Spectrum of Aminoglycoside Modifying Enzymes in Gram-Negative Bacteria Causing Human Infections

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Abstract

Introduction Aminoglycosides are formidable broad-spectrum antibiotics used in clinical settings; woefully their usage has been reduced by the emergence and distribution of resistance mainly due to aminoglycoside modifying enzymes (AME).

Purpose This study was performed to determine the diverse prevalence of AME and their pattern of occurrence in the clinical isolates of gram-negative bacteria. This study also aimed to detect the presence of AMEs that are prevalent in gram-positive bacteria, among gram negatives.

Materials and Methods A total number of 386 clinical isolates were included in this study. Polymerase chain reaction revealed the prevalence rate of AMEs screened [aac(6′)-Ib, aac(3′)-I, aac(3′)-II, aac(3′)-VI, ant(2′)-I, ant(4′)-IIb, aac(3′)-III, aac(3′)-IV, aph(2′)-Ib, aph(2′)-Ic, aph(2′)-Id, aac (6′)-Ie- aph(2′)-Ia, and aph(3′)-IIIa]. Conjugation experiment was performed for the clinical isolates which harbored any one of the AME which was prevalent in gram-positive bacteria [aph(3′)-IIIa, aac(6′)-Ie-aph(2′)-Ia].

Results aac(6′)-Ib is the most prevalent AME, followed by aac(3′)-I, aph(3′)-VI, aac(3′)-VI, and aac(3′)-II. The AMEs such as ant (2′)-I, ant(4′)-IIb, aac(3′)-III, aac(3′)-IV, aph(2′)-Ib, aph(2′)-Ic, and aph(2′)-Id were not established in our study isolates. The rate of prevalence of aph(3′)-IIIa, aac(6′)-Ie-aph(2′)-Ia—the AMEs encountered in gram-positive and their co-existence was 19.68% and the conjugation experiment revealed their transfer via plasmids.

Conclusion This is the first report from India revealing the presence and prevalence of AMEs which are often encountered among gram-positive bacteria in gram negatives and their presence on conjugative plasmids.

Keywords
- AME
- conjugative plasmid
- GP AME
- gram-negative bacteria

Introduction

Aminoglycosides (AG) play an important and adjunctive role in the treatment of life-threatening infections owing to their synergetic and broad-spectrum activity against both gram-positive and gram-negative bacteria. This group of antibiotics bind to the ribosomes of the bacteria thereby leading to inhibition of protein synthesis and consequent bacterial cell death. Their extensive use has resulted in development and dissemination of resistance to this class of antimicrobials.

The mechanisms of aminoglycoside resistance are diverse. The most common mechanism is the inactivation of the antibiotic by a family of enzymes named aminoglycoside modifying enzymes (AME).[1] The AMEs catalyze the modification of the AGs at–OH or–NH_2 groups of the 2-deoxystreptamine nucleus or sugar moieties via acetyltransferases, nucleotidyltransferases, and phosphotransferases[2] which modify the drug, resulting in poor binding to the ribosome thereby allowing the bacteria to survive in the presence of the drug.[3]

Besides the AMEs, other resistance mechanisms include change in bacterial membrane permeability, expression of

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efflux pumps, and methylation through 16S ribosomal RNA methyltransferases. These 16S rRNA methyltransferases are often encountered in multidrug-resistance gram negatives especially among New Delhi Metallo betalactamase producers.[4]

There are more than 85 AMEs reported in both gram-positive and gram-negative bacteria. A few among them, particularly ant(2′)-I, aac(6′)-I, aac(3′)-I to IV, and aac(3′)-VI, undergo continuous mutation[5] leading to the generation of new AME variants which utilize a variety of AGs as substrates, cleave them, and make them ineffective. These AMEs virtually spread to all bacterial types through conjugative plasmids, natural transformation, or transduction.[6]

Dissemination of AMEs via plasmids has been reported in developed countries.[7] Since there is a paucity of information from India regarding the prevalence and type of AMEs in gram-negative bacteria, the present study was undertaken to determine their prevalence among clinical isolates of gram-negative bacteria in a tertiary care center.

There are some AMEs which were originally detected in gram-positive (GP AMEs) bacteria and characteristically occur in them. They are aph(3′)-IIIa that is the most prevalent and a bifunctional AME aac(6′)-Ie-aph(2′)-Ia. They confer resistance to a broad spectrum of AGs. There have been reports of their presence in gram-negative clinical isolates from Slovakia and Germany.[8] Hence, this study was aimed to detect their presence in gram-negative bacteria.

Materials and Methods

Ethical Approval

The study was approved by the Institutional Ethical Committee (IEC-NI/15/APR/6/18).

Bacterial Isolates

A total of 386 amikacin-resistant gram-negative bacteria which were clinically significant and nonduplicate were collected over a period of 3 years from June 2015 to September 2018. All the isolates were speciated based on conventional or VITEK-2 system (Vitek-2 GN-card; BioMerieux).The bacterial isolates included were obtained from different clinical sources such as blood (14), urine (176), exudate (162), and respiratory secretion s(34)

Antibiotic Susceptibility Testing

Disk diffusion test was performed in accordance with the Clinical Laboratory Standard Institute (CLSI, 2016).[9] The AGs tested were amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), and netilmicin (10 µg) (HiMedia Laboratories).

Polymerase Chain Reaction

Nine sets of uniplex and two sets of multiplex polymerase chain reactions (PCRs) were performed for AMEs using previously described primers and conditions.[10-14] The primers used for different sets of genes, their annealing temperatures, and the amplicon sizes are listed in ►**Table 1**.

Seven sets of uniplex PCRs were performed for 16SrRNA methyltransferase using primers established in our earlier study.[15]

Each reaction volume contained 2 µL of the deoxyribonucleic acid (DNA) template added to the master mix which includes 10 pmol of the forward and reverse primers (Sigma-Aldrich), 10 mm deoxyribonucleotide triphosphate (Takara), 5U Taq polymerase (Takara), and 10× buffer with $MgCl_2$ (Takara).

Amplification of the reactions was performed under the following conditions: initial denaturation at 95°C for 4 minutes, followed by 32 cycles of denaturation at 94°C for 30 seconds, annealing based on the primer employed for 30 seconds with an extension at 72°C for 50 seconds, and a final extension for one cycle at 72°C for 5 minutes. The PCR product was then run on a 1.5% agarose gel for detection of the amplified fragment.

Template DNA Preparation

A single bacterial colony was inoculated into Luria-Bertani broth (HiMedia Laboratories) and incubated overnight at 37°C, and it was then centrifuged at 10,000 rpm for 10 minutes. The pellet was re-suspended in 250 µL of Millipore water, boiled at 100°C for 10 minutes, and cooled and centrifuged at 10,000 rpm for 10 minutes. The supernatant served as the template DNA.[16]

DNA Sequencing

PCR-positive amplicons were purified and sequenced. The sequenced strains served as positive controls. Sequencing was done by BigDye 3.1 cycle sequencing kit using Sanger AB13730 XL DNA analyzing instrument (AgriGenome). The nucleotide sequences were aligned using the Bioedit sequence program (product version 7.0.5.3) and were compared with the basic alignment search tool available at the National Center for Biotechnology Information website (www.ncbi.nIm.nih.gov).

Conjugation Assay

Bacterial conjugation was performed at 37°C for the clinical isolates which harbored either one of the GP AMEs [aph(3′)-IIIa and aac(6′)-Ie-aph(2′)-Ia]. Azide-resistant *Escherichia coli* J53 served as recipient. The transconjugants were selected on MacConkey agar plate containing 100 µg of sodium azide (HiMedia Laboratories) along with 4 µg of amikacin.[17] The transferability of the AMEs through plasmid in transconjugants was confirmed by PCR.

Result

The study isolates includes *E. coli* (n = 79), *Klebsiella pneumonia* (n = 149), *Klebsiella oxytoca* (n = 4), *Citrobacter freundii* (n = 2), *Enterobacter cloacae* (n = 11), *Proteus mirabilis* (n = 5), *Proteus vulgaris* (n = 2), *Providencia rettgeri* (n = 16), *Morganella morganii* (n = 12), *Pseudomonas aeruginosa* (n = 63), *Pseudomonas fluorescens* (n = 4), *Pseudomonas putida* (n = 1), and *Acinetobacter baumannii* (n = 38). All the

Table 1 Primers used for performing PCR for amplifying AME genes

Simplex set	Gene	Primer	Annealing temperature (°C)	Amplicon size (bp)
1	aac(6′)-Ib	F-TTG CGA TGC TCT ATG AGT GGC TA R-CTC GAA TGC CTG GCG TGT TT	60	482
2	aph(3′)-VI	F-ATGGAATTGCCCAATATTATT R-TCAATTCAATTCATCAAGTTT	55	780
3	aac(3′)-I	F-TTC ATC GCG CTT GCT GCY TTY R-GC CAC TGC GGG ATC GTC RCC RTA	56	239
4	aac(3′)-II	F-GCG CAC CCC GAT GCM TCS ATG G R-GGC AAC GGC CTC GGC GTA RTG SA	58	370
5	aac(3′)-III	F-GAC AAT GGC GTG CTA SCS GAR T R-C CAG ATG CTC GGC ATG RTG SAG	58	241
6	aac(3′)-IV	F-GAC GAC GAG CCG TTC GAY CC R-C CT CAA CTC GGC AAG ATG SAG	58	280
7	aac(3′)-VI	F-GCC CT CCC GAC GCA TCS ATG G R-CGC CAC CGC TTC GGC ATA RTG SA	55	780
8	ant(2′)-I	F-TGG GCG ATC GAT GCA CGG CTR G R-AA AGC GGC ACG CAA GAC CTC MAC	58	428
9	ant(4′)-IIb	F TAT CTC GGC GGT CGA GT R CAC GCG GGG AAA CGC GAG AA	60	364
Multiple × 1	aac(6′)-Ie-aph(2′)-Ia	F-CAGGAATTTATCGAAAATGGTAGAAAAG R-CACAATCGACTAAAGAGTACCAATC	55	369
	aph(3′)-IIIa	F-GGCTAAAATGAGAATATCACCGG R-CTTTAAAAAATCATACAGCTCGCG		523
Multiple × 2	aph(2′)-Ib	F-CTTGGACGCTGAGATATATGAGCAC R-GTTTGTAGCAATTCAGAAACACCCTT	58	867
	aph(2′)-Ic	F-CCACAATGATAATGACTCAGTTCCC R-CCACAGCTTCCGATAGCAAGAG		444
	aph(2′)-Id	F-GTGGTTTTTACAGGAATGCCATC R-CCCTCTTCATACCAATCCATATAACC		641

Abbreviations: AME, aminoglycoside modifying enzymes; PCR, polymerase chain reaction.

study isolates exhibited resistance to all the tested AGs as determined by disk diffusion method.

PCR identification revealed the prevalence of 16SrRNA methyltransferases and AMEs, of which all the clinical isolates carried one or more than one 16SrRNA methyltransferases (data were not disclosed in this study). Of the study isolates, 46.63% harbored single AME and 38.86% harbored more than one AME. The distributions of these enzymes among the different gram-negative species were tabulated (►**Table 2**).

Interestingly GP AMEs namely aph(3′)-IIIa and aac(6′)-Ie-aph(2′)-Ia were identified in this study isolates. They were detected in 3.88 and 8.03% of the study isolates, respectively (►**Table 3**). Co-occurrence of both of these enzymes was encountered in 7.77%.

However, AMEs such as ant(2′)-I, ant(4′)-IIb, aac(3′)-III, aac(3′)-IV, aph(2′)-Ib, aph(2′)-Ic, and aph(2′)-Id were not encountered in any of the study isolates.

Conjugation assay was successful in all the clinical isolates tested which harbored the GP AMEs.

Discussion

AGs play a vital role as monotherapy and in combination for the treatment of majority of bacterial infection. The resistance to AGs in bacteria is predominantly due to the AMEs.[1]

All the 386 clinical isolates were resistant to all the tested AGs. They did not exhibit any substrate-specific hydrolyzing profile which is commonly encountered in AME. This is attributable to the presence of 16S rRNA methyltransferases which confer resistance to all AGs.[4]

The prevalence of aac(6′)-Ib singly and in combinations with other AME is higher (►**Table 2**) when compared with previous reports from Iran, China, and Spain which had 31. 6, 19.6, 4.2% of aac(6′)-Ib, respectively.[18-20] The AME aac(3′)-I was the second most prevalent gene singly and in also combination(6.21 and 17.61%). This enzyme has been reported in large number of gram-negative clinical isolates previously.[2] The enzyme aph(3′)-VI, first identified in *A. baumannii* in 1988,[21] was the third most prevalent gene.

Table 2 Distribution of AME genes and their combination among different gram negatives

Aminoglycoside modifying enzyme genes	Total number of gram negatives (*n* = 386)								
	E. coli (*n* = 79)	Klebsiella (*n* = 153)	Citrobacter (*n* = 2)	Enterobacter (*n* = 11)	Proteus (*n* = 7)	Providencia (*n* = 16)	Morganella (*n* = 12)	Pseudomonas (*n* = 68)	Acinetobacter (*n* = 38)
aac(6′)-Ib (*n* = 135)	22	59	2	5	4	4	6	21	12
aac(3′)-I (*n* = 24)	7	4	–	1	3	3	1	4	1
aph(3′)-VI (*n* = 13)	–	5	–	–	–	–	1	7	–
aac(3′)-VI (*n* = 5)	–	–	–	3	–	–	–	–	2
aac(3′)-II (*n* = 3)	–	3	–	–	–	–	–	–	–
aac(6′)-Ib+ aac(3′)-I(*n* = 68)	30	24	–	1	–	1	–	10	2
aac(6′)-Ib+ aph(3′)-VI (*n* = 47)	6	24	–	1	–	–	2	7	7
aac(6′)-Ib+ aac(3′)-II (*n* = 8)	3	5	–	–	–	–	–	–	–
aph(3′)-VI + aac(3′)-I (*n* = 3)	–	–	–	–	–	–	–	–	3
aac(6′)-Ib+ aph(3′)-VI + aac(3′)-I (*n* = 20)	2	7	–	–	–	–	–	7	4
aac(6′)-Ib+ aac(3′)-I+ aac(3′)-II (*n* = 1)	1	–	–	–	–	–	–	–	–
aac(6′)-Ib+ ac(3′)-I+ aac(3′)-II + aph(3′)-VI (*n* = 3)	3	–	–	–	–	–	–	–	–

Abbreviations: AME, aminoglycoside modifying enzymes.

Table 3 Study isolates associated with AMEs prevalent in gram-positive bacteria

Gram-positive AMEs	Total number of gram negatives (n = 386)								
	E. coli (*n* = 79)	Klebsiella (*n* = 153)	Citrobacter (*n* = 2)	Enterobacter (*n* = 11)	Proteus (*n* = 7)	Providencia (*n* = 16)	Morgonella (*n* = 12)	Pseudomonas (*n* = 68)	Acinetobacter (*n* = 38)
aph(3′)-IIIa (*n* = 15)	–	7	–	–	–	–	–	8	–
aac(6′)-Ie-aph(2′)-Ia (*n* = 31)	4	5	–	–	2	5	2	8	5
aph(3′)-IIIa + aac(6′)-Ie-aph(2′)-Ia (*n* = 30)	6	13	–	3	–	–	–	8	–

Abbreviation: AME, aminoglycoside modifying enzymes.

AMEs such as ant(2′)-I, ant(4′)-IIb, aac(3′)-III, and aac(3′)-IV were not encountered in our study isolates but their presence was widely reported in countries like Iran,[22] France[23] and China.[24] The GP AMEs aph(2′)-Ib, aph(2′)-Ic, and aph(2′)-Id were not encountered in our study; however, their presence was significantly reported in *Enterococci* and *Staphylococcus*.[14,25] This significant difference in their presence of AMEs may be due to usage of antibiotics and other geographical factors involved.[19]

The bifunctional enzyme aac(6′)-Ie-aph(2′)-Ia that confers high-level resistance to gentamicin, amikacin, tobramycin, netilmicin and is considered more prevalent in *Enterococci*[26]has been identified in the present study; there are two previous reports citing its presence and transferability in gram-negative bacteria.[8] The prevalence of these AMEs in this study is 19.68% and their transfer indicates their location on conjugative plasmids. However, the prevalence rate is significantly less compared with their rate of occurrence in *Enterococci* (38.20%).[27]

Conclusion

Our findings throw light on the distribution of the different AMEs and their combination among the clinical isolates of gram-negative bacteria. To the best of our knowledge, this is the first report to study the presence of GP AMEs in gram-negative bacteria from India. Considering the transferability potential of these resistance genes between

gram-positive and gram-negative bacteria frequent surveillance studies are required to study the changing pattern and evolution of resistance among bacteria.

Conflict of Interest

None.

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