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

Ayan Aden Moussa
Amirah Fatihah Md Nordin
Rukman Awang Hamat
Azmiza Syawani Jasni

Department of Medical Microbiology and Parasitology, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, UPM Serdang 43400, Malaysia

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’)-IIb, aph(2’)-Ic, aph(2’)-Id, aph(3’)-IIa]. 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 (66.8%), 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’)-IIa contributed to the HLAR E. faecalis and E. faecium. The other AME genes [aph(2’)-IIb, 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

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. 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...
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 10µg, 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 microdilution for high level gentamicin 512 µg/mL and streptomycin 1024 µg/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 QIAamp 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 (Tm) of the two primers, extension at 72°C for a 1 min/kb of the expected size.
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 SPSS 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. 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’)-Illa.

Table 1 Primers Used In PCR Assay For 16s rRNA And Detection Of Aminoglycosides Resistance Genes

| Genes          | Primer Sequences (5’ – 3’)                                                                 | Product Size (bp) |
|----------------|--------------------------------------------------------------------------------------------|------------------|
| 16s rRNA       | F: GTGCTGGAGAGGTGATCCTGGCTCAG R: CACAGATCCTACGGGTACCTTGTTACGACTT                              | 1465             |
| aac(6’)-Ie-aph(2”)-Ia | F: CAGGAATTATCGAAAAATGGTACAAAAAG R: CACAATCGACTAAAGAGTACCCATT                                  | 369              |
| aph(2’)-Ib     | F: CTTCGACGCTGAGATATATGAGCAG R: GTTGTAGAATTTCCAGAAACACCTT                                     | 867              |
| aph(2’)-Ic     | F: CCACAAATGATAATGACGTTCCC R: CCACAGTCCTCCGATACGAG                                          | 444              |
| aph(2’)-Id     | F: GTGGTTTTTACAGGAATGCTACATC R: CCCCTTTCTATACAAATCATATAACC                                   | 641              |
| aph(3’)-Illa   | F: GGCTAAAATGAGATATATCACC GG R: CTTCATACACCGCTCC                                            | 523              |

Table 2 Distribution Of Enterococcus Species

| Specimen | E. faecalis (n=50), n (%) | E. faecium (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.
(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 healthcare 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 al\(^ {20}\) (74.4%) and Padmasini et al\(^ {10}\) (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.\(^ {21}\)

### 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.
Distribution of HLAR genes depends on the geographical region, and the same gene is not necessarily found in the same enterococci species.\textsuperscript{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.\textsuperscript{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.\textsuperscript{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 al\textsuperscript{10} (77%), Li et al\textsuperscript{20} (56%), and Ramin et al\textsuperscript{7} (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.\textsuperscript{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.

**References**

1. O’Driscoll T, Crank CW. Vancomycin-resistant enterococcal infections: epidemiology, clinical manifestations, and optimal management. *Infect Drug Resist*. 2015;8:217–230. doi:10.2147/IDR.S54125
2. Arias CA, Murray BE. Emergence and management of drug-resistant enterococcal infections. *Expert Rev Anti Infect Ther*. 2008;6(5):637–655. doi:10.1586/14787210.6.5.637
3. Weng PL, Ramli R, Shamsudin MN, Cheah YK, Hamat RA. High genetic diversity of Enterococcus faecium and Enterococcus faecalis clinical isolates by pulsed-field gel electrophoresis and multilocus sequence typing from a hospital in Malaysia. *Biomed Res Int*. 2013:2013:938937.
4. Ibrahim R, Mohamad M, Rahman M. Enterococci: emerging drug resistant bacteria in hospital acquired infections at Hospital Kuala Lumpur, Malaysia. Internet J Microbiol. 2010;9(2).

5. Galindo JA, Tejada YG, Cerezo SG, Salazar OM. High level aminoglycoside resistance Enterococcus spp in a tertiary care hospital in Mexico. Electron J Biomed. 2005;1:40–45.

6. Baddour LM, Wilson WR, Bayer AS, et al. Infective endocarditis in adults: diagnosis, antimicrobial therapy, and management of complications: a scientific statement for healthcare professionals from the American Heart Association. Circulation. 2015;132(15):1435–1486. doi:10.1161/CIR.0000000000000296

7. Ramin B, Asadpour L, Tehrani HF, Amirmozafari N. Detection and distribution of various HLAR gene in Enterococcus faecalis and Enterococcus faecium by multiplex-PCR. Mod Med Lab J. 2017;1(2):68–76. doi:10.30699/nnllj17

8. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. Drug Resist Updat. 2010;13(6):151–171. doi:10.1016/j.drup.2010.08.003

9. Shente V, Grover N, Kumar M. Analysis of aminoglycoside modifying enzyme genes responsible for high-level aminoglycoside resistance among enterococcal isolates. J Pathog. 2017;2017:3256952

10. Padmasini E, Padmaraj R, Ramesh SS. High level aminoglycoside resistance and distribution of aminoglycoside resistant genes among clinical isolates of Enterococcus species in Chennai, India. Sci World J. 2014;1–5. doi:10.1155/2014/329157

11. Hollenbeck BL, Rice LB. Intrinsic and acquired resistance mechanisms in Enterococci. Virulence. 2012;3(5):421–569. doi:10.4161/viru.21282

12. Vakulenko SB, Donabedian SM, Voskresenskiy AM, et al. Multiplex PCR for detection of aminoglycoside resistance genes in enterococci. Antimicrob Agents Chemother. 2003;47(4):1423–1426. doi:10.1128/aac.47.4.1423-1426.2003

13. Simonsen GS, Smâbrekke L, Monnet DL, et al. Prevalence of resistance to ampicillin, gentamicin and vancomycin in Enterococcus faecalis and Enterococcus faecium isolates from clinical specimens and use of antimicrobials in five Nordic hospitals. J Antimicrob Chemother. 2003;51(2):323–331. doi:10.1093/jac/dkg052

14. Zarrilli R, Tripodi MF, Di Popolo A, et al. Molecular epidemiology of high-level aminoglycoside-resistant enterococci isolated from patients in a university hospital in southern Italy. J Antimicrob Chemother. 2005;56(5):827–835. doi:10.1093/jac/dki347

15. Conwell M, Daniels V, Naughton PJ, Dooley JSG. Interspecies transfer of vancomycin, erythromycin and tetracycline resistance among Enterococcus species recovered from agrarian sources. BMC Microbiol. 2017;17(191):1–8. doi:10.1186/s12866-016-0921-2

16. Jain S, Kumar A, Kashyap B, Kaur IR. Clinico-epidemiological profile and high-level aminoglycoside resistance in enterococcal septicaemia from a tertiary care hospital in east Delhi. Int J Appl Basic Med Res. 2011;1(2):80–83. doi:10.4103/2229-516X.91149

17. Cai JC, Hu YY, Zhang R, Zhou HW, Chen GX. Linezolid-resistant clinical isolates of methicillin-resistant coagulase-negative staphylococci and Enterococcus faecium from China. J Med Microbiol. 2012;61(11):1568–1573. doi:10.1099/jmm.0.043729-0

18. Hendrickx AP, van Luit-Ambroek M, Schapendonk CM, et al. SgrA, a nodulin-binding LPXTG surface adhesin implicated in biofilm formation, and EcBA, a collagen binding MSCRAMM, are two novel adhesins of hospital-acquired Enterococcus faecium. Infect Immun. 2009;77(11):5097–5106. doi:10.1128/IAI.00275-09

19. Schouten MA, Voss A, Hoogkamp-Korstanje JA; for The European VRE Study Group. Antimicrobial susceptibility patterns of enterococci causing infections in Europe. Antimicrob Agents Chemother. 1999;43(10):2542–2546.

20. Li W, Li J, Wei Q, et al. Characterization of aminoglycoside resistance and virulence genes among Enterococcus spp. isolated from a hospital in China. Int J Environ Res Public Health. 2015;12(3):3014–3025. doi:10.3390/ijerph12030314

21. Tam J, Lee SJ, Shah V, Morris SK. Successful treatment of high-level aminoglycoside-resistant Enterococcus faecalis bacteremia in a preterm infant with ampicillin and cefotaxime. Case Rep Infect Dis. 2018;2018:7567914.

22. Kobayashi N, Alam MM, Nishimoto Y, Urasawa S, Uehara N, Watanabe N. Distribution of aminoglycoside resistance genes in recent clinical isolates of Enterococcus faecalis, Enterococcus faecium and Enterococcus avium. Epidemiol Infect. 2001;126(2):197–204. doi:10.1017/S0093311800025710

23. Udo EE, Al-Sweih N, John P, Jacob LE, Mohanakrishnan S. Characterization of high-level aminoglycoside-resistant enterococci in Kuwait hospitals. Microb Drug Resist. 2004;10(2):139–145. doi:10.1089/1076629041310037

24. Amini F, Krimpour HA, Ghaderi M, et al. Prevalence of aminoglycoside resistance genes in Enterococcus strains in Kermanshah, Iran. Iran J Med Sci. 2018;43(5):487–493.

25. Tian Y, Yu H, Wang Z. Distribution of acquired antibiotic resistance genes among Enterococcus spp. isolated from a hospital in Baotou, China. BMC Res Notes. 2019;12(1):27. doi:10.1186/s13104-019-4064-z

26. Niu H, Yu H, Hu T, et al. The prevalence of aminoglycoside-modifying enzyme and virulence genes among enterococci with high-level aminoglycoside resistance in Inner Mongolia, China. Braz J Microbiol. 2016;47(3):691–696. doi:10.1016/j.bjm.2016.04.003

27. Sahm DF, Boonlayangoor S, Schulz JE. Detection of high-level aminoglycoside resistance in enterococci other than Enterococcus faecalis. J Clin Microbiol. 1991;29(11):2595–2598.