MOLECULAR CHARACTERIZATION OF SELECTED *ENTEROCOCCUS* STRAINS (PREVIOUSLY *STREPTOCOCCUS*) USING GENOTYPING TECHNIQUES

By

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PREFACE

The experimental work described in this dissertation for M.Sc was carried out in the Department of Genetics, School of Biochemistry, Genetics, Plant Pathology and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, January 2006 to December 2007, under the direct supervision of Doctor M. Beukes.

I hereby declare that, unless specially indicated to the contrary in the text, this thesis is my own original work and has not been submitted to any university for a similar or any other degree.

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A.G. Jugdave

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Dr M. Beukes (Supervisor)

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ABSTRACT

The genus *Enterococcus* comprises of a group of commensal organisms of the human gut which has been associated with cases of endocarditis and urinary tract infections. In the present study, 12 *Enterococcus* isolates were obtained from clinical specimens and characterized using genotyping techniques that have become an integral part of clinical research. There were three different genotyping methods used to identify the enterococci to species level and to determine the level of genetic diversity among the selected strains. These techniques were, **r**andomly **a**mplified **p**olymorphic **D**NA-PCR (RAPD-PCR), 16S rDNA ribotyping analysis and **p**ulse **f**ield **g**el **e**lectrophoresis (PFGE) respectively. The **m**inimum **i**nhibitory **c**oncentration (MIC) to penicillin and vancomycin were also determined using a disc diffusion assay and a microtitre plate dilution assay. All twelve strains were found to be **v**ancomycin **r**esistant **e**nterococci (VRE) at a MIC value greater than 100μg/ml. Penicillin growth inhibition based on MIC values were categorized into three groups, susceptible (< 0.25 μg/ml), intermediate (≤ 3μg/ml) and resistant (≥ 4μg/ml) respectively. RAPD-PCR was performed using four random primers. Primers yielding the highest discriminative power were used for phylogenetic analysis. The phylogenetic analysis indicated that all 12 strains yielded clonal dissemination, therefore a low genetic diversity between them. The 16S rDNA of all strains were used to identify the enterococci at species level. The rDNA were sequenced and analysed using the NCBI BLAST algorithm and found to belong to three species of *Enterococcus.* These were *E.faecalis*, *E.faecium* and *E.durans*. PFGE analysis was performed by restriction of all 12 strain's genomic DNA with the restriction enzyme *Sma*I. The PFGE patterns were divided into two groups with low genetic diversity. Compared with the RAPD PCR patterns PFGE gives a higher discriminatory power as a higher dissimilarity between the strains was observed. Similar penicillin MICs for each of the strains in the three categories are grouped together in the phylogenetic trees for both PFGE and RAPD-PCR. RAPD-PCR is a sensitive, faster, specific and cost effective technique, PFGE analysis has given a higher discriminatory power, higher reproducibility of the results and the polymorphism seen in the patterns suggest that PFGE has a potential of being an essential tool in clinical diagnostics.

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

16S rDNA	16 Subunit Ribosomal Dideoxyribose Nucleic Acid
A	Adenine
BLAST	Basic Local Alignment Search Tool
bp	Base Pair
C	Cytosine
CHEF	Contour Clamped Homogeneous Electric Field
CO_2	Carbon dioxide
dATP	Dideoxy Adenosine Triphosphate
dCTP	Dideoxy Cytosine Triphosphate
ddNTPs	Dideoxy Nucleotide Triphosphates
dGTP	Dideoxy guanine Triphosphate
DNA	Dideoxyribose Nucleic Acid
dTTP	Dideoxy thymine Triphosphate
E.avium	*Enterococcus avium*
E.coli	*Escherichia coli*
E.durans	*Enterococcus durans*
E.faecalis	*Enterococcus faecalis*
E.faecium	*Enterococcus faecium*
EDTA	Ethylene Diamine Tetra Acetic Acid
ET	Energy Transfer
erm	Erythromycin Ribosome Methylase
FIGE	Field Inversion Gel Electrophoresis
G	Guanine
Kb	Kilobase
LB	Luria Bertoni Broth
LMP	Low Melting Point Agarose
mA	Milliamp
mef	Macrolide Efflux
MIC	Minimum Inhibitory Concentration
Min	Minutes
MLS	Macrolides, Lincosamides and Streptogramins
mM	Millimolar

MWM	Molecular Weight Marker
NAG	N-Acetyl Glucosamine
NAM	N-Acetyl-Muramic Acid
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
nm	Nanometres
nM	Nanomolar
PAGE	Poly Acrylamide Gel Electrophoresis
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
RGE	Rotating Gel Electrophoresis
RNA	Ribose Nucleic Acid
S.pneumonia	*Streptococcus pneumonia*
T	Thymine
TBE	Tris, boric acid, EDTA
Tm	Melting Temperature
TSB	Tryptone Soy Broth
UCT	University of Cape Town
UPGMA	Unweighted-Pair Group Arithmetic Mean
UTIs	Urinary Tract Infections
UV	Ultra Violet
V	Volts
VRE	Vancomycin Resistant Enterococci

TABLE OF CONTENTS

CHAPTER ONE

LITERATURE REVIEW

1.1. Introduction

Enterococcus is a genus of bacteria that belongs to the phylum *Firmicutes*. Some members of this group were previously designated as Group D *Streptococcus* until 1984 when genomic DNA analysis indicated that a separate genus classification was required. *Enterococcus* strains are separated into four distinct species, namely, *Enterococcus avium, Enterococcus durans, Enterococcus faecalis* and *Enterococcus faecium.* There are beta-haemolytic strains for each of the three species. enterococci are classified as facultative anaerobic, Gram positive diplococci bacteria. (Schouls, *et al.*, 2003).

Enterococcus strains have shown to be an important human pathogen involved in several ominous infections and abscesses. In the past two decades, virulent strains of *Enterococcus* have been gaining antibiotic resistance to vancomycin. Some of the virulent clinical infections caused by *Enterococcus* strains are urinary tract infections (cystitis), bacteremia, bacterial endocarditis, diverticulis, bladder infections, wound infections and meningitis. The *Enterococcus* group form part of the normal flora of the human intestinal and upper respiratory, gastro-intestinal and female urogenital tracts (Schouls, *et al.*, 2003).

As an epidemiological tool, strain typing can be used to detect the spread of hospital-associated infections, food and water contamination and veterinary infections. Strain typing also plays a role in research and industry. Genotyping methods have increasingly become a comprehensive part in clinical and research microbiology laboratories. Microbial genotyping techniques are used in molecular biology to reliably distinguish between different bacterial strains. Genotyping methods include plasmid analysis, restriction endonuclease analysis, PCR assays, multilocus enzyme electrophoresis, multilocus sequence typing, pulse field gel electrophoresis (PFGE), DNA sequencing, ribotyping, PCR ribotyping, restriction fragment length polymorphism, randomly amplified polymorphism (RAPD) PCR and amplified polymorphic DNA analysis (Poyart, *et al.,* 1998).

Genotyping profiles can be used to determine the genetic diversity among different species and strains. The genetic diversity within a particular group or species can be derived from inversions, deletions, insertions, translocations, and rearrangement of chromosomes, acquisition or loss of prophage and transposable elements in plasmids. The greater the genetic diversity among the *Enterococcus* group, the greater the rate of antibiotic resistance (Healy, *et al.*, 2005).

The genotyping techniques chosen in this study were PFGE, 16S rDNA ribotyping RAPD PCR. Among other techniques, PFGE has been widely accepted as a well established method for typing bacteria in terms of discriminatory power and reproducibility (Kearns *et al*., 2002). RAPD PCR is widely known to be cheaper, faster and less laborious with a high discriminatory power (Quale *et al*., 2001). There are alternative genotyping techniques that could have been used , such as specific and random amplification PCR, amplified fragment length polymorphism, rep-PCR, PCR ribotyping, PCR , PCR amplification of intergenic rRNA spacer regions and amplified ribosomal DNA restriction analysis (Nguimbi, *et al*., 2004).

The main aims of this study were to molecularly characterize twelve selected enterococcal strains to species level and to determine the genetic diversity among them using genotyping techniques. There were three main genotyping techniques used to characterize the enterococcal strains. These were randomly amplified polymorphic DNA RAPD) polymerase chain reaction (PCR) to detect the amount of genetic diversity among the *Enterococcus* group, pulse field gel electrophoresis for comparative profiling analysis and 16S rDNA ribotyping to identify the strains to species level. The final outcome was to produce a comparative profiling analysis with all three techniques and to verify which of the typing techniques was an adequately discriminatory technique (Healy, *et al.*, 2005).

1.2. Phenotypic Analysis and Mechanisms of Resistance in *Enterococcus Species*

1.2.1. *Enterococcus Species*

The majority of bacteria fall under 0.75μm to 4 μm. They are unicellular structures which may occur cylindrical (rod-shaped) or spherical (coccoid) forms. The bacterial cells are capable of forming aggregates in its coccoid form. They may exist as pairs (diplococci) or in chains like a string of beads (streptococci or enterococci) (Figure 1.1). The aggregates are often so characteristic as to give a generic name, hence *Enterococcus* are sub divided into four species. These are *Enterococcus faecium, Enterococcus faecalis, Enterococcus durans* and *Enterococcus avium*. These bacteria are non-sporing and can grow aerobically or non-aerobically. The *Enterococcus* groups are a major human pathogen and causative agent in several diseases (Hugo & Russell, 1992).

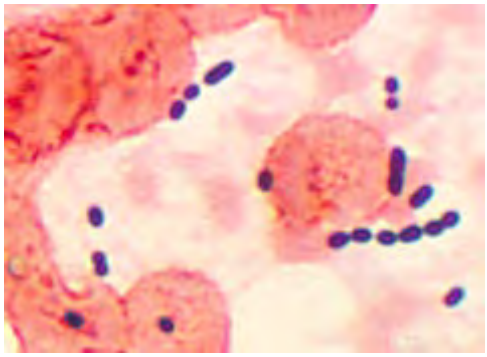

Figure 1.1. An illustration of *Enterococcus durans* diplococci forming chain like structures in pulmonary tissue (Hugo & Russell, 1992).

There are three fundamental structures of the bacterial cell. These are the cell wall, cell membrane and the cytoplasm. The cell wall consists of alternating N-acetyl glucosamine (NAG) and N-acetyl-muramic acid (NAM) molecules giving rise to a polysaccharide backbone. This provides the mechanical strength and is the target for a group of antibiotics that work to inhibit the biosynthesis occurring during cell growth and division (Bourne, *et al.,* 2001).

Strains from *Enterococcus* can be identified phenotypically initially among other techniques by using the Gram stain. The peptidoglycan layer contains chemical structures that differ between two types of bacteria, Gram positive and Gram negative. Gram staining is a well established method that consists of treating bacteria on a slide with crystal violet and Iodine dye. The dye is easily washed off by alcohol in Gram-negative bacteria such as *E.coli* but stains Gram-positive bacteria purple such as *Enterococcus durans* (Figure1. 2b). These strains are classified as Gram-positive bacteria because Gram positive bacteria have a much thicker peptidoglycan layer than Gram negative bacteria (Figure1.3) (Hugo & Russell, 1992).

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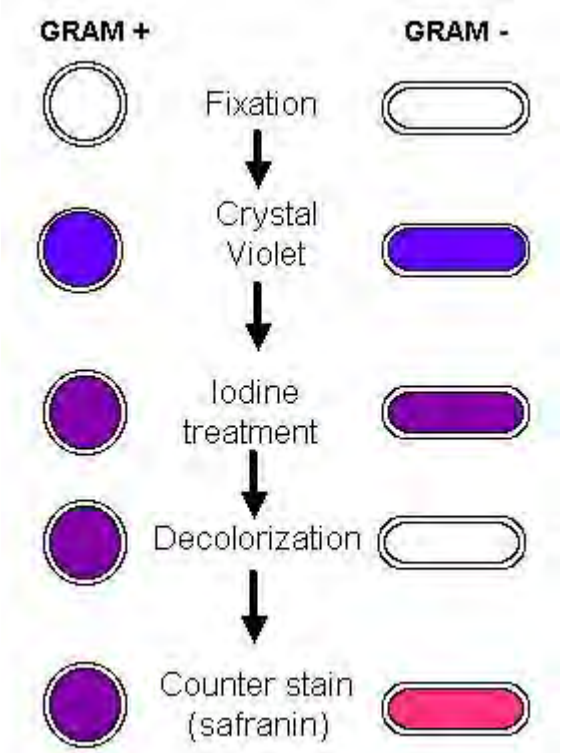

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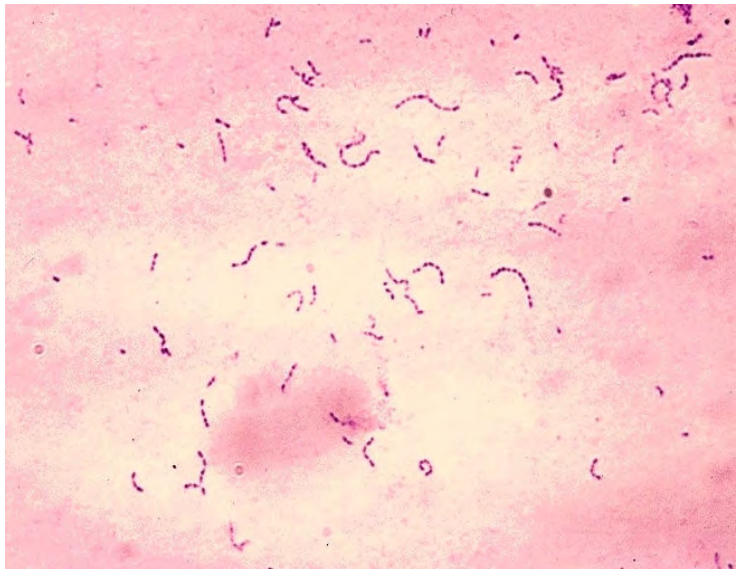


(b)

Figure 1.2. An illustration of the Gram stain technique to distinguish between Gram positive (purple stain) and Gram negative (pink stain) bacteria (Hunt, 2006).

(a) The chemical treatment of the bacterial cells to attain a stain depending on the structure of the cell wall.

(b) The Gram stain of a species belonging to the genus *Enterococcus* isolated from a clinical specimen.

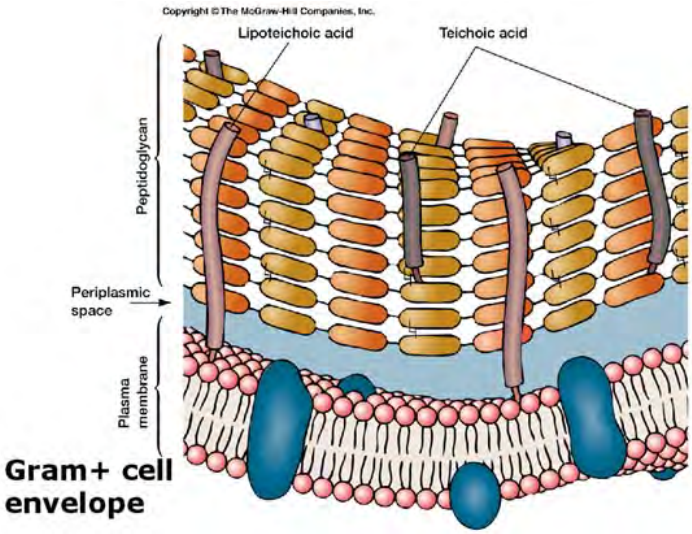


Figure 1.3. A diagram illustrating the structure of the peptidoglycan layer of Gram-positive bacteria such as *Enterococcus faecalis* (Hugo & Russell, 1992).

1.2.2. Clinical Disease

Enterococci are gram positive cocci, facultative anaerobic organisms which often occur in pairs and are difficult to distinguish from streptococci on physical characteristics alone. They are distinguished into four species: *E.avium, E.durans, E.faecalis* and *E faecium. Enterococcus* forms a normal part of the human oral cavity, upper respiratory, gastro-intestinal and female urogenital tract. Two species are common commensal organisms in the intestines of humans: *E. faecalis* and *E. faecium.* These isolates have been found to cause many severe infections (Clarridge, *et al.,* 2001). Organisms from *Enterococcus* are isolated from purulent infections such as mouth, internal organs, liver, intestines, lungs, kidneys and spleen. *Enterococcus* results in diseases such as appendicitis, peritonitis, endocarditis, meningitis, obstetric and neonatal, skin and soft tissue infections (Merriam, *et al.,* 2003; Ahmet, *et al.,* 1995).

Enterococci are responsible for many virulent infections and rank among the top four pathogens to cause nosocomial infections. The most common infections are urinary tract infections (UTIs) and bacteremia. A complication of bacteremia caused is known as

endocarditis. Other infections include wound infections, infections within the peritoneal cavity, intra- abdominal abscesses, kidney infections as an impediment of UTIs, prostate infections and skin infections (Hovart, *et al.,* 1998).

1.2.3. Mode of Infection

Their mode of infection is through the intestinal or urinary tract where they transgress into the bloodstream and thereby result in bacteremia. Some strains are able to transverse the blood brain barrier resulting in meningitis (Whiley, *et al.,* 1999).

Their pathogenesis is via many mechanisms to the attachment of the host cell. Fimbriae like proteins found on the cytoplasmic membrane promote the attachment to epithelial cells. Cell wall adhesions facilitate the binding to cells of the intestinal tract (Dorman, 1994). The secretion of cytolysin inhibits the growth of other intestinal gram positive bacteria to facilitate enterococcal colonization that induces local tissue damage. Bacteria are able to secrete a pheromone that is a chemo attractant for neutrophils and thereby promote inflammatory reactions (Jarvis & Martone, 1992). Secreted gelatinase are produced that hydrolyzes collagen and hemoglobin. The emergence of antimicrobial resistance by many β-lactam antibiotics such as penicillin G or other cephalosporins has increased drastically over the years (Ivanov, 2005). They are also able to acquire resistant genes to aminoglycosides and vancomycin (VRE or vancomycin-resistant enterococci.) The enterococci are among the most antibiotic resistant of all bacteria, with some isolates resistant to all known antibiotics (Zorzi, *et al*., 1996).

1.2.4. Level of Resistance

Enterococcus was once completely susceptible to penicillin and vancomycin. Their antibiotic resistance has recently been categorized in three groups. These are susceptible, intermediate and resistant. The development and spread of enterococcal strains with reduced susceptibility to vancomycin is partially due to the inappropriate use of penicillin or vancomycin antibiotics. This may be due to the side effects of such antimicrobial agents. Some patients refuse to intake antimicrobial agents once they have experienced

the side effects. The long periods of non-intake results in the bacterial organism gaining resistance over the β-lactam or aminoglycoside antimicrobial agent. *Enterococcus* strains minimum inhibitory concentrations and therefore level of resistance can be screened using disc diffusion assays and microtitre plate dilutions. It was found that the zone of inhibition was ≤0.12μg/ml for all β-lactam susceptible bacteria (Table1.1) (Tracy, *et al.*, 2001).

Table 1.1. The minimum inhibitory concentrations of *Enterococcus* indicating interpretation of the strains to penicillin and vancomycin (Tracy, *et al*., 2001).

Minimum inhibitory concentration (MIC)	**Interpretation**
≤ 0.12 μg/ml	Susceptible
0.25 – 2 μg/ml	Intermediate resistant
≥ 4 μg/ml	Resistant

1.2.4.1. The Action of Penicillin to Penicillin Binding Proteins

Penicillin is a β-lactam antibiotic. β-Lactams are derived from two amino acids, valine and cysteine via a tripeptide intermediate (Duez, *et al.,* 2001). The penicillin structure consists of a β-lactam ring and a thialzolidine ring. There is also a "R" group or side chain attached to the β-lactam ring that gives penicillin its name, for example benzyl penicillin (Figure 1.4). The β-lactam ring is the active moiety of penicillin and binds together with the thialzolidine ring to the serine active site of penicillin binding proteins (PBPs) (Al-Tatari, *et al*., 2006.).

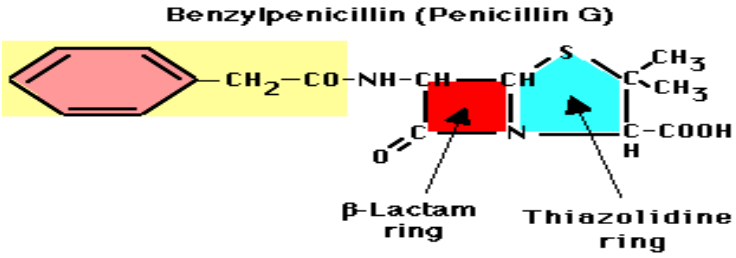


Figure 1. 4. Benzyl penicillin, a β-lactam antibiotic (Bryan, 1982).

These high molecular weight penicillin-binding proteins constitute a class of essential transpeptidase and transglycosylase enzymes that catalyze the final steps of cell wall peptidoglycan cross-linking and elongation respectively (Figure 1.5). The high molecular weight PBPs is made up of an N-terminal hydrophobic region, central penicillin binding domain, and a C-terminal domain. The β-lactam antibiotics inhibit the transpeptidase activity of PBPs (Nagai, *et al.,* 2002; Fontana, *et al.,* 1985).

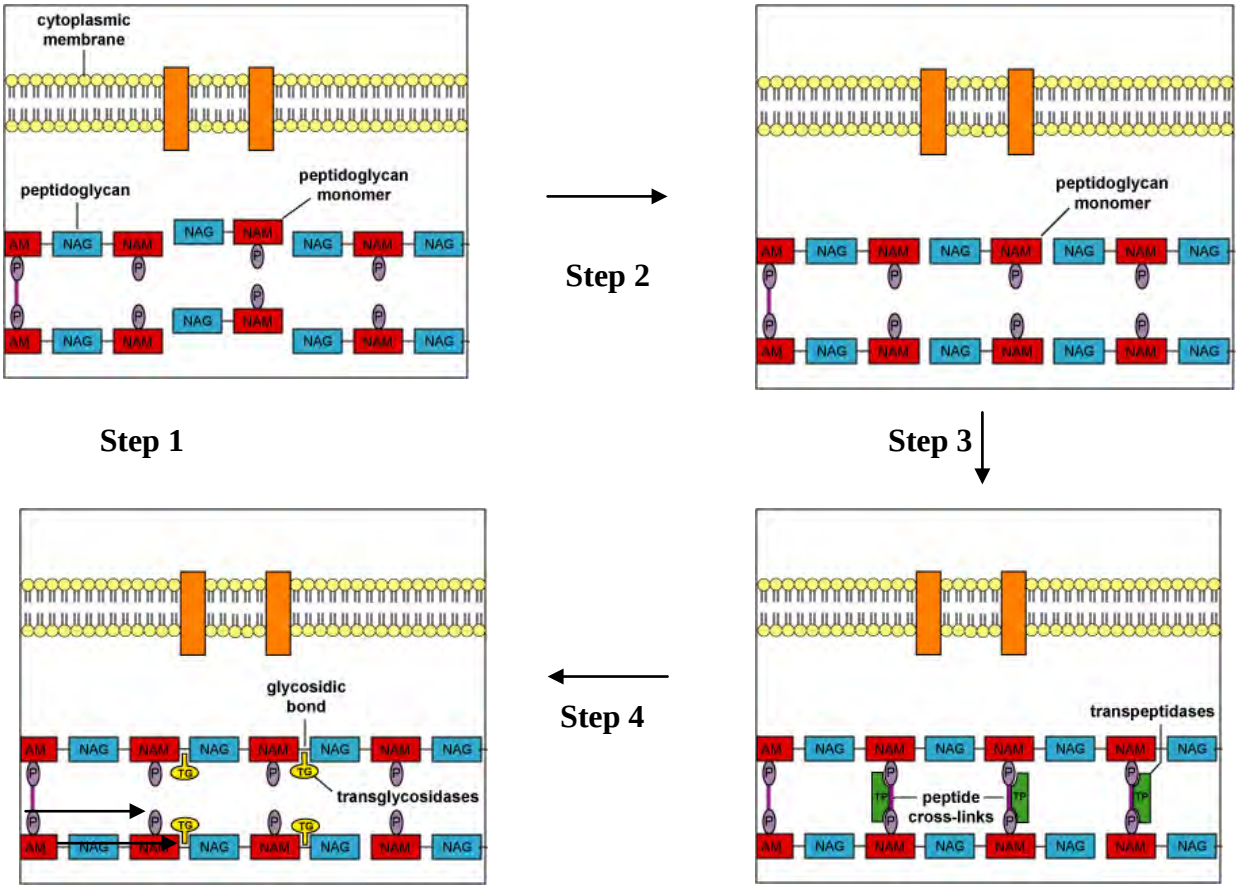


Figure 1.5. PBPs as essential transpeptidase and transglycosylase enzymes catalyzing peptidoglycan layer in *Enterococcus* (Poyart, *et al*, 1998). Where PBP = Penicillin Binding Protein, NAM = N-Acetyl-Muramic Acid, NAG = N-Acetyl Glucosamine, P = Peptide, TP = Transpeptidase and TG = Transglycosylase.

When treated with low levels of penicillin, bacterial cells change shape and grow into long filaments. As the antibiotic concentration is increased, the cell surface loses its integrity, as it puffs, swells, and ultimately ruptures. Penicillin attacks enzymes that build a strong network of carbohydrate and protein chains; called peptidoglycan that braces the outside of bacterial cells. Bacterial cells are under high osmotic pressure; because they are concentrated with proteins, small molecules and ions are on the inside and the environment is dilute on the outside. Without this bracing corset of peptidoglycan,

bacterial cells would rapidly burst under the osmotic pressure (Al-Tatari, *et al*., 2006; Du Plessis, *et al*., 1999).

Penicillin acts, as a blocking construction that prevents binding to PBPs. Penicillin is chemically similar to the modular pieces that form the peptidoglycan, and when used as a drug, it blocks the enzymes that connect all the pieces together. As a group, these enzymes are called PBPs. Some assemble long chains of sugars with little peptides sticking out in all directions. The D-alanyl-D-alanine carboxypeptidase/transpeptidase then cross-link to these little peptides to form a two-dimensional network that surrounds the cell like fishing net (Bryan, 1982).

1.2.4.2. The Action of Vancomycin to Penicillin Binding Proteins

All different microbial strains have variable resistance to different antibiotics. Vancomycin is considered the most efficient antibiotic used in the treatment of Gram positive bacterial infections. Vancomycin is sometimes the only available antibiotic left that is effective against such microbes (Al-Tatari, *et al.*, 2006). About 40 years ago, vancomycin was one of the first chemically related antibiotics that were isolated in Eli Ellis Laboratories (USA) from a *Streptomyces orientalis*, a species found in soils obtained from Borneo and India. . The bacterial species presently known for the production of vancomycin is *Amycolatopsis orientalis* (Aracil, *et al.,* 1999).

Resistance to vancomycin (MIC 0. 32 to 64 μg/ml) in *E.faecium* and *E.faecalis* results from increased production of a low-affinity (PBP), PBP5 that is thought to be fundamental to all *Enterococcus strains* and can assume the functions of all of the other PBPs in cell wall synthesis. In *Enterococcus hirae*, a closely related species to *E. faecium* has increased production of PBP5 that has been due to a deletion in *psr*, a repressor of *pbp5* expression. Similar regulatory mutations in an *E. faecium psr* are assumed to be important in an increased expression of PBP5. Mutations in the structural *pbp5* gene resulting in a decrease in PBP5 penicillin binding affinity have been found in *E. faecium* strains with high-level ampicillin resistance MICs of 128 μg/ml to 512 μg/ml (Jayaratne, *et al.,* 1999).

Vancomycin inhibits cell wall (peptidoglycan) synthesis by binding to the terminal D-alanyl-D-alanine of the pentapeptide precursors, preventing the polymerization and cross-linking that are important for peptidoglycan structural stability. Glycopeptide (vancomycin) resistance in enterococci results from the acquisition of resistance operons, expression of which results in the synthesis of precursors terminating in D-alanine-D lactic acid that bind glycopeptides with low affinity and in the destruction of normal pentapeptide precursors (Plessis, *et al.,* 1995 (Figure 1.6). Two operons, *vanA* and *vanB*, have been identified. The close association between ampicillin and vancomycin resistance phenotypes in VRE is not explained by synergistic or duplicated mechanisms of resistance (Hammerum, *et al.,* 2000; Dukta-Malen, *et al.,* 1995; Miele & Goldstein, 1995).

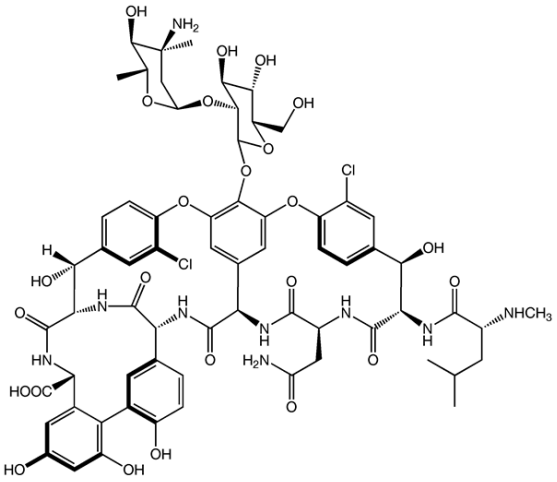


Figure 1.6. A chemical representation of the structure of vancomycin antibiotic used in the treatment of Gram positive microorganisms (Bryan, 1982).

1.2.4.3. Penicillin Binding Proteins Mechanisms of Antibiotic Resistance

Via point mutations, PBPs can become resistant by alteration of the amino acid sequence. This has been shown to occur in *Enterococcus* species (Poyart, *et al*., 1998).

Apart from their physiological properties, enterococci also differ from streptococci in that they generally are naturally 10- to 1,000-fold less susceptible to penicillin than streptococci. It was demonstrated that the natural low susceptibility of enterococci to

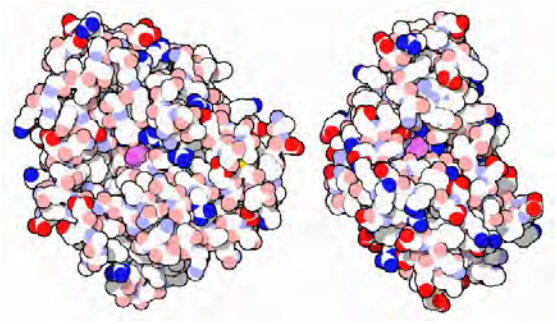
penicillin is linked to the presence of at least one high-molecular-mass PBP which has a low affinity for β-lactams. *Enterococcus faecium* appears to be the enterococcal species most resistant to β-lactam antibiotics, for which there are a wide range of benzylpenicillin MICs (0.5 to 64 mg/ml) for clinical isolates. Recently it became obvious that a new population of clinical *E. faecium* isolates for which the MICs of benzylpenicillin were very high (256 to 512 mg/ml) had emerged in different countries (Ferroni, *et al.,* 2001).

The biosynthesis of the peptidoglycan layer consists of four critical steps. The new peptidoglycan synthesis occurs at the cell division plane by way of a collection of cell division machinery known as the divisome. The bacterial enzymes that break down both the glycosidic bonds at the point of growth along the present peptidoglycan layer as well as the peptide cross-bridges that link the rows of sugars together, is known as autolysins. These autolysins are found in the divisome, a collection of cell division machinery. The transglycosidase enzymes produced by PBP 2A then insert and link new peptidoglycan monomers into the breaks in the peptidoglycan. Finally, the transpeptidase enzymes produced by PBP 2B reform the peptide cross-links between the rows and layers of peptidoglycan to make the wall mechanically strong (Nichol, *et al.*, 2002).

The PBPs are associated with the peptidoglycan layer of the bacterial cell wall. The peptidoglycan layer of *Enterococcus* is comprised of linear glycan strands containing alternating units of NAG and NAM that are cross-linked by short peptide bridges that give the polymer its mechanical strength. If the biosynthesis and assembly of cross-linked peptidoglycan is inhibited by antibiotic action, the peptidoglycan layer is unable to support the cell wall and therefore the cells take on abnormal shape and eventually lyse and die (Nichol, *et al.*, 2001).

Resistance occurs in the essential PBPs that occur on the cell wall. These PBPs are altered in such a way that interaction with β-lactam antibiotics takes place at much higher antibiotic concentrations than with PBPs of susceptible strains, and hence biological activity of the drug is greatly reduced (Asahi, *et al.*, 1999). These PBPs are able to alter their forms in such a way that they are unable to bind to the β-lactam antibiotic but able to perform their function normally (Figure1.7) (Hugo & Russell, 2002). They ultimately

conform resistance by altering or blocking the active site that these antibiotics bind to resulting in penicillin unsuccessfully binding to the active site (Ferroni & Berche, 2002).



(a) **(b)**

Figure 1.7. An illustration of a normal penicillin binding protein which due to alteration at the active moiety becomes resistant to penicillin but maintains function. **(a)** illustrates the substrate binding site and **(b)** the mutant-type protein (Ligozzi & Fontana., 1996).

The PBPs are associated with the peptidoglycan layer of the bacterial cell wall. The peptidoglycan layer of *S. pneumonia* is comprised of linear glycan strands containing alternating units of NAG and NAM that are cross-linked by short peptide bridges which give the polymer its mechanical strength. If the biosynthesis and assembly of cross-linked peptidoglycan is inhibited by antibiotic action, the peptidoglycan layer is unable to support the cell wall and therefore the cells take on abnormal shape and eventually lyse and die (Asahi, *et al.,* 1998).

The emerging resistance of bacteria to penicillin is a terrifying problem in a number of common pathogenic bacterial species. *Enterococcus*, previously designated as part of the *streptococcus* group is of growing concern as a major human pathogen of the urinary and respiratory tract, and has developed resistance to many β-lactams, an important class of antibiotics. The resistance to these β-lactam antibiotics is due to alterations to PBPs, interspecies recombination events that have occurred between PBP genes and point mutations in PBPs resulting in decreased affinity for penicillin. The worldwide incidence of infections caused by *Enterococcus* isolates resistance to penicillin has increased vastly over the past two decades (Beall, *et al.*, 1998).

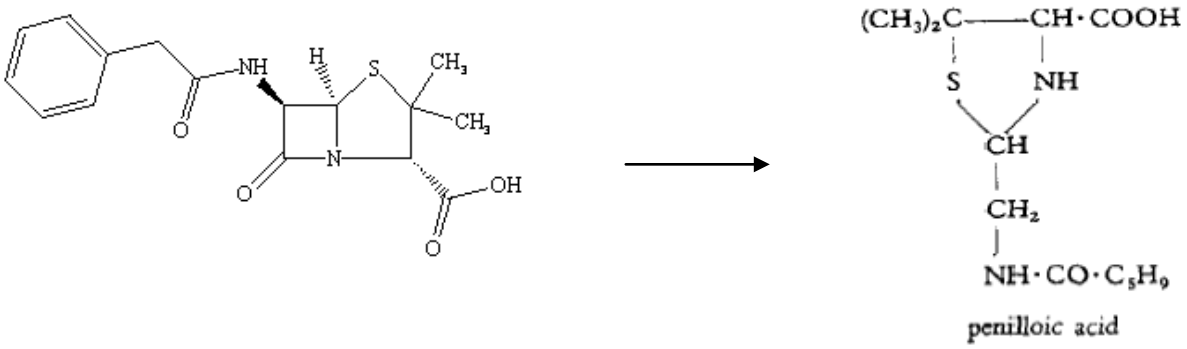
PBPs interact with β-lactams enzymatically by forming a covalent complex via the active site serine. The active site of transpeptidase activity is formed by three-conserved amino acid motifs. These are SXXK, SXN, and KT (S) G. These motifs occur at specific amino acid positions in the different amino acids. Changes in these motifs or in the positions flanking these motifs result in decreased affinity to penicillin (Asahi, *et al.*, 1999). These changes are due to point mutations in strains or recombination between PBP genes with PBP genes of other strains of *Enterococcus* form mosaic genes (Nagai, *et al.*, 2002). These alterations result in the blocking of the target site of β-lactam antibiotics and result in resistance.

1.2.4.4. Production of β- lactamases

There are several established biochemical methods to determine resistance, found to be associated with *Streptococcus pneumonia*. The conversion of an active drug to an inert product by an enzyme found only in resistant microorganisms. A change in the antibiotic target site leading to drug resistance (Poyart, *et al.*, 1998). Acquisition of resistance via gene transfer of the target enzyme and the reduction in cellular permeability to the antibiotic resulting in its exclusion from the bacterium leads to resistance (Hugo & Russell, 1992).

Resistance to β-lactam antibiotics is associated with the hydrolysis of the β-lactam ring. Soon after the introduction of penicillin, penicillin resistance was noted. It was found that these bacteria converted the drug into an inactive product, penicilloic acid (Figure 1.8). Enzymes called β-lactamases catalyze the reaction. These resistant strains produce β-lactamase controlled by many plasmids genes (Hugo & Russell, 1992). There is considerable homology amongst the amino acid sequences of the β–lactamases of gram positive bacteria suggesting a common evolutionary origin of these enzymes (Holzapfel, *et al.*, 2001). These enzymes function to lyse the active β-lactam and thialzolidine rings involved in binding to an active serine residue on PBPs. It has been shown that some of the enzymes involved in peptidoglycan synthesis may slowly release bound penicillin.

The released penicillin is degraded into an inactive penilloic acid (Smith & Klugman, 2003).

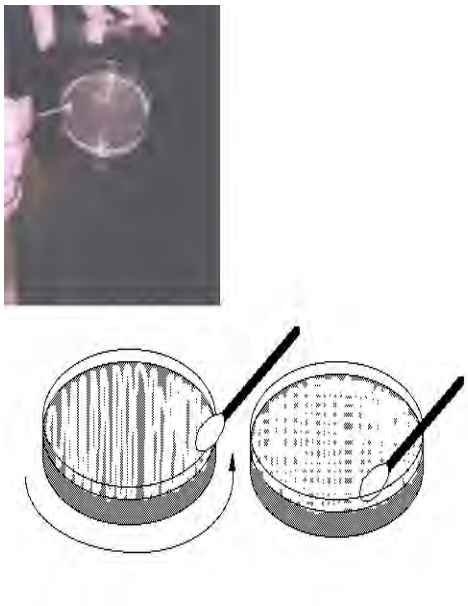


Penicillin

Figure 1.8. Action of β-lactamase (penicillinase) on penicillin resulting in degradation of the β-lactam antibiotic into inactive penilloic acid (Hugo & Russell, 1992).

1.2.5. Disc Diffusion Assay

The main aim of disc diffusion assay is to determine the minimum inhibitory concentrations (MIC) in a particular microorganism. The MIC is the minimum concentration of a particular antibiotic that is used to inhibit the microorganism. This technique can be applied to determine the MIC of penicillin in *Enterococcus*. The final result of this technique is a zone of inhibition for a particular antibiotic and microorganism. Resistance is determined as the larger the concentration of the antibiotic, the larger the zone of inhibition. There are many important steps that need to be followed for optimum results (Figures 1.9).The MIC in penicillin sensitive *Enterococcus* group has been noted to be greater than 2μg/ml, one of the highest noted resistances to penicillin (Aracil, *et al.*, 1999, Kohner, *et al.,* 1997, Russell & Chopra, 1996).



(a) **(b)** **(c)**

Figure 1.9. An illustration of the disc diffusion assay and the steps taken to determine the MIC value of a strain (Russell & Chopra, 1996).

(a) The inoculation of a culture onto agar using aseptic technique.

(b) The application of sterilized discs onto the streaked medium.

(c) The observation of zones of inhibition to determine minimum inhibitory concentration of an antibiotic such as penicillin in *Enterococcus* group.

1.3. Characterization of *Enterococcus* Strains Using DNA Fingerprinting Analysis Techniques

1.3.1. Randomly Amplified Polymorphic DNA polymerase Chain Reaction (RAPD PCR)

The polymerase chain reaction (PCR) is used to amplify specific regions on a gene. PCR is used to amplify random regions along the genomic DNA of the *Enterococcus* species. The PCR procedure can be done when at least one short DNA segments on each side of the region of known interest. The PCR reaction requires synthetic oligonucleotides complementary to these known sequences to prime enzymatic amplification of the PBP segment DNA in a test tube (Figure 1.10) (Wilkie & Simon, 1991).

The PCR protocol involves three main steps. These are:

1. The genomic DNA to be amplified is denatured by heating to 95^{o}C-97^{o}C for 15-30 seconds.
2. The denatured DNA is annealed to an excess of the synthetic oligonucleotide primers by incubating them together at 50^{o}C-60^{o}C for 30 seconds.
3. *Taq* polymerase is isolated from the thermophillic eubacterium *Thermus aquaticus* and is used to synthesize the strand. *Taq* polymerase is used because it is a heat stable synthesizing enzyme and serves as a much efficient enzyme during denaturation, i.e. does not lose its activity (Snustad & Simmons, 2000).

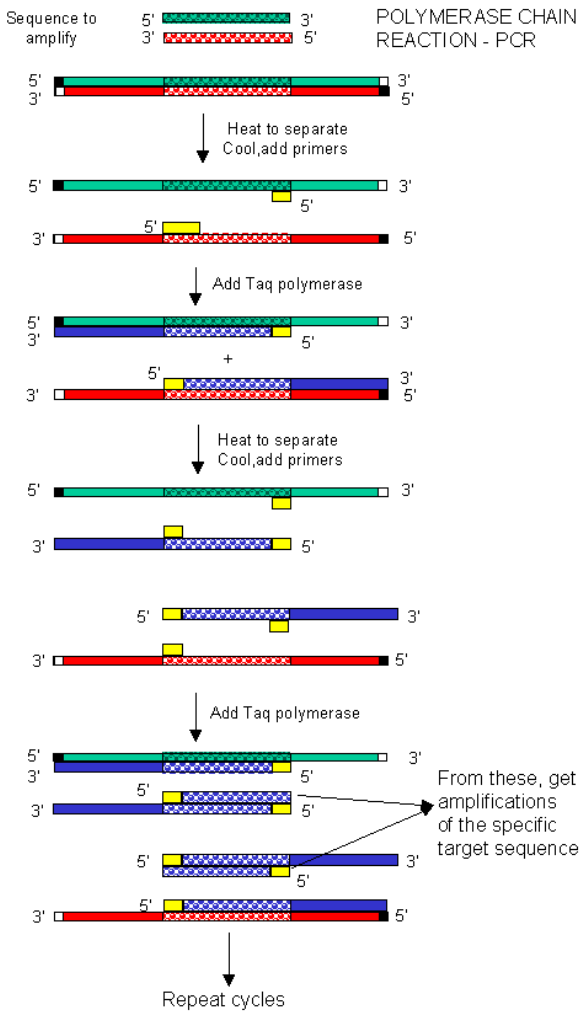


Figure 1.10. An illustration of PCR in the amplification of target sequences in a test tube (Snustad & Simmons, 2000).

Randomly amplified polymorphic DNA is PCR reactions that amplify segments of DNA at random. A normal PCR (Figure 1.10, 1.11) will amplify a known DNA sequence. The primers designed in a normal PCR flanks the gene of interest and amplifies that particular gene. A particular product will be expected, i.e., a single band. The primers used in RAPD PCR are designed to bind randomly to segments of DNA along the *Enterococcus* genome (Kearns, *et al.*, 2002).

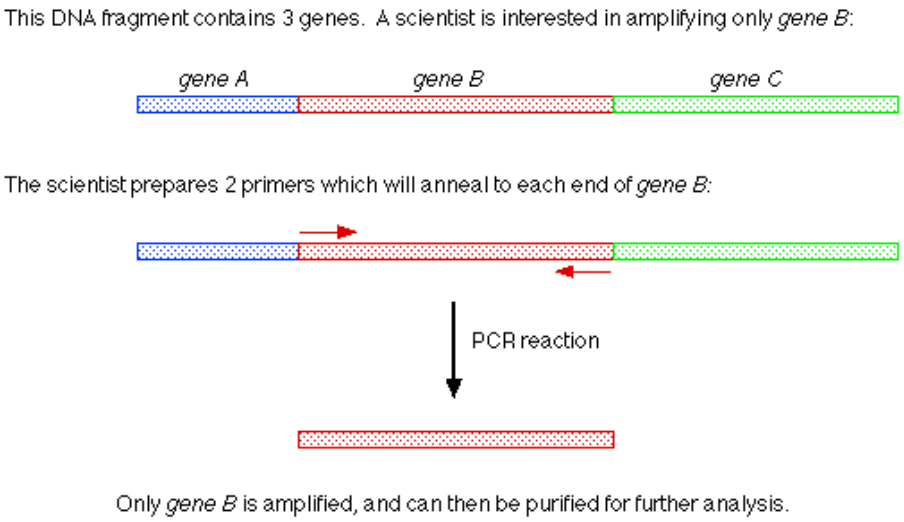


Figure 1.11. An illustration of a standard PCR (Snustad & Simmons, 2000).

RAPD analysis involves an unknown target sequence (Figure 1.12). The primer used is about ten base pairs in length and will bind randomly along the genome and will amplify that particular sequence. The PCR product expected will be a high number of bands. In figure 1.13 a large genomic DNA is subjected to RAPD PCR primers like that of *Enterococcus* isolates.

RAPD Reaction #1:

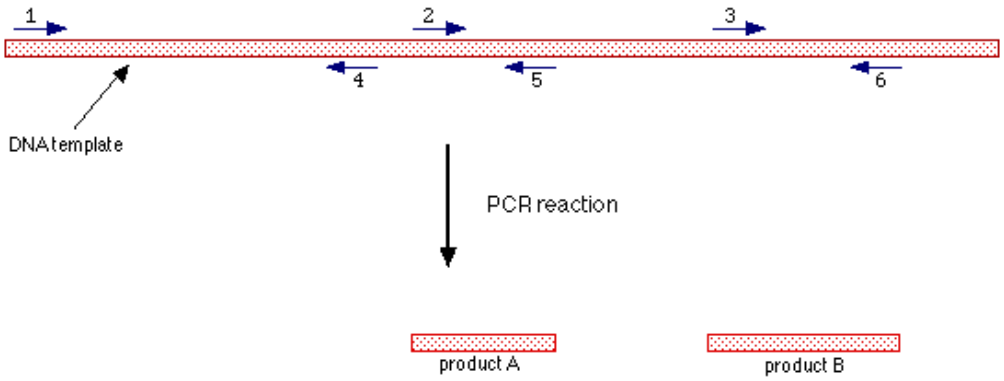


Figure 1.12. A RAPD PCR reaction illustrating the random amplification of genes as in *Streptococcus anginosus* group (Snustad & Simmons, 2000).

In figure 1.13, 2 RAPD PCR products are produced. Product A is produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 2 and 5. Product B is the produced by PCR amplification of the DNA sequence which lies in between the primers bound at positions 3 and 6. There are no PCR product is produced by the primers bound at positions 1 and 4 because these primers are too far apart to allow completion of the PCR reaction.

Note that no PCR products are produced by the primers bound at positions 4 and 2 or positions 5 and 3 because these primer pairs are not oriented towards each other.

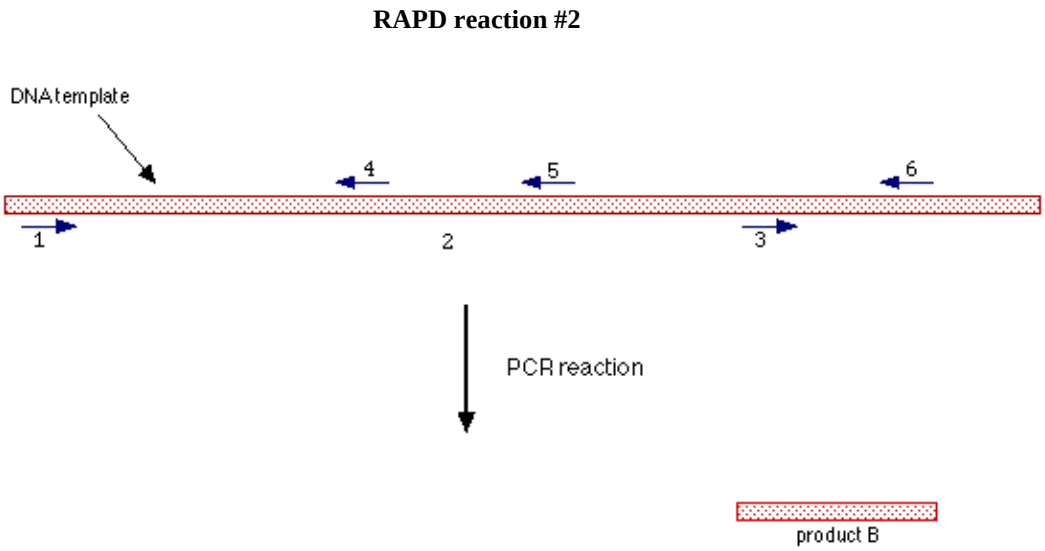


Figure 1.13. A RAPD PCR reaction illustrating a change in PCR primer will produce a different band (Snustad & Simmons, 2000).

In figure 1.16, the primer is no longer able to anneal to site #2, and thus the PCR product A is not produced. Only product B is produced. If the 2 RAPD PCR reactions in figure 1.12 and 1.13 are run on an agarose gel electrophoresis and viewed under a UV transilluminator, the bands will be analyzed as in figure 1.14.

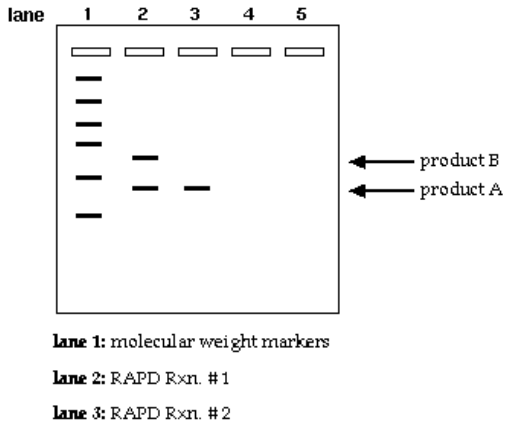


Figure 1.14. A 1.5% [w/v] agarose gel illustrating RAPD PCR reactions 1 and 2 products (Snustad & Simmons, 2000).

RAPDs are PCR -based molecular markers that may substantially reduce time, labour, and cost required for molecular mapping. RAPDs involve the use of a single DNA primer to direct amplification under PCR based amplification of random sequences. This technique can be used to detect polymorphisms. RAPD PCR is very useful in different objectives. These are assessment of genetic variation in populations and species, to study the phylogenetic relationships among species and subspecies, to construct and understand genetic linkage maps, gene tagging, and identification within species such as *Enterococcus*, any fingerprinting application to characterize a particular species.

There are many advantages to RAPD PCR technology. There are more polymorphisms than restriction fragment length polymorphism, fast and simple, a large number of bands produced per primer and differentially amplifies DNA samples based on mutations. There are some disadvantages to RAPD PCR technology. Detection of polymorphisms are still limited, reproducibility of results is inconsistent, poor profile resolutions of RAPDs on agarose gel resulting in very few bands, only detects dominant markers.

1.3.2. Design of RAPD PCR Primers

There are specific criteria that should be followed when designing primers. These important criteria are primer length, melting temperature, and specificity, complementary sequences, G/C content and 3' end sequence. The primers should be at least 18 bases in length. Primer length is proportional to annealing efficiency, the longer the primer the greater the chance of non-specific binding. However a RAPD PCR primer is about ten base pairs long hence annealing efficiency is not as effective. The annealing temperature is usually calculated as less than 5^{o}C than the estimated melting temperature (Tm) (Table 1.2). If the temperature is too low then non-specific binding may occur. There are two oligonucleotide sequences added to the reaction, hence they should have a similar Tm. The two primers are a random sequence within the template DNA of interest but not to each other. The base composition of primers should be between 45% and 55% GC content. The primer sequence must be such that there is no poly C or poly G that can enable non-specific binding. The 3' end terminal position is essential for the control of mis-priming (Snustad & Simmons, 2000). All of these criteria need to be optimized for best results.

The PCR primers designed will be specifically made to bind randomly along the genomic DNA of each species belonging to *Enterococcus*. The assumption will be made that if the primers bind randomly to the DNA, the bands will be analyzed and compared to each species indicating the similarities and dissimilarities between the species and thereby indicate the genetic diversity of the group. This will then aid the characterization of each species within *Enterococcus*.

Table 1.2. The annealing temperature for different primer lengths (Kearns, *et al*, 2002).

Primer Length	**Tm = 2(AT) + 4 (GC) – 5**
17	46 ^{o}C
18	49 ^{o}C
19	52 ^{o}C
20	55 ^{o}C

1.3.3. Screening RAPD PCR: Agarose Gel Electrophoresis

Electrophoresis is the migration of charged molecules in solution in response to an electric field. The rate of migration depends on the strength of the field, on the net charge, size and shape of the molecules and the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is simple, and highly sensitive. It is used analytically to study the properties of a single charged species and as a separation technique based on molecular weight (Schnitzer & Grunberg, 1990).

The technique requires particular equipment and reagents such as an electrophoresis chamber and power supply, gel casting trays, electrophoresis buffer, loading buffer, ethidium bromide and a transilluminator for viewing product (Figure 1.15). The ethidium bromide is used to stain the DNA fragments an orange-reddish colour for efficient viewing under transilluminator. These equipment is essential for optimum results (Schnitzer & Grunberg, 1990).

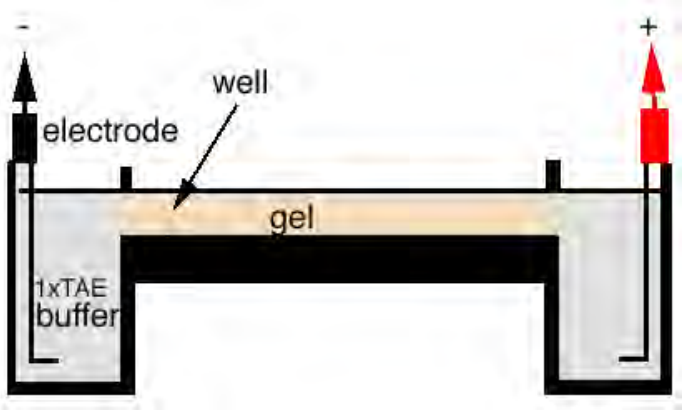


Figure 1.15. An illustration of an agarose gel electrophoresis apparatus (Hugo, *et al.*, 1992).

Agarose gel electrophoresis is an important tool for separation and characterization of DNA fragments. Agarose is a powder that can be used at different amounts depending on the size of the DNA fragment required to be separated. The higher the percentage of agarose used, the smaller the pores of the matrix and therefore the smaller the fragments of DNA that will run on the gel. The PCR products of the randomly amplified genomic DNA will be analyzed using agarose gel electrophoresis based on the separation of the product. There will be a variety of bands expected indicating the genetic diversity of the *Enterococcus* group.

1.3.4. Microbial Typing: 16S rRNA Ribotyping Analysis

The technique involves bacterial genomic DNA's being digested using rare cutting endonuclease such as *SmaI* (Table1.3). The digested DNA is then subjected to polymerase chain reaction. Specific primers that target specific conserved domains of the 16S ribosomal RNA coding sequences are used to detect the band patterns. Analysis will be carried out using pulse field gel electrophoresis (PFGE). The homology between the three species can then be determined by sequencing the 16S rDNA and using NCBI BLAST algorithm to characterise to species level. (Koneman, *et al.*, 1997; Godillo, *et al.*, 1993).

Table 1.3. Restriction endonucleases suitable for 16S ribotyping and pulse field gel electrophoresis (Smith, *et al.,* 1998).

Restriction endonuclease (Enzyme)	Recognition Sequence(5'-3')
ApaI	GGGCC/C
ClaI	AT/CGAT
NotI	GC/GGCCGC
EcoRI	GAATTC
SalI	G/TCGAC
SmaI	CCC/GGG
XhoI	C/TCGAG

1.3.5. DNA Sequencing Techniques

There are many different types of sequencing methods and kits available in molecular biology. Sanger dideoxy sequencing technique is one of the more established manual sequencing techniques and dye terminator s equencing technique (DYEnamic ET terminator cycle sequencing) being a more advanced method using energy transfer dye labeled terminators. A sequencing technique will be used to determine the sequence of the amplified genes from the *Enterococcus* species.

1.3.5.1. Sanger Dideoxy Sequencing Technique

DNA sequencing has become a powerful technique in molecular biology that allows the analysis of genes at the nucleotide level. This technique has been applied to many areas of research. A use of Sanger sequencing is with the polymerase chain reaction, a method that rapidly produces numerous copies of a desired piece of DNA requires first knowing the flanking sequences of this piece. This technique may also be applied to sequencing proteins directly and amino acid sequences can be determined more easily by sequencing a piece of cDNA and finding an open reading frame (Hall, *et al.,* 1992).

Dideoxynucleotide sequencing represents only one method of sequencing DNA. This technique utilizes 2', 3'-dideoxynucleotide triphospates (ddNTPs); molecules that differ from deoxynucleotides by the having a hydrogen atom attached to the 3' carbon rather than an OH group (Snustad & Simmons, 2000). These molecules terminate DNA chain elongation because they cannot form a phospho-diester bond with the next deoxynucleotide (Trzcinski, *et al*., 2004).

1.3.5.2. DYEnamic ET Terminator Cycle Sequencing

DYEnamic ET terminator cycle sequencing is a kit designed for sensitive and vigorous sequencing using energy transfer dye-labelled terminators. To sequence, a combination of the sequencing reagent, template DNA and primer are premixed and are thermally cycled. The reaction products are precipitated with ethanol so that the dye-labelled terminators that are not incorporated are separated (Sensen, 2002). The samples are then mixed with a suitable laoding solution for separation and detection using the sequencing instruments such as ABI 373, ABI310 or ABI 3700 (Ju, *et al*., 1996).

This technique is based on the modification of the Sanger method, where terminators are labeled with fluorescent dyes for automated detection. However in this method the four dideoxy terminators (ddNTPs) are labeled with two dyes, a florescein and one of four different rhodamine dyes. The flourescein dye behaves as a donor that absorbs energy from the incident laser light and transfers it to the rhodamine acceptor dye on the same terminator molecule. Each acceptor dye emits light at a particular wavelength for detection of the next nucleotide resulting in chain termination (Sensen, 2002).

1.3.6. NCBI Data Analysis

The sequenced genes from the *Enterococcus* will be entered into BLAST (Basic Local Alignment Search Tool) algorithm on NCBI (National Center for Biotechnology Information). NCBI is a bioinformatics search tool that enables the comparison of stored sequenced data to strains sequenced by Sanger- Dideoxy method. NCBI provides a search tool that compares sequences to a wide range of organisms including bacteria, viruses,

etc. This bioinformatics tool will be used to analyze and characterize the *Enterococcus* group into their original species being *E.faecium, E.faecalis, E.avium* and *E.durans.*

1.3.7. Pulse Field Gel Electrophoresis (PFGE)

Conventional electrophoresis has been ineffective in influencing the size dependent migration of large DNA. The cause of this size dependent migration of DNA is most likely related to the rod like shape of the DNA molecules (Koneman, *et al.,* 1997). Different sizes of DNA fragments differ in the lengths of their long axes, but not in the diameter of their short axes. In an electrical field the large DNA orientate themselves along their long axes as they enter the gel matrix. Since all large molecules have the same charge to mass ratio and they all travel along their long axes and have identical short axes, they therefore move at the same speed in the gel (Bartie, *et al*., 2000).

The analysis and manipulation of DNA are fundamental in molecular biology. The separation of DNA mixtures into different sized fragments by electrophoresis has been well established in the early 1970's. DNA was isolated intact and then treated with restriction enzymes to generate pieces small enough to resolve by electrophoresis in agarose or acrylamide. Although this procedure still forms the core of DNA separation and analysis in current laboratories, the rules of the separation have changed (Chu, 1986).

In 1984, Schwartz and Cantor described pulse field gel electrophoresis (PFGE), introducing a new way to separate chromosomal fragments produced by enzymatic digestion of intact bacterial genomic DNA (Figure 1.16). PFGE resolved extremely large DNA for the first time, raising the upper size limit of DNA separation in agarose from 30-50 kb to well over 10 Mb (10,000 kb). In contrast to the conventional gel electrophoresis, PFGE is multi-directional, continually changing the location of the positive charge (Georing, 1993). The DNA molecules respond by continually reorienting their direction of migration through the agarose gel. In addition, an electric pulse of different duration favours the separation of different sizes of DNA fragments (Elliot, *et al.,* 1993). With each reorientation of the electric field relative to the gel, smaller sized DNA will begin moving in the new direction more quickly than the larger DNA. Thus, the large DNA lags behind, providing a separation from the smaller DNA molecules (Coque & Murray, 1995).

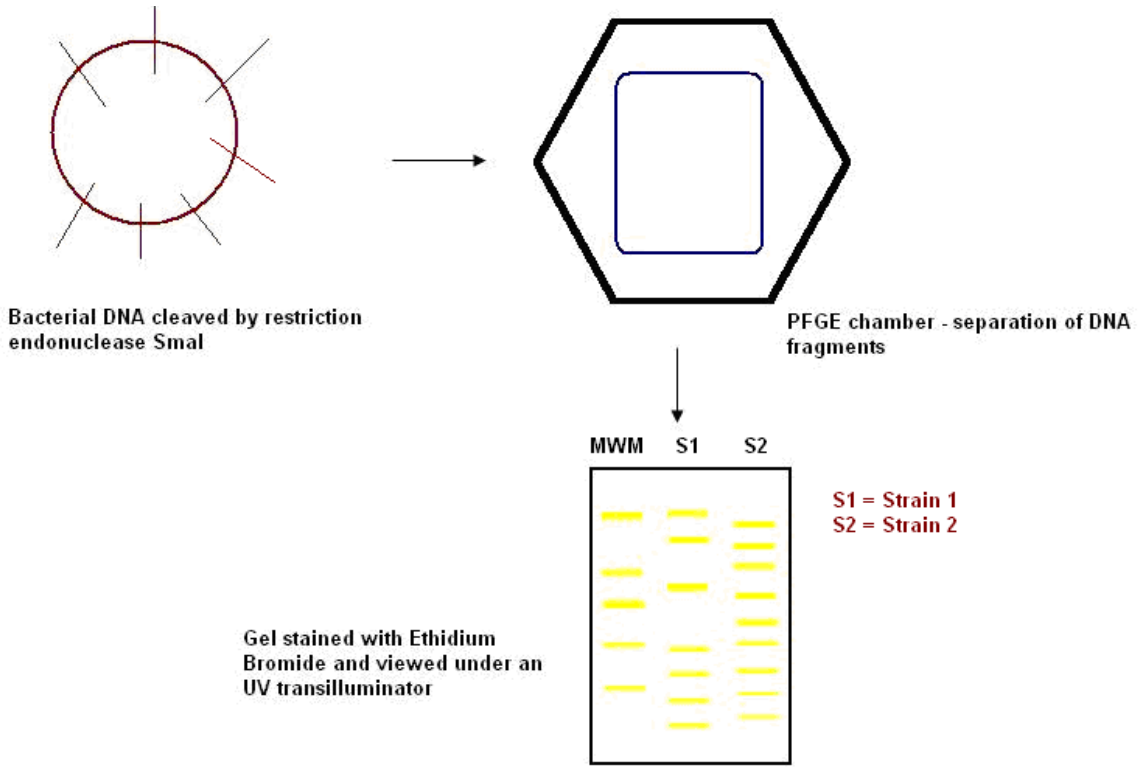


Figure 1.16. A sketch to illustrate the process of pulse field gel electrophoresis.

The size of the molecule that will separate from the others depends strongly on the length of the pulse in each direction. A fast pulse rate-short pulse time, causes smaller molecules to separate, whereas a slow pulse rate-long pulse time, causes even larger molecules to separate (Chu, 1986). If the pulse rate is increased, known as ramping, during the period of a run, a larger size range of molecules will separate out very sharply (Sahm, 1996). By varying both the direction and the duration of the electric field, PFGE allows the resolution of DNA molecules greater than 1000 kb in length.

Other physical factors have shown to be important. Changes in parameters such as temperature, voltage, agarose concentration and ionic strength will act inter-dependently, but non-uniformly, on the mobility of different sizes of DNA. Thus changes in one parameter might affect resolution adversely, but making an adjustment in another parameter can restore resolution. The agarose concentration used most widely is 1%

[w/v], with the size and the shape of the gel being determined by the precise method and design of the apparatus used, but most pulse field methods use horizontal agarose gels in submerged mode (Tikoo, *et al.,* 2001)). Buffers are circulated continuously and cooled normally to a constant temperature between 10^{o}C and 15^{o}C (Chu, 1991). Electrical conditions and separation times depend on the precise method being used and the size range of the DNA molecules being separated (Chu, 1986).

After completion of the electrophoresis, gels are stained with ethidium bromide (0.5 mg/ml) for an hour, destain with distilled water as necessary, and the DNA visualized in the same manner as for conventional agarose gel electrophoresis. The effective use of PFGE requires accurate and reliable size standards for estimating the sizes of the DNA fragments being studied.

There are different types of PFGE methods that differ in the way the pulse field is delivered to the agarose gel. These are contour clamped homogeneous electric field (CHEF); field inversion gel electrophoresis (FIGE) and rotating gel electrophoresis (RGE) (Carle, *et al*, 1986). FIGE and CHEF methods display different but complementary ends of the PFGE spectrum.

RGE is one of the most recent commercial introductions of pulsed field equipment and combines variable angles with a homogeneous electric field (Figure 1.17). The electrodes are positioned along opposite sides of the buffer chamber with their polarity fixed. Briefly, the gel is cast on a circular running plate and then placed in the buffer chamber. The gel is coupled to a magnetic drive beneath the buffer chamber to eliminate the possibility of leakage that a direct connection might cause. To force the migrating DNA to a new direction, the magnetic drive simply rotates the gel to the new angle. Because the reorientation angle of the DNA is determined by a straightforward mechanical coupling, RGE offers a lot of flexibility at a reduced cost. Voltage, angle, and pulse times are varied with the program stored into memory of the unit.

Figure 1.17. Electrode configuration of RGE, the positive charge orientation is indicated by the arrows in the middle of the diagram (Carle, *et al.,* 1986).

Field inversion gel electrophoresis (FIGE) utilizes any conventional electrophoresis chamber box that has temperature control and periodically inverts the positive charge by 180^{o}, hence the term field inversion. During electrophoresis FIGE subjects DNA molecules to an 180^{o} re-orientation. As a result, DNA molecules spend a certain amount of time moving backward but with the net movement being forward (Figure 1.18). FIGE has the potential for rapid electrophoretic separation, in the range of a few hours, which is preparative in nature because DNA fragments more than 300 to 400 kb tend to have a thicker, more diffuse appearance (Carle, *et al*., 1986).

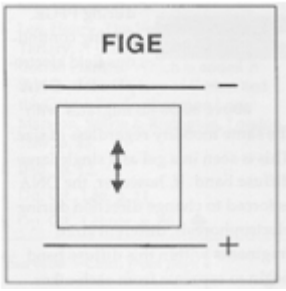


Figure 1.18. Electrode configuration of FIGE, the positive charge orientation is indicated by the middle arrow in the diagram (Carle, *et al*., 1986).

Cantour clamped homogeneous gel electrophoresis (CHEF) uses a more composite electrophoresis chamber, with multiple electrodes arranged in a polygonal cantour and clamped to predetermine electric potentials to achieve a highly uniform electrophoretic

field, usually reorienting the DNA molecules over 96^{o} to 120^{o} angle. The method applies the principles of electrostatics to gel electrophoresis (Figure 1.19) (Chu, *et al*., 1986).

In particular, the electric field vector is confined to two dimensions and has two components, Ex (x, y) and Ey (x, y). To simplify the problem, the electric field may be expressed as the negative gradient of a single function, the scalar potential field $\phi(x, y)$ (Chu, *et al*., 1986).

$$Ex\ (x, y) = -\partial\phi\ (x, y)/\ \partial x \qquad (1a)$$

$$Ey\ (x, y) = -\ \partial\phi\ (x, y)/\ \partial y \qquad (1b)$$

A homogeneous electric field is generated by two parallel, infinitely long electrodes in such a way that if one electrode is located along the x axis (y = 0) and the other is separated by a fixed distance (y = a), the potential field between the electrodes is,

$$\phi\ (x, y) = \phi_0\ y/a \qquad (2)$$

where ϕ_0 is the voltage applied across the electrodes (Chu, *et al*., 1986). Substitution of equation 2 into equation 1 shows that the corresponding electric field is homogeneous and oriented perpendicular to the electrode,

$$Ex\ (x, y) = 0 \qquad (3)$$

$$Ey\ (x, y) = \phi_0 \qquad (3b)$$

It is impractical to use infinitely long electrodes but it is possible to produce a homogeneous electric field with a finite system. A solution is to use multiple electrodes arranged along a polygonal contour in which two faces of the polygon coincide with the positions of the infinite electrodes (Chu, *et al*., 1991). The electrodes along y = 0 and x = a are clamped to the potentials 0 and ϕ_0 respectively, whereas the remaining electrodes located at intermediate positions are clamped to intermediate potentials, as determined by equation two (Chu, *et al*., 1986).

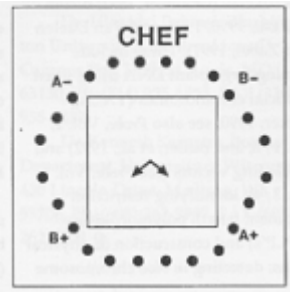


Figure 1.19. Electrode configuration of CHEF, the positive charge orientation is indicated by arrows in the middle of the diagram (Carle, *et al*., 1986).

The DNA preparation and digestion using a restriction endonuclease is a core requisite of microbial typing and pulse field gel electrophoresis. Along with the ability to separate large DNA came the need for new sample preparation and handling procedures. Large DNA is easily sheared and also difficult to pipette due to its high viscosity. The solution to this problem is to first embed the bacteria in agarose plugs and then treat the plugs with enzymes to digest away the cell wall and proteins, thus leaving the naked DNA undamaged in the agarose. The plugs then are cut to size, treated with restriction enzymes if necessary, loaded in the sample well, and sealed into place with agarose (Table 1.3). PFGE is a very formidable, simple approach in the separation of large DNA and plays an important role in DNA finger printing analyses in molecular biology.

1.3.8. Microbiological Interpretation of PFGE and RAPD-PCR Analysis

The interpretations of DNA fragments or banding patterns generated by PFGE and RAPD analysis and translate them into useful epidemiology (Miranda, *et al.,* 1991). The comparative analysis of PFGE and RAPD patterns are imperative as well as the understanding of altered patterns via genetic events. Both PFGE and RAPD patterns of isolates representing the outbreak strain would be indistinguishable from one another and distinctly different from those of epidemiologically unrelated strains (Tenover, *et al.,* 1997).

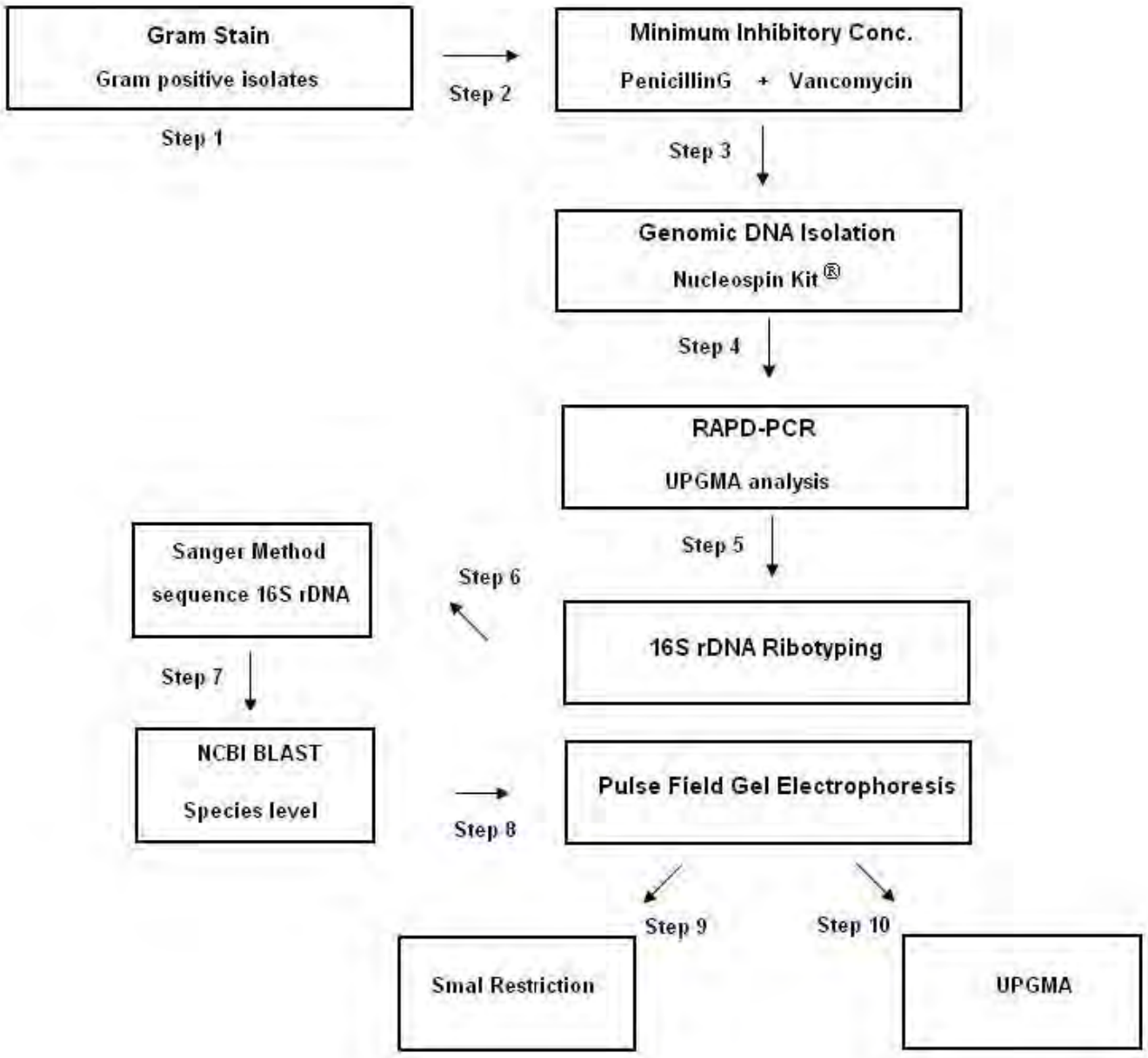
Many random genetic events such as point mutations, transversions, inversions, deletions and insertions result in a change in genetic variability and therefore affect the band patterns for PFGE and RAPD-PCR. These genetic events allows for the interpretation of each isolate into four categories (Tenover, *et al*., 1997).These are indistinguishable, closely related, possibly related and unrelated to the outbreak pattern (Table 1.4).

Table 1.4. A microbiological interpretation of DNA profiles from PFGE and RAPD-PCR analysis (Tenover, *et al.,* 1997).

Microbiological Interpretation based on typing results	**No. of genetic differences compared to outbreak pattern**	**Typical no. of fragments compared to outbreak pattern**	**Epidemiological correlation**
Indistinguishable	0	0	Isolate is part of the outbreak
Closely related	1	2-3	Isolate is probably part of the outbreak
Possibly related	2	4-6	Isolate is possibly part of the outbreak
Different	3	≥7	Isolate is not part of the outbreak

1.4. Molecular Characterization of Selected *Enterococcus* Isolates to Species Level: *E.faecium, E.faecalis, E.avium* and *E.durans*

The following procedure will be undertaken to characterize the *Enterococcus* into its four distinct species.



CHAPTER TWO

MATERIALS AND METHODS

2.1. Bacterial Strains

The bacterial strains used in this study were selected *Enterococcus* and *Escherichia coli (E. coli)* strains. All enterococcal strains were supplied by Dr M.Beukes and are clinical isolates of the culture collection at the microbiological laboratory of the University Of Maastricht, the Netherlands. The *E. coli* strain used was JM109 (ATCC). Twelve strains of *Enterococcus* were used, designated 21, 175, 301, 382, 406, 430, 468, 859, 904, 908, 943 and 1393. All enterococcal strains were cultured at 37^{o}C overnight in Tryptone Soy Broth (TSB) [Merck Laboratories, South Africa]. The *E. coli* JM109 strain was grown at 37^{o}C in Luria Bertoni Broth (LB) [Merck Laboratories, South Africa].

2.2. Bacterial Identification.

The Gram types of the strains were confirmed using the Gram stain technique. The Gram stain technique was performed as follows; a small drop of water is placed on a microscope slide. A colony of the strain that is being tested is picked up with an inoculating loop, mixed with the water and spread evenly onto the slide to make a homogeneous film. The film is allowed to dry, and heat fixed over a Bunsen flame. After the slide has cooled down, oxalate crystal violet stain [a mixture of 0.004g/L Crystal violet, 95% Ethanol, 0.08g/L Ammonium Sulfate in distilled water] is covered onto the slide for 1 minute and washed off gently under a stream of water. The slide was then covered with Iodine [2g Potassium Iodine and 1g Iodine in 20 ml water] for 1 minute and washed as before. In the following step the smear was decolorized with 95% Ethanol for 30 seconds and the ethanol wash off as before. The smear was counterstained with Safranin [10 ml of saturated solution of Safranin in 95% Ethanol in 100 ml water] for 1-2 minutes. Finally the slide is blotted dry with filter paper. Slides were viewed under an oil immersion microscope at 100x magnification.

2.3. Disc Diffusion Assay

Bacterial strains were grown on Tryptone Soy agar (TSA) [Merck Laboratories, South Africa] [38g Tryptone Soy Agar in 1 litre distilled water, autoclaved at 121^{o}C for 15 minutes] overnight at 37^{o}C in 5% CO_2 atmosphere. The antibiotics used for minimum inhibitory concentration (MIC) determination were Penicillin G (benzyl penicillin) and Vancomycin [Sigma, Germany]. Penicillin G and Vancomycin concentrations used were, 0.1 μg/ml, 0.2μg/ml, 0.4 μg/ml, 0.6 μg/ml, 0.8 μg/ml, 1 μg/ml, 2 μg/ml, 4 μg/ml, 6 μg/ml, 8 μg/ml and 10 μg/ml. Over-night cultures (200μl), of the test strains, were inoculated into 5ml top layer agar (half strength agar, 7% w/v), and spread onto the surface of TSA plates using a hockey stick spreader. The relative antibiotic susceptibilities were tested using sterile paper disks containing penicillin and vancomycin, ranging from 0.1μg/ml to 60μg/ml. The control organism used in this study was *Escherichia coli* (*E. coli*). After over-night incubation at 37°C, plates were checked for inhibition zones. The relative size of the inhibition zones were measured and tabulated. The experiments were duplicated for each strain.

2.4. Microtitre Plate Assay

Selected *Enterococcus* strains and *E. coli* JM109 were grown at 37^{o}C overnight in TSB. Penicillin and Vancomycin were used as antibiotics with concentrations ranging from 0.1 μg to 100 μg per ml. These were prepared from a 1mg/ml stock of both antibiotics. Each well contained a 180 μl of TSB media, a specific amount of the antibiotic working stock and made up the total volume of 200 μl with sterile distilled water. A 2 μl inoculation of each corresponding strain is added to each well, resulting in 1% inoculums (v/v) in a total volume of 200 μl.

Table 2.1. Experimental layout of the microtitre plate assay using antibiotics vancomycin and penicillin.

Well no.	1	2	3	4	5	6	7	8	9	10	11	12
μg/ml	70	60	50	40	30	20	10	5	1	0.5	(+)	(-) Control
Antibiotic stock (μl)	14	12	10	8	6	4	2	1	0.4	0.1	-	-
d H_2O	6	8	10	12	14	16	18	19.0	19.8	19.9	20	20
TSB	Add 180 μl of TSB into each well											

2.5. Genomic DNA Isolation

Genomic DNA from all strains was isolated using the NucleoSpin® Tissue Kit [Macherey-Nagel, Easton, PA, USA] according to manufacturer's instructions. Briefly; each bacterial strain was grown overnight in TSB at 37^{o}C in a shaking water bath. Following overnight incubation 1 ml of each culture was centrifuged in a micro centrifuge [Biocentrifuge, South Africa] at 13000 x g for 1 min at room temperature, and the supernatant removed. Due to the toughness of gram positive organisms, a pre-lysis step was included. The pellet was resuspended in lysis buffer [20mM Tris-Cl, 2mM EDTA, 1% Triton X-100(pH8), and 20mg/ml lysozyme] instead of T1 buffer. The mixture was incubated at 37^{o}C in a water bath for 30 minutes. Proteinase K (25 μl of a 6 mg/ml stock) was added to the mixture, and further incubated at 56^{o}C for 1 hour. To prevent sheering of the genomic DNA all the vortexing steps were replaced by finger-tapping and inverting the tube. Buffer B3 (200 μl) was added to the mixture and incubated at 70^{o}C in a shaking water bath for 10 minutes. The DNA's binding ability to the column's silica membrane is increased by adding 210 μl of 98% Ethanol to the samples. The sample is applied to the NucleoSpin® Tissue kit column and the DNA allowed to bind. The column containing the sample and collection tube was centrifuged at 11000 x g, for 1 min at room temperature. After discarding the flow-through, the silica membrane is washed twice, first with 500 μl buffer BW by centrifugation at 11000 x g

for 1 min at room temperature and the flow- through discarded as before. The second wash was with 600 ml of buffer B5, centrifuged at 11000 x g for1 min at room temperature]. The silica membrane is then dried by centrifuging the columns at 11000 x g for 2 min at room temperature. The elution of the pure DNA is carried out by placing the NucleoSpin ® tissue columns in a 1.5 ml microfuge tube, adding 50µl prewarmed BE buffer (70^{o}C) to the column and centrifuging it at 11000 x g for 1 min at room temperature. This step is repeated to give a final volume of a 100 µl of the DNA sample. A solution of RNAse A [20 mg/ml] of 20 µl is added to the DNA samples. Samples are stored at -20^{0}C until further use.

2.6. Analysis of DNA Using Agarose Gel Electrophoresis

Genomic DNA samples were analysed on a 0.8 % [w/v] agarose gel [0.4g agarose, 50 ml TAE buffer, TAE (4.85g/l of Tris, 0.41g/l of anhydrous sodium acetate and 0.37g/l EDTA), pH7.8]. Ethidium Bromide was added at a concentration of 5 µg/ml for visualizing the DNA in the gel. The samples were prepared as follow, each sample is prepared containing 5 µl of DNA sample, 5 µl of TE buffer [10mM Tris-Cl, pH 8.0, 1mM EDTA, pH 8.0] and 2 µl of loading buffer [0.25% (v/v) bromophenol blue, 0.25% (v/v) xylene cyanol, 30% (v/v) glycerol in distilled water]. Molecular weight marker (MWM) III (Roche, South Africa) was used as a reference. Electrophoresis was carried out at 80 volts (V) at maximum current (mA) for 90 minutes and visualized on a UV transilluminator. Images were captured using a Versadoc (BioRad, CA) documentation system.

2.7. Quantification of DNA Using UV Spectrophotometry

Spectrophotometric quantification was performed using a Carey®50 Bio UV spectrophotometer at a wavelength of 260nm. Samples were diluted in TE (Tris-EDTA) buffer at a 1:100 dilution with TE also used as a blank reference. Each DNA sample contained 5µl of DNA diluted into 495 µl of TE buffer. Samples are placed in a quartz cuvette and read at wavelengths of 260nm and 280nm respectively. The DNA

concentrations were calculated using the equation: A_{260} x dilution factor x 50 = X μg/ml, where A_{260} refer to the absorbance reading at 260 nm. The dilution factor used was 100. The DNA samples were also read at the wavelength of 280nm to determine the amount of protein contamination in samples. The ratio between the readings at 260nm and 280nm (OD_{260}/OD_{280}) provides an estimate of the purity of the DNA samples. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of between 1.8 and 2.0.

2.8. RAPD PCR (Randomly Amplified Polymorphic DNA) Primer Design

Primers used in RAPD PCR are generally designed to be approximately 10 to 15 nucleotides in length. In this study, four different random primers were chosen previously used to screen for genetic diversity among the selected *Enterococcus* species (Desai, 2005). These primers were named MBPZ1, MBPZ2, MBPZ3, and MBPZ4 (Table 2.2).

Table 2.2. Short random oligonucleotide primers used in RAPD analysis of *Enterococcus* strains (Desai, 2005).

Primer	Sequence (5'→3')	%GC	Expected product size (Kb)
MBPZ1	AGGGGGTTCC	70	0.6 - 3
MBPZ2	AACGCGCAAC	60	0.6 - 4
MBPZ3	GCATACAATC	40	0.5 – 1.5
MBPZ4	AGTCGGGTGG	70	0.1 – 4.5

The optimal annealing temperature (Tm) for each primer were determined by calculating the melting temperatures from the following equation, Tm = $2^{o}C$ (A + T) + $4^{o}C$ (G + C).

2.9. RAPD PCR (Randomly Amplified Polymorphic DNA) Optimization

The RAPD PCR was optimised by systematically altering the variables involved in the PCR reaction. The magnesium concentration in the reaction was optimized by titration, whilst keeping all other variables constant. Initially four primers were used to determine the most discriminative one of the four. The RAPD PCR experiments were run in triplicates per primer used.

Table 2.3. Parameters used during $MgCl_2$ profiling for optimization of RAPD-PCR Assay.

Reagent	**Initial Concentration**	**Required Concentration**
SdH2O	-	-
10X reaction Buffer without $MgCl_2$	10X	1X
$MgCl_2$	25 mM	A
dNTP	10 mM	0.2 mM
Primer	100 μM	2 μM
DNA Template	10 μg/ml	100 ng
Taq (Hot Start)	5 U/μl	1 U

A – Concentration of $MgCl_2$ needed

The concentration of $MgCl_2$ used ranged from 1.0 mM to 3.5 mM. Each concentration was tested with template from the twelve selected species of enterococci. Reaction mixtures were made up to a final volume of 25 μl per reaction containing a single primer in each reaction. A negative control consisting of the same reaction mixture, but with sterile distilled water, instead of template DNA and a negative enzyme control consisting of the same reaction mixture but distilled water in place of the enzyme was also used.
PCR reactions was performed using a Gene Amp® PCR systems (Applied biosystems) model 9700.

Table 2.4. RAPD PCR Profile for screening genetic differences within genome of the *Enterococcus* strains used in this study.

Cycle	Temperature	Time
1 Cycle	94°C	4min
44 Cycles	94°C	1 min
	36°C	1 min
	72°C	2 min
Last Cycle	72°C	10 min
	4°C	∞

2.10. Agarose Gel Analysis of RAPD PCR Profiles

Agarose gel electrophoresis was performed as previously described (section 2.6), with minor modifications. Amplification products were analysed on a 1% agarose gel prepared with 0.5x TBE buffer [5.4g/l Tris, 2.5g/l boric acid, 0.45g/l EDTA], containing a final concentration of 0.5 μg/ml of ethidium bromide.

The electrophoresis parameters were 100V and 50mA constant current for 6.5 hours. Molecular weight marker III (Roche) was used as molecular standards. Following electrophoresis gels were visualized with a UV transilluminator and images captured using a Versadoc (BioRad) documentation system.

2.11. Computer Analysis of Banding Pattern Data for RAPD PCR

Gel images obtained by RAPD analysis contains discrete band patterns that in most cases can be automatically identified and digitized by computer assisted systems. The gel patterns are compared on the basis of presence or absence of a band. Many different genetic measurements, or similarity coefficients, exist. The present study uses the Euclidean formula for genetic distance, which takes into consideration the presence and the absence of a band.

The data for banding patterns were synopsized into binary values, 1 and 0. Zero indicates an absence of a band at a particular position and one indicates the presence of a band at a particular position. Euclidean distance measure discard negative matches between pairs of isolates and provides a more accurate picture of relatedness. Euclidean distance measure is described by , Di_{AB} = square root ($(a_A - a_B)^2 + (b_A\text{-}b_B)^{2)}$, where Di_{AB} is the shortest distance between species A and B , a_A is a band that exists in species A, a_B is a band that exists in species B, b_A is a band that exists in species B. The phylogenetic trees were constructed using unweighted-pair group arithmetic mean (UPGMA).

2.12. 16S Ribosomal DNA Analysis using Polymerase Chain Reaction (Ribotyping)

Primers 16S8FE and B-16S1523RB (Table 2.5) were used to amplify the 16S rDNA of selected *Enterococcus* species. Amplification was done in a final reaction volume of 25 µl. Each sample contained: 0.3 µM of both primers, and 1 U of super *Taq* DNA polymerase (Roche, South Africa). The standard amounts of amplification reagents; 2mM $MgCl_2$, PCR Buffer (1x), 0.5 µg DNA template,0.2mM DNTP (Roche, South Africa) and sterile distilled water up a final reaction volume of 25 µl were used (Table 2.6). The thermocycler used was a Gene Amp® model 2700 (ABI).

Table 2.5. Primers used to amplify the 16S rDNA region of the *Enterococcus* strains (Schouls, *et al.*, 2003).

Primers	Primer Sequence (5'→ 3')	Expected product size
16S8FE	AGA GTT TGA TC (CA) TGG (CT) TC AG	1.3 Kb pairs
B-16S15223RB	AAG GAG GTG ATC CA (CTG) CC (CT) CA	1.2 Kb pairs

Table 2.6. The amplification cycles of the 16S rDNA of *Enterococcus* strains, used in this study.

Cycle	Temperature	Time
1 Cycle	94°C	3 Min
25 Cycles	94°C	20 Seconds
	55°C	1 Min
	72°C	1 Min
Final Cycle	72°C	7 Min
	4°C	∞

The melting temperatures ™ were determined for each primer to attain optimum annealing temperatures. The equation used was $T_m = 2^oC\ (A + T) + 4^oC\ (G + C)$.

2.13. Visualization of 16s rDNA amplicons by Agarose Gel Electrophoresis

Agarose gel electrophoresis was performed as previously described (section 2.6), with minor modifications. A 1% [w/v] agarose gel was prepared in 1 x TAE buffer. A final concentration of 0.5 μg/ml ethidium bromide was added to the agarose gel. The products were viewed under a UV transilluminator.

2.14. Purification of PCR Products

The twelve PCR products, obtained from 16s rDNA amplification, were extracted from the 1% agarose gel and purified using the Nucleospin® ExtractII Kit [Macherey-Nagel, Easton, PA, USA]. A clean scalpel was used to excise the DNA fragment from the agarose gel. The slice containing the fragment is weighed out and transferred to a clean tube. For each 100mg of agarose gel 200 µl buffer NT was added. The sample was incubated at 50°C for between 5-10 minutes, with periodic vortexing every 2 to 3 minutes, until the gel slices are completely dissolved.

A Nucleospin® Extract II column (silica membrane) is placed into a 2ml collecting tube, the samples loaded, and centrifuged [Beckman Centrifuge, South Africa] [JA 18.1 rotor, 11,000 x g, for 1 minute at 4°C]. The flow through was discarded and the Nucleospin® Extract II column was replaced into a collecting tube.

The silica membrane is washed by adding 600 µl buffer NT3 to the Nucleospin® Extract II column and centrifuged [JA 18.1 rotor, 11,000 x g, 1 minute at 4°C]. The flow through was discarded, the membrane dried, and the Nucleospin® Extract II column was placed back into the collecting tube.

The membrane was centrifuged [JA 18.1, 11,000 x g, 2 minutes at 4°C] to remove any residual buffer NT3. A volume of 15 µl to 50 µl of elution buffer NE was added to each sample and incubated at room temperature for 1 minute to increase the yield of DNA eluted. The samples were then centrifuged [JA 18.1, 11,000 x g, 1 minute at 4°C]. The DNA was eluted from the Nucleospin® Extract II column into a sterile micro centrifuge tube.

2.15. Sanger Dideoxy-nucleotide Sequencing

The purified 16S rDNA PCR products were sent to the University Of Cape Town, South Africa for sequencing. The DNA sequences were obtained, however the method used was not supplied at the time.

The DNA template is denatured under high temperature conditions into single strands. A primer is annealed to one single template strand. The primer is specific and is designed so that the 3'OH of the primer is next to the DNA sequence of interest. The primer is fluorescently labelled so the final product can be viewed on an agarose gel. Once the primer is bound to the DNA strands, the solution is separated into four tubes in which different reagents are added (Table2.7). The four tubes are labelled: G, A, T, and C.

Table 2.7. List of the reagents added to each tube, G, A, T and C (Russel, 2002).

Tube	Reagents Added
Tube G	4 DNTP's + ddGTP + DNA Polymerase
Tube C	4 DNTP's + ddCTP + DNA Polymerase
Tube A	4 DNTP's + ddATP + DNA Polymerase
Tube T	4 DNTP's + ddTTP + DNA Polymerase

All tubes contain a specific ddNTP (0.01* DNTP concentration) = Final concentration of ddNTP

An example of the tube A in Table 2.7

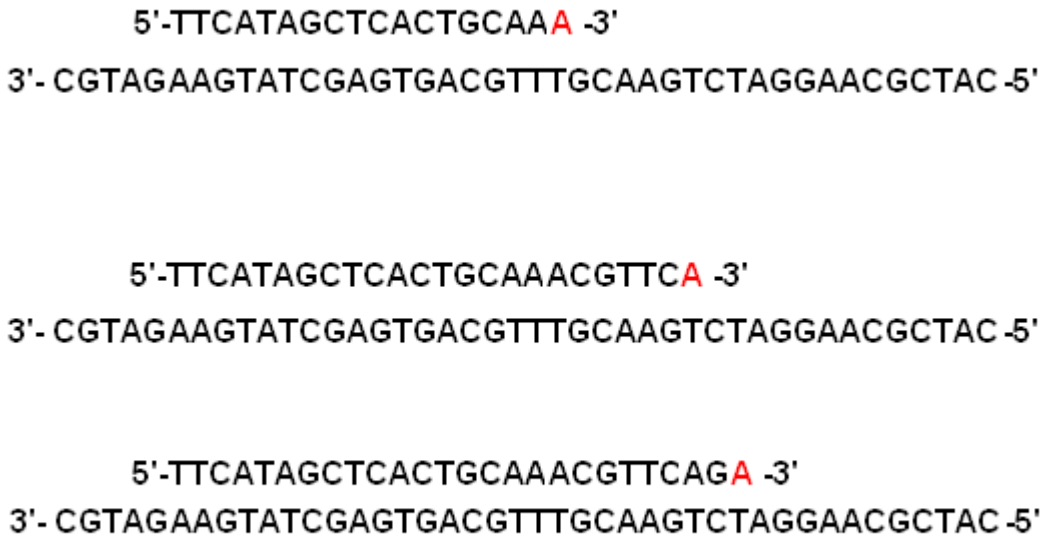


Figure 2.1. An example of the solution in Tube A where the nucleotide **A** is labeled.

All the reactions begin with a particular nucleotide and end with a specific base. In a solution the DNA synthesis has the potential to terminate every time a nucleotide for that particular position is replaced by a ddNTP. The termination of the DNA synthesis results in different band lengths. Once all four reactions are complete, all of the DNA are denatured so that separation may occur on a polyacrylamide gel. Each tube is run on a separate lane on the gel. The result of the electrophoresis is viewed under an UV transilluminator. The smaller fragments are formed due to the dideoxynucleotide being integrated near the primer and migrate faster towards the end of the gel. The sequence is read off the gel in the 5' to 3' direction from the bottom of the gel up guided by the banding patterns produced (Russell, 2002).

2.16. National Centre for Biotechnology Information: Basic Local Alignment Search Tool

The sequencing results of the 16S rDNA regions were submitted to a BLAST (Basic local alignment search tool) search at NCBI (www.ncbi.nlm.nih.gov/). NCBI is a bioinformatics research tool that contains stored data from previous research and allows for the comparison of the nucleotide sequences to other species of microorganisms sequences and therefore allow for the characterization of the four species that belong to *Enterococcus*, that is *E. avium, E. durans, E. faecalis* and *E. faecium*.

2.16.1. Computational Analysis of 16S Ribosomal DNA Sequences Using a Bioinformatics Phylogenetic Package

The 16S rDNA sequences were analyzed using a bioinformatics package which consists of four main programs to produce and analyze a phylogenetic tree. These programs are BioEdit Sequence Alignment Editor, ClustalX, Phylip and Tree view. The sequences of the 13 strains were exported as a text file. The sequence data were aligned using ClustalX (Appendix1). Thereafter the sequences were exported to Phylip for further analysis and the final production of a consensus phylogenetic tree was analyzed using Tree view (Figure 2.2). Refer to Appendix B to view the program settings used for the generation of the unrooted dendogram.

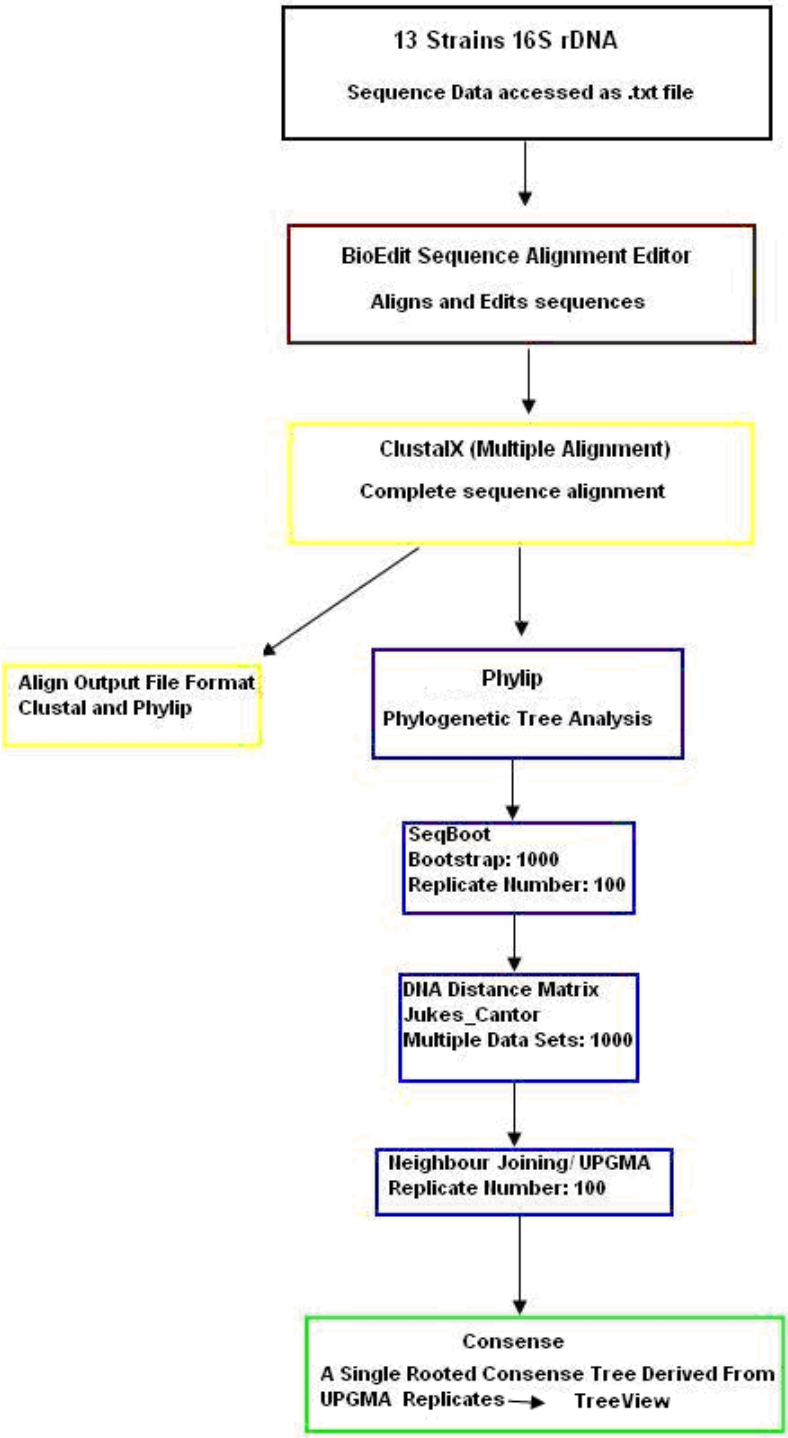


Figure 2.2. A schematic presentation to show the procedure used to analyze sequence data using the Bioinformatics package to produce a consensus phylogenetic tree.

2.17. Pulse Field Gel Electrophoresis (PFGE)

Pulse field gel electrophoresis is a method based on the separation of large DNA greater than 10Mb. This experiment was replicated in triplicate for statistical analysis.

2.17.1. Lysis of Agarose Plugs

A 5ml culture of enterococci grown in TSB is left overnight in a 37°C in a CO_2 (5%) incubator. The cells are harvested by centrifugation [JA 18.1, 5000 x g, 10minutes at 10°C]. A Beckman centrifuge was used. The supernatant is discarded and the pellet is resuspended 1.5-2 ml of PIV [10mM Tris-HCl, pH7.6 and 1M NaCl, sterile distilled water (dH_2O) used to make up final volume) solution.

Low melting point agarose (LMP) [Bio-Rad, South Africa] was used to prepare an agarose plugs [1.6% low melting point agarose made up in 100ml sterile distilled water, stored at 50°C before use]. The plug molds comb apparatus were set up where each strain had two to four molds each.

In a separate sterile tube, equal amounts of resuspended cells in solution and 1.6% LMP agarose were quickly mixed together to bring about a final concentration plugs to 0.8% [600 µl of 1.6% LMP agarose in 600 µl of resuspended cells]. The OD_{578} values of the bacterial suspension were determined by diluting the suspension 100 times (10µl of bacterial suspension was mixed with 990µl PIV solution). The OD_{578} values were adjusted to be between 0.01 and 0.025 using PIV solution. PIV was used as the blank. The wells of the plug molds are filled without creating bubbles in it. The molds are stored at 4°C for 15 minutes. Usually 600µl of cells and 600 µl of LMP agarose is enough for 4 plugs per organism. Four plugs are enough for 12-15 PFGE gels. *Enterococcus* lysis solution (Table 2.8) was prepared in a sterile flask while harvesting the cells and was aliquoted in 6-10ml in a 15 ml capacity tube, capped and incubated on ice.

Table 2.8. *Enterococcus* lysis solution made up to 100 ml.

Reagent	Final Concentration
Tris-HCl	6mM
NaCl	1M
EDTA Ph7.5	100mM
Brij-58	0.5% (v/v)
Deoxycholate	0.2% (v/v)
Sarkosyl	0.5% (v/v)
RNAse A	20μg/ml
Lysozyme	1mg/ml

After 15 minutes of incubation, the plug molds are opened and gently scraped into the EC lysis solution. Incubation was followed for 4 – 6 hours at 37°C on a rotator. Both *E. faecalis* and *E faecium* lyse well during this period.

The EC lysis solution was decanted and replaced with 10ml ESP (0.5M EDTA, pH 9.5, 1% N-lauroyl sarkosine and 50 μg/ml Proteinase K, recombinant , sterile distilled water was used to bring up to final volume] solution .Samples were incubated in tubes overnight at 50°C in shaking water bath.

2.17.2. Deproteinization of Agarose Plugs

The ESP solution was decanted after the overnight incubation. The plugs were washed three times with 10ml of TE [10mMTris, 1mM EDTA, pH8] at 37°C on a rotator for 40min for each wash. The plugs can be saved in fresh TE buffer at 4°C till further use. Plugs remain in good condition for 2-3 years. When ready to run the gel, restriction digestion of plugs was carried out.

2.17.3. Restriction Digestion Endonuclease of Agarose Plugs

The TE solution (not the plugs) was transferred from the agarose plug tube to another sterile tube. The plugs are placed on a clean glass slide carefully as the plugs are almost transparent. A sterile scalpel was used to cut at 3-4 mm wide slice from each plug (1/4 work well). The slices are then transferred to a sterile micro centrifuge tube containing 1 ml sterile distilled water. The tubes are inverted a few times and incubated at room temperature (25°C) for 5minutes. The 1ml of dH_2O was replaced with 0.2ml sterile dH_2O, 25ul of 10X reaction buffer was added to 10-20U of *SmaI* restriction enzyme. The enzyme was mixed well by flicking the tube gently. Overnight incubation was carried out at the temperature recommended for that particular enzyme (*SmaI* digestion 25°C). All twelve enterococcal strains and control strain *E. coli* JM109 was digested using the restriction enzyme *SmaI*. The enzyme *Sma1* was used because it recognizes G/C rich sequences, and because enterococci are A/T rich it will produce less frequent bands.

2.17.4. CHEF (Clamped Homogenous Gel Electrophoresis) of Agarose Plugs

The reaction mix was replaced with 1ml of TE and samples were incubated for 1hour at 37°C. During the 1 hour period, pulse field gel electrophoresis agarose (Bio-Rad, South Africa) [1.6% PFGE agarose, 0.5 x TBE and 0.5 μg/ml ethidium bromide]. Before the agarose gel was poured into the PFGE agarose tray, 3-5ml was removed to fill the wells after loading the plugs in to the wells.

Each sample is transferred, one into each well, with the use of a small spatula. The formation of bubbles while loading was prevented. The samples were all loaded against one side of the wells. The wells of all lanes were covered with the remaining of the PFGE gel. The gel was removed from the casting tray and loaded on Pulsed Field Gel Electrophoresis (CHEF) [CHEFIII Systems, Biorad, South Africa]. The gel was covered with 0.5X TBE buffer. Pulse field gel electrophoresis was carried out using three programs listed in Table 2.9.

Table 2.9. Preferred PFGE conditions for resolving all sizes of DNA fragments.

E. faecium	*E.faecalis*
1% IDNA agarose	1% IDNA agarose
0.25X TBE	0.25 X TBE
14°C temperature	14°C
15-16h run time	15 – 16h
2-28s pulse ramping time	5-35s pulse ramping time

2.18. Computer Analysis of Banding Pattern Data for PFGE

The banding analysis was compiled using the bioinformatics program package to develop a phylogenetic tree using the component program UPGMA as before (Section 2.11).

CHAPTER THREE

RESULTS AND DISCUSSION

3.1. Bacterial Strains

All bacterial strains were identified phenotypically to be Gram positive using the Gram stain technique (Figure 3.1). This indicates that these bacterial strains have a much thicker peptidoglycan layer than Gram negative bacteria (Hugo & Russell, 1992). Once the Gram stain analysis confirmed a purple stain and that the bacterial cultures were diplococci, isolation of the DNA and disc diffusion assay could be done.

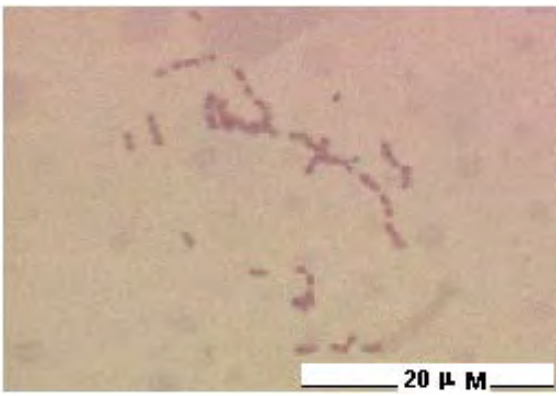


Figure 3.1. An illustration of the Gram stain technique used to determine the morphological structure of the microorganism, viewed under a light microscope at 40X magnitude, where the microbe diameter was determined to be 20µm. The bacterial strains were grown at 37^{o}C overnight in TSB, a loopful of inoculant was placed on a slide and heat fixed. Crystal violet stain [4g crystal violet, 0.8% ammonium oxalate and 20ml of 95% ethanol] was used as the primary stain, and then the slide was washed with sterile dH2O and covered with Iodine solution [2g Potassium Iodine and 1g Iodine in 200ml water]. Decolourization was with 95% Ethanol and counterstained with secondary stain safarin. The purple stains indicate Gram positive microorganisms, *Enterococcus*.

3.2. Disc Diffusion Assay

The main aim of the disc diffusion assay is to determine the minimum inhibitory concentrations (MIC) of a particular antibiotic. The MIC is the minimum concentration of a particular antibiotic that is needed to inhibit the microorganism. This technique was applied to determine the MIC of penicillin for the enterococcal species tested. Visualization of the result for this technique is a zone of inhibition

around the antibiotic disc when a particular microorganism is seeded on a media plate. The principle is that the higher the concentration of the antibiotic, the larger the zone of inhibition. However the size of the zone is sometimes related to the aqueous solubility of the antibiotic. The MIC of penicillin in resistant *Enterococcus* species has been noted to be greater than 2μg/ml, one of the highest noted resistances to penicillin (Mouz, *et al.*, 1999). The MIC values for all strains were determined using penicillin G as the antibiotic (Table 3.1).

Table 3.1. MIC values of *Enterococcus* strains and *E.coli* JM109 to the antibiotic Penicillin G using the disc diffusion assay.

Strain	**MIC value(μg/ml)**	**Interpretation**
21	80	Resistant
175	80	Resistant
301	1.5	Intermediate Resistant
382	1.5	Intermediate Resistant
406	80	Resistant
430	80	Resistant
468	30.2	Resistant
859	80	Resistant
904	3	Intermediate Resistant
908	<1	Susceptible
943	1	Intermediate Resistant
1393	<1	Susceptible
*E.coli*JM109 (Control)	>80	Resistant

The bacterial strains tested were categorized into three groups, susceptible (< 0.25 μg/ml), intermediate (≤ 3μg/ml) and resistant (≥ 4μg/ml). The highest MIC value observed was 80 μg/l for strains 21, 175, 406, 430, 468 and 849. Strains 908 and 1393 had very low MIC values, which indicated complete susceptibility to benzyl penicillin. The MIC values for strains 301, 382, 904 and 943 were ≤3 g/ml, indicating an intermediate to high resistance level for penicillin (Table 3.1).

These strains indicate intermediate to high resistance or decreased sensitivity to penicillin; hence these strains have low affinity PBPs. Strains 908 and 1393 are susceptible to penicillin and therefore do not carry low affinity PBPs. The low affinity PBPs confers resistance due to alterations or mutations on the serine active site, the site at which penicillin exhibit its action. This occurs via the folding of the protein blocking the binding site of penicillin to the PBP. The positive control used was *E. coli* JM109. *E. coli* was used as a control to confirm the efficiency activity of the antibiotic used, i.e. penicillin G. The control strain did not have any zones of inhibition from 1.0 μg/ml to 10 μg/ml. In literature *E. coli* JM109 has a MIC value of 22.3 μg/ml (Froger, *et al.*, 2001). But mutations could have occurred such as deletions, or the partial truncation of the PBPs found on the peptidoglycan layer rendering *E.coli* resistant to penicillin G. Therefore, since the antibiotic concentration ranged from 1.0 μg/ml to 100 μg/ml *E. coli* JM109 did not have any zones of inhibition. The concentration needed to increase to observe zones of inhibition.

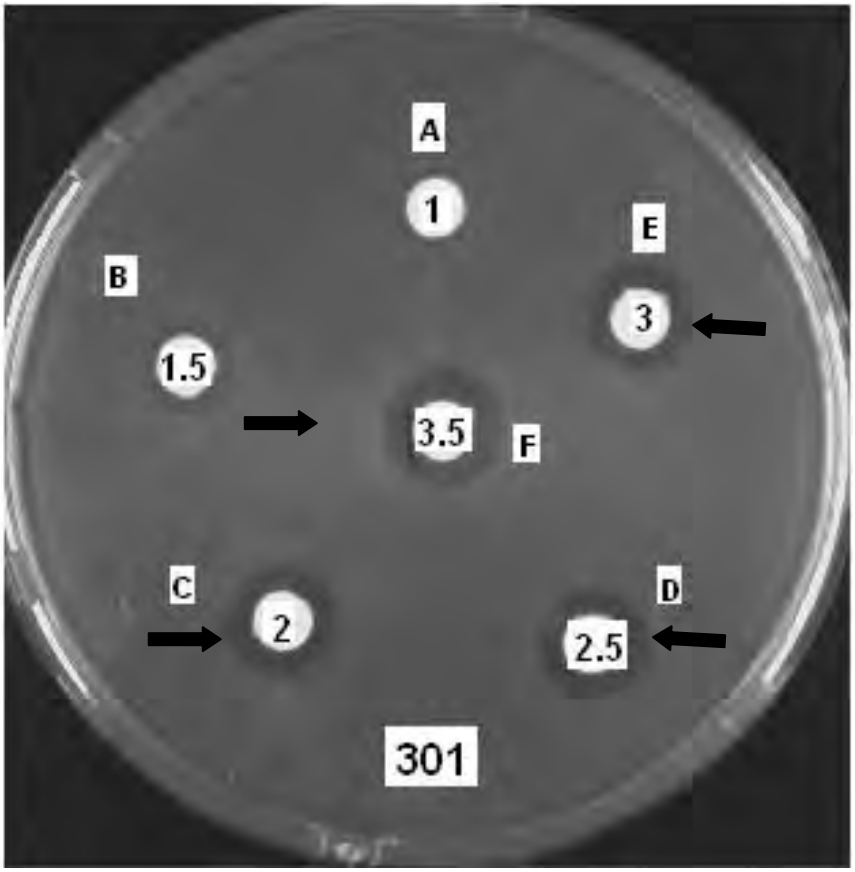


Figure 3.2. An illustration of the disc diffusion assay for strain 301 were intermediate resistance was observed. The antibiotic concentration ranged from 1 – 3.5 μg/ml. The MIC value determined was 1.5 μg/ml. The strain was grown at 37^{o}C in a 5% CO_2 incubator overnight, and observation of the zones was analyzed. The arrows indicate the zones of inhibition observed.

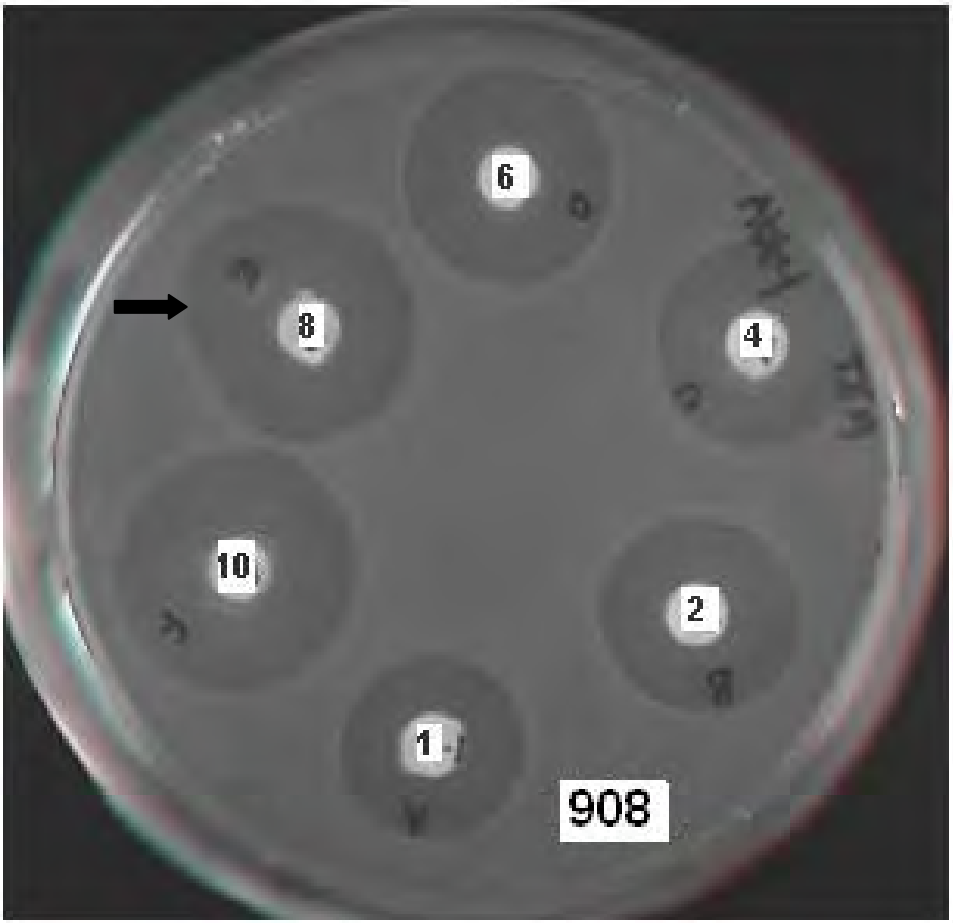


Figure 3.3. An illustration of the disc diffusion assay for strain 908 indicating complete susceptibility to penicillin G. The experiment was carried out at 37^{o}C in a 5% CO_2 incubator overnight. The arrow indicates the zones of inhibitions that were observed for all antibiotic concentrations ranging from 1- 10 μg/ml. The MIC value for strain 908 was determined to be less than 1 μg/ml.

In figure 3.4, strain 21 was observed to have a zone of inhibition at 100μg/ml. The observed results indicate a high level of resistance to penicillin G. The high level resistance could be due to an overproduction of PBP 5, an elimination of the repressor gene (*psr* element) (Depardieu, *et al.*, 2004).

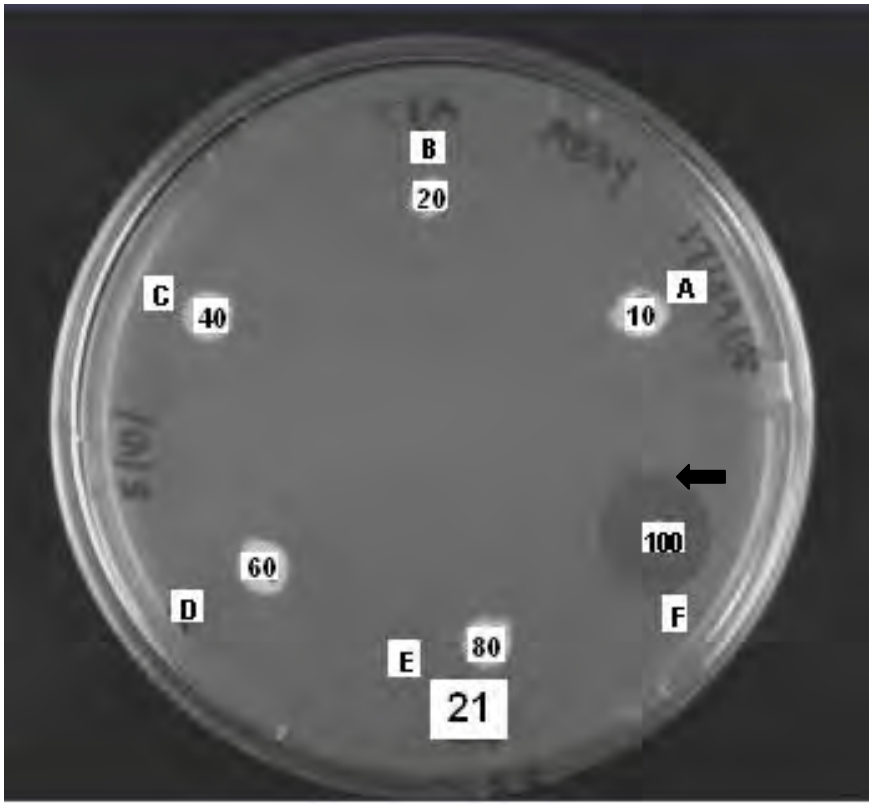


Figure 3.4. An illustration of disc diffusion assay of strain 21 where penicillin concentration ranged from 10 – 100μg/ml. A zone of inhibition was observed at 100 μg/ml. The MIC value was determined to be 80 μg/ml – 100 μg/. This is an indication of high resistance to Penicillin G. The strain was grown overnight in a 5% CO_2 incubator and observation of the zones was carried out. The arrow indicates the zone of inhibition at 100 μg/ml.

In figure 3.5, strain 175 indicated complete resistance to vancomycin. The vancomycin antibiotic concentration ranged from 10-100 μg/ml for all twelve selected strains. These experiments were repeated several times for a statistically confirmation. Therefore one can conclude all 12 selected strains conferred complete resistance to vancomycin (Depardieu, *et al*., 2004).

The results indicate that these strains are highly resistant to vancomycin. This could be due to many factors such as the production of modified precursor molecules ending in D-Ala-D-Lac (VanA, VanB and VanD) or D-Ala-D-Ser (VanC, VanE and VanG). These genotypes exhibit low binding affinities to glycopeptides. The deletion of the high affinity D-Ala-D-Ala ending precursors resulting in resistance (Miele, *et al.,* 1995).

The acquired resistance to glycopeptides in the three $_{D}$-Ala-$_{D}$-Lac types, VanA, VanB and VanD are categorized depending on the level of resistance to vancomycin. VanA type strains display high level inducible resistance , VanB strains have various level of resistance (intermediate) and VanD type strains are characterized by constitutive resistance to moderate levels of vancomycin (susceptible) (Depardieu, *et al*., 2004). Therefore, the disc diffusion assay with the enterococcal strains in figure 3.6 indicate a very high level of resistance to vancomycin hence a high probability of carrying alterations within the VanA genotype, possibly mutations in the D-Ala-D-Lac precursor endings (Miele, *et al.,* 1995).

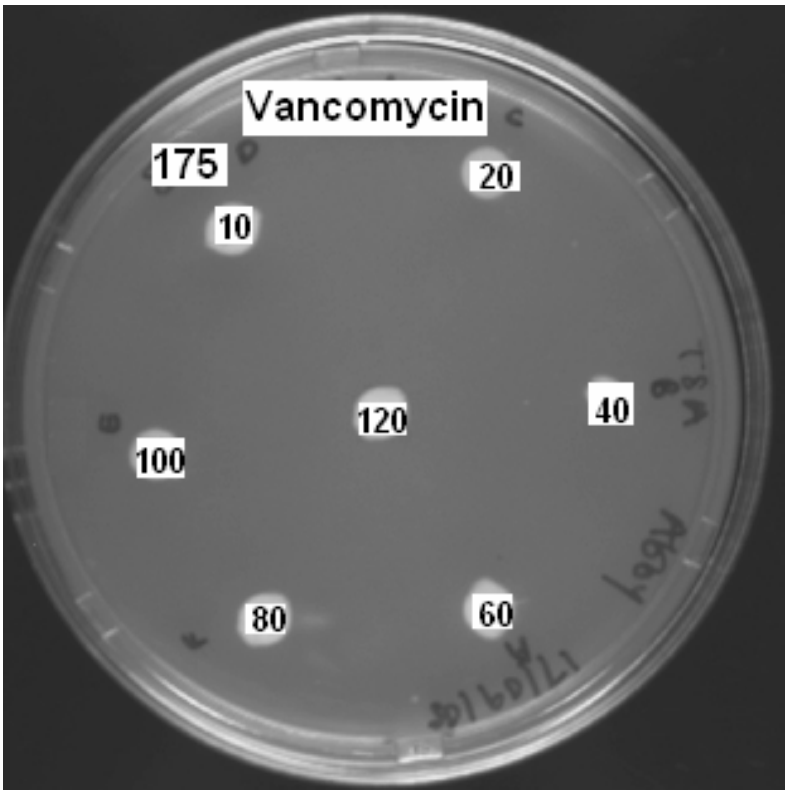


Figure 3.5. An illustration of the disc diffusion assay for strain 175, the antibiotic used was vancomycin were it ranged from 10 – 120 μg/ml. The strain was grown overnight at 37^{o}C in a 5% CO_2 incubator and checked for zones of inhibition. All 12 enterococcal strains were subjected to these conditions and all 12 strains indicated complete resistance to vancomycin.

3.3. Microtitre Plate Assay

Microtitre dilution was used as an alternative to disc diffusion assay to determine the MIC values of all enterococcal strains used in this study. All 12 strains were tested against the glycopeptide antibiotic, vancomycin.

The antibiotic concentration ranged from 0.1 to 100 μg/ml. The control strain used was *E.coli* JM109. From the observed results (Table 3.2), growth was observed for all 12 enterococcal strains. This result suggests that all the selected strains are vancomycin resistant (VRE). The control strain *E.coli* JM109 had an observed MIC value of 70 μg/ml (Depardieu, *et al*., 2004)

Enteroccocci are bacteria that are normally present in the human intestine, the female genital tract and are also often found in the environment. These bacteria can sometimes cause infections. Vancomycin is an antibiotic that is often used to treat infections caused by enterococci. In some instances, enterococci have become resistant to this drug and are called vancomycin-resistant enterococci (VRE) (Carias, *et al*., 1998).

Antimicrobial resistance to vancomycin has increased steadily over the years, mostly in hospitals or health care facilities. All Gram positive micro organisms are treated with vancomycin as the last line of defence. However, with the growing resistance rate, fatal bacterial infections are on the increase. The emergence of VRE came about during the late 1980's (Carias, *et al*., 1998).

There are several mechanisms of resistance to these antibiotics. Vancomycin inhibits cell wall (peptidoglycan) synthesis by binding to the terminal D-alanyl-D-alanine of the pentapeptide precursors thereby preventing the polymerization and cross linking that are vital for the structural stability of the organism. Vancomycin resistance can be acquired via the alteration of the terminal amino acid residues of the NAM/NAG-peptide subunits, normally D-alanyl-D-alanine, which vancomycin binds to. A type of variation is D-alanyl-D-lactate and D-alanyl-D-serine which result in only a 4-point hydrogen bonding interaction being possible between vancomycin and the peptide.

The loss of just one point of interaction results in a 1000-fold decrease in affinity (Carias, *et al*., 1998).

Table 3.2. A microtitre dilution plate assay analysis to determine the MIC values of *Enterococcus* and *E.coli JM109* strains against vancomycin, where the antibiotic concentration ranged from 0.1-100 μg/ml.

Strain	MIC Value (μg/ml)	Observation
21	100	Growth in all wells (0.1-100μg/ml)
175	100	Growth in all wells (0.1-100μg/ml)
301	100	Growth in all wells (0.1-100μg/ml)
382	100	Growth in all wells (0.1-100μg/ml)
406	100	Growth in all wells (0.1-100μg/ml)
430	100	Growth in all wells (0.1-100μg/ml)
468	100	Growth in all wells (0.1-100μg/ml)
859	100	Growth in all wells (0.1-100μg/ml)
904	100	Growth in all wells (0.1-100μg/ml)
908	100	Growth in all wells (0.1-100μg/ml)
943	100	Growth in all wells (0.1-100μg/ml)
1393	100	Growth in all wells (0.1-100μg/ml)
E.coli JM109	70	Growth in all wells (0.1-60μg/ml)

3.4. DNA Isolation

DNA was isolated from the enterococcal strains using the NucleoSpin® tissue kit.

3.4.1. Analysis of Genomic DNA by Agarose Gel Electrophoresis

From all selected strains of *Enterococcus* and *E.coli* DNA was isolated. DNA isolation was confirmed by the presence of bright bands on the agarose gel (Figure 3.6). The DNA bands were viewed under a UV transilluminator. The bright bands indicated a high concentration of DNA. The concentration of each DNA sample was analyzed using spectrophotometry at a wavelength of 260 nm. This is the wavelength at which nucleic acids absorb. The concentration of the DNA was therefore determined for use in polymerase chain reaction. The highest concentration noted was for strain 406 at 455 μg/ml. The protein contamination was determined by measuring the absorbance at 280nm. The purity of all samples were high, ranging from 1.8 to 2.1(Table 3.3).The purity was calculated by the ratio of the 260nm over 280nm OD readings.

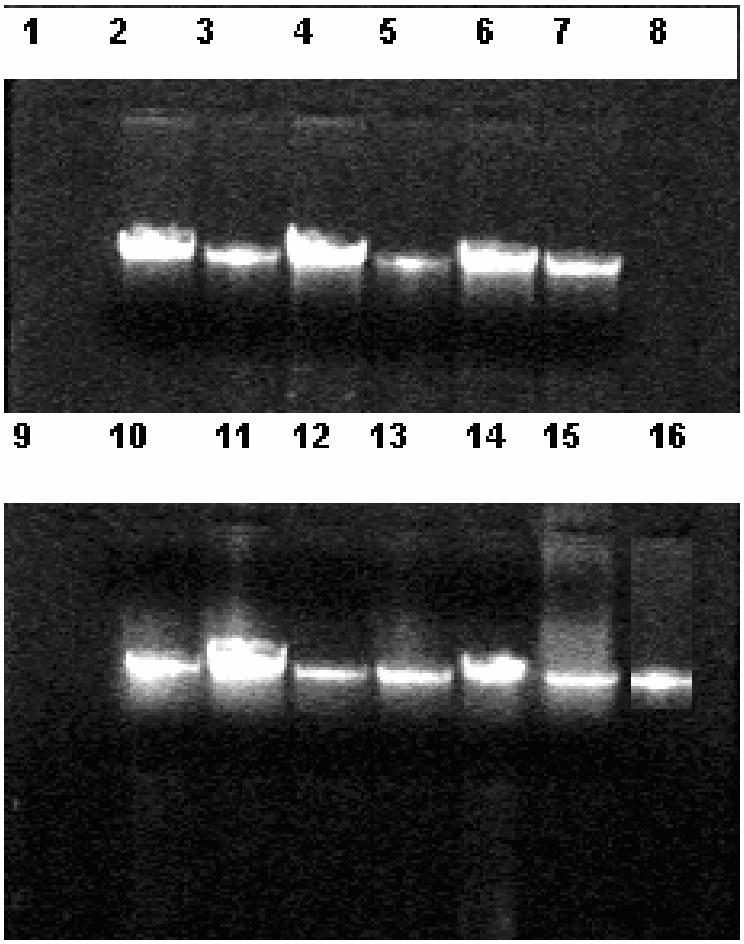


Figure 3.6. DNA extractions using the NucleoSpin ® tissue kit resolved in a 0.8% [w/v] agarose gel of all 12 *Enterococcus* strains, run at 80 volts, maximum current and for 90 minutes in 1 x TAE running buffer. The bands were viewed under a UV transilluminator. Lane 1: blank, lane 2: strain 21, lane 3: strain 175, lane 4: strain 301, lane 5: strain 382, lane 6: strain 406, lane 7: strain 430, lane 8: blank, lane 9: blank, lane10: strain 468, lane 11: strain 859, lane 12: strain 904, lane 13: strain 908, lane 14: strain 943, lane 15: strain 1393 and lane 16: *E.coli* JM109.

3.4.2. Quantification of DNA using UV Spectrophotometry

Table 3.3. An overview of the DNA concentration and purity obtained using UV spectrophotometery at wavelengths 260nm and 280nm respectively to determine DNA concentration and protein contamination.

Strains	A260nm	A280nm	*DNA Concentration (μg/ml)	DNA Purity (OD_{260}/OD_{280})
21	0.0144	0.0080	72	1.8
175	0.0194	0.0102	97	1.9
301	0.0070	0.0035	35	2.0
382	0.0330	0.0165	165	2.0
406	0.0091	0.0507	455	1.8
430	0.0029	0.0145	145	2.0
468	0.0277	0.0154	138.5	1.8
859	0.0173	0.0075	86.5	2.3
904	0.01365	0.0065	68.3	2.1
908	0.0038	0.0020	100	1.9
943	0.0139	0.0077	69.5	1.8
1393	0.0378	0.0210	189	1.8
E.coli JM109	0.0321	0.0153	160.5	2.0

*DNA concentration: A260 x 100 x 50 = Xμg/ml

The DNA purity is determined by value ≥ 1.8

3.5. Genetic Diversity of Selected Isolates Using RAPD PCR as a Tool for DNA Fingerprinting Analysis

Traditional typing methods for bacteria based on phenotypic characteristics are often regarded as less discriminatory and have lower levels of reproducibility than methods based on direct analysis of the bacterial genome. As an epidemiological tool, DNA genotyping techniques are used to assist in tracking the spread of hospital associated infections, food and water contamination and veterinary infections (Frey, 2003). These techniques include a wide variety of PCR based methods that have been designed for fingerprinting analysis. RAPD analysis has evolved as the most popular method for DNA profiling of infectious pathogens (Kuhn, *et al*., 1995).

There were four different primers used consisting of 10-15 base pairs in length to randomly amplify genes along the enterococcal genome (Table 2.2). The primers used were MBPZ-1, MBPZ-2, MBPZ-3 and MBPZ-4 to fingerprint the genome of the selected strains, and generate output data via 1% [w/v] agarose gel electrophoresis (Figure 3.7, 3.8, 3.9, and 3.10).

Although RAPD-PCR has been further developed over the years, there are several disadvantages attached to the technique. RAPD-PCR has a high discriminatory power, however it has poor inter-and intralaboratory reproducibility, due to short random primer sequences and generally low PCR annealing temperatures. RAPD PCR is less laborious than other standard molecular techniques and the ease of processing is much higher. Results can be obtained within a day. It is a low cost experiment with a high discriminatory power and can be applied in any standard molecular biology laboratory (Hunter, 1990).

Virtually every part of a PCR technique can affect the rate of reproducibility. There were three replicate trial runs per primer used; however the reproducibility rate for these primers was moderately low. This can be due to small changes in the essential parameters, such as the elongation temperatures, the concentration of magnesium and the ratio of primer to template concentration. The changes of these variables affect low intensity bands but have been known to affect the position and concentration of high intensity bands (Healy, *et al.,* 2004).

In figure 3.7 (a), (b), (c) and (d) the RAPD PCR patterns were repeated several times at different times using the same genomic DNA to test for the rate of reproducibility. The verification of the reproducibility of patterns between the four primers was carried out a minimum of three times under similar conditions. All four primers exhibited a high reproducibility rate. The rate of reproducibility is dependent on the number of trial runs per primer used at separate times in a constant laboratory environment. A statistic analysis is deduced from these results. The most informative and reproducible fingerprint patterns were obtained with 3.5mM $MgCl_2$. An increase in $MgCl_2$ resulted in a more evident background, without additional major bands. The number of amplified DNA bands viewed ranged from 8 to 13 fragments (Hunter, 1990).

Primers MBPZ-1 and MBPZ-2 yielded 70%-75% reproducible patterns. Primers MBPZ-3 and MBPZ-4 yielded reproducible rate of 80%-90%. This test confirmed the reproducibility power of the RAPD PCR method for the enterococcal strains. Therefore all four primers were used in the experiment.

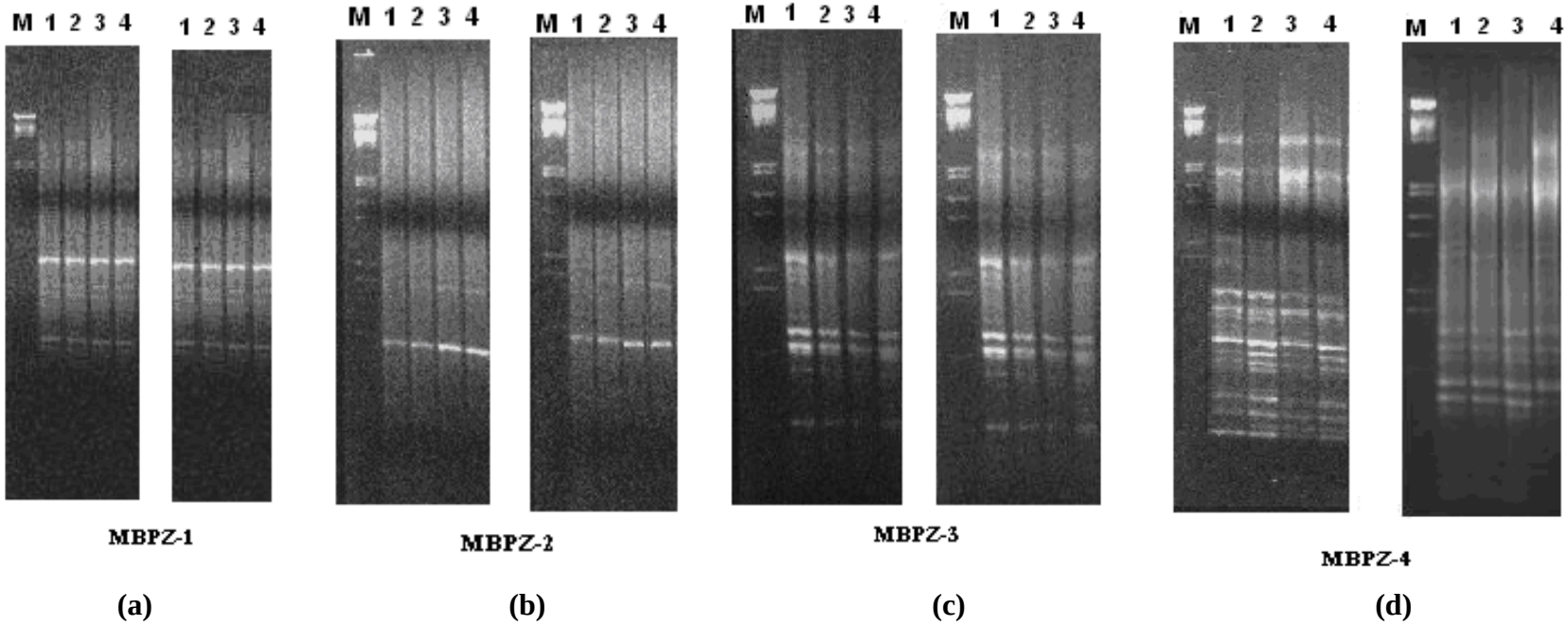


Figure 3.7. An illustration of a 1% [w/v] agarose gel to show the reproducibility rate of the four primers used producing RAPD patterns for the enterococcal strains. There were three trial runs for each primer, of which two were randomly selected. Lane M: Molecular weight marker, lane 1: strain 21, lane 2: strain 175, lane 3: strain 301 and lane 4: strain 382.

(a) RAPD patterns for MBPZ-1 are illustrated at different times (days) with the same genomic DNA

(b) RAPD patterns of MBPZ-2 illustrated at different times (days) with the same genomic DNA.

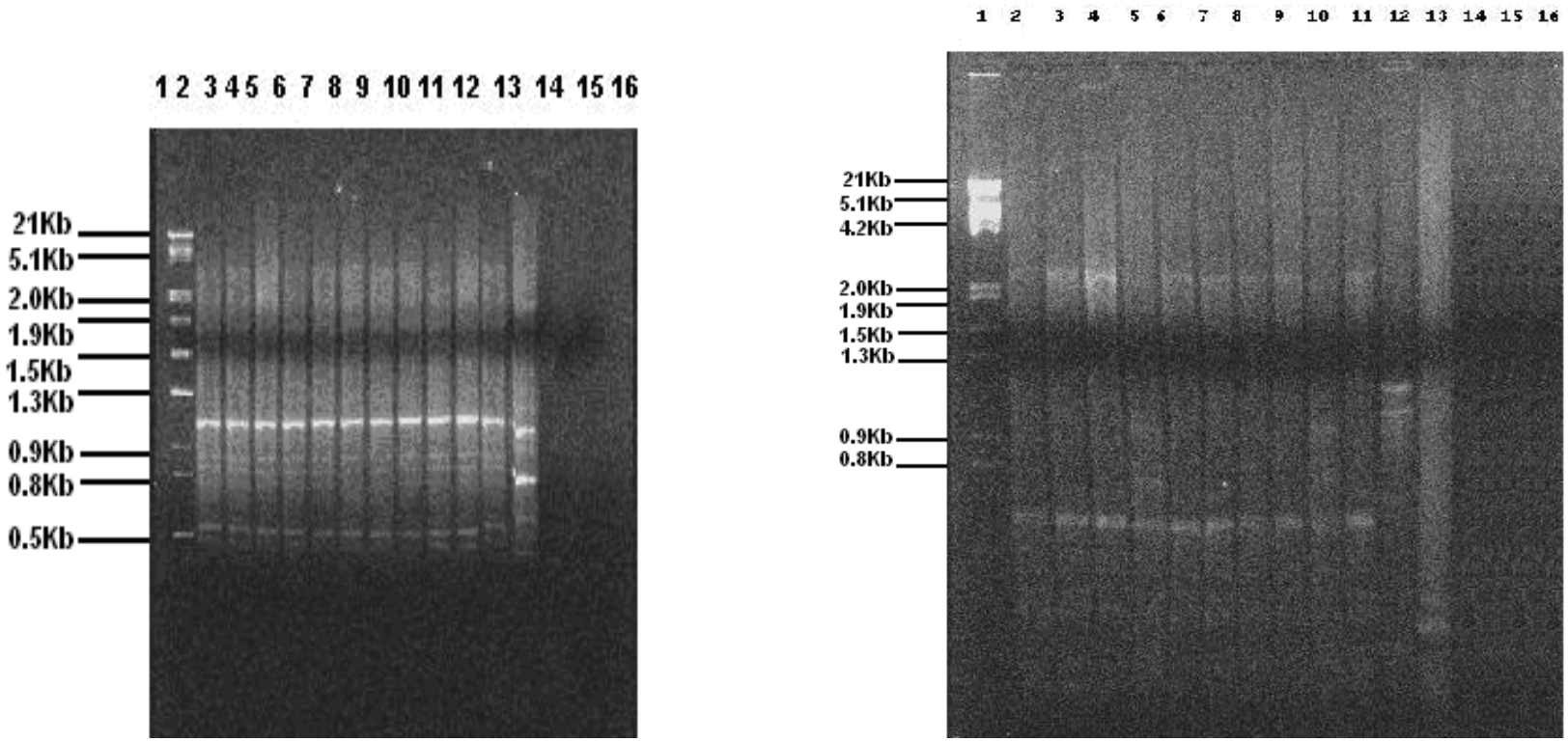
(c) RAPD patterns of MBPZ-3 illustrated at different times (days) with the same genomic DNA.

(d) RAPD patterns of MBPZ-4 illustrated at different times (days) with the same genomic DNA. .

The primers that yielded the highest reproducibility as well as the highest discriminatory power to produce the most reliable banding patterns for phylogenetic analysis were selected. Primers MBPZ-3 and MBPZ-4, with a GC content of 40%-70% were selected as they produced a higher reproducibility and discriminatory banding patterns than MBPZ-1 and MBPZ-2. These primers had exhibited a clear definition between the different enterococcal species and the control strain *E.coli*.

3.5.1. Analysis of RAPD Banding Patterns using Four Different Random Primers

MBPZ-1 and MBPZ-2 RAPD patterns in figure 3.8 (a) and (b) were reproducible but not very discriminatory. Only a few bands were amplified using these primers. These bands were indistinguishable between the enterococcal isolates and the *E.coli* control strain. The banding patterns suggested that these primers were not frequent throughout the genomic DNA of the selected strains and therefore produced a low discrimination between the selected enterococcal strains. The few bands observed for all 13 strains comprised of a small range, a single high intensity band and a small number of low intensity bands that were therefore inadequate for phylogenetic analysis.



(a) **(b)**

Figure 3.8. (a) and **(b)** RAPD PCR patterns of genomic DNA fragments of *Enterococcus* strain obtained with primer MBPZ-1 and resolved on a 1% [w/v] agarose gel in 0.5 X TBE buffer. Lane 1: MWMIII, lane 2: strain 21, lane 3: strain 175, lane 4: strain 301, lane 5: strain 382, lane 6: strain 406, lane 7: strain 430, lane 8: strain 468, lane 9: strain 859, lane10: strain 904, lane 11: strain 908, lane 12: strain 943, lane 13: strain 1393, lane 14: Blank, lane 15: No template control and lane 16: No enzyme control.

MBPZ-3 RAPD patterns were observed using a 1% [w/v] gel viewed under an UV transilluminator after ethidium bromide staining (Figure 3.9). Banding patterns were observed with a high discriminatory power. There were many high intensity bands and a few low intensity bands observed that ranged from 0.2kb to 1.0kb in size. However, all enterococcal strains exhibited a very similar RAPD patterns between them. This banding pattern indicated that all the selected enterococcal strains are closely related with a low genetic diversity. The *E.coli* strain can be clearly distinguished as the out-group control strain.

The 13 isolates RAPD banding patterns for primer MBPZ-3 were scored based on the presence or absence of a band yielding a 1 or 0 score respectively (Table 3.4). The scores were then generated into a distance matrix based on a dissimilarity coefficient. The distance matrix is generated via the Euclidean distance (ED) coefficient measure that takes into account the discrete values 1 or 0 depending on the presence or absence of a band (Mueller, *et al.,* 2001). The dissimilarity between each isolate was calculated to produce the distance matrix (Table 3.4).

The genetic relationship between the 13 strain's RAPD patterns was generated by the UPGMA-neighbour joining computer software package to produce a consensus tree (section 2.11). The dendogram in Figure3.10 indicated a very close relationship between the selected *Enterococcus* strains. The dendogram was separated into group I and Group II. Group I consisted of the out-group *E.coli*. Group II was separated into two clusters, A (11 isolates) and B (1 isolate). The genetic relationship between the *Enterococcus* isolates indicated an 86% similarity between them. These results suggest that all of the selected enterococcal strains have a clonal distribution between them and therefore have a very low genetic diversity and may belong to the same or closely related subspecies.

The enterococcal isolates with similar minimal inhibitory concentrations to penicillin were found to belong to the same cluster. Strains 21,175,406, 486 and 859 were shown to share a very close linkage and all exhibited resistance. Strains 301 and 382 were closely linked and had the same MIC value of 1.5 μg/ml rendering them intermediately resistant. Strains 943 and 908 indicated 10% dissimilarity between

them and were categorized as susceptible isolates. These results indicate that the selected strains are very closely related to each other.

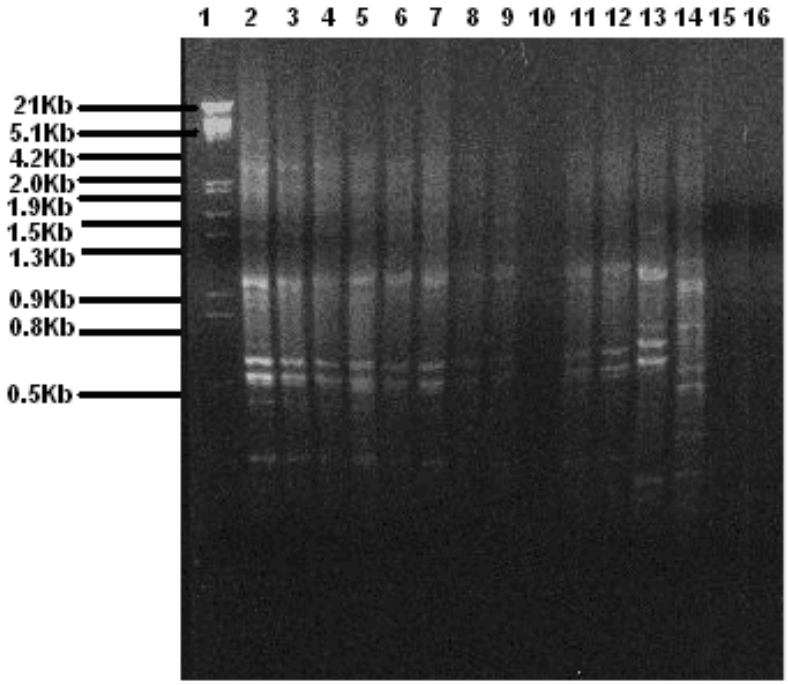


Figure 3.9. RAPD PCR patterns of genomic DNA fragments of *Enterococcus* strain obtained with primer MBPZ-3 and resolved on a 1% [w/v] agarose gel in 0.5 X TBE buffer. Lane 1: MWMIII, lane 2: strain 21, lane 3: strain 175, lane 4: strain 301, lane 5: strain 382, lane 6: strain 406, lane 7: strain 430, lane 8: train 468, lane 9: strain 859, lane10: blank, lane 11: strain 904, lane 12: strain 908, lane 13: strain 943, lane 14: strain 1393, lane 15: No template control and lane 16: No enzyme control.

Table 3.4. An illustration of the scores of bands obtained from RAPD PCR amplification of primer MBPZ-3.

Strains	A	B	C	D	E	F	G	H
21	1	1	0	0	1	1	0	1
175	1	1	0	0	1	1	1	1
301	1	1	0	0	1	0	0	1
382	1	0	0	0	1	0	0	1
406	1	1	0	0	1	0	0	1
430	1	1	0	0	1	1	0	0
468	1	1	0	0	0	1	0	0
859	0	1	0	0	0	1	0	0
904	0	1	0	0	1	0	0	0
908	1	1	0	0	0	0	0	0
943	1	1	0	1	1	1	0	0
1393	1	1	0	1	1	1	0	0
E.coli	1	1	1	1	1	1	1	1

A-H are random variables used to indicate each band.

Table 3.5. Distance matrix generated from the scores of bands from RAPD PCR amplification of primer MBPZ-3 based on a dissimilarity coefficient.

	21	175	301	382	406	430	468	859	904	908	943	1393	*E.coli*
21	0												
175	1	0											
301	1	1	0										
382	1	1	0	0									
406	1	1	0	2	0								
430	1	1	2	2	2	0							
468	2	2	3	3	3	1	0						
859	3	3	4	4	4	2	1	0					
904	3	2	2	2	1	2	3	2	0				
908	3	2	2	1	2	2	1	2	2	0			
943	2	2	3	3	2	1	2	3	3	3	0		
1393	2	2	3	3	3	1	2	3	4	3	0	0	
E.coli	4	4	4	4	4	4	5	6	6	3	3	3	0

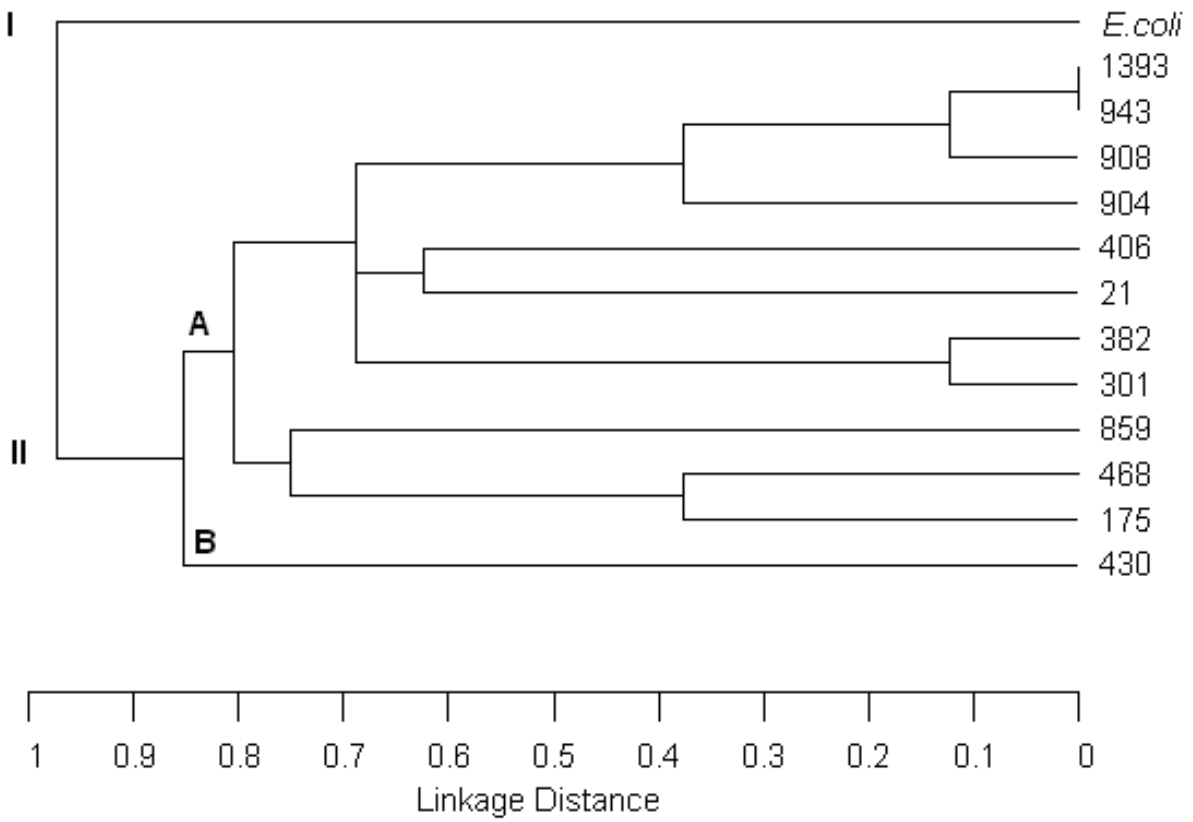


Figure 3.10. A dendogram to determine the genetic relationship between the 12 selected enterococcal strains and *E.coli* JM109 strain using data retrieved from RAPD patterns from primer MBPZ-3 with a high discriminatory power with UPGMA-neighbour joining software.

MBPZ-4 RAPD patterns were observed on a 1% [w/v] agarose gel viewed under an UV transilluminator in figure 3.11. Of the 13 strains RAPD patterns were observed with a high discriminatory power. There were several high intensity bands and a few low intensity bands observed that ranged from 0.2kb to 1.9 kb in size. Similarly to the RAPD patterns for MBPZ-3, the enterococcal strains displayed very similar RAPD patterns between them. The almost identical RAPD patterns indicate that the selected strains are very closely related to each other or may belong to a genetically similar subgroup. The control out-group *E.coli* showed a distinct RAPD pattern variation from the other 12 strains.

The 13 banding patterns observed were scored using a binomial method depending on the presence or absence of a band to yield a 1 or 0 respectively (Table 3.6). A distance matrix was generated similarly to MBPZ-3 (Table 3.7) using the data from table 3.6. The distance matrix was generated on a dissimilarity coefficient (Rao, 1989).

The genetic relationship between the 13 isolates RAPD patterns were determined using UPGMA-neighbour joining computer software to generate a consensus dendogram. The dendogram represented in figure 3.14 show that all strains are very closely related with a low level of genetic diversity. The enterococcal strains showed an 80% similarity between each other, while *E.coli* showed almost 100% dissimilarity among the 12 isolates. The dendogram was separated into group I and group II. Group I belonged to the enterococcal strains and group II belonged to the out-group strain *E.coli*. Group I was divided into two clusters, A and B. Cluster a (5 isolates) showed a very close genetic linkage and same genetic distance between the 5 strains therefore displaying clonal dissemination. Cluster B (7 isolates) also showed a close genetic relationship between the strains. These strains displayed a similarity of 60%-90% between each other. Their close genetic relationship based on the dissimilarity coefficient indicated a low genetic variability between them.

The MIC values to penicillin G were observed to be similar to the clustering of most of the isolates. In group I, clustal a, strains 382 and 301 grouped closely together and exhibit an intermediate resistance MIC value. In clustal B, the resistant strains 859, 468,430 and 406 group closely together and show a very low 10%- 30% dissimilarity.

The results indicate that these isolates are very closely related and hence have clonal relationships between them.

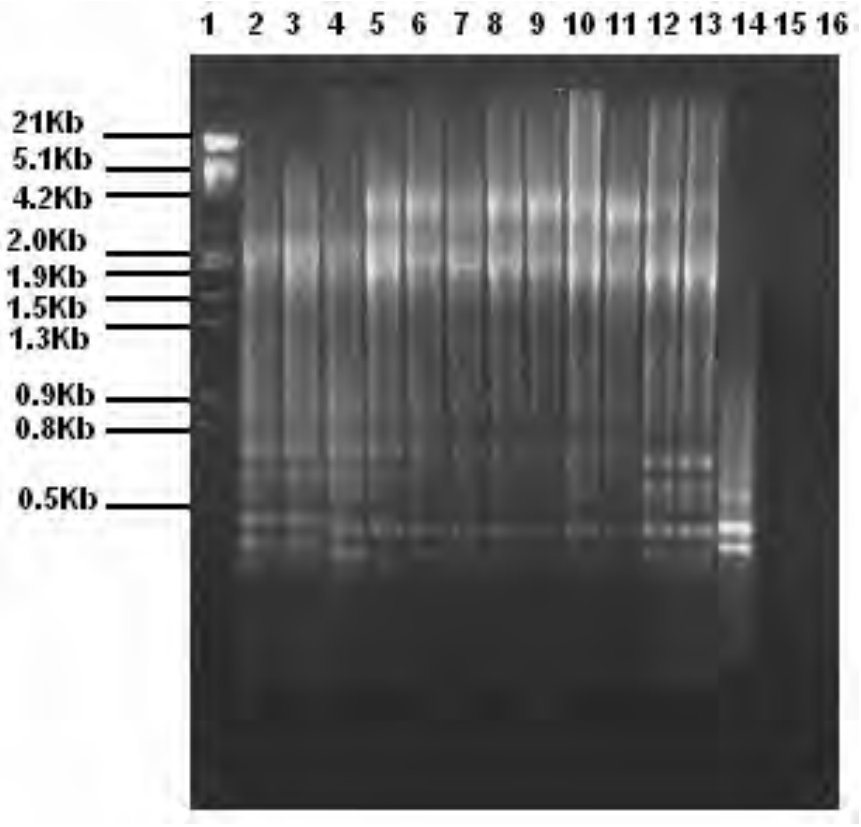


Figure 3.11. RAPD PCR patterns of genomic DNA fragments of *Enterococcus* strain obtained with primer MBPZ-4 and resolved on a 1% [w/v] agarose gel in 0.5 X TBE buffer. Lane 1: MWMIII, lane 2: strain 21, lane 3: strain 175, lane 4: strain 301, lane 5: strain 382, lane 6: strain 406, lane 7: strain 430, lane 8: strain 468, lane 9: strain 859, lane10: strain 904, lane 11: strain 908, lane 12: strain 943, lane 13: strain 1393, lane 14: *E.coli* JM109, lane 15: blank and lane 16: No enzyme control.

Table 3.6. An illustration of the scores of bands obtained from RAPD PCR amplification of primer MBPZ-4.

Strains	A	B	C	D	E	F	G	H	I	J	K	L	M
21	0	1	1	0	1	1	1	1	1	1	0	0	0
175	1	1	1	1	1	1	1	1	1	0	0	0	0
301	0	1	1	1	1	1	1	1	1	0	0	0	0
382	1	1	1	1	1	1	1	0	0	1	0	0	0
406	1	1	0	0	1	1	1	1	1	0	0	0	0
430	1	1	1	1	1	1	1	1	1	0	0	0	0
468	1	1	0	0	1	1	1	0	1	1	0	0	0
859	1	1	0	1	1	1	1	1	1	1	0	0	0
904	1	1	1	0	1	1	1	1	1	1	0	0	0
908	1	1	1	0	1	1	0	1	1	1	0	0	0
943	1	1	1	0	1	1	1	1	1	1	0	0	0
1393	1	1	1	1	1	1	1	1	1	0	0	0	0
E.coli	0	0	0	0	1	1	1	1	1	0	1	1	1

A to M are arbitrary variables used to signify each band observed in each lane on a 1% [w/v] agarose gel.

Table 3.7. Distance matrix generated from the scores of bands from RAPD PCR amplification of primer MBPZ-4 based on a dissimilarity coefficient.

	21	175	301	382	406	430	468	859	904	908	943	1393	*E.coli*
21	0												
175	3	0											
301	2	1	0										
382	4	3	4	0									
406	3	2	3	5	0								
430	3	0	1	3	2	0							
468	3	4	5	3	2	4	0						
859	2	2	3	2	1	2	2	0					
904	1	2	3	3	2	2	2	2	0				
908	2	3	4	4	3	3	3	3	1	0			
943	1	1	3	3	2	2	2	2	1	1	0		
1393	3	2	1	3	2	1	1	1	2	3	2	0	
E.coli	6	7	6	7	6	7	7	7	7	8	7	7	0

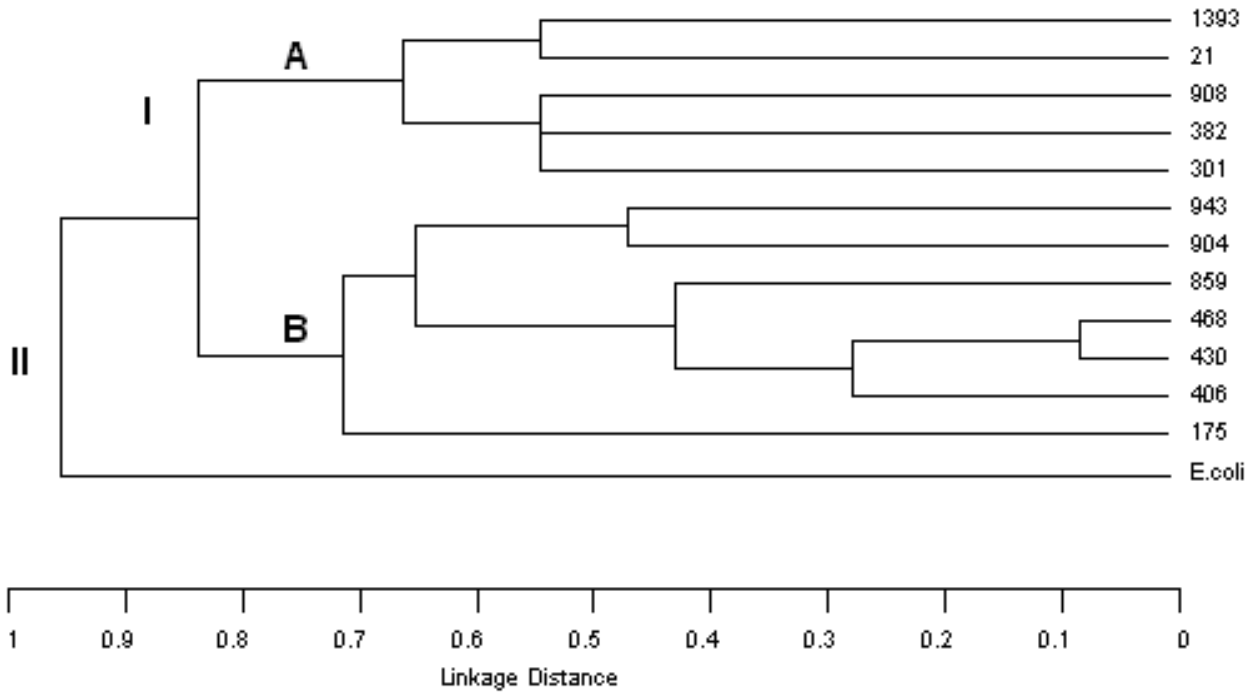


Figure 3.12. A dendogram to determine the genetic relationship between the 12 selected enterococcal strains and *E.coli* JM109 strain using data retrieved from RAPD patterns from primer MBPZ-4 with the highest discriminatory power with UPGMA-neighbour joining software.

3.6. 16S Ribosomal DNA Ribotyping Analysis using Polymerase Chain Reaction (PCR)

The 16S rDNA ribotyping technique consisted of the amplification of the 16SrDNA gene and the extraction was carried out using the Nucleospin® PCR purification kit. The products were sequenced at the University of CapeTown. The sequences were submitted to NCBI BLAST for analysis

3.6.1. 16S Ribosomal DNA Extraction

The twelve selected strains were initially known to belong to the *Streptococcus* group D species acquired from the University Of Maastricht, Netherlands. The primers used were 16S8FE at nucleotide position 8-17 base pairs and 16S1523RB at nucleotide position 1536-1555 base pairs (Schouls, *et al*., 2003) designed to amplify specifically the entire 16S rDNA gene of the selected strains. The 16S rDNA was amplified because there are variable regions along the 16S rDNA that are specific to a particular species and allows for identification of the species via ribosomal DNA sequencing (Schouls, *et al*., 2003). The expected size of the 16S rDNA strands was 1.5 Kb pairs. After amplification of the 16S rDNA, the PCR product was run on a 1.5% agarose gel for analysis. The observed PCR product size was approximately 1.45Kb pairs (Figure 3.13). This indicated that the PCR amplification of the 16S rDNA was successful, and the amplicon was at a high concentration as bright bands were viewed on the gel (Limia, *et al.,* 2000; Pryce, *et al.,* 1999).

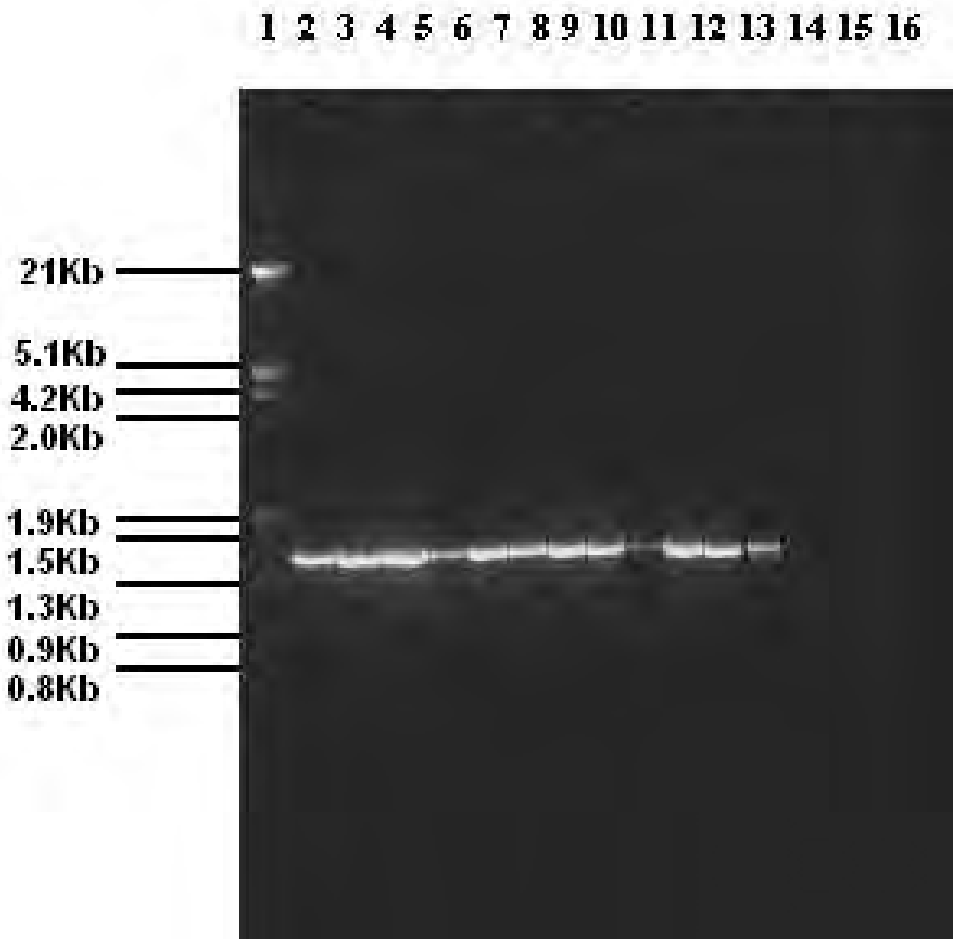
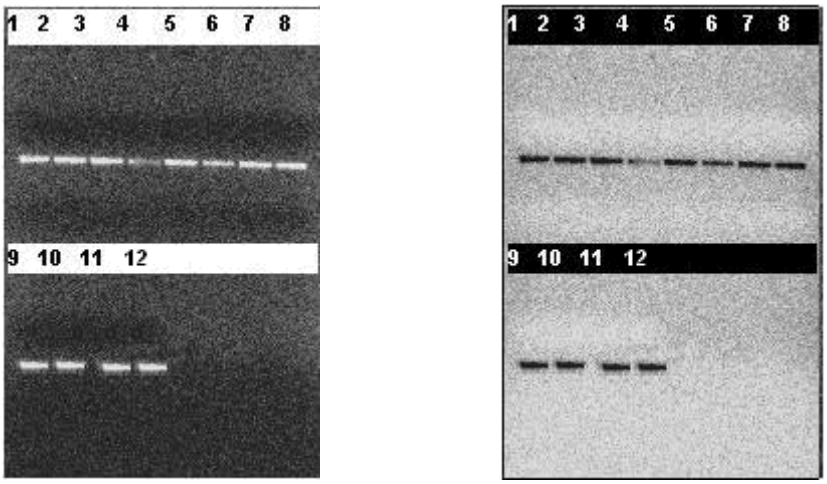


Figure 3.13. A 1.5% [w/v] agarose gel indicating the products of 16S rDNA PCR of all the *Enterococcus* strains using the primers 16S8FE and 16S1523RB in 0.5 x TBE buffer, run at 80 Volts at maximum current for 90 minutes. Lane 1: MWM III, lane 2: strain 21, lane 3: strain 175, lane 4: strain 301, lane 5: strain 382, lane 6: strain 406, lane 7: strain 430, lane 8: strain 468, lane 9: strain 859, lane 10: strain 904, lane 11: strain 908, lane 12: strain 943, lane 13: strain 1393, lanes 14- 16: blank.

3.6.2. Nucleospin Extraction ® for Purification of Product

Purification of the PCR products was carried out using the Nucleospin® ExtractII Kit to remove the primers and other reagents. The purified PCR products (16S rDNA) were run on a 2% [w/v] agarose gel to verify the purified PCR products of all twelve selected strains (Figure 3.14). A band was observed for all twelve strains therefore the 16S rDNA amplification and purification was successful.



(a) **(b)**

Figure 3.14. (a) A 2% [w/v] agarose gel of the purified 16s rDNA amplicon of *Enterococcus* strains obtained after purification using the Nucleopsin® Extract II. Lane 1: strain 21, lane 2: strain 175, lane 3: strain 301, lane 4: strain 382, lane 5: strain 406, lane 6: strain 430, lane 7: strain 468, lane 8: strain 859, lane 9: strain 904, lane 10: strain 908, lane 11: strain 943 and lane 12: strain 1393.
(b) A colour inversion of A to view 16S rDNA pure PCR products bands more clearly.

3.6.3. Sanger Dideoxy-nucleotide Sequencing Of 16S rDNA

The purified PCR products were sent for DNA sequencing using the Sanger Dideoxy-nucleotide method at the University Of Cape Town, South Africa (Figure 3.15). The DNA sequences of all twelve strains were run on NCBI BLAST algorithm, a nucleotide Genbank. A comparison of the 16S rDNA was made to all the data stored on Genbank from previous research to give an output of the closest similarity to the selected strains 16S rDNA (Figure 3.16). The twelve strains were found to have the highest similarity (90%-100%) to the genus *Enterococcus*. The genus *Enterococcus* consists of Gram-positive cocci and all strains were confirmed to be Gram positive cocci. The twelve strains were then characterized into species based on the 16S rDNA ribotyping results. These are *E.faecium, E.faecalis* and *E.durans* (Table 3.8).

175
CRWKWGSGGSKKCCTAATSATGSAGTCGACGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGA
GAAAGAGGAGTGGCGAACGGGTGAGTAACACGTGGGTAACCTGCCCATCAGAAGGGGATAAC
ACTTGGAAACAGGTGCTAATACCGTATAACAATCRAAACCGCATGGTTTTGATTTGAAAGGCGC
TTTCGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGKTGGTGAGGTAACGGCTCAC
CAAGGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGC
CCAAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGGCAATGGACGAAAGTCTGACCGAGC
AACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGA
TGAGAGTAACTGTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG
GCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGG
AGACTTGAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATG
GAGGAACACCAGKGCGAAGCGGCTCTCTGGTCTGWACTGACSCTGAGCTCGAAAGCGTGGGA
GCAAMAGATTARAWACCCTGTAGTCMCGCCGTAAACGATGAGKGCTAAGTGKTGGAGGTTCC
GCCTTMAKGCTGCAGCTACGSMATAGCACTCCGCCTGGGAGTAYMGAATCGCCAGGTTGRAA
CTCCAAGGAATTGGYWCGGGSTC

Figure 3.15. A DNA sequence obtained by Sanger Dideoxy-nucleotide sequencing of strain 175 to be run on NCBI BLAST (National Centre for Biotechnology Information: Basic Local Alignment Search Tool) algorithm.

```
LOCUS       AY653231   1332 bp    DNA     linear   BCT 28-DEC-2004
DEFINITION  Enterococcus faecium 16S ribosomal RNA gene, partial
sequence.
ACCESSION   AY653231
VERSION     AY653231.1  GI:55975493
KEYWORDS    .
SOURCE      Enterococcus faecium
  ORGANISM  Enterococcus faecium
            Bacteria; Firmicutes; Lactobacillales; Enterococcaceae;
            Enterococcus.
REFERENCE   1  (bases 1 to 1332)
  AUTHORS   Ballard,S.A., Grabsch,E.A., Johnson,P.D. and Grayson,M.L.
  TITLE     Comparison of three PCR primer sets for identification of
            vanB gene carriage in feces and correlation with carriage
            of vancomycin-resistant enterococci: interference by anB-
            containing anaerobic bacilli.
  JOURNAL   Antimicrob. Agents Chemother. 49 (1), 77-81 (2005)
   PUBMED   15616278
REFERENCE   2  (bases 1 to 1332)
  AUTHORS   Ballard,S.A., Johnson,P.D.R. and Grayson,M.L.
  TITLE     Direct Submission
  JOURNAL   Submitted (15-JUN-2004) Infectious Diseases, Austin
            Hospital, Studley Road, Heidelberg, Victoria 3085,
            Australia
FEATURES    Location/Qualifiers
     source          1..1332
                     /organism="Enterococcus faecium"
                     /mol_type="genomic DNA"
                     /strain="MLG856-2"
                     /db_xref="taxon:1352"
                     /note="genotype: vanB"
     rRNA            <1..>1332
                     /product="16S ribosomal RNA"
```

Figure 3.16. An illustration of the data output from NCBI BLAST of strain 175 identified to have >99.5% 16S rRNA sequence homology to species from the genus *Enterococcus*.

Table 3.8. Characterization of all 12 selected strains to their genus and species level using 16S rDNA ribotyping and NCBI BLAST analysis.

Strain	NCBI BLAST Homology Match
21	*E.faecium* 16S rRNA
175	*E.faecium* 16S rRNA
301	*E.faecium* 16S rRNA
382	*E.faecium* 16S rRNA
406	*E.faecium* 16S rRNA
430	*E.faecium* 16S rRNA
438	*E.faecium* 16S rRNA
859	*E.durans* 16S rRNA
904	*E.faecium* 16S rRNA
908	*E.faecium* 16S rRNA
943	*E.faecium* 16S rRNA
1393	*E.faecalis* 16S rRNA

3.6.4. Phylogenetic Analysis Using BioEdit, ClustalX and Phylip Version 3.36

A dendogram of the 16S rDNA, 13 isolates sequences were generated using a bioinformatics program (Figure 3.17). The package consists of three main programs these are BioEdit sequence alignment editor, ClustalX and Phylip. BioEdit sequence alignment allows for the alignment and modification of the 13 sequences. The *E.coli* strain 16S rDNA sequence was extracted from GenBank, accession number AB269763. The program ClustalX allows for the complete alignment of nucleotides for each strain therby indicating sequence similarity (Appendix A). The ClustalX multiple alignment showed a large number of areas with high sequence homology. A (*) indicates that the codon belongs to a fully conserved group. The enterococcal isolates used in the comparison revealed a nucleotide identity of greater than 90%. The high homology between the nucleotides indicated a close relationship between the enterococcal strains. The program Phylip is separated into many sub programs. The sub programs used were seqboot, DNA distance, neighbour joining and consense. SeqBoot allows the user to bootstrap the tree repetitively by creating multiple data sets. Distance matrix then uses the multiple data sets to create replicating distance

matrices. The matrices are then subjected to neighbour joining where the type of phylogenetic analysis was chosen to be unweighted-pair group arithmetic mean (UPGMA). The number of bootstrap chosen, usually 1000 produces a wide range of trees up to a 1000. A final consensus tree is then derived from the multiple trees produced by neighbour joining to give a statistically arithmetic mean tree or dendogram (Farris, 1972).

The genetic relationships between the isolates were clearly distinguishable between them in the dendogram (Figure 3.17). The dendogram was divided into group I and group II where group I consisted of the enterococcal strains and group II consisted of the out-group *E.coli*. Group I was sub-divided into cluster A (1 isolate) and Cluster B (11 isolates). The 11 isolates in cluster B showed a 0 %– 60% dissimilarity between them which confirmed the NCBI BLAST results as these strains were very closely related with a low genetic variability between them. The isolates that were linked with the closest similarity showed greater genetic relatedness as they belonged to the same MIC category for penicillin. Strain 859 found to be *E.durans* in group I, cluster B showed a greater dissimilarity (15%) among the other strains. These results confirm the NCBI BLAST results in that all the strains do belong to the same genus, *Enterococcus*, and that they belong to very closely related species, *E.faecium*, *E.faecalis* and *E.durans*.

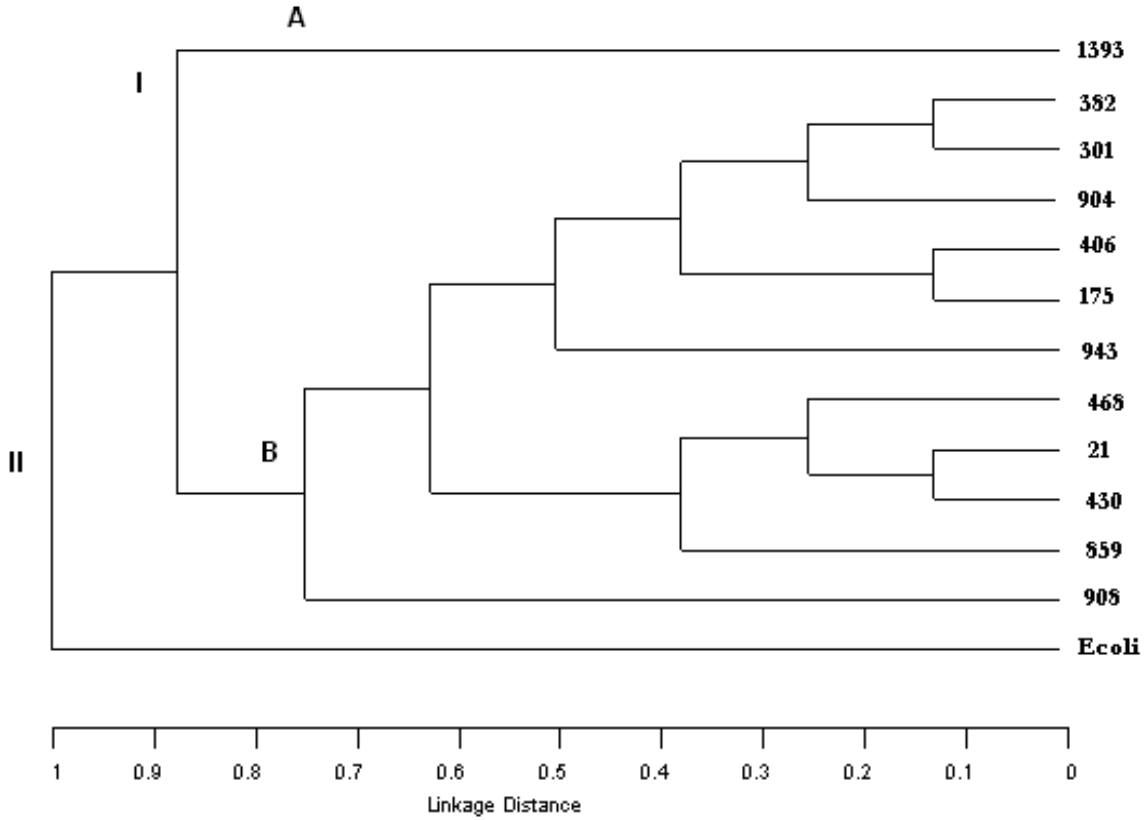


Figure 3.17. A dendogram to show the genetic relationships between the selected *Enterococcus* isolates and out-group *E.coli* 16SrDNA sequences using UPGMA computer software with arithmetic means.

3.7. Analysis of Selected *Enterococcus* Strains Using the Pulse Field Gel Electrophoresis (PFGE) Genotyping Technique

Although PFGE is considered the 'gold standard' for *Enterococcus* typing its use is limited because it's very time consuming (approximately 1 week), expensive and laborious. Among other techniques, PFGE has a very high discriminatory power. The experiment has a high inter-and intralaboratory reproducibility rate and can be applied in any standard molecular biology laboratory. Due to its high discriminative power, it is much easier to process than RAPD PCR (Coque, *et al*., 1995)

In figure 3.18, all the bands were very easily distinguishable from each another. There were a high number of high intensity bands and a few low intensity bands observed which allow for a higher discriminatory power. The PFGE patterns for all 13 isolates yielded a slightly higher polymorphism than the RAPD patterns. There were 4 different PFGE patterns identified for all 13 isolates. The control strain JM109 was clearly distinguished from the 12 other isolates. The 12 *Enterococcus* strains however did exhibit clonal relationships between them that suggest and confirms that these strains are very closely linked and belong to very similar subgroups (Nallapareddy, *et al.,* 2002).

The 13 strains PFGE band patterns observed were scored based on the presence or absence of a band as binomial values (Table3.9). The distance matrix was generated using the Euclidean distance equation using the scored bands on a dissimilarity coefficient (Table 3.10).A very low genetic diversity among the isolates was observed as the strains yielded almost 80% similarity between them.

The genetic relationship between the 13 isolates was compiled using UPGMA-neighbour joining computer software to produce a consensus dendogram (Figure 3.19). The dendogram of the 13 isolates show that there is considerable polymorphism among the PFGE patterns, which is important because this allows the inference that these isolates that have the same genomic pattern are likely to belong to a single strain. The dendogram was separated into group I and group II. Group I consisted of the control out-group strain *E.coli.* Group II consisted of all the selected enterococcal strains and was divided into two clusters, A (9 isolates) and B (3 isolates). The strains

in group II, exhibited a much higher genetic diversity as compared to RAPD-PCR. However strains 1393, 301, 382, and175 in cluster A and strains 904 and 908 from cluster B displayed clonal relationships. The strains genetic relatedness was compared to their relative MIC values to penicillin and it was found that most strains belonging to similar MIC categories shared a dissimilarity of 0-40% These results suggest that all strains showed a low genetic diversity which confirmed that these strains do belong to the same genus and share close inter-relationships with each other. PFGE is a well developed DNA profiling method that allows for a much greater discrimination between the 13 strains and should be used as a clinical diagnostic tool for infectious diseases.

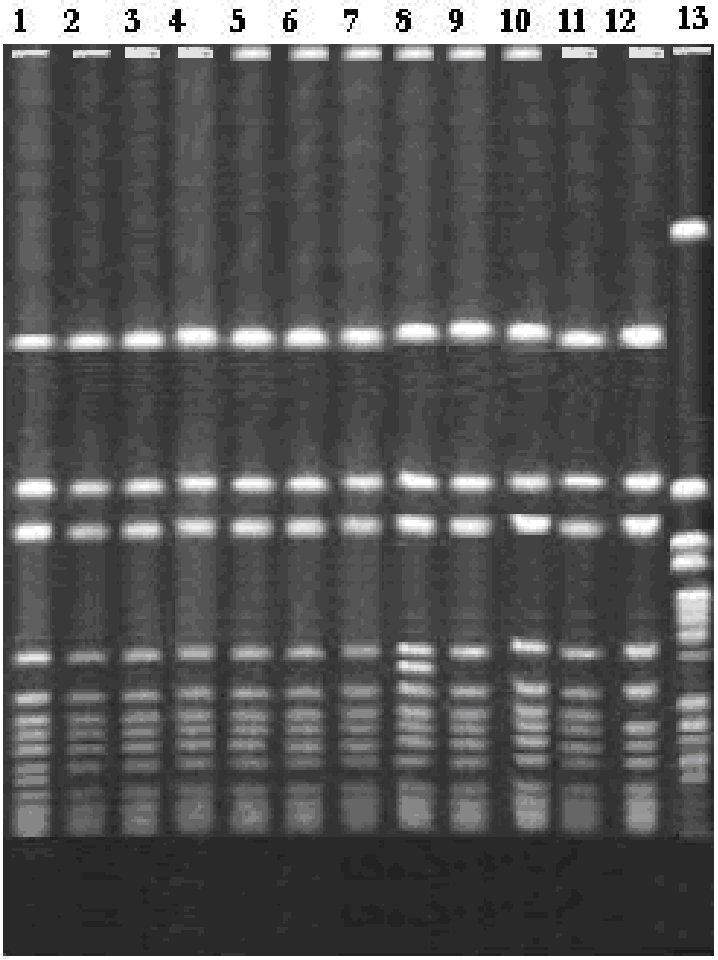


Figure 3.18. *SmaI* microrestriction patterns of enterococcal isolates analyzed by PFGE on a 1% [w/v] agarose gel electrophoresis in 0.25 x TBE buffer. Lane1: strain 21, lane 2: strain 175, lane 3: strain 301, lane 4: strain 382, lane 5: strain 406, lane 6: strain 430, lane 7: strain 468, lane 8: strain 859, lane 9: strain 904, lane 10: strain 908, lane 11: strain 943 lane 12: strain 1393, and lane 13: *E.coli* JM109

Table 3.9. An illustration of the scores of bands obtained from PFGE using *SmaI* restriction enzyme.

Strains	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
21	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1
175	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
301	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
382	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
406	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
430	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
468	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
859	0	1	1	0	0	0	0	0	0	0	1	1	1	1	1
904	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
908	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
943	0	1	1	0	0	0	0	0	0	0	0	1	1	1	1
1393	0	1	1	0	0	0	0	0	0	0	0	1	0	1	1
E.coli	1	0	0	1	1	1	1	1	1	1	0	0	1	1	0

Table 3.10. A distance matrix generated from the scores of bands obtained from PFGE microrestriction using *SmaI* restriction enzyme.

Strains	21	175	301	382	406	430	468	859	904	908	943	1393	*E.coli*
21	0												
175	1	0											
301	1	0	0										
382	1	0	0	0									
406	1	0	0	0	0								
430	1	0	0	0	0	0							
468	1	0	0	0	0	0	0						
859	0	1	1	1	1	1	1	0					
904	1	0	0	0	0	0	0	0	0				
908	1	0	0	0	0	0	0	0	1	0			
943	1	0	0	0	0	0	0	0	1	1	0		
1393	1	1	1	1	1	1	1	2	1	1	1	0	
E.coli	10	10	10	10	10	10	10	12	10	10	10	12	0

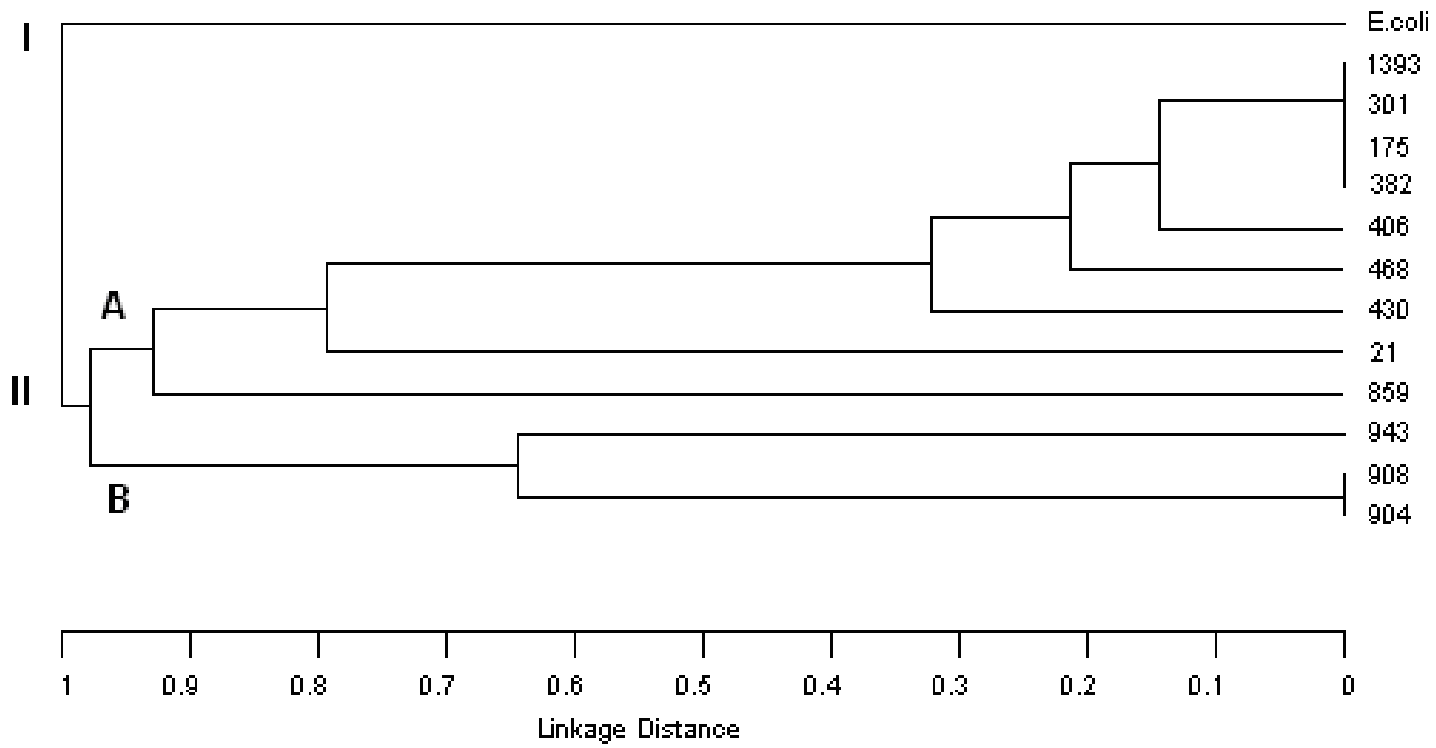


Figure 3.19. A dendogram to show the genetic relationships between all 12 enterococcal strains and *E.coli* JM109 using UPGMA-neighbour joining computer software to determine the genetic diversity and homology attained from the clustering analysis of PFGE patterns using *SmaI* restriction enzyme.

CHAPTER FOUR

CONCLUSION
AND FUTURE DIRECTIONS

The emergence and progression of *Enterococcus* infections has increased steadily over the past years. The taxonomy of this species has been difficult to characterize due to its biochemical, serological and genetically heterogeneous traits. DNA fingerprinting techniques such as RAPD PCR, 16S rRNA ribotyping (PCR) and pulse field gel electrophoresis has proven useful in the identification and characterization of bacterial species such as *Enterococcus* consisting of *E. avium E. durans, E. faecalis and E. faecium*.

Enterococcus strains have been compared by using phenotypic, biochemical, carbohydrate utilization, antibiotic resistance and serotyping (Facklam, *et al*., 1989). Genotyping techniques will allow for the comparison between the 3 distinct species indicating the genetic diversity among the selected isolates. With the use of genotyping techniques, the patterns provided illustrate the genetic relationship between the strains. A genotyping pattern that is shared with another isolate suggests that both those isolates represent the same strain.

The present study illustrated the penicillin and vancomycin resistance of selected *Enterococcus* isolates using the disc diffusion assay and the microtitre dilution methods. All strains were determined to be vancomycin resistant enterococci (VRE) and exhibited a high resistance to penicillin G. VRE has been related to nosocomial infections. Both methods used proved to be a reliable source for MIC evaluation.

The strains were then characterized using genotyping techniques RAPD-PCR using primers MBPZ-3 and MBPZ-4. These primers were chosen to profile the selected enterococcal strains and the unrelated *E. coli* strain to produce RAPD patterns based on their reproducibility and discriminatory power. The RAPD analysis indicated a clonal dissemination among most of the selected enterococcal strains which suggested they belong to the same strain or a closely related species. The efficiency of the RAPD analysis was defined by the same species being grouped together on the phylogenetic tree.

Genetic variability is of great importance as it allows the inference that the isolates yielding similar banding patterns belong to the same strain (Machete, *et al.,* 2001). The clustering patterns for strains 301 and 382 exhibited almost 100% homology for

both primers MBPZ-3 and MBPZ-4 RAPD patterns and shared the same MIC for penicillin G and therefore represent the same strain. These results were confirmed using 16S rDNA ribotyping analysis were the strains were identified as belonging to the same strain *E. faecium*. The 16S rDNA ribotyping technique was used because the 16S rDNA is strain specific (Jacobs, *et al*., 1996). Strain 1393 grouped differently in RAPD_PCR and PFGE. These results indicated a genetic variance between strain 1393 and the other *E.faecium* strains. These results were confirmed using 16S rDNA ribotyping where the NCBI BLAST proved a 100% homology to strain *E.faecium*. The rest of the *Enterococcus* strains showed a very close relation and those strains that grouped together in a cluster exhibited similar MIC values for penicillin G. The bacterial grouping results attained were similar with those of PFGE analysis.

PFGE genotyping technique requires a week to complete, whilst RAPD analysis, after optimization of the primers and reaction conditions for the production of well defined bands are determined, is less laborious. Although the RAPD application does not have a good intra-laboratory, and inter-laboratory reproducibility it is less predictable (Miranda, *et al*., 1991). The differences in the number of patterns obtained by each method actually reflect the difference of principle on which these methods are based. Therefore RAPD and PFGE analysis are useful genotyping tools that are discriminatory DNA-based techniques for differentiation of clinical *Enterococcus* isolates (Morandi, *et al.,* 2006).

RAPD analysis is more specific, faster, has a high discriminatory power and is less laborious. Results can be inconsistent due to low rate of reproducibility. PFGE provides results that are reproducible and have a high discriminatory power (Quale, *et al.,* 2001). RAPD-PCR should be used as a first screening genotyping technique for *Enterococcus* clinical isolates as it is much faster and simple. PFGE yields a much higher polymorphism and should be used as a tool for screening epidemiological bacterial strains in future clinical diagnostics.

Further biochemical and molecular characterization of these strains can be done to determine the enterococcal strains that are resistant to various β-lactam antibiotics such as penicillin, ampicillin and other cephalosporins (Al-Tatari, *et a*l., 2006). These strains can be further characterized by using whole cell wall proteins and analyzing

the data with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. This is an important molecular epidemiological tool because it generates a profile that can distinguish between strains that otherwise may have very similar characteristics based on phenotypic and biochemical tests (Ahmet, *et al*., 1995). Further analysis of these strains that can be done is proteomics, mass spectrophotometry analysis and whole cell fingerprints analysis (Zhou, *et al*., 2002).

CHAPTER FIVE

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APPENDIX A

CLUSTAL X (1.81) Multiple Sequence Alignment

21 --------GCYATSGGCA--KGTMGAAGTCTGACCGAGCAC--GCCGCGTGAGTGAAGA-

859 -------GGTWAYSGGCA--KG--AGAGTCTGACCGAGCAC--GCCGCGTGAGTGAAGA-

1393 -------GRWCATSGGCA--TGGMGAAGTCTGACCGAGCAC--GCCGCGTGAGTGAAGA-

430 -------TRTCMSSSSSMAKGGCCGAAGTCTGMC-GASCMC--GCCGCGTGAGTGAAGA-

301 ----------TRAKGSGGSKCT---ATRCATGCA-GTCGA-

904 ----------CMGKKGSGGGKCT---ATR-ATGCAAGTCGAA

943 ----------CSGKGGGGSRKCT---ATR-MTGCAAGTCGA-

175 ----------CRWKWGSGGSKKCCT---AATSATGSA-GTCGA-

406 ----------CMAKKGSSGSCKTCT---ATRCRTGSA-GTCGAA

382 ----------CYAKKSGGSWKCCT---ATG-STGSARGTCGA-

908 ----------CYKKGSSGCGKCT---ATAMATGCA-GTCGAG

E.coli

AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCT-AACACATGCAAGTCGAA

468 ----------

CSKKSCMWWTTTTGKTMCGACTTCACCCCAGT

*****

21

AGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGA-GAGTAACTGTTC

859

AGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGA-GAGTAACTGTTC

1393

AGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGATGA-GAGTAACTGTTC

430

AGGTTTTCGGATCGTAAAACTCTGTTGWTAGAGAAGAACAAGGATGA-GAGTAACTGTTC

301

CGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAAAAAGAGGAGTGG CGAACGGGTGAGT

904

CGSTTCTTTTTCCACCGGAGCTTGCTCCACCGGAGAAAGAGGAGTGG CGAACGGGTGAGT

943

CGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAGAAAGAGGAGTGG CGAACGGGTGAGT

175

CGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAGAAAGAGGAGTGG CGAACGGGTGAGT

406

CGCTTCTTTTTCCACCGGAGCTTGCTCCACCGGAGAAAGAGGAGTGG CGAACGGGTGAGT

382

YGSTTCTTTTTCCACCGGAGCTTGCTCCACCGGAGAAAGAGGAGTGG GGAACGGGTGAGT

908 CGAACAGACGA-----GGAGCTTGCTCCTCTG----- ACGTTAGCGGCGGACGGGTGAGT

E.coli **CGGTAACAGAAA----GCAGCTTGCTGCTTTG---- CTGACGAGTGGCGGACGGGTGAGT**

468 CATGAACCCTGCCGT-

GGTAATCGCCCTCCTTGCGGTAGGCTCANNACTTCTG---GYRW

*** ***

21

ATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA

GCAGCCGCGGTAA

859

ATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA

GCAGCCGCGGTAA

1393

ATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA

GCAGCCGCGGTAA

430

ATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCA

GCAGCCGCGGTAA

301 AACACGTG--GGTAACCTGCCCATCAGAAGGGGATAACACT-

TGGAAACAGGTGC--TAA

904 AACACGTG--GGTAACCTGCCCATCAGAAGGGGATAACACT-

TGGAAACAGGTGC--TAA

943 AACACGTG--GGTAACCTGCCCATCAGAAGGGGATAACACT-

TGGAAACAGGTGC--TAA

175 AACACGTG--GGTAACCTGCCCATCAGAAGGGGATAACACT-

TGGAAACAGGTGC--TAA

406 AACACGTG--GGTAACCTGCCCATCAGAAGGGGATAACACT-

TGGAAACAGGTGC--TAA

382 ARCACGTG--GGKAACSTGCCCATCAGAAGGGGATAACRCK-

KGKAARCWGGTGC--TAA

908 AACACGTG--GATAACCTACCTATAAGACTGGGATAACTTC-

GGGAAACCGGAGC--TAA

E.coli **AATGTCTG--GG-AAACTGCCCGATGGAGGGGGATAACTAC-**

TGGAAACGGTAGC--TAA

468

WACCCACTCCCATGGTGTGACGGGCGGTGTGTACAAGACCCGGGAA

CGTATTCACCGCGG

*** * * * ***

21 TACGTAGGTGGCAAGCGTTGTC-

CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTC

859 TACGTAGGTGGCAAGCGTTGTC-

CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTC

1393 TACGTAGGTGGCAAGCGTTGTC-

CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTC

430 TACGTAGGTGGCAAGCGTTGTC-

CGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTC

301 TAC-

CGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGG

GTGTCGCT

904 TAC-

CGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGG

GTGTCGCT

943 TAC-

CGTATAACAATCRAAACCGCRTGGTTTTGATTTGAAAGGCGCTTTCGG

GTGTCGCT

175 TAC-

CGTATAACAATCRAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGG

GTGTCGCT

406 TAC-

CGTATAACAATCAAAACCGCATGGTTTTGATTTGAAAGGCGCTTTCGG

GTGTCGCT

382 KRC-

CKTAKAACAAKCRAAACCGYRTGGTTTTGATTTGAARGGCGSKTTCSG

GTGKYGYT

908 TAC-

CGGATAATATATTGAACCGCATGGTTCAATAGTGAAAGACGGTTTTGC

-TGTCACT

E.coli **TAC-CGCATAACG-------TCGCAAGACC--AAAGAGGGGGAC-CTTGGGCCTCTTGCC**

468 CAT---GCTGATCCGCGATTACTAGCGATTCCAGCTTCACGTA---GTCGAGTTGCAGAC

*****

21 TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG--TCATTGGAAACTGGGAGA

859 TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG--TCATTGGAAACTGGGAGA

1393 TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG--TCATTGGAAACTGGGAGA

430 TTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGG--TCATTGGAAACTGGGAGA

301 GATGGATGGACCCGCGGTGCATTAGCT--AGTTGGTGAGG--TAACGGCTCACCAAGGCC

904 GATGGATGGACCCGCGGTGCATTAGCT--AGGTGGTGAGG--TAACGGSTCACCAAGGCC

943 GATGGATGGACCCGCGGTGCATTAGCT--AGKTGGTGAGG--TAACGGCTCACCAAGGCC

175 GATGGATGGACCCGCGGTGCATTAGCT--AGKTGGTGAGG--TAACGGCTCACCAAGGCC

406 GATGGATGGACCCGCGGTGCATTAGCT--AGGTGGTGAGG--TAACGGSTCRCCAAGGSC

382 GATGGATGGWCCSSCGGTGCWTTWKYT--AGGKGGKGRGG--TRACGGSTCRSCAAGGSC

908 TATAGATGGATCCGCGCCGCATTAGCT--AGTTGGTAAGG--TAACGGCTTACCAAGGCA

E.coli **ATCGGATGTGCCCAGATGGGATTAGCT--AGTAGGTGGGG--TAAAGGCTCACCTAGGCG**

468

TACGATCCGGACTACGATGCGTTTTCTGGGATTAGCTCCCCCTCGCGGGTTGGCAACCCT

*** * * ***

21 CTT-
GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGG---
TGAAATGCG---TAG
859 CTT-
GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGG---
TGAAATGCG---TAG
1393 CTT-
GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGG---
TGAAATGCG---TAG
430 CTT-
GAGTGCAGAAGAGGAGAGTGGAATTCCATGTGTAGCGG---
TGAAATGCG---TAG
301 ACG-
ATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAA
904 ACG-
ATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAA
943 ACG-
ATGCATAGCCGACCTGAGAGGGTGATCGGCCACCTTGGGACTGAGAC
ACGGCCCAA
175 ACG-
ATGCATAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGAC
ACGGCCCAA
406 ASG-
RTGCRTAGCCGACCTGAGAGGGTGATCGGCCRCRTTGGGACTGAGAC
ACGGCCCAA
382 ASS-
RTGCRTAKYSGAYSTGAGAGGGTGRTCGGCCRCRTTGGKACTGAGAC
ACGGYCCAA

908 ACG-

ATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGAACTGAGAC

ACGGTCCAG

***E.coli* ACG-**

ATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAACTGAGAC

ACGGTCCAG

468

CTGTACGCACCATTGTATGACGTGTGAAGCCCTACCCATAAGGGCCA

TGAGGACTTGACG

*** ** * * ***

21 ATATATG-GAGGAACACCAGTGGCGAAGGCGGCT-

CTCTGGTCTGTAA-CTGAC---GCT

859 ATATATG-GAGGAACACCAGTGGCGAAGGCGGCT-

CTCTGGTCTGTAA-CTGAC---GCT

1393 ATATATG-GAGGAACACCAGTGGCGAAGGCGGCT-

CTCTGGTCTGTAA-CTGAC---GCT

430 ATATATG-GAGGAACACCAGTGGCGAAGGCGGCT-

CTCTGGTCTGTAA-CTGAC---GCT

301 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

904 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

943 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

175 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

406 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

382 MYTYMTACGGGAGGCAGCAGTAGGGAATCTTCGG-

CAATGGACGAAAGTCTGACCGAGCA

908 ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCG-

CAATGGGCGAAAGCCTGACGGAGCA

E.coli **ACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA-**

CAATGGGCGCAAGCCTGATGCAGCC

468

TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCTCTCTAGAGTGC

TCTTGYGTAGCA

*** * ** * * * ** ****

21 GAGGCTCGAAAGCGTGGGGAGCAAA-

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAA

859 GAGGCTCGAAAGCGTGGGGAGCAAA-

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAA

1393 GAGGCTCGAAAGCGTGGGRAGCAAA-

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAA

430 GAGGCTCGAAAGCGTGGGGAGCAAA-

CAGGATTAGATACCCTGGTAGTCCACGCCGTAAA

301 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

904 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

943 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

175 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

406 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

382 ACGCCGCGTGAGTGAAGAAGGTTTT-

CGGATCGTAAAACTCTGTTGTTAGAGAAGAACAA

908 ACGCCGCGTGAGTGATGAAGGTCTT-

CGGATCGTAAAACTCTGTTATTAGGGAAGAACAA

E.coli **ATGCCGCGTGTATGAAGAAGGCCTT-**

CGGGTTGTAAAGTACTTTCAGCGGGGAGGAA-GG

468

ACTAGAGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC

ACGACACGAGC--

*** * * * ***

21 CGATGAGTGCTAAGT-
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGC--ATTA
859 CGATGAGTGCTAAGT-
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGC--ATTA
1393 CGATGAGTGCTAAGT-
GTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGC--ATTA
430 CGATGAGTGCTAAGT-
GTTGGAGGSTTTCCGCCCTTCAGTGCTGCAGCTAACGC--ATTA
301 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
904 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
943 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
175 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
406 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
382 GGATGAGAGTAAC-T-
GTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTA
908 ATGTGTAAGTAAC-T-
ATGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTA
E.coli **GAGTAAAGTTAATAC-**
CTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTC
468 TGACGACAGCCATGCAGTACCTGTGTCCACTTTCCCT-
TTCGGGMACSTAATGC-ATCTC

*** * * * * ** ***

APPENDIX B

Program parameters used in the SEQBOOT program for bootstrapping analysis.

Bootstrapping algorithm, version 3.66

Settings for this run:

D	Sequence, Morph, Rest., Gene Freqs?	Molecular sequences
J	Bootstrap, Jackknife, Permute, Rewrite?	Bootstrap
%	Regular or altered sampling fraction?	regular
B	Block size for block-bootstrapping?	1 (regular bootstrap)
R	How many replicates?	1000
W	Read weights of characters?	No
C	Read categories of sites?	No
S	Write out data sets or just weights?	Data sets
I	Input sequences interleaved?	Yes
0	Terminal type (IBM PC, ANSI, none)?	IBM PC
1	Print out the data at start of run	No
2	Print indications of progress of run	Yes

Y to accept these or type the letter for one to change

y

Random number seed (must be odd)?

5

Program parameters used in the DNADIST program for distance matrix analysis.

Nucleic acid sequence Distance Matrix program, version 3.66

Settings for this run:

D	Distance (F84, Kimura, Jukes-Cantor, LogDet)?	Jukes-Cantor
G	Gamma distributed rates across sites?	No
C	One category of substitution rates?	Yes
W	Use weights for sites?	No
L	Form of distance matrix?	Square
M	Analyze multiple data sets?	Yes, 1000 data sets
I	Input sequences interleaved?	Yes
0	Terminal type (IBM PC, ANSI, none)?	IBM PC
1	Print out the data at start of run	No
2	Print indications of progress of run	Yes

Y to accept these or type the letter for one to change

Y

Program parameters used in the NEIGHBOUR-JOINING program for phylogenetic analysis.

Neighbor-Joining/UPGMA method version 3.66

Settings for this run:

N	Neighbor-joining or UPGMA tree?	UPGMA
L	Lower-triangular data matrix?	No
R	Upper-triangular data matrix?	No
S	Subreplicates?	No
J	Randomize input order of species?	Yes (random number seed = 5)
M	Analyze multiple data sets?	Yes, 1000 sets
0	Terminal type (IBM PC, ANSI, none)?	IBM PC
1	Print out the data at start of run	No
2	Print indications of progress of run	Yes
3	Print out tree	Yes
4	Write out trees onto tree file?	Yes

Y to accept these or type the letter for one to change

Y

Program parameters used in the CONSENSUS program to produce a single consensus tree.

Consensus tree program, version 3.66

Settings for this run:

C	Consensus type (MRe, strict, MR, Ml):	Majority rule (extended)
O	Outgroup root:	No, use as outgroup species 1
R	Trees to be treated as Rooted:	No
T	Terminal type (IBM PC, ANSI, none):	IBM PC
1	Print out the sets of species:	Yes
2	Print indications of progress of run:	Yes
3	Print out tree:	Yes
4	Write out trees onto tree file:	Yes

Are these settings correct? (Type Y or the letter for one to change)

y