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Research Article

Molecular Characterization of High-Level Gentamicin Resistance in Enterococcal Isolates from Clinical Samples in South India

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ABSTRACT

Background: High-level gentamicin resistance (HLGR) in Enterococci compromises the synergistic efficacy of aminoglycosides combined with cell wall-active antibiotics, creating significant treatment challenges.

Objective: To determine the prevalence of HLGR phenotypically and to characterize the underlying resistance genes in Enterococcus isolates from clinical specimens at a tertiary care center in South India.

Methods: A total of 104 Enterococcus isolates were tested for HLGR using the Kirby-Bauer disc diffusion method with high-concentration gentamicin discs. PCR assays were performed on HLGR isolates to detect aminoglycoside resistance genes: aac(6')-Ie-aph(2")-Ia, aph(2")-Ib, aph(2")-Ic, and aph(2")-Id.

Results: HLGR was observed in 64 (61.5%) isolates. Molecular analysis revealed that all HLGR isolates harbored the bifunctional aminoglycoside-modifying enzyme gene aac(6')-Ie-aph(2")-Ia. None of the isolates carried aph(2")-Ib, aph(2")-Ic, or aph(2")-Id genes.

Conclusion: The aac(6')-Ie-aph(2")-Ia gene is the predominant mechanism of HLGR among Enterococcus isolates in this region. Routine molecular screening for this gene is recommended to enhance antimicrobial resistance surveillance and guide effective therapeutic and infection control strategies.

Keywords: High-Level Gentamicin Resistance, Enterococcus, Aminoglycoside-Modifying Enzyme, Aac(6')-Ie-Aph(2")-Ia Gene, Molecular Characterization.

INTRODUCTION

Enterococci, traditionally regarded as low-virulence commensals of the gastrointestinal tract, have emerged over recent decades as significant nosocomial pathogens [1]. They are increasingly implicated in a wide spectrum of hospital-acquired infections, including urinary tract infections (UTIs), bacteremia, intra-abdominal infections, surgical site infections, and endocarditis [2, 3]. Their ability to survive harsh environmental conditions and acquire resistance to multiple antibiotics has made Enterococcus species a formidable challenge in clinical settings [4]. Aminoglycosides, particularly gentamicin, have long been employed as part of combination therapy with cell wall-active agents such as beta-lactams (ampicillin) or glycopeptides (vancomycin) to treat serious enterococcal infections. This combination exploits the synergistic effect where cell wall-active antibiotics facilitate aminoglycoside penetration, leading to enhanced bactericidal activity [5, 6]. However, the clinical utility of this synergism is threatened by the rise of high-level aminoglycoside resistance (HLAR) among enterococci, which negates the synergy and results in treatment failures [7, 8]. High-level gentamicin resistance (HLGR) is especially concerning because it renders gentamicin ineffective even at high concentrations, eliminating the option of combination therapy with aminoglycosides [7]. The most common and clinically significant mechanism of HLGR involves the production of aminoglycoside-modifying enzymes (AMEs). These enzymes chemically modify aminoglycosides, preventing their binding to bacterial ribosomes and thereby conferring resistance [9, 10]. Among the AMEs, the bifunctional enzyme encoded by the aac(6′)-Ie-aph(2″)-Ia gene is the most widely reported and clinically relevant. This gene encodes a bifunctional enzyme that combines acetyltransferase and phosphotransferase activities, conferring resistance to gentamicin and several other aminoglycosides. Its presence is often associated with high-level resistance phenotypes and is linked to treatment failure in enterococcal infections [11]. Other aminoglycoside resistance genes, including aph(2″)-Ib, aph(2″)-Ic, and aph(2″)-Id, have also been identified, though less frequently, and contribute variably to aminoglycoside resistance profiles. Understanding the prevalence and molecular basis of HLGR in *Enterococcus* species is critical for guiding appropriate antimicrobial therapy and infection control strategies [12-14]. Despite the clinical importance, data on the molecular characterization of HLGR among enterococcal isolates from South India remain limited. This study was therefore undertaken to determine the prevalence of phenotypic HLGR in clinical Enterococcus isolates and to characterize the underlying resistance genes, with a focus on the detection of the aac(6′)-Ie-aph(2″)-Ia gene and other AME genes, using polymerase chain reaction (PCR). The findings aim to provide valuable insights into the local epidemiology of HLGR and assist in the development of effective antimicrobial stewardship policies.

MATERIALS AND METHODS

Study Design and Setting:

A prospective observational study was conducted over an eight-month period from January to August 2017 at the Institute of Microbiology, Madurai Medical College, a tertiary care center located in South India.

Sample Collection and Identification:

A total of 104 clinical isolates of Enterococcus species were included in the study. These isolates were obtained from various clinical specimens such as urine, blood, pus, wound swabs, and other body fluids. Initial identification of Enterococcus species was carried out by standard microbiological methods, including culture on selective media, Gram staining, catalase test, and biochemical tests such as bile esculin hydrolysis and growth in 6.5% NaCl broth. Species-level identification was confirmed using conventional biochemical reactions or automated systems where applicable.

Phenotypic Detection of High-Level Gentamicin Resistance (HLGR):

HLGR was detected by the Kirby-Bauer disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Enterococcal isolates were tested against high-concentration gentamicin discs (120 µg) and streptomycin discs (300 µg) placed on Mueller-Hinton agar plates inoculated with bacterial suspensions standardized to 0.5 McFarland turbidity. Plates were incubated aerobically at 35±2°C for 16–18 hours. Absence of an inhibition zone around the discs was interpreted as high-level resistance to gentamicin or streptomycin, indicating resistance that compromises synergistic therapy.

DNA Extraction:

Genomic DNA was extracted from all phenotypically confirmed HLGR isolates using a standard boiling method or commercial DNA extraction kits, following the manufacturer's protocols. Briefly, bacterial colonies were suspended in sterile distilled water, heated to lyse cells, and centrifuged to obtain DNA-containing supernatants, which were stored at –20°C until further use.

Molecular Characterization by PCR:

Polymerase chain reaction (PCR) assays were performed to detect the presence of aminoglycoside resistance genes associated with HLGR. Specific primers targeting the following genes were used:

- aac(6′)-Ie-aph(2″)-Ia (encoding the bifunctional aminoglycoside-modifying enzyme)
- aph(2″)-Ib
- aph(2″)-Ic
- aph(2″)-Id

PCR amplification conditions were optimized for each target gene, including initial denaturation, annealing, extension, and final extension steps. Amplified PCR products were subjected to electrophoresis on 1.5% agarose gels stained with ethidium bromide or a safe alternative and visualized under UV transillumination. The presence of specific amplicons of expected sizes confirmed the presence of respective resistance genes.

Data Analysis:

Phenotypic and genotypic data were compiled and analyzed descriptively. The prevalence of HLGR and the distribution of resistance genes among Enterococcus species were calculated as percentages. Results were correlated to determine the concordance between phenotypic resistance and genotypic profiles.

RESULTS

Among the 104 Enterococcus isolates analyzed, 61.5% (64/104) demonstrated high-level gentamicin resistance (HLGR) based on phenotypic testing, while 27.8% (29/104) exhibited high-level streptomycin resistance (HLSR). Resistance to both gentamicin and streptomycin was observed in 23.07% (24/104) of the isolates (Table 1). Species-wise distribution showed that HLGR was slightly more prevalent in *E. faecium* (66.6%) compared to *E. faecalis* (62.3%). Similarly, HLSR affected 33.3% of *E. faecium* isolates and 25.97% of *E. faecalis* isolates. Concurrent resistance to both aminoglycosides was seen in 29.6% of *E. faecium* and 20.77% of *E. faecalis* isolates. Vancomycin resistance was detected in 7.6% (8/104) of the isolates, including 5.19% (4/77) of *E. faecalis* and 14.8% (4/27) of *E. faecium*. Importantly, 7 out of these 8 vancomycin-resistant isolates also exhibited HLGR, indicating a significant overlap between vancomycin and aminoglycoside resistance. Molecular analysis by PCR confirmed the presence of the aac(6′)-Ie-aph(2″)-Ia gene in all 64 HLGR isolates. None of the isolates were positive for aph(2″)-Ib, aph(2″)-Ic, or aph(2″)-Id genes, demonstrating the predominance of the bifunctional aminoglycoside-modifying enzyme gene in conferring high-level gentamicin resistance in this cohort.

Table 1: Distribution of High-Level Aminoglycoside Resistance and Vancomycin Resistance among Enterococcus Isolates

Species	**Total Isolates (n)**	**HLGR (n, %)**	**HLSR (n, %)**	**Resistance to Both (n, %)**	**Vancomycin Resistant (n, %)**	**VRE with HLGR (n)**
E. faecalis	77	48(62.3%)	20 (25.97%)	16(20.77%)	4 (5.19%)	3
E. faecium	27	18(66.6%)	9 (33.3%)	8(29.6%)	4(14.8%)	4
Total	104	64(61.5%)	29 (27.8%)	24(23.07%)	8(7.6%)	7

DISCUSSION

The high prevalence of high-level gentamicin resistance (HLGR) observed in this study (61.5%) among clinical Enterococcus isolates is concerning, particularly because aminoglycosides like gentamicin play a vital role in combination therapy with cell wall-active agents to achieve synergistic bactericidal effects. The widespread resistance significantly compromises the efficacy of these therapeutic regimens and poses a challenge to clinical management of enterococcal infections.

Our findings corroborate earlier studies that have identified the bifunctional aminoglycoside-modifying enzyme encoded by the aac(6′)-Ie-aph(2″)-Ia gene as the predominant mechanism driving HLGR in Enterococci [15, 16]. The consistent presence of this gene in all HLGR isolates highlights its critical role in inactivating gentamicin and related aminoglycosides by enzymatic modification. Notably, other aminoglycoside resistance genes such as aph(2″)-Ib, aph(2″)-Ic, and aph(2″)-Id were not detected in this cohort, suggesting a possible regional variation or selective antibiotic pressure that favors the dominance of the bifunctional enzyme gene. This pattern aligns with global epidemiological data, which identify the aac(6′)-Ie-aph(2″)-Ia gene as the chief contributor to HLGR worldwide [17, 18]. Species-wise, *E. faecium* exhibited a slightly higher frequency of HLGR (66.6%) compared to *E. faecalis* (62.3%). This difference mirrors the well-documented trend that *E. faecium* often displays greater multidrug resistance, complicating treatment options and infection control measures [19, 20]. The co-occurrence of vancomycin resistance and HLGR in several isolates further exacerbates the clinical challenge, as it restricts the available therapeutic arsenal and increases the risk of treatment failure [21]. The study underscores the critical importance of incorporating routine phenotypic and molecular screening for HLGR in clinical microbiology laboratories. Detection of the aac(6′)-Ie-aph(2″)-Ia gene via PCR provides a rapid, sensitive, and specific method for identifying resistant strains, enabling timely infection control interventions and guiding targeted antimicrobial therapy. Enhanced surveillance and molecular characterization of resistance determinants are essential to curb the spread of multidrug-resistant enterococci in healthcare settings.

CONCLUSION

High-level gentamicin resistance is highly prevalent among clinical Enterococcus isolates in this region of South India, posing a significant challenge to effective antimicrobial therapy. The aac(6′)-Ie-aph(2″)-Ia gene is the predominant mechanism responsible for this resistance. Incorporating molecular diagnostic methods such as PCR into routine laboratory workflows is essential for the early and accurate detection of HLGR, enabling timely intervention and appropriate treatment. Furthermore, reinforcing antimicrobial stewardship programs and stringent infection control policies is crucial to limit the dissemination of these multidrug-resistant pathogens in healthcare settings.

Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

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