UNIVERSITI PUTRA MALAYSIA

CHARACTERIZATION OF ANTIBIOTIC SUSCEPTIBILITY AND HIGH LEVEL AMINOGLYCOSIDE RESISTANT GENES IN Enterococcus faecalis AND Enterococcus faecium CLINICAL ISOLATES

AYAN ADEN MOUSSA

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By

AYAN ADEN MOUSSA

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirements for the degree of Master of Science

May 2018
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DEDICATION

To my parents
CHARACTERIZATION OF ANTIBIOTIC SUSCEPTIBILITY AND HIGH LEVEL AMINOGLYCOSIDE RESISTANT GENES IN Enterococcus faecalis AND Enterococcus faecium CLINICAL ISOLATES

By

AYAN ADEN MOUSSA

May 2018

Chairman : Azmiza Syawani Jasni, PhD
Faculty : Medicine and Health Sciences

Enterococcus faecium and Enterococcus faecalis are among the predominant species causing hospital-acquired infections, with high mortality and morbidity rates. Enterococci which exhibit high level aminoglycoside (HLAR) and vancomycin resistance (VRE) possess a significant problem in therapeutic treatment. These resistance strains are becoming more widespread in Europe, USA and Asia. Studies have shown that the different distributions of aminoglycoside modifying enzymes (AMEs) among HLAR are based on the geographical regions. In Malaysia, however, data on the prevalence of HLAR and the distribution of AMEs are still limited. Hence, the aims of this study were to determine the antibiotic susceptibility patterns of the clinical isolates E. faecalis and E. faecium and the distribution of HLAR genes among the resistant isolates. Seventy-five clinical enterococci isolates used in this study were originally obtained from a tertiary centre, in the year 2009 and 2010. These isolates were isolated from different sources including pus (50%), blood (32%), urine (11%) and other sources such as CSF and HVS (7%). Re-identification of these isolates was carried out using several methods including sub-culturing on selective medium and Gram staining, followed by confirmatory tests using a commercial biochemical profiling (API20 strep) and 16s rRNA PCR amplification. Antimicrobial susceptibility tests were performed using disc diffusion, E-test and broth microdilution methods. The antibiotics used were aminoglycosides (gentamicin, streptomycin, kanamycin, amikacin, tobramycin) and other antibiotics which are commonly...
used in the hospital to treat enterococcal infections such as ampicillin, vancomycin, erythromycin, tetracycline, chloramphenicol and linezolid. Detection of HLAR genes was performed on resistance isolates using single PCR amplifications. Out of 25 (33.3%) isolates of *E. faecium*, 84% and 68% showed high level gentamicin resistance (HLGR) and high level streptomycin resistance (HLSR), respectively. Resistance against erythromycin and tetracycline were 100%, while ampicillin and chloramphenicol showed 84% and 32% resistance rates, respectively. None of the *E. faecium* isolates showed resistance towards vancomycin and linezolid. On the other hand, all 50 (66.7%) *E. faecalis* isolates were resistance to all aminoglycosides tested with the following percentages; amikacin (100%), kanamycin (90%), tobramycin (70%), HLGR (36%) and HLSR (46%). These isolates were also resistance to tetracycline (98%), erythromycin (96%), chloramphenicol (46%), ampicillin (24%), linezolid (8%), and vancomycin (4%). Interestingly, the *E. faecalis* isolates that were resistant to vancomycin were previously reported as susceptible. In this study, *E. faecium* exhibited higher resistance rates to all antibiotics except vancomycin and linezolid compared to *E. faecalis*. Additionally, 46% of *E. faecalis* showed MIC of streptomycin up to 1042 μg/mL while another 36% showed MIC of gentamicin equal to 512 μg/mL. The MIC of aminoglycosides determined in this study showed similar level as observed in other countries, despite the variation of the methods used. The presence of aminoglycoside modifying enzyme (AME) genes \[\text{aac}(6)-\text{Ie-aph}(2)-\text{Ia}, \text{aph}(2)-\text{Ib}, \text{aph}(2)-\text{Ic}, \text{aph}(2)-\text{Id}, \text{aph}(3)-\text{IIIa}\] were detected using PCR amplification. The results demonstrated that the HLGR gene, \text{aac}(6)-\text{Ie-aph}(2)-\text{Ia} and the HLSR gene, \text{aph}(3)-\text{IIIa} were detected in 32% and 40% of *E. faecalis* and *E. faecium* resistance isolates, respectively. The spread of these genes were responsible for high level resistance to gentamicin and streptomycin among enterococci isolated in this study. As HLAR genes are highly transferable to not only among the enterococci species but also among various bacterial species, continuous antibiotic surveillance in Malaysian hospitals is warranted in future and preventive measures can be implemented accordingly. **Keywords:** High level aminoglycosides resistance, Enterococci
Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PEMPROFILAN KERENTANAN ANTIBIOTIK DAN PENYEBARAN GEN KETAHANAN AMINOGLIKOSIDA TAHAP TINGGI DI KALANGAN Enterococcus faecalis DAN Enterococcus faecium

Oleh

AYAN ADEN MOUSSA

Mei 2018

Pengerusi : Azmiza Syawani Jasni, PhD
Fakulti : Perubatan dan Sains Kesihatan

Enterococcus faecium dan Enterococcus faecalis merupakan antara spesis utama yang menyebabkan infeksi dapatan hospital, dengan kadar kematian dan morbiditi yang tinggi. Enterococci yang rintang terhadap aminoglikosida (HLAR) dan vankomisin (VRE) tahap tinggi merupakan masalah yang signifikan di dalam rawatan terapeutik. Strain tersebut semakin meluas di Eropah, USA dan Asia. Kajian telah menunjukkan bahawa perbezaan penyebaran bagi enzim pengubahsuaian aminoglikosida (AMEs) di kalangan HLAR adalah berdasarkan kawasan geografi. Di Malaysia, walau bagaimanapun, data mengenai kelaziman HLAR dan penyebaran AME masih lagi terhad. Oleh itu, kajian ini bertujuan untuk menentukan pola kerintangan antibiotik bagi pencilan klinikal E. faecalis dan E. faecium dan penyebaran gen HLAR di kalangan pencilan yang rintang ini. Tujuh puluh lima pencilan enterococci klinikal yang digunakan di dalam kajian ini telah diperoleh daripada pusat tertier, pada tahun 2009 dan 2010. Pencilan tersebut telah dipencilan daripada sumber yang berbeza termasuk nanah (50%), darah (32%), air kencing (11%) dan sumber lain seperti CSF dan HVS (7%). Pengenalpastian semula pencilan tersebut telah dijalankan menggunakan pelbagai kaedah daripada sumber yang berbeza termasuk nanah (50%), darah (32%), air kencing (11%) dan sumber lain seperti CSF dan HVS (7%). Pengenalpastian semula pencilan tersebut telah dijalankan menggunakan pelbagai kaedah daripada sumber yang berbeza termasuk nanah (50%), darah (32%), air kencing (11%) dan sumber lain seperti CSF dan HVS (7%). Pengenalpastian semula pencilan tersebut telah dijalankan menggunakan pelbagai kaedah daripada sumber yang berbeza termasuk nanah (50%), darah (32%), air kencing (11%) dan sumber lain seperti CSF dan HVS (7%). Pengenalpastian semula pencilan tersebut telah dijalankan menggunakan pelbagai kaedah daripada sumber yang berbeza termasuk nanah (50%), darah (32%), air kencing (11%) dan sumber lain seperti CSF dan HVS (7%).
aminoglikosida (gentamisin, streptomisin, kanamisin, amikasin, tobramisin) dan antibiotik lain yang biasanya digunakan di hospital bagi merawat infeksi enterococcal, seperti ampicilin, vankomisin, eritromisin, tetrasiklina, kloramfenikol dan linezolid. Pengesanan gen HLAR telah dijalankan ke atas pencilan yang rintang dengan menggunakan kaedah amplifikasi PCR tunggal. Daripada 25 (33.3%) pencilan E. faecium, 84% dan 68% masing-masing menunjukkan tahap ketahanan gentamisin (HLGR) dan ketahanan streptomisin (HLSR) tahap tinggi. Kerintangan terhadap eritromisin dan tetrasiklina ialah 100%, manakala ampicilin dan kloramfenikol, masing-masing menunjukkan 84% dan 32% kadar kerintangan. Tiada pencilan E. faecium menunjukkan kerintangan terhadap vankomisin dan linezolid. Di samping itu, kesemua 50 (66.7%) pencilan E. faecalis adalah rintang terhadap semua aminoglikosida yang diuji dengan peratusan berikut; amikasin (100%), kanamisin (90%), tobramisin (70%), HLGR (36%) dan HLSR (46%). Pencilan-pencilan tersebut juga rintang terhadap tetrasiklina (98%), eritromisin (96%), kloramfenikol (46%), ampicilin (24%), linezolid (8%), dan vankomisin (4%). Yang menariknya, pencilan E. faecalis yang rintang pada vankomisin sebelum ini telah dilaporkan sebagai rentan. Dalam kajian ini, E. faecium memperlihatkan kadar kerintangan yang tinggi terhadap semua antibiotik kecuali vankomisin dan linezolid berbanding dengan E. faecalis. Tambahlan lagi, 46% E. faecalis menunjukkan MIC streptomisin sehingga 1042 μg/mL manakala selainnya 36% menunjukkan MIC gentamisin bersamaan 512 μg/mL. MIC aminoglikosida yang ditentukan dalam kajian ini menunjukkan tahap yang sama seperti yang didapati di negara lain, meskipun kelpelbagaia kaedah yang digunakan. Kewujudan gen enzim pengubahsuai aminoglikosida (AME) [aac(6)-Ie-aph(2)-Ia, aph(2)-Ib, aph(2)-Ic, aph(2)-Id, aph(3)-IIIa] telah dikesan dengan menggunakan amplifikasi PCR. Keputusan menunjukkan bahawa gen HLGR, aac(6)-Ie-aph(2)-Ia dan gen HLSR, aph(3)-IIIa telah dikesan, masing-masing di dalam 32% dan 40% pencilan E. faecalis dan E. faecium yang rintang. Penyebaran gen tersebut bertanggungjawab terhadap kerintangan tahap tinggi pada gentamisin dan streptomisin dalam kajian ini. Disebabkan gen HLAR berkeupayaan untuk pindah bukan sahaja di kalangan spesis enterococci yang sama tetapi juga pelbagai spesis bakteria yang berlainan, pengawasan antibiotik yang berterusan di hospital di Malaysia adalah perlu agar langkah-langkah pencegahan dapat dilaksanakan dengan sewajarnya.

Kata kunci: Tahap kerintangan, aminoglikosida tahap tinggi, Enterococci
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I certify that a Thesis Examination Committee has met on 16 May 2018 to conduct the final examination of Ayan Adan Moussa on his thesis entitled "Characterization of Antibiotic Susceptibility and High Level Aminoglycoside Resistant Genes in Enterococcus faecalis and Enterococcus faecium Clinical Isolates" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Master of Science.

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Name of Member of Supervisory Committee: Associate Professor Dr. Rukman Awang Hamat
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>ABSTRACT</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRAK</td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>v</td>
</tr>
<tr>
<td>APPROVAL</td>
<td>vi</td>
</tr>
<tr>
<td>DECLARATION</td>
<td>viii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xiv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>xvi</td>
</tr>
</tbody>
</table>

CHAPTER

1 INTRODUCTION

1.1 Background 1
1.2 Problem statement 2
1.3 Objectives 3

2 LITERATURE REVIEW

2.1 Nomenclature of Enterococci 4
2.2 Identification of Enterococci 5
   2.2.1 Morphological characteristics of Enterococci 5
   2.2.2 Biochemical Tests 5
   2.2.3 Molecular Based Method 6
2.3 Species Distribution 6
2.4 Clinical Significance of Enterococci 7
2.5 Management of Enterococcal Infections 7
2.6 Pathogenicity of Enterococci 8
   2.6.1 Biofilm Formation 8
   2.6.2 Other Virulence Factors 8
2.7 Antimicrobial Resistance in Enterococci 10
   2.7.1 Intrinsic Resistance 10
   2.7.2 Acquired Resistance 10
2.8 Horizontal Gene Transfer 11
   2.8.1 Transformation 12
   2.8.2 Transduction 12
   2.8.3 Conjugation 12
2.9 Mechanism of Antimicrobial Resistance in Enterococci 13
2.10 Antibiotic Resistance in Enterococci 16
   2.10.1 Mechanisms of Aminoglycosides and HLAR in Enterococci 16
   2.10.2 Aminoglycoside Resistance 17
   2.10.3 Based on biochemical mechanism of resistance 18
3 MATERIALS AND METHODS

3.1 Study Ethics 19
3.2 Bacterial Strains 19
3.3 Bacterial Growth Conditions 19
3.4 Bacterial Stock Preparation and Storage 19
3.5 Re-identification of Enterococci Isolates 20
  3.5.1 Subculturing on Selective Agar 20
  3.5.2 Gram Staining 20
  3.5.3 Analytical Profile Index for Species Identification 20
  3.5.4 DNA Extraction 21
  3.5.5 PCR Amplification 22
  3.5.6 Gel electrophoresis 23
  3.5.7 Sequence Analysis 23
3.6 Antibiotic Susceptibility Test (AST) 23
  3.6.1 Disc Diffusion 23
  3.6.2 Epsilometer Test (E-test) 24
  3.6.3 Broth Microdilution Method 25
3.7 Data analysis 25

4 RESULTS

4.1 Characterization of Enterococci Isolates 26
4.2 Re-identification of Enterococci Species 27
  4.2.1 Growth on Bile Esculin Agar 27
  4.2.2 Gram Staining 28
  4.2.3 Analytical Profile Index for Species Identification 29
  4.2.4 Specific Identification Using 16S rRNA 29
4.3 Antimicrobial Susceptibility Profiling of Enterococci Species 33
  4.3.1 Disc Diffusion 33
4.4 Minimum Inhibitory Concentration (MIC) 36
4.5 Amplification of HLAR Genes Using polymerase chain reaction (PCR) 44
  4.5.1 DNA Sequencing Analysis result 47

5 DISCUSSION

5.1 Re-identification of Enterococci Species 49
5.2 Antibiotic Susceptibility Profile of Enterococci Species 51
5.3 Distribution of High Level Gentamicin and Streptomycin Resistance 54
5.4 Distribution of Aminoglycoside Modified Enzymes (AME) Genes 55
6 CONCLUSION AND RECOMMENDATION 57
6.1 Recommendations on The Basis of This Study 58
6.2 Limitation of Study 58

REFERENCES 59
APPENDICES 72
BIODATA OF STUDENT 83
**LIST OF TABLES**

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Scientific Classification of <em>Enterococci</em></td>
<td>4</td>
</tr>
<tr>
<td>2.2 Type of resistances to antibiotics in <em>Enterococci</em></td>
<td>11</td>
</tr>
<tr>
<td>2.3 Mode of action and biological mechanism of resistance to some class of antibiotics</td>
<td>15</td>
</tr>
<tr>
<td>3.1 Primers targeting aminoglycoside resistance genes</td>
<td>22</td>
</tr>
<tr>
<td>3.2 PCR condition used in amplification cycle</td>
<td>23</td>
</tr>
<tr>
<td>3.3 Antibiotics and results interpretation</td>
<td>24</td>
</tr>
<tr>
<td>4.1 Species discrimination using API20 Strep System</td>
<td>29</td>
</tr>
<tr>
<td>4.2 Isolate Distribution by sources among enterococci species</td>
<td>33</td>
</tr>
<tr>
<td>4.3 Antibiotic susceptibility profile of <em>E. faecalis</em> clinical isolates</td>
<td>34</td>
</tr>
<tr>
<td>4.4 Antibiotic Susceptibility Profile of <em>E. faecium</em> Clinical Isolates</td>
<td>35</td>
</tr>
<tr>
<td>4.5 MIC of high level aminoglycoside among <em>E. faecalis</em> and <em>E. faecium</em></td>
<td>36</td>
</tr>
<tr>
<td>4.6 Distribution of HLAR genes of the source of isolates and their MIC</td>
<td>48</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Enterococci determinants that have been shown to cause virulence phenotypic in vivo</td>
</tr>
<tr>
<td>2.2</td>
<td>Mechanisms of Horizontal gene transfer that include transformation, transduction and conjugation</td>
</tr>
<tr>
<td>2.3</td>
<td>General mechanism of antimicrobial resistance in enterococci</td>
</tr>
<tr>
<td>3.1</td>
<td>The API Test strip. The combinations numbers observed are subjected to an online interpretation system, apiweb™</td>
</tr>
<tr>
<td>4.1</td>
<td>Distribution of enterococci isolates based on different sources (n=75; n= frequency)</td>
</tr>
<tr>
<td>4.2</td>
<td>Distribution of isolates based on the hospital wards (n=75; n= frequency)</td>
</tr>
<tr>
<td>4.3</td>
<td>Colonies morphology of pure enterococci grown for 24 hrs on bile esculin agar</td>
</tr>
<tr>
<td>4.4</td>
<td>Morphology of enterococci as observed under oil emersion (1000X magnification)</td>
</tr>
<tr>
<td>4.5</td>
<td>API strip inoculated with API strep medium after 24 hours of incubation</td>
</tr>
<tr>
<td>4.6</td>
<td>Agarose gel electrophoresis of the amplified PCR products for detection of enterococci species</td>
</tr>
<tr>
<td>4.7</td>
<td>Confirmation of <em>E. faecalis</em> isolate with percentage identity of 99% to the sequence from GenBank</td>
</tr>
<tr>
<td>4.8</td>
<td>Confirmation of isolated <em>E. faecium</em> isolate with percentage identity of 97% to the sequence from GenBank</td>
</tr>
<tr>
<td>4.9</td>
<td>Antibiotic resistance profile of <em>E. faecalis</em> (n=75 strains)</td>
</tr>
<tr>
<td>4.10</td>
<td>Antibiotic resistance profile of <em>E. faecium</em></td>
</tr>
<tr>
<td>4.11</td>
<td><em>E. faecalis</em> isolates with high level gentamicin resistance isolated from pus sample</td>
</tr>
<tr>
<td>4.12</td>
<td><em>E. faecalis</em> Isolates with high level gentamicin resistance isolated from blood sample</td>
</tr>
</tbody>
</table>
4.13 *E. faecium* isolates with high level gentamicin resistance isolated from pus sample

4.14 *E. faecium* isolates with high level gentamicin resistance isolated from blood sample

4.15 *E. faecium* isolates with high level gentamicin resistance isolated from urine sample

4.16 *E. faecalis* isolates with high level streptomycin resistance isolated from pus sample

4.17 *E. faecium* isolates with high level streptomycin resistance isolated from pus sample

4.18 *E. faecalis* isolates with high level streptomycin resistance isolated from blood sample

4.19 Agarose gel electrophoresis of the amplified PCR products for detection of HLGR gene, aac(6)-Ie- aph(2)-Ia *E. faecalis* (expected size: 369bp)

4.20 Agarose gel electrophoresis of the amplified PCR products for detection of HLGR gene, aac(6)-Ie- aph(2)-Ia *E. faecalis* and *E. faecalis* (expected size: 369 bp)

4.21 Agarose gel electrophoresis of the amplified PCR products for detection of HLSR gene aph(3)-Illa *E. faecalis* (expected size: 529pb)

4.22 Agarose gel electrophoresis of the amplified PCR products for detection of HLSR gene aph(3)-Illa *E. faecalis* and *E. faecium* (expected size: 529bp)

4.23 Multi Sequence Alignment of HLGR Genes. Sequence 58_FS represents isolate number 58 which is *E. faecalis* resistance to gentamicin while sequence RC represents *E. faecalis* plasmid pKUB3007-1 KUB3007 DNA, GenBank: (Accession no: AP018544.1), both sequences 99% are aligned

4.24 Multi Sequence Alignment of HLSR Genes. Sequence 59_FS represents *E. faecium* resistance to streptomycin, while sequence 59_RCS represents *E. faecium* plasmid pKUB3006-1 KUB3006 DNA, GenBank: (Accession no: AP018539) both sequences 99% are aligned
### LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control and Prevention</td>
</tr>
<tr>
<td>IMR</td>
<td>Institute for Medical Research</td>
</tr>
<tr>
<td>AAC</td>
<td>Aminoglycoside N-acetyltransferase se</td>
</tr>
<tr>
<td>AME</td>
<td>Aminoglycosides modifying enzyme</td>
</tr>
<tr>
<td>ANT</td>
<td>Aminoglycoside O- adenyltransferase</td>
</tr>
<tr>
<td>APH</td>
<td>Aminoglycoside O-Phosphotransferase</td>
</tr>
<tr>
<td>AST</td>
<td>Antibiotic susceptibility testing</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>BHI</td>
<td>Brain heart infusion agar</td>
</tr>
<tr>
<td>Blast</td>
<td>Basic local alignment search tool</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming unit</td>
</tr>
<tr>
<td>CLSI</td>
<td>Clinical Laboratory Standard Institute</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>HLAR</td>
<td>High level aminoglycoside resistance</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi drug resistance</td>
</tr>
<tr>
<td>MHA</td>
<td>Muller Hinton agar</td>
</tr>
<tr>
<td>MIC</td>
<td>Minimal inhibitory concentration</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Centre for Biology Information</td>
</tr>
<tr>
<td>PBP</td>
<td>Penicillin binding protein</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TBE</td>
<td>Mixture of tris base, boric acid, EDTA and water</td>
</tr>
<tr>
<td>VRE</td>
<td>Vancomycin resistant enterococci</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Background

Enterococcal infections are significant hospital and community acquired infections due to multidrug resistance as widely reported (Arias & Murray, 2012). They have potential capacity to persist under various environmental conditions such as extreme temperature and osmolality (Garrido et al., 2014). These organisms are able to persist on various surfaces in hospitals and on hands of healthcare workers (Kuch et al., 2011). The Centre for Disease Control and Prevention (CDC) and prevention have estimated about 4 million patients in USA have hospital acquired infections (HAIs) annually (Weng et al., 2013). Recent report indicated that enterococci were placed as a second most common cause of hospital acquired infection after *Staphylococcus aureus* (Arias et al., 2010). In Malaysia between 2006 and 2007 about 2263 and 2647 patients respectively, were reported with HAIs which were due to enterococcal infections (Weng et al., 2013).

Enterococci are Gram positive bacteria which are found mostly in the gastrointestinal tract as normal flora (McBride et al., 2007). Presently, 54 various species have been described (O’Driscoll & Crank, 2015). The enterococci infection which include *Enterococcus faecium* and *Enterococcus faecalis* are predominantly associated with human diseases such as urinary tract infection, bacteremia, endocarditis, wound infections such as surgical burns, intra-abdominal abscess, septicemia, pelvic infection, meningitis as well as skin and soft tissue infections that result in high morbidity and mortality (Wang et al., 2013;Vu & Carvalho 2011). It is estimated that about 60% of enterococcal infections are associated with *E. faecalis*. However, infections due to *E. faecium* have risen over the last few decades in Europe, USA as well as Asia. This leads to a growing burden of enterococcal infections in general, and to a decreased ratio of *E. faecalis* to *E. faecium* infections (down to 60:40). Although *E. faecalis* remains generally less resistant to antibiotics than *E. faecium*, antimicrobial resistance in the former poses an increasing challenge to therapy as earlier reported (Kuch et al., 2011).

Enterococci are able to withstand harsh environmental conditions and acquire antibiotic resistant determinants. Enterococci are subject to horizontal gene transfer (HGT) elements, a characteristic which empowers bacterial organisms to develop rapidly through rapid exchange and distribution of beneficial trait-
encoding components, including antibiotic resistance genes (Fisher & Phillips, 2009).

Recently, several resistance determinants were reported to lead to the development of resistance to the seven groups of antibiotics by enterococci. Other determinants are encoded genes substances such as insertion sequence, integrons, transposon and plasmids (Li et al., 2015). Horizontal gene transfer has been reported to be the main route through which 25% of genomic determinants are acquired by the enterococcus species (Garcia-Solache et al., 2016). According to Patel et al (2008), enterococci are a normal microbial flora of the gut which is responsible for the spread of antibiotic resistant determinants. However, management of infection due to E. faecium today is highly challenging to the clinician due to multidrug resistance (MDR) which is currently on the increase in the population (Miller et al., 2014). Presently, enterococci are one of the leading etiologies of HAIs which are reported by National Healthcare Safety Network from CDC.

Management of enterococcal infections involves the actual synergistic effect of aminoglycosides and cell wall active antibiotics such as glycopeptides (vancomycin) or beta-lactam (ampicillin). To date, enterococci have exhibited multidrug-resistance (MDR) with high level of aminoglycoside resistance (HLAR) and vancomycin resistance (VRE). This has caused significant problem in clinical anti-infective therapy (Lebreton et al., 2016; Li et al., 2015). The strains of enterococci are becoming more widely spread in Europe, USA and Asia (Lebreton et al., 2016). Several studies have documented different distribution of aminoglycoside modifying enzymes (AMEs) and encoding genes differently based on the geographical regions (Zarrilli et al., 2005; Padmasini et al. 2014; Udo et al., 2004). In Malaysia, the data regarding the prevalence of HLAR and the distribution of AMEs are still limited. Hence, this study is aimed to determine the distribution of AMEs genes among enterococci isolates from clinical sample.

1.2 Problem statement

The Centre for Disease Control and Prevention (CDC, 2014) has reported that approximately 4 million hospitalized patients had nosocomial infections which are caused by antimicrobial resistance organisms. Among the pathogens, enterococci have been reported as the second most common cause of hospital acquired infections due to their wide-range of antimicrobial resistance and synergistic effects with beta-lactams, glycopeptides and aminoglycosides, which are currently being used in clinical practices.
Enterococci are inherently resistant to aminoglycosides due to aminoglycoside modified enzymes. Nevertheless, the types and distribution of resistance genes are different and geographically dependent. In Malaysia, data on the prevalence of HLAR and distributions of the resistance genes are still limited.

1.3 Objectives

General Objective

The aims of this study are to determine the antibiotic susceptibility patterns of the clinical isolates *Enterococcus faecalis* and *Enterococcus faecium* and the distribution of HLAR genes among the resistant enterococci isolates.

Specific Objectives

1. To re-identify *E. faecalis* and *E. faecium* using phenotypic and genotypic approaches.
2. To determine the antibiotic susceptibility profiles of *E. faecalis* and *E. faecium* by using disc diffusion.
3. To determine the minimum inhibitory concentrations of the resistant enterococci isolates using microdilution method.
4. To determine the distribution of aminoglycoside resistance encoding genes in the resistance enterococci isolates using molecular biology approaches.
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