




Molecular Characterization of High-Level Aminoglycoside Resistance among *Enterococcus* Species

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Abstract

Background *Enterococci* are nosocomial pathogen. They can develop high-level resistance to aminoglycoside by producing aminoglycoside modifying enzymes (AMEs). In enterococci, high level resistance to aminoglycosides is mediated by acquisition of plasmid mediated genes encoding for aminoglycoside modifying enzymes (AMEs). High level gentamicin resistance (MIC \geq 500 μ g/mL) is predominantly mediated by *aac(6′)-Ie-aph(2′)-Ia*, encoding the bifunctional aminoglycoside modifying enzyme AAC(6′)-APH(2′). This enzyme eliminates the synergistic activity of gentamicin when combined with a cell wall active agent. Other AME genes such as *aph(2′)-Ib*, *aph(2′)-Ic*, *aph(2′)-Id* and *ant(4′)-Ia* have also been detected in enterococci.

Objective This study was carried out to determine the diverse prevalence of AME and their pattern of occurrence in the clinical isolates of *Enterococci*.

Materials and Methods A total number of 150 clinical isolates were included in this study. Susceptibility to various antibiotics was determined by disc diffusion. Minimum Inhibitory Concentration (MIC) was ascertained by agar dilution method. Polymerase chain reaction was done to screen the following AMEs (*aac(6′)-Ie-aph(2′)-Ia*; *aph(2′)-Ib*; *aph(2′)-Ic*; *aph(2′)-Id* and *aph(3′)-IIIa* genes).

Results 51.3% of the study isolates exhibited high level gentamicin resistance. Polymerase chain reaction revealed that *aph(3′)-IIIa* is the most prevalent AME, followed by *aac(6′)-Ie-aph(2′)-Ia*. The combination of both the genes were detected in 44.1% of the study isolates. The rest of the AMEs and their combinations were not encountered in this study. 8.6% of the study isolates did not harbour any AME genes screened for, but was phenotypically resistant to gentamicin. In contrast 31.3% anchored the AME genes but phenotypically appeared susceptible to gentamicin.

Conclusion This study indicates the high-level aminoglycoside resistance disseminated among *Enterococci* in our geographical region. It also emphasizes the detection of AMEs by PCR is mandatory because strains that appear susceptible by disc diffusion and/or MIC method may harbour one or more AMEs genes leading to therapeutic failure.

Keywords

- ▶ AMEs
- ▶ *Enterococcus* species
- ▶ high-level aminoglycoside resistance
- ▶ encoding genes

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Introduction

Enterococci have emerged as an important multidrug-resistant nosocomial pathogen causing health-care-associated infections ranging from urinary tract infection, to surgical site infection, prosthetic valve endocarditis, and sepsis. They are highly resilient and versatile, which make them adaptive and survive in the health care environments.¹

Two species *Enterococcus faecalis* and *Enterococcus faecium* cause the majority of enterococcal infections. They exhibit multidrug resistance by both intrinsic and extrinsic mechanisms. Intrinsically they are resistant to common antibiotics like cephalosporins, penicillinase-resistant penicillin, low-level aminoglycosides, clindamycin, sulfamethoxazole, and trimethoprim. Extrinsically they acquire resistance to high-level aminoglycoside, high-level ampicillin, and vancomycin either through mutations or horizontal transfer of resistant genes.¹

Enterococci can develop resistance to aminoglycoside by two different mechanisms, one is the low-level resistance which is due to reduced cell wall permeability and this type can be overcome by using a combination of aminoglycoside and cell-wall-acting agents. Another mechanism is the high-level resistance (HLR) which is due to the production of aminoglycoside-modifying enzymes (AMEs). This enzyme in enterococci negates the synergistic activity of aminoglycoside when it is being combined with a cell-wall-acting agent.^{2,3}

Previously *aac(6')-Ie-aph(2'')-Ia* was the only gene found to be associated with high level gentamicin resistance (HLGR). But in recent years three new AME genes that mediate HLGR in enterococci have been detected, namely *aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id*. Resistance to other aminoglycosides like high level streptomycin and high level kanamycin are usually mediated by *aph(3')-IIIa* gene but not to gentamicin. *Ant(4')-Ia* gene is also usually associated with high level Aminoglycoside resistance (HLAR).^{2,4-9}

Studies on prevalence of these resistance genes are limited. The purpose of this study is to determine the rate of HLARs and their genetic mechanism in clinical isolates of enterococci. Also, to screen for other common genes that encode for HLAR.

Materials and Methods

Study Setting

This study was conducted in a 1,600-bedded university teaching hospital from August 2018 to February 2019. The study protocol was approved by the institutional ethics committee (REF: CSP-MED/18/AUG/45/113).

Bacterial Strains

The study included 150 clinically significant, consecutive, and nonrepetitive enterococcal isolates recovered from clinical specimens of hospitalized patients. The isolates were obtained from clinical specimens such as blood, pus, and urine. The organisms were identified up to species level either by conventional biochemical tests or by an automated

method (Micro scan Walk Away 96, Gram-positive panels). Care was taken to differentiate commensals from pathogens for isolates obtained from nonsterile sites (urinary tract and wound swabs). The significance of the isolates was based on clinical history, presence of the organism in the Gram stain, presence of intracellular forms of the organism, and growth in culture with a significant colony count.

Antimicrobial Susceptibility Testing

Susceptibility to various classes of antibiotics was determined by the disc diffusion method in accordance with Clinical Laboratory Standard Institute (CLSI 2018) guidelines. The antibiotics tested were ampicillin (10 µg), high-level gentamicin (120 µg), erythromycin (15 µg) (for isolates from exudates), vancomycin (30 µg), linezolid (30 µg), nitrofurantoin (300 µg) (for urinary isolates), and ciprofloxacin (5 µg) (for urinary isolates). The antimicrobial agents were procured from Himedia Laboratories (Mumbai, Maharashtra, India).

Minimal Inhibitory Concentration

The overnight bacterial culture was inoculated in a nutrient broth and incubated for 20 minutes. The turbidity was adjusted to 0.5 McFarland standard. An amount of 1 µL of this inoculum containing 10 cfu/spot was spotted on a nutrient agar plate containing gentamicin at a concentration of 500 µg/mL. Growth of organism in the media was indicative of HLGR.

Template DNA Preparation

A single bacterial colony was inoculated into Luria-Bertani broth (Himedia Laboratories, Mumbai, Maharashtra, India) 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.

Polymerase Chain Reaction

Two sets of multiplex and one simplex polymerase chain reactions (PCRs) were performed using the previously described primers and conditions for all the study isolates. The multiplex primers used for different sets of genes, their annealing temperature, and the amplicon size are listed in ►Table 1.

Each reaction volume contained 2 µL of the DNA template added to the master mix which includes 10 pmol of the forward and reverse primers (Sigma-Aldrich, Missouri, United States), 10 Mm dNTPs (Takara, Shiga, Japan), 5 U taq polymerase (Takara, Shiga, Japan), and 10X buffer with MgCl₂ (Takara, Shiga, Japan).

Amplification reactions were performed under the following conditions: initial denaturation at 95°C for 5 minutes, followed by 32 cycles of denaturation at 95°C for 60 seconds, annealing at 58°C for 45 seconds, and elongation at 72°C for 60 seconds, repeated for 32 cycles and a final extension at 72°C for 5 minutes. The PCR product was then run on a 1.5% agarose gel for detection of the amplified fragment (►Fig. 1).

Table 1 Genes and their sequences for aminoglycoside-modifying enzymes used in PCR

Multiplex set	Gene	Primer sequence (5' → 3')	Amplicon size (bp)	Annealing temperature (°C)
1	<i>aac(6')-Ie-aph(2'')-Ia</i>	F-CAGGAATTTATCGAAAATGGTAGAAAAG R-CACAATCGACTAAAGAGTACCAATC	369	58
	<i>aph(3')-IIIa</i>	F-GGCTAAAATGAGAATATCACCGG R-CTTTAAAAAATCATACAGCTCGCG	523	58
2	<i>aph(2'')-Ib</i>	F-CTTGGACGCTGAGATATATGAGCAC R-GTTTGTAGCAATTCAGAAACACCCTT	867	58
	<i>aph(2'')-Ic</i>	F-CCACAATGATAATGACTCAGTTCCC R-CCACAGCTTCCGATAGCAAGAG	444	58
	<i>aph(2'')-Id</i>	F-GTGGTTTTTACAGGAATGCCATC R-CCCTCTTCATACCAATCCATATAACC	641	58
Simplex	<i>ant(4')-Ia</i>	F-CAAACGCTAAATCGGTAGAAAGCC R-GGAAAGTTGACCAGACATTACGAACT	294	58

Abbreviation: PCR, polymerase chain reaction.

Nucleotide Sequencing

PCR-positives were purified and sequenced. Sequencing was performed using the BigDye 3.1 cycle sequencing kit in Sanger AB13730 XL DNA analyzing instrument (AgriGenome). The aligned sequences were then analyzed with the Bioedit sequence program. Similarity searches for the nucleotide sequences were performed with the BLAST program and sequences were submitted for the accession numbers (<http://www.ncbi.nlm.nih.gov>). These sequenced strains served as positive controls.

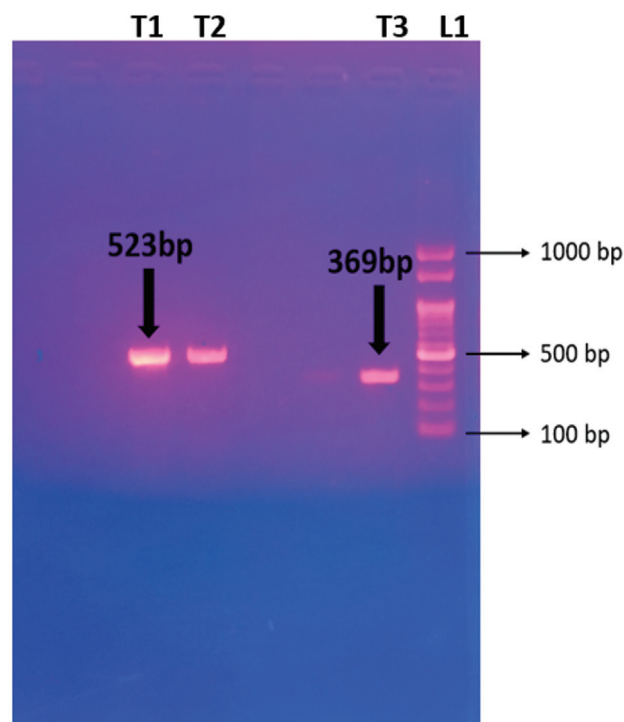


Fig. 1 Image of gel electrophoresis of PCR for detecting aminoglycoside modifying enzyme (AME) gene. Band at 523bp (T1&T2) represents the presence of *aph(3')-IIIa* gene and band at 369bp (T3) represents presence of *aac(6')-Ie-aph(2'')-Ia* gene. L1 is the 100bp ladder. PCR, polymerase chain reaction.

Results

Out of the 150 isolates, 130 (86.6%) were *E. faecalis* and 20 (13.3%) were *E. faecium*. They were obtained from exudates (pus and wound swabs) 95 (63.3%), urine 52 (34.6%), and blood 3 (2%) (► **Table 2**).

By the Kirby–Bauer disc diffusion method, sensitivity percentages to antibiotics tested are as follows: ampicillin 75% (113/150), high-level gentamicin 48.7% (73/150), erythromycin for isolates from exudates 13% (12/95), vancomycin 98% (147/150), linezolid 100% (150/150), nitrofurantoin 98% (51/52), and ciprofloxacin 46% (24/52).

Minimal inhibitory concentration (MIC) by the agar dilution method revealed high-level gentamicin (> 500 µg/mL) resistance in 51.3% (77/150) isolates (► **Fig. 2**).

PCR screening for AME genes revealed that 111 of the 150 isolates harbored one or more AME-encoding genes. This distribution is as follows: 11.3% (17/150) isolates harbored *aac(6')-Ie-aph(2'')-Ia* gene alone, 18.6% (28/150) isolates carried *aph(3')-IIIa* gene alone, and 44.1% (66/150) isolates co-harbored both the above genes. The other AME genes were not detected in this study (► **Table 3**).

Discussion

Of the 150 isolates collected 130 (86.6%) were *E. faecalis* and 20 (13.3%) were *E. faecium*. This reflects the preponderance of *E. faecalis* over *E. faecium* among the pathogenic enterococcal species. Almost all the studies had *E. faecalis* as the

Table 2 Sample wise distribution of the species

Source of the isolates	<i>Enterococcus faecalis</i>	<i>Enterococcus faecium</i>	Total
Exudate	82	13	95
Urine	46	6	52
Blood	2	1	3
Total	130	20	150

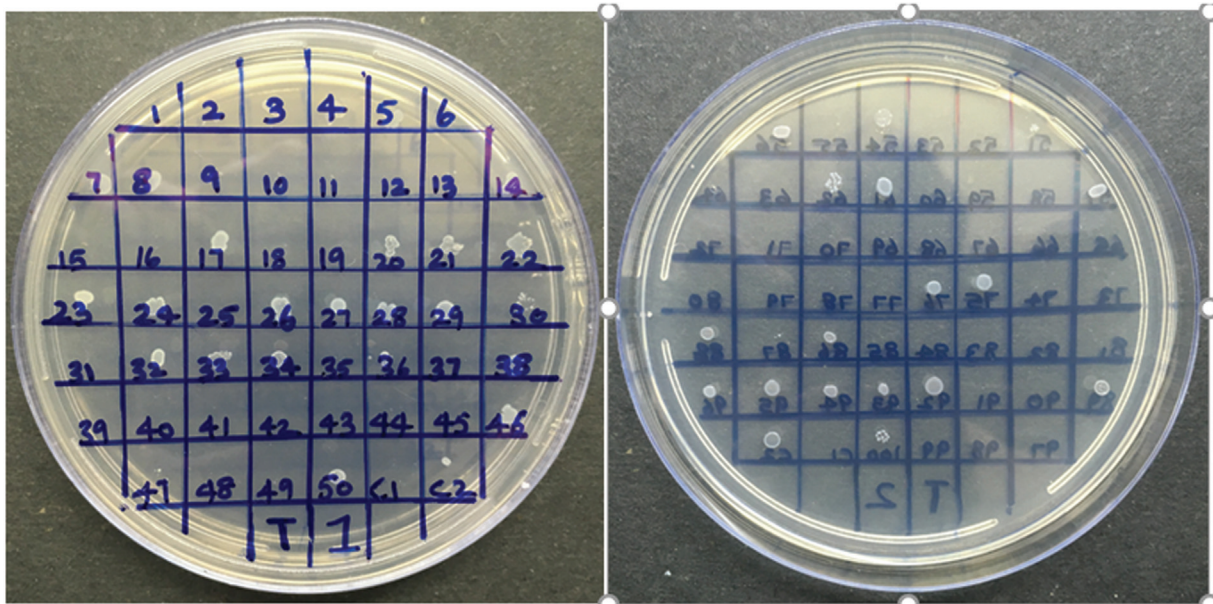


Fig. 2 Image of minimum inhibitory concentration (MIC) testing for high level gentamicin (> 500 µg/mL); done by the agar dilution method.

predominant isolate except few studies, like a study conducted in Michigan by Vakulenko et al⁴ in which *E. faecium* was the predominant species. This is in alignment with various other studies conducted across India where *E. faecalis* has been predominant accounting for 80 to 85%.

Of the 150 isolates, 77 (51.3%) were identified to be resistant to high-level gentamicin phenotypically. Majority of the HLGR enterococci were from urine 47 (61%), followed by exudates 30 (39%). There was no HLGR enterococci isolated from blood stream. The speciation revealed that 60 (78%) were *E. faecium* and 17 (22%) were *E. faecalis*. This is in concordance with the previous study from Chennai by Padmasini et al² where *E. faecium* 39 (51.3%) was found to have higher rates of HLGR than *E. faecalis* 32 (42%).

The percentage of HLGR in this study was 51.3%, while a few study had lower incidence of HLGR ranging from 27.7 to 49.2%. Others reported higher incidence of 60% to 68%.

Out of the 150 study isolates, 77 (51.3%) were found to be resistant to high-level gentamicin by the agar dilution method. The results of MIC by the agar dilution method and the Kirby-Bauer disc diffusion method were in concordance. No discrepancy was noted, hence the disc diffusion method can be used as a reliable screening test to detect HLGR in enterococci in a clinical laboratory.

Although a spectrum of AME genes are known to be responsible for HLAR status among *Enterococcus* species, in

this study only *aac(6')-1e-aph(2'')-1a* and *aph(3')-IIIa* were encountered among the six genes screened for. Among the study isolates, 55.3% (83/150) isolates had *aac(6')-1e-aph(2'')-1a* gene and 62.6% (94/150) isolates had *aph(3')-IIIa* gene. Varying distribution of both the genes has been cited in the medical literature: *aac(6')-1e-aph(2'')-1a* (38.5–80%); *aph(3')-IIIa* (40–40.4%). The coexistence of these genes was noted in 44% (66/150) isolates in the current study, which is twice that of the previous study from Chennai by Padmasini et al,² which had only 20.2%. Other major AME genes like *aph(2'')-1b*, *aph(2'')-1c*, *aph(2'')-1d*, and *ant(4')-1a* were not detected in this study. Previous studies from India had also reported similar AME gene profile with only *aac(6')-1e-aph(2'')-1a* and *aph(3')-IIIa* genes being detected.^{2,10} This observation emphasizes the restricted gene distribution and transfer of resistance gene confined to a geographical region.

Studies from abroad, like the study by Diab et al from Egypt, have observed the presence of aminoglycoside-modifying gene *aac(6')-1e-aph(2'')-1a* only in 66.7% of their HLGR isolates and *aph(3')-IIIa* gene in 86.5% of high-level streptomycin resistance isolates. *aph(2'')-1b*, *aph(2'')-1c*, and *aph(2'')-1d* were not detected.¹¹

Moussa et al characterized HLAR for the presence of AME. The bifunctional AME gene *aac(6')-1e-aph(2'')-1a* that confers HLR to gentamicin was detected in 40% of *E. faecalis* and *E. faecium* isolates, whereas 32% carried *aph(3')-IIIa*. Other AME

Table 3 Distribution pattern of various AME genes

No. of strains with gene(s)	Presence of gene					
	<i>aac(6')-1e-aph(2'')-1a</i>	<i>aph(2'')-1b</i>	<i>aph(2'')-1c</i>	<i>aph(2'')-1d</i>	<i>aph(3')-IIIa</i>	<i>ant(4')-IIIa</i>
17	+	–	–	–	–	–
28	–	–	–	–	+	–
66	+	–	–	–	+	–

Abbreviation: AME, aminoglycoside-modifying enzyme.

genes such as *aph(2'')-Ib*, *aph(2'')-Ic*, and *aph(2'')-Id* were not detected in their study as well.¹²

Elsewhere in Michigan, Vakulenko et al⁴ detected the presence of all majorly prevalent AME genes: *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*, and *ant(4')-Ia*. Of the 93 gentamicin-resistant isolates, all contained either the *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, or *aph(2'')-Id* gene and one isolate carried both *aac(6')-Ie-aph(2'')-Ia* and *aph(2'')-Ic*. The *aph(3')-IIIa* gene was present in 80 of 113 isolates, and the *ant(4')-Ia* gene was present in 26 of 113 isolates. Five of the 20 isolates with low-level resistance to gentamicin contained none of the six genes studied.

In the present study, though 77 (51.3%) of the isolates were phenotypically resistant to gentamicin, 13(8.6%) did not harbor the majorly prevalent AME genes. It may be proposed that they may harbor genes other than those screened for in this study. In contrast, 47 (31.3%) harbored the AME genes but phenotypically appeared susceptible to gentamicin. Possibly they were not expressed, and this observation is in concordance with a previous study from Chennai.²

Conclusion

Enterococcus faecalis is more common than *E. faecium* among clinical isolates of enterococci. For detection of HLGR, the performance of the disc diffusion susceptibility test is similar to MIC determination by agar dilution. Hence the disc diffusion test can be used as a reliable screening test for HLGR in clinical microbiology laboratory. The most common AMEs mediating HLGR are *aac(6')-Ie-aph(2'')-Ia* and *aph(3')-IIIa*. This indicates that HLAR genes are widely disseminated among enterococci in our geographical region. This study also emphasizes that the detection of AMEs by PCR is mandatory because strains that appear susceptible by disc diffusion and/or MIC method may harbor one or more AME genes leading to therapeutic failure. And also frequent surveillance studies should be conducted among *Enterococcus* isolates to document the resistant gene profile.

Conflict of Interest

None.

Reference

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