Molecular Characterization of Enterococci Isolates from Northeastern and Southern Regions of Thailand

Seinn So Lwin

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Master of Science in Microbiology (International Program)

Prince of Songkla University

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I hereby certify that this work has not been accepted in substance for any degree and is not being currently submitted in candidature for any degree**.**

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ABSTRACT

Enterococci have been becoming one of the most important pathogens due to resistance to common-used antibiotics and prolonged persistence in hospital settings. In addition, biofilm formation and various virulence factors activities are supported for colonization and pathogenicity of enterococci. Therefore, the study aimed to determine the occurrence of antibiotic resistance, biofilm formation and virulence determinants in clinical enterococcal isolates from two tertiary care hospitals in Northeastern and Southern Thailand. Information of antibiotic resistance and virulence factors activities in enterococcal infections are required to develop the control plan for emerging antibiotic resistance. However, data related with enterococcal infection in Thailand is limited. Seven hundred eighty-four enterococcal clinical isolates were obtained from 2 tertiary care hospitals in Thailand; Sunpasitthiprasong Hospital (SS), Northeastern Thailand and Hat Yai Hospital (HTY), Southern Thailand between 2015 and 2018. Species and vancomycin-resistant genes identification were achieved by multiplex PCR. *E. faecalis*, *E. faecium*, *E. casseliflavus*, *E. gallinarum,* and *Enterococcus* spp. from urine, pus, blood, body fluid, and sputum found at 73.2%, 25.5%, 0.1%, 0.1% and 1.0%, respectively. Kirby-Bauer disc diffusion method for susceptibility testing was carried out using 10 antibiotics in common enterococci isolates. Five additional antibiotics were tested for VRE isolates. MIC of linezolid, tigecycline and vancomycin were determined for VRE isolates by broth microdilution method. MDR revealed in 79.9% and 80.5% of isolates from SS and HTY, exhibiting greater resistance to erythromycin (85.2% and 75.0%), tetracycline (76.8% and 77.6%), and ciprofloxacin (71.9% and 72.8%), respectively. High-level aminoglycoside resistance (HLAR) and VRE were demonstrated in 60% and 4.7% of isolates from SS, and 59.6% and 5.1% of isolates from HTY, respectively. High-level vancomycin

resistance gene, *vanA* gene was revealed in 32 out of 38 VRE isolates and no *van* gene was revealed in 4 VRE isolates from two hospitals. *vanB*, *vanD* and *vanM* genes were not detected. Low-level vancomycin resistance gene, $vanC_{1/2}$ gene was carried in *E. casseliflavus* and *E. gallinarum* isolates. All VRE isolates were susceptible to linezolid and tigecycline. Biofilm formation, hemolysin, gelatinase, caseinase and lipase were detected. Biofilm, hemolysin, gelatinase, caseinase, and lipase were produced in 40.8%, 50.5%, 55.1%, 46.3%, and 1.6% of isolates from SS and 34.6%, 86.1%, 44.9%, 25.7%, and 14.7% of isolates from HTY, respectively. Moreover, correlation between antibiotic resistance and virulence factors activities were explored using Chi-square test in SPSS software. Species distribution and antibiotic-resistant patterns were similar in two hospitals. However, gelatinase and caseinase enzymes were more produced in isolates from SS, hemolysin and lipase were more produced in isolates from HTY. Enterococci isolated in urinary tract were significantly more produced biofilm ($p<0.05$). Resistance to ciprofloxacin, chloramphenicol, high-level aminoglycosides, and tetracycline were statistically associated with biofilm formation and secreted virulence factors activities in isolates from two hospitals ($p<0.05$). When the clonal relationship of all VRE isolates were evaluated using random amplified polymorphic DNA (RAPD) with four primers sets such as AB1-15, AP4 plus ERIC1R, AP4 and M13, AP4 plus ERIC1R primer gave rise the informative clonal clusters among VRE isolates from two hospitals. The clusters based on 80% similarity in RAPD dendrogram using AP4 plus ERIC1R primer revealed VRE infection was clonally disseminated in an individual hospital especially between June 2017 and January 2018 in SS and between January 2015 and August 2015 in HTY.

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LIST OF ABBREVIATIONS AND SYMBOLS

%	percent
°C	degree Celsius
α	alpha
β	beta
γ	gamma
µg	microgram
µg/ml	microgram per milliliter
µl	microliter
µM	micromolar
ATCC	American Type Culture Collection
bp	base pair
CFU	colony-forming unit
CLSI	Clinical and Laboratory Standards Institute
cm	centimeter
D-Ala-D-Ala	D-alanyl-D-alanine
D-Ala-D-Ser	D-alanyl-D-serine
dATP	deoxyadenosine 5'- triphosphate
dCTP	deoxycytosine 5'- triphosphate
dGTP	deoxyguanosine 5'- triphosphate
dNTPs	deoxyribonucleic 5'- triphosphate
dTTP	deoxythymidine 5'- triphosphate
DI	deionized water
DNA	deoxyribonucleic acid
E	erythromycin
EDTA	ethylenediamine tetra acetic acid
E. faecalis	*Enterococcus faecalis*
E. faecium	*Enterococcus faecium*
E. casseliflavus	*Enterococcus casseliflavus*
E. gallinarum	*Enterococcus gallinarum*

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

EUCAST	The European Committee on Antimicrobial Susceptibility Testing
g	gram
HCl	hydrogen chloride
hr	hour
HTY	Hat Yai Hospital
kb	kilo base pair
L	liter
lb	pound
mg	milligram
mg/ml	milligram per milliter
MIC	minimal inhibitory concentration
MDR	multidrug resistance
ml	milliliter
mm	millimeter/millimolar
min	minute
MRSA	methicillin-resistant *Staphylococcus aureus*
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
ODc	cut-off optimal density
PCR	polymerase chain reaction
RAPD	random amplified polymorphic DNA
RNase	ribonucleic acidase
rRNA	ribosomal ribonucleic acid
rpm	rotation per minute
RT	room temperature

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

S. aureus	*Staphylococcus aureus*
SDS	sodium dodecyl sulfate
sodA	superoxide dismutase A
spp.	species
Taq	thermus aquaticus
TAE	tris-acetate EDTA
TBE	tris-borate EDTA
TE	tris-EDTA / tetracycline
U	unit
UPGMA	unweighted pair group method with arithmetic mean
UV	ultraviolet
v	volume
V	volt
VRE	vancomycin-resistant enterococci
w	weight
w/v	weight by volume

CHAPTER 1

INTRODUCTION

1.1. Background and rationale

Enterococci, Gram-positive and diplococci or short chain, are ubiquitous in nature, but also normal inhabitants in the digestive tract of human and animals. However, enterococci have been emerging as one of the most important opportunistic pathogens relating with urinary tract infection, bacteremia, endocarditis, and soft tissue infections because of the ability to produce various virulence factors and acquisition of antibiotic-resistant genes. Moreover, they can be notably resistant to adverse conditions in the environment such as the wide range of temperature from 10°C to 45°C, and extreme pH (O'Driscoll & Crank, 2015). Enterococci can transmit to human not only from medical equipment and hands of medical staff in the hospital environment but also from contaminated food, and water in the community (Daniel *et al.*, 2015). Among over forty *Enterococcus* species, particularly *E. faecalis* and *E. faecium* are the most frequently isolated pathogens in human. Additionally, infrequent cases of human infections due to other enterococci species including *E. gallinarum, E. casseliflavus, E. durans, E. dispar, E. hirae,* and *E. raffinosus* have reported (Adhikari, 2010; Monticelli *et al.*, 2018).

Enterococci can be resistant to various classes of antibiotics intrinsically or acquired by transferring resistance genes. Resistance to glycopeptides (vancomycin resistance), β-lactams and aminoglycosides (high-level) in enterococcal infections are a major great concern because glycopeptides and β-lactams alone or combined with aminoglycosides are common treatments for enterococcal infections (Gozalan *et al.*, 2015). In addition, enterococci can be transferred the resistance determinants to bacteria of different genera. For instance, vancomycin-resistant genes can be transferred from enterococci to more pathogenic Gram-positive bacteria, *Staphyloccus aureus* without no barrier (Guido Werner *et al.*, 2013).

Vancomycin-resistant enterococci (VRE) is in the rank of the important antibiotic-resistant bacteria (Raza *et al.*, 2018). There are 9 types of vancomycin resistance depending on transforming of peptidoglycan precursors (glycopeptide

binding sites). Vancomycin-resistant genes - *vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN* transformed the N-terminal D-Ala-D-Ala residue to D-lactate or D-serine. vanA- and vanB-types are the most abundant resistant types in clinical enterococci strains worldwide (Bender *et al.*, 2018). In recent years, the prevalence of vanM- and vanD-type resistance was also highlighted as the important vancomycin resistance and dissemination in clinical enterococci strains (Zhang *et al.*, 2018). Although new antibiotics including quinupristin/dalfopristin, linezolid, tigecycline or daptomycin had been approved for VRE treatment, therapeutic treatments for multidrug-resistant VRE infections were still limited (Lee, Pang, & Abraham, 2019). Because resistance to same class antibiotics or antibiotics with the same mechanism is consequently resistant to new antibiotics. Therefore, VRE have been regarded as a superbug because of consequent resistance to the vast important antibiotics and challenging to treat. (Hancock, 2005; O'Driscoll & Crank, 2015).

In addition to drug resistance, enterococci well adapted for living in biofilm and carried various kinds of virulence factors. Biofilm formation may provide enterococci survival and persistence in hospital environments as well as in infections (Lewis, 2001). And, bacteria in the biofilm were more resistant to antibiotics than planktonic bacteria. The acquisition of numerous virulence factors of enterococci is also supported to the rise of pathogenesis and biofilm formation (Heidari *et al.*, 2016). As the first mechanism of pathogens is to colonize to the host tissues and invade the host immune system, secreted pathogenic factors of enterococci including cytolysin, gelatinase, caseinase, hyaluronidase, and lipase are essential factors for this mechanism. These secreted factors supported the bacterial adhesion, colonization, and persistence in hospitalized patients, and environment leading to increase antibiotic resistance (Heidari *et al.*, 2016). Cytolysin contributes to haemolytic activity and toxin production. Serine protease such as gelatinase and casinease enzymes are capable to hydrolyze gelatin, collagen, casein, haemoglobin and other peptides (Upadhyaya *et al.*, 2009). Like other opportunistic pathogens, enterococcal lipase that disintegrates membrane structures of the host, lyses RBCs and contributes the nutrient supply and growth of enterococci (Hasan *et al.*, 2018).

According to the literature, enterococci are responsible for a wide range of infections and rapid acquisition of resistant and virulent determinants. Therefore,

reliable information and accurate data on resistance and virulence factors of enterococci are essential to control the rate of enterococci infections and supply suitable treatment strategies. This study was in part an attempt to examine vancomycin-resistant enterococci and multidrug-resistant enterococci, and to investigate biofilm formation and virulence factors of enterococci. Moreover, the association of antibiotic resistance, biofilm formation, and virulence factors of enterococci from clinical isolates are analysed. Molecular typing method based on the analysis of DNA fingerprinting, random amplified polymorphism DNA (RAPD) was provided in VRE isolates to gain useful information for the epidemiology of VRE and for the development of effective strategies to limit the spread of VRE. This study will be useful to understand the emergence of enterococci infections and characteristics of biofilm formation and virulence factors to predict the persistence of enterococci and to innovate the infection control and treatment.

LITERATURE REVIEW

1.2. Habitat of enterococci

1.2.1. Characteristics of enterococci

Enterococci are commensal in gastrointestinal tract of mammals including humans, reptiles, insects, and birds. They are also found in plants, soil, and water. Moreover, they are one of the important starters in food manufacture and probiotics but also the cause of serious infection in human and other animals as typical opportunistic pathogen (Clewell *et al.*, 2014).

Enterococci are facultative anaerobic Gram-positive cocci that appear single, in pairs and sometimes in short chains. They are catalase negative but some *E. faecalis* grown on blood agar produce pseudocatalase. The optimum growth temperature of enterococci is range from 10 °C to 45 °C. They are heat tolerance and salt tolerance. They can grow in 6.5% NaCl and hydrolyze esculin in the presence of 40% bile salts (Clewell *et al.*, 2014).

Conventional methods such as phenotypic characterization on different media, motility and pigmentation can be used for enterococcal species identification. Carbohydrate fermentation and enzyme patterns with mannitol, sorbitol, arabinose, sucrose, raffinose and maltose are useful to identify *Enterococcus* spp. as shown in Figure 1 (Manero & Blanch, 1999). In later years, these indicator-based sugar utilization methods had been modified as test kits for timesaving and simplify. However, Konrad *et al.* reported that these test kits can only use for limited number of *Entercoccus* spp. and other tests are required for higher level identification (Konrad J Domig *et al.*, 2003). For instance, *E. casseliflavus* and *E. gallinarum* are difficult to separate from other enterococci, especially from *E. faecium*. Only motility and pigments on agar can be differentiated for these three species. *E. casseliflavus* and *E. gallinarum* are motile but *E. faecium* is non-motile while *E. casseliflavus* produces yellow pigment but *E. gallinarum* and *E. faecium* do not produce (Cartwright *et al.*, 1995).

To solve the problems of conventional methods, PCR-based species identification methods had been designed to differentiate *Enterococcus* species. There

are many developed PCR-based genus specific and species specific methods such as *ddl* (D-Ala, D-Ala ligase) (Dutka-Malen *et al.*, 1995), 16s rRNA gene (Monstein *et al.*, 2001), the *tuf* (elongation factor EF-Tu) genes (Ke *et al.*, 1999) and the *sodA* (superoxide dismutase) gene (Jackson *et al.*, 2004). Among different housekeeping genes for species identification, 16S rDNA sequences may have identical sequences in closely related species for example *E. gallinarum* and *E. casseliflavus* (Patel *et al.*, 1998) where *sodA* gene consists of more discriminative target sequence than 16S rRNA gene for differentiating closely related species (Poyart *et al.*, 2000). According to Moore *et al.* report (Moore *et al.*, 2006), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) can also use for species and genus identification of enterococci within minutes. This method is useful for rapid detection of high-risk enterococci.

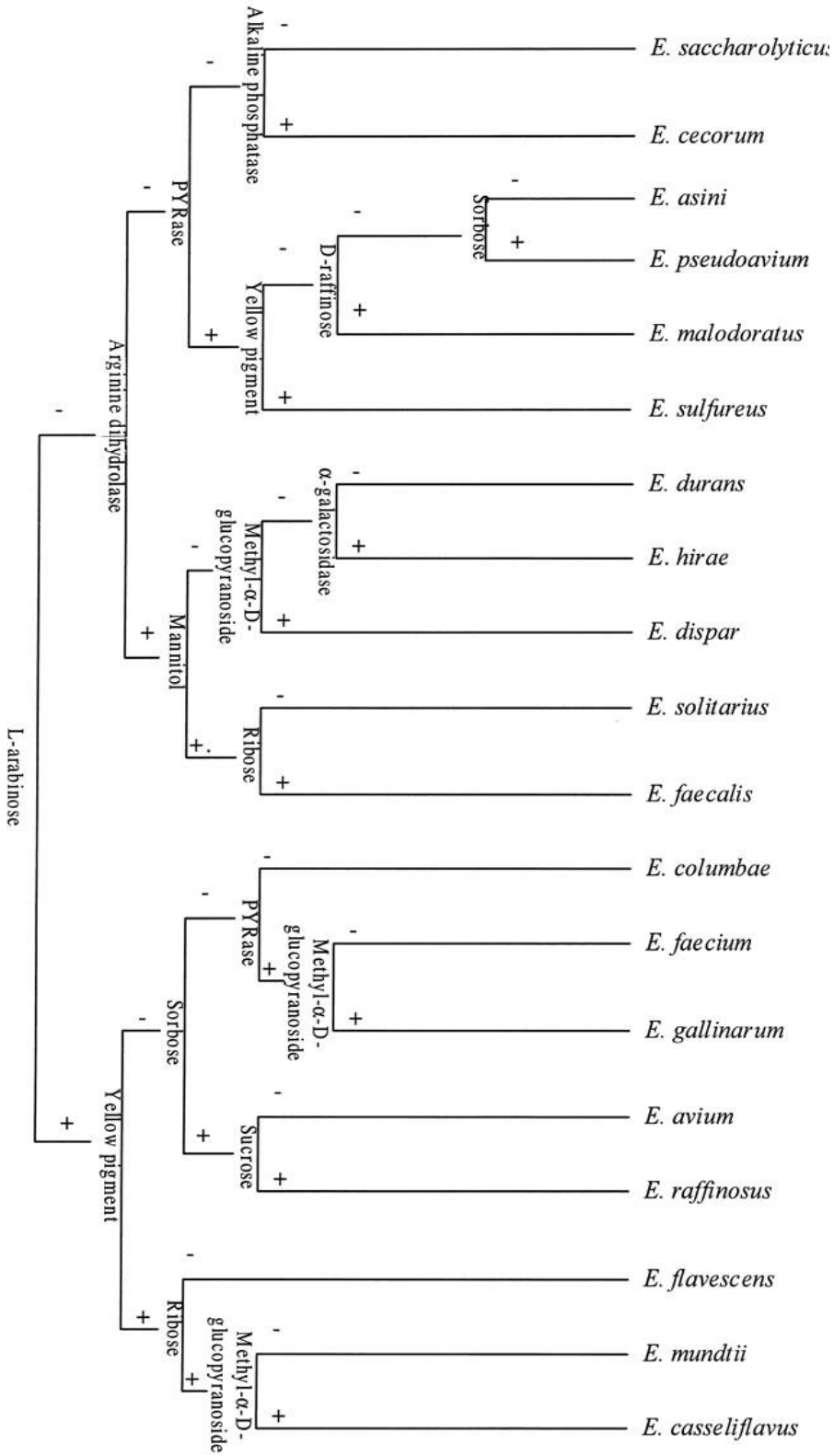


Figure 1. A flowchart of *Enterococcus* spp. identification using biochemical keys (Manero & Blanch, 1999)

1.2.2. Taxonomy of enterococci

At first, enterococci were classified as enteric Gram-positive cocci in origin and involved in the genus *Streptococcus*. The term *Enterococcus* was used firstly in 1899 (Clewell *et al.*, 2014). When the Lancefield serological typing system was established in 1933, enterococci were classified as group D streptococci or fecal streptococci (Cetinkaya *et al.*, 2000). According to genetic differences, *Streotococcus faecalis* and *Streptococcus faecium* were reclassified as *Enterococcus faecalis* and *Enterococcus faecium* respectively in 1984 (Hardie & Whiley, 1997). Over forty new species had been described by improving differentiation methods (Clewell *et al*., 2014).

1.3. Clinical significance of enterococci

Enterococci have been thought to be insignificant bacteria in medical field for a long time. Later and later, enterococci are recognized as important nosocomial pathogens worldwide. They rank as the second most common Gram-positive bacteria of hospitalized infections after staphylococci in many diseases such as urinary tract infection, bacteremia, endocarditis, wound infection, cholecystitis, peritonitis, sepsis and neonatal meningitis (Kashef *et al.*, 2017). According to National Healthcare Safety Network (NHSN) report for four years between 2011- 2014, enterococci stood as the second most healthcare-associated infections in USA. Especially, enterococci associated with the most infectious organisms in the central line-associated bloodstream infection and the third in the catheter-associated urinary tract infection in NHSN report (Weiner *et al.*, 2016).

The prevalence of *E. faecalis* and *E. faecium* are more predominant accounting for 80 - 90% of all clinical infections. Furthermore, *E. faecalis* is more isolated than *E. faecium* (Peng *et al.*, 2018). Although the infections related with non-*faecalis* non-*faecium* species can be found less than 5% of clinical isolates *(Clewell et al*., 2014), the Centre for Disease Control-endorsed National Healthcare Safety Network (NHSN) also reported that non-*feacalis* non-*faecium* infections were 10th rank among healthcare-associated pathogens in the 2011 - 2014 (Weiner *et al*., 2016). This prevalence was indicated that colonization and infection of other *Enterococcus* spp. have been increasing and needed to concern (Monticelli *et al*., 2018). One of the study

in India was proved that non-*faecalis* non-*faecium* enterococcal infections have been increasing with 14.8% and 19%, respectively (Desai *et al*., 2001; Prakash *et al*., 2005).

1.3.1. Urinary tract infection (UTI)

Enterococci were related with catheters and other urological abnormality rather than infecting symptomatic infection (Walsh & Collyns, 2017), which were second leading cause in hospital settings accounting to 10% of cases. This catheter-associated enterococci UTIs was higher in European and western countries (>12%) than Southeast Asia (8%) (Higuita & Huycke, 2014; Peng *et al.*, 2018). In addition, most of *E. faecalis* UTIs are intrinsically resistant to common antibiotics and acquired resistance to gentamycin and vancomycin (Abat *et al*., 2016).

1.3.2. Bacteremia

The translocation of enterococci through an intact intestinal epithelial barrier is leading to many bacteremia with no identifiable source (Jett *et al.*, 1994). Enterococci is the third leading cause of bacteremia, can be usually found as a member of polymicrobial bacteremia (Chirouze *et al.*, 2013). The most frequent sources of enterococci bacteremia are the urinary tract (15.9%), respiratory tract (14.3%), gastrointestinal tract (12.7%) and soft tissue infection (7.9%). According to *Enterococcus* spp., *E. faecium* bacteremia is related with higher mortality rate with 75% than *E. faecalis* (Higuita & Huycke, 2014). One of the most serious enterococcal infection, VRE. *faecium* bacteremia infected 5 to 35% in Europe and up to 60% in North America (Coombs *et al*., 2014).

1.3.3. Endocarditis

Initial attachment of enterococci leads to septic vegetation by forming matrix of platelets and fibrin which causes valvular destruction, congestive heart failure and death (Chuang *et al.*, 2009). Enterococcal endocarditis is the third leading cause of endocarditis after streptococci and staphylococci. They are responsible for 5-20 % of all infective endocarditis and this rate has not changed significantly over several years. Approximately 90% of enterococcal infective endocarditis are caused by *E. faecalis*,

E. faecium, E. avium, E. casseliflavus, E. durans, E. gallinarum and *E. raffinosus* (Chirouze *et al.*, 2013).

1.3.4. Skin and soft tissue infections

Enterococci are infected only in previously damaged tissues and are not responsible for primary cellulites. They are frequently caused in wound infections after abdominal surgery (Fluit *et al.*, 2001). Enterococcal skin and soft tissue infections are associated with other pathogens, therefore, their pathogenicity is unclear in these infections. More than 5% of skin and soft tissue infection have been isolated by *E. faecalis* (Higuita & Huycke, 2014).

1.3.5. Uncommon infections

Other infections such as menigitis, hemoatogenous osteomyelitis, septic arthritis and pneumonia are less or rarely enterococci infections. Enterococci are always isolated with mixing other microbial flora and rarely monomicrobial infection in pelvic and intra-abdominal infections. However, they can cause monomicrobial infection in patients who get treatment with peritoneal dialysis or liver cirrhosis (Higuita & Huycke, 2014).

1.4. Virulence factors of enterococci

1.4.1. Mechanism of virulence factors

Bacteria have a diverse arrangement of virulence factors that attack host defense system for their survival. Enterococci also possess numerous virulence factors associated with a risk of acquired enterococcal infections (Biswas *et al*., 2014). Enterococcal virulence factors can enhance the pathogenicity of enterococci in different ways such as biofilm formation, adherence to host tissues, invasion and abscess formation, resistance against defense mechanisms of the host, evading the host's immune response and toxin secretion (Rathnayake *et al.*, 2012).

(i) Biofilm formation

Biofilm formation is associated with approximately 65% of all bacterial infection (Jamal *et al.*, 2018). The complex community of biofilm created prolonged persistence of bacteria in living or non-living surface. Biofilm production is an important role in the pathogenesis of enterococcal infections such as urinary tract infection, endocarditis and wound infections but also contamination in the environment. Various virulence factors especially extracellular surface protein, aggregation substance (AS), secreted enzymes, pili and adhesion factors are supported to enterococcal biofilm formation (Fallah *et al.*, 2017). Enterococci are also known to produce slime, an amorphous extracellular polysaccharide which is one of the major components of biofilm formation which act to escape detection and clearance by the host immune system (Thurlow *et al.*, 2009). Because of the complex structure of biofilm, biofilm associated enterococcal infections are more difficult to be eradicated and more resistant to antibiotics than planktonic bacteria. In addition, biofilm environment supports the dissemination of resistance and virulence genes among bacteria in biofilm (Strateva *et al.*, 2016).

Otherwise, enterococcal infections could be suggested association with biofilm according to previous studies worldwide, 95.5% of clinical isolates were produced biofilm in Egypt between 2009 and 2015 (Hashem *et al.*, 2017), 50.4% in China, and 60 – 90% in Europe (Jin-Xin *et al.*, 2017). Most of studies reported that *E. faecium* specie was less produced compared to *E. faecalis* although the clinical outcome of infections and antibiotic resistance caused by *E. faecium* may be worsening (Mohamed & Huang, 2007).

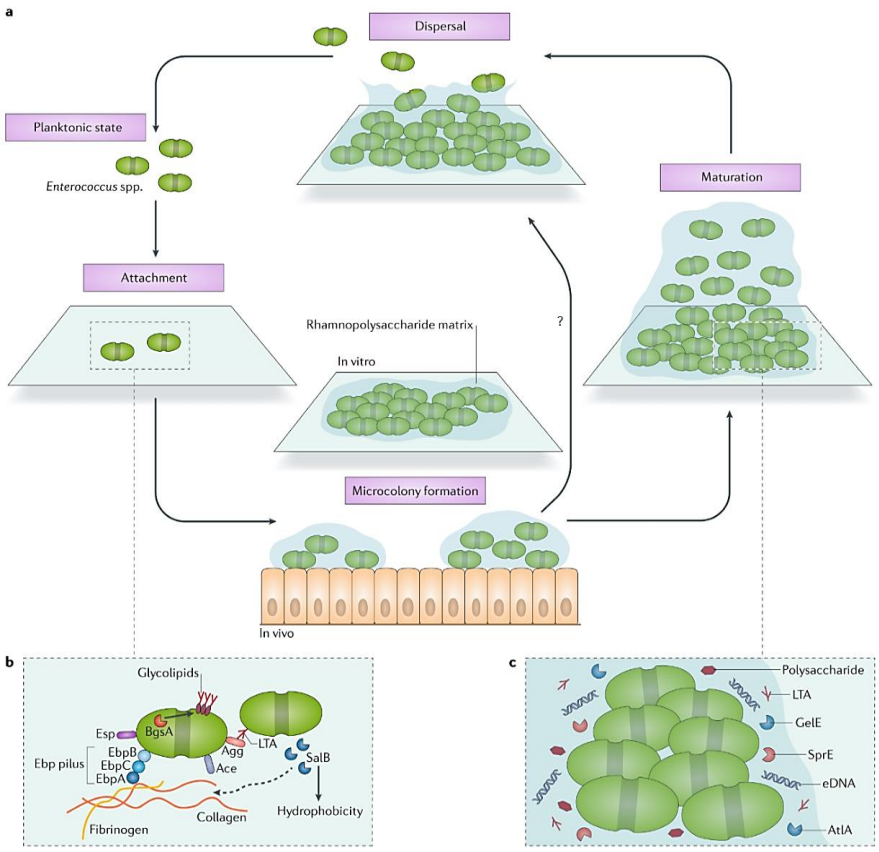


Figure 2. Stages of biofilm formation in enterococci (Ch'ng *et al*., 2019)

(a) *E. faecalis* biofilm formation; planktonic stage, dispersal stage, maturation stage and microcolony formation stage

(b) Planktonic cells attach to a surface with the help of adhesins such as endocarditis and biofilm associated pilus (Ebp), aggregation substance (Agg), enterococcal surface protein (Esp), adhesion to collagen (Ace), proteases and glycolipids, biofilm-associated glycolipid synthesis A (BgsA), SagA-like protein B (SalB)

(c) Mature enterococcal biofilms are characterized by the accumulation of extracellular DNA (eDNA), polysaccharides, extracellular proteases, including autolysin (AtlA), gelatinase (GelE) and serine protease (SprE), and lipoteichoic acid (LTA) in the matrix

(ii) Adherence to host tissues

Adhesions of enterococci play an important role in binding with eukaryotic receptors on mucosal surfaces to overcome the peristalsis. Moreover, they play diverse roles as reducing local inflammatory responses, effector molecules for phagocytosis or acting as toxins (Jett *et al.*, 1994). They can induce platelet aggregation and tissue factor-dependent fibrin production, which are associated with the pathogenesis of enterococcal endocarditis. Aggregation substance (AS), cell wall adhesion (*EfaA*), collagen-binding protein and enterococcal surface protein (Esp), exotoxin, and exoenzymes serve as adhesions to promote the colonization and support the nourishment to pathogens (Chajęcka-Wierzchowska *et al.*, 2017).

(iii) Invasion to host tissues or abscess formation

Enterococci produce extracellular enzymes to withstand the constant pressure of the host defenses. Secreted exoenzymes such as DNase, hyaluronidase, gelatinase, caseinase, and lipase have been related with tissue damage and resistance to host immune response (Jett *et al.*, 1994). These secreted enzymes, especially gelatinase are capable of hydrolyzing collagen, casein, hemoglobin, lipid and other peptides which assist in the advancement and further survival in newly infected places (Upadhyaya *et al.*, 2009).

(iv) Evading the host's immune response

Infections can promote if the host defense system is neutralized, or restricted. Therefore, the factors to modulate the host immune response and tissue damage are crucial for pathogenesis. Membrane structures such as lipoteichoic acid (LTA), wall teichoic acid (WTA) and capsular polysaccharides (Cps) are immunogenic cell wall components of enterococci (Gao *et al.*, 2018). DNase, cytolysin, gelatinase and enterococcal surface protein are also served this survival mechanism (Jett *et al.*, 1994).

(v) Toxin secretion

Toxins of pathogens are recognized as virulence factors because they can destroy and inactivate one or more vital component of the host. Especially, bacterial toxins are interfered cellular homeostasis of the host (Jett *et al.*, 1994). Cytolysin is the important exotoxin of enterococci (Chajęcka-Wierzchowska *et al.*, 2017). Normally, enterococci are not produced hemolysin (γ-hemolysin type) but some enterococci can produce α- or β- hemolysin which are associated with severe infections (Coburn & Gilmore, 2003). Cytolysin is a bacteriocin-type exotoxin, which lyses to erythrocytes, leucocytes and macrophages by destroying cell membrane as pore forming and supply the nourishments to enterococci. Cytolysin secretion in enterococci inhibits the growth of other intestinal Gram-positive bacteria to promote enterococcal dissemination (Chajęcka-Wierzchowska *et al.*, 2017).

1.4.2. Secreted virulence factors

(i) Gelatinase

Gelatinase, a zinc-dependent endopeptidase encoded by *gelE* gene that hydrolyzes gelatin, casein, hemoglobin and other bioactive compounds (Medeiros *et al.*, 2014). Production of gelatinase may interfere host defense mechanism and allow further dissemination of bacteria. Moreover, they can degrade antimicrobial peptides. And also this enzyme is involved in the first step of biofilm formation by mediating signals arriving through the quorum-sensing *fsr* system, composing with *fsrA*, *fsrB* and *fsrC* genes (L. E. Hancock & Perego, 2004). Moreover, gelatinase production in *E. faecalis* is supported in translocation within host's intestine (Zeng, Jing, *et al*, 2005).

(ii) Lipase

The mechanism of microbial extracellular lipase greatly concern enterococcal infections since the lipid of the host, which is essential to store energy and being part of cell membrane structural components and biological effectors of enterococci (Stehr *et al*., 2003). The important consequences of host cellular lipids digestion are sticking to host tissue and neighboring cells and interrupting the defense

mechanism of host's phagocytic cells (Furumura *et al*., 2006). Isolates from deep infections exhibit higher lipase activity than those from superficial infections which indicate that lipase activity may be important for bacteria in the center of biofilm (Stehr *et al.*, 2003).

(iii) Hyaluronidase (Hyl)

Hyaluronidase is a degradative enzyme which is encoded by *hyl* gene. The effect of its mechanism is tissue damage by depolymerizing hyaluronic acid and spreading their toxins through host tissue. Another function of hyaluronidase is providing degradation products of its target substrates as nutrients to bacteria (Kafil *et al.*, 2013).

1.4.3. Cell surface associated virulence factors

Enterococci comprise of complicated cell surface components which serve as virulence factors. These factors are associated with protecting bacteria from neutrophil phagocytosis, increasing biofilm formation and adhesion to host surface (Gao *et al.*, 2018). Critical cell wall surface components such as lipoteichoic acid, wall teichoic acid (WTA) and capsular structure are associated with increasing biofilm formation, resisting to phagocytosis and facilitating adhesion to host surface. These acids also contribute antibiotic resistance (Gao *et al.*, 2018). Several enterococcal genes which are encoded with cell wall anchored LPxTG surface proteins are involved in enterococcal pathogenesis. These proteins are assisted host tissue adhesion for colonization and persistence. Enterococcal surface protein (esp), aggregation substance (AS) and pili are important virulence determinants to anchor cell surface (Hendrickx *et al.*, 2009).

(i) Enterococcal surface protein (Esp)

Enterococcal surface protein is the largest protein which is encoded by *esp* gene. This protein can evade to the immune response of the host and antibiotics (Rathnayake *et al.*, 2012). This putative virulence determinant is situated in a pathogenicity island which can be transferred between strains of *E. faecium* by plasmid

conjugation and between *E. faecalis* by chromosome to chromosome transposition (Chajęcka - Wierzchowska *et al.*, 2017). Moreover, enterococci clinical isolates are more frequently revealed *esp* gene than non-clinical isolates which indicated this gene is associated in initial adherence to human gut and biofilm formation (Hendrickx *et al.*, 2009).

(ii) Aggregation substance (AS)

Aggregation substance (AS) is one of sex pheromone-inducible surface protein which contribute adhesion in terms of many functions. It can transfer resistance and virulence genes to neighboring cells in conjugation and adhere to host tissue by mating between donor and recipient cells (Chajęcka-Wierzchowska *et al*., 2017). AS is also invaded in cells derived from the colon and duodenum, indicating that AS might play a role in the translocation through the intestinal wall, leading to systemic infection (Hendrickx *et al*., 2009). The most suitable studied genes for AS proteins are *asa1*, *asa10* and *acp1* which are encoded on conjugated plasmids, pAD1, pCF10 and pPD1, respectively (Kafil *et al*., 2013). Cytolysin genes are also encoded in the same plasmids, therefore, they can act synergistically and support to increase the virulence (Chajęcka-Wierzchowska *et al*., 2017).

(iii) Sex pheromones

Another important LPxTG associated virulence factors are sex pheromones (chromosomally encoded genes such as *cpd, cob, ccf, cad*), which are short, hydrophobic peptides and interact with specific conjugative plasmid. They have regulatory roles in bacterial mating and involve in eliciting an inflammatory response (Chajęcka-Wierzchowska *et al.*, 2017). They are also participated in the spreading of antibiotic resistance and commonly isolated in patients with bacteremia and wound infections (Wardal *et al.*, 2010).

(iv) Collagen binding protein

Collagen binding protein is a member of microbial surface component recognize adhesive matrix molecules (MSCRAMM). It is encoded by the *ace* gene

(adhesion of collagen of *E. faecalis*) and *acm* gene (adhesion of collagen of *E. faecium*) (Chajęcka-Wierzchowska *et al.*, 2017). The important role of MSCRAMMs is in colonization of host tissues by binding to specific proteins in the extracellular matrix. *Ace* can bind with collagen types I and IV, laminin and dentin. *Acm* cohere to collagen type I and lesser extent to collagen type IV. Both of these genes are involved in the pathogenesis of experimental endocarditis (Sava *et al.*, 2010).

(v) Pili

Pili are filamentous protein surface structures which are involved in biofilm formation, and in adherence to multiple types of human cells (Gao *et al*., 2018). This filamentous protein increases the attachment in urinary tract leading to UTIs. *E. faecalis* keeps two pili gene clusters (PGCs) including *ebp* locus (endocarditis and biofilm associated pili) and *bee* locus (biofilm enhancer in enterococci). Likewise, *E. faecium* keeps four PGCs, PGC 1 to 4 (Kafil *et al.*, 2013).

1.4.4. Detection of virulence factors

Virulence factors of enterococci can be detected with phenotypic and genotypic methods. Some virulence factors of enterococci such as hemolysin, lipase, slime layer formation, DNase and serine proterase (gelatinase and caseinase) can be detected by phenotypic testing using specific culture. Biofilm production can also be detected phenotypically by microtiter plate assay with crystal violet stain, Congo red agar method, tissue culture plate assay and fluorescent microscopic examination. Among biofilm assay, microtitre plate method is recommended for screening of biofilm producing bacteria. This method is simple to use and reliable method (Hassan *et al*., 2011). In 2004, Vankerckhoven *et al.* had developed the multiplex PCR to detect potential virulence genes of enterococci such as aggregation substance (*asa1),* gelatinase (*gelE),* cytolysin (*cylA),* enterococcal surface protein (*esp*), and hyaluonidase (*hyl).* This method provides reliable and rapid alternative to phenotypic methods and single PCRs for virulence factors identification (Vankerckhoven *et al.*, 2004). Detection of other virulence genes with multiplex PCR has been developed in recent years.

1.4.5. Prevalence of virulence factors in enterococci

The prevalence of virulence factors from clinical enterococcal isolates may vary depending upon the sample origins and geographic nature. As their prominent level, hemolysin and gelatinase identification are recorded worldwide. From previous studies in India (Banerjee & Anupurba, 2015; Fernandes & Dhanashree, 2013), gelatinase and hemolysin activities in clinical enterococcal isolates were 40.6% and 33% in 2013 whereas 9.03% and 31.6% in 2015. In one study from Brazil, over 70% of *E. faecalis* isolates revealed hemolysin, caseinase and lipase activity whereas no isolates revealed gelatinase activity (Furumura *et al*., 2006). Biofilm producing *E. faecalis* isolates form root canal infections were carried *ebpR* (91%), *ace* (85%), *efaA* (82%), *gelE* (81%), *esp* (56%), *asa1* (33%) and *hyl* (2%) in Iran (Aghdam et al., 2017).

1.5. Antibiotics: Specify for enterococci

1.5.1. Mechanisms of antibiotics

(i) Inhibition of bacterial cell wall synthesis

Bacterial cell wall is the outermost component, composed of peptidoglycan, polysaccharides, lipids, lipoproteins, proteins and several glycoconjugates. Gram-positive bacteria are generally more susceptible to cell wall synthesis inhibitor than Gram-negative bacteria because the peptidoglycan layer of Gram-positive bacteria is thicker than that of Gram-negative bacteria. β-lactams and glycopeptides antibiotics have the ability to prevent the synthesis of peptidoglycan layer of bacteria to bacterial lysis (Yoneyama & Katsumata, 2006).

(ii) Inhibition of bacterial protein synthesis

Protein synthesis is a complex process with many enzymes and conformational alignment. Antibiotics terminate the proliferation of cells by disturbing the protein synthesis at the 30S subunit or 50S subunit of the bacterial ribosome. Tetracycline and aminoglycosides interact with the conserved sequences of 16S rRNA of the 30S subunit and macrolides, streptogramins and chloramphenicol interact with the 23S rRNA of 50S ribosomal subunit (Yoneyama & Katsumata, 2006).

(iii) Inhibition of nucleic acid synthesis

The production and regulation of nucleic acids (DNA and RNA) are essential for survival and replication of organisms. Antibiotics interrupt in the steps of RNA transcription such as initiation, elongation and termination and disrupt in DNA replication. Rifampin antibiotic combines with DNA dependent RNA polymerase to inhibit the initiation of RNA transcription. Rifampin motif is specific for RNA polymerase of Gram-positive bacteria and some Gram-negative bacteria. Fluoroquinolones such as ciprofloxacin, norfloxacin, levofloxacin block DNA synthesis by obstructing topoisomerase, especially topoisomerase II, an enzyme included in DNA replication. Topoisomerases are present in all organisms but, quinolones are specific obstructers of bacterial topoisomerase II (Yoneyama & Katsumata, 2006).

(iv) Injuring the plasma membrane

The plasma membrane is permeable to molecules and ions selectively and controls the movement of things in and out of cells. Injuring plasma membrane makes rapid depolarization and loss of membrane potential leading to obstruction of the protein, RNA and DNA synthesis. Daptomycin, a member of lipopeptide, is interfering bacterial cell membrane functions by binding to the membrane to become fast depolarization (Yoneyama & Katsumata, 2006).

(v) Inhibiting essential metabolite synthesis

All living cells depend on anabolism and catabolism chemical processes within the cells to maintain homeostasis. Antibiotics serve as antimetabolite by preventing the use of required substance for metabolism of bacteria. This antimetabolite has toxic effects on cells and has the ability of halting cell division or cell growth. Moreover, these antibiotics serve as antifolates which damage the capacity of folic acid to disrupt in the synthesis of DNA and RNA. Sulfonamides serve as competitive inhibitors of dihydropeteroate synthetase (DHPS) enzyme, is involved in folate synthesis of bacteria. Trimethoprim is a folate antagonistic. These antibiotics show

inactivity in vitro for enterococcal infection treatment but they can be used (Yoneyama & Katsumata, 2006).

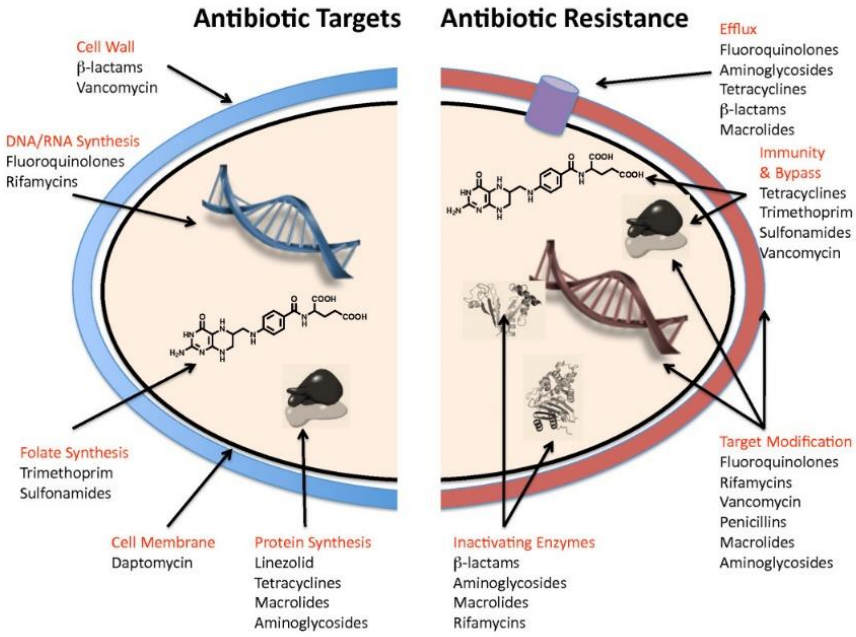


Figure 3. Mechanism of action of common antibiotics and antibiotic resistance (Wright, 2010)

β-lactams and glycopeptides (vancomycin) disrupt cell wall synthesis, Fluoroquinolones interrupts DNA replication, Rifampin blocks RNA synthesis, Trimethoprim/ Sulfonamides damage the capacity of folic acid, Daptomycin targets to injure cytoplasmic membrane, Aminoglycoside, Tetracycline, Chloramphenicol, Clindamycin, Linezolid, Macrolides and Quinpristin/ Dalfopristin inhibit protein synthesis

1.5.2. Antibiotics used for enterococci infections

1.5.2.1. Glycopeptides

Glycopeptides are glycosylated non-ribosomal peptides which are produced by various groups of soil actinomycetes. Vancomycin and teicoplanin are the first generation of clinically important glycopeptide antibiotics. Vancomycin was synthesized from *Amycolatopsis orientalis* and first introduced in 1958. Teicoplanin

was produced from *Actinoplanes terichomyceticus* and first introduced in 1978, initially used in Europe (1988) and Japan (1998).

Glycopeptide (vancomycin and teicoplanin) are cell wall active agents by binding to the D-Ala-D-Ala of peptidoglycan precursors located on the outer surface of the cell wall. They inhibit the synthesis of enzymes that used as substrates for transpeptidation and transglycosylation to decrease cell wall integrity (Kristich *et al*., 2014). In spite of possessing similar antimicrobial activity in vancomycin and teicoplanin, teicoplanin presents more potency because of different fatty-acid sustituents to *Enterococcus, Staphylococcys* and *Streptococcus* genera (Binda *et al*., 2014). In addition, teicoplanin has lower toxicity and longer half-life than vancomycin (Economou *et al.*, 2013). These antibiotics are not useful to treat Gram-negative bacteria because lipopolysaccharide membrane in Gram-negative bacteria is protected to be permeable to large biomolecules (Binda *et al*., 2014). Because of increasing dramatically in high-level resistance to β-lactams and aminoglycosides, usage of glycopeptide antibiotics to treat enterococcal and other Gram-positive infections had been increased (Shepard & Gilmore, 2002).

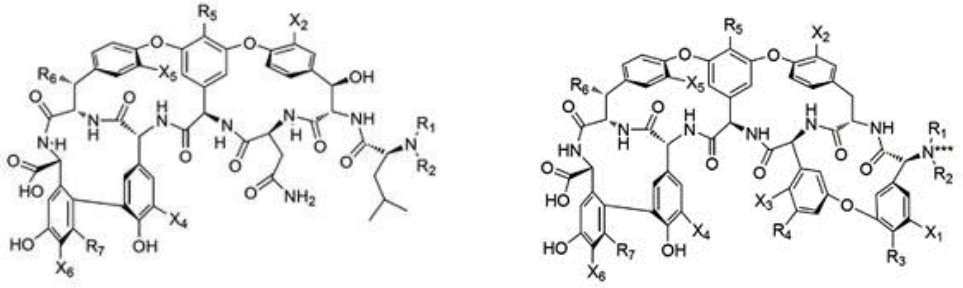


Figure 4. Chemical structures of vancomycin and teicoplanin (Yim *et al.*, 2014)

1.5.2.2. β-lactams

β-lactams antibiotics are the most widely used broad-spectrum antibiotics. Antibiotics including penicillin, ampicillin, methicillin, cephalosporins, monobactams, carbapenem and other related compounds possess beta-lactam ring in their molecular structures, are so-called beta-lactams. The function of beta-lactams is bactericidal by binding with penicillin binding protein (PBPs) to inhibit the final

transpeptidation of peptidoglycan layer (Miller *et al.*, 2014). New β-lactam antibiotics have been developed to overcome β-lactamase enzymes by combining clavulanic acid (e.g. Augmentin) (Buynak, 2006). Combination of β-lactams with aminoglycoside is a synergistic bactericidal treatment for severe enterococcal infections including meningitis and endocarditis (Shepard & Gilmore, 2002). Because of the nephrotoxic effect of aminoglycosides, the combined use of ceftriaxone and ampicillin is recently detected as an alternative agents (Gagetti *et al.*, 2018).

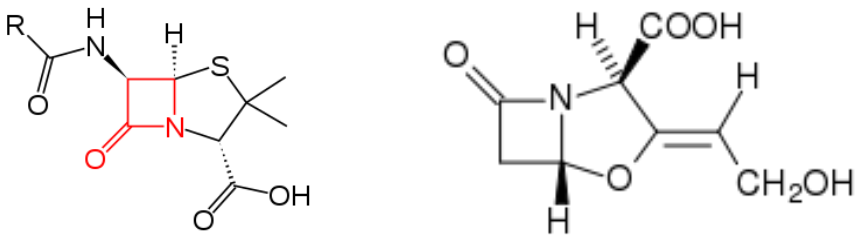


Figure 5. Chemical structures of penicillin and clavulanic acid (Page, 2012)

1.5.2.3. Aminoglycosides

Aminoglycoside are multifunctional hydrophilic sugars that possess several amino and hydroxy functionalities. Streptomycin, the first generation of aminoglycosides produced and used against tuberculosis in 1944. After that, gentamicin, kanamycin and tobramycin introduced in the 1970s as the second generation. Amikacin, semisynthetic aminoglycoside was introduced in the 1970s and more effective for strains with developed resistance mechanisms towards former aminoglycosides. Aminoglycosides are performed by joining to the 30S ribosomal subunit, but these antibiotics are not inhibited the binding of mRNA and placing a tRNA in the P site. However, aminoglycosides are inhibited joining of 50S subunit to the 30S subunit and interrupting protein synthesis in bacteria (Kotra *et al.*, 2000). Even though the protein synthesis inhibition of aminoglycosides is bacteriostatic, high concentration of aminoglycosides may effect nonspecific membrane toxicity leading to losing bacterial cell wall integrity and bacterial cell lysis (Wilson, 2004).

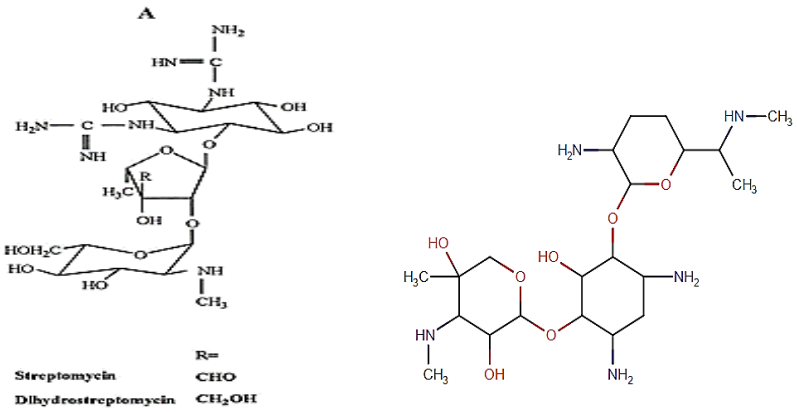


Figure 6. Chemical structures of streptomycin and gentamicin (Mingeot-Leclercq *et al.*, 1999)

1.5.2.4. Chloramphenicol

Chloramphenicol was introduced in 1947 for against typhoid. Now it can use for severe infections, multidrug-resistant infections including VRE and unknown causative infections (Kristich *et al.*, 2014). Chloramphenicol has been used successfully for treatment of meningitis, complicated skin and soft tissue infections and community-acquired pneumonia (R. E. Hancock, 2005). It is lipid soluble that can diffuse bacterial cell wall. Chloramphenicol is a bacteriostatic antibiotic that inhibits protein synthesis binding to the 50S subunit of bacterial ribosome to inhibit peptidaltransferase step and prevent the transpeptidation process of peptide chain elongation (Modi & Chaudhary, 2017).

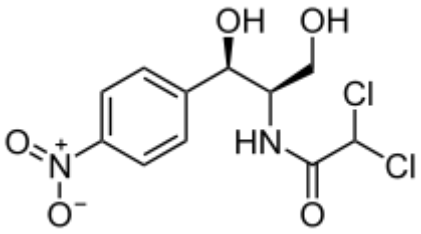


Figure 7. Chemical structure of chloramphenicol (Modi & Chaudhary, 2017)

1.5.2.5. Fluoroquinolones

Quinolones, broad-spectrum bactericidal antibiotics, introduced in 1962 for treatment of UTIs. The majority used quinolones, fluoroquinolones which are the

second generation of quinolones which contain a fluorine atom in their structure and are effective against both Gram-positive and Gram-negative bacteria. These antibiotics are widely used as first-line treatment for many infections, including acute sinusitis, acute bronchitis, and uncomplicated urinary tract infections. Quinolones interrupt DNA replication to prevent the bacterial growth by combining with controllers of DNA supercoiling; topoisomerases II (DNA gyrase) and topoisomerase IV. The primary targets for quinolones in Gram-positive organisms are both of enzymes and in Gram-negative organisms are DNA gyrase only. Quinolones can enter easily into the cells, so they are often used for intracellular pathogens treatment (Kristich *et al.*, 2014).

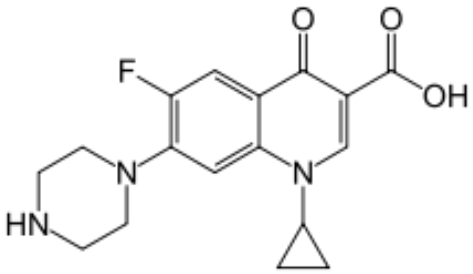


Figure 8. Chemical structure of ciprofloxacin (Blondeau, 2004)

1.5.2.6. Macrolides

Erythromycin, the first generation of macrolides, was produced from *Streptomyces erythraeus* in 1950s. Synthetic derivatives of erythromycin were developed in the 1970s and 1980s. Ketolides are structurally related with macrolides and included two ribosomal binding sites. Therefore, ketolides can use to treat macrolide-resistant respiratory tract infections (Lonks *et al.*, 2005). Macrolides can be used against Gram-positive bacterial infections and limited Gram-negative infections. Macrolides are also protein synthesis inhibitors by binding to the 23S rRNA of the 50S ribosomal subunit to block the translocation reduction of polypeptide chain elongation and inhibit ribosomal translation. This mechanism causes disassociation of peptidyl tRNA from the ribosome. This mechanism is bacteriostatic but they cannot effect at very high concentrations and in some unusual situations (Fair & Tor, 2014).

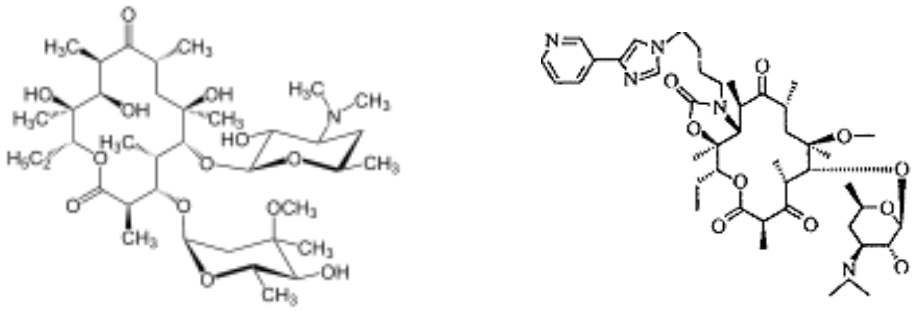


Figure 9. Chemical structures of erythromycin and telithromycin (first ketolide antibiotic) (Lonks *et al.*, 2005)

1.5.2.7. Tetracycline

Tetracycline was produced in 1953 and commercially available in 1978. It is regarded as the most effective and safe antibiotic in health system. There are two groups of tetracycline dividing by mode of action: tetracycline, doxycycline or minocycline have bacteriostatic activity whereas some tetracycline derivatives are bactericidal (Schnappinger & Hillen, 1996). Bacteriostatic tetracycline is broad-spectrum antibiotics for Gram-positive and Gram-negative bacteria, atypical organisms such as chlamydia and protozoan parasites. Its mechanism is binding to the 30S subunit of the ribosome and disrupting with the anchoring of aminoglycocyl-tRNA to the ribosomal acceptor (A) site (Fair & Tor, 2014). Other bactericidal tetracycline derivatives such as anhydrotetracycline, chelocardin, and thiatetracycline are poor inhibitors of protein synthesis with the incorporation of nucleic acids into DNA and RNA and to disrupt the cytoplasmic membrane (Bockstael & Aerschot, 2009).

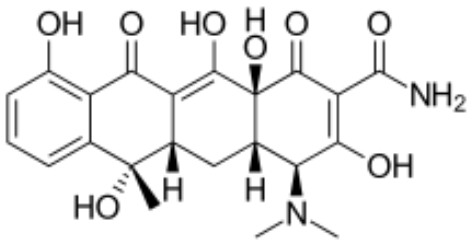


Figure 10. Chemical structure of tetracycline (Chopra & Roberts, 2001)

1.5.2.8. Nitrofurantoin

Nitrofurantoin was introduced in 1953, being drug of choice for urinary tract infections (UTIs) especially uncomplicated cystitis caused by Gram-negative and Gram-positive bacteria because it has been no significant resistance for long time. This may be because of its multiple mechanisms and sites of actions reduce the bacterial ability to develop nitrofurantoin resistance. Especially it is useful treatment for ampicillin or vancomycin-resistant enterococci UTIs (G. G. Zhanel *et al.*, 2001). Nitrofurantoin is bactericidal or bacteriostatic depends on the concentration by inhibiting bacterial enzymes involved in synthesis of DNA, RNA and cell wall protein of bacteria (Guay, 2001). At high concentration, it inhibits protein synthesis with ribosomal protein and rRNA. Bacteria nitro-reductases convert nitrofurantoin to highly reactive electrophilic intermediates. These electrophilic intermediates attack nucleophilic protein sites of bacteria ribosomal proteins (McOsker & Fitzpatrick, 1994).

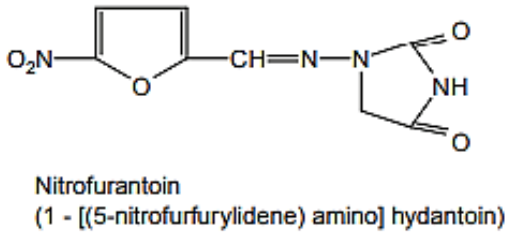


Figure 11. Chemical structure of nitrofurantoin (Guay, 2001)

1.5.2.9. Rifampin

Rifampin was explored in 1965 and approved in 1971. It has been used as part of antibiotic combination treatment for *Mycobacterium tuberculosis* infection for decades. In recent years, it had been found that it was useful for staphylococcal infections. Nowadays, it is widely used for bacterial infections by combing with other class antibiotics. Rifampin binds to the beta subunit of RNA polymerase (RpoB) and prevents initiation of transcription to inhibit bacterial growth (Kristich *et al.*, 2014).

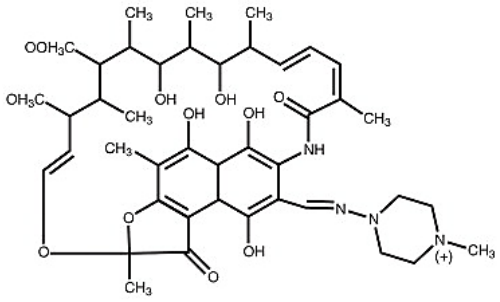


Figure 12. Chemical structure of rifampicin (Darst, 2004)

1.5.2.10. Antibiotics for VRE

(i) Oxazolidinones

The first oxazolidinones, linezolid was discovered in 1990s and accepted to treat VRE and MRSA in 2000 (Praharaj *et al*., 2013). Linezolid is bacteriostatic antibiotic against Gram-positive bacteria which can be used to treat complicated skin and soft-tissue infections, health-care associated and community-acquired pneumonia (Kristich *et al*., 2014; Peppard & Weigelt, 2006). It joins to the 50S subunit of 23S rRNA to interrupt the putting of the aminoacyl-tRNA in the A site of the ribosome. Hence, this action prevents the transportation of peptides and the subsequent elongation of the polypeptide chain (Miller *et al*., 2014). It can use intravenously and orally against both *E. faecalis* and *E. faecium* infections. The next generation oxazolidinone is tedizolid which has broad spectrum bacteriostatic activity. It was approved to use for skin infections in 2014. It can use against resistant Gram-positive bacteria such as vanA- and vanB-type VRE and also linezolid resistance with cfr mutations (Rybak *et al*., 2014).

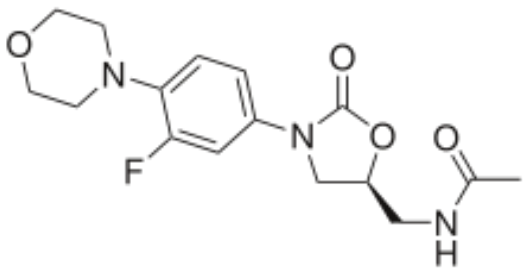


Figure 13. Chemical structure of linezolid (Ford *et al*., 2001)

(ii) Tigecycline

Tigecycline, a new derivative of tetracycline, was broad-spectrum only approved glycylcycline in 2005. It is more effective to treat antibiotic resistance Gram-positive and Gram-negative bacteria including VRE, MRSA and many MDR infections than tetracycline because modified structure N,N-dimethyglycylamido (DMG) moiety attached to the 9-position of tetracycline ring D (Nguyen *et al*., 2014). Even though the mechanism of tigecycline is similar to the mechanism of tetracycline, it overcomes resistant mechanisms of tetracycline including efflux pumps and ribosomal protection (Pournaras *et al*., 2016). The combination of tigecycline and daptomycin has successful mechanism against infective endocarditis due to VRE. However, tigecycline is unsuitable for the treatment of bacteremia because of its low serum concentration (Meagher *et al*., 2005).

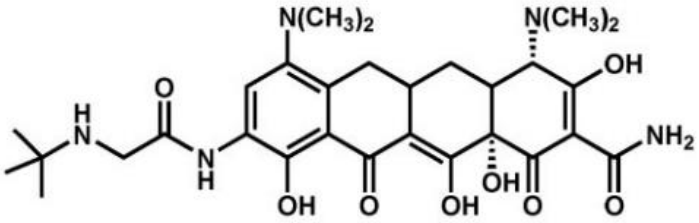


Figure 14. Chemical structure of tigecycline (Olson *et al*., 2006)

(iii) Quinupristin/dalfopristin (Q/D)

Quinupristin/dalfopristin was approved to use in 1998. This antibiotic is combining of two different antibiotics within the class of streptogramins for synergic effect; type A (70% dalfopristin) and type B (30% quinupristin). The mechanism of streptogramins is blocking the translation of mRNA into protein by binding to the different target sites on the 50S subunit of ribosome for better effective. Dalfopristin prevents binding of aminoacyl-tRNA to the ribosomal A site and formation of peptide bond. Quinupristin performs the permanent inhibition of the ribosome complex by stimulating the dissociation of peptidyl-tRNA from the ribosome. When two types are used as the combination, actions of dalfopristin induce structural change in the ribosome that exposes high-affinity binding site for quinupristin. This antibiotic can be

used for E. *faecium* VRE infections but unable to against *E. faecalis* due to efflux pumps (Higuita & Huycke, 2014).

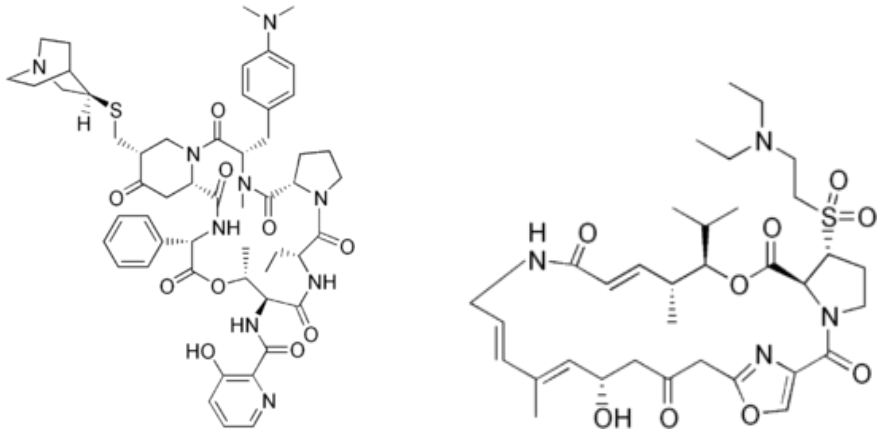


Figure 15. Chemical structures of quinupristin and dalfopristin (Kehoe *et al*., 2003)

(iv) Daptomycin

Daptomycin was discovered in the late 1980s, but it was approved to use in people in 2003. Daptomycin is the lipopeptide, has unique bactericidal mechanism by disrupting several parts of bacteria cell membrane function. It is part of the calcium-dependent insertion into the cytoplasmic membrane that caused to membrane depolarization and produce intracellular potassium ions and support to rapid cell death (Kristich *et al*., 2014). Due to unique mechanism of its action, daptomycin is useful for multidrug-resistant Gram-positive bacterial infection treatment including VRE and skin and soft tissues infections (O'Driscoll & Crank, 2015).

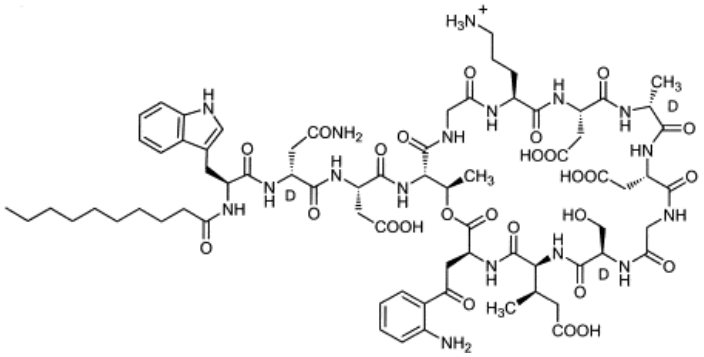


Figure 16. Chemical structure of daptomycin (Micklefield, 2004)

(v) Lipoglycopeptides

Telvancin was approved as the first lipoglycopeptides in 2009, carried on dalbavancin and oritavancin in 2014. Lipoglycopeptides, second-generation glycopeptides are semisynthetic glycopeptides that consist of lipophilic side chains to anchor to the cell membrane of bacteria and promote their half-life, leads to more effective than glycopeptides (Stegmann *et al.*, 2010). The mechanisms of lipoglycopeptide inhibit cell-wall formation by blocking the transglycosylation step in peptidoglycan synthesis similar to glycopeptide. However, they are 4 to 8 times more potent than vancomycin against Gram-positive organisms (Klinker & Borgert, 2015). Telavancin and dalbavancin have bactericidal activity depending concentration to treat antibiotic resistant Gram-positive bacteria including vanB-type VRE but less activity against vanA-type VRE (O'Driscoll & Crank, 2015). Oritavancin has the broadest spectrum bactericidal activity against nearly all resistant Gram-positive bacteria including both vanA- and vanB-type VRE. The combination of oritavancin and gentamicin has the synergy effect for the treatment of bacteremia, endocarditis and skin and soft tissue infection (O'Driscoll & Crank, 2015).

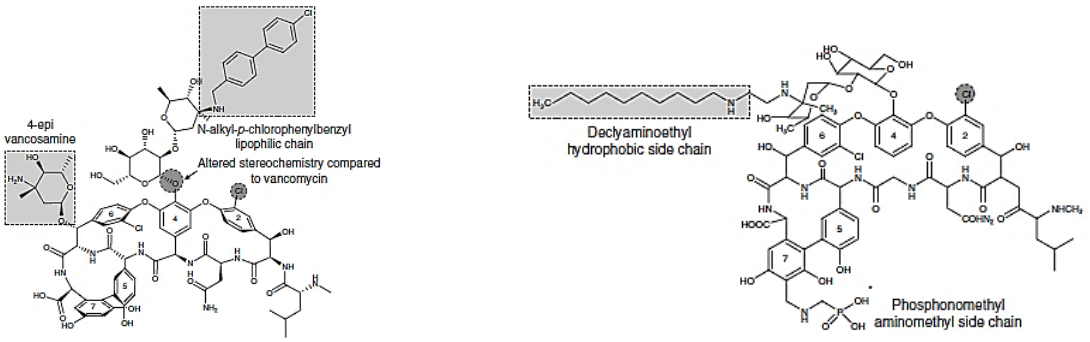


Figure 17. Chemical structures of oritavancin and telavancin (George G Zhanel *et al.*, 2010)

1.5.3. Development of resistance to antibiotics

Treatment of enterococcal infections can be difficult because they are resistant to intrinsically many antibiotics and easily acquired antibiotic resistant determinants (Shepard & Gilmore, 2002). Intrinsic resistance is natural resistance that

depends upon the structural characteristics of bacteria. This resistance is low-level resistance to many agents with various mechanisms but still important in the health care (Shepard & Gilmore, 2002). *Enterococcus* spp. are intrinsically resistant to low-level aminoglycosides by low permeability to cell wall, to bramycin and kanamycin by modifying ribosome methyltransferase, low-level penicillin and moderate to high-level cephalosporin by production *pbp*4/5 or alteration of cell wall, clindamycin and low-level streptogramin B by ABC efflux pump (Hollenbeck & Rice, 2012).

Acquired resistance is the resistance to antibiotics through chromosomal exchange, mutation, and transfer of mobile genetic elements including plasmids, transposons and integrons. These mobile genetic elements are transferred by conjugation, transduction and transformation (Jain *et al.*, 2016). These acquired resistance mechanisms are more important than chromosomally resistance since they are easily transferrable among homo-species or hetero-species in environment. For instance, plasmid transfer of glycopeptide resistance genes from enterococci to more pathogenic bacteria, such as *Staphylococcus* spp. and *Streptococcus* spp. is virulent (Courvalin, 2006).

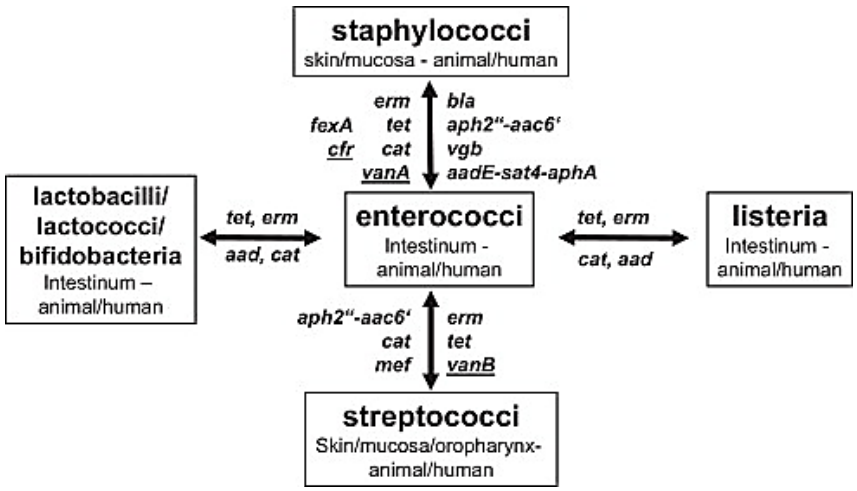


Figure 18. Resistance genes shared in Gram-positive bacteria (Guido Werner *et al.*, 2013)

The second line in the box describes the habitat of the corresponding bacterium. Resistance genes represents *aad*, streptomycin; *aac6-aph2*, gentamicin/tobramycin; *bla*, penicillins; *cat*, chloramphenicol; *cfr*, florfenicol/linezolid; *erm*, macrolide-lincosamide-streptogramin B (MLSB); *fexA*, florfenicol/chloramphenicol, *mef*, macrolides; *sat4*, nourseothricin; *tet*, tetracycline; *vanA*, vancomycin/teicoplanin; *vanB*, vancomycin; *vgb*, streptogramin B. Some determinants represent several classes and types of resistance genes such as *erm* for ermA/B/C or *tet* for tetM/O/W (ribosomal protection) and tetK/L (efflux pumps).

1.5.4. Mechanism of resistance to antibiotics

There are three main mechanisms of antibiotic resistance in bacteria such as specific modification of antibiotic targets, inactivation of the drug by modification of its active parts and blocking antibiotics from getting their targets by decreasing uptake or overexpression of antibiotic efflux pumps (Miller *et al*., 2014). In addition, the roles of bacterial enzymes involve several key mechanisms in antibiotic resistance. Modification of bacterial enzymes is also one of the resistance mechanisms because the enzymes related with cell wall synthesis and nucleic acid synthesis are target for antibiotics (Egorov, A.M, *et al*, 2018). Common mechanism of antibiotic

resistance is changing in the antimicrobial target sites that inhibit antibiotic binding (Lambert, 2005).

1.5.4.1. Resistance to glycopeptide

Glycopeptide resistance mediated by efflux pumps is rare, but *acrF* efflux pump has been known to cause resistance (Fair & Tor, 2014). Bacteria are resistant to glycopeptide antibiotics by modification of peptidoglycan precursors in acyl-D-alanyl-D-alanine (D-Ala-D-Ala) termini into D-alanyl-D-Lactate (D-Ala-D-Lac) or D-alanyl-D-Serine (D-Ala-D-Ser) (Arias & Murray, 2012). However, these modified precursors can still act as substrates for the construction of functional peptidoglycan (O'Driscoll & Crank, 2015).

Nine different glycopeptide-resistant gene clusters providing in enterococci can differentiate into acquired and intrinsic resistance. These determinants are different in phenotypically and genetically, depends on mobile genetic elements or in the core genome (Kristich *et al.*, 2014). Namely, acquired resistance genes are *vanA, vanB, vanD, vanE, vanG, vanL, vanM* and *vanN* and the intrinsic low-level resistance gene is *vanC* (Xu *et al*., 2010). *vanA* and *vanB* genes are found in the most common clinical isolates and *vanA* gene is investigated in a wide variety of enterococcal species (Salem-Bekhit *et al*., 2012).

(i) High-level acquired resistance to vancomycin and teicoplanin (vanA-type resistance)

vanA-type resistance is acquired resistance to high levels of vancomycin and teicoplanin by modification of peptidoglycan precursor from D-Ala-D-Ala to D-Ala-D-Lac. This resistance is induced by either vancomycin or teicoplanin (Xu *et al.*, 2010). *vanA* gene cannot perform its mechanism by itself, because D-hydroxy acids such as D-Lac are neither present in the enterococci environment naturally nor produced by enterococci normally (Cetinkaya *et al.*, 2000). It is needed to cooperate with *vanR* and *vanS* (regulators of resistance gene expression), *vanH* (synthesis of the D-Ala-D-Lac) and *vanX* and *vanY* (hydrolysis of peptidoglycan precursors) and *vanZ* (its function is unknown) (Ranotkar *et al.*, 2014). This *vanA* gene cluster is enconded in Tn1546 transposon and it can be transferrable.

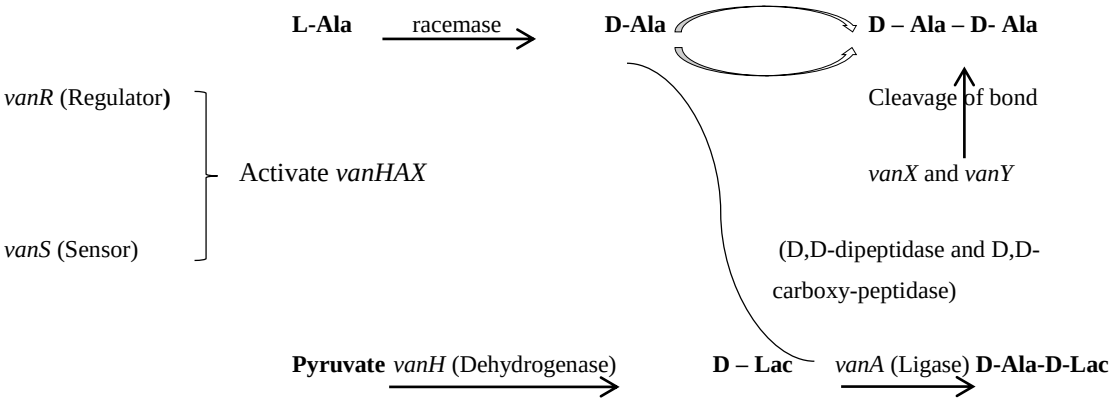


Figure 19. Regulation of *vanA* gene cluster in vanA-type glycopeptide resistance (Ranotkar *et al.*, 2014)

(ii) High-level acquired resistance to vancomycin (vanB-type resistance)

The structure and function of vanB phenotype is similar to that of vanA-type, in synthesis of D-Ala-D-Lac ending peptidoglycan precursors instead of D-Ala-D-Ala but different in regulation. vanB-type makes moderate to high-level resistance to vancomycin, but susceptible to teicoplanin (Xu *et al.*, 2010). The dehydrogenase, ligase, and a dipeptidase encoding genes of *vanB* operon have high-level identity (67% - 76%) with those of the vanA-type. The mechanism of additional *vanW* gene in *vanB* gene cluster is unknown and is not related to *vanZ* from vanA-type. *vanB* gene cluster is located on plasmids or in chromosome which acquired on Tn5382/ Tn1547 transposons and it can be transferrable (Kristich *et al.*, 2014).

(iii) Variable-level acquired resistance to vancomycin and teicoplanin (vanM-type resistance)

vanM-type glycopeptide resistance is the moderate to high-level of vancomycin resistance and the moderate-level of teicoplanin resistance. *vanM* gene is encoded D-Ala-D-Lac ligase as *vanA*, *vanB* and *vanD* genes. The nucleotide of vanM-type is identical 81.8%, 72.5%, 67.7%, and 78.2% with *vanA*, v*anB*, *vanD*, and *vanF* respectively. No genes from *vanM* gene cluster are similar to *vanZ* or *vanW* from the *vanA* and v*anB* gene clusters. The regulatory system of *vanM* gene cluster, $vanR_M$ and

vanS$_M$ are identical with *vanR$_F$* and *vanS$_F$*. This gene cluster is located on plasmid or chromosome and transferred by conjugation (Xu *et al.*, 2010).

(iv) Moderate-level acquired resistance to vancomycin (vanD-type resistance)

Acquired vanD-type resistance is also due to the synthesis of peptidoglycan precursors ending in D-Ala-D-Lac. The vanD-type makes constitutive resistant to moderate-level of vancomycin and low level of teicoplanin (Xu *et al.*, 2010). The gene responsible for vanD-type is located on the chromosome and is non-transferable. The amino acid sequence of *vanD* gene is similar with those of *vanA* and v*anB* genes. However, the genes as *vanZ* and *vanW* from the *vanA* and v*anB* operons are absent in *vanD* operon (Ranotkar *et al.*, 2014).

(v) Low-level intrinsic resistance (vanC-type resistance)

vanC-type resistance is the production of peptidoglycan precursors ending in D-Ala-D-Ser. This resistance is intrinsic low-level resistance to vancomycin and susceptible to teicoplanin. *vanC* gene cluster is consist of *vanT, vanC* and *vanXY$_c$*. There are three *vanC* genes, *vanC$_1$, vanC$_2$ and vanC$_3$,* are specific to *E. gallinarum, E. casseliflavus and E. flavescens* respectively. The genotypes of *vanC$_2$* and *vanC$_3$* are closely related (Kristich *et al.*, 2014). The *vanC* operon is chromosomally located and is not transferrable (Courvalin, 2006).

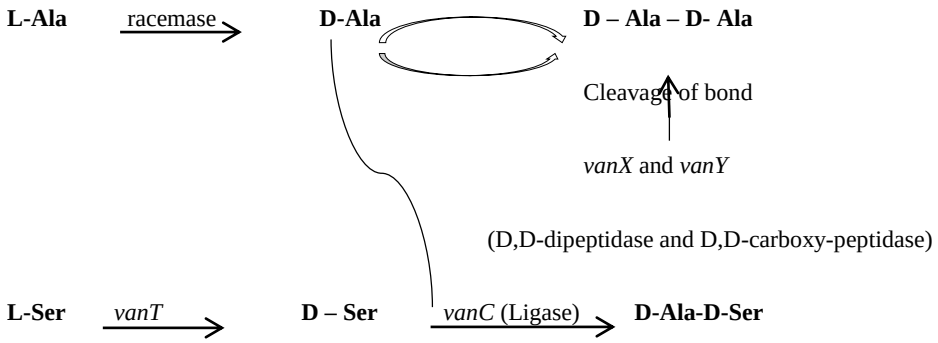


Figure 20. Regulation of *vanC* gene cluster in vanC-type glycopeptide resistance (Courvalin, 2006)

(vi) Low-level acquired resistances (vanE, vanG, vanL and vanN-type resistances)

vanE, vanG, vanL and vanN-type resistances are the synthesis of peptidoglycan precursors terminating in D-Ala-D-Ser. These acquired resistance types are low-level resistance to vancomycin and susceptible to teicoplanin. *vanE* amino acid sequence is similar to vanC (55%) than to *vanA* (45%), *vanB* (43%), or *vanD* (44%) (Bhatt *et al*., 2015). Difference from other van operons in the *vanG* cluster is that the cluster consists of three genes (*vanR*$_G$, *vanS*$_G$ and *vanU*$_G$) and the additional *vanU* gene encodes a predicted transcriptional activator. The regulatory genes of vanG-type resistance have the highest similarity to these of vanD-type (Courvalin, 2006). *vanL* gene cluster is composed as similar as the *vanC* and *vanE* operons except serine racemase activity is encoded by two genes, *vanTm*$_L$ and *vanTr*$_L$. The *vanL* gene exhibited 51% and 49% identity to the *vanE* and *vanC* ligases, respectively (Boyd *et al.*, 2008). *vanXYN* is identical (61%) with *vanXYL* and *vanTN* gene is 52% to *vanTC*. Deduced protein *vanRN* exhibited 74% identity with *vanRL* and *vanRC* and *vanSN* exhibited 61% identity with *vanSL* (Lebreton *et al*., 2011). These resistance genes are located on the chromosome except *vanN* gene. They are not transferrable and not frequently isolated in clinical conditions.

(vii) vanF-type resistance

vanF gene cluster has been described in a biopesticide, *Paenibacillus popilliae*, but has not been found in enterococci (Xu *et al.*, 2010).

(viii) Vancomycin dependent enterococci (VDE)

Some vanA-type and vanB-type VRE are VDE, need vancomycin for their growth and are resistant to vancomycin as well. In this phenomenon, vancomycin performs as an inducer to induce *vanA* and *vanH* genes for constructing D-Ala-D-Lac. If vancomycin is removed, the synthesis of D-Ala-D-Lac is stopped. Bacteria will dead in the absence of the precursor of cell wall (Raza *et al*., 2018).

(ix) Prevalence of vancomycin resistance enterococci

Vancomycin-resistant enterococci are emerging in healthcare environment worldwide; one third of all healthcare associated infections in US and one fifth in some European countries (Sparo *et al*., 2018). Although resistant prevalence of vancomycin was different, most of VRE isolates were reported as MDR. Dissemination of VRE was rapid in greater than twenty-fold increase (from 0.3 to 7.9%) in the United States between the period of 1989 and 1993 due to the extensive use of vancomycin and other broad-spectrum antibiotics (Cetinkaya *et al.*, 2000). According to CDC report in USA, 1300 deaths from over 20,000 vancomycin-resistant enterococcal infection exposures in 2013, mainly *E. faecium*, in hospitalized patients are estimated (Kohinke & Pakyz, 2017). At the same time, Greece and Portugal have the higher prevalence of VRE (45%) and Netherland and Scandinavia have the lowest prevalence of VRE (<1%) (Both et al., 2017). The incidence of VRE was revealed increasingly from 1.6% in 2001 to 11.5% in 2012 in Czech (Oravcova *et al.*, 2017). The prevalence of VRE in Asia was noted in China, Taiwan, Japan and India and was less than 2% in other countries such as The Philippines, Saudi Arabia and Thailand (Daniel *et al.*, 2015). The rate of VRE from clinical isolates in Thailand was not more than 2% until 2014 but had been increasing to reach 2.2% in 2015 and 4.6% in 2018 according to National Antimicrobial Resistance Surveillance Centre, Thailand (NARST) (NARST, 2018).

Among nine van-type resistance in enterococci, vanA and vanB are the most prevalent vancomycin-resistance type in most of countries. The prevalence of vanB-type is more prevalent than vanA-type in Europe in recent years (Hammerum *et al.*, 2017). In contrast, all 101 isolates except 2 isolates had vanA phenotype in China during 6 years period between 2005 and 2011 (Sun *et al.*, 2012). Interestingly, Schouten *et al.* was reported vanC-type was the highest resistance in Latyia (14.3%) and Turkey (11.7%) (Schouten *et al.*, 2000) and 11 out of 14 isolates were detected vanC-type resistance in the first report of Nigeria (Ekuma *et al.*, 2016). In Thailand, all VRE isolates were vanA-type during the period between 1999 to 2002 in Rajavithi Hospital whereas all were vanB-type between 2005 to 2009 in King Chulalongkorn Memorial Hospital (Chongthaleong, 2003; Thongkoom *et al.*, 2012)

Table 1. Characteristics of glycopeptides-resistance in enterococci (O'Driscoll & Crank, 2015)

	Acquired								Intrinsic
	High		Variable	Moderate	Low				Low
	vanA	vanM	vanB	vanD	vanE	vanG	vanL	vanN	vanC
Vancomycin MIC(µg/ml)	64 - 1,000	>256	4 - 1,000	64 - 128	8-32	≤16	8	16	2-32
Teicoplanin MIC (µg/ml)	16 - 512	96	0.5 - 1	4- 64	0.5	≤0.5	≤0.5	≤0.5	0.5 - 1
Modification	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Lac	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser	D-Ala-D-Ser
Location	Plasmid/ Chromosome	Plasmid/ Chromosome	Plasmid/ Chromosome	Plasmid/ Chromosome	Chromosome	Chromosome	Chromosome	Plasmid	Chromosome
Transferrable	Yes	Yes	Yes	No	No	No	No	Yes	No
Expression	Inducible	Inducible	Inducible	Constitutive or inducible	Inducible	Inducible	Inducible	Constitutive	Constitutive or inducible
Species	*E. faecalis*, *E. faecium*, *E. gallinarum*, *E. casseliflavus*, *E. durans*,	*E. faecium*	*E. faecalis*, *E. faecium*	*E. faecalis*, *E. faecium*	*E. faecalis*	*E. faecalis*	*E. faecalis*	*E. faecium*	*E. gallinarum*, *E. casseliflavus*, *E. flavescens*

1.5.4.2. Resistance to β-lactams

Enterococci are low-level resistant intrinsically to β-lactams by producing of low-affinity penicillin binding protein (PBPs). They are acquired resistant by mutations in chromosomal DNA (*pbp*5 gene) that cause overproduction of modified pbp5 leading to low affinity to β-lactam antibiotics (Ligozzi *et al.*, 1996). β-lactamase production in enterococci is infrequent reported that β-lactamase producing *E. faecium* strains were reported in Richmond in 1992 and epidemiology unrelated strains in Modena in 2010 (Gagetti *et al.*, 2018).

1.5.4.3. Resistance to aminoglycosides

Enterococci can be resistant to low-level aminoglycoside intrinsically by reducing the cellular permeability and efflux pump. There are two mechanisms for high-level acquired resistance to aminoglycosides: production of aminoglycoside-modifying enzymes and single mutations within a protein of 30S ribosomal subunit. Aminoglycoside-modifying enzymes can be distinguished into three main classes according to the type of modification; acetyltransferases (AAC.), adenyltransferases (ANT) and phosphotransferases (APH). *aac(6')-Ie-aph(2')-Ia* gene situated on transposons or plasmids which codes the most important bi-functional (acetylating and phosphorylating) enzymes is resistant to most of the aminoglycosides (90%) such as gentamicin, amikacin except streptomycin (Shepard & Gilmore, 2002). The other high-level resistance to streptomycin is due to ribosomal mutations which usually changse the S12 ribosomal protein (Arias & Murray, 2012).

1.5.4.4. Resistance to chloramphenicol

Chloramphenicol resistance in enterococci is mediated by chloramphenicol acetyltransferases, which are encoded by plasmid-mediated or chromosomally integrated *cat* genes. The *cat* genes are shown little homology enzymes in Gram-positive and Gram-negative bacteria. This chloramphenicol acetyltransferase enzymes help to link one or two acetyl groups and hydroxyl groups on the chloramphenicol molecule to inhibit the binding of chloramphenicol and ribosome (Woodford, 2005).

1.5.4.5. Resistance to fluoroquinolones

The first mechanism of quinolone resistance in enterococci is mutation in the regions of genes encoded with DNA gyrase and topoisomerase IV. This mutation prevents the binding of antibiotic to the enzyme but is able to continue DNA replication despite the presence of antibiotic (Hawkey, 2003). Alteration in *gyrA* gene of the DNA gyrase and *parC* gene of the toiposomerase IV are mainly resistant to quinolones. The next mechanism of quinolone resistance is efflux function. Another mechanism is that the members of the Qnr protein family are protected DNA gyrase and topoisomerase IV from inhibition by quinolones. However, the natural functions of Qnr proteins are still unclear (Kristich *et al.*, 2014).

1.5.4.6. Resistance to macrolides

The common type of acquired resistance to macrolides is the production of methylases enzymes that coded with the erythromycin resistance methylase (*erm*B) gene in the 23S rRNA of the 50S ribosomal subunit. This enzyme which is reduced the binding capacity of the macrolide to the ribosome. Other acquired macrolide resistance mechanism such as production of drug-inactivation enzymes are rare. Mutation in 23S rRNA and the ribosomal protein can be resistant to macrolides (Kristich *et al.*, 2014).

1.5.4.7. Resistance to tetracyclines

Tetracycline resistance in bacteria is mediated by multiple genes; twenty-nine tetracycline resistance genes and three oxytetracycline resistance genes. The presence of these genes is resistant to tetracycline under two mechanisms; efflux pump and ribosomal protection. Efflux pumps were mediated by plasmid-borne determinants such as *tetK* and *tetL* in Gram-positive bacteria, and *tetA* to *tetE, tetG,* and *tetH* in Gram-negative bacteria. Protection proteins encoded by *tetM, tetO, tetQ, tetS, tetT* and *tetW* genes are bound to the ribosome and prevented binding of tetracycline and minocycline to ribosome (Zahid *et al.*, 2017). *tetU* is resistant to low-level tetracycline through an unknown mechanism (Chopra & Roberts, 2001).

1.5.4.8. Resistance to nitrofurantoin

Nitrofurantoin has no significant resistance for long time because of its multiple actions and sites of attack. Mutations in nitroreductase genes (*nfsA* and *nfsB*) that converted nitrofurantoin into toxic intermediate compound to be resistant. Moreover, a plasmid-encoded efflux pump, *oqxAB* can be resistant to nitrofurantoin. This o*qxAB* genes is located in *Tn*6010 and has the broad substrate specific for olaquindox, chloramphenicol, ciprofloxacin, trimethoprim and disinfectants. Actually, the veterinary use of olaquindox (OLA) has banned in many countries because of the high prevalence of *oqxAB* genes. However, agents chemically related with OLA were used for animal feeding in China. The wide use of these compounds contributes to the rapid emergence of *oqxAB* genes. Consequently, the acquisition of *oqxAB* genes carrying plasmid reduce nitrofurantoin MIC markedly and also efflux pump facilitate the development of nitrofurantoin resistance (Ho *et al.*, 2016).

1.5.4.9. Resistance to rifampin

Rifampin has not been used extensively for enterococci infection treatment. However, rifampin resistance in enterococci is the consequence of rifampin exposed during non-enterococcal infections treatment. Reduction of rifampin affinity is the consequence of specific mutation in the gene encoding the beta subunit of the RNA polymerase (RpoB). Additionally, enzymatic inactivation of rifampin is leading to rifampin resistance. Other factors such as alteration of rifampin transport by subinhibitory concentrations of daptomycin is also caused to rifampin resistance (Kristich *et al.*, 2014).

1.5.4.10. Resistance to antibiotics for treatment of VRE infections

(i) Resistance to oxazolidinones

Linezolid resistance is caused by two main mechanisms; G2576T mutation and developing of *cfr* (chloramphenicol – florfenicol resistance) gene through horizontal transmission. The first mechanism, a point mutation in the domain V of the 23S rRNA gene leads to nucleotide change from Guanine (G) to Uracil (U) at position

2576 in the 23S sub-unit, the binding site of the oxazolidinone antibiotics (Souli *et al.*, 2009). The second resistant mechanism is that preventing of antibiotic binding by a plasmid-borne determinant, *cfr* gene which encodes for methyltransferase and modifies adenosine in the linezolid-binding site on the 23S rRNA. This multidrug resistance gene can be resistant to phenicols, lincosamides, oxazolidinones, pleuromutilins and streptogramin A compounds (O'Driscoll & Crank, 2015). In recent, the new oxazolidinone resistance gene *optrA* has been indicated in enterococci isolates of human and animal origin, which is resistant to oxazolidinone and phenicol only (Wang *et al.*, 2015). Mutations in the bacterial ribosomal proteins L3 and L4 have also been associated with resistance to linezolid (Li *et al.*, 2016). Among them, mutations in the 23sRNA gene is the most common mechanism of linezolid resistance in enterococci (Cho *et al.*, 2018).

(ii) Resistance to tigecycline

Few data are available for tigecycline resistance in Gram-positive bacteria. In 2015, S. Fiedler from Germany reported that two tetracycline resistance determinants *tetL* and *tetM* were reduced tigecycline susceptibility in enterococcal clinical isolates (Fiedler *et al.*, 2015).

(iii) Resistance to quinupristin/dalfopristin

Enterococci are resistant to streptogramins by target modification, enzymatic degradation or active efflux. *E. faecalis* is intrinsically resistance to this antibiotic due to active efflux pump. Resistance to one antibiotic from this cocktail is dramatically reduced the potency of synergy effect. Resistance to dalfopristin can be occurred by the plasmid-mediated dissemination of genes encoding by a series of virginiamycin acetyltransferases (*vatD* and *vatE*). Resistance to quinupristin is mediated by 23S rRNA target methylation with members of erythromycin resistance methylase gene (*erm*) (R. E. Hancock, 2005). These *erm* genes are encoded an enzyme to decrease the binding of macrolides, lincosamides and quinupristin. On the other hand, quinupristin resistance can be occurred the enzymatic cleaveage of ring structure of quinupristin due to the lactonases *vgbA* and *vgbB* (Miller *et al.*, 2014).

(iv) Resistance to daptomycin

Daptomycin resistance in enterococci is associated with mutations in *liaSFR*, a three-component regulatory system controlling cell-envelope stress response, genes encoding proteins involved in phospholipid metabolism including glycerolphosphoryl diester phosphodiesterase (*gdpD*) and cardiolipin synthase genes. These resistant mechanisms performed with two main functions such as managing the cell envelope to become more positively charged for repulsion of antibiotic molecules from cell surface and preventing the combining of antibiotic molecules to cell membrane (Tran *et al*., 2013).

1.5.5. Prevalence of antibiotic resistance in enterococci clinical isolates

The first line antibiotic resistance rates including β-lactams, aminoglycosides, tetracycline and quinolones are higher in enterococci infections all over the world with different frequencies. In Latin American surveillance of antimicrobial resistance (LAVRA) report of Argentina, 85% of *E. faecium* and 1.8% of *E. faecalis* isolates were resistant to ampicillin in 2015 (Gagetti *et al.*, 2018). In Malaysia, ciprofloxacin and penicillin resistance were increased from 20.6% and 84.4% in 2012 to 21.1% and 89.6% in 2013 (Daniel *et al.*, 2015). Antibiotics used in Iran also indicated that uncontrolled usage with higher resistance rates of common antibiotics in enterococcal infections; tetracycline (71.1%), gentamicin (75.1%), erythromycin (69%), ciprofloxacin (53.7%), chloramphenicol (33.4%), vancomycin (32.2%), penicillin and ampicillin (32%) (Arbabi *et al.*, 2016). Antibiotic-resistant *Enterococcus* cases in Myanmar were reported resistance to ampicillin (30.8%) and erythromycin (68.8%) during 2009 to 2013 (Daniel *et al.*, 2015). The previous study in India also reported as *E. faecalis* strains were resistant to vancomycin (77.63%), gentamicin (64.47%) and oxacillin (55.26%) (Oli & Rajeshwari, 2017).

Among antibiotics used for VRE treatment, linezolid and tigecycline antibiotics are commonly used in recent years. The susceptibility rate of these antibiotics are still 100% in most of studies (Goudarzi *et al.*, 2018; Oravcova *et al.*, 2017). However, resistance to these antibiotics in VRE are also reported infrequently; resistance to linezolid with 5% in Brazil (Sacramento *et al.*, 2017), and 0.7% in Iran

(Moosavian *et al.*, 2018). Quinupristin/dalfopristin resistance in *E. faecalis* is natural whereas resistance in VRE. *faecium* is reported as 24% in Brazil (Sacramento *et al.*, 2017), and 11.3% in Korea (Cha *et al.*, 2012). Daptomycin antibiotic is one of a few bactericidal antibiotics for VRE which are approved recently while daptomycin non-susceptible VRE strains have been reported. Significantly, the ratio of daptomycin susceptible and non-susceptible VRE strains in USA within Jan 2008 to Dec 2009 was equal (1:1) (Judge *et al.*, 2012). Resistance to lipoglycopeptides in VRE have not been reported yet. The resistance of antibiotics used for VRE pointed out to be great care for VRE prevention and treatment.

1.6. Epidemiological evaluations of enterococci infections

The main purpose of using typing is to assess the relationship between bacterial isolates that is important to determine the routes of infections, source, confirmation outbreaks or transmission of nosocomial pathogens. Conventional typing methods such as antibiogram, protein profiling, serotyping, and phage typing have been used in the epidemiology study of microbial infections. However, these methods are demonstrated little diversity in epidemiological investigations (Ranjbar *et al.*, 2014).

Consequently, DNA-based typing methods without amplification; pulsed-field gel electrophoresis (PFGE), and restriction fragment length polymorphism (RFLP), DNA-based typing methods with amplification; random amplified polymorphic DNA (RAPD), and amplified fragment length polymorphism (AFLP), and sequence-based typing methods; multi-locus sequence typing (MLST), multi-locus variable number tandem repeat analysis (MLVA) and whole genome sequencing have been improved to study epidemiology. Moreover, ribotyping and plasmid typing are used for specific typing. Most of currently used molecular typing methods are electrophoretic separation of DNA fragments with or without amplification. In these methods, the relatedness between strains were determined analyzing the DNA fingerprint patterns of each organism and combined with epidemiologic data. However, most DNA fingerprinting methods have major limitations such as lack of standardization, require skilled personnel and need significant time to analyze the data (Brisse *et al.*, 2002). The use of gene sequencing data for the determination of genomic relatedness at the intra- and inter- species levels has recently been advocated because

of its advantages in reproducibility and portability over the banding pattern techniques. However, these sequence-based method have still less discriminatory power than fingerprinting methods (Zeigler, 2003). From various range of methods, reliable and suitable method needs to decide depending upon several variables such as inter-laboratory portability, intra- and inter- laboratory reproducibility, interpretation, high differentiation power, cost and ease to use (Olive & Bean, 1999; G Werner, 2013).

1.6.1. Methods without amplification

(i) Pulsed-field gel electrophoresis (PFGE)

PFGE is a method separating large DNA fragments (larger than 20kb) by cutting with restriction enzymes in a conventional gel electrophoresis using static electric field. The rare cutting restriction enzymes for strain differentiation and VRE are *Sma*I and *Apa*I (Persing, 2004). This method is useful for the classification and identification of enterococci from a variety of sources. It has high discriminatory power, reproducible and standard criteria for interpretation. It is useful to investigate local outbreaks during short time periods (Tenover *et al.*, 1995a). Even one single deletion or insertion of a base pair in the genome of the bacteria expressed different banding patterns in this method. Although this method is time-consuming, requiring high technical standard and qualified persons and less portable intra-laboratory comparability, PFGE typing is still considered as the gold standard for genotyping in molecular epidemiology including VRE (G Werner, 2013). To overcome the drawbacks of PFGE, more advanced genetic typing methods such as AFLP and MLST have been developed. These methods are also labor intensive and high-cost methods but they are suitable to study clonal relations in an evolutionary sense rather than clonal spread in an outbreak situation (Homan *et al.*, 2002).

(ii) Restriction fragment length polymorphism (RFLP)

RFLP is a method by analyzing the various lengths of fragments after cutting with restriction enzymes. Although the patterns produced by RFLP are less clear than those produced by PFGE, this method has been used to study of the epidemiology

of enterococci. Because this method is more rapid than PFGE and the equipment requirements are less (Chiew & Hall, 1998).

1.6.2. Methods with amplification

(i) Random amplified polymorphic DNA (RAPD) PCR

RAPD is one of the PCR-based DNA fingerprinting methods, is used arbitrary single primer (not more than 7-10 bp) that is not targeted to any known sequence of the bacterial genome. Unlike with normal PCR is that RAPD primer is designed to flank randomly to DNA segments along the genome, which give a high number of bands. Therefore, this method can be used to determine polymorphisms. One of the advantages of this method is that short RAPD primer overcomes the problem of non-specific binding in longer primer. Moreover, RAPD PCR is a well-accepted, fast, cheap and less laborious and time consumption tool for differentiation and characterization of enterococci (Konrad J. Domig *et al.*, 2003). Study of A.Weiss *et al.* proved that *E. faecium* isolates can differentiate with RAPD, giving superior discriminatory power to the other PCR-based methods (Weiss *et al.*, 2010). Furthermore, this method is also a useful method for investigation of epidemiological VRE. Although the discriminatory power of RAPD method for VRE is less than PFGE, fingerprint results of RAPD were highly congruent with PFGE fingerprints (Barbier *et al.*, 1996). However, RAPD PCR has some disadvantages such as limited detection of polymorphisms and only detects dominant markers (Bardakci, 2001).

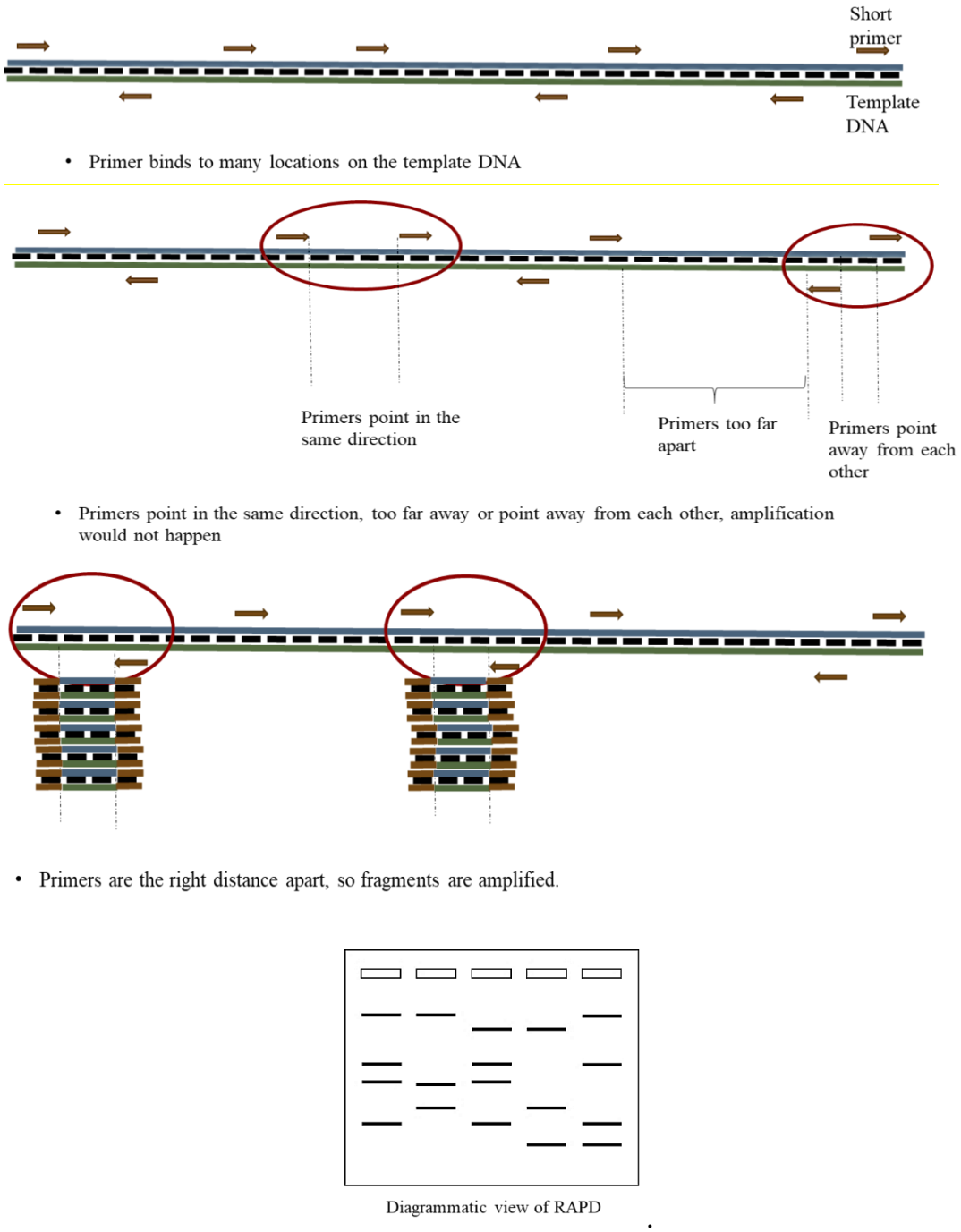


Figure 21. RAPD PCR reaction and agarose gel illustration (Bardakci, 2001)

(ii) Amplified fragment length polymorphism (AFLP)

AFLP is the selective PCR amplification of restriction fragments after total digest of target genomic DNA. This method digests target DNA using two different restriction enzymes, followed by ligation of oligonucleotide adaptors to the sticky-ended DNA fragments, selective amplification of restriction fragments sets and

gel analysis of amplified fragments. This technique can be used for subspecies but cannot be used to discriminate among genera. The drawbacks of AFLP method are expensive, require high specific instruments and additional software for data analysis (Antonishyn *et al.*, 2000).

1.6.3. Sequence-based typing methods

(i) 16S rRNA gene sequencing

16S rRNA gene sequence (~1500 bp) has highly conserved and variable regions. After amplification using the bacterial universal primers, sequences were assembled using sequencer and analyzed using BLAST. Despite 16s rRNA gene sequencing is considered as the gold standard for species identification, the capacity of this gene sequencing is limited in identifying of closely related organisms. They suggested that 16S rRNA gene sequencing was incapable of differentiating among the known strains of *E. faecalis* and *E. faecium,* even these two species were readily discriminated. Moreover, the 16S rRNA gene sequences of *E. faecium* and *E. mundtii* were approximately 98% similar and formed a single cluster in the phylogenetic tree (Nayak *et al.*, 2011).

(ii) Multi-locus sequence typing (MLST)

MLST identifies genetic variation in the internal fragment sequences of seven house-keeping genes. The DNA sequences of seven housekeeping genes are determined and sequence variation identified within each gene is termed alleles. Each allele is given a number to produce allelic profile. The allelic profile at each of the seven loci decides the sequence type (ST) of each organism. Allele assignments are portable between laboratories and the data can be stored, shared and updated from a central database (Urwin & Maiden, 2003). The advantages of MLST are lacking biased results and available exchange data (Homan *et al.*, 2002). MLST is the standard method for epidemiological investigations for large scale international comparisons. This technique can be overcome the difficulties for comparisons of DNA fingerprinting patterns between laboratories. In addition, this method is highly reproducible. The disadvantage of this method may be the high cost for DNA polymerase, sequencing reaction components and equipment operation (Persing, 2004). Although MLST is

regarded as the standard method for typing isolates, clinical *E. faecium* isolates with the same ST revealed considerable sequence diversity. This phenomenon may be due to *E. faecium* could not be typed by MLST owing to loss of the required housekeeping gene *pstS* (Carter *et al*, 2016).

(iii) Multi-locus variable number of tandem repeat analysis (MLVA)

The principle of MLVA is determining DNA repeat regions present at multiple loci on the chromosome of bacteria and its variations in number and composition. This method is rapid to detect variation of tandem repeats by using specific primers targeting the flanking regions of the tandem repeats. The results of this method are also compared between laboratories by exchanging an unambiguous numerical result from the gain or loss of discrete repeats (Top, LM Schouls, *et al.*, 2004). The discrimination ability of this method for *E. faecium* is as similar as that of AFLP and MLST and less than PFGE (Top *et al.*, 2008). However, the MLVA typing is useful for strain characterization and outbreak analysis (Borgmann *et al.*, 2007).

(iv) Whole genome sequencing (WGS)

The principle of WGS is the process of complete DNA sequencing of the genome and discrimination the stains; even only single nucleotide is different. This method provides the greater resolution. Therefore, this method is a suitable for molecular epidemiologic analysis in outbreak investigations. Moreover, the data from this method can be shared worldwide. WGS has an increased discriminatory power as PFGE and MLST and better for epidemiological study (Lytsy *et al.*, 2017).

(v) Single-strand conformational polymorphism (SSCP)

PCR-SSCP method is detecting conformational changes in the DNA molecules. This method is used to observe the mobility of single-stranded DNA by identifying sequence changes in amplified DNA in polyacrylamide gel. Because of its simplicity and ease to use, it is useful for screening of inherited mutation and detecting somatic mutations in cancer cells. It can also be used to classify virus strains. In

antibiotic resistance detection, it is widely used to detect point mutations in target genes (Konstantinos *et al.*, 2008).

1.6.4. Ribotyping

Ribotyping is a technique by identifying conserved rRNA genes such as 23S and 16S rRNA using oligonucleotide probes after treatment of genomic DNA with restriction endonucleases. Distinct enzymes that used in ribotyping of enterococci are *Eco*RI, *Hind*III, *Pvu*II, *Ban*HI, and *Bsc*I. The result of ribotyping has slightly less discriminatory power than PFGE (Brisse *et al.*, 2002). Ribotyping is useful for inter- and intra- species discrimination of enterococci because the genes encoding rRNA are highly conserved and a single probe can be used for subtyping all enterococci. For VRE typing, its discriminatory power is limited and required some modifications for VRE epidemiological study according to the comparison data of PFGE and automated ribotyping system (Price *et al.*, 2002). In addition, this method is laborious because of complicated steps including DNA isolation, restriction endonuclease digestion, electrophoresis, and transferring to nitrocellulose or nylon membranes.

1.6.5. Plasmid typing

Plasmid can be used as a marker for comparing strains in studying epidemiology of bacteria. However, this method has many disadvantages. The main drawbacks are that plasmid can be lost or gained by conjugation and spread rapidly from one strain to other (Tenover *et al.*, 1997). For epidemiological study of enterococci, plasmid typing is commonly used for horizontal *vanA* cluster dissemination. However, the composition of enterococcal plasmids are involved two or more genotypes in each individual. It leads to encounter the problems when only single plasmid marker gene is used in plasmid typing (Guido Werner *et al.*, 2011).

OBJECTIVES

The overall objectives of this research are to study epidemiological role of enterococcal infections from two tertiary care hospitals in Southern and Northeastern Thailand and of their possible pathogenetic factors in human infections.

This work attempt to achieve the following specific objectives:

1. To investigate the incidence of vancomycin resistance and multidrug resistance in enterococci
2. To explore the virulence factors including biofilm formation and secreted factors of enterococci
3. To investigate a possible relationship between antibiotic resistance and virulence factors of enterococci
4. To determine the genetic relatedness of vancomycin-resistance enterococci from Southern and Northeastern Thailand

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

2.1.1. Bacterial isolates

- Clinical enterococci isolates from two hospitals
- *Enterococcus faecalis* ATCC 29212
- *Enterococcus faecium* DMST 14756
- *Enterococcus casseliflavus* ATCC 25788
- *Enterococcus gallinarum* ATCC 49573
- *Staphylococcus aureus* ATCC 25923
- *E. faecium* ATCC 51559 (*vanA* positive)
- *E. faecalis* ATCC 51299 (*vanB* positive)

2.1.2. Antimicrobial agents

(i) Antibiotic discs (Oxoid, UK)

- Ampicillin, 10 µg
- Ciprofloxacin, 5 µg
- Chloramphenicol, 30 µg
- Erythromycin, 15 µg
- Gentamicin, 120 µg
- Linezolid, 30 µg
- Nitrofurantoin, 300 µg
- Penicillin G, 10 iU
- Quinupristin/dalfopristin, 15 µg
- Rifampin, 5 µg
- Streptomycin, 300 µg
- Teicoplanin, 30 µg
- Tetracycline, 30 µg

- Tigecycline, 15 µg
- Vancomycin, 30 µg

(ii) Antibiotics

- Linezolid, 600 mg/300 ml (Zyvox, Pfizer, USA)
- Tigecycline, 50 mg (Tygacil, Wyeth, USA)
- Vancomycin, 500 mg (CJ Pharma, Korea)

2.1.3. Culture media (HiMedia, Mumbai)

- Bile esculin salt agar
- Brain Heart Infusion agar (BHIA)
- Brain Heart Infusion broth (BHIB)
- Muller Hinton agar (MHA)
- Muller Hinton broth (MHB)
- Nutrient gelatin

2.1.4. Supplement for culture preparation

- 50% egg-yolk emulsion
- 10% Glucose
- Human blood
- Sterile skimmed milk

2.1.5. Chemical and biological reagents

(i) Reagents for genomic DNA extraction

- PBS buffer, 1x
- TE buffer, 1x
- Lysozyme, 10 µg/ml (Sigma-Aldrich, USA)
- Sodium dodecyl sulfate (SDS), 10% (Merck, USA)
- RNase, 10 µg/ml (Thermo Fisher Scientific, USA)
- Phenol, saturated (Thermo Fisher Scientific, USA)
- Chloroform (Merck, USA)

- Absolute ethanol (Merck, USA)
- Sterile deionized water

(ii) Reagents for PCR and gel electrophoresis

- PCR buffer, 10x (Thermo Fisher Scientific, USA)
- $MgCl_2$, 25 mM (Thermo Fisher Scientific, USA)
- dNTPs mix, 100 mmol (Thermo Fisher Scientific, USA)
- Taq polymerase, 5 U (Thermo Fisher Scientific, USA)
- Molecular weight marker, 100 bp (Thermo Fisher Scientific, USA)
- Specific forward and reverse primers, 100mM (Sigma-Aldrich, USA)
- Loading dye, 6x (Thermo Fisher Scientific, USA)
- Agarose gel (Merck, USA)
- TAE buffer, 1x
- Ethidium bromide solution, 10 mg/ml (AppliChem, USA)

(iii) Reagents for RAPD

- PCR buffer, 10x with 2 mM $MgCl_2$ (Takara, Japan)
- dNTPs, 2.5 mmol (Takara, Japan)
- Taq polymerase, 5 U (Takara, Japan)
- Molecular weight marker, 1kb (Thermo Fisher Scientific, USA)
- TBE buffer, 1x

(iv) Other chemical reagents

- 0.85% normal saline
- 0.1% crystal violet
- 40% glycerol
- 1M hydrogen chloride (HCl)
- 3% hydrogen peroxide (H_2O_2)

- 0.04% resazurin
- 1M sodium hydroxide (NaOH)
- Acetone
- Gram iodine
- Immersion oil
- Safranin

2.1.6. Equipment and instruments

- Micron filter, 0.22 µm (Merck, USA)
- Multimode plate reader (Enspire^{TM}, PerkinElmer, USA)
- Autoclave, ES315 (Tomy, Japan)
- Automatic pipette, 0.2 – 2 µl, 2 – 20 µl, 20 – 100 µl, 100 – 1000 µl (Eppendorf, Germany)
- Colony counter, Co C-110 (New Brunswick Scientific,USA)
- Cotton swab
- Centrifuge, microliter, EBA21 (Hettich Germany)
- Centrifuge, high speed, 5404/5804R (Eppendorf, Germany)
- Cryo box and rack
- Duran bottles (Pyrex)
- Dropper
- Electrophoresis set, Horizontal, Wide Mini-Sub Cell GT Cell (Bio-Rad, USA)
- Flask (Pyrex)
- Falcon, centrifuge tube 15 ml, 50 ml (NEST, USA)
- Freezer, CH 150 (Panasonic, Japan)
- Genovanano microvolume spectrophotometer (Jenway, UK)
- Genegenius gel light imaging system, 31415 (Syngene, India)
- Glass slide, Ground edges, Plain slide
- Hot air oven, T410340 (Binder, Germany)

- Incubator, B5100E (Heraeus, Germany)
- Incubator shaker, 4518 (Thermo Forma, USA)
- Laminar air flow cabinet, BH43AS (Gelman, Australia)
- Light microscope, CX31RBSFA (Olympus, Japan)
- Microcentrifuge tube, 1.5 ml (NEST, USA)
- Microtiter plate 96 wells, polystyrene (NEST, USA)
- Multichannel automatic pipette, 30 – 300 µl (Axygen, USA)
- PCR tubes, 0.2 ml (NEST, USA)
- Petri dish, 150 x 15 mm (Pyrex)
- Pipette tips, 10 µl, 20 – 200 µl, 1000 µl (NEST, USA)
- Refrigerator, NR-BL308PSPH (Panasonic, Japan)
- Spectrophotometer, UV-1201V (Shimadsu, Japan)
- Test tube 3 x 100 mm, 25 x 150 mm (Pyrex)
- Test tube racks
- Thermal cycler (Bio-Rad, USA, T100TM)
- Vernier caliper, 0 - 150 mm (Whale, China)
- Vortex mixer (Vortex Genie 2, USA)
- Water bath, TW 20 (Julabo, Germany)

2.2. Methods

2.2.1. Sources of enterococci isolates

The sources of enterococci isolates from two tertiary care hospitals were urine, pus, blood, sputum and body fluid, which were collected between January 2015 and February 2018. The data of species identification, antibiotic resistance, biofilm formation and secreted virulence factors activities of 272 enterococci isolates from Hat Yai Hospital (640-bed capacity in 2017), Songkhla province, Southern Thailand were obtained from previous study (Kanitta Muangngam, 2017). Furthermore, 512 isolates from Sunpasitthiprasong Hospital (1,218-bed capacity in 2017), Ubon Ratchathani Province, Northeastern Thailand were obtained from the microbiological laboratory of this hospital. The isolates were prepared with BHI broth and kept with 20% glycerol in -80 °C for further studies.

Figure 22. Location of the sample collection areas on Thailand map

2.2.2. Identification of enterococci isolates

The isolates were inoculated onto Brain Heart Infusion (BHI) agar and incubated at 35 ± 2 °C overnight. *Enterococcus* genus identification had been already tested in the hospital laboratories. However, Gram staining, catalase test, and bile

esculin test were used to confirm enterococci. Multiplex PCR using superoxide dismutase (*sodA*) gene sequences for *E. faecalis* and *E. faecium* and using $vanC_1$ and $vanC_{2/3}$ gene sequences for *E. casseliflavous* and *E. gallinarum* identified. The primer sequences used in this study listed in Table 6.

2.2.3. Antimicrobial susceptibility testing

2.2.3.1. Kirby-Bauer disc diffusion assay

The antibiotic susceptibility screening was examined using Kirby-Bauer disc diffusion method according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2017). Ten antibacterial agents listed in Table 2. were tested in all enterococci isolates. High-level aminoglycoside resistance (HLAR) also screened in all isolates with high content discs of gentamicin and streptomycin. In addition, five more antibiotics (Table 2) were tested in VRE isolates. *S. aureus* ATCC 25923 and *E. faecalis* ATCC 29212 were used as the control strains. If the results of control strains were acceptable, the tested results were interpreted according to CLSI guideline (Table 2).

Table 2. Disc diffusion zone diameter breakpoints and quality control ranges

Antibiotic	Disc content (µg)	Zone diameter breakpoints (mm)			
		S	I	R	*S. aureus* ATCC 25923
Vancomycin	30	≥17	15 - 16	≤14	17 - 21
Teicoplanin	30	≥14	11 - 13	≤10	15 - 21
Ampicillin	10	≥17	-	≤16	27 - 35
Penicillin G	10 (iU)	≥15	-	≤14	26 - 35 ± 2
Gentamicin (High-level)	120	≥10	7 - 9	≤6	16 - 23*
Streptomycin (High-level)	300	≥10	7 - 9	≤6	14 - 20*
Chloramphenicol	30	≥18	13 - 17	≤12	19 - 26
Ciprofloxacin	5	≥21	16 - 20	≤15	22 - 30
Erythromycin	15	≥23	14 - 22	≤13	22 - 30
Tetracycline	30	≥19	15 - 18	≤14	24 - 30
Antibiotics used in VRE isolates					
Linezolid	30	≥23	21 - 22	≤20	25 - 32
Nitrofurantoin	300	≥17	15 - 16	≤14	18 - 22
Quinupristin/Dalfopristin	15	≥19	16 - 18	≤15	21 - 28
Rifampin	5	≥20	17 - 19	≤16	26 - 34
Tigecycline	15	≥19	-	≤18	20 - 25

S, susceptible; I, intermediate; R, resistant, **E. faecalis* ATCC 29212 was used as control strain for high-level gentamicin and high-level streptomycin susceptibility in disc diffusion assay (CLSI, 2017)

2.2.3.2. Vancomycin agar screen testing

BHI agar supplemented with 6 µg/ml vancomycin used to detect intermediate vancomycin resistance (MIC ≥8 µg/ml) because strains with vancomycin MICs ≤32 µg/ml were often revealed as susceptible in disc diffusion. *E. faecalis* ATCC 29212 was used as the control strain. If bacterial growth (>1 colony) was examined on the screening agar, minimum inhibitory concentrations (MIC) of vancomycin was confirmed using broth microdilution assay according to CLSI guideline.

2.2.3.3. Broth microdilution assay

The MIC of vancomycin, tigecycline and linezolid was determined in the isolates that grown on vancomycin screen agar according to CLSI guideline. Broth microdilution assay with the concentration of vancomycin (2 - 1,024 µg/ml), tigecycline (0.0625 - 1 µg/ml), and linezolid (1 - 16 µg/ml) conducted with two-fold dilution. *E. faecalis* ATCC 29212 used as the reference strain. Breakpoints of vancomycin and linezolid established according to the CLSI guideline (Table 3). EUCAST recommended breakpoint used for tigecycline (EUCAST, 2019). The MIC results were interpreted after incubation of 16 - 20 hrs for tigecycline and linezolid, and 24 hrs for vancomycin at 35 ± 2 °C.

Table 3. CLSI and EUCAST-recommended vancomycin, linezolid and tigecycline MIC breakpoints

Antibiotic	Solvent	Range of concentration (µg/ml)	MIC breakpoints (µg/ml)			
			S	I	R	*E. faecalis* ATCC 29212
Vancomycin	D/W	2 - 1,024	≤4	8 - 16	≥ 32	1 - 4
Linezolid	D/W	1 - 16	≤ 2	4	≥ 8	1 - 4
Tigecycline*	D/W	0.0625 - 1	≤ 0.25	-	-	0.03 - 0.12

*Tigecycline MIC breakpoint was followed by EUCAST, 2019;
D/W, sterile distilled water; S, susceptible; I, intermediate; R, resistant

2.2.4. Biofilm formation assay using microtitre plate method

Quantitative biofilm production determined by using a modified microtitre plate method as described previously (Biswas *et al.*, 2014). Briefly, the inoculum prepared with BHI broth supplemented with 0.5% glucose at 35 ± 2 ºC. After that, the inoculum was diluted into 1:100 with BHI broth with 0.5% glucose and added to the wells of a flat-bottomed polystyrene microtiter plate. After incubation at 35 ± 2°C for 24 hrs, broth was carefully drawn off and the wells were washed 2 times with sterile 1x phosphate buffered saline (PBS, pH - 7.4) to remove unbounded bacteria. After that, the plate was stained with 200 µl of 0.1% crystal violet for 15 mins. Finally, 200 µl of ethanol-acetone (80:20, v/v) was added to solubilize the stain attached with biofilm and leave for 15 mins at room temperature. The optical density (OD) of the re-solubilized stain was measured at 570 nm by using a microtiter plate reader. The mean OD of 3 times of negative control plus 3 times of standard deviation was used as optical density cut-off (ODc) value to interpret the biofilm formation level. Biofilm formation of isolates was analyzed into 4 groups based on ODc value showing in the Table 4 (Stepanović *et al.*, 2007). Biofilm quantification for each isolate was performed in triplicate. Medium without bacteria was used as the negative control and *Staphylococcus aureus* ATCC 25923 was used as the positive control.

Table 4. Classification of biofilm-forming ability in microtitre plate method

Cut-off value calculation	Biofilm forming ability
$OD_{570} > 4 \times ODc$	Strong
$2 \times ODc < OD_{570} \leq 4 \times ODc$	Moderate
$ODc < OD_{570} \leq 2 \times ODc$	Weak
$OD_{570} < ODc$	None

2.2.5. Evaluation of secreted virulence factors by phenotypic tests

The expressions of four secreted virulence factors including hemolysin, gelatinase, caseinase and lipase were determined using phenotypic tests according to the previous study (Biswas *et al*., 2014). *Staphylococcus aureus* ATCC 25923 used as positive control.

2.2.5.1. Hemolysin assay

Detection of hemolysin was performed by streaking the colony from overnight culture on 5% human blood agar. After incubation at 35 ± 2 °C for 24 - 48 hrs, the colourless area around the streak was exhibited as the presence of β-hemolytic activity, greenish zones around the streak as α-hemolytic activity and the absence of zones as γ-hemolytic activity (negative hemolysis).

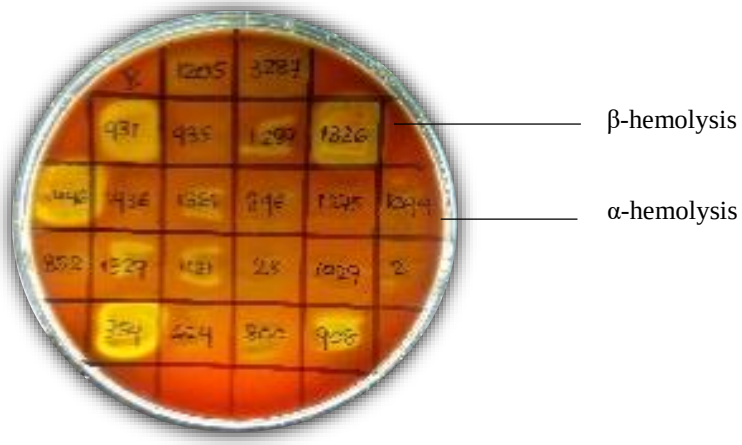


Figure 23. Hemolysis activity of enterococci on blood agar

2.2.5.2. Gelatinase assay

The colony from overnight culture was picked with straight wire loop and stabbed into the 3% gelatin agar tubes. Gelatin tube without inoculum was used as negative control. After incubation at 35 ± 2 °C for 24 hrs, tubes were taken out from the incubator without shaking and put into the refrigerator for 30 mins or until the negative control tube was solidified. After taking out from the refrigerator, liquefaction of cultured media was assumed as gelatinase production of the isolates.

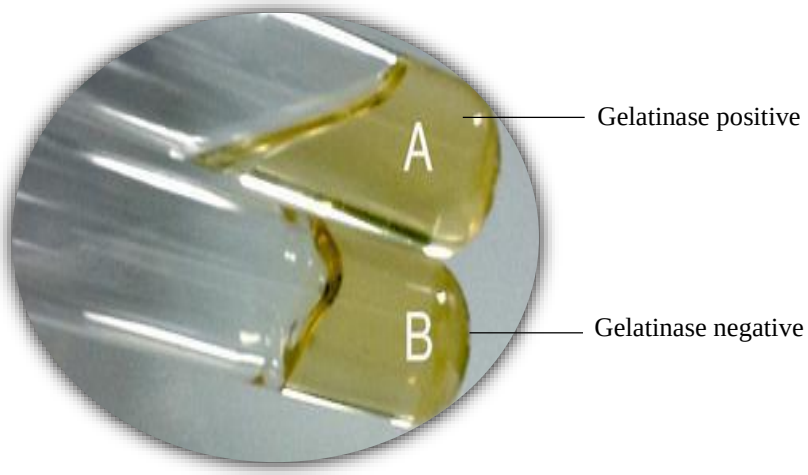


Figure 24. Gelatinase activity of enterococci in gelatin tube

2.2.5.3. Caseinase assay

Casein hydrolysis was detected by streaking the isolates on 3% skimmed milk agar. After incubation at 35 ± 2 °C for 24 hrs, the presence of a colourless zone around the streak was pointed out the caseinase activity.

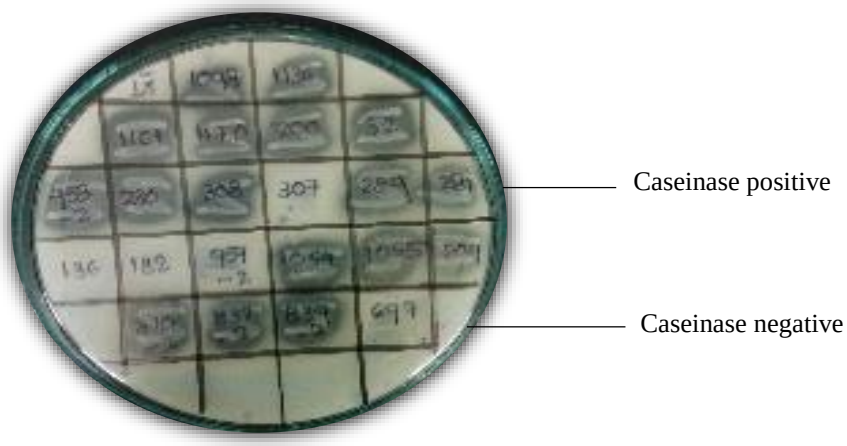


Figure 25. Caseinase activity of enterococci on 3% skimmed milk agar

2.2.5.4. Lipase assay

To determine lipase production, the isolates were streaked on 5% egg-yolk agar and then incubated at 35 ± 2 °C for 24 - 48 hrs. The formation of thin iridescent pearly layer on the streak was recorded as lipase enzyme production.

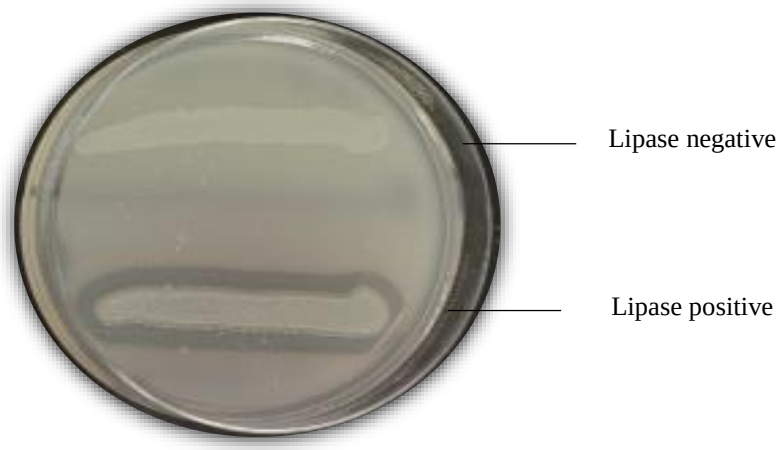


Figure 26. Lipase activity of enterococci on 5% egg yolk agar

2.2.6. Genomic DNA extraction

The inoculum was prepared with 10 ml of BHI broth and incubated at 35 ± 2 ºC overnight. After incubation, the bacterial pellets were harvested by centrifugation at 8,000 rpm for 2 mins and washed with 1x PBS (pH - 7.4). Five different combinations of lysis enzymes with lysozyme, proteinase K and SDS, listed in Table 5 were optimized by systematically altering the variables to extract the genomic DNA from *Enterococcus* isolates. The initial condition of lysis reaction (lysis enzymes combination 1) was established as described previously with some modifications (Depardieu, Perichon, & Courvalin, 2004). After digestion of bacterial cell wall and protein with different combinations of lysis enzymes, the genomic DNA was extracted by adding equal volume of phenol and chloroform (200 µl of each). The genomic DNA was precipitated using twice volumes of ice-cold absolute ethanol with gentle mixing and kept on ice at least 15 mins. The DNA pellet was washed with 70% ethanol and air-dried. The final pellet was dissolved in 60 - 100 µl of sterile deionized water. The activity of 100 µg/ml RNase was tested in two conditions; in the second step of cell lysis and in the last step of elution with 15 mins incubation at 35 ± 2 °C.

Table 5. Phenol-chloroform DNA extraction

Lysis enzymes combination	1st step	Final concentration of enzymes	Incubation time	2nd step	Final concentration of enzyme	Incubation time
1	200 µl 1x TE buffer + 50 µl 10 mg/ml lysozyme	lysozyme - 2 mg/ml	35 ± 2 °C, 30 mins	20 µl 20mg/ml proteinase K + 50 µl 10% SDS	proteinase K - 1.3 mg/ml SDS - 1.6%	60 °C, 30 mins
2	197 µl 1x TE buffer + 23 µl 10 mg/ml lysozyme	lysozyme - 1 mg/ml	35 ± 2 °C, 30 mins	25 µl 1mg/ml proteinase K + 6 µl 40% SDS	proteinase K - 100 µg/ml SDS - 1%	35 ± 2 °C, 30 mins
3	227 µl 1x TE buffer + 23 µl 10 mg/ml lysozyme	lysozyme - 1 mg/ml	35 ± 2 °C, 30 mins	2 µl 20 mg/ml proteinase K	proteinase K - 200 µg/ml	35 ± 2 °C, 30 mins
4	202 µl 1x TE buffer + 23 µl 10 mg/ml lysozyme	lysozyme - 1 mg/ml	35 ± 2 °C, 30 mins	25 µl 10% SDS	SDS - 1%	35 ± 2 °C, 30 mins
5	225 µl 1x TE buffer + 25 µl 10 mg/ml lysozyme	lysozyme - 1 mg/ml	35 ± 2 °C, 30 mins	-	-	-

2.2.7. Analysis of DNA quality

(i) Gel electrophoresis

The quality and purity of DNA checked with gel electrophoresis in 1x TAE buffer and 1% agarose gel with 100V for 45 mins. The gel stained with 0.5 µg/ml ethidium bromide for 1 min and de-stained with distilled water for 30 mins. DNA bands checked under UV light using UV transilluminator (Syngene UV SYDR-1875, Model Gene Genius).

(ii) Spectrophotometry

DNA quality also checked by measuring with Genova nano microvolume spectrophotometer at wavelength 260 nm and 280 nm. The ratio between the readings at 260 nm and 280 nm provided an estimate purity of DNA sample. The value of the ratio between 1.8 and 2.0 suggested as pure DNA. After checking the DNA quality, DNA stored at -20 ºC till further analysis.

2.2.8. Molecular characterization of *Enterococcus* spp. and vancomycin-resistant genes

Multiplex PCR conditions for species identification and vancomycin-resistant genes detection followed from previous study (Kanitta Muangngam, 2017). *E. gallinarum* and *E. casseliflavus* usually harbored $vanC_1$ and $vanC_{2/3}$ genes which were intrinsically low-level resistance to vancomycin. Therefore, $vanC_1$ and $vanC_{2/3}$ gene sequences were used for identification of *E. gallinarum* and *E. casseliflavus*. Molecular characterization of Enterococcus spp. (*E. faecalis, E. faecium, E. gallinarum* and *E. casseliflavus*) and vancomycin-resistant genes (*vanA, vanB, vanD,* and *vanM*) were performed using 3 multiplex PCR sets according to the primer sequences listed in Table 6, master mix condition in Table 7 and PCR conditions in Table 8. Negative control was carried out with deionized water instead of DNA template. Clinical isolates from previous study with known *vanA* and v*anB* gene sequences, *E. faecalis* ATCC 29212, *E. faecium* DMST 14756, *E. casseliflavus* ATCC 25788 and *E. gallinarum*

ATCC 49573 were used as positive controls for tested genes. Moreover, 16S rRNA primer added as internal positive control in all reactions.

Table 6. Primer sequences for *Enterococcus* spp. identification and vancomycin-resistant genes detection

Primer set	Name of primer	Sequence (5' - 3')	Product size (bp)	Genes	References
Set I	*E. faecalis* - F	ACT TAT GTG ACT AAC TTA ACC	360	*sodA*	(Jackson, Fedorka-Cray, & Barrett, 2004)
	E. faecalis - R	TAA TGG TGA ATC TTG GTT TGG			
	E. faecium - F	GAA AAA ACA ATA GAA GAA TTA T	215	*sodA*	(Jackson, Fedorka-Cray, & Barrett, 2004)
	E. faecium - R	TGC TTT TTT GAA TTC TTC TTT A			
	vanA - F	GGG AAA ACG ACA TTG C	732	*vanA*	(Dutka-Malen, Evers, & Courvalin, 1995)
	vanA - R	GAT CAA TGC GGC CGT TA			
	vanB - F	ATG GGA AGC CGA TAG TC	635	*vanB*	(Dutka-Malen, Evers, & Courvalin, 1995)
	vanB - R	GAT TTC GTT CCT CGA CC			
Set II	$vanC_1$ - F	GGT ATC AAG GAA ACC TC	822	$vanC_1$	(Patidar, Gupta, & Singh, 2013)
	$vanC_1$ - R	CTT CCG CCA TCA TAG CT			
	$vanC_{2/3}$ - F	CTC CTA CGA TTC TCT TG	439	$vanC_{2/3}$	(Patidar, Gupta, & Singh, 2013)
	$vanC_{2/3}$ - R	CGA GCA AGA CCT TTA AG			
Set III	*vanD* - F	TGG AAT CAC AAA ATC CGG CG	311	*vanD*	(Nomura, *et al.* 2013)
	vanD - R	TCC CGC ATT TTT CAC AAC			
	vanM - F	GGC AGA GAT TGC CAA CAA CA	425	*vanM*	(Nomura, *et al.* 2013)
	vanM - R	AGG TAA ACG AAT CTG CCG CT			
Internal PCR control	16S - F	CTA GTA ATC GCG GAT CAG CAT	174	16S rRNA	(Okolie, Wooldridge, & Turner, 2015)
	16S - R	GAT ACG GCT ACC TTG TTA CGA CTT			

F, forward primer; R, reverse primer; bp, base pair

Table 7. Preparation of multiplex PCR for *Enterococcus* spp. and vancomycin-resistant genes detection

Component	Final concentration	20 µl reaction (µl)
10x amplification buffer	1x	2.0
25 mM $MgCl_2$	1.5 mM	1.2
10 µM forward primer	0.5 - 2.0 µM*	1.0
10 µM reverse primer	0.5 - 2.0 µM *	1.0
10 µM 16S rRNA - F	0.1 µM	0.2
10 µM 16S rRNA - R	0.1 µM	0.2
Taq DNA polymerase	0.4 U	0.08
Each dNTP	0.2 mM	2.0
Template DNA	100 - 200 ng	1.6
Nuclease-free water	Up to 20 µl	

***** Primer concentration 0.5 µM was used for *E. faecalis* and *vanB*, 1.0 µM for *E. faecium, vanC₁, vanC₂, vanD* and *vanM*, and 2.0 µM for *vanA* gene. For 16S rRNA primer, 0.1 µM was used in all reactions.

Table 8. Condition of multiplex PCR for *Enterococcus* spp. and vancomycin-resistant genes detection

Step	Temperature	Time (mins)	Cycle	Stage
1	95°C	5	1	Initial denaturation
2	95°C	1	30	Denaturation
3	53°C	1		Annealing
4	72°C	1		Elongation
5	72°C	5	1	Final extension

2.2.9. Random amplified polymorphic DNA (RAPD) method

The genetic diversity among VRE isolates determined using RAPD method. The discriminatory power of this method can be enhanced using different random primers. For this reason, RAPD with following four different primers from previous studies were chosen, AB1-15 (Issack *et al*., 1996), AP4 (Andrighetto, *et al*. 2016), AP4 plus ERIC1R (Barbier *et al*., 1996) and M13 (Andrighetto, *et al*. 2016). PCR amplifications were carried out separately according to the corresponding conditions in previous studies, briefly listed in Table 9. Concentration of MgCl2 according to literature and concentration included in PCR buffer; 2.5 mM and 2 mM in AP4 RAPD, 1.5 mM and 2 mM in M13 RAPD and 2 mM and 4 mM in AP4 plus ERIC1R RAPD were evaluated. Reproducibility of RAPD was confirmed by triplicate trial runs using the reference strains, *E. faecalis* ATCC 29212 and *E. faecium* DMST 14756. Moreover, duplicate PCR for all VRE strains with independent preparation were conducted in AP4 plus ERIC1R and AB1-15 RAPD. Master mix without DNA template was added in all reactions as negative control. RAPD products were electrophoresed at 100 V for 7 mins (initial run) and at 55 V for 130 mins (for better separation) with 1.5% agarose gel in 1x TBE buffer. One kb DNA ladder was used as a molecular size standard. Gel was stained in 0.5 µg/ml of ethidium bromide for 5 mins and de-stained with distilled water for 1 hr. Gel images were captured using UV transilluminator (Syngene UV SYDR-1875, Model Gene Genius).

Table 9. PCR conditions for RAPD with four different primers

	RAPD primer set			
	AB1-15	AP4	AP4 plus ERIC1R	M13
Reference	(Issack *et al.*, 1996)	(Akpaka *et al.*, 2016) (Andrighetto, *et al.* 2016)	(Barbier *et al.*, 1996)	(Andrighetto, *et al.* 2016)
Primer sequence	5' GGA GGG TGT T 3'	5' AAG AGC CCG T 3'	AP4 - 5' AAG AGC CCG T 3' ERIC1R – 5' ATG TAA GCT CCT GGG GAT TCA C 3'	5' GAG GGT GGC GGT TCT 3'
PCR preparation				
PCR buffer	1x	1x	1x	1x
$MgCl_2$	2 mM	2 mM/2.5 mM	2 mM/ 4mM	1.5 mM/ 2 mM
dNTPs	200 µM	200 µM	200 µM	200 µM
Ex Taq polymerase (Takara)	2.5 U	1 U	0.65 U	1 U
primer	0.3 µM	2 µM	0.4 µM (AP4) and 0.1 µM (ERIC1R)	0.2 µM
DNA template	20 ng	20 ng	20 ng	100 ng
D/W	up to 20 µl	up to 20 µl	up to 20 µl	up to 20 µl
PCR condition				
Initial denaturation	94°C, 2 mins	94°C, 2 mins	94°C, 4 mins	94°C, 5 mins
No. of cycles	50	45	44	35
Denaturation	94°C, 5 secs	94°C, 1 min	94°C, 30 secs	94°C, 1 min
Annealing	36°C, 30 secs	36°C, 1 min	35°C, 1 min	40°C, 20 secs
Elongation	72°C, 1 min	72°C, 2 mins	72°C, 2 mins	72°C, 2 mins
Final extension	72°C, 10 mins	72°C, 5 mins	72°C, 7 mins	72°C, 5 mins

(i) Computer analysis of banding patterns from RAPD PCR

Gel images obtained by RAPD analysis were automatically identified with the assist of the Bionumeric software version 7.6 (Applied Maths, Austin, TX, USA). Dice correlation coefficient and cluster analysis by the unweighted pair group method with arithmetic average (UPGMA) were used to compare the banding patterns and strain grouping coefficients of similarity.

(ii) Calculating diversity index (D.I)/ discriminatory power (D.P)

The D.I/ D.P of each RAPD method estimated by Simpson's diversity index (Struelens, M.J. *et. al*, 1996). The D.I is the probability of tested isolates will distinguish by that typing method. D.I depends on the number of groups and the homogeneity of the frequency distribution of isolates into groups. According to the

guidelines, a typing system should achieve a D.I of >0.95 for reliable assessment of the clonal relatedness of isolates.

The following equation was used to calculate the D.I,

$$D.I = 1 - \frac{1}{N\,(N-1)} \sum_{j=1}^{S} nj\,(nj-1)$$

N = the total number of strains

nj = the number of strains belonging to the j^{th} type

2.2.10. Statistical analysis

Comparison of the associations between the data especially species, sources, antibiotic resistance, biofilm formation and virulence factors activities were analyzed by the Chi-square test or Fisher's exact test based on data. *p*-value of <0.05 was considered as the statistically significant level with confidence interval of 95% and that of <0.001 was considered as highly significant with confidence interval of 99.9%. All statistical analyses were performed using the SPSS software version 23 (SPSS Inc, Chicago, IL, USA).

CHAPTER 3

RESULTS

3.1. Identification of *Enterococcus* spp.

3.1.1. Gram stain and biochemical tests

Gram-positive short-chain appearance under oil immersion microscope, presented in Figure 27 revealed in isolates. No bubbles in 3% hydrogen peroxide that indicated catalase test negative (Figure 28). Black esculetin deposited on the slant of bile esculin agar tube (Figure 29).

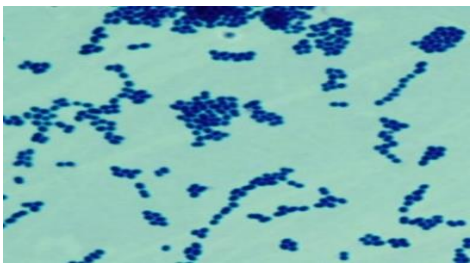


Figure 27. *E. faecalis* appearance in Gram stain under oil immersion (100x) microscope

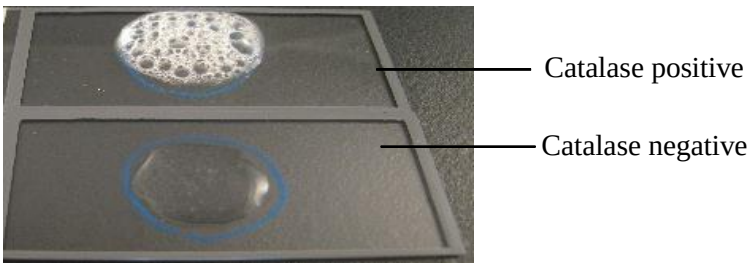


Figure 28. Catalase activity in slide method

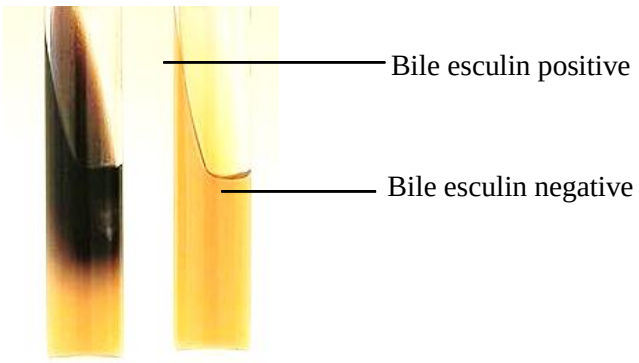


Figure 29. Hydrolysis of esculin in bile esculin slant tube

3.1.2. Optimization of enzymes concentrations in phenol-chloroform DNA extraction method

When four different concentrations of lysis enzymes were optimized in extraction method, the most suitable enzyme combination was combination 4 (1 mg/ml lysozyme and 1% SDS enzyme). This protocol let to have a necessary amount of DNA template without producing smear band in most cases (see Figure 30 B, Lane 3). When 100 µg/ml RNase was added in 100 µl of eluted DNA, both DNA and RNA were degraded without appearing bands in gel photo (Figure 30 C, Lane 2). When 100 µg/ml RNase was added in SDS lysis step and stopped the RNase activity incubating at 70 °C for 15 mins, RNA free DNA elute was achieved as shown in Figure 30 C, Lane 1.

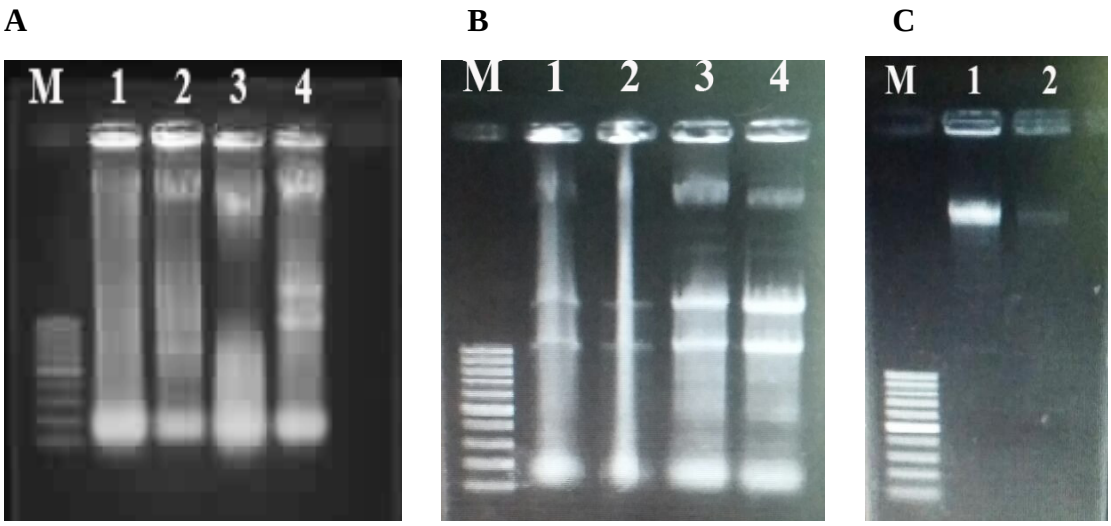


Figure 30. Gel electrophoresis of lysis enzymes and RNAse enzyme optimization in DNA extraction

A. Gel photo of initial DNA extraction according to the previous study
   From left to right, Lane M; 100 bp DNA marker, Lane 1 to 4; *Enterococcus* ATCC 29212

B. Gel photo of lysis enzymes optimization
   From left to right, Lane M; 100 bp DNA marker, Lane 1; enzymes combination of lysozyme, proteinase K and SDS, Lane 2; enzymes combination of lysozyme and proteinase K, Lane 3; enzymes combination of lysozyme and SDS, Lane 4; only lysozyme enzyme

C. Gel photo of RNase enzyme optimization
   From left to right, M; 100 bp DNA marker, Lane 1; 100 µg/ml RNase added in lysis condition, Lane 2; 100 µg/ml RNase added in elution

3.1.3. Prevalence rate of *Enterococcus* spp. among clinical isolates

Gel photos of two multiplex PCR sets for the identification of four *Enterococcus* spp. and two vancomycin-resistant genes presented in Figure 31 and 32. Species distributions in urine, pus and blood sources were comparable in two hospitals, presented in Table 10. All 12 body fluid and 5 sputum samples obtained from HTY. *E. faecalis* was significantly more isolated among five different sources followed by *E. faecium* and other *Enterococcus* spp. ($p<0.05$). One *E. casseliflavus* and one *E. gallinarum* isolate from blood samples observed from SS.

Table 10. Distribution of *Enterococcus* spp. among different sources in two hospitals

Source of specimen	Total numbers of isolates		Number of isolates (%)											
			E. faecalis		*E. faecium*		*E. casseliflavus*		*E. gallinarum*		Other *Enterococcus* spp.		*p*-value	
	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY
Urine	406	187	279 (68.7)	138 (73.8)	126 (31)	49 (26.2)	-	-	-	-	1 (0.2)	-		
Pus	77	54	64 (83.1)	48 (88.9)	10 (13)	4 (7.4)	-	-	-	-	3 (3.9)	2 (3.7)		
Blood	29	14	19 (65.5)	13 (92.9)	6 (20.7)	1 (7.1)	1 (3.5)	-	1 (3.5)	-	2 (6.9)	-	0.001	0.02
Fluid	-	12	-	9 (75)	-	3 (25)	-	-	-	-	-	-		
Sputum	-	5	-	4 (80)	-	1 (20)	-	-	-	-	-	-		
Total	512	272	362 (70.7)	212 (77.9)	142 (27.7)	58 (21.3)	1 (0.2)	-	1 (0.2)	-	6 (1.2)	2 (0.7)		

SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hospital

The correlation between sources and species distribution was analyzed using Chi-square test. *p*-value ≤0.001 was regarded as highly significance and *p*-value <0.05 as significance.

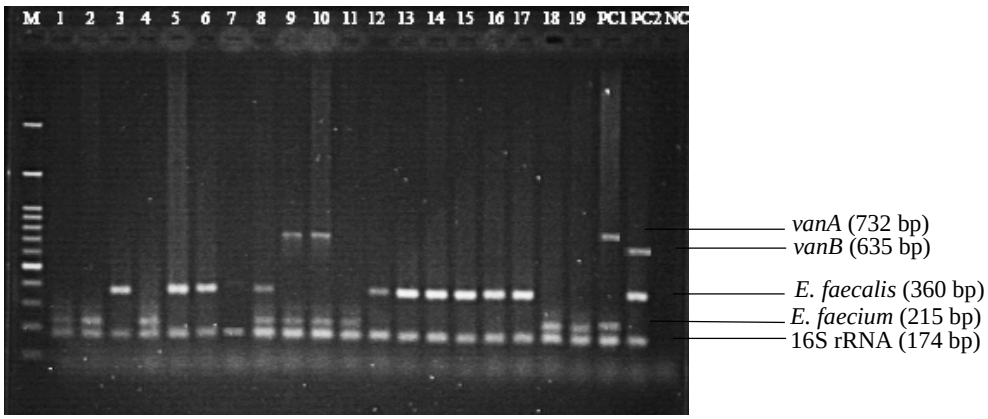


Figure 31. Gel electrophoresis of vancomycin-resistant genes (*vanA* and *vanB*) and *Enterococcus* spp. genes (*E. faecalis* and *E. faecium*) detection
From left to right: Lane M, 100 bp DNA marker; Lane 1 to 19, enterococci clinical isolates; Lane PC1, positive control of *E. faecium* (215 bp) and *vanA* gene (732 bp); Lane PC2, positive control of *E. faecalis* (360 bp) and *vanB* gene (635 bp); Lane NC, negative control

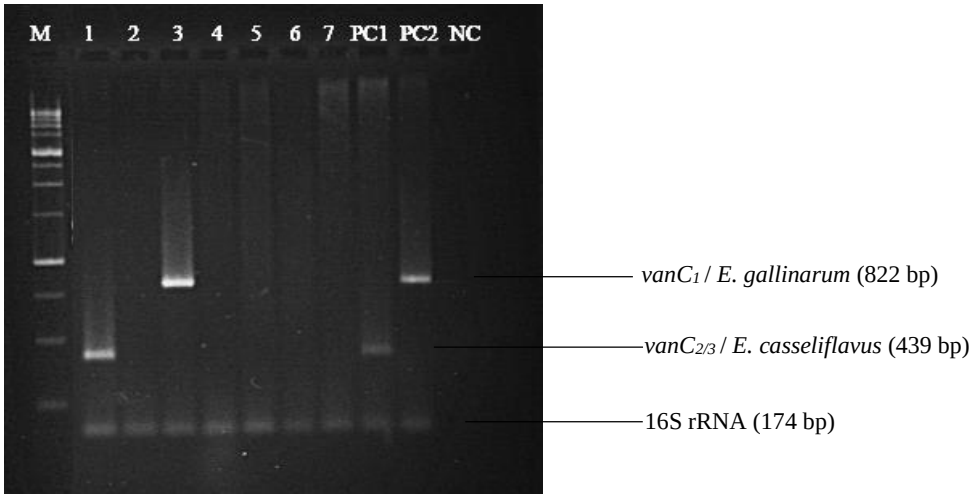


Figure 32. Gel electrophoresis of *E. casseliflavus* and *E. gallinarum* spp. detection
From left to right: Lane M, 1 kbp DNA marker; Lane 1 to 7, enterococci clinical isolates; PC1, *E. gallinarum* ATCC 49573 (822 bp); PC2, *E. casseliflavus* ATCC 25788 (439 bp); NC, negative control

3.2. Antibiotic-resistant patterns among enterococci isolates

The comparative antibiotic resistance patterns from two hospitals were summarized in Table 11. The majority resistant antibiotics were erythromycin (85.2% and 75%), tetracycline (76.8% and 77.6%) and ciprofloxacin (71.9% and 72.8%) in SS and HTY, respectively. Isolates from SS and HTY were resistant to vancomycin as the least (4.7% and 5.1%). A statistically higher prevalence of resistance to tested antibiotics except high-level aminoglycosides, chloramphenicol and tetracycline in *E. faecium* isolates was exhibited than that in *E. faecalis* isolates in both hospitals ($p<0.05$). Non-*faecalis* non-*faecium* isolates were less resistant to antibiotics. *E. casseliflavus* isolate was susceptible to all tested antibiotics where *E. gallinarum* isolate was resistant to chloramphenicol and tetracycline.

Regarding the combination of aminoglycoside with β-lactams or glycopeptides as essential synergistic antibiotics for enterococci infections, high-level gentamicin (HLGR), high-level streptomycin (HLSR), resistance to β-lactams and glycopeptides were studied as presented in the Table 11. HLGR was found in 64.6% and 58.5% of *E. faecalis* and 51.4% and 48.3% of *E. faecium* isolates in SS and HTY, respectively. Similar distribution of HLSR in *E. faecalis* isolates from SS and HTY was found with 64.4% and 58% where HLSR *E. faecium* isolates were more common in HTY (65.5%) than in SS (24.6%). Resistance to β-lactams antibiotics (ampicillin and penicillin) in SS and HTY were less than 50% in *E. faecalis* and more than 75% in *E. faecium* isolates.

Analyzing the resistant prevalence among sources, isolates from urine samples in both hospitals were the most resistant to antibiotics (except vancomycin and teicoplanin in SS and high-level gentamicin, chloramphenicol and ciprofloxacin in HTY) followed by blood and pus origins (Table 11). The prevalence of antibiotic resistance except vancomycin and teicoplanin from different sources in SS were statistically significant ($p<0.05$) while the resistance rates of antibiotics except penicillin, high-level streptomycin and erythromycin from different sources in HTY were not different ($p>0.05$).

Table 11. Incidence of antibiotic-resistant enterococci isolates in two hospitals

Species	Source	Tested strains No.		AMP (%)		P (%)		CN (%)		S (%)		VAN (%)		TEC (%)	
		SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY
E. faecalis	Urine	279	138	38 (13.6)	36 (26.1)	159 (57.0)	56 (40.6)	200 (71.7)	84 (60.9)	199 (71.3)	92 (66.7)	6 (2.2)	3 (2.2)	6 (2.2)	3 (2.2)
	Pus	64	48	7 (10.9)	9 (18.8)	14 (21.9)	11 (22.9)	24 (37.5)	22 (45.8)	25 (39.1)	20 (41.7)	-	2 (4.2)	-	2 (4.2)
	Blood	19	13	2 (10.5)	3 (23.1)	6 (31.6)	5 (38.5)	10 (52.6)	8 (61.5)	9 (47.4)	3 (23.1)	-	-	-	-
	Fluid	-	9	-	2	-	4	-	6	-	4	-	1	-	1
	Sputum	-	4	-	-	-	1	-	4	-	3	-	-	-	-
E. faecium	Urine	126	49	116 (92.1)	39 (79.6)	121 (96.0)	42 (85.7)	68 (54.0)	23 (46.9)	32 (25.4)	32 (65.3)	15 (11.9)	7 (14.3)	15 (11.9)	7 (14.3)
	Pus	10	4	8 (80.0)	2 (50.0)	8 (80.0)	2 (50.0)	3 (30.0)	2 (50.0)	2 (20.0)	3 (75.0)	1 (10.0)	1 (25.0)	1 (10.0)	1 (25.0)
	Blood	6	1	4 (66.7)	1 (100.0)	5 (83.3)	1 (100.0)	2 (33.3)	1 (100.0)	1 (16.7)	1 (100.0)	-	-	-	-
	Fluid	-	3	-	2	-	2	-	2	-	2	-	-	-	-
	Sputum	-	1	-	1	-	1	-	-	-	-	-	-	-	-
E. casseliflavus	Urine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	-	-	-	-	-	-	-	-	1	-	-	-
E. gallinarum	Urine	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	-	-	-	-	-	-	-	-	1	-	-	-
Enterococcus spp.	Urine	1	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	3	2	-	1	-	1	-	-	-	1	-	-	-	-
	Blood	2	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	512	272	175 (34.2)	96 (35.3)	313 (61.1)	126 (46.3)	307 (60.0)	152 (55.9)	268 (52.3)	161 (59.2)	24 (4.7)	14 (5.1)	22 (4.3)	14 (5.1)
	p-value (species)			0.001	0.001	0.001	0.001	0.005	0.120	0.001	0.482	0.001	0.001	0.001	0.001
	p-value (sources)			0.002	0.15	0.001	0.017	0.001	0.333	0.001	0.003	0.285	0.859	0.285	0.859

Species	Source	Tested strains No.		C (%)		CIP (%)		E (%)		TE (%)		MDR (%)	
		SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY
E. faecalis	Urine	279	138	174 (62.4)	59 (42.8)	202 (72.4)	99 (71.7)	245 (87.8)	106 (76.8)	241 (86.4)	115 (83.3)	227 (81.4)	112 (81.2)
	Pus	64	48	17 (26.6)	14 (29.2)	21 (32.8)	28 (58.3)	39 (60.9)	27 (56.3)	41 (64.1)	36 (75.0)	32 (50.0)	29 (60.4)
	Blood	19	13	11 (57.9)	7 (53.8)	9 (47.4)	10 (76.9)	15 (78.9)	9 (69.2)	17 (89.5)	10 (76.9)	12 (63.2)	10 (76.9)
	Fluid	-	9	-	3	-	6	-	5	-	5	-	5
	Sputum	-	4	-	1	-	3	-	4	-	4	-	4
E. faecium	Urine	126	49	16 (12.7)	10 (20.4)	122 (96.8)	45 (91.8)	122 (96.8)	45 (91.8)	82 (65.1)	32 (65.3)	125 (99.2)	49 (100.0)
	Pus	10	4	-	2 (50.0)	9 (90.0)	3 (75.0)	9 (90.0)	4 (100.0)	4 (40.0)	4 (100.0)	8 (80.0)	4 (100.0)
	Blood	6	1	1 (16.7)	-	5 (83.3)	1 (100.0)	5 (83.3)	1 (100.0)	4 (66.7)	1 (100.0)	5 (83.3)	1 (100.0)
	Fluid	-	3	-	1	-	2	-	3	-	2	-	3
	Sputum	-	1	-	-	-	1	-	-	-	1	-	1
E. casseliflavus	Urine	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	-	-	-	-	-	-	-	-	-	-
E. gallinarum	Urine	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	1	-	-	-	-	-	1	-	-	-
Enterococcus spp.	Urine	1	-	-	-	-	-	-	-	1	-	-	-
	Pus	3	2	-	-	-	-	-	-	1	1	-	1
	Blood	2	-	-	-	-	-	1	-	1	-	-	-
	Total	512	272	220 (43.0)	97 (35.7)	368 (71.9)	198 (72.8)	436 (85.2)	204 (75.0)	393 (76.8)	211 (77.6)	409 (79.9)	219 (80.5)
	p-value (species)			0.001	0.061	0.001	0.001	0.001	0.003	0.001	0.001	0.001	0.573
	p-value (sources)			0.001	0.415	0.001	0.067	0.001	0.037	0.004	0.744	0.001	0.379

SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hospital; AMP, ampicillin; P, penicillin; CN, gentamicin; S, streptomycin; VAN, vancomycin; TEC, tecoplanin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; TE, tetracycline

The correlation between antibiotic resistance and species distribution was analyzed using Chi-square test. *p*-value ≤0.001 was regarded as highly significance and *p*-value <0.05 as significance.

3.2.1. Incidence of multidrug resistance (MDR)

The prevalence of multidrug resistance (MDR) (at least 3 antibiotics class resistance) was high in both hospitals, 79.9% in SS and 80.5% in HTY, respectively (Table 11). The incidence of multidrug resistance among species distribution or source distribution in two hospitals were comparable. The resistance patterns in MDR isolates from two hospitals were diverse with 52 patterns in SS and 57 patterns in HTY. Furthermore, multidrug resistance was more prevalent in *E. faecium* isolates (97.2% and 100%) compared to *E. faecalis* isolates (74.9% and 75.7%) in SS and HTY.

Regarding the infection sites, the most incidence of multidrug resistance isolates in SS and HTY were from urine origins (86.7% and 86.1%), followed by blood (58.6% and 78.6%) and pus (50.6% and 59.3%), respectively. All five sputum isolates and 9 out of 12 body fluid isolates from HTY were also revealed multidrug resistance.

The most frequent MDR pattern in isolates from SS was TE-E-CIP-C-S-CN-P (20.3%, 104/512) including urine isolates (23.2%, 94/406), pus (6.5%, 5/77), and blood (17.2%, 5/29), respectively. Among MDR isolates from HTY, the most frequent pattern was TE-E-CIP-C-S-CN (10.3%, 28/272) including urine (10.2%, 19/187), pus (13%, 7/54), blood (7.1%, 1/14), and fluid (8.3%, 1/12).

3.2.2. Phenotypic and genotypic characterization of vancomycin-resistant enterococci isolates

Vancomycin resistance was observed in 6 *E. faecalis*, 16 *E. faecium*, 1 *E. casseliflavus* and 1 *E. gallinarum* isolates among 512 enterococci isolates from SS. Similarly, vancomycin resistance was revealed in 2 *E. faecalis* and 12 *E. faecium* isolates from HTY. Species diversity of vancomycin resistant isolates among different sources was presented in Table 12.

The results of disc diffusion and vancomycin screening methods were indifferent in *E. faecalis* and *E. faecium* isolates. However, *E. casseliflavus* and *E. gallinarum* isolates grown on vancomycin screening agar which were susceptible in disc diffusion method with 20 mm zone diameter (Susceptible $\geq$ 17 mm). All vancomycin-resistant *E. faecalis* and *E. faecium* strains were resistant to teicoplanin,

whereas *E. gallinarum* and *E. casseliflavus* strains were susceptible. Broth microdilution method to determine MIC of vancomycin was conducted in all 38 vancomycin-resistant enterococci isolates which were grown on vancomycin screening agar. Various vancomycin MIC in VRE. *faecalis* and VRE. *faecium* isolates were presented in Table 12. The highest MIC of vancomycin in *E. faecalis* isolates from two hospitals was 512 µg/ml where that in *E. faecium* isolates was 1,024 µg/ml. *E. casseliflavus* and *E. gallinarum* isolates were low-level resistance to vancomycin with 8 µg/ml in MIC.

The vancomycin resistant genes including *vanA*, *vanB*, and *vanC*$_1$/*C*$_2$ were significantly incident in enterococci isolates from two hospitals (p<0.001), presented in Table 12. Among high-level vancomycin resistant isolates, *vanA gene* was carried in 32 out of 36 isolates from two hospitals. *vanB* gene was not observed in VRE isolates. When *vanD* and *vanM* genes were detected in four high-level vancomycin resistant isolates carried non-*vanA* non-*vanB* genes, there was no band in these VRE isolates. *E. casseliflavus* and *E. gallinarum* isolates from SS were showed intrinstic *vanC*$_1$/*C*$_2$ genes.

Except one *E. faecalis* isolate from pus origin and low-level vancomycin-resistant *E. casseliflavus* and *E. gallinarum*, 35 out of 38 VRE isolates were multidrug resistance. All VRE isolates were susceptible to linezolid and tigecycline in disc diffusion and broth microdilution methods as presented in Table 12.

3.2.2.1. Correlation between vancomycin-resistant phenotype and corresponding-resistant genes

Susceptibility test for vancomycin antibiotic in enterococci strains showed the significant correlation with *van* genes (p<0.001). High-level vancomycin-resistant *E. faecalis* and *E. faecium* isolates (88.9%) were correlated with *vanA* gene. Low-level MIC showing *E. casseliflavus* and *E. gallinarum* isolates were completely corresponded with *vanC* gene.

Table 12. Phenotypic and genotypic characters of vancomycin-resistant enterococci isolates from two hospitals (A) Sunpasitthiprasong Hospital, (B) Hat Yai Hospital

(A)

No.	Code	Species	Collection date	Source	Genotype	Phenotype		MIC (µg/ml)			Biofilm	Virulence factors	Resistance pattern
						VAN	TEC	VAN	LZD	TGC			
1	SS0002	*E. faecalis*	Jun, 2017	Urine	*vanA*	R	R	512	2	0.125	Weak	γ, Cas	AMP, P, CIP, E, TE, F, RD
2	SS0014	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	512	2	0.125	Weak	γ, Cas	AMP, P, CN, CIP, E, TE, F, RD
3	SS0050	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	1024	2	0.125	Weak	γ, Cas	AMP, P, CN, CIP, E, TE, F, RD
4	SS0066	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	1024	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
5	SS0086	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	1024	2	0.125	Weak	γ	AMP, P, CN, CIP, E, F, RD, QD
6	SS0090	*E. casseliflavus*	Jun, 2017	Blood	$vanC_2$	S	S	8	2	0.125	Weak	γ , Cas	-
7	SS0118	*E. gallinarum*	Jun, 2017	Blood	$vanC_1$	S	S	8	2	0.125	Weak	γ	C, TE
8	SS0126	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	512	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
9	SS0159	*E. faecium*	Jun, 2017	Urine	*vanA*	R	R	512	2	0.125	Weak	γ	AMP, P, CIP, E, TE, F, RD
10	SS0171	*E. faecalis*	Jul, 2017	Urine	*vanA*	R	R	128	2	0.065	Weak	γ, Gel, Cas	AMP, P, CN, CIP, E, TE, F, RD, QD
11	SS0172	*E. faecium*	Jul, 2017	Urine	*vanA*	R	R	256	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
12	SS0190	*E. faecalis*	Jul, 2017	Urine	*vanA*	R	R	128	2	0.25	Weak	α, Gel, Cas	AMP, P, CN, S, C, CIP, E, TE, QD
13	SS0203	*E. faecium*	Jul, 2017	Urine	*vanA*	R	R	256	2	0.25	Weak	α, Cas	AMP, P, CN, CIP, E, F, RD, QD
14	SS0253	*E. faecalis*	Aug, 2017	Urine	-	R	R	512	2	0.125	Moderate	α , Gel, Cas	S, CIP, E, RD, Q/D
15	SS0257	*E. faecalis*	Sep, 2017	Urine	-	R	R	256	2	0.125	Weak	α , Gel, Cas	E, TE, F, RD
16	SS0321	*E. faecalis*	Sep, 2017	Urine	*vanA*	R	R	256	2	0.125	Weak	γ, Cas	AMP, P, CN, S, CIP, E, RD, QD
17	SS0323	*E. faecalis*	Sep, 2017	Urine	*vanA*	R	R	128	2	0.25	Weak	α, Cas	AMP, P, CN, S, C, CIP, E, TE, RD, QD
18	SS0330	*E. faecium*	Sep, 2017	Urine	*vanA*	R	R	128	2	0.25	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
19	SS0335	*E. faecium*	Sep, 2017	Urine	*vanA*	R	R	256	2	0.125	Moderate	γ	AMP, P, CN, S, CIP, E, TE, F, RD
20	SS0375	*E. faecium*	Dec, 2017	Urine	*vanA*	R	R	128	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
21	SS0376	*E. faecium*	Dec, 2017	Pus	*vanA*	R	R	1024	2	0.25	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
22	SS0401	*E. faecium*	Dec, 2017	Urine	*vanA*	R	R	128	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD
23	SS0419	*E. faecium*	Dec, 2017	Urine	*vanA*	R	R	256	2	0.125	Weak	γ	AMP, P, CN, CIP, E, TE, F, RD, QD
24	SS0481	*E. faecium*	Jan, 2018	Urine	*vanA*	R	R	128	2	0.125	Weak	γ	AMP, P, CN, S, CIP, E, TE, F, RD

(B)

No.	Code	Species	Collection date	Source	genotype	phenotype		MIC (μg/ml)			Biofilm	Virulence factors	Resistance pattern
						VAN	TEC	VAN	LZD	TGC			
1	HTY0028	*E. faecium*	Jan, 2015	Urine	*vanA*	R	R	512	2	0.125	Moderate	α, Gel, Cas	AMP, P, S, CIP, F, RD, QD
2	HTY0074	*E. faecium*	Feb, 2015	Pus	*vanA*	R	R	512	2	0.125	Weak	α, Lip	CN, CIP
3	HTY0085	*E. faecium*	Feb, 2015	Pus	*vanA*	R	R	256	2	0.125	Weak	α, Lip	AMP, P, CIP, E, TE, F, RD, QD
4	HTY0088	*E. faecalis*	Feb, 2015	Pus	-	R	R	512	2	0.125	Moderate	α	CIP
5	HTY0175	*E. faecalis*	May, 2015	Urine	*vanA*	R	R	512	2	0.125	Weak	α	AMP, P, CN, S, CIP, E, TE, RD, QD
6	HTY0195	*E. faecium*	May, 2015	Urine	-	R	R	512	2	0.125	Weak	α, Lip	AMP, P, CIP, E, F, RD, QD
7	HTY0196	*E. faecalis*	May, 2015	Urine	*vanA*	R	R	512	2	0.125	Weak	α	AMP, P, CN, S, CIP, E, TE, RD, QD
8	HTY0206	*E. faecium*	May, 2015	Urine	*vanA*	R	R	512	2	0.125	Moderate	α	AMP, P, CN, S, CIP, E, TE, RD, QD
9	HTY0207	*E. faecium*	May, 2015	Urine	*vanA*	R	R	512	2	0.125	Weak	α	AMP, P, CN, S, CIP, E, TE, RD, QD
10	HTY0254	*E. faecium*	Jun, 2015	Urine	*vanA*	R	R	1024	1	0.065	Moderate	α	AMP, P, S, CIP, E, RD
11	HTY0256	*E. faecium*	Jun, 2015	Urine	*vanA*	R	R	1024	2	0.065	Strong	β	AMP, P, S, CIP, E, RD, QD
12	HTY0164	*E. faecium*	Jul, 2015	Urine	*vanA*	R	R	256	2	0.125	Moderate	α	CIP, E, TE, F, RD
13	HTY0325	*E. faecalis*	Aug, 2015	Fluid	*vanA*	R	R	128	2	0.065	Moderate	α, Cas	AMP, P, CN, CIP, E, RD, QD
14	HTY0815	*E. faecium*	Dec, 2017	Urine	*vanA*	R	R	128	2	0.25	Weak	γ, Cas	AMP, P, CN, S, CIP, E, F, RD, QD

R, resistance; S, sensitive; AMP, ampicillin; P, penicillin; CN, gentamicin; S, streptomycin; VAN, vancomycin; TEC, tecoplanin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; TE, tetracycline; F, nitrofurantoin; LZD, linezolid; RD, rifampin; TGC, tigecycline, QD, quinipristin/dalfopristin, α, α-hemolysin; β, β-hemolysin; γ, γ-hemolysin; Gel, gelatinase; Cas, caseinase; Lip, lipas

3.3. Biofilm formation in enterococci isolates

Biofilm producing activities of enterococci isolates were calculated from triplicate results of each isolate and described as strong, moderate, weak or non-biofilm formation. Strong and moderate biofilm were more produced in isolates from SS (40.8%) than isolates from HTY (34.2%). When association of biofilm formation and species distribution were analyzed, *E. faecalis* isolates from SS and HTY (45.9% and 36.5%) were significantly more produced biofilm than *E. faecium* isolates (28.9% and 24.2%) ($p<0.05$). Among 10 non-*faecalis* non-*faecium* isolates, strong and moderate biofilm were produced in only two isolates from SS as presented in Table 13.

Biofilm formation was significantly associated with sources in two hospitals ($p<0.05$) as shown in Table 13. Isolates from urine (44.1% and 42.2%) were the most likely to exhibit biofilm formation in SS and HTY, followed by isolates from pus (27.3% and 14.8%) and blood (31.0% and 14.3%), respectively. In addition, biofilm was formed in 4 out of 12 body fluid isolates and 1 out of 5 sputum isolates from HTY.

Table 13. Incidence of biofilm formation and virulence factors activities in enterococci isolates

Species	Source	Tested strains No.		Biofilm				Hemolysin				Gelatinase		Caseinase		Lipase	
				Strong		Moderate		α		β							
		SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY	SS	HTY
E. faecalis	Urine	279	138	19 (6.8)	24 (17.4)	121 (43.4)	35 (25.4)	132 (47.3)	91 (65.9)	57 (20.4)	36 (26.1)	164 (58.8)	40 (29.0)	203 (72.8)	69 (50.0)	4 (1.4)	21 (15.2)
	Pus	64	48	1 (1.6)	1 (2.1)	17 (26.6)	6 (12.5)	13 (20.3)	26 (54.2)	20 (31.3)	14 (29.2)	39 (60.9)	17 (35.4)	42 (65.6)	21 (43.8)	3 (4.7)	5 (10.4)
	Blood	19	13	2 (10.5)	1 (7.7)	6 (31.6)	1 (7.7)	7 (36.8)	10 (76.9)	4 (21.1)	2 (15.4)	12 (63.2)	2 (15.4)	13 (68.4)	6 (46.2)	-	-
	Fluid	-	9	-	-	-	3	-	8	-	-	-	4	-	7	-	1
	Sputum	-	4	-	-	-	1	-	1	-	1	-	2	-	1	-	-
E. faecium	Urine	126	49	16 (12.7)	11 (22.4)	22 (17.5)	9 (18.4)	21 (16.7)	29 (59.2)	1 (0.8)	8 (16.3)	18 (14.3)	4 (8.2)	20 (15.9)	14 (28.6)	1 (0.8)	11 (22.4)
	Pus	10	4	1 (10.0)	-	1 (10.0)	1 (25.0)	1 (10.0)	3 (75.0)	1 (10.0)	1 (25.0)	2 (20.0)	-	1 (10.0)	1 (25.0)	-	2 (50.0)
	Blood	6	1	-	-	1 (16.7)	-	-	1 (100.0)	-	-	1 (16.7)	-	1 (16.7)	-	-	-
	Fluid	-	3	-	-	-	1	-	1	-	-	-	1	-	3	-	-
	Sputum	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-	-
E. casseliflavus	Urine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-
E. gallinarum	Urine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Blood	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Enterococcus spp.	Urine	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Pus	3	2	-	-	-	-	-	-	-	1	1	-	-	-	-	-
	Blood	2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Total	512	272	39 (7.6)	37 (13.6)	168 (32.6)	57 (21.0)	174 (33.8)	174 (64.0)	83 (16.1)	63 (23.2)	237 (46.0)	70 (25.7)	281 (54.6)	122 (44.9)	8 (1.6)	40 (14.7)
	p-value (species)			0.001	0.585	0.001	0.585	0.001	0.501	0.001	0.501	0.001	0.539	0.001	0.420	0.889	0.341
	p-value (sources)			0.012	0.001	0.012	0.001	0.002	0.620	0.002	0.620	0.874	0.688	0.289	0.830	0.176	0.411

SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hospital

The correlation between biofilm formation and species distribution was analyzed using Chi-square test. *p*-value ≤0.001 was regarded as highly significance and *p*-value <0.05 as significance.

3.4. Secreted virulence factors producing in enterococci isolates

Phenotypic activities of virulence factors such as hemolysin, caseinase, gelatinase and lipase in clinical isolates were studied. Hemolysin activities in isolates from HTY (86.0%) were the most prevalent virulent determinants among enterococci isolates where lipase activity in isolates from SS (1.6%) was the least virulent determinant. Gelatinase and caseinase enzymes were more produced in isolates from SS than isolates from HTY where hemolysin and lipase were less produced in isolates from SS than isolates from HTY as presented in Table 13. Very low percentage of lipase enzyme was produced in enterococci isolates from two hospitals. However, ten times higher rate of lipase activities was produced in isolates from HTY (14.7%) than isolates from SS (1.6%).

Regarding the virulence activities according to species, tested virulence factors except lipase enzyme were more significantly secreted in *E. faecalis* isolates than *E. faecium* isolates in SS ($p<0.05$). However, virulence factors producing isolates from HTY were not statistically significant in species distribution, even gelatinase and lipase were more secreted in *E. faecalis* isolates than *E. faecium* isolates in HTY ($p>0.05$), as presented in Table 13. Secreted virulence factors were less produced in non-*faecalis* non-*faecium* isolates.

Analyzing the virulence factors activities among different sources, there was no statistically significance between the production of secreted virulence factors (except hemolysin activity in isolates from SS) and origins of isolates ($p>0.05$), as presented in Table 13.

3.5. Correlation of antibiotic resistance, biofilm formation and virulence factors activities in enterococci isolates

Table 14 was represented the relationship between antibiotic resistance and biofilm formation or virulence factors activities. Among antibiotic-resistant isolates from SS, biofilm producing isolates were more resistant to tested antibiotics except ampicillin, vancomycin and teicoplanin than non-biofilm producing isolates. Similarly, resistance to tested antibiotics except ampicillin and penicillin were more prevalent in biofilm producers than non-producers in HTY. Generally, the frequency of hemolysin, gelatinase and caseinase activities was higher in the isolates from SS resistant to high-level gentamicin, high-level streptomycin, chloramphenicol, erythromycin and tetracycline compared to isolates not expressing virulence factors. Similarly, isolates producing hemolysin, gelatinase and caseinase from HTY were more resistant to high-level gentamicin, high-level streptomycin, chloramphenicol, ciprofloxacin and erythromycin than non-producing isolates. Moreover, hemolysin producing isolates from HTY were more resistant to vancomycin and teicoplanin than non-producing isolates where hemolysin positive isolates from SS were less resistant to vancomycin. Ampicillin and penicillin resistances were frequent in lipase producing isolates in both hospitals where these resistant isolates were infrequently produced biofilm and other virulence factors. In addition, ciprofloxacin and erythromycin resistant isolates were more produced lipase enzyme in both hospitals even they were not significant.

MDR isolates from SS were associated with hemolysin production and MDR isolates from HTY were associated with biofilm formation ($p<0.05$). Although MDR isolates from SS were more produced biofilm, they were not statistically significant. Similarly, MDR isolates from HTY were more produced gelatinase and caseinase but they were not associated statistically.

Table 14. Correlation between antibiotic resistance and biofilm formation or virulence factors activities among enterococci isolates in two hospitals, (A) Sunpasitthiprasong Hospital, (B) Hat Yai Hospital

(A)

Viruelnce factors		Number of antibiotic-resistant isolates (%)										
		Amp	P	CN	S	VAN	TEC	C	CIP	E	TE	MDR
Biofilm	+ (n=209)	30 (14.4) **	136 (65.1)	149 (71.3) **	124 (59.3) **	8 (3.8)	8 (3.8)	113 (54.1) **	161 (77.0)	184 (88.0)	184 (88.0) **	174 (83.3)
	- (n=303)	116 (38.3)	177 (58.4)	158 (52.1)	144 (47.5)	16 (5.3)	16 (5.3)	107 (35.3)	207 (68.3)	252 (83.2)	209 (69.0)	236 (77.9)
Hemolysin	+ (n=257)	53 (20.6) **	142 (55.3) **	180 (70.0) **	176 (68.5) **	5 (1.9)*	5 (1.9)*	155 (60.3) **	180 (70.0) **	231 (89.9)*	214 (83.3) **	214 (83.3)*
	- (n=255)	122 (47.8)	171 (67.1)	127 (49.8)	92 (36.1)	19 (7.5)	19 (7.5)	65 (25.5)	188 (73.7)	205 (80.4)	179 (70.2)	196 (76.9)
Gelatinase	+ (n=237)	46 (19.4) **	141 (59.5)	158 (66.7) *	154 (65.0) **	4 (1.7)*	4 (1.7)*	132 (55.7) **	176 (74.3)*	200 (84.4)	192 (81.0) **	184 (77.6)
	- (n=275)	129 (46.9)	172 (62.5)	149 (54.2)	114 (41.5)	20 (7.3)	20 (7.3)	88 (32.0)	192 (69.8)	236 (85.8)	201 (73.1)	226 (82.2)
Caseinase	+ (n=282)	52 (18.4) **	168 (59.6)	199 (70.6) **	189 (67.0) **	11 (3.9)	11 (3.9)	162 (57.4) **	213 (75.5)*	242 (85.8)	226 (80.1) **	224 (79.4)
	- (n=230)	123 (53.5)	145 (63.0)	108 (47.0)	79 (34.3)	13 (5.7)	13 (5.7)	58 (25.2)	155 (67.4)	194 (84.3)	167 (72.6)	226 (82.2)
Lipase	+ (n=8)	5 (62.5)	6 (75.0)	5 (62.5)	3 (37.5)	-	-	3 (37.5)	7 (87.5)	8 (100.0)	6 (75.0)	7 (87.5)
	- (n=504)	170 (33.7)	307 (60.9)	302 (59.9)	265 (52.6)	24 (4.8)	24 (4.8)	217 (43.1)	361 (71.6)	428 (84.9)	387 (76.8)	403 (80.0)

(B)

Viruelnce factors		Number of antibiotic-resistant isolates (%)										
		Amp	P	CN	S	VAN	TEC	C	CIP	E	TE	MDR
Biofilm	+ (n=94)	30 (31.9)	43 (45.7)	56 (59.6)	62 (66.0)	6 (6.4)*	6 (6.4)*	42 (44.7)	73 (77.7)	80 (87.2)*	76 (80.9)	151 (84.4)*
	- (n=178)	66 (37.1)	83 (46.6)	96 (53.9)	99 (55.6)	8 (4.5)	8 (4.5)	55 (30.9)	125 (70.2)	122 (68.5)	135 (75.8)	69 (74.2)
Hemolysin	+ (n=234)	79 (33.8)**	107 (45.7)*	135 (57.7)	142 (60.7)*	13 (5.6)	13 (5.6)	93 (39.7)**	177 (75.6)**	183 (78.2)**	189 (80.8)**	188 (77.4)
	- (n=38)	17 (44.7)	19 (50.0)	17 (44.7)	19 (50.0)	1 (2.6)	1 (2.6)	4 (10.5)	21 (55.3)	21 (55.3)	22 (57.9)	32 (84.2)
Gelatinase	+ (n=70)	6 (8.6)**	22 (31.4)*	49 (70.0)*	53 (75.7)*	-	-	39 (55.7)**	53 (75.7)	55 (78.6)	62 (88.6)*	62 (88.6)
	- (n=202)	90 (44.6)	104 (51.5)	103 (51.0)	108 (53.5)	14 (6.9)	14 (6.9)	58 (28.7)	145 (71.8)	149 (73.8)	149 (73.8)	158 (78.2)
Caseinase	+ (n=122)	23 (18.9)**	47 (38.5)*	78 (63.9)	88 (72.1)**	2 (1.6)	2 (1.6)	61 (50.0)**	94 (77.0)	91 (74.6)	94 (77.0)	99 (81.1)
	- (n=150)	73 (48.7)	79 (52.7)	74 (49.3)	73 (48.7)	12 (8.0)	12 (8.0)	36 (24.0)	104 (69.3)	113 (75.3)	117 (78.0)	121 (80.7)
Lipase	+ (n=40)	26 (65.0)**	26 (65.0)*	17 (42.5)	15 (37.5)**	4 (10.0)	4 (10.0)	8 (20.0)*	36 (90.0)*	34 (85.0)	29 (72.5)	31 (77.5)
	- (n=232)	70 (30.2)	100 (43.1)	135 (58.2)	146 (62.9)	10 (4.3)	10 (4.3)	89 (38.4)	162 (69.8)	170 (73.3)	182 (78.4)	189 (81.5)

AMP, ampicillin; P, penicillin; CN, gentamicin (high-level); S, streptomycin (high-level); VAN, vancomycin; TEC, teicoplanin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; TE, tetracycline

Correlation of antibiotic resistance and virulence factors was analyzed using Chi-square test. **p*-value <0.001 was regarded as highly significance, ** *p*-value <0.05 as significance

3.6. Correlation of biofilm formation and virulence factors activities in enterococci isolates

The biofilm formation process is complicated, and many factors are supported to this mechanism. Therefore, the correlation of biofilm formation and virulence factors activities were sought to find out in the study (Table 15). Hemolysin, gelatinase and caseinase positive isolates in SS had a significantly greater prevalence in strong or moderate biofilm formation than negative isolates ($p<0.001$) However, lipase producing was not significantly correlated with biofilm formation ($p>0.05$) even lipase enzyme was produced more in strong and moderate biofilm producers. Similarly, biofilm formation was detected more frequently in hemolysin and caseinase producing isolates from HTY (36.7% and 35.3%) than in non-producing isolates (21.1% and 34.0%), respectively, but there was no statistically significant ($p>0.05$). In contrast, biofilm formation was detected higher in gelatinase and lipase negative isolates (35.2% and 33.2%) than positive isolates (22.8% and 32.5%) in HTY.

Table 15. Correlation between biofilm formation and virulence factors activities among enterococci isolates in two hospitals, (A) Sunpasitthiprasong Hospital, (B) Hat Yai Hospital

(A)

Virulence factors		Number of biofilm producers (%)				*p* - value
		Strong	Moderate	Strong/ Moderate	Weak/ non-biofilm	
Hemolysin	+ (n=257)	19 (7.4)	109 (42.4)	128 (49.8)	129 (50.2)	0.001
	- (n=255)	21 (8.2)	60 (23.5)	81 (31.7)	174 (68.2)	
Gelatinase	+ (n=237)	9 (3.8)	100 (42.2)	109 (46.0)	128 (54.0)	0.001
	- (n=275)	31 (11.3)	69 (25.1)	100 (36.4)	175 (63.6)	
Caseinase	+ (n=282)	11 (3.9)	123 (43.6)	134 (46.5)	148 (52.5)	0.001
	- (n=230)	29 (12.6)	46 (20.0)	75 (32.6)	155 (67.4)	
Lipase	+ (n=8)	2 (25.0)	3 (37.5)	5 (62.5)	3 (37.5)	0.249
	- (n=504)	38 (7.5)	166 (32.9)	204 (40.4)	300 (59.5)	

(B)

Virulence factors		Number of biofilm producers (%)				*p* - value
		Strong	Moderate	Strong/ Moderate	Weak/ non-biofilm	
Hemolysin	+ (n=94)	34 (14.5)	52 (22.2)	86 (36.7)	148 (63.2)	0.285
	- (n=178)	3 (7.9)	5 (13.2)	8 (21.1)	30 (78.9)	
Gelatinase	+ (n=234)	12 (17.1)	11 (15.7)	23 (22.8)	47 (67.1)	0.342
	- (n=38)	25 (12.4)	46 (22.8)	71 (35.2)	131 (64.9)	
Caseinase	+ (n=70)	18 (14.8)	25 (20.5)	43 (35.3)	79 (64.8)	0.881
	- (n=202)	19 (12.7)	32 (21.3)	51 (34.0)	99 (66.0)	
Lipase	+ (n=122)	7 (17.5)	10 (25.0)	17 (32.5)	23 (57.5)	0.511
	- (n=150)	30 (12.9)	47 (20.3)	77 (33.2)	155 (66.8)	

Correlation of biofilm formation and virulence factors was analyzed using Chi-square test. *p*-value <0.001 was regarded as highly significance, *p*-value <0.05 as significance.

3.7. DNA fingerprints investigation using RAPD method in VRE isolates

3.7.1. Optimization of $MgCl_2$ concentration in AP4, M13 and AP4 plus ERIC1R RAPD

The similar banding patterns from AP4 RAPD with 2.5 mM and 2 mM $MgCl_2$ concentration and M13 RAPD with 1.5 mM and 2 mM $MgCl_2$ concentration were obtained in the reference strains (Figure 33, Lane 3, 4, 5 and 6). Therefore, 2 mM $MgCl_2$ concentration was used in these two RAPD. For AP4 plus ERIC1R RAPD, additional bands were disappeared in 2 mM $MgCl_2$ concentration (Figure 33, Lane 1). The most informative and reproducible fingerprint patterns for AP4 plus ERIC1R primer were obtained in 4 mM $MgCl_2$ concentration (Figure 33, Lane 2). Therefore, 4 mM $MgCl_2$ concentration was used in AP4 plus ERIC1R RAPD for all isolates.

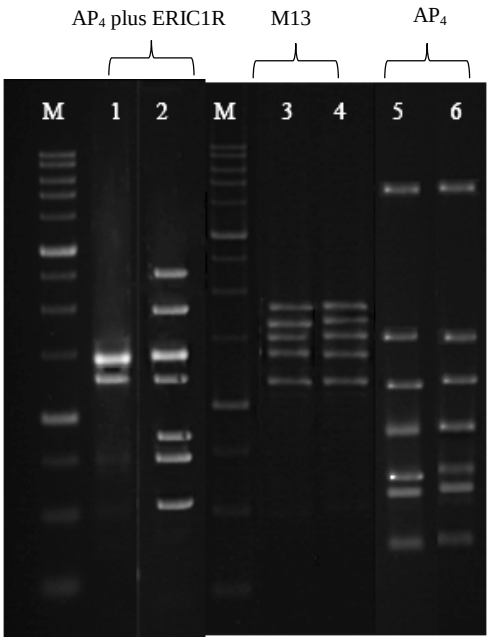


Figure 33. Optimization of $MgCl_2$ concentration in RAPD with AP4 plus ERIC1R, M13 and AP4 primer sets

Lane M, 1 kb DNA marker; Lane 1 and 2, 2 mM and 4 mM $MgCl_2$ in AP4 plus ERIC1R; lane 3 and 4, 1.5 mM and 2 mM $MgCl_2$ in M13 primer; lane 5 and 6, 2.5 mM and 2 mM $MgCl_2$ in AP4 primer

3.7.2. Reproducibility and typeability of RAPD using four primers

This test confirmed the reproducibility of RAPD method for enterococcal strains. For *E. faecalis* and *E. faecium* strains in each method, the similarity among three independent repetitions of the same reference strain was reproducible as presented in the Figure 34 A to D. In addition, the similar banding patterns were revealed in duplicate running of all VRE. *faecalis* and VRE. *faecium* isolates with AB-15 and AP4 plus ERIC1R RAPD as presented in Figure 35 and 36. Moreover, all tested strains were typeable in all RAPD methods.

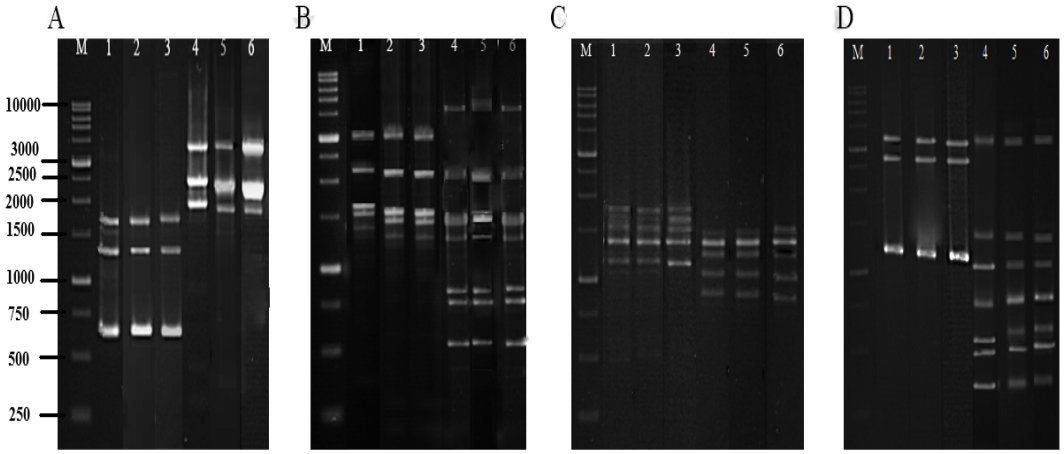


Figure 34. Reproducibility of RAPD with four primers sets obtained amplifying three times of *E. faecalis* ATCC 29212 and *E. faecium* DMST 14756 reference strains (A) AB1-15, (B) AP4 plus ERIC1R, (C) M13, (D) AP4
Lane M, 1 kb DNA marker; Lane 1 to 3, *E. faecalis* ATCC 29212; Lane 4 to 6, *E. faecium* DMST 14756

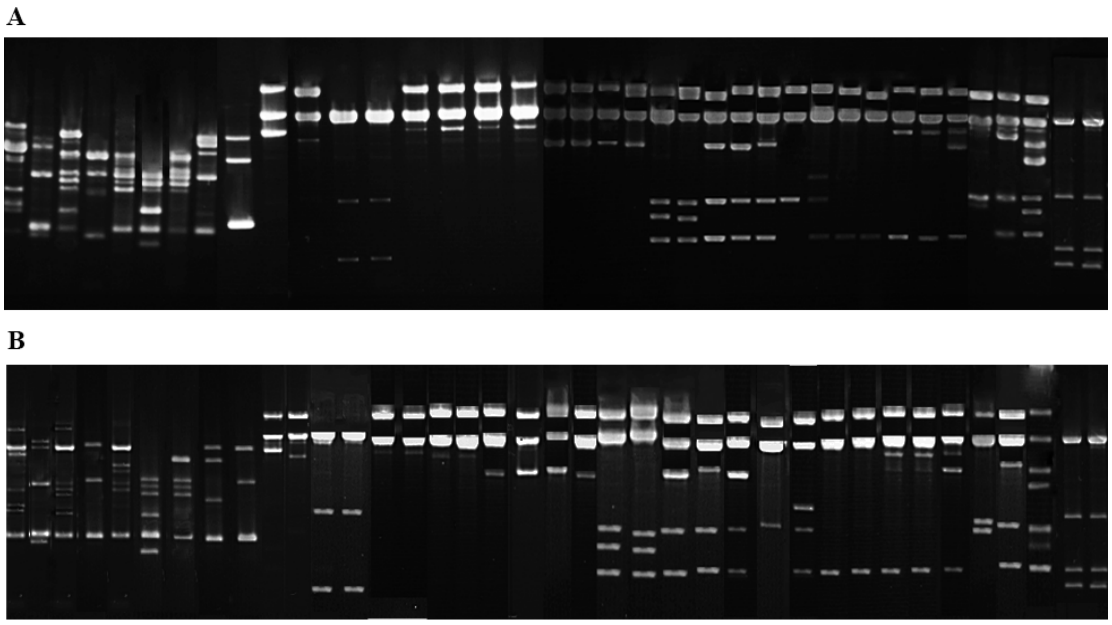


Figure 35. Duplicate RAPD with AB1-15 primer for 38 VRE. *faecalis* and VRE. *faecium* strains (A) first time, (B) second time

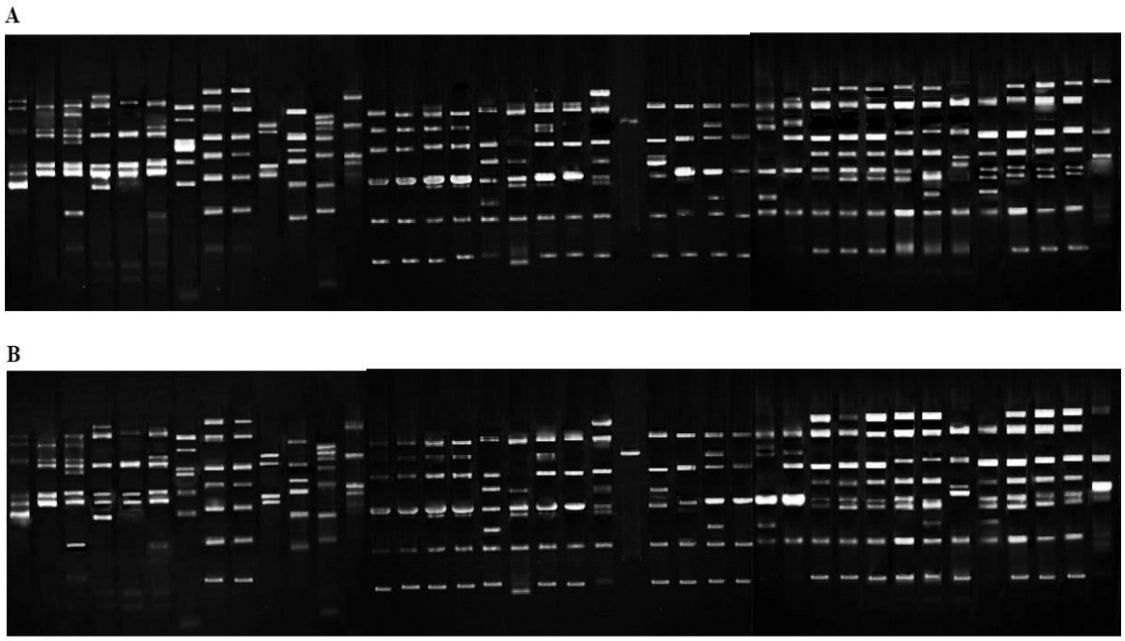


Figure 36. Duplicate RAPD with AP4 plus ERIC1R primer sets for 38 VRE. *faecalis* and VRE. *faecium* strains (A) first time, (B) second time

3.7.3. Discriminatory index (D.I)/ discriminatory power (D.P) of RAPD with four primers

The number of clusters and D.I of each primer were calculated after cluster analysis of RAPD patterns using Bionumerics software which were summarized in Table 16. All tested strains revealed fingerprint patterns in four RAPD. However, one *E. faecium* isolate (SS0330) revealed only one band in AP4 plus ERIC1R and M13 RAPD in duplicate run.

AP4 plus ERIC1R primer and AP4 primer turned out to be the highest-performance techniques for *E. faecalis* with unique single patterns. RAPD with AB1-15 primer gave rise the highest D.I (0.931) for *E. faecium* with 14 patterns. Even AP4 RAPD method gave rise the highest value of D.I for *E. faecalis* (D.I = 1.0), the lowest value of D.I for *E. faecium* was obtained with this method (D.I = 0.471, 7 patterns). Because of lower discrimination in M13 and AP4 RAPD, clusters from these RAPD were subdivided into different clusters in AB1-15 and AP4 plus ERIC1R RAPD. However, two main species were significantly separated in RAPD with all four primers.

Table 16. Results of cluster analysis performed on RAPD with four primers separately

		E. faecalis	*E. faecium*
AB1-15 primer	No. of isolates	9	29
	No. of patterns	8	14
	D.I/ D.P for each species	0.972	0.931[a]
	% of typeability	100%	
		E. faecalis	*E. faecium*
AP4 plus ERIC1R primer	No. of isolates	9	29
	No. of patterns	9	15
	D.I/ D.P for each species	1[a]	0.897
	% of typeability	100%	
		E. faecalis	*E. faecium*
M13 primer	No. of isolates	6	26
	No. of patterns	5	8
	D.I/ D.P for each species	0.933	0.74
	% of typeability	100%	
		E. faecalis	*E. faecium*
AP4 primer	No. of isolates	6	26
	No. of patterns	6	7
	D.I/ D.P for each species	1[a]	0.471
	% of typeability	100%	

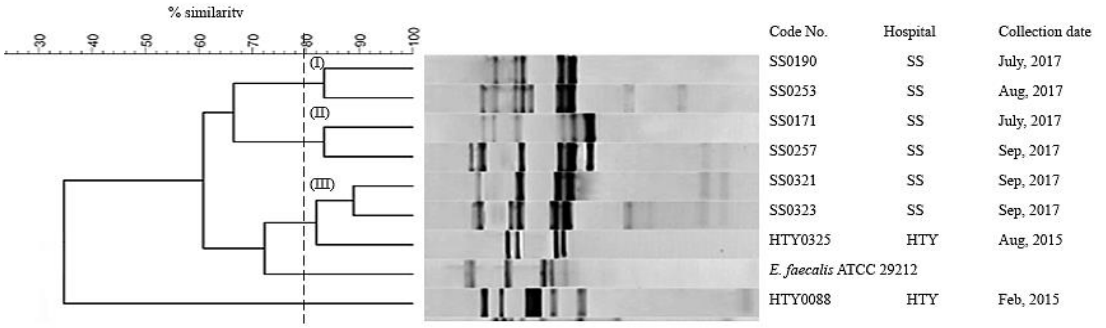
-, not performed; a, best D.I obtained for each species, discrimination power was calculated with 100% similarity threshold for each species in each method

3.7.4. Analysis of RAPD banding patterns using four different primers

(i) AP4 plus ERIC1R RAPD

Even the highest discriminatory power for *E. faecium* species were given by AB1-15 primer, the reliable results with phenotypic characters and comparable banding patterns were given by AP4 plus ERIC1R primer set. Therefore, the dendrogram from AP4 plus EICR1R RAPD was analyzed for epidemiological information for VRE isolates (Figure 37). The banding patterns of AP4 plus ERIC1R RAPD yielded 4 to 10 bands ranging from 0.25 kbp to 6 kbp. Based on the banding patterns of AP4 plus EICR1R RAPD, 3 important clonal groups among *E. faecium* isolates with 80% similarity were observed (Figure 37B). *E. faecium* isolates from SS except SS0203 were classified into group I and isolates from HTY were grouped into Group II and III. Of 16 *E. faecium* isolates from SS, 5 isolates collected in June 2017 were identical in group I (100% similarity). One isolate obtained in July 2017 and two isolates obtained in December 2017 from SS also were revealed 100% similarity in cluster I. In group II, 6 isolates from HTY obtained within January 2015 and June 2015 were identical. Two *E. faecium* isolates collected in May 2015 which possessed the same antibiotic resistance patterns and virulence factors activities were grouped in group III with 100% similarity. Eight *E. faecalis* isolates from two hospitals presented 3 clonal groups and 1 single pattern based on 80% similarity. Six *E. faecalis* isolates from SS collected within July and September 2017 were grouped into three clusters (Figure 37A).

A



B

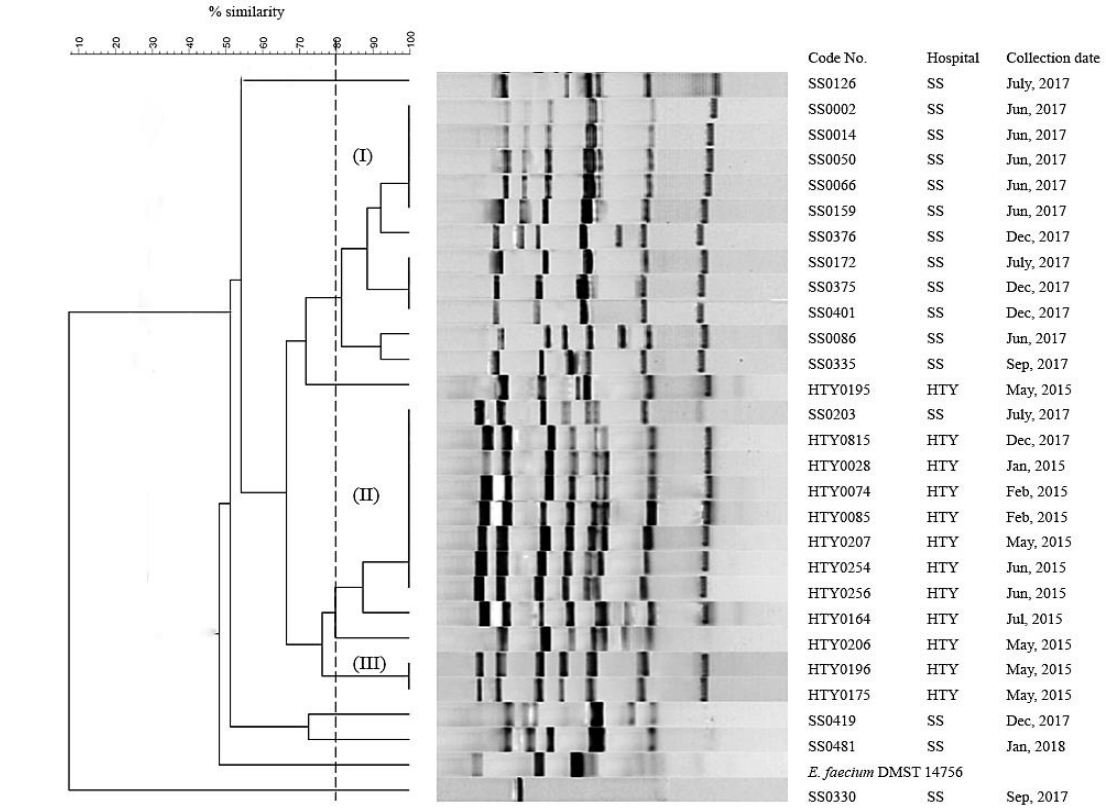


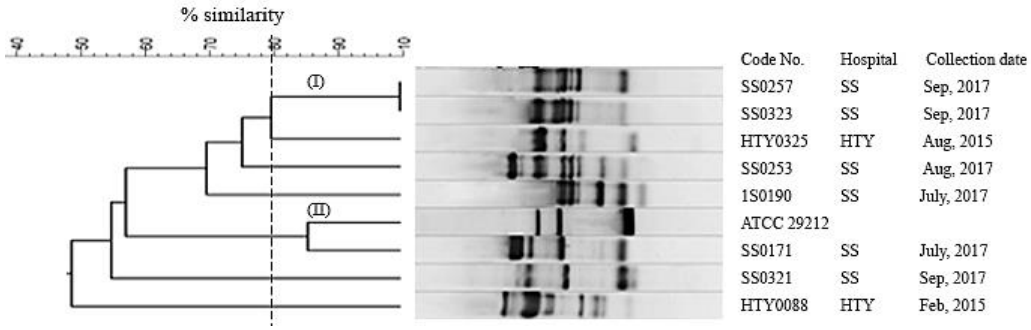
Figure 37. Dendrogram for AP4 plus ERIC1R illustrating the relation between VRE isolates (A) VRE. *faecalis* (B) VRE. *faecium*

The similarities between the fingerprints were calculated using the Dice coefficient (optimization, 1%; position tolerance, 1%), and the fingerprints were clustered according to their similarities using UPGMA algorithm. SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hospital

(ii) AB-15 RAPD

In the dendrogram of AB1-15 RAPD, 2 clusters for *E. faecalis* and 8 clusters for *E. faecium* including several groups were revealed as shown in Figure 38. There were 2 to 7 high intensity bands observed that ranged from 0.3 to 4 kbp in size for all VRE strains in AB1-15 RAPD. Four *E. faecium* strains from SS isolated between June 2017 and December 2017 were revealed the same pattern with 100% similarity. In the same way, *E. faecium* strains from HTY collected within February 2015 and July 2015 were revealed the same pattern with 100% similarity. Four *E. faecium* strains from HTY obtained in May 2015 were grouped into two branches with 100% similarity. VRE. *faecium* isolates from two hospitals collected in different collection period were grouped together in group III, IV, VI and VIII. Two *E. faecalis* strains from SS in September 2017 were revealed the same pattern.

A



B

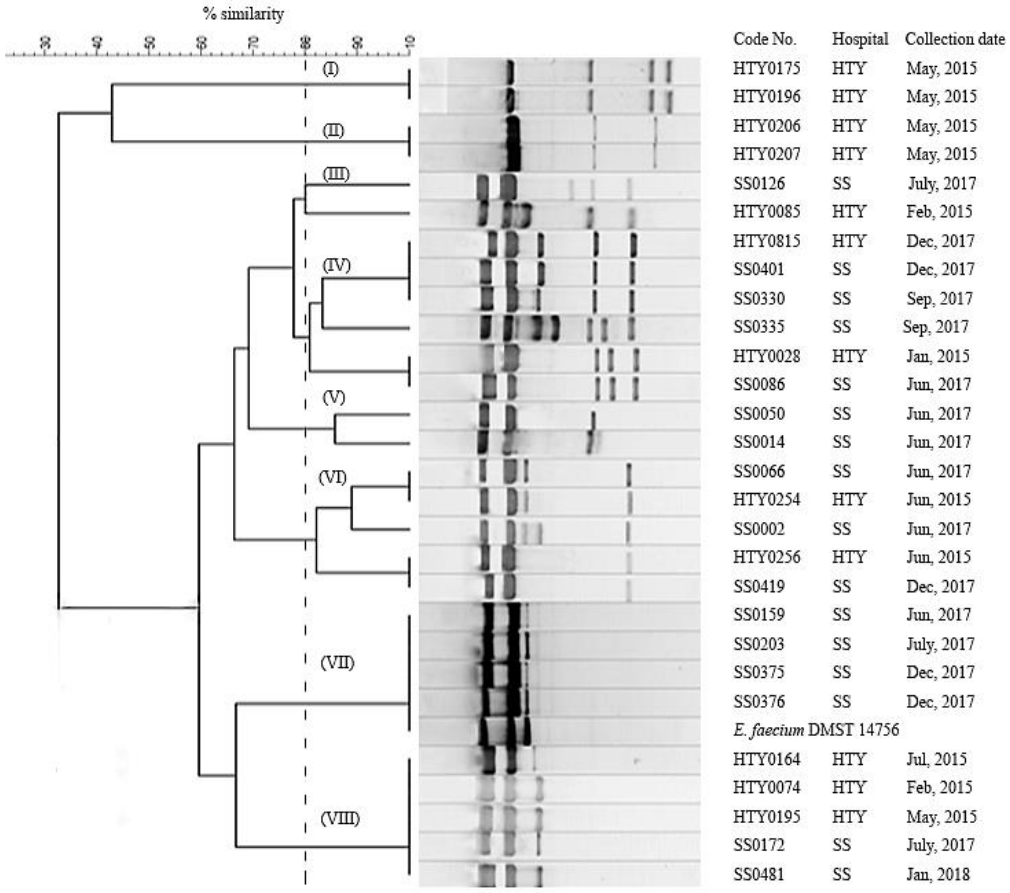


Figure 38. Dendrogram for AB1-15 RAPD illustrating the relation between VRE isolates (A) VRE. *faecalis* (B) VRE. *faecium*

The similarities between the fingerprints were calculated using the Dice coefficient (optimization, 1%; position tolerance, 1%), and the fingerprints were clustered according to their similarities using UPGMA algorithm. SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hospital

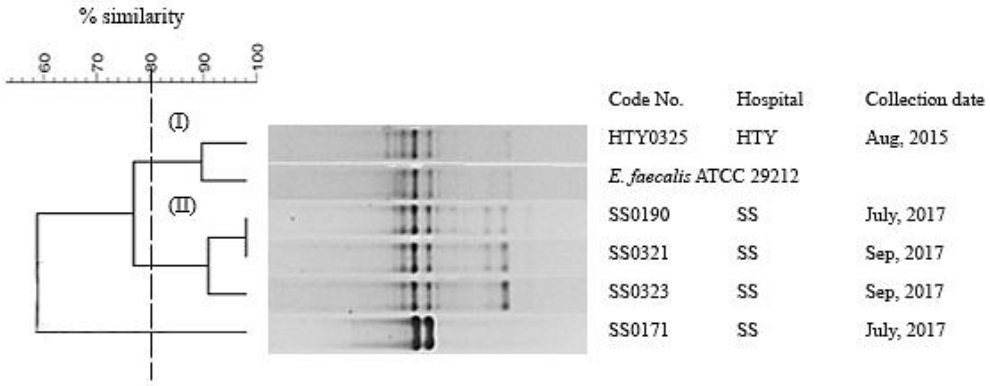
(iii) M13 RAPD and AP4 RAPD

The use of M13 and AP4 fingerprinting alone came out to be not suitable to discriminate *Enterococcus* isolates at strain level, as a low number of groups with lower D.I was obtained. Thirteen different patterns were distinguished among selected 30 VRE. *faecalis* and VRE. *faecium* using M13 and AP4 RAPD.

The bands from M13 RAPD were 2 to 6 fragments ranged from 0.55 kbp and 3 kbp in size (Figure 39). Three VRE. *faecalis* isolates from SS collected in July and September 2017 were grouped in 80% similarity. SS0330 isolate showing one band in AP4 plus ERIC1R RAPD that also showed one band in M13 RPAD. The largest group (Group IV) in M13 RAPD was constituted by 12 *E. faecium* isolates from SS isolated within June 2017 and January 2018 (37.5%). VRE. *faecium* isolates from HTY collected within January 2015 and July 2015 revealed identical banding patterns in Group II as the second largest group in M13 RAPD. One isolate from SS collected in September 2017 and one isolate from HTY collected in December 2017 were accompanied in Group II.

Similarly in AP4 RAPD, the bands from AP4 RAPD were 3 to 7 fragments ranged from 0.35 kbp and 3.5 kbp in size (Figure 40). The largest group was constituted by 20 *E. faecium* strains from different hospitals in different collection periods (62.5%).

A



B

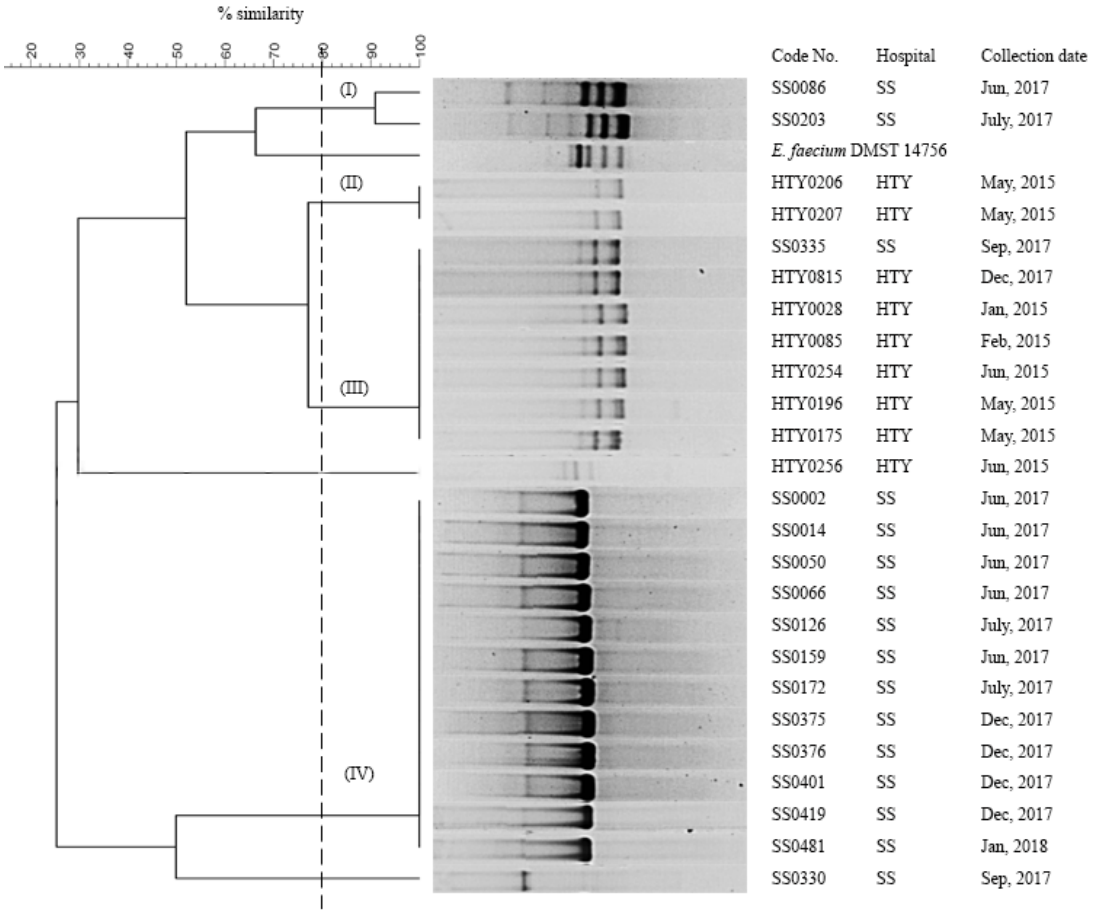
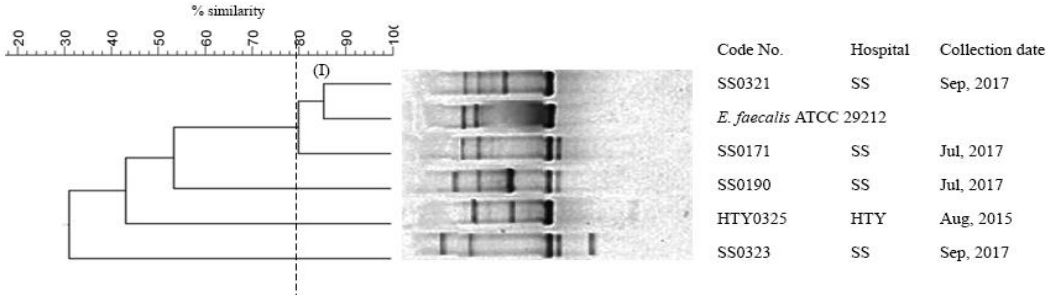


Figure 39. Dendrogram for RAPD using M13 primer illustrating the relation between 30 selected VRE isolates (A) VRE. *faecalis* (B) VRE. *faecium*

The similarities between the fingerprints were calculated using the Dice coefficient (optimization, 1%; position tolerance, 1%), and the fingerprints were clustered according to their similarities using UPGMA algorithm.

A



B

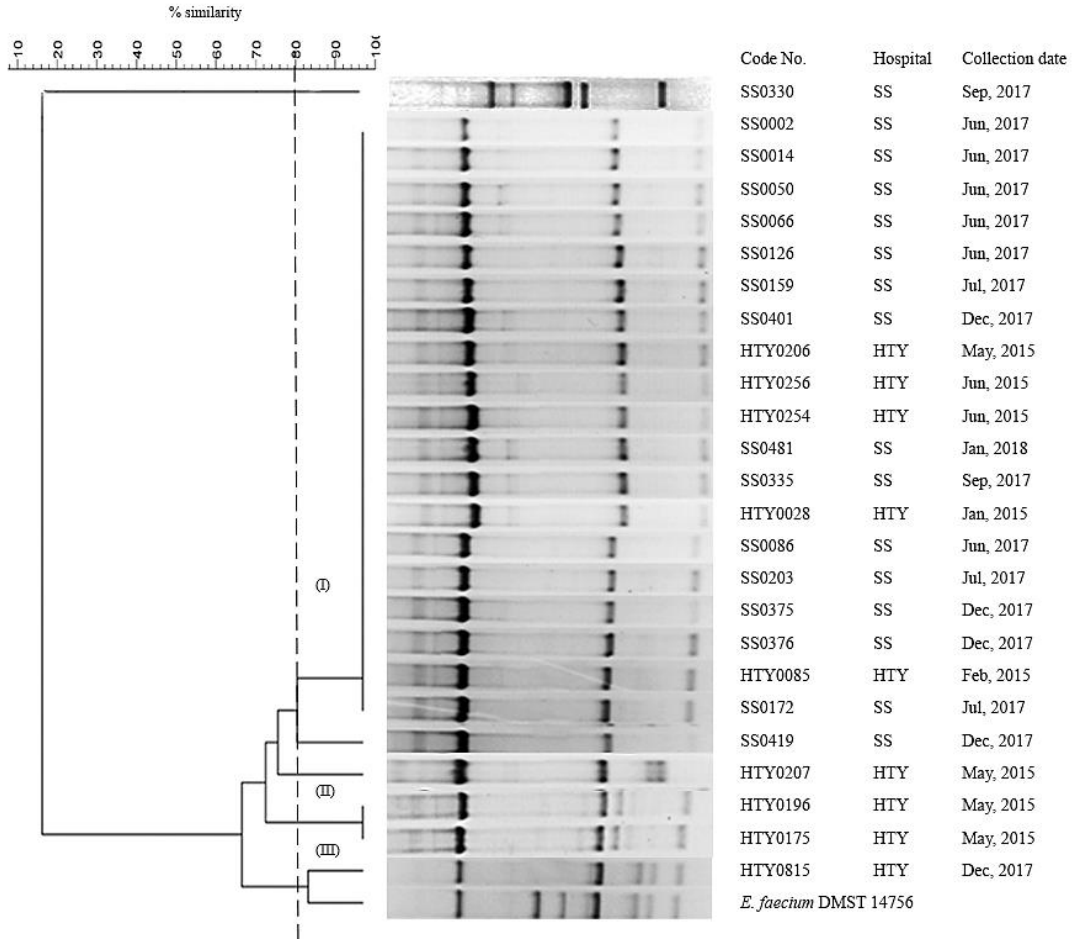


Figure 40. Dendrogram for RAPD using AP4 primer illustrating the relation between 30 selected VRE isolates (A) VRE. *faecalis* (B) VRE. *faecium*

The similarities between the fingerprints were calculated using the Dice coefficient (optimization, 1%; position tolerance, 1%), and the fingerprints were clustered according to their similarities using UPGMA algorithm.

CHAPTER 4

DISCUSSION

4.1. Molecular identification of *Enterococcus* spp.

The predominant *Enterococcus* spp. isolates in this study were *E. faecalis* followed by *E. faecium*, similar to the distribution of the previous studies in clinical isolates (Kashef, *et al.* 2017; Arbabi, *et al.*2016). Although *E. faecalis* is regarded to be the most prevalent species in clinical isolates, the prevalence of *E. faecium* and another *Enterococcus* spp. are also increasing. Other *Enterococcus* spp. distribution in the study (1.3%) was the similar rate with previous studies, as less than 5% in *E. casseliflavus/flavescens*, *E. gallinarum*, etc. (Jia, Li, & Wang, 2014; Thapa *et al.*, 2014) .

The most frequent source of *Enterococcus* isolates was urine followed by pus and blood in both two hospitals. That is pointing out enterococci are the critical opportunistic pathogens of urinary tract infection, wound infection, and bacteremia and are less pathogenic in other infected sites, same as previous studies (Arabestani *et al.*, 2017; Bhatt *et al.*, 2015).

4.2. Antibiotic-resistant patterns of enterococci isolates

As similar to several studies from different countries (Fernandes & Dhanashree, 2013; Golia & Nirmala, 2014; Jia *et al.*, 2014), *E. faecium* isolates from two hospitals were more resistant to antibiotics than *E. faecalis* isolates except HLAR, chloramphenicol and tetracycline. This resistance rate might be because of the easily acquisition of resistance genes in *E. faecium* that resulted from the adaptation of *E. faecium* clones and to overcome stressful conditions in host (Lee, Pang, Abraham, & Coombs, 2019).

The high incidence of MDR (79.9% and 80.5%) in two hospitals observed may be due to prior exposure and high consumption and usage of antibiotics. However, MDR enterococci in this study was slightly lower than that reported in Northeastern Thailand (93.1%) (Thapa *et al.*, 2014). Previous studies also exhibited

that erythromycin, tetracycline and ciprofloxacin were the most resistant antibiotics in enterococcal infections in other countries (Arabestani *et al.*, 2017; Daniel *et al.*, 2015). In addition, National Antimicrobial Resistance Surveillance Centre Thailand (NARST) reported that enterococci isolates from 68 hospitals in Thailand gave high-rate resistance to tetracycline (86%) and erythromycin (60%) in 2018 (NARST, 2018). This high-level emergence of antibiotic resistance is a warning for hospital environments to the widespread use of these antibiotics and derivatives for the treatment of various infections.

The resistance to β-lactams and high-level aminoglycosides were still higher incidence as previous study in Thailand, ampicillin (43.3%), penicillin (51.3%) and high-level gentamicin (57.7%) (Thapa *et al.*, 2014). These resistance rates were indicated poor efficacy of synergic therapy between aminoglycosides and β-lactams antibiotics for enterococcal infections.

4.3. Vancomycin resistance in enterococci isolates

VRE dissemination has been increasing worldwide since their first report of VRE isolation in 1988. The spread of VRE in hospital settings is a serious problem because not only they are resistant to a wide variety of antibiotics but also they can serve as a potential reservoir of resistance genes and transfer to other strains, including MRSA (Ahmed & Baptiste, 2018). The epidemiology of VRE varies widely between different geographical areas (Abdallah & Al-Saafin, 2018; Raza *et al.*, 2018). The prevalence of VRE in Europe and America (20 – 50%) was still higher than in Asian countries. However, the prevalence of VRE was noted with over 10% in China, India, Korea, Japan, and Taiwan but was less than 2% in other Asian countries such as The Philippines, and Saudi Arabia (Kim *et al.*, 2018). The prevalence of VRE in Thailand is still low, even the rates of VRE infection have increased from 1.9% in 2009 to 2.8% in 2014 (Thongkoom *et al.*, 2012; D Chotiprasitsakul *et al.*, 2016). VRE incidence (4.8%) in this study was higher than previous studies in Thailand. However, higher rate of VRE in Thailand (8.0%) reported in the previous study in Songkhalanagrind Hospital within 2011 and 2018 (Saengsuwan, Phanvasri, *et al*., 2018). Similar tendencies were reported in neighboring countries, for instance, 0.3% (2014) in long-time care hospitals in Singapore were reached to 1.1% (2016) and 5.1%

(2014) in intermediate care hospitals were reached to 7.4% (2016) (Tan *et al.*, 2018), and in similar way, 1% (2008) were increased to 2.88% (2013) in Malaysia (Daniel *et al.*, 2015).

Of the nine vancomycin-resistant types, vanA and vanB-type were accounted for the most prevalent vancomycin resistant types in clinical isolates. Recently vanM type has been reported in China and Singapore (Lee, Pang, & Abraham, 2019). Regarding the previous study in Thailand, 100% *vanA* gene and no *vanB* gene in VRE isolates in an outbreak of 2013 – 2014 period in Bangkok (Chotiprasitsakul *et al.*, 2016). In contrast, the findings of the study in King Chulalongkon Memorial Hospital, Bangkok within 1995 - 1999 period was not consistent with the results of this study, all VRE isolates (0.81%, 15/1854) carried *vanB* gene (Chongthaleong, 2003). Four VRE isolates revealed neither *vanA* nor *vanB* gene even though they were high-level resistant to vancomycin (≥256 μg/ml) and resistant to teicoplanin. The similar findings from Iran had reported that it might be the point mutations in orf_1, *vanS, vanA, vanX* or *vanY* of Tn1546 were happened the inability to detect the *vanA* gene expression (Ali & Mohammad, 2018). On the other hand, high-level vancomycin resistance without *vanA* and *vanB* genes may be vanM or vanD-type resistance, which is also resistant to vancomycin with high-level (Chen *et al.*, 2015).

The most of VRE isolates (92.1%) were multidrug resistance compared to other studies (Moosavian, Ghadri, & Samli, 2018; Oravcova *et al.*, 2017). The use of newer antibiotics such as linezolid and tigecycline, for complicated infections including VRE and MRSA has increased because of the rapid emergence of multidrug-resistant VRE infections (Ali & Mohammad, 2018). Among therapeutic options against VRE infections, the activity of linezolid, quinupristin/dalfopristin and tigecycline were examined. All VRE isolates were susceptible to linezolid and tigecycline antibiotics is concordant with other study (Oravcova *et al.*, 2017) where linezolid resistance was revealed in Thailand (Diaz L, Kiratisin P, *et al.*, 2012). Quinupristin/dalfopristin is the combination of two drugs in the same class streptogramin. Resistance to this antibiotic was observed as 75% of *E. faecalis* and 42.9% of *E. faecium.*

4.4. Characterization of biofilm formation and secreted virulence factors activities in enterococci isolates

Biofilm formation in enterococci is one of the several defense mechanisms to evade the action of antibiotics and help in persistence of infections (Sieńko, Wieczorek, & Al, 2015). Maximum number of biofilm formation seen in urine samples which is consistent with another study (Tsikrikonis, Maniatis, & Labrou, 2012). This result proved that biofilm was potential virulence determinant for colonization and prolonged existence in the urinary tract. Different profiles of virulence factors are important for understanding the enterococcal pathogenicity and their effects on the host (Furumura, Figueiredo, & Carbonell, 2006). The secreted hemolysin and exoenzymes such as gelatinase, caseinase and lipase activities were examined in enterococcal isolates. Although the activities of caseinase and gelatinase in enterococcal infections were conflicted in previous studies (Biswas, Dey, & Adhikari, 2014; Furumura, Figueiredo, & Carbonell, 2006), activities of both protease enzymes were revealed similar prevalence in this study. Data relating lipases as bacterial virulence factors from various clinical isolates are scarce. This enzyme can catalyze both the hydrolysis and synthesis of ester bonds of triacylglycerols that make them a useful enzyme in food industry. Nevertheless, high incidence of lipase enzyme in clinical *E. faecalis* samples (23 out of 32 samples) has been reported (Furumura, Figueiredo, & Carbonell, 2006) and 78.5% of clinical enterococci isolates produced in Egypt (Aladarose & Said, 2019). The production of lipase enzymes was observed very low incidence (1.6% in SS and 14.7% in HTY) in this study. However, incidence of lipase enzyme in clinical isolates assumed that this enzyme might support to enterococci for nutrient acquisition, adhere to host tissue and interrupting the defense mechanism of host's phagocytic cells. *E. faecalis* was revealed as the most biofilm formation and virulence factors producer among *Enterococcus* spp. as other studies (Fernandes & Dhanashree, 2013; Soares, Fedi, & Al, 2014; Tsikrikonis, Maniatis, & Labrou, 2012). This may be one of the factors why *E. faecalis* infection is more prevalent than *E. faecium* infection. In addition, the incidence of biofilm formation and virulence factors activities in clinical isolates indicates that the necessary of these

determinants for tissue invasion, adhesion and pathogenicity in urinary tract infection, wound infection and bacteremia.

4.5. Correlation of antibiotic resistance, biofilm formation and secreted virulence factors activities in enterococci isolates

The biofilm formation process is complicated (Sieńko, Wieczorek, & Al, 2015). Therefore, many investigators have sought to find out the correlation of biofilm formation and virulence factors activities. Hemolysin, gelatinase, caseinase and lipase producing isolates appeared to be linked significantly with biofilm formation. This finding was supported that secreted virulence factors were correlated with biofilm formation because nutrient availability is an important determinant in shape and size of biofilm (Hancock and Perego, 2004). Sienko *et al.* also reported that α-hemolysin was related with biofilm formation in their study (Sieńko, Wieczorek, & Al, 2015). Tsikrikonis *et al.* from Turkey reported hemolysin was associated with biofilm formation in clinical isolates but not in gelatinase production (Tsikrikonis, Maniatis, & Labrou, 2012). It was similar with this biofilm-producing isolates from HTY which were not correlated with gelatinase producing. In contrast, one study from Egypt reported that hemolysin, and lipase productions were not correlated with biofilm formation where gelatinase production was correlated (Aladarose & Said, 2019).

Another focus of this study was the mutual effects between antibiotic resistance and biofilm formation or secreted virulence factors activities. Biofilm formation, hemolysin, gelatinase and caseinase activities were correlated with antibiotic resistance except β-lactams and glycopeptides antibiotics in isolates from two hospitals. One of the study from India also suggested that biofilm formation was correlated with high-level aminoglycosides resistance but not correlated with vancomycin resistance (Shridhar & Dhanashree, 2019). One study from Iran reporting biofilm formation was higher in antibiotic resistance except vancomycin that was also supported the findings of this study (Talebi, Moghadam, & Al, 2015). Furthermore, the study of Sonbol *et al.* who reported that their gelatinase production was associated with chloramphenicol and multidrug resistance except ampicillin, in consistence with this study (Sonbol, El-Banna, & Al, 2013). They reported that pheromone responsive plasmids contained *gelE* gene which possibly contributed to vancomycin and chloramphenicol resistance.

However, vancomycin resistance was low incidence in gelatinase producers in this study. In contrast, biofilm formation of enterococcal clinical isolates in Brazil were not correlated with antibiotic resistance. They suggested that antibiotic resistance might be due to bacterial fitness and not because of biofilm formation (Soares, Fedi, & Al, 2014). Additionally, findings in Banerjee *et al.* study were revealed biofilm formation and virulence factors were not correlated with antibiotic resistance (Banerjee & Anupurba, 2015). According to the studies in different parts of the world, the relation between antibiotic resistance and virulence factors still conflict. It might be bacteria fitness may vary according to different geographic area. Overall, virulence determinants producing in clinical enterococci isolates may promote the emergence of infections and persistence of pathogens which consequently lead to the spread of antibiotic-resistant genes. Therefore, antibiotics combination to inhibit virulence factors activities of enterococci should be considered in treatment for enterococcal infections.

Non-*faecalis* non-*faecium* isolates were less resistant to antibiotics and less incident of biofilm formation and virulence factors. It might be the consequence of infrequent reservation of plasmid-borne antibiotic resistance and virulence genes in these species. Therefore, they have not yet become the virulent dissemination in human infections. (Tuhina, Anupurba, & Karuna, 2016).

4.6. DNA fingerprinting of VRE isolates

The genotypic typing of VRE isolates using RAPD method was assessed in this study. Barbier *et al*. showed that RAPD with AP4 plus ERIC1R primer was well-suited for epidemiological typing of enterococci (Barbier *et al*., 1996). When they analyzed the ability of RAPD with AP4 plus ERIC1R primer set for 60 VRE isolates comparing with PFGE, fingerprint results of RAPD (30 patterns) were highly congruent with PFGE fingerprints (36 patterns). One study from India also reported that RAPD using AP4 plus ERIC1R can be used to determine clonal relatedness and to trace possible sources (Banerjee. T, 2013). Supporting to these previous studies of RAPD method in VRE isolates, RAPD using AP4 plus ERIC1R primer set gave a high discriminatory ability in VRE isolates in this study. RAPD using AB1-15 primer also revealed the high discrimination in VRE isolates in this study. Issack *et al.* also reported that AB1-15 primer using RAPD was given the good results in VRE isolates when they

tested 26 different random primers for RAPD (Issack, Power, & French, 1996). However, the clusters in the dendrogram of AB-15 RAPD were not represented for epidemiologic relatedness when the genotypic and phenotypic characters of VRE isolates were analyzed. According to Coenye *et al.*, the effectiveness of molecular typing method is not exclusively determined based on the higher discriminatory power of this method. Ease of interpretation, reproducibility, typeability and the ability to represent meaningful epidemiologic information also need to consider the usefulness of this method (Coenye, *et al.* 2002).

Based on the clustering patterns in AP4 plus ERIC1R RAPD, meaningful clustering for VRE isolates with three main clusters was identified in AP4 plus ERIC1R RAPD at 80% similarity. Meanwhile, AB1-15 RAPD presented 8 different clusters, grouping isolates from two hospitals with different collection period. As such, AB1-15 RAPD was not chosen to analyze clonal dissemination. Comparison between AP4 plus ERIC1R RAPD and AB1-15 RAPD was observed that a few isolates were grouped into the same cluster of each dendrogram.

The analysis of AP4 plus ERIC1R RAPD dendrogram revealed three main clusters with 80% similarity for *E. faecium* isolates grouped with 11 isolates from SS, 10 isolates from SS and HTY, and 2 isolates from HTY, respectively (Figure 37B). Similarly, 8 *E. faecalis* isolates from two hospitals grouped into three clusters (Figure 37A). The genetic similarity of VRE isolates from SS and HTY in RAPD method was low. The clusters based on 80% similarity in RAPD dendrogram revealed VRE infection was clonally disseminated in an individual hospital especially between June 2017 and January 2018 in SS and between January 2015 and August 2015 in HTY. There was no clear association between the virulence factors activities and fingerprinting profile. In addition, strains with different resistant profiles and virulence determinants revealed in the same branch. Based on these characteristics, it was quite possible that these pathogenic determinants frequently exchanged among clinical strains.

Although RAPD using AP4 primer gave the superior discriminatory power than other five PCR-based type methods in differentiating *E. faecium* strains in the literature (Weiss *et al.*, 2010), RAPD with AP4 primers in this study produced some uniform bands in tested strains. It indicated that they were able to bind in conserved genomic

regions of strains. Therefore, M13 and AP4 RAPD gave rise low discriminatory power were not effective to perform VRE strain typing. The clustering patterns from AP4 plus ERIC1R RAPD achieved the suitable performance among the primers used. This primer had exhibited the most reliable banding patterns for phylogenetic analysis. The results of RAPD highlighted the determination of suitable random primer for RAPD may give the efficient information for epidemiology. Furthermore, RAPD method is useful for epidemiology study and outbreak to evaluate the dissemination of infection. This method is less time-consuming and cost effective which can apply any molecular laboratory.

CHAPTER 5

CONCLUSION

Through the investigation of enterococcal clinical strains characters including multidrug resistance and virulence factors activities, proper antibiotics treatment for infection can be approached. Multidrug resistance was observed in three quarters of clinical enterococci strains from both two hospitals in the study. That indicates the uncontrol use of antibiotics in enterococci infections. The incidences of antibiotic resistance pattern and biofilm formation in clinical isolates were similar in both two hospitals from Northeastern and Southern of Thailand, whereas virulence factors activities of isolates were varied. The incidence of secreted virulence factors such as hemolysin, gelatinase, caseinase and lipase in clinical isolates were supported the pathogenicity and antibiotic resistance of enterococcal infections. Especially, biofilm formation is associated with urinary tract infecitons. That indicated biofilm formation is essential factors of enterococci for prolonged existence in urinary tract. The correlation between resistance to different antibiotics classes and biofilm formation or virulence factors activities supposed to consider the treatment strategies for virulence determinants of enterococci to prevent the acquisition of antibiotic resistance. Moreover, multidrug-resistant vanA-type VRE was predominant in Thailand within 2015 - 2018 period. There are no vanB, vanD and vanM-type vancomycin resistance detected in this study. These VRE isolates were still susceptible to linezolid and tigecycline. RAPD banding patterns of VRE isolates were revealed the clonal dissemination of VRE infection in the same hospital in a particular time. Analyzing the results of RAPD with different random primers, RAPD typing is revealed as a quick and cost-effective molecular typing method that can be used to trace the clonal relatedness of VRE for epidemiological purposes. However, for the better informative patterns, two or more primers should be provided in RAPD. Overall, the information of this study may support analyzing the characteristics of enterococcal infections in Thailand.

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APPENDIX

Culture media preparation

1. Brain heart infusion broth (BHI broth)

HM infusion powder	
(Calf brain infusion form)	12.5 g
BHI powder	5.0 g
Proteose peptone	10.0 g
Dextrose (Glucose)	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g

Final pH (at 25 °C) 7.4 ± 0.2

Suspend 37.0 g in 1000 ml of D/W. Heat to dissolve the medium completely. Dispense into bottles or tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

10% glucose preparation

Glucose powder	10 g
D/W	100 ml

Suspend 10 g of glucose in 100 ml of D/W. Sterilize by autoclaving at 15lbs pressure (115 °C) for 10 mins. For BHI broth with 0.5% glucose, mix 50 ml of glucose and 950 ml of BHI broth aseptically.

2. Brain Heart Infusion agar (BHI agar)

HM infusion powder	
(Calf brain infusion form)	12.5 g
BHI powder	5.0 g
Proteose peptone	10.0 g
Dextrose (Glucose)	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Agar	1.5 g
Final pH (at 25 °C) 7.4 ± 0.2	

Suspend 52.0 g in 1000 ml of D/W. Heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool at 45 - 50°C. Mix well and pour into sterile petri plates.

3. Muller Hinton Broth (MHB)

Beef infusion	300 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Final pH (at 25 °C) 7.3 ± 0.1	

Suspend 21 g in 1000 ml of D/W. Heat to dissolve the medium completely. Dispense into bottles or tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

4. Muller Hinton Agar (MHA)

Beef infusion	300 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Final pH (at 25 °C) 7.4 ± 0.1	

Suspend 38.0 g in 1000 ml of D/W. Heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool at 45 – 50 °C. Mix well and pour into sterile petri plates.

5. Bile Esculin Azide agar

Tryptone	17.0 g
Beef extract	5.0 g
Proteose peptone	3.0 g
Oxgall	10.0 g
Esculin	1.0 g
Ferric ammonium citrate	0.5 g
Sodium chloride	5.0 g
Sodium azide	0.150 g
Agar	15.0 g
Final pH 7.1 ± 0.2 (at 25 °C)	

Suspend 56.65 g in 1000 ml of D/W. Heat to dissolve the medium completely. Dispense into tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

6. Blood Agar

Beef infusion	300 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Blood	50 ml
Final pH 7.4 ± 0.1 (at 25 °C)	

Prepare the blood agar base as suspended 38.0 g in 950 ml of D/W. Heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool at 45 – 50 °C and add 50 ml of sterile blood aseptically. Mix well gently to avoid air bubbles formation and pour into sterile petri plates.

7. Egg yolk agar

Beef infusion	300 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Final pH 7.4 ± 0.1 (at 25 °C)	

Prepare the egg yolk agar base as suspended 38.0 g in 920 ml of D/W. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool at 45 – 50 °C.

Egg yolk emulsion

Soak eggs in 70% ethanol for 1 hr. Crack eggs aseptically. Retain yolks and mix yolk to equal volume of sterile 0.85% saline.

Add 80 ml of 50% egg yolk emulsion into agar base at 45 – 50 ºC. Mix well gently to avoid air bubbles formation. Pour into sterile petri plates.

8. Nutrient gelatin agar

Peptic digest of animal tissue	5 g
Beef extract	3 g
Gelatin	120 g
Final pH 6.8 ± 0.2 (at 25°C)	

Suspend 128 g in 1000 ml of D/W. Heat to dissolve the medium completely. Dispense into test tubes. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Allow the medium tube to cool in an upright position.

9. Skimmed milk agar

Beef infusion	300 g
Casein acid hydrolysate	17.5 g
Starch	1.5 g
Agar	17.0 g
Final pH 7.4 ± 0.1 (at 25°C)	

Prepare the skimmed milk agar base as suspended 38.0 g in 970 ml of D/W. Heat to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins. Cool at 45 – 50 °C and add 30 ml of sterile skimmed milk aseptically and mix well gently to avoid air bubbles formation. Pour into sterile petri plates.

Reagent preparation

1. **Catalase Reagent: 3% H_2O_2**

30% H_2O_2	33.3 ml
D/W	66.7 ml

Mix and keep at 4°C.

2. **Gram stain reagent**

2.1. Crystal violet staining reagent

Solution A: 2.0 g of crystal violet mix with 20 ml of 95% ethanol
Solution B: 0.8 g of ammonium oxalate mix with 80 ml of D/W
Solution A and B mix and store at least 24 hrs and filter prior to use.

2.2. Mordant: Gram's iodine

Mix 1.0 g of iodine and 2.0 g of potassium iodide with 300 ml of D/W.
Iodine and potassium iodide were grinded in a mortar and water was added slowly with continuous grinding until the iodine is dissolved. Store at room temperature with amber bottle.

2.3. Decolorizing agent

95% ethanol

2.4. Counterstain: Safranin

Stock solution: 2.5g of safranin O mix with 100 ml of 95% ethanol
Working Solution: 10 ml of stock solution mix with 90 ml of D/W.

3. **0.04% Resazurin**

Resazurin	0.16 g
D/W	100 ml

Mix and filter prior to use. Store in amber bottle in cool place.

4. 0.85% Normal saline solution

Sodium chloride	0.85 g
D/W	100 ml

Mix and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins.

5. 0.1% Crystal violet stain

Crystal violet	1 g
95% ethanol	20 ml
D/W	adjust to 1 L

Dissolve 1 g of crystal violet powder into 20 ml of 95% ethanol. Adjust volume to 1 L with D/W and filter prior to use.

6. 10X Phosphate buffered saline (PBS)

NaCl	80 g
KCl	2 g
Na_2HPO_4	14.4 g
KH_2PO_4	2.4 g

Dissolve in 800ml of D/W. Adjust pH to 7.4. Adjust volume to 1 L with additional D/W. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins and keep at room temperature. To prepare 1X PBS, dilute 1:10 with sterile D/W.

7. 10X Tris-EDTA (TE) buffer

1M Tris-HCl, (pH 8.0)	100 ml
0.5M EDTA (pH 8.0)	20 ml
D/W	880 ml

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins and keep at room temperature. To prepare 1X TE, dilute 1:10 with sterile D/W.

8. 10X Tris-acetate EDTA (TAE) buffer

Tris base	48.4 g
Glacial acetic acid	11.4 ml
0.5M EDTA (pH – 8.0)	20 ml

Adjust volume to 1 L with D/W. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins and keep at room temperature. To prepare 1X TAE, dilute 1:10 with sterile D/W.

9. 10X Tris-borate EDTA (TBE) buffer

Tris base	108 g
Boric acid	55 g
EDTA	9.3 g

Adjust volume to 1 L with D/W. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins and keep at room temperature. To prepare 1X TBE, dilute 1:10 with sterile D/W.

10. 40% glycerol

Absolute glycerol	40 ml
D/W	60 ml

Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 mins and keep at room temperature.

11. 1M Sodium hydroxide (NaOH)

Sodium hydroxide	40 g
Distilled water	1 L

Mix and keep at room temperature.

12. 1M Hydrogen chloride (HCl)

Concentrated HCl	8.3 ml
D/W	91.7 ml

Mix and keep at room temperature.

13. 10 mg/ml lysozyme

Lysozyme	0.01 g
D/W	1 ml

Mix and keep at -20ºC

14. 10% SDS

Sodium dodecyl sulfate	0.1 g
D/W	1 ml

Mix and dissolve at 60ºC for 20 mins, keep at room temperature

15. 1% agarose gel

Agarose agar	0.6 g
1x TAE buffer	60 ml

Mix and dissolve with medium heat in microwave for 1.5 mins, pour into the gel tray and leave to solidify at least 30 mins

Supplementary Table 1. Antibiotic resistance profiling among multidrug-resistant enterococci isolates according to sources in two hospitals

Number of resistant antibiotics	Antibiotic resistance profiling										Number of isolates from different sources									
											Urine		Pus		Blood		Fluid	Sputum	Total	
											SS	HTY	SS	HTY	SS	HTY	HTY	HTY	SS	HTY
10	TE	E	CIP	C	TEC	VAN	S	CN	P	AMP	2								2	
9	TE	E	CIP	TEC	VAN	S	CN	P	AMP		1	4							1	4
8	TE	E	CIP	C	S	CN	P	AMP			22	5	1	1	1				24	6
8	TE	E	CIP	TEC	VAN	CN	P	AMP			10		1						11	
8	TE	E	CIP	TEC	VAN	S	P	AMP				1								1
8	E	CIP	TEC	VAN	S	CN	P	AMP			2	1							2	1
7	TE	E	CIP	C	S	CN	P				94	12	5	1	5	1	2	1	104	17
7	TE	E	CIP	C	S	P	AMP					2								2
7	TE	E	CIP	C	CN	P	AMP					2								2
7	TE	E	C	S	CN	P	AMP				1								1	
7	TE	E	CIP	S	CN	P	AMP				7	10	1	1		1	2		8	14
7	TE	E	CIP	TEC	VAN	P	AMP				2			1					2	1
7	E	CIP	C	S	CN	P	AMP					1								1
7	E	CIP	TEC	VAN	S	P	AMP					1								1
7	E	CIP	TEC	VAN	CN	P	AMP				2						1		2	1
6	TE	E	CIP	C	S	CN					30	19	3	7		1	1		33	28
6	TE	E	CIP	C	S	P					1	2							1	2
6	TE	E	CIP	C	CN	P					2	1				1	1		2	3
6	TE	E	CIP	C	CN	AMP									1				1	
6	TE	E	CIP	C	P	AMP					2	2		1					2	3
6	TE	E	CIP	S	CN	P					18	6		1					18	7
6	TE	E	CIP	S	P	AMP					1	4	1	2		1			2	7
6	TE	E	CIP	CN	P	AMP					26	9	1	2	1	1			28	12
6	TE	CIP	S	CN	P	AMP						1								1

Number of resistant antibiotics	Antibiotic resistance profiling										Number of isolates from different sources									
											Urine		Pus		Blood		Fluid	Sputum	Total	
											SS	HTY	SS	HTY	SS	HTY	HTY	HTY	SS	HTY
6	E	CIP	C	S	CN	P					5								5	
6	E	CIP	C	S	P	AMP					2								2	
6	E	CIP	S	CN	P	AMP					3	4	2				1		5	5
6	CIP	VAN	TEC	S	P	AMP						1								1
5	TE	E	CIP	S	CN						7	7		1				2	7	10
5	TE	E	CIP	S	P						1		2						3	
5	TE	E	CIP	P	AMP						25	3	2	1	2				29	4
5	TE	E	CIP	C	P						2								2	
5	TE	E	CIP	C	CN							5	1	1		2			1	8
5	TE	E	CIP	C	S							3	1		2				3	3
5	TE	E	CIP	CN	P							1				1			1	1
5	TE	E	C	S	CN						6	3	4	1	2				12	4
5	TE	E	C	P	AMP							1								1
5	TE	E	CN	P	AMP							1								1
5	TE	E	S	P	AMP						2								2	
5	TE	CIP	S	P	AMP							4								4
5	TE	CIP	C	S	CN							1								1
5	E	CIP	S	P	AMP						7	5							7	5
5	E	CIP	S	CN	P						1								1	
5	E	CIP	C	S	CN						1	3							1	3
5	E	CIP	C	P	AMP							1		1						2
5	E	CIP	CN	P	AMP						14	1	2	1					16	2
4	TE	E	CIP	C							3	1							3	1
4	TE	E	CIP	S							1	4							1	4
4	TE	E	CIP	CN							1	2		1					1	3
4	TE	E	C	S							5	3		1					5	4
4	TE	E	C	CN							6		1		1				7	1

Number of resistant antibiotics	Antibiotic resistance profiling										Number of isolates from different sources									
											Urine		Pus		Blood		Fluid	Sputum	Total	
											SS	HTY	SS	HTY	SS	HTY	HTY	HTY	SS	HTY
4	TE	E	S	CN							2	5	3	2					5	7
4	TE	CIP	P	AMP							4							1	4	
4	TE	E	P	AMP								2								2
4	TE	CIP	P	AMP							4							1	4	
4	TE	CIP	CN	P							1								1	
4	TE	S	P	AMP								1								1
4	E	CIP	P	AMP							18	6	3		1				22	6
4	E	CIP	S	AMP							1								1	
4	E	CIP	C	S								1								1
4	CIP	S	P	AMP							1								1	
4	CIP	CN	P	AMP								1								1
3	TE	E	S								3	1	3	1					6	2
3	TE	E	C								1					1	1		1	2
3	TE	E	CIP									1	1						1	1
3	TE	E	CN								2		1	2	1			1	4	3
3	TE	CIP	C													1				1
3	TE	CIP	CN								1	1		1					1	2
3	TE	CIP	S									2								2
3	TE	C	S								2								2	
3	TE	S	P									1								1
3	TE	P	AMP									1								1
3	E	CIP	C											1						1
3	E	CIP	S								1								1	
3	E	CIP	CN									1								1
Total											352	161	39	32	17	11	9	5	408	215

SS, Sunpasitthiprasong Hospital; HTY, Hat Yai Hosptial; AMP, ampicillin; P, penicillin; CN, gentamicin (high-level); S, streptomycin (high-level); VAN, vancomycin; TEC, teicoplanin; C, chloramphenicol; CIP, ciprofloxacin; E, erythromycin; TE, tetracycline

VITAE

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Educational Attainment

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Scholarship Awards during Enrolment

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2018 Graduate School of Prince of Songkla University Research Fund

List of Publication and Proceeding

Seinn So Lwin, Kanitta Muangngam, Wipawadee Sianglum. 2018. Antibiotic resistance, biofilm formation and virulence factors among enterococci clinical isolates collected from two hospitals in different parts of Thailand. Joint International Tropical Medicine Meeting (JITMM, 2018), December 12 – 14, 2018, Amari Watergate Hotel, Bangkok (*Abstract, Oral Presentation*)

Seinn So Lwin, Kanitta Muangngam, Amornrat Sianglum, Jiraphorn Nilsakul, Wipawadee Sianglum. 2019. Characterization of antimicrobial resistance, biofilm formation and virulence factors in vancomycin-resistant enterococci isolated from tertiary care hospitals in Thailand (*Submitted*)