR showed that 95.2% of the isolates (179/188) were positive for at least one aminoglycoside resistance gene: 153 isolates (81.4%) had one gene, 26 (13.8%) had two genes, and one (0.5%) had three genes. Overall, 171 isolates (91%) had *aac(3)-II*, 23 (12.2%) had *aac(6′)-Ib/Ib-cr* (including 12 *aac(6′)-Ib* and 11 *aac(6′)-Ib-cr*), eight (4.3%) had *rmtB*, four (2.1%) had *ant(2′′)-Ia*, and two (1.1%) had *armA*. The prevalence of *aac(3)-II* gene among isolates collected in 2004–2008 and 2009–2010 were 65.5% (19/29) and 95.6% (152/159), respectively ($p < 0.001$). All 10 isolates positive for *rmtB* or *armA* exhibited high-level resistance to both gentamicin and amikacin with the isolates growing to the edges of the discs. By comparison, only three of the *aac(6′)-Ib/Ib-cr*-positive isolates had no inhibition zones around the amikacin disc. The remaining *aac(6′)-Ib/Ib-cr*-positive isolates ($n = 20$) had amikacin inhibition diameters that ranged from 9 mm to 23 mm, including five isolates with inhibition diameters that fall into the susceptible range (17–23 mm). The proportions of ESBL producers among isolates positive or negative for the 16s rRNA methylase genes were similar [33.3% (3/9) vs. 33.0% (59/179), $p = 0.98$]. By contrast, significantly more *aac(6′)-Ib/Ib-cr*-positive isolates than *aac(6′)-Ib/Ib-cr*-negative isolates were ESBL producers [69.6% (16/23) vs. 27.9% (46/165), $p < 0.00$]. CTX-M-type genes were detected in 90.3% of the ESBL-producers (56/62): 46 isolates had *bla*_{CTX-M-9G}, eight had *bla*_{CTX-M-1G}, and two had both *bla*_{CTX-M-9G} and *bla*_{CTX-M-1G}. Six isolates were negative for all *bla*_{CTX-M} subgroup genes. MLST analysis revealed that the 37 Gen-R/Ak-R isolates belong to 18 different sequence types (STs). Six STs including ST405 ($n = 8$), ST131 ($n = 6$), ST95 ($n = 4$), ST38 ($n = 3$), ST648 ($n = 2$), and ST354 ($n = 2$) comprised 25 isolates. The remaining 12 STs had one isolate each (ST12, ST46, ST88, ST117, ST167, ST173, ST224, ST393, ST453, ST964, ST1287, and ST2003). The 10 isolates with the 16s rRNA methylase genes belonged to seven STs (ST95, ST117, ST224, ST405, ST453, ST964, and ST1287).

Table 1 Occurrence of AMG genes according to aminoglycoside resistance phenotypes

	No. (%) of isolates			<i>P</i> ^b
	Total	Gen-R/Ak-S	Gen-R/Ak-R	
Number of isolates	188	151	37	
Coresistance				
ESBL	62 (33.0)	45 (29.8)	17 (45.9)	0.06
Cotrimoxazole	151 (80.3)	122 (80.8)	29 (78.4)	0.7
Ciprofloxacin	106 (56.4)	71 (47.0)	35 (94.6)	< 0.001
fosfomycin	7 (3.7)	7 (4.6)	0 (0)	0.4
chloramphenicol	67 (35.6)	43 (28.5)	24 (64.9)	< 0.001
MDR	68 (36.2)	44 (29.1)	24 (64.9)	< 0.001
Aminoglycoside resistance genes				
<i>aac(3)-II</i>	171 (91.0)	146 (96.7)	25 (67.6)	< 0.001
<i>ant(2'')-Ia</i>	4 (2.1)	0 (0)	4 (10.8)	0.001
<i>aac(6')-Ib</i>	11 (5.9)	1 (0.7)	10 (27.0)	< 0.001
<i>aac(6')-Ib-cr</i>	12 (6.4)	4 (2.6)	8 (21.6)	< 0.001
<i>rmtB</i>	8 (4.3)	0 (0)	8 (21.6)	< 0.001
<i>armA</i>	2 (1.1)	0 (0)	2 (5.4)	0.2
Number of aminoglycoside resistance genes				
None	8 (4.3)	5 (3.3)	3 (8.1)	
1	153 (81.4)	141 (93.4)	12 (32.4)	
2 or more	27 (14.4) ^a	5 (3.3)	22 (59.5)	

^a Including *aac(3)-II* + *aac(6')-Ib* (*n* = 9), *aac(3)-II* + *aac(6')-Ib-cr* (*n* = 8), *aac(3)-II* + *rmtB* (*n* = 5), *aac(6')-Ib-cr* + *ant(2'')-Ia* (*n* = 3), *aac(6')-Ib* + *ant(2'')-Ia* (*n* = 1), *aac(3)-II* + *aac(6')-Ib-cr* + *armA* (*n* = 1).

^b For Gen-R/Ak-S versus Gen-R/Ak-R comparisons.

Ak-R = amikacin-resistant; Ak-S = amikacin-susceptible; Gen-R = gentamicin-resistant; MDR = multidrug resistant (as defined by c-resistance involving three or more of ESBL, cotrimoxazole, ciprofloxacin, fosfomycin, and chloramphenicol).

Discussion

This study showed that a substantial proportion of the gentamicin resistance in *E. coli* blood culture isolates was attributed to *aac(3)-II*. In our region, the annual prevalence of gentamicin and amikacin resistance among clinical isolates of *E. coli* ranged 30–33% and 1–3%, respectively during 2000–2010.⁷ We previously demonstrated that 84.1% of gentamicin-resistant *E. coli* collected from patients with urinary tract infection in 2002–2004 was positive for *aac(3)-II*.² Therefore, this AME gene has been the predominant resistance determinant in different *E. coli* populations for the past decade. This may be explained by the fact that gentamicin is the workhorse aminoglycoside in local clinical practices. The *aac(3)-II* gene has at least five sequence alleles, from *aac(3)-IIa* to *aac(3)-IIe*.^{2,3} In China, the prevalent alleles were *aac(3)-IIc* and *aac(3)-IIe*, whereas in other parts of the world the main alleles were from *aac(3)-IIa* to *aac(3)-IIc*.^{2,3} The *aac(3)-II* produces resistance to gentamicin, tobramycin, and netilmicin.³ Given its stronger affinity for gentamicin than tobramycin and netilmicin, strains with *aac(3)-II* are highly resistant to gentamicin but may only exhibit low-level resistance to the latter two aminoglycosides.³ In the current collection, 71.3% of the *aac(3)-II* positive isolates (122/171) had netilmicin inhibition zones larger than the CLSI breakpoint (i.e. ≥ 15 mm inhibition zone, equivalent to MIC ≤ 8 μ g/mL) and would be classified as susceptible. In areas where *aac(3)-II* are common, sensitive result for tobramycin and

netilmicin in gentamicin-resistant *E. coli* should be suppressed or be reported with a caution statement.⁶

Among the amikacin-resistant isolates, one or more resistance determinants (*aac(6')-Ib/Ib-cr*, *rmtB*, and *armA*) were identified in 73.0% (27/37). The mechanism of amikacin resistance in the remaining 10 isolates was unknown. Our findings showed that the gentamicin and amikacin coresistant isolates were genetically diverse, suggesting that horizontal spread rather than expansion of clones are playing more important roles in the dissemination of the resistance determinants. Among *E. coli* isolates, previous studies have highlighted the common occurrence of *aac(6')-Ib/Ib-cr*, *armA*, or *rmtB* genes co-carried with *bla_{CTX-M}* on the same plasmids.⁹ In addition, *aac(6')-Ib/Ib-cr*, *rmtB*, and *bla_{CTX-M}* could also occur as integron cassettes.⁹ Whether those genes are also carried on the same plasmids in our isolates deserve further investigation in the future. Our results demonstrated that most gentamicin and amikacin coresistant *E. coli* were resistant to ciprofloxacin. The association may be explained by the high prevalence of *aac(6')-Ib-cr* among the gentamicin- and amikacin-coresistant *E. coli*. *Aac(6')-Ib-cr* reduces the activity of ciprofloxacin by acetylation, and presence of this enzyme also increased the frequency of selection of chromosomal mutants upon exposure to ciprofloxacin.⁹

In conclusion, this work shows the prevalence of *aac(6')-Ib-cr* and *rmtB* among blood culture isolates with coresistance to aminoglycosides and multiple other antibiotic classes. Emerging multidrug resistance involving drugs

considered to be critically important for serious human infections highlight the need to expedite integration of rapid diagnostic testing, such as matrix-assisted laser desorption/ionization time-of-flight with antimicrobial stewardship team intervention.

Conflicts of interest

All authors declare no conflicts of interest.

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