DEVELOPMENT OF MULTIPLEX POLYMERASE CHAIN REACTION (PCR) ASSAY FOR THE SIMULTANEOUS DETECTION OF VANCOMYCIN AND LINEZOLID RESISTANT GENES IN *ENTEROCOCCUS*

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by

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LIST OF SYMBOLS

℃	Degree Celcius
G	Gram
M	Molar
%	Percentage
µl	Microlitre
µM	Micromolar
pg	picogram
ng	nanogram
Kg	Kilogram
g	gravitational force
Tm	Melting temperature
Ta	Annealing temperature
G	Guanine
C	Cytosine
A	Adenine
T	Thymine
V	Volume
V	Volts
U	Units

LIST OF ABBREVIATIONS

ABC	adenosine triphosphate-binding cassette
Ace,acm	adhesion to collagen
efaAfs, efaAfm	adhesion-like endocarditis antigens
Agg	aggregation substances
ATCC	American Type Culture Collection
BCCM	Belgian Co-ordinated Collections of Micro-organisms
BHI	Brain heart infusion
BLAST	Basic Local alignment search tools
CDC	Centre for Disease Control
CFU	Colony forming units
CNS	central nervous system
CSF	cerebrospinal fluid
Cyl	cytolysin
ddl	D-alanine:D-alanine Ligases
DNA	Deoxyribonucleic Acid
dNTPs	deoxynucleotide triphosphates
dsDNA	Double-stranded synthetic DNA
EDTA	ethylenediaminetetraacetic acid
EHEC	Enterohemorrhagic
EPEC	Enteropathogenic
Esp	extracellular surface protein
FDA	Food and Drug Administration
FP	False positive
FN	False negative
GelE	gelatinase
GI	gastrointestinal
GU	Gastro urinary
IAC	internal amplification control
IMR	Institute for Medical Research
LAB	lactic acid bacteria
LOD	Limit of Detection

LZRE	Linezolid-Resistance *Enterococcus*
$MgCl_2$	Magnesium chloride
MIC	Minimum inhibitory concentration
Min	Minutes
MLS	macrolide antibiotics, lincosamides, and streptogramins
Ml	Millilitre
MRCNS	methicillin-resistant coagulase-negative staphylococci
MRSA	methicillin-resistant *Staphylococcus aureus*
MRSE	methicillin-resistant *Staphylococcus epidermis*
NaCl	Sodium Chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NCBI	National Centre for Biotechnology Information
NPV	Negative predictive value
PAC	Positive amplification control
PAI	pathogenicity island
PCR	Polymerase Chain Reaction
PhLOPSA	phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A
PO_3^-	Phosphate
PPV	Positive predictive value
RAPD	randomly amplified polymorphic DNA
RPM	Revolutions per minute
rRNA	Ribosomal Ribonucleic Acid
SAM	S-adenosylmethionine
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
Tn	transposons
TP	True positive
TN	True negative
USM	Universiti Sains Malaysia

UTI	Urinary tract infection
UV	Ultra violet
VP	Voges-Proskauer
VRE	Vancomycin-Resistant *Enterococcus*
WHO	World Health Organization

LIST OF APPENDICES

PEMBANGUNAN ASAI TINDAK BALAS RANTAI POLIMERASE MULTIPLEKS (PCR) BAGI PENGESANAN SERENTAK GEN RINTANGAN VANCOMYCIN DAN LINEZOLID DI DALAM *ENTEROCOCCUS*

ABSTRAK

Enterococci adalah bakteria Gram-positif kokus yang boleh didapati dalam usus manusia dan haiwan. Kehadiran dan penyebaran *Enterococcus* rintang Vacomycin (VRE) dan *Enterococcus* rintang Linezolid (LZRE) dalam pusat penjagaan kesihatan telah meningkatkan risiko dan kesukaran pengurusan pesakit. Tiada PCR multipleks telah dibangunkan untuk pengesanan serentak kedua-dua gen tahan vancomycin dan linezolid dalam *Enterococcus*. Matlamat penyelidikan ini ialah untuk membangunkan assai PCR multipleks yang dapat mengesan genus *Enterococcus*, empat gen VRE, dan tiga gen LZRE semuanya pada masa yang sama. Primer yang digunakan dalam kajian ini direka khusus bagi pengesanan gen rintang vancomycin dan linezolid di dalam *Enterococcus*. Gen-gen ini adalah; 16S rRNA genus *Enterococcus*, *vanA* – *vanB* – *vanC* - *vanD* bagi vancomycin, *cfr* methyltransferase, *optrA* dan *poxtA*; pengangkut kaset pengikat adenosin trifosfat (ABC) bagi linezolid. *Vibrio cholerae ctxA* (kawalan amplifikasi dalaman) turut disertakan. Pengoptimuman kepekatan primer, $MgCl_2$, dNTPs, DNA polimerase *Taq* dan suhu penyepuhlindapan primer juga telah dilakukan. Ini kemudiannya diikuti dengan menilai sensitiviti dan spesifisiti PCR multipleks yang dioptimumkan serta sensitiviti analitikal pada peringkat genomik dan jumlah bakteria. Kepekatan akhir primer yang telah dioptimumkan adalah seperti berikut; 16S rRNA ialah 1.0 pmol/µl, *vanA* 1.0 pmol/µl, *optrA* 1.0 pmol/µl, *cfr* 1.0 pmol/µl, *poxtA* 0.1 pmol/µl, *vanB* 0.08 pmol/µl, *ctxA* 0.07 pmol/0µl, *vanC* 0.8 pmol/0µl, dan *vanD* 0.1 pmol/µl. Selanjutnya, kepekatan optimum bagi $MgCl_2$, dNTPs dan DNA polimerase *Taq* ialah masing-

masing, 2.5mM, 0.16mM dan 0.75 unit. Suhu penyepuhlindapan 64.5 °C, LOD 100 pg pada tahap genomik dan serendah 10^5 CFU/ml pada tahap bakteri telah digunakan dan dinilai dalam assai PCR multipleks yang dibangunkan. Dalam kajian ini, sensitiviti, spesifisiti, NPV, PPV dan ketepatan ujian PCR multipleks yang dibangunkan dalam pengesanan gen VRE ialah 76.32% (CI: 59.76% - 88.56%), 100% (CI: 87.23% - 100.00%), 75% (CI: 62.90% - 84.15%), 100% dan 86.15% (CI: 75.34% - 93.47%) masing-masing. Begitu juga, sensitiviti, spesifisiti, NPV, PPV dan ketepatan ujian PCR multipleks yang dibangunkan dalam pengesanan gen LZRE sebagai 88.89% (CI: 51.75% - 99.72%), 100% (CI: 86.77% - 100.00%), 96. % (CI: 80.38% - 99.40%), 100% dan 97.14% (CI: 85.08% - 99.93%) masing-masing. PCR multipleks yang dibangunkan ini adalah sensitif, spesifik spesies, pantas dan mampu mengesan gen *Enterococcus* tahan vancomycin dan linezolid dalam tetapan klinikal dan persekitaran. Pembangunan ujian PCR multipleks yang akan mengambil kira semua gen VRE yang diketahui dan mutasi linezolid sangat disyorkan.

DEVELOPMENT OF MULTIPLEX POLYMERASE CHAIN REACTION (PCR) ASSAY FOR THE SIMULTANEOUS DETECTION OF VANCOMYCIN AND LINEZOLID RESISTANT GENES IN *ENTEROCOCCUS*

ABSTRACT

Enterococci are Gram-positive cocci found in the guts of humans and animals. The introduction and dissemination of vancomycin-resistant *Enterococcus* (VRE) and Linezolid-Resistant *Enterococcus* (LZRE) in healthcare settings has increased patient management risks and difficulties. No multiplex PCR has been developed for the simultaneous detection of both vancomycin and linezolid resistant genes in *Enterococcus*. The goal of this research is to develop a multiplex PCR assay that can detect the *Enterococcus* genus, four VRE genes, and three LZRE genes all at the same time. Primers used in this study were specifically designed for the detection of vancomycin and linezolid resistant genes in *Enterococcus*. These genes are; 16S rRNA of *Enterococcus* genus, *van*A – *van*B – *van*C - *van*D for vancomycin, *cfr* methyltransferase, *optr*A *and poxt*A*;* an adenosine triphosphate-binding cassette (ABC) transporter for linezolid. A *Vibrio cholerae ctx*A (Internal amplification control) was included. Optimization of primer concentrations, $MgCl_2$, dNTPs, *Taq* DNA polymerase and primers annealing temperature was also done. This was followed by evaluating the sensitivity and specificity of the optimized multiplex PCR and their analytical sensitivity both at the genomic and bacteria level. Final Primer concentrations was optimized as follows; 16S rRNA is 1.0 pmol/µl, *van*A 1.0 pmol/µl, *optr*A 1.0 pmol/µl, *cfr* 1.0 pmol/µl, *poxt*A 0.1 pmol/µl, *van*B 0.08 pmol/µl, *ctx*A 0.07 pmol/µl, *van*C 0.8 pmol/µl and *van*D 0.1 pmol/µl.

Further, a $MgCl_2$, dNTPs and *Taq* DNA polymerase optimized concentration was 2.5 mM, 0.16 mM and 0.75 units respectively. An annealing temperature of 64.5℃, a LOD of 100 pg at the genomic level and as low as 10^5 CFU/ml at the bacteria level were utilized and evaluated in the developed multiplex PCR assay. In this study, the sensitivity, specificity, NPV, PPV and accuracy of the developed multiplex PCR assay in the detection of VRE genes were 76.32% (CI: 59.76% - 88.56%), 100% (CI: 87.23% - 100.00%), 75% (CI: 62.90% - 84.15%), 100% and 86.15% (CI: 75.34% - 93.47%) respectively. Similarly, the sensitivity, specificity, NPV, PPV and accuracy of the developed multiplex PCR assay in the detection of LZRE genes were 88.89% (CI: 51.75% - 99.72%), 100% (CI: 86.77% - 100.00%), 96.30% (CI: 80.38% - 99.40%), 100% and 97.14% (CI: 85.08% - 99.93%) respectively. This developed multiplex PCR is sensitive, species-specific, rapid and capable of detecting vancomycin and linezolid resistant *Enterococcus* genes in clinical and environmental settings. The development of a multiplex PCR assay that will take into account all known VRE genes and linezolid mutation so that they would not be missed during routine laboratory diagnosis is highly recommended.

CHAPTER 1

INTRODUCTION

1.1 Enterococci

Enterococci are Gram-positive bacteria that are present in the guts of humans and animals (Wada et al., 2019). While they are an important component of the microbiome, they are capable of causing serious illnesses, particularly in hospitalized individuals with disrupted gut microbiota. Thiercelin discovered a saprophytic bacterium found in the intestine which is capable of causing infection towards the end of the 19th century, which gave rise to the term " *entérocoque*." (Devriese et al., 2006; Lebreton et al., 2014).

Following the isolation and characterization of *Micrococcus zymogenes* now known as *Enterococcus faecalis* from a cytolytic strain, MacCallum and Hastings documented in detail the pathogenesis of *Enterococcus* from a patient with acute endocarditis (Fiore et al., 2019; Singh et al., 2019). MacCallum and Hastings designated *Enterococcus* as an important pathogen of animals and humans based on the reproduction of the endocarditis element in animal models when samples from patients were cultured, which was consistent with Koch's hypothesis. Later, *Enterococcus* was recognized not only as a commensal organism but also as one of the commonest causes of hospital infection around the world (Cho et al., 2020; Krawczyk et al., 2021).

Urinary tract infections, bacteraemia, intra-abdominal infections, and endocarditis are illnesses caused by microorganisms of the genus *Enterococcus* either *E. faecium* or *E. faecalis* that primarily infect patients in hospital settings

(Kristich et al., 2014; Wada et al., 2020). For a long time now, enterococci have been one of the most occurring hospital-acquired organism ranked third in America and other parts of the world, accounting for noticeable proportions of all hospital-acquired infections (Maillard et al., 2020; Weiner et al., 2016).

Enterococci are accountable for a significant proportion of community-acquired endocarditis, in addition to hospital-acquired infections (Angsutararux & Angkasekwinai, 2019; Nakagawa et al., 2014; Slipczuk et al., 2013; Vogkou et al., 2016). Enterococci are cocci that are Gram-positive and exist in short chains or pairs, catalase and oxidase-negative, are non-spore-forming, and are facultative anaerobic (Ben Braïek & Smaoui, 2019; Courvalin, 2006). With about 37 species characterized based on phylogeny utilising 16S rRNA sequencing and DNA to DNA hybridization, *Enterococcus* is classified under the lactic acid bacteria (LAB) family which after *Lactobacillus* and *Streptococcus*, is the third largest LAB family. (Albayrak & Duran, 2021; de Sousa et al., 2020).

1.2 Taxonomic Hierarchy of Enterococci

After being categorized as streptococci, enterococci were classified individually in 1984 (Ludwig et al., 1985). These authors advocate reclassifying some taxa due to inadequate distinctions among *Enterococcus* and characterized as distinct groups, like *E. casseliflavus* and *E. flavescens*, or regrouping species due to similar traits, such as *E. porcinus* and *E. avillorum*. The classification of Enterococci is shown below;

Kingdom Bacteria – bacteria, bactéries, bacterias, bactérias (Cavalier-Smith, 2002)

Subkingdom; Posibacteria, (Cavalier-Smith, 2002)

Phylum; Firmicutes corrig. (Gibbons & Murray, 1978)

Class; Bacilli (Wolfgang Ludwig et al., 2010)

Order; Lactobacillales (Wolfgang Ludwig et al., 2010)

Family; Enterococcaceae (Wolfgang Ludwig et al., 2010)

Genus; *Enterococcus* (ex Thiercelin and Jouhaud, 1903) (Schleifer & Kilpper-Balz, 1984) Species *Enterococcus faecium* (Orla-Jensen, 1919) (Schleifer & Kilpper-Balz, 1984)

Table 1.1 shows other *Enterococcus* species and the researchers that discovered them.

Table 1.1 All recorded *Enterococcus* species discovered by various researchers

S/No	***Enterococcus* species**	**References**
1	*E. alcedinis*	(Frolková et al., 2013)
2	*E. aquimarinus*	(Švec, Vancanneyt, Devriese, et al., 2005)
3	*E. asini*	(De Vaux et al., 1998)
4	*E. avium*	(Collins et al., 1984)
5	*"E. bovis"*	(Udo et al., 2003)
6	*E. bulliens*	(Kadri et al., 2016)
7	*"E. burkinafasonensis"*	(Gouba et al., 2020)
8	*E. caccae*	(Carvalho et al., 2006)
9	*E. canintestini*	(Naser et al., 2005)
10	*E. camelliae*	(Sukontasing et al., 2007)
11	*E. casseliflavus*	(M. D. Collins et al., 1984)
12	*E. canis*	(De Graef et al., 2003)
13	*"E. coli"*	(Ho et al., 2003)
14	*E. cecorum*	(Williams et al., 1989)
15	*E. columbae*	(Devriese et al., 1993)
16	*E. crotali*	(McLaughlin et al., 2017)
17	*E. devriesei*	(Švec, Vancanneyt, Koort, et al., 2005)
18	*E. diestrammenae*	(J. Y. Kim et al., 2013)
19	*E. dispar*	(Collins et al., 1991)
20	*E. dongliensis*	(Li & Gu, 2019)
21	*E. durans*	(Collins et al., 1984)
22	*E. eurekensis*	(Cotta et al., 2013)
23	*E. faecium*	(Schleifer & Kilpper-Balz, 1984)
24	*E. faecalis*	(Schleifer & Kilpper-Balz, 1984)
25	*E. flavescens*	(Pompei et al., 1992)
26	*E. florum*	(Techo et al., 2019)
27	*E. gallinarum*	(Collins et al., 1984)
28	*E. gilvus*	(Tyrrell et al., 2002)
29	*E. haemoperoxidus*	(Švec et al., 2001)
30	*"E. hawaiiensis"*	(Ben Belgacem et al., 2009)
31	*E. hirae*	(Farrow & Collins, 1985)
32	*E. hulanensis*	(Li & Gu, 2019)
33	*E. hermanniensis*	(Koort et al., 2004)
34	*E. italicus*	(Fortina et al., 2004)
35	*"E. lacertideformus"*	(Agius et al., 2021)
36	*E. lemanii*	(Cotta et al., 2013)
37	*E. lactis*	(Morandi et al., 2012)
38	*E. mundtii*	(Collins et al., 1986)
39	*E. malodoratus*	(Collins et al., 1984)
40	*"E. massiliensis"*	(Le Page et al., 2016)
41	*"E. mediterraneensis"*	(Takakura et al., 2019)
42	*E. moraviensis*	(Švec et al., 2001)
43	*E. nangangensis*	(Li & Gu, 2019)
44	*E. olivae*	(Lucena-Padrós et al., 2014)
45	*"E. pernyi"*	(LinLing et al., 2010)
46	*E. pallens*	(Tyrrell et al., 2002)

Table 1.1 Continued

47	*E. plantarum*	(Švec et al., 2012)
48	*E. phoeniculicola*	(Law-Brown & Meyers, 2003)
49	*E. pingfangensis*	(Li & Gu, 2019)
50	*E. pseudoavium*	(Collins et al., 1989)
51	*E. porcinus*	(Teixeira et al., 2001)
52	*E. quebecensis*	(Sistek et al., 2012)
53	*E. raffinosus*	(Collins et al., 1989)
54	*E. ratti*	(Teixeira et al., 2001)
55	*"E. rattus"*	(Zhang et al., 1999)
56	*E. rivorum*	(Maarit Niemi et al., 2012)
57	*E. rotai*	(Sedláček et al., 2013)
58	*E. saccharominimus*	(Marc Vancanneyt et al., 2004)
59	*E. saccharolyticus*	(Rodrigues & Collins, 1990)
60	*E. sulfureus*	(Martinez-Murcia & Collins, 1991)
61	*E. silesiacus*	(Švec et al., 2006)
62	*"E. sanguinicola"*	(Carvalho et al., 2008)
63	*E. seriolicida*	(Kusuda et al., 1991)
64	*E. songbeiensis*	(Li & Gu, 2019)
65	*E. solitarius*	(Collins et al., 1989)
66	*E. saigonensis*	(Harada et al., 2016)
67	*E. thailandicus*	(Tanasupawat et al., 2008)
68	*E. termitis*	(Švec et al., 2006)
69	*"E. timonensis"*	(Fonkou et al., 2018)
70	*"E. timonensis"*	(Fonkou et al., 2019)
71	*E. ureilyticus*	(Sedláček et al., 2013)
72	*E. ureasiticus*	(Sistek et al., 2012)
73	*E. villorum*	(Vancanneyt et al., 2001)
74	*E. viikkiensis*	(Rahkila et al., 2011)
75	*E. wangshanyuanii*	(Jin et al., 2017)
76	*E. xinjiangensis*	(Ren et al., 2016)
77	*E. xiangfangensis*	(Li et al., 2014)

" ": not validly published

1.3 Microbiological, Biochemical and Physiological Characteristics

Enterococci are Gram-positive and exist in short chains or pairs, catalase and oxidase-negative, are non-spore-forming, and are facultative anaerobic. Enterococci can thrive at temperatures ranging from 10 to 45 ℃, with an ideal temperature range of 30 to 35 ℃, thus, are mesophilic in nature. Further, they can also survive in a pH from 4.4 to 9.6, as well as in hypersaline conditions containing

6.5% NaCl. Enterococci are distinguished from streptococci by their ability to tolerate heat at 60°C for 30 minutes, proliferate in enriched broth composed of 40% salts, and esculin hydrolysation (Ferchichi et al., 2021; Raza et al., 2018). When it is cultured on horse blood agar, enterococci can show both alpha and beta-hemolysis and no hemolysis. They form 1 to 2 mm colonies with a wet appearance. Given their metabolic capabilities, different selective culture media have been developed for the isolation of enterococci, containing bile salts, antibiotics, esculin salts or tetrazolium. The most clinically relevant species grow well on these media. Clinical tests for identification of enterococci include the catalase production test, the pyrrolidonyl arylamidase/pyrrolidonyl aminopeptidase (PYR) test and the bile esculin hydrolysis test (Ferchichi et al., 2021; Raza et al., 2018).

1.4 Enterococci Pathogenicity

Endocarditis, intra-abdominal, bacteremia, pelvic infections, infections of the central nervous system, and urinary tract infection (UTI), are among the most common nosocomial diseases caused by enterococci. (Levitus et al., 2020; Tang et al., 2021). *E. faecalis* was found to be responsible for almost 80% of these infections and once thought to be minor clinical pathogens, have suddenly emerged as prevalent opportunistic infections in humans (Deng et al., 2021).

Enterococci, which were long considered unimportant clinical pathogens, have unexpectedly become common opportunistic infections in humans. So much so that virulence factors and the emergence of resistant strains of antibiotic, particularly VRE, have been linked to their pathogenicity. Therefore, when *Enterococcus* spp. is recognized as the causative agent of disease or sickness,

especially in patients with compromised immune systems, they provide a significant task to health care providers (Rosselli Del Turco et al., 2021). Enterococcal infections are caused by strains that originate in the patient's intestinal microbiota which could be transmitted from person to person or acquired by consuming contaminated food and water (Iseppi et al., 2020).

1.4.1 Virulence Mechanisms

These are active protein that increases a microorganism's ability to cause disease. Enterococcal virulence factors or genes have an important influence on the pathogenesis of enterococcal infections. These genes in enterococci include cytolysin (*cyl*), extracellular surface protein (*esp*), aggregation substances (*agg*, *asa1*), adhesion to collagen (*ace*, *acm*), adhesion-like endocarditis antigens (*efaAfs* and *efaAfm*) and gelatinase (*gelE*) (Freitas et al., 2018).

Agg and *asa*1 are virulence genes eliciting surface protein of *Enterococcus* which facilitate aggregation development in bacterial reproduction. It facilitates the binding affinity to cells which are epithelial for infection, transfer of plasmids bearing virulence and resistant genes (Hashem et al., 2021; Jett et al., 1994). In addition, proteins on the outer surface such as thrombospondin, fibronectin, collagen type I, and may attach to the aggregation substances (Ben Braïek & Smaoui, 2019). The *agg* gene enhances the hydrophilic nature of the enterococcal membrane, which causes cholesterol to be transported to phagosomes and delays binding with lysosomal vesicles (Hashem et al., 2017). The *agg* determinant has only been detected in *E. faecalis* isolates so far (Igbinosa & Beshiru, 2019; Landete et al., 2018).

The protein bacteriocin/hemolysis bifunctionality or cytolysin (or -haemolysin) in Enterococci, is the most researched virulence mechanism. It's a peptide toxin that can destroy cells through creating holes within the targeted cells in the bacterial cytoplasmic structures. The likelihood of dying from an enterococcal infection producing cytolysin is five folds higher compared to the risk of death from a non-cytolysin-producing enterococcal infection. This likelihood is a dynamic relationship between the *cyl* and *agg* genes, according to studies on endocarditis (Rahman et al., 2021).

Gelatinase is an extracellular Zn-metallo-endopeptidase that hydrolyzes collagen, haemoglobin, gelatin, casein, insulin, and other peptides (Ben Braïek & Smaoui, 2019). Gelatinase may break fibrin and disrupt host tissue, permitting bacteria to migrate and propagate, raising its importance in enterococci pathogenicity, notably *E. faecalis*. In addition, this protease allows enterococci to infiltrate tissues thereby remaining in some sites of infection through biofilm production (Wada et al., 2019).

Esp is a pathogenic gene marker linked to binding, notably attachment to eukaryotic cells, as well as evading the host's immune response (Ch'ng et al., 2018). This infection-promoting gene is predominantly found in *E. faecium* and is found in an evolutionarily preserved chromosome site (Tendolkar et al., 2004).

E. faecalis and *E. faecium's ace/acm*, link to collagen types I and IV, respectively, with laminin additionally attaching to *acm*. The *acm* is also documented as a member of the bacteria adhesion subfamily that stick particularly to the membrane protein of the host's cytoskeleton (Ch'ng et al., 2018).

Endocarditis is significantly linked to the *efaA* virulence gene. For *E. faecium* and *E. faecalis*, the most well-known genes includes *efaAfs* and *efaAfm* (Johnson, 2017). Other virulence determinants that are associated with enterococcal infections are less well known and less thoroughly characterized. The *sag* gene, which is released by *E. faecium* and capable of broad-spectrum attachment to cytoskeletal proteins is one of these virulence factors less thoroughly characterized (Ch'ng et al., 2018). Another *E. faecium* adhesion, *scm*, was described to attach to collagen type IV effectively. In addition, the *ebp* gene is designed to boost the production of biofilm in *E. faecalis* and codes for biofilm-associated pili and endocarditis (Ch'ng et al., 2018).

In addition, the *bee* gene (*Enterococcus* biofilm producer) is known to impart a strong biofilm-producing *E. faecalis* phenotype. Furthermore, another virulent component called *hyl*, which encodes a hyaluronidase hydrolyzing hyaluronic acid, possibly indicating some translocation function. On the plasmid, this virulence determinant was shown to be linked to resistant and pilin genes (Johnson, 2017).

In general, most of these virulence factors were less common in *E. faecalis* than in *E. faecium* isolates, and enterococcal virulence may not be clarified solely by the occurrence of virulence mechanisms as antibiotic-resistant genes also contribute to enterococcal pathogenicity.

1.4.2 Antibiotic Resistance

Another important virulence trait in enterococci is their resistance to commonly prescribed antibiotics, which boosts *Enterococcus* ability to cause

disease, rendering them an ideal hospital-acquired infections opportunistic in nature (Opalska et al., 2020; Raza et al., 2018; Wada et al., 2020). Prolonged exposure to antibiotics and their widespread use as preventative agents or growth promoters in human and veterinary medicine has resulted in a boost in the incidence of enterococcal isolates showing resistance to multiple antibiotic groups. This is possibly due to genetic mutations bestowing antibiotic resistance and allowing enterococci to survive. As a result, drug resistance has emerged as a significant public health issue. Enterococcal drug resistance can be developed by a number of mechanisms, including target alteration, enzymatic drug inactivation and drug access modifications (García-Solache & Rice, 2019).

Enterococci have encoded chromosomes that provide inherent antibiotic-resistance to sulphonamides, cephalosporins, lincosamides, aminoglycosides and lactams (van Harten et al., 2017). Acquired resistance to aminoglycosides, fluoroquinolones, chloramphenicol, penicillin, erythromycin, tetracycline, ampicillin, and glycopeptides, particularly vancomycin, has been observed in enterococci through plasmids or transposons (Ferchichi et al., 2021; R. S. Lee et al., 2018). Vancomycin resistance is of particular interest because VRE has been linked to major infections and illnesses that are resistant to standard antibiotics. VRE provided a significant issue for clinicians because it is frequently utilized to substitute aminoglycosides, penicillin and ampicillin in allergic individuals, making it the "drug of last resort" in the treatment of enterococcal infections. Daptomycin and other novel medications like oxazolidinones, quinupristin-dalfopristin, and everninomycins, have been studied as vancomycin alternatives.

*van*A, *van*B, *van*C, *van*D, *van*E, *van*G, *van*L, *van*M, *van*N, and most recently *van*P are all known enterococci glycopeptide-resistant genes. The *vanA* operon is characterised by isolates with high levels of resistance to teicoplanin and vancomycin, and has *E. faecium* as its major reservoir (Asgin & Otlu, 2020). Vancomycin resistance is induced by the *van*B operon, except resistance to teicoplanin. v*an*A and *van*B are the only genes capable of vertical and horizontal gene transfer and they give substantial degrees of resistance. Low vancomycin resistance and inherent teicoplanin sensitivity are mostly caused by *van*C gene (Levitus et al., 2020; Sujatha & Praharaj, 2012). Vancomycin resistance is encoded via the *van*D, *van*E, and *van*G operons. In general, the genes *van*A, -B, -D, -E, and -G are inherited traits of motile enterococci, but the gene *van*C is an inherent characteristic of motile enterococci.

Several investigations conducted have found that VRE colonization exists in the community in addition to human repositories; animal, environmental, and food reservoirs could all serve as community reservoirs for VRE outside of the health-care context (Wada et al., 2020). Avoparcin is being added to feeds in farms giving rise to the detection of VRE with the *van*A gene in farms. Avoparcin was extensively employed to promote growth in Australia, Europe, and several other nations in 1975, except the United States or Canada as it did not get approval for use (Wada et al., 2019). Surprisingly, elevated levels of VRE were found in European animal farms, whereas no VRE was found in animal farms in the United States. As a result, the utilization of the avoparcin to promote animal growth was banned in Europe, leading to a quick drop in VRE in European farms, but not complete elimination. Several hypotheses have been proposed to understand the persistence of VRE; the first suggests that the utilization of the macrolide tylosin

may co-select for vancomycin resistance since both resistance factors are on the same plasmid, and that plasmid adhesion systems may be involved in resistance maintainance (Sujatha & Praharaj, 2012).

VRE could also be found outside of hospitals, indicating that a transfer of resistance genes from animals to humans or a clonal expansion of resistant bacteria could be to blame (Wada et al., 2021). Furthermore, VRE could enter foods by environmental contaminants from a variety of sources, including sewage treatment wastewater, cattle faeces, and poultry farm manure. Globally, resistant enterococci have been detected not only in the environment but also in food animals (Biggel et al., 2021; Elghaieb et al., 2019; McDonald et al., 1997; Reyes et al., 2016).

Enterococci (*E. faecalis, E. faecium, E. casseliflavus,* and *E. gallinarum*) isolated show high gentamicin, kanamycin, streptomycin, tetracycline, and glycopeptide resistance in bovine mastitis chickens, pigs, food derived from animals, uncooked food, sewage, and water. In a broad sense, the advent of this high level of resistance to antibiotics in all of these different reservoirs and habitats shows that resistant traits are transmitted between strains.

Both *E. faecalis* and *E. faecium* are still linezolid susceptible (Yang et al., 2020; Zurenko et al., 2001). Mutations in the rRNA genes are a common cause of resistance. *E. faecalis* contains four of these rRNA genes, whereas *E. faecium* have six, thus, the amount of resistance expressed is determined by the rRNA genes number carrying the necessary mutations (Hashemian et al., 2018). Following a single mutation, continuing linezolid preference stress has been linked to "gene conversion," where additional genes express same mutation with mutant genes utilising same linkage. In the dearth of antibiotics, however, if a single wild-type

gene exists, gene flipping can restore susceptibility (Papadimitriou-Olivgeris et al., 2020; Ruiz-Ripa et al., 2020), showing that these mutations have some selective disadvantages in the loss of selective stress. Modifications of L22, L4 and L3 rRNA appear to be particularly an unusual causes of resistance.

The *cfr* or *cfr*(B) gene, which expresses a methyltransferase that changes the position of A2503 in bacterial 23S rRNA, can also help enterococci build resistance to linezolid. Resistance to phenicols, lincosamides, oxazolidinones, pleuromutilins, and streptogramin A, as well as diminished susceptibility to the 16-membered macrolides spiramycin and josamycin, is bestowed by this enzyme (Park et al., 2020; Schwarz et al., 2000). The gene *cfr* is plasmid expressed and transferrable and has been linked to linezolid resistance infections in a number of Gram-positive bacteria. Finally, the acquisition of *optr*A and *poxt*A, which expresses a putative ABC transporter, has been linked to plasmid-mediated resistance (Bin Kim et al., 2021; Mališová et al., 2021).

1.4.2(a) Transfer of Virulence Factors and Antibiotic Resistant Genes

The genomic plasticity of enterococci is well known. They may integrate and exploit migratory genetic material such as plasmids, transposons, prophages, and insertion sequences, permitting them to readily transmit intrinsic characteristics between isolates of the same species, genus, or other harmful and non-pathogenic bacteria (Cattoir, 2022). Enterococcal virulence mechanisms and antibiotic-resistant genes are known to link up to extremely transferable plasmids in this setting. Gene horizontal or vertical transmission methods, as well as the capacity to accept genetic material, have earlier been linked to virulence features and antibiotic resistance in enterococci. In this context, a transfer, horizontal in

nature known as a "pathogenicity island" (PAI) (Coburn et al., 2007; Selleck et al., 2019; Zischka et al., 2015) that contains roughly 100 operons, mostly coding virulent genes, earlier identified in *E. faecalis* was observed.

A plasmid was used to carry the pathogenicity island horizontally in reaction to pheromones (De Leener et al., 2005). A genetic marker (*ermB*) to show that the resistant genes for lincosamides, macrolide antibiotics, and streptogramins were horizontally transferred from an *E. faecium* isolates of animal origin to an isolate of human origin (Wang et al., 2020; Wang et al., 2016). This route of antibiotic resistance strain proliferation through the exchange of genetic elements which are either transposons or plasmids are usually essential than clonal spread. These tests were carried out on experimental animals and did not compensate for the natural environment, which has a significant impact on the dissemination of moving materials. Enterococcal transconjugation poses a significant threat to an enterococcal strain that is relatively safe and is devoid of virulent factors, which raises severe concerns in both human and nonhuman reservoirs (Coburn et al., 2007; Tyson et al., 2018; Xu et al., 2010a).

1.5 Detection of *Enterococcus*

1.5.1 Genotyping Methods

The ability of many genotypic approaches to classify enterococcal strains to species level has been tested. Williams et al. (1991) and Patel et al. (1998) completed full sequencing of the 16S-rRNA gene. A phylogenetic tree based on these sequences was created, allowing species groups to be recognized. For a 1,452-nucleotide region, similarity values within the genus *Enterococcus* varied from 93.7% to 99.8%. (Williams et al., 1991). Because this procedure is expensive and

time-consuming, various tools have been investigated for their capacity to identify *Enterococcus*.

A PCR assay that is particular to each species has been designed (Dutka-Malen et al., 1995). Four primer pairs are utilized in this multiplex PCR experiment. The genes expressing D-alanine-D-alanine ligases (*ddl* genes) are targeted by two primer pairs, one complementary to *ddl E. faecium* and the other to *ddl E. faecalis*. *E. gallinarum* and *E. casseliflavus* have their *van*C-1 and *van*C-2 genes, respectively. Inherent vancomycin resistance is expressed by these genes.

To distinguish enterococcal isolates to species, Tyrrell et al. (1997) employed intergenic ribosomal PCR, which increases the noncoding region between the 16S and 23S rRNA genes. *E. avium, E. raffinosus, E. malodoratus,* and *E. pseudoavium*, as well as *E. faecalis* and some *E. hirae* strains, have remarkably identical profiles. Except between *E. avium* and *E. pseudoavium*, differentiation was facilitated by the processing of the amplification products with *Sau*3A.

The utility of randomly amplified polymorphic DNA (RAPD) in confirmation of enterococci was observed by Descheemaeker et al. (1997) and Quednau et al. (1998). For the species, *E. faecalis, E. faecium, E. hirae, E. durans, E. gallinarum*, and *E. casseliflavus*, the utilization of primer D11344 resulted in various amplification profiles (Descheemaeker et al., 1997). Without the utilization of computer-based assessment, Quednau et al. (1998) were capable of visibly recognizing all clinically significant species purely on their fingerprint.

E. flavescens isolates revealed same profile as *E. casseliflavus* in both experiments, indicating that the former nomenclature should be ignored. Speciation

is limited to *E. faecalis*, *E. faecium, E. casseliflavus* and *E. gallinarum*, in the multiplex PCR of Dutka-Malen et al. (1995). The RAPD, including other methods can be utilized as long as the method's discriminating capability and inter-laboratory reproducibility are acceptable.

1.5.2 Phenotyping Methods

1.5.2(a) Genus Identification

The Gram-positive, facultatively anaerobic cocci, catalase-negative *E. faecium* and *E. faecalis*, as well as the species that form a species group with the latter, share a number of features that help to distinguish them from other Gram-positive, catalase-negative, facultatively anaerobic cocci: propensity to develop in a 6.5% NaCl broth at pH 9.6, 10°C, and 45°C; occurrence of group D antigen. These features, which have been ascribed to enterococci in the past, are not shared by other enterococcal species (Devriese et al., 2006).

In all of these analyses, the newer species, in particular, are typically determined to be negative. A significant variety of additional traits are present in practically all enterococci, but they are not unique to the genus, with a few important but not definite exceptions. With reference to streptococci, the VP (Voges-Proskauer or acetoin reaction) and acid generation from ribose have a large difference value. Only *S. agalactiae*, *S. uberis*, and the -hemolytic *S. porcinus*, as well as all enterococci, except *E. saccharolyticus* (VP-), *E. asini*, and a few *E. casseliflavus* isolates, respond favorably in both tests (Devriese et al., 1993a).

Even though no one phenotypic test or set of tests can effectively define the *Enterococcus* genus, certain useful techniques could be applied. Enterococcal

colonies capable of developing in 6.5% NaCl broth and growing to "normal" colony size on media containing 0.04% sodium azide selective for enterococci are most likely enterococci. VP and/or ribose testing can be added if there is any question (Devriese et al., 2006). On these media, only streptococci belonging to the *Streptococcus bovis* species group have colony properties that are analogous to those of enterococci. These streptococci are invariably ribose-negative and do not grow in broths containing 6.5% NaCl.

This approach is only applicable when "classical" enterococci are searched for and the newer species can be ignored.

1.5.2(b) Species Identification

Species identification is more challenging, and inconsistencies within groups are more common than across groupings. Devriese et al. (1993b) provide more information. Presumptive identifications based on growth parameters are frequently validated in conventional diagnostic bacteriology, and phenotypic identifications are made utilizing identification galleries or brief identification methods like the one developed by Facklam & Collins (1989).

1.6 Vancomycin

An organic chemist discovered vancomycin from *Streptomyces orientalis*, a fungus in the remote rainforest of Borneo in 1957 (Mühlberg et al., 2020). In broth fermentation, samples from the rainforest soil where the fungus was discovered produced a chemical that was extremely efficient and bactericidal against Staphylococci. The first chemical was designated as 05865, and preliminary research revealed that staphylococci did not build resistance to it after multiple crossings in media containing this drug. Because drug-resistant Staphylococci were on the rise at the time, the US Food and Drug Administration (FDA) awarded 05865 a "fast track approval" based on open-label experiments submitted to the agency in 1958. After then, 05865 was dubbed "vancomycin," a phrase taken from the word vanquish. The original product, vancomycin, was generated through fermentation and had significant amounts of impurities (up to 70%) as well as a brown color, earning it the nickname "Mississippi Mud" (Kisil et al., 2021; Patel et al., 2020).

Vancomycin kills Gram-positive aerobic cocci and bacilli such as Staphylococci, Streptococci, Enterococci, and Pneumococci, as well as *Corynebacterium*, *Listeria*, *Bacillus* spp., Clostridia, and oral Gram-positive anaerobes. Vancomycin is effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and methicillin-resistant *Staphylococcus epidermidis* (MRSE), and also penicillin-resistant *Corynebacterium jeikeium*, *Streptococcus pneumoniae*, and *Clostridium difficile*. Vancomycin resistance is present in *Leuconostoc*, *Lactobacillus*, *Pediococcus*, and *Erysipelothrix* strains (Patel et al., 2021; Werner et al., 2020).

For methicillin-resistant coagulase-negative and coagulase-positive staphylococcal infections, such as bacteremia, endocarditis, pneumonia, cellulitis, and osteomyelitis, vancomycin is nevertheless the first-line treatment. It's also used to manage significant Gram-positive illnesses in those who are allergic to cephalosporins or semi-synthetic penicillin (Devrim et al., 2022).

Although vancomycin is bactericidal against all susceptible Gram-positive infections, it is only bacteriostatic against enterococci and requires the addition of another drug, normally an aminoglycoside, to acquire bactericidal activity. Vancomycin also has efficacy against Gram-positive anaerobes like *Clostridium* species, and also oral anaerobes like *Peptostreptococcus*, *Propiobacterium,* and others, but not Gram-negative bacilli. Vancomycin has a "slow bactericidal" action against methicillin-susceptible *Staphylococcus aureus* (MSSA) when compared to *β*-lactam, which is evident in the poor clinical likelihood of MSSA bacteremia and pneumonia incidents managed with vancomycin (Patel et al., 2020, 2021).

Vancomycin is a tricyclic glycopeptide (Figure 1.1) with a tricyclic architecture made up of seven-membered peptide chains and an associated disaccharide made up of vancosamine and glucose. Vancomycin has a molecular weight of 1449.2 g/mol, making it heavier than other *β*-lactam antibiotics but similar to the molecule daptomycin. Teicoplanin and its precursor dalbavancin, as well as the telavancin and the more remotely linked lipoglycopeptide oritavancin, have comparable molecular weights (Gandomkarzadeh et al., 2020).

1.6.1 Mechanism of Action of Vancomycin

Vancomycin hinders the polymerization of peptidoglycans in the bacteria cell wall, which is composed of complex compounds termed N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) placed in a solid layer. It is exclusively effective against Gram-positive bacteria (Lee et al., 2019). Vancomycin binds to D-alanyl D-alanine and blocks the carrier's glucosyltransferase and P-phospholipid, preventing NAM and NAG from fusing and polymerizing. As a result, intracellular materials leak out of the weakening cell wall, causing the bacteria's cell wall to die (Bartoletti et al., 2018).

1.6.2 Pharmacokinetics of Vancomycin

Vancomycin is a less bactericidal antibiotic that is administered intravenously, orally, and via rectal injection (Patel et al., 2020). When given orally, it has a 10% bioavailability or less, however, after infusing vancomycin intravenously, action commences immediately after a serum peak concentration (Durand et al., 2018). Vancomycin binds to 55% of proteins and circulates in substantial amounts in fluids and tissues, with the exception of normal meninges and cerebrospinal fluid (CSF) (Colin et al., 2021). Furthermore, unlike linezolid, it has no evident metabolism and clearance rates ranging from 0.71 mL/min/kg to 1.31 mL/min/kg in persons with healthy renal tubules, as well as a bi-phasic elimination half-life of 4 to 6 hours at the terminal end and a quick initial half-life. Patients with renal impairment should be observed around the clock due to the elimination of half-life (Durand et al., 2018). Furthermore, unlike linezolid, intravenous vancomycin is eliminated 75% through urine and 25% through the glomeruli,

whereas oral vancomycin is excreted largely through faeces (Patel et al., 2020, 2021).

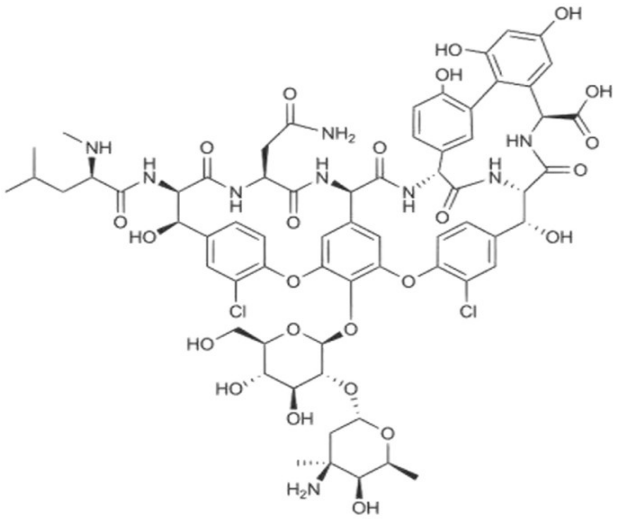

Figure 1.1 Chemical Structure of Vancomycin ($C_{66}H_{75}Cl_2N_9O_{24}$;1449.2 g/mol).

Vancomycin is extensively utilized in clinical practice, with regular usage in the skin and soft tissue infections, in addition to osteomyelitis. It's used for these reasons based on experience, before culture results are available, and when MRSA is the culprit. It's also commonly utilized in the treatment of bacteremia and endocarditis in cases when MRSA is suspected of being the culprit. In this case, after MSSA has been established as the causal agent, vancomycin has been associated with poor results and ought not to be utilized in the treatment of MSSA bacteremia or endocarditis. Vancomycin is also used to manage gram-positive pneumonia, especially in the situation of nosocomial infections and bacterial meningitis caused by penicillin-resistant *Streptococcus pneumonia*. In the 1970s, the widespread of MRSA and MRSE reintroduced vancomycin to the spotlight. Pharmacokinetics gained popularity at this time, and blood levels were measured

along with the first nomograms with dosage modifications for individuals with renal damage.

1.7 Vancomycin-Resistant *Enterococcus*

In the 1980s, vancomycin-resistant *Enterococcus* strains were discovered in Europe, probably as a result of the use of the avoparcin to boost growth in farm animals. *E. faecalis* and *E. faecium* resistant to vancomycin in England were documented. Similar strains were discovered in hospitals in the eastern part of the United States soon after the first strains of (VRE) were documented by researchers in France and the United Kingdom. Consequently, VRE has advanced at an unforeseen rate and is now found in hospitals in nearly every nation (Wada et al., 2019)

The increased use of vancomycin as a treatment option is probable to have aided transmission in the United States. Several epidemics affected hospitals all through the 1990s and 2000s on account of person-to-person transfers. Vancomycin-resistant *Enterococcus* is detected on the skin after fecal shedding and propagation through bare or unclean surfaces. The Center for Disease Control (CDC) designated vancomycin-resistant *Enterococcus* as a "serious danger" in 2013, indicating the essence for intensified surveillance and preventative efforts.

1.7.1 Clinical Manifestations

Enterococcus can cause a range of medical problems. Bacteriuria is conceivably the most prevalent clinical manifestation, albeit it is getting obvious that several of these cases are a result of colonization instead of infection. Bacteremia without endocarditis is another well-known source of infection,

followed by endocarditis. The following are some of the other clinical manifestations of VRE.

1.7.1(a) Urinary tract infection (UTI)

Enterococcus is usually mentioned as one of the three most likely causes of both mild and complex UTIs, particularly those related to healthcare and a significant proportion of them are *E. faecalis*, whereas *E. faecium* strains comprise most of the vancomycin-resistant strains (Dimitrijevic et al., 2021; Lichtenberger & Hooton, 2008). Peripherally inserted central catheters and equipment are commonly linked with it. The disease can range in severity from simple cystitis to complex cystitis, pyelonephritis, perinephric abscess, or prostatitis. Many reported UTIs are actually colonization, according to a growing body of evidence and except the patient has indications and/or signs of a UTI or sepsis, asymptomatic pyuria and bacteriuria should not be given attention (Wang et al., 2019). Remschmidt et al., 2018 in their study reported that the proportion of VRE causing UTI was 9.9% and 11.2% in the ICU and core groups respectively in Germany. In East India, Das et al., 2022 reported that the proportion of VRE causing UTI was 8.97%.

1.7.1(b) Intra-abdominal and pelvic infections

Vancomycin-resistant *Enterococcus* can be detected from intra-abdominal and pelvic infections (Bondi et al., 2020) and abscesses, wounds, and peritonitis are common problems. They are commonly found as part of a polymicrobial illness with gram-negative or anaerobic bacteria (Reinseth et al., 2019). Conversely,

enterococcal bacteremia is frequently linked with intra-abdominal and pelvic sores, necessitating antibiotic therapy against *Enterococcus*.

1.7.1(c) Bacteremia

Bacteremia is a potentially fatal complication of vancomycin-resistant *E. faecalis*. Intravascular catheters and urine catheters are common sources of nosocomial bacteremic infections. Therefore, the common sources for community-aquired bacteremia are from the gastrointestinal and genitourinary tracts. Increased mortality is linked to *E. faecium* in the bloodstream and it most likely correlates strongly with increasing levels of resistance (Aslam et al., 2020; Jumah et al., 2018).

1.7.1(d) Infective endocarditis

Enterococci are the second leading cause of Infective endocarditis which contributed 5 – 20% of cases (Hill et al., 2007). Presence of central lines, surgical procedures involving gastro intestinal (GI) or gastro urinary (GU) tracts, valvular heart disease (damaged mitral or aortic valve infections, and liver transplants) are also prominent causes or riks (Barnes et al., 2021). In patients with no risk, community-acquired endocarditis can develop, and it is mainly caused by *E. faecalis* (Dahl et al., 2019). They show clinically as sub-acute fevers and clinical signs. Fever or a fresh murmur are common indicators of infection. Endocarditis' usual indications, such as petechiae, Osler nodes, and Roth spots, are uncommon and, like other causes, are more regular with subacute illness than acute infection (Levitus et al., 2020).