DEVELOPMENT OF A DNA-BASED MOLECULAR METHOD FOR THE RAPID DETECTION OF ENTEROCOCCUS SPECIES AND ANTIMICROBIAL RESISTANCE GENOTYPES

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by

CHAN YEAN YEAN

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DEDICATIONS

This thesis is dedicated to my mother and my husband for their encouragement and patience, and to my late father, Chan Chai Teck, who has been a great source of inspiration and motivation.
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LIST OF SYMBOLS

1.1 % Percentage
1.2 ~ Approximately
1.3 µg Micro gram
1.4 µl Micro liter
1.5 µM Micro Molar
1.6 $\beta$ Beta
1.7 < Less than
1.8 ≤ Equal and/or less than
1.9 > More than
1.10 ≥ Equal and/or more than
1.11 λ Lambda (wavelength)

LIST OF ABBREVIATIONS

1.1 A Adenosine
1.2 BLAST Basic Local Alignment Search Tool
1.3 bp Base pair
1.4 C Cytosine
1.5 CaCl₂ Calcium chloride
1.6 CFU Colony forming unit
1.7 cm Centimeter
1.8 dH₂O Distilled water
1.9 DNA Deoxyribonucleic acid
1.10 dNTP Deoxynucleotide triphosphate
1.11 e.g. *Eumpli gratia* or for example
1.12 EDTA Ethylenediamine tetraacetic acid
1.13 *et al.* *Et alii*
1.14 g Gravity
1.15 G Guanine
1.16 g gram
1.17 hr Hour
1.18 *i.e.* *Id est* or that is
1.19 kb kilobase
1.20 L Liter
1.21 mg Miligram
1.22 MgCl₂ Magnesium chloride
1.23 min Minute
1.24 ml Mililiter
1.25 mM Milimolar
1.26 N Normal
1.27 NCBI National Centre of Biotechnology Information
1.28 CLSI Clinical and Laboratory Standards Institute
1.29 ºC Degree Celcius
1.30 PCR Polymerase chain reaction
1.31 pg Pico gram
1.32 pmole Pico mole
1.33 Prof. Professor
1.34 rpm Revolution per minute
1.35 sec Seconds
1.36 T Thymine
1.37 Ta Annealing temperature
1.38 Taq *Thermus aquaticus*
1.39 Tm Melting temperature
1.40 U Unit
1.41 U.S.A United States of America
1.42 V Volt
1.43 vol. Volume
1.44 wt. Weight
1.45 X Times or multiplication
1.46 PFGE Pulse field gel electrophoresis
1.47 S Susceptible
1.48 UV Ultra violet
1.49 Ltd Limited
1.50 n Nano
1.51 p Plasmid

**LIST OF APPENDICES**

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<tr>
<th>Appendix</th>
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</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>
LIST OF PUBLICATIONS, SEMINARS & AWARDS

1.0 PUBLISHED ARTICLES


2.0 GENBANK SEQUENCES SUBMISSION


3.0 SEMINARS OR CONFERENCES


4.0 AWARDS


4.2 **GOLD MEDAL**: M. Ravichandran, P. Lalitha, Chan Y.Y., Lim kun Lee, Lee Su Yin, Melissa Chan Li Ann, Habsah bt Hassan, Azian bt. Harun, Mohd Radzi Johari. **EZVRE Amp**. SALON INTERNATIONAL DES INVENTIONS GENEVE, Geneve, 5-9 April **2006**.

PEMBANGUNAN KAEDAH MOLEKUL BERASASKAN DNA UNTUK PENGESEANAN GENOTIP SPESIS ENTEROCOCCUS DAN RINTANGAN ANTIMIKROBIA

ABSTRAK

Enterococci muncul sebagai penyebab jangkitan nosokomial yang penting di kebanyakan negara di dunia semenjak kurun yang lepas. Strain enterococci yang paling biasa dijumpai dalam isolat klinikal ialah E. faecalis dan E. faecium yang telah memperoleh kerintangan terhadap gentamicin atau vancomycin. Ujian kultur konvensional mengambil 2-5 hari untuk mendapat maklumat lengkap tentang jenis organisma dan corak kepekaan antibiotiknya. Oleh sebab itu, kajian ini tertumpu terhadap pembangunan suatu ujian reaksi berantai polimerasi berganda untuk pengesanan pants enterococci yang rintang vancomycin dan gentamicin. Ujian ini mampu mengesan 8 gen serentak, iaitu 16S rRNA daripada genus Enterococcus, ddl daripada E. faecalis dan E. faecium, aacA-aphD yang mengkodkan kerintangan tinggi terhadap gentamicin, kerintangan vancomycin yang pelbagai peringkat, iaitu vanA, vanB, vanC dan vanD serta satu gen kawalan dalaman. Pasangan pencetus khusus dan unik direka supaya julat pengamplifikasi produk berada diantara 150 ke 1200 bp. Pengkhususan pencetus disah berdasarkan urutan jujukan DNA produk reaksi berantai polimerasi berganda and analisa mengecam. Seterusnya, reaksi berantai polimerasi berganda ini dijadikan stabil suhu dan ujian kestabilan pants dijalankan pada suhu bilik, 37°C dan 10°C. Kepekaan dan kekhususan ujian ini telah dibandingkan deangan kaedah kultur konvensional. Kepekaan analitikal ujian diperikat DNA ialah 1 ng manakala kekhususan analitikal yang dinilai dengan 43 strain rujukan enterococci dan bukan enterococci adalah 100%. Ujian kestabilan
pantas bagi campuran stabil suhu reaksi berantai polimerasi berganda yang disimpan pada 10°C adalah stabil sehingga dua tahun dan enam bulan. Kejuitan diagnostik yang ditentukan dengan menggunakan sejumlah 543 sampel daripada isolat klinikal, ladang ternakan ayam, sampel air dan najis, mendapati 3.9% daripada sampel adalah rintang vancomicin dan 16.0% ialah enterococci yang mempunyai kerintangan aminoglikosida dwi-fungsi. Kehadiran kawalan dalaman dalam ujian reaksi berantai polimerasi berganda membantu dalam penentuan kes negatif palsu. Ujian reaksi berantai polimerasi berganda adalah tegap dan boleh memberikan maklumat tentang 8 gen penting untuk pengesanan spesis Enterococcus yang paling biasa dan corak kerintangan antibiotiknya. Ujian reaksi berantai polimerasi yang dibangunkan dalam kajian ini boleh digunakan sebagai alat pemantauan yang berkesan untuk kajian prevalens enterococci dan corak rintangan antibiotiknya dalam hospital dan haiwan ternakan.
Enterococci have emerged as a significant cause of nosocomial infections in many parts of the world over the last decade. The most common enterococci strains present in clinical isolates are *E. faecalis* and *E. faecium*, which have acquired resistance to either gentamicin or vancomycin. The conventional culture test takes 2-5 days to yield complete information of the organism and its antibiotic sensitivity pattern. Hence, our present study was focused on developing a multiplex PCR assay for the rapid detection of vancomycin and bifunctional aminoglycoside resistant enterococci. This assay simultaneously detects 8 genes namely 16S rRNA of *Enterococcus* genus, * ddl* of *E. faecalis* and *E. faecium*, *aacA-aphD* that encodes high level gentamicin resistance (HLGR), multilevel vancomycin resistant genotypes such as *vanA*, *vanB*, *vanC* and *vanD* and one internal control gene. Unique and specific primer pairs were designed to amplify the 8 genes with the PCR products ranging from 150 to 1200 bp. The specificity of the primers were confirmed by DNA sequencing of the multiplex PCR products and BLAST analysis. The sensitivity and specificity of multiplex PCR assay was evaluated against the conventional culture method. The multiplex PCR was thermostabilized and an accelerated stability test was evaluated at room temperature, 37°C and 10°C. The analytical sensitivity of the assay was found to be 1 ng at the DNA level while the analytical specificity was evaluated with 43 reference enterococci and non-enterococcal strains and was found to be 100%. The thermostabilized multiplex PCR mix stored at 10°C was stable up to two and a half
years by the accelerated stability test. The diagnostic accuracy was determined using a total of 543 samples from clinical isolates, poultry farms, water samples and stool samples, which showed that 3.9% of the samples were vancomycin resistant and 16.0% were bifunctional aminoglycoside resistant enterococci. The presence of an internal control in the multiplex PCR assay helped to rule out false negative cases. The multiplex PCR assay is robust and can give information about the 8 genes that are essential for the identification of the most common *Enterococcus* species and their antibiotic susceptibility pattern. The PCR assay developed in this study can be used as an effective surveillance tool to study the prevalence of enterococci and their antibiotic resistance pattern in hospitals and farm animals.
CHAPTER ONE: INTRODUCTION

1.1 INTRODUCTION

1.1.1 History and significance of Enterococcus

Enterococci are Gram-positive cocci that occur singly, in pairs, or as short chains (Facklam et al., 2002). Their complex interaction with humans in food manufacturing, ever-present in humans and animals alimentary tract, in soil, water and food causes serious human and animal infections. These diverge characteristics make it an important group of bacteria in human life. Enterococcus is closely related to the streptococci (Facklam et al., 2002). In the 1930s, the Lancefield serological typing system was established, which classified enterococci as group D streptococci and were differentiated from non-enterococcal group streptococci (Streptococcus bovis) (Lancefield, 1933, Cetinkaya et al., 2000). The enterococcal group was introduced and used by Sherman in 1938, to describe streptococci that grew at 10 to 45°C, in broth with pH 9.6 and in broth containing 6.5% NaCl, and survived at heating to 60°C for 30 min (Sherman, 1938, Cetinkaya et al., 2000). In 1970, the enterococcal streptococci genus was proposed by Kalina based on cellular arrangement and phenotypic characteristics, whereby S. faecalis and S. faecium of these two taxons be named Enterococcus (Kalina, 1970, Facklam et al., 2002). However, the proposal was not accepted and the use of Streptococcus genus continued. Finally in 1984, Schleifer and Kilpper-Balz provided sufficient genetic evidence of the difference of S. faecalis and S. faecium from the other members of Streptococcus genus to merit a separate genus (Schleifer and Kilpper-Balz, 1984, Cetinkaya et al., 2000, Facklam et al., 2002). The streptococcal species S. faecalis and S.
faecium were excluded from Streptococcus to form the Enterococcus genus (Schleifer and Kilpper-Balz, 1984).

Presently, based on standard laboratory procedures for species identification using phenotypic tests and molecular-based methods such as DNA-DNA reassociation (Niemi et al., 1993), 16S rRNA gene sequencing (Devriese et al., 1995, Vandamme et al., 1996) and whole-cell protein analysis (Teixeira et al., 1995); a total of 27 Enterococcus species have been included in the Enterococcus genus (Table 1.1) (Facklam et al., 2002). The phylogenetic analysis of catalase-negative, Gram-positive cocci based on the comparison of 1,400 bases of the 16S rRNA gene has revealed that the Enterococcus is more closely related to Vagococcus, Tetragenococcus and Carnobacterium than to the phenotypically-associated Streptococcus and Lactococcus genera (Collins et al., 1989, Aguirre and Collins, 1992, Devriese et al., 1993, Facklam et al., 2002).
Table 1.1. Taxonomic studies of the genus *Enterococcus*.

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Year identified (method)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>E. faecalis</em></td>
<td>1983 (DNA analysis); 1993 (protein analysis)</td>
</tr>
<tr>
<td>2</td>
<td><em>E. faecium</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>3</td>
<td><em>E. avium</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>4</td>
<td><em>E. casseliflavus</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>5</td>
<td><em>E. durans</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>6</td>
<td><em>E. gallinarums</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>7</td>
<td><em>E. malodoratus</em></td>
<td>1983 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>8</td>
<td><em>E. hirae</em></td>
<td>1985 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>9</td>
<td><em>E. mundtii</em></td>
<td>1986 (DNA analysis); 1993 (protein analysis)</td>
</tr>
<tr>
<td>10</td>
<td><em>E. raffinosus</em></td>
<td>1989 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>11</td>
<td><em>E. pseudoavium</em></td>
<td>1989 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>12</td>
<td><em>E. cecorum</em></td>
<td>1989 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>13</td>
<td><em>E. columbae</em></td>
<td>1990 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>14</td>
<td><em>E. saccharolyticus</em></td>
<td>1984 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>15</td>
<td><em>E. dispers</em></td>
<td>1991 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>16</td>
<td><em>E. sulfureus</em></td>
<td>1986 (DNA analysis); 1994 (protein analysis)</td>
</tr>
<tr>
<td>17</td>
<td><em>E. asini</em></td>
<td>1998 (DNA analysis); 2001 (protein analysis)</td>
</tr>
<tr>
<td>18</td>
<td><em>E. villorum</em></td>
<td>2001 (DNA analysis); 2001 (protein analysis)</td>
</tr>
<tr>
<td>19</td>
<td><em>E. haemoperoxidus</em></td>
<td>2001 (DNA analysis)</td>
</tr>
<tr>
<td>20</td>
<td><em>E. moraviensis</em></td>
<td>2001 (DNA analysis)</td>
</tr>
<tr>
<td>21</td>
<td><em>E. ratti</em></td>
<td>1998 (DNA analysis); 2001 (protein analysis)</td>
</tr>
<tr>
<td>22</td>
<td><em>E. porcinus</em></td>
<td>2001 (DNA analysis); 2001 (protein analysis)</td>
</tr>
<tr>
<td>23</td>
<td><em>E. pallens</em></td>
<td>(DNA analysis); (protein analysis)</td>
</tr>
<tr>
<td>24</td>
<td><em>E. gilvus</em></td>
<td>(DNA analysis); (protein analysis)</td>
</tr>
<tr>
<td>25</td>
<td><em>E. seriolicida</em></td>
<td>1991 (DNA analysis); 1991 (protein analysis)</td>
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<tr>
<td>26</td>
<td><em>E. solitarius</em></td>
<td>1998 (DNA analysis); (protein analysis)</td>
</tr>
<tr>
<td>27</td>
<td><em>E. flavescens</em></td>
<td>1992 (DNA analysis); 1997 (protein analysis)</td>
</tr>
</tbody>
</table>

Note: Adapted and slightly modified from reference (Facklam *et al.*, 2002) (Appeared as table 2 and page 6-7 in the source of original).
1.1.2 Epidemiology of Enterococcus

In the past decades, enterococci have emerged as important nosocomial pathogens in many countries around the world (Moellering, 1992). Most enterococci exhibit a high extent of antimicrobial resistance, particularly to vancomycin. Vancomycin resistant enterococci (VRE) strains also show resistance to penicillins and high-level resistance to aminoglycosides (Malani et al., 2002). *E. faecium* is more resistant than *E. faecalis* that accounted for 5-15% of enterococcal infections (Cetinkaya et al., 2000).

The *E. faecalis* species was reported to be responsible for most infections in the community, long-term care unit and hospitals (Graninger and Ragette, 1992, Patterson et al., 1995, Malani et al., 2002). *E. faecalis* is more likely to be susceptible to vancomycin, but resistant to high-level gentamicin and other aminoglycosides that make patients with serious infections like endocarditis difficult to treat.

Currently, enterococci that accounted for around 20% of nosocomial infections isolated from intensive care units (ICU) patients and approximately 15% from non-ICU patients are VRE (Fridkin and Gaynes, 1999, Malani et al., 2002). Fortunately, in Malaysia, only 3 cases of VRE isolated from clinical specimens from year 1996 to 2006 have been reported (Riley et al., 1996, Raja et al., 2005, Zubaidah et al., 2006). It is very important to identify the reservoirs for colonization and the routes of transmission of enterococci since there are limited therapeutic options for treatment of VRE infections (Malani et al., 2002). The role of widespread and indiscriminate use of antimicrobials is obviously encouraging the colonization of VRE.
1.1.3 Classification

Current classification and identification of enterococci are done by conventional or molecular tests.

1.1.3.1 Conventional methods for identification of enterococcal species

The enterococcal species are separated into five groups based on conventional (biochemical) phenotypic tests such as acid formation in mannitol and sorbose broths and hydrolysis of arginine (Tables 1.2 and 1.2.1). However, this method may require long incubation time (2-10 days) (Facklam et al., 2002).
Table 1.2. Phenotypic characteristics used for identification of *Enterococcus* species.

| Species                  | MAN | SOR | ARG | ARA | SBL | RAF | TEL | MOT | PIG | SUC | PYU | MGP |
|--------------------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| **Group I**              |     |     |     |     |     |     |     |     |     |     |     |     |     |
| *E. avium*               | +   | +   | -   | +   | +   | -   | -   | -   | -   | +   | +   | v   |     |
| *E. malodoratus*         | +   | +   | -   | -   | +   | +   | -   | -   | -   | +   | +   | v   |     |
| *E. raffinosus*          | +   | +   | -   | +   | +   | +   | -   | -   | -   | +   | +   | v   |     |
| *E. pseudoavium*         | +   | +   | -   | +   | +   | -   | -   | -   | +   | +   | +   |     |     |
| *E. saccharolyticus*     | +   | +   | -   | +   | +   | +   | -   | -   | -   | +   | -   | +   |     |
| *E. pallens*             | +   | +   | -   | -   | +   | -   | -   | +   | +   | -   | +   |     |     |
| *E. gilvus*              | +   | +   | -   | -   | +   | -   | -   | +   | +   | -   | -   | +   |     |
| **Group II**             |     |     |     |     |     |     |     |     |     |     |     |     |     |
| *E. faecalis*            | +   | -   | +   | -   | +   | +   | -   | -   | -   | +*  | +   | -   |     |
| *E. faecium*             | +   | -   | +   | -   | -   | v   | +   | -   | -   | +*  | +   | -   | -   |
| *E. casseliflavus*       | +   | -   | +   | +   | v   | +   | -*  | -*  | +   | +*  | +   | v   | +   |
| *E. mundtii*             | +   | -   | +   | +   | v   | +   | -   | -   | +   | +   | -   | -   |     |
| *E. gallinarum*          | +   | -   | +   | +   | -   | -   | -   | -*  | -   | +   | -   | +   |     |

*a.*
Table 1.2. Continued.

<table>
<thead>
<tr>
<th>Species</th>
<th>MAN</th>
<th>SOR</th>
<th>ARG</th>
<th>ARA</th>
<th>SBL</th>
<th>RAF</th>
<th>TEL</th>
<th>MOT</th>
<th>PIG</th>
<th>SUC</th>
<th>PYU</th>
<th>MGP</th>
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<td>All 7 species refer to table 1.2.1)</td>
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<td><em>E. asini</em></td>
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<td><em>E. sulfureus</em></td>
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<tr>
<td><em>E. cecorum</em></td>
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<tr>
<td><em>E. casseliflavus</em></td>
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<tr>
<td><em>E. gallinarum</em></td>
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<tr>
<td><em>E. columbae</em></td>
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</tbody>
</table>

Note: Adapted from reference (Facklam et al., 2002) (Appeared as table 3 and page 10 in the source of original).

* MAN, mannitol; SOR, sorbose; ARG, arginine; ARA, arabinose; SBL, sorbitol; RAF, raffinose; TEL, 0.04% tellurite; MOT, motility; PIG, pigment; SUC, sucrose; PYU, pyruvate; MGP, methyl-α-D-glucopyranoside; +, >90% positive; -, <10% positive; V, variable; +* or -*, occasional exceptions (<3% of strains show aberrant reactions).
Table 1.2.1. Phenotypic characteristics used for identification of *Enterococcus* species **Group III**.

<table>
<thead>
<tr>
<th>Species</th>
<th>LM</th>
<th>PYU</th>
<th>HIP</th>
<th>TEL</th>
<th>ARA</th>
<th>GYL</th>
<th>RAF</th>
<th>SUC</th>
<th>TRE</th>
<th>XYL</th>
<th>MGP</th>
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</thead>
<tbody>
<tr>
<td><em>E. durans</em></td>
<td>A/C</td>
<td>-/0</td>
<td>+/-82</td>
<td>-/0</td>
<td>-/0</td>
<td>-/0</td>
<td>-/0</td>
<td>+/-100</td>
<td>-/0</td>
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</tr>
<tr>
<td><em>E. porcinus</em></td>
<td>A/-</td>
<td>+/-6</td>
<td>-/0</td>
<td>-/0</td>
<td>-/0</td>
<td>-/0</td>
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<td>+/-100</td>
<td>+/-100</td>
<td>-/0</td>
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</tr>
<tr>
<td><em>E. ratti</em></td>
<td>+/-6</td>
<td>-/0</td>
<td>+/-60</td>
<td>-/0</td>
<td>-/0</td>
<td>-/0</td>
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<td>-/20</td>
<td>-/0</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>E. hirae</em></td>
<td>A/-</td>
<td>+/-100</td>
<td>+/-100</td>
<td>-/0</td>
<td>+/-100</td>
<td>+/-100</td>
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<td>-/0</td>
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<td></td>
</tr>
<tr>
<td><em>E. dispar</em></td>
<td>A/C</td>
<td>+/-76</td>
<td>-/13</td>
<td>+/-88</td>
<td>-/0</td>
<td>-/12</td>
<td>-/0</td>
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<td>-/0</td>
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<td></td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>A/C</td>
<td>+/-100</td>
<td>+/-100</td>
<td>-/6</td>
<td>+/-100</td>
<td>-/6</td>
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<td>v/38</td>
<td>+/-75</td>
<td>-/0</td>
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<tr>
<td><em>E. faecium</em></td>
<td>A/v</td>
<td>+/-56</td>
<td>-/6</td>
<td>+/-100</td>
<td>-/6</td>
<td>+/-100</td>
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<td>v/38</td>
<td>+/-75</td>
<td>-/0</td>
</tr>
</tbody>
</table>

Note: Adapted from reference (Facklam *et al.*, 2002) (Appeared as table 4 and page 11 in the source of original).

*LM*, litmus milk; *A*, acid; *C*, clot formation; *PYU*, pyruvate utilization; *HIP*, hippurate hydrolysis; *TEL*, tolerance to 0.04% tellurite; *ARA*, GYL, RAF, SUC, TRE, XYL, MGP, acid formation in broth containing 1% arabinose, glycerol, raffinose, sucrose, trehalose, xylose, methyl-α-D-glucopyranoside, respectively; + or – or v/ number, interpretation/ percent positive; +, 85% or more of the strains positive; -, 15% or less than the strains positive; v, variable reactions (16 to 84% positive); **b.** *E. villorum* has similar phenotypic characteristics but has not been tested in conventional tests; **c.** Mannitol-negative variants.
1.1.3.2 Molecular tests for identification of *Enterococcus* species

During the last decade, attempts to develop more rapid and accurate identification methods by application of molecular techniques have expanded dramatically. Molecular-based tests such as DNA-DNA hybridization, 16S rRNA genes sequencing and gas-liquid chromatography of fatty acids to analyze different *Enterococcus* cells molecules for taxonomic purposes have the potential to be adaptable for use in microbiology laboratories (Facklam *et al.*, 2002).

Other molecular methods that have been applied for the identification of *Enterococcus* species are WCP profile analysis, vibrational spectroscopic analysis, RAPD analysis, sequencing analysis of the 16S rRNA gene, fragment length polymorphism analysis of amplified 16S rRNA, sequencing of the domain V of 23S rRNA gene, amplification of the rRNA intergenic spacer, sequencing of *ddl* genes, amplification of *ddl* and *van* genes, sequencing of the *sodA* gene, sequencing and probing of the *cpm60* gene, amplification and probing of the *efaA* gene, amplification and probing of the *ace* gene, amplification and sequencing of the *tuf* gene and lastly, amplification of the pEM1224 gene.
1.1.4 Properties of *Enterococcus*

1.1.4.1 Physical characteristics

1.1.4.1.1 Phenotypic typing methods

Phenotypic characterization methods that were previously used in epidemiological investigation have included biotyping and antibiotyping, based on physiological (Kuhn *et al.*, 1995), antimicrobial resistance characteristics (Luginbuhl *et al.*, 1987, Murray, 1992) and serotyping (Sharpe and Shattock, 1952, Sharpe, 1964, Smyth *et al.*, 1987 Maekawa *et al.*, 1992). Although these approaches yielded useful information, they are complicated, highly irreproducible, gave insufficient discrimination, time-consuming and difficult to interpret. These drawbacks have limited their value in epidemiological studies (Facklam *et al.*, 2002).

1.1.4.1.2 Molecular typing methods

Among the earliest molecular techniques developed and used were the analysis of plasmid profiles and restriction enzyme analysis of genomic DNA by electrophoresis (Hall *et al.*, 1992, Lacoux *et al.*, 1992, Bodnar *et al.*, 1996, Savor *et al.*, 1998, Quednau *et al.*, 1999, Facklam *et al.*, 2002). Yet, the inconsistencies in plasmid yield and difficulties in accurate interpretation of electrophoretic profiles that failed to discriminate among enterococcal strains were noted (Facklam *et al.*, 2002). Currently, the gold standard for the epidemiological analysis of nosocomial enterococcal infections is the PFGE profiles of *SmaI*-digested genomic DNA. This is the single most useful and reliable typing method for enterococci. However, the use of specialized equipments, lack of standardized procedures and difficult interpretation of PFGE banding profiles limit the extensive application of this technique (Facklam *et al.*, 2002).
1.1.4.2 Growth and culture characteristics

Enterococci are facultative anaerobes that grow optimally at 35°C. The growth of enterococci can be supported by brain-heart infusion with 5% sheep blood and any blood agar base containing 5% animal blood. Some strains of *E. faecalis* are beta-hemolytic on agar bases containing rabbit, horse, or human blood but not on agar containing sheep blood. However, some *E. durans* strains are beta-hemolytic regardless of the type of blood used. The other species are alpha or gamma-hemolytic (Facklam *et al.*, 2002). If the sample to be cultured may contain Gram-negative bacteria, bile-esculin azide (Enterococcusel agar) (Sabbaj *et al.*, 1971), Pfizer selective *Enterococcus*, Enterococci broth (Merck, Darmstadt, Germany) and other commercially prepared medium containing azide are primary isolation media (Facklam *et al.*, 2002). The azide inhibits Gram-negative bacteria, and enterococci appear as black colonies of esculin hydrolysis. Besides that, Columbia colistin-nalidixic acid agar (CNA) (Ellner *et al.*, 1966) and phenylethyl alcohol agar (PEA) (Dayton *et al.*, 1974) have been used successfully to isolate enterococci (Facklam *et al.*, 2002).

The rise in the increase of vancomycin resistance among enterococci makes it important and necessary to isolate VRE for the control of the spread of these organisms in hospital environments (Ieven *et al.*, 1999, Cetinkaya *et al.*, 2000, Facklam *et al.*, 2002). A variety of different selective-enrichment media have been used for the isolation of VRE from fecal specimens (stool samples and rectal swabs) like Mueller-Hinton agar (MHA) (Willey *et al.*, 1992), brain-heart infusion agar (BHI) (Swenson *et al.*, 1994), Enterococcosel agar (Ieven *et al.*, 1999, Roger *et al.*, 1999) and Enterococci broth supplemented with 6 µg/ml of vancomycin.
1.2 CLINICAL MANIFESTATIONS, PATHOGENESIS, TREATMENT AND PREVENTION OF ENTEROCOCCUS

1.2.1 Reservoirs and mode of transmission

Enterococci are normal flora in the gastrointestinal tract of humans and many other animals. *E. faecalis* is most common and abundant of enterococci followed by *E. faecium*. Even though enterococci are found throughout the whole gastrointestinal tract, both *E. faecalis* and *E. faecium* are found in the highest number in the colon and also a low number in the oral cavity (Chenoweth, 1990). In addition, *E. faecalis* is a predominant normal inhabitant in the genital tract (Malani et al., 2002).

The emergence of VRE has made it important to understand its colonization pattern, because once it colonizes the host, it may persist for months or years (Lai et al., 1997, Roghmann et al., 1997b, Bonten et al., 1998, Malani et al., 2002).

In the 1980s, the rise in nosocomial acquisition and the subsequent spread of aminoglycoside resistant enterococci and VRE in the 1990s have demonstrated the transmission of enterococci among patients in the hospital ward (Handwerger et al., 1993, Boyce et al., 1994, Saurina et al., 1997, Malani et al., 2002). The spread of VRE into the environment is likely to occur in the diarrhea patients ward (Boyce et al., 1994, Cetinkaya et al., 2000). The carriage of enterococci by the hands of health care workers is the presumed mode of transmission from patient to patient and by way of contaminated medical equipments (Cetinkaya et al., 2000, Hayden, 2000, Mayhall, 1999, Malani et al., 2002). Contaminated environmental surfaces and medical equipments in the hospital ward may serve as a reservoir for the bacteria in the hospital (Cetinkaya et al., 2000). Another suggested mode of transmission of enterococci is by direct inoculation onto intravenous or urinary catheters, but this is more likely to result
in gastrointestinal tract colonization with the acquired strain, which subsequently become the patient’s normal flora (Zervos et al., 1987a, Malani et al., 2002). The use of broad-spectrum antimicrobials in hospitals causes the normal flora to acquire antibiotic resistance and persist in the gastrointestinal tract. Subsequently, the infections that arise could be due to this acquired strain (Malani et al., 2002). The spread of VRE within a hospital unit (Karanfil et al., 1992, Handwerger et al., 1993, D'Agata et al., 2001), between hospitals (Moreno et al., 1995, Donskey et al., 1999b) and even from state to state (Chow et al., 1993) has been documented. This may be caused by the increased movement of colonized patients among different health care centers. Although it is uncommon, non-hospitalized individuals may acquire VRE through contaminated animal-based food products that serve as a reservoir (Moreno et al., 1995, Trick et al., 1999, Cetinkaya et al., 2000, Malani et al., 2002).
1.2.2 Pathogenesis and immune responses

Enterococcal is a commensal organism in the gastrointestinal tracts of various organisms, from humans to insects. However, 100 years ago enterococci were recognized as capable of causing serious infections and in the past two decades, it has become resistant to many groups of antimicrobial including the antimicrobial of last resort, vancomycin. In this era of modern medicine, the balance in commensalism is disrupted, whereby organisms acquiring new traits can result in the breakdown of the host’s ability to hold commensal organisms in check, and this enables the organism to colonize new niches of the host that the host is incapable of adequately defending (Gilmore et al., 2002).

Enterococci occur as a commensal in the human colon (~ $10^8$ CFU/g of feces) (Noble, 1978, Huycke et al., 1998) and also can be isolated from the environment (fecal material contamination). The emerging literature evidence suggests that enterococci may be highly host adapted (enterococci colonizing specific hosts), many clinical isolates are virulent species that are distinct from those that colonize the GI tracts of healthy individuals. The factors that cause enterococci-adapted host specificity include species-specific mucin characteristics, co-resident GI tract flora composition, diet and motility rates (Kararli, 1995). Based on Escherichia coli that have both commensal and pathogenic roles, an emerging concept that human commensal enterococcal strains are genetically distinct from pathogenic isolates was built (Gilmore et al., 2002).

The virulent enterococcal lineages are able to cause hospital ward outbreaks involving multiple patients as a clonal outbreak (Huycke et al., 1991, Murray et al., 1991, Thorisdottir et al., 1994, Chow et al., 1993, Suppola et al., 1999, Van Den Braak et al., 1999), while commensal strains do not. The acquisition of additional traits on
mobile genetic elements enhanced its ability to destabilize the commensal relationship and cause disease (Hacker and Kaper, 2000). Enterococci traits that caused clonal outbreaks are antimicrobial resistances (Murray et al., 1991, Thorisdottir et al., 1994, Chow et al., 1993, Suppola et al., 1999, Van Den Braak et al., 1999) and expression of enterococcal cytolysin (Huycke et al., 1991, Gilmore et al., 2002).

The effect of antimicrobial introduction and its widespread usage on enterococcal infection is unknown, but literature reviews showed that enterococcal infection could have occurred prior to the introduction and widespread use of antimicrobials (Gilmore et al., 2002).

Enterococci possess an intermediate level of virulence compared to streptococci and lactococci, thus Enterococcus was termed as “facultative parasite” (Andrewes, 1906). Since the pre-antibiotic era, E. faecalis and E. faecium are the major cause of enterococcal infections (Huycke et al., 1998). Translocation of enterococci from intra-abdominal abscesses, intravenous catheters, genitourinary tract and most GI tract are causes of enterococcal bacteremia (Cheung et al., 1997, Shlaes et al., 1981, Mundy et al., 2000, Gilmore et al., 2002).

Nosocomial isolates analysis reveals that different patient isolates are clonally related (Zervos et al., 1987a). Colonization of GI tract upon hospitalization by virulent lineages eliminates many endogenous flora and caused overgrowth of pathogenic enterococcal strains. The indigenous enterococci and nosocomial enterococci do not compete for the same location and this perhaps explain why nosocomial strains with particular traits are able to colonize certain GI tract location that indigenous enterococci cannot. GI tract serves as area for multiplication of numbers of nosocomial enterococci, and when opportunities arise, the organism will spread from the primary colonization
site to urinary tract, postsurgical wound site or bloodstream, where nosocomial strain with its additional traits enable it to colonize, evade host clearance mechanisms and induce pathology (Gilmore et al., 2002).

Among bacterial virulence factors that influence the host-parasite relationship are:

(A) Secreted factors:

(i) Cytolysin encoded in a complex operon (cyl) consisting of 8 genes and it is a bacterial toxin expressed by some strains of \textit{E. faecalis} that displays both hemolytic and bactericidal activity in the pathogenesis of enterococcal infection. Enterococcal infections have two critical components; inflammation, which is treatable with corticosteroid, and toxin-mediated organ destruction, which is untreatable by either corticosteroid or antimicrobial. Cytolysin caused pathogenicity of \textit{E. faecalis} by direct tissue damage. The hemolytic toxin enables \textit{E. faecalis} to manufacture cytochromes from exogenous hemin by hemolysin erythrocytes that allow for aerobic respiration and greater growth yield to explain the enhanced presence of cytolytic strains in the bloodstream (Ritchey and Seeley, 1974, Pritchard and Wimpenny, 1978, Janda and Abbott, 1993);

(ii) Bacterial proteases function to provide peptide nutrients to organisms and work as virulence factors, which cause direct and indirect host tissue damage, indirect degradation of host connective tissues by activating host matrix metalloproteases (Burns et al., 1996, Okamoto et al., 1997); deregulating critical host processes to facilitate microbial invasion and survival in host environments (Maeda and Yamamoto, 1996); deregulating key components of the host immune system by degrading either immunoglobulins or complement pathways (Schultz and Miller, 1974, Plaut, 1983,
Sundqvist et al., 1985, Prokesova et al., 1992); degrading specific host proteins resulting in production of toxins (Tonello et al., 1996, Duesbery et al., 1998); activating viruses either directly or indirectly (Tashiro et al., 1987); processing other bacterial virulence factors (Booth et al., 1984, McGavin et al., 1997); and mediating direct degradation of host connective tissues or tissue proteins (Lantz et al., 1991, Travis et al., 1994, Gilmore et al., 2002). E. faecalis secrete two proteases, namely, gelatinase or coccolysin, (gelE, a metalloprotease) to inactivate human endothelin (a vasoactive peptide). Hospital isolates show high frequency compared with fecal isolates from healthy volunteers and studies found that E. faecium strains are not gelatinase-producer (Coque et al., 1995, Elsner et al., 2000). Serine proteases (sprE gene) are also secreted that down-regulate the expression of surface proteins, such as protein A, coagulase and fibronectin-binding protein (Recsei et al., 1986, Novick et al., 1993, Lebeau et al., 1994, Cheung et al., 1997, Ji et al., 1997, Gilmore et al., 2002).

(B) Enterococcal adhesions:

(i) Aggregation substances are surface-localized protein encoded by pheromone-responsive, self-transmissible plasmids that mediate binding or interaction between donor and recipients bacterial cells (Clewell, 1993), mediating enterococcal binding to eukaryotic cells and as a binding factor to resistance to immune clearance by interfering with PMN-mediated killing that caused E. faecalis to survive within polymorphonuclear neutrophils (PMNs) following phagocytosis (Rakita et al., 1999). In summary, aggregation substances have multiple roles for enterococcal virulence: it disseminates plasmid-encoded virulence factors, such as enterococcal cytolysin and antimicrobial resistance determinants, promoting adherence of E. faecalis to epithelial cell surface,
promote internalization and survival within intestinal macrophages and PMNs. The cytolsin and aggregation substances act synergistically to enhance virulence by facilitating quorum achievement and activate cytolysin, resulting in tissue damage and deeper tissue invasion.

(ii) Enterococcal surface protein (esp) expressed by enterococci as a large-molecular-weight cell-surface-localized protein; that mediates biofilm formation (Toledo-Arana et al., 2001) – esp has been linked to epidemic vancomycin-resistant strains of E. faecium (Willems et al., 2001) and were found to be responsible for hospital outbreaks in the United States, Europe and Australia. Esp functions as an adhesive that mediates binding to bladder uroepithelial cells (Shankar et al., 2001). The esp gene is located closely to cytolsin operon on the chromosome of the enterococci. The clustering of these and other virulence related genes, such as regions encoding several transposases and recombinases are characteristics of pathogenicity islands and were observed to be highly dynamic (Hacker and Kaper, 2000).

(iii) Ace, an adhesion of E. faecalis to host cells collagen (Patti et al., 1994, Casolini et al., 1998, Joh et al., 1999) fiber that are produced during infection, but the role in causing infection is still unknown.

(iv) E. faecalis antigenA (EfaA) shown to have adhesion, virulence and ATP-binding cassette transporter systems (Jenkinson, 1994), but its role in pathogenesis of enterococcal infection is still unknown (Gilmore et al., 2002).

(C) Enterococcal capsule and cell wall:

Cell wall components (carbohydrates and teichoic acids), composition and structure that drive the complex interactions between bacteria and the host immune system (Knirel,
1990, Nilsson et al., 1997, Thakker et al., 1998, Portoles et al., 2001, Tzianabos et al., 2001) and the capsules that protect the organism from phagocytosis and vary under immune selection (Roche and Moxon, 1995, Claverys et al., 2000);

(i) variable capsular carbohydrate may be compositionally and immunologically distinct. The virulence of cps pathway showed ability to persist within lymphatic tissue;

(ii) common cell wall polysaccharide - enterococcal polysaccharide antigen (epa) are reported to be involved in biosynthesis of an enterococcal polysaccharide antigen during infection in humans (Gilmore et al., 2002).

(D) Toxin metabolic products of enterococci:

Enterococci are rarely able to produce quantities of extracellular superoxide that rival activated neutrophil (Huycke et al., 1996). Thus, isolates from human bloodstream have more of this metabolic activity capacity and are more common in the species faecalis than faecium. The production of superoxide results in the human colonic epithelial cells chromosomes fragmentation and may relate to colon carcinoma etiology or overt tissue damage, which may facilitate bloodstream access by enterococci (Gilmore et al., 2002).

Although E. faecium increasingly causes infections in human, the genetic determinants coding for its virulence factors remain poorly characterized. Only the esp gene is common among E. faecium strains infection (Willems et al., 2001). Other traits that work as virulence factors in E. faecalis (hemolysin, aggregation substance and gelatinase or serine protease) are rare in E. faecium (Gilmore et al., 2002).
1.2.3 Clinical disease and complications

Enterococci cause a variety of clinical infections. In some infections, other microorganisms are frequently implicated, making it difficult to assess whether the disease manifestations are a result of infection of the tissue by enterococci or whether these comparatively avirulent organisms are simply playing a minor role in the infection (Gilmore et al., 2002). However, in endocarditis, enterococci are clearly the cause of this life-threatening disease and accounted for 10 to 15% of endocarditis cases (Gilmore et al., 2002). The resistance of enterococci to vancomycin and high level aminoglycosides make treatment of endocarditis difficult (Cetinkaya et al., 2000, Gilmore et al., 2002). An effective treatment requires the synergistic effect of these two drugs (Gilmore et al., 2002). Thus, in VRE or high-level aminoglycoside resistant enterococcal endocarditis, antimicrobial treatment often failed, and the surgery to remove the infected valve is critical (Gilmore et al., 2002). *E. faecalis* is the most common cause of endocarditis, especially in older men. Valvular infections are usually caused by bacteremia arising from the genitourinary or gastrointestinal tract. The increase in left sided involvement is much more common. The increase in prosthetic valve enterococcal endocarditis cases are caused by the increasing use of valvular prostheses on older men (high risk enterococcal bacteremia) and the mortality rate is at 15 to 20% (Rice et al., 1991, Megran, 1992, Gilmore et al., 2002). In the older age, presence of multiple underlying illnesses and multidrug-resistant enterococci cause the most problem in management of endocarditis (Landman and Quale, 1997, Gilmore et al., 2002).

Besides that, enterococci are the third leading cause of nosocomial bacteremia (Cetinkaya et al., 2000) and VRE cause 25% of enterococcal bacteremia in ICU (Gilmore et al., 2002). The overall mortality rate resulting from enterococcal bacteremia
is 30 to 75% and some studies have shown that *E. faecium* bacteremia has a higher mortality rate than *E. faecalis* (Maki and Agger, 1988, Noskin et al., 1995a, Gilmore et al., 2002). The urinary tract is the most common source of bacteremia. In addition, intra-abdominal or biliary tract, infected intravenous catheter or a soft tissue infection can also lead to polymicrobial infection (Maki and Agger, 1988, Patterson et al., 1995, Gilmore et al., 2002). Enterococci seed at single organs and never cause metastatic abscesses after a bacteremia episode (Gilmore et al., 2002). Older people who have multiple underlying diseases, like cancer, heart disease, diabetes mellitus and prior surgery are at risk of developing enterococcal bacteremia (Maki and Agger, 1988, Terpenning et al., 1988, Patterson et al., 1995, Gilmore et al., 2002).

Urinary tract infection is the most common infection by enterococci (Gilmore et al., 2002). Enterococcal lower urinary tract infections (cystitis, prostatitis, and epididymitis) and upper urinary tract infections that can lead to bacteremia are common in older men (Gilmore et al., 2002). In ICU, enterococci cause almost 15% of nosocomial urinary tract infections (Fridkin and Gaynes, 1999, Gilmore et al., 2002,). The enterococcal urinary tract infections acquired from hospitals or long-term care settings are more likely to be resistant to many antimicrobials, especially vancomycin (Terpenning et al., 1994, Gilmore et al., 2002). The second most common source for enterococcal bacteremia is intra-abdominal or pelvic wounds or abscesses (Maki and Agger, 1988, Graninger and Ragette, 1992, Noskin et al., 1995a, Patterson et al., 1995) and it is isolated from 15% of surgical site infection wounds among ICU patients (Fridkin and Gaynes, 1999, Gilmore et al., 2002). Enterococci are frequently isolated as a module of mixed microbial flora from intra-abdominal, pelvic and soft-tissue infections specimens, and rarely cause monomicrobial infection at these sites (Gilmore
et al., 2002). The importance of enterococci in wounds and abscesses is yet unclear. However, in cases of peritonitis occurring secondary to cirrhosis or to chronic peritoneal dialysis, enterococci can cause monomicrobial infection. Meningitis, hematogenous osteomyelitis, septic arthritis and pneumonia caused by enterococci, are less common but these are caused by broad-spectrum antibiotic use in debilitated or immunocompromised patients (Gilmore et al., 2002).

1.2.4 Antimicrobial resistance

Enterococci are intrinsically resistant to broad-spectrum antimicrobial agents and this limits the choice of drugs for treatment. The increased prevalence of enterococci pathogens in nosocomial infections worldwide has resulted in antimicrobials being used in greater frequency in hospitals (Kak and Chow, 2002). In animal husbandry, the same antimicrobial agents have been used widely as growth promoters (Kak and Chow, 2002). The acquisitions of antimicrobial resistance genes on plasmids or transposons from other organisms or spontaneous mutation have made enterococci increasingly resistant to various antimicrobial agents (Table 1.3) (Kak and Chow, 2002).
Table 1.3. Major patterns and mechanisms of resistance to antimicrobial agents in enterococci.

<table>
<thead>
<tr>
<th>High-level resistance to aminoglycosides&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Enzymatic (production of aminoglycoside-modifying enzymes)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Gentamicin</td>
<td>● AAC(6’)-Ie + APH(2’’)-Ia</td>
</tr>
<tr>
<td>● Kanamycin</td>
<td>● AAC(6’)-Ii</td>
</tr>
<tr>
<td>● Streptomycin</td>
<td>● APH(2’’)-Iba; APH(2’’)-Ic; APH(2’’)-IId; APH(3’’)-IIIa</td>
</tr>
<tr>
<td></td>
<td>● ANT(3’)-Ia; ANT(4’)-Ia; ANT(4’)-Ia; Alteration of the target (leading to decreased ribosomal binding)</td>
</tr>
</tbody>
</table>

Resistance to glycopeptides<sup>c</sup>

<table>
<thead>
<tr>
<th>Vancomycin</th>
<th>VanA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Teicoplanin</td>
<td>VanB VanC VanD VanE VanG</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Resistance to β-lactams</th>
<th>Alteration of the target (modification of the peptidoglycan biosynthetic pathway)</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Penicillin</td>
<td>Alteration of the target (altered penicillin-binding proteins)</td>
</tr>
<tr>
<td>● Ampicillin</td>
<td>Enzymatic (production of β-lactamase)</td>
</tr>
</tbody>
</table>

Resistance to quinolones

<table>
<thead>
<tr>
<th>Resistance to chloramphenicol</th>
<th>Enzymatic (production of chloramphenicol acetyl transferase)</th>
</tr>
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</table>

<table>
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<tr>
<th>Resistance to the MLS group</th>
<th>Enzymatic (production of methylating enzymes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>● Macrolides (erythromycin)</td>
<td></td>
</tr>
<tr>
<td>● Lincosamides (clindamycin)</td>
<td></td>
</tr>
<tr>
<td>● Streptogramin B</td>
<td></td>
</tr>
</tbody>
</table>

Note: Adapted from reference (Facklam <i>et al.</i>, 2002) (Appeared as table 6 and page 27 in the source of original).

<sup>a</sup>Found in increasing frequencies in <i>E. faecalis</i> and <i>E. faecium</i>; <sup>b</sup>AAC, acetyltransferases; APH, phosphotransferases; ANT, nucleotidyltransferases; <sup>c</sup>VanA and VanB phenotypes are usually found in <i>E. faecalis</i> and <i>E. faecium</i>; VanC is usually associated with <i>E. gallinarum</i> (VanC1) and <i>E. casseliflavus</i> (VanC2/C3). VanD resistance has been described in <i>E. faecium</i> strains and VanE was found in <i>E. faecalis</i>.
1.2.4.1 Epidemiology and emergence of VRE and HLGR enterococci

Since the initial recovery of VRE from patients in the United Kingdom and France, VRE have spread to other countries, including Australia, Canada, Denmark, Germany, Italy, Malaysia, The Netherlands, Spain, Sweden and the United States (Woodford et al., 1995, Cetinkaya et al., 2000). Molecular typing of enterococcal strains developed in the last two decades and serious problems arising from the emergence of VRE have intensified studies on epidemiology, colonization and infection of enterococci (Gilmore et al., 2002). Due to limited therapeutic options to treat VRE infections, it has become increasingly urgent to identify reservoirs for colonization and the routes of transmission of enterococci (Gilmore et al., 2002).

Antimicrobial-susceptible enterococci and VRE seem to share the same environmental reservoirs (Gilmore et al., 2002). The hospital setting appears to play an important role in transmission of VRE, whereby medical equipment such as bed rails, tables, bed linen, urinals, blood pressure cuffs and stethoscopes can be easily contaminated (Bonilla et al., 1997, Bonten et al., 1998, Gilmore et al., 2002). One early VRE outbreak was caused by a contaminated electronic rectal thermometer that transmitted VRE from patient to patient (Livornese et al., 1992, Gilmore et al., 2002). Several studies have shown that environmental strains and strains isolated from patients are highly related (Bonten et al., 1996, Bonilla et al., 1997, Gilmore et al., 2002). Colonized patients with diarrhea may increase the risk of environment contamination with VRE as the density of VRE in their feces is increased due to the selective pressure of anti-anaerobic antimicrobials (Roghmann et al., 1997a, Donskey et al., 2000, Gilmore et al., 2002). VRE can persist for at least 2 months on laboratory bench, fabric and