Introduction

Aminoglycosides (AG) play an important and adjunctive role in the treatment of life-threatening infections owing to their synergistic 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–NH2 groups of the 2-deoxystreptamine nucleus or sugar moieties via acetyltransferases, nucleotidyltransferases, and phosphotransferases2 which modify the drug, resulting in poor binding to the ribosome thereby allowing the bacteria to survive in the presence of the drug.1

Besides the AMEs, other resistance mechanisms include change in bacterial membrane permeability, expression of
Aminoglycoside Modifying Enzymes in Gram-Negative Bacteria  Aishwarya et al.

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 (34).

Antibiotic Susceptibility Testing

Disk diffusion test was performed in accordance with the Clinical Laboratory Standard Institute (CLSI, 2016). 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. 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. 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 MgCl2 (Takara).

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′)-le-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. 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 morgani (n = 12), Pseudomonas aeruginosa (n = 63), Pseudomonas fluorescens (n = 4), Pseudomonas putida (n = 1), and Acinetobacter baumannii (n = 38). All the
study isolates exhibited resistance to all the tested AGs as determined by disk diffusion method.

PCR identification revealed the prevalence of 16S rRNA methyltransferases and AMEs, of which all the clinical isolates carried one or more than one 16S rRNA methyltransferase (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′)-le-aph(2′)-la 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′)-lc, and aph(2′)-ld 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.\(^2\)

The prevalence of aac(6′)-Ib singly and in combinations (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.\(^3\) The AME aac(3′)-I was the second most prevalent gene singly and in combination (6.21 and 17.61%). This enzyme has been reported in large number of gram-negative clinical isolates previously.\(^4\) The enzyme aph(3′)-VI, first identified in *A. baumannii* in 1988,\(^5\) was the third most prevalent gene.
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,
France
and China.

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.

This significant difference in their presence of AMEs may be due to usage of antibiotics and other geographical factors involved.

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
has been identified in the present study; there are two previous reports citing its presence and transferability in gram-negative bacteria.

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%).

**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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