

High Level Aminoglycoside Resistance And Distribution Of The Resistance Genes In *Enterococcus faecalis* And *Enterococcus faecium* From Teaching Hospital In Malaysia

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Background: *Enterococcus faecium* and *Enterococcus faecalis* are among the predominant species causing hospital-acquired infections. Currently, enterococcal infections are treated using combination therapy of an aminoglycoside with cell-wall active agents, which led to high level aminoglycoside resistance (HLAR) and vancomycin resistance (VRE) among enterococci. The aim of this study was to determine the prevalence of HLAR and the distribution of the resistance genes among clinical *E. faecalis* and *E. faecium* isolates in Malaysia.

Materials and methods: Seventy-five enterococci isolates recovered from different clinical sources were re-identified by subculturing on selective medium, Gram staining, biochemical profiling (API 20 Strep), and 16s rRNA sequencing. Antimicrobial susceptibility testing (AST) was performed using Kirby-Bauer disc diffusion, E-test, and broth microdilution methods. PCR amplification was used to detect the presence of aminoglycoside modifying enzyme (AME) genes [*aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, *aph(3')-IIIa*]. Descriptive data analysis was used to analyze the antibiotic susceptibility profiles and the distribution of HLAR genes.

Results: The majority of the isolates recovered from the clinical samples are *E. faecalis* (66.7%), with the highest recovery from the pus. The prevalence of HLGR (51%) is higher when compared to HLSR (45–49%). Analysis of the resistance genes showed that bifunctional genes *aac(6')-Ie-aph(2'')-Ia* and *aph(3')-IIIa* contributed to the HLAR *E. faecalis* and *E. faecium*. The other AME genes [*aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*] were not detected in this study.

Conclusion: This study provides the first prevalence data on HLAR and the distribution of the AME genes among *E. faecalis* and *E. faecium* isolates from Malaysia. These highlight the need for continued antibiotic surveillance to minimize its emergence and further dissemination.

Keywords: enterococci, high level aminoglycosides resistance, aminoglycoside modifying enzyme

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Introduction

Enterococci are Gram-positive, non-motile, non-spore forming bacteria that constitute a major part of the human normal flora, mainly in the gastrointestinal tract and vagina. They comprise of over 50 distinct species with different characteristics including habitats and phenotype.¹ Enterococci have emerged as one of the most common causes of hospital and community acquired infections due to their adaptability to various environmental conditions and the limited treatment options of

multidrug resistant strains.² In Malaysia, there were 2,263 and 1,647 enterococcal infections cases reported in 2006 and 2007, respectively.^{3,4}

The most common clinical manifestations of enterococcal infections were urinary tract and wound infections, septicemia, and endocarditis. The recommended management of enterococcal infections involves a synergistic combination of aminoglycosides such as streptomycin and gentamicin with cell wall active inhibitors such as glycopeptides or beta-lactams. The emergence of high-level aminoglycoside resistant (HLAR) enterococci has posed significant challenges for infection management due to production of aminoglycosides modifying enzymes (AME) that inactivate aminoglycosides.⁵ Acquisition of AME genes such as *aac(6')-Ie-aph(2'')-Ia*, *aph(2'')-Ib*, *aph(2'')-Ic*, *aph(2'')-Id*, and *aph(3'')IIIa* by enterococci has seriously affected the treatment for enterococcal infections.⁶ High-level aminoglycosides resistance (HLAR) in enterococci was first detected in 1980s and is defined by MIC \geq 512 $\mu\text{g/mL}$.⁷

The AME that confer HLAR in enterococci catalyze the modification either at amino- or hydroxyl groups of the 2-deoxystreptamine nucleus or the sugar moieties of aminoglycosides.⁸ The three classes of AMEs are N-Acetyltransferases [AAC (6') and AAC (6')-II], which catalyze acetyl-CoA-dependent acetylation of an amino group, O-Adenyltransferases [ANT (4') and ANT (6')], which catalyze ATP-dependent adenylation of hydroxyl group, and O-Phosphotransferases [APH (2'') and APH (3'')], which catalyze ATP-dependent phosphorylation of a hydroxyl group.^{7,9}

The distribution of HLAR genes varies depending on the different geographical areas and is widely distributed across Asia, North America, and Europe.¹⁰ These genes have been reported to be either integrated within the mobile genetic elements or plasmid-encoded, in which both are transferable via horizontal gene transfer.¹¹ In Malaysia, the prevalence of HLAR and the distribution of AME in enterococcal isolates are still limited despite the high incidence rates. This study is therefore aimed at determining the prevalence of HLAR and the distribution of AME genes among enterococcal isolates from clinical samples obtained from a teaching hospital in Malaysia. The findings from this study provide the first Malaysian data on the prevalence of HLAR and AME genes distribution which will highlight the needs for continual monitoring of antibiotic surveillance in the country.

Materials And Methods

Ethics Approval

Ethical clearance to conduct this study was obtained from the Ethics Committee for Research Involving Human Subjects, Universiti Putra Malaysia [JKEUPM Ref. No. FPSK (FR16) P030].

Bacterial Isolates

A total of 75 *Enterococcus* isolates which were part of the routine hospital laboratory procedure were collected from Hospital Kuala Lumpur, Malaysia. These isolates were previously recovered from pus, blood, urine, and other miscellaneous sources such as cerebrospinal fluid (CSF) and high vaginal swab (HVS). The *E. faecalis* reference strains used in this study were ATCC 51299 and ATCC 29212. All *Enterococcus* isolates were phenotypically and genotypically re-identified using different techniques including subculturing on bile esculin agar, Gram staining, biochemical profiling using API 20 Strep (BioMerieux, Inc., USA) and 16s rRNA sequencing.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility profile of *Enterococcus* was carried out using Kirby-Bauer disc diffusion methods according to Clinical and Laboratory Standards Institute (CLSI, 2016) guidelines. The antibiotics for disc diffusion test were obtained from Oxoid, UK in the following concentrations; gentamicin 120 μg , streptomycin 300 μg , ampicillin 10 μg , vancomycin 30 μg , linezolid 30 μg , tetracycline 30 μg , chloramphenicol 30 μg , and erythromycin 15 μg . Minimum inhibitory concentrations (MIC) were determined using E-test for vancomycin, and broth micro-dilution for high level gentamicin 512 $\mu\text{g/mL}$ and streptomycin 1024 $\mu\text{g/mL}$. The experiment was carried out in triplicate, with *E. faecalis* ATCC strains ATCC 51299 and ATCC 29212 used as resistant and susceptible controls, respectively.

Polymerase Chain Reaction (PCR)

DNA extraction was performed using the QIAmp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. PCR was subsequently performed in conditions consisting of an initial denaturation and denaturation steps between 94°C and 98°C, for 1–3 mins, annealing for 1 min at 45–65°C which was calculated as 5°C below the melting temperature (T_m) of the two primers, extension at 72°C for a 1 min/kb of the expected size

Table 1 Primers Used In PCR Assay For 16s rRNA And Detection Of Aminoglycosides Resistance Genes^{9,12}

Genes	Primer Sequences (5' – 3')	Product Size (bp)
16s rRNA	F: GTGCTGCAGAGAGTTGATCCTGGCTCAG R: CACGATCCTACGGGTACCTTGTACGACTT	1465
<i>aac(6')-Ie-aph(2'')-Ia</i>	F: CAGGAATTTATCGAAAATGGTAGAAAAG R: CACAATCGACTAAAGAGTACCAATC	369
<i>aph(2'')-Ib</i>	F: CTTGGACGCTGAGATATATGAGCAC R: GTTTGTAGCAATTCAGAAACACCCTT	867
<i>aph(2'')-Ic</i>	F: CCACAATGATAATGACTCAGTTCCC R: CCACAGCTTCCGATAGCAAGAG	444
<i>aph(2'')-Id</i>	F: GTGGTTTTTACAGGAATGCCATC R: CCCTCTTCATACCAATCCATATAACC	641
<i>aph(3')-IIIa</i>	F: GGCTAAAATGAGAATATCACCGG R: CTTTAAAAAATCATAACGCTCGCG	523

of PCR product, final extension at 72°C for 4 min, and preservation at 4°C. Primers used in this study are listed in Table 1. The PCR components were 12.5 µL of 2 x EconoTaq® PLUS GREEN master mix (10 µM), 0.5 µL of forward and reverse primers, and 10.5 µL of sterile milli-Q water and DNA template.

Data Analysis

The descriptive data analysis (frequency and percentage) on the distribution of HLAR genes and antibiotic susceptibility profiling were analyzed using SSPS version 22 and Prism (one-way ANOVA). *P*-value <0.05 was considered as significant.

Results

Re-identification of the enterococci isolates showed that the predominant species was *E. faecalis* (n=50), with the remaining isolates identified as *E. faecium* (n=25). The majority of the *E. faecalis* isolates were recovered from pus (38.7%), 18.6% from blood, 2.7% from urine, and 6.7% from miscellaneous sources. In contrast, the highest percentage of *E. faecium* was recovered from blood (13.3%), followed by pus (12%) and urine (8%) specimens (Table 2).

Preliminary antibiotic screening showed that a total of 48% and 46% of *E. faecalis* isolates were HLGR and HLSR, respectively. The resistance prevalence was also observed for tetracycline (98%), erythromycin (96%), chloramphenicol (46%), ampicillin (24%), vancomycin (6%), and linezolid (4%), as displayed in Table 3 and Figure 1. Conversely, up to 84% and 68% of *E. faecium*

Table 2 Distribution Of *Enterococcus* Species

Specimen	<i>E. faecalis</i> (n=50), n (%)	<i>E. faecium</i> (n=25), n (%)	Total Isolates (n=75), n (%)
Pus	29 (38.7)	9 (12)	38 (50)
Blood	14 (18.6)	10 (13.3)	24 (32)
Urine	2 (2.7)	6 (8)	8 (11)
Others	5 (6.7)	0 (0)	5 (7)
Total	50 (66.7)	25 (33.3)	75 (100)

Abbreviation: n, number of occurrence.

isolates were found to be HLGR and HLSR, respectively. All *E. faecium* isolates showed resistance to tetracycline and erythromycin; 84% of the isolates were resistant to ampicillin, whereas 32% were chloramphenicol-resistant. None of the *E. faecium* isolates were resistant to vancomycin or linezolid (Table 4 and Figure 1).

The isolates were confirmed as HLGR and HLSR using broth microdilution at concentrations 512 µg/mL for gentamicin and 1,024 µg/mL for streptomycin. Out of 47 HLAR *E. faecalis* isolates, 24 (51%) and 23 (49%) of them showed MICs up to 512 µg/mL and 1024 µg/mL of gentamicin and streptomycin, respectively. From a total of 38 HLAR *E. faecium* isolates, 21 (55%) showed MIC of 512 µg/mL of gentamicin, while another 17 (45%) showed MIC of 1,024 µg/mL of streptomycin.

All HLAR isolates were analyzed for the presence of aminoglycoside modifying enzyme coding genes. The bifunctional AME gene *aac(6')-Ie-aph(2'')-Ia* that confers high level resistance to gentamicin was detected in 40% of *E. faecalis* and *E. faecium* isolates, whereas 32% carried *aph(3')-IIIa*

Table 3 Antimicrobial Patterns Of *E. faecalis* Isolates

Antibiotic Class	Antibiotic Agents	Susceptible, n (%)	Resistance, n (%)
Aminoglycosides	Gentamicin 120 µg	26 (52)	24 (48)
	Streptomycin 300 µg	27 (54)	23 (46)
Beta-lactams	Ampicillin 30 µg	32 (76)	12 (24)
Glycopeptides	Vancomycin 10 µg	47 (94)	3 (6)
Macrolides	Erythromycin 15 µg	2 (4)	48 (96)
Chloramphenicol	Chloramphenicol 30 µg	27 (54)	23 (46)
Tetracyclines	Tetracycline 30 µg	1 (2)	49 (98)
Oxazolidinones	Linezolid 30 µg	48 (96)	2 (4)

Note: Antimicrobial breakpoints were interpreted according to CLSI (2016) guidelines.

(Figures 2A and B). Other AME genes such as *aph(2'')-Ib*, *aph(2'')-Ic* and *aph(2'')-Id* were not detected among the study isolates. Sequencing data showed 100% identity with bifunctional aminoglycoside modifying enzyme in the database.

Discussion

In this study, the predominant enterococcal species that was recovered from various clinical samples is *E. faecalis* in which the majority of these isolates were recovered from the pus. A similar occurrence was reported in other studies in the USA, Europe, and the Middle East.^{13,14} On the contrary, some countries reported high occurrence of *E. faecium* which explains that the distribution of the predominant enterococcal species varies from one country to another depending on various contributing factors such as host dynamism, environmental conditions,¹⁵ or due to clinical conditions that were presented to the hospitals, genetic diversity, as well as the presence of specific virulence factors.^{3,16,17}

Enterococci are considered as multidrug resistant organisms that may be either as a result of the intrinsic

factors of the species or acquired resistance.^{11,18} Resistance to aminoglycosides is contributed by both intrinsic and acquired factors; resistance to low level amikacin, tobramycin, and kanamycin are normally due to intrinsic factors, whereas resistance to high level gentamicin and streptomycin is acquired through genetic transfer of the resistant determinants.

HLAR has become a very serious problem in most health-care facilities. High levels of resistance to gentamicin and streptomycin have been reported in many European countries, with occurrence ranging from 1% to 48% with little or no difference in geographical prevalence among these countries where the studies were conducted.¹⁹ The National Surveillance of Antimicrobial Resistance in Malaysia has reported an increasing trend of HLGR *E. faecalis* rate in 2016 of 20.2%, as compared to 19.4% in 2013. In this study, we have observed about 50% HLAR enterococci isolates, whereas other findings have reported higher rates, including Li et al²⁰ (74.4%) and Padmasini et al¹⁰ (72.5%). It is also indicated that HLGR is more common in both species as compared to HLSR. Other studies also reported higher occurrence of HLGR compared to HLSR, including a recent study which reported up to 60% from a total of 100 enterococcal isolates exhibiting HLGR.⁹

Treatment of HLAR enterococci requires synergistic combination therapy with beta-lactam and aminoglycoside antibiotic, but a consistent rise in resistance rate against antibiotics commonly used for the treatment poses a growing threat to the treatment and control of the infections. Despite the fact that enterococci are intrinsically resistant to cephalosporin, a recent study by Tam et al has reported an effective treatment of HLAR *E. faecalis* infection in a neonate using ampicillin and cefotaxime antibiotics.²¹

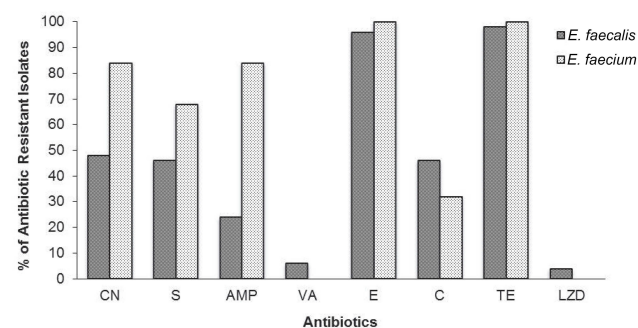


Figure 1 Antibiotic resistance profile of *E. faecalis* and *E. faecium*. **Abbreviations:** CN, gentamicin; S, streptomycin; AMP, ampicillin; VA, vancomycin; E, erythromycin; C, chloramphenicol; TE, tetracycline; LZD, linezolid.

Table 4 Antimicrobial Patterns Of *E. faecium* Isolates

Antibiotic Class	Antibiotic Agents	Susceptible, n (%)	Resistance, n (%)
Aminoglycosides	Gentamicin 120 µg	4 (16)	21 (84)
	Streptomycin 300 µg	8 (32)	17 (68)
Beta-lactams	Ampicillin 30 µg	4 (16)	21 (84)
Glycopeptides	Vancomycin 10 µg	25 (100)	0 (0)
Macrolides	Erythromycin 15 µg	0 (0)	25 (100)
Chloramphenicol	Chloramphenicol 30 µg	17 (68)	8 (32)
Tetracyclines	Tetracycline 30 µg	0 (0)	25 (100)
Oxazolidinones	Linezolid 30 µg	25 (100)	0 (0)

Note: Antimicrobial breakpoints were interpreted according to CLSI (2016) guidelines.

Distribution of HLAR genes depends on the geographical region, and the same gene is not necessarily found in the same enterococci species.¹⁴ The prevalence data obtained from this study revealed that the HLGR gene *aac(6)-Ie-aph(2)-Ia* was detected in 40% of the HLAR *E. faecalis* and *E. faecium* isolates. This is in agreement with data from Japan that showed a 42% detection rate and in contrast with another study in Kuwait Hospital that reported up to a 93% detection rate.^{22,23} Moreover, as reported in many other studies, high prevalence of the *aac(6)-Ie-aph(2)-Ia* AME gene was found in HLAR *Enterococcus* isolates.^{24–26}

High level streptomycin resistance (MIC 1024 µg/mL) in enterococci could be due to a single mutation in

ribosomal protein or enzymatic inactivation by AMEs encoded by *aph(3')-IIIa* genes. We have detected the gene in 32% of our isolates, which is lower than those reported by Padmasini et al¹⁰ (77%), Li et al²⁰ (56%), and Ramin et al⁷ (49%). The differences in the detection rate could possibly be due to the horizontal transfer of the resistance factors, since HLAR genes are located on plasmid and conjugative transposons.^{11,27}

In conclusion, our results demonstrate that *E. faecalis* is more predominant than *E. faecium*. The prevalence of HLGR is more common than HLSR among the enterococci isolates. Bifunctional AME gene *aac(6)-Ie-aph(2)-Ia* is the main factor responsible for HLAR. As the genes encoding AME are usually found on plasmids and transposons, thus augmenting to the high emergence of HLAR in enterococci is the natural ability of bacteria to acquire the resistant genes and is becoming an urgent issue. Continual antibiotic surveillance including a better stewardship is therefore warranted for an efficient implementation of preventive measures.

Disclosure

The authors declare that there is no competing interest regarding the publication of this paper.

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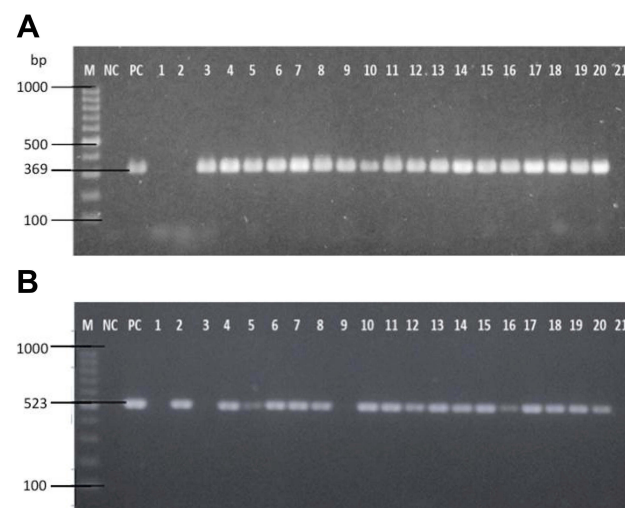


Figure 2 (A) Representative image of the amplified PCR products of HLGR gene, *aac(6)-Ie-aph(2)-Ia* of *Enterococcus* isolates (expected size: 369 bp). (B) Representative image of the amplified PCR products of HLSR gene, *aph(3')-IIIa* of *Enterococcus* isolates (expected size: 523 bp).

Abbreviations: Lane M, 100 bp ladder; NC, negative control (DNA-free template); PC, positive control (ATCC strain 51299); Lanes 1–21, *Enterococcus* isolates.

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