MOLECULAR ANALYSIS OF VANCOMYCIN – RESISTANT *ENTEROCOCCUS* ISOLATED FROM SURFACE AND GROUND WATER SAMPLES

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DECLARATION

I, **Pheeha Daniel Matlou** hereby declare and confirm that the work done for this mini dissertation is my own work, unless where acknowledged. It has not been submitted to any institution for the purpose of obtaining a qualification. All materials used and quoted herein have been duly acknowledged.

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DEDICATION

I dedicate this project to my Mother (The late Miss M.T Matlou) and my family

"Honor your father and mother.

Then you will live a long,

full life in the land the LORD your God will give you"

Exodus 20:12

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ABSTRACT

Vancomycin Resistant *Enterococcus* (VRE) has been responsible for numerous outbreaks of serious infections in humans worldwide. *Enterococcus faecium* and *Enterococcus faecalis* are the two main species that usually habour high levels of vancomycin resistance determinants, and cause both hospital and community acquired infections in humans. The difficulty in managing infections caused by antibiotic resistant bacteria particularly VREs has resulted in extensive surveillance studies with this pathogen. In addition, the high interest in VRE surveillance studies is also due to the fact that the pathogen is most often isolated from untreated water that is intended for human consumption. The aim of this study was to isolate VREs from ground and surface water as well as to determine their virulence capabilities using phenotypic and genotypic assays. A total of 170 ground and surface water samples collected between August 2015 and April 2016 were analyzed and 81 potential isolates were screened for characteristics of *Enterococcus* species using biochemical tests, species specific PCR analysis and sequence analysis. Antimicrobial susceptibility tests were performed to determine the antibiotic resistance profiles of the isolates particular VREs. A total of 56 isolates were confirmed as *Enterococcus* species through amplification of *ddl* genes and sequencing data. The distribution of species comprised *Enterococcus faecium* (38), *Enterococcus faecalis* (17) and *Enterococcus saccharolyticus* (1). Phenotypic characterization by cluster analysis of the isolates using their antibiotic inhibition zone diameter data was used to determine the similarities as well as resolve differences between isolates from the different water sources and/or locations revealed that the isolates derived from these samples have originated from similar progeny. Large proportions (78.6-83%) of isolates were resistant to vancomycin and nalidixic acid. Forty four VREs isolates were detected phenotypically and most of these isolates (56. 8%) were derived from surface water samples. In addition, a large proportion of the isolates (80.4%) were resistant to multiple antibiotics and the MAR phenotype VAN-NAL-AMP was dominant among these isolates. A total of 17.9 % of the isolates harboured VRE genes. The *vanA* and *vanB* genes were detected in 16% and 3.6% of the isolates respectively and this was most prevalent among *E. faecalis*. The presence of virulence factors in VRE isolates was determined. Virulence determinants were detected in 56.8% of the VRE and 4 of the virulence factors (*asa1, esp, gel* and *hyl*). A large proportion (79.5%) of the isolates showed great similarities based on the DNA banding patterns. The findings of this study have demonstrated that enterococci from environmental water of South Africa are resistant to multiple antimicrobial agents, some of which are commonly used for treatment. Furthermore, these isolates harbor significant genes coding for virulence factors, which frequently enhance their pathogenic potential. This study also highlights the need for continuous monitoring for virulent VRE strains in water that is intended for human consumption and household activities.

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LIST OF ABBREVIATIONS AND SYMBOLS

API	: Analytical Profile Index
BEA	: Bile Esculin Agar
Blast	: Basic Local Alignment Search Tool
E.	: *Enterococcus*
EDTA	: Ethylenediamine-tetraacetic acid
ICU	: Intensive Care Unit
MAR	: Multiple Antibiotic Resistance(s)
MIC	: Minimum Inhibition Concentration(s)
mL	: Milliliter(s)
mM	: Millimeter(s)
NNIS	: National Nosocomial Infectious Surveillance
NaCl	: Sodium Chloride
PBP	: Penicillin-Binding Protein
PCR	: Polymerase Chain Reaction
rRNA	: Ribosomal Ribonucleuotide Acid
RSB	: Resuspension Buffer
TAE	: Tris acetic acid EDTA buffer
UTI	: Urinary Tract Infections
µg	: Microgram(s)
µL	: Microlitre(s)

μm	: Micrometer(s)
v/v	: Volume per volume
v/w	: Volume per weight
van	: Vancomycin
VRE	: Vancomycin Resistant *Enterococcus*
VSE	: Vancomycin Susceptible *Enterococcus*
w/v	: Weight per volume
WHO	: World Health Organization

DEFINITION OF CONCEPTS

Antibiotic resistance: The ability of a given bacteria to survive the exposure to a defined concentration of an antimicrobial agent.

Bacteraemia: is the presence of bacteria in the bloodstream, which can occur spontaneously during certain tissue infections, wound-care and other associated procedures.

Cluster analysis: A comparative analysis of typing data collected for a variety of bacterial isolates in order to group them based on the similarity of their data.

Fingerprint: A specific banding pattern displayed by an isolate on application of one or more typing methods.

Nosocomial infections: these are hospital-acquired infections that are mainly caused by bacterial, viral, and fungal pathogens; the most common types are bloodstream infections (BSI), pneumonia, urinary tract infection (UTI), and surgical site infection (SSI).

Multiplex PCR: A convenient variant method of polymerase chain reaction in which more than one gene is simultaneously amplified in the same reaction mixtures.

Phylogeny: A process in which lineage of organisms evolved by separation from common ancestors.

Plasmid: A circular DNA molecule that is able to replicate independently from the chromosome and promote lateral transfer among different species of bacteria through the conjugation process.

Polymerase Chain Reaction: A molecular method that is used to amplify specific regions of DNA many times over using primers.

Primer: A short strand of nucleic acid sequences (generally about 10 base pairs) that serves as a starting point for DNA synthesis. It is required for DNA replication because the enzymes that catalyze this process, DNA polymerases, can only add new nucleotides to an existing strand of DNA.

Species: A collection of bacterial cells which share an overall similar pattern of traits in contrast to other bacteria whose pattern differ significantly.

Teicoplanin: An antibiotic used in the prophylaxis and treatment of serious infections caused by Gram-positive bacteria, including methicillin-resistant *Staphylococcus aureus* and resistant *Enterococcus* species.

Vancomycin: An antibiotic used to treat a number of bacterial infections. It is a member of the glycopeptides antibiotic class and is effective mostly against Gram-positive bacteria.

CHAPTER ONE

INTRODUCTION AND PROBLEM STATEMENT

CHAPTER 1

INTRODUCTION AND PROBLEM STATEMENT

1.1 GENERAL INTRODUCTION

Enterococci are Gram-positive, facultative anaerobic bacteria that usually occur as normal flora in gastrointestinal and genitourinary tracts of humans and animals (Zirakzadeh and Patel, 2006). Despite that there are more than a dozen species that have been identified and fully characterized, strains belonging to the species *Enterococcus faecalis* and *Enterococcus faecium* are the two most frequently identified isolates especially in food, water and clinical samples and therefore account for the majority of human Enterococcal infections worldwide (Bonten *et al.*, 1996; Fisher and Phillips, 2009; Ateba and Maribeng, 2011; Ranotkar *et al.*, 2014)

Enterococcus species are generally known to possess low intrinsic virulence traits which explain why they are most often associated with opportunistic infections (Lin and Hayden, 2010). Despite this, in recent years a number of highly pathogenic *Enterococcus* species have been isolated from food products, animals and water that is intended for human consumption as well as from infected humans (Frieden *et al.*, 1993; Harwood *et al.*, 2001; CDC, 2011; Mohapi and Ateba, 2013; Nam *et al.*, 2013; Goldstein *et al.*, 2014). In addition, most of these isolates possess virulence gene determinants which account for their pathogenesis (Harwood *et al.*, 2001; Mohapi and Ateba, 2013; Goldstein *et al.*, 2014). These virulent strains have been associated with a variety of infections, including bacteraemia, endocarditis, skin infections, intra-abdominal tract and genitourinary tract infections in humans (Mohapi and Ateba, 2013; Lisboa *et al.*, 2015). These infections present severe complications in infants, immuno-compromised as well as elderly individuals. In a country like South Africa where the incidence of HIV/AIDS is high, the impact of these pathogens cannot be overemphasized.

The public health significance of virulent *Enterococcus* species has been amplified by the constant increase in the prevalence of hospital-acquired Enterococcal infections worldwide (Ramsey and Zilberberg, 2009). However, the treatment of infections caused by bacteria species including enterococci is usually achieved through the administration of antibiotics (Stranden *et al.*, 2003; Fraser, 2012). Unfortunately, the success rate achieved with antimicrobial agents is greatly affected by the fact that most *Enterococcus* isolates are highly resistant to commonly recommended drugs and also that some enterococci possess intrinsic resistance against a variety of antimicrobial agents (Borgen *et al.*, 2001; Butterworth *et al.*, 2002), thereby significantly limiting therapeutic options.

In the past, most Enterococcal infections in humans were treated with ampicillin, penicillin, or vancomycin with or without an aminoglycoside (Cohen *et al.*, 1992). However, during the past two decades, enterococci have adapted and acquired resistance to most of the above mentioned agents resulting with the development of multidrug-resistant strains (Valenzuela *et al.*, 2008). This has greatly reduced treatment options for infections caused by vancomycin-resistant *Enterococcus* (VRE) strains (Nannini *et al.*, 2005; Valenzuela *et al.*, 2008). The isolation of Multidrug resistant enterococci from water poses a serious health threat.

Water is a very important resource for human and animal life and access to safe drinking water is a significant human need (WHO, 2003). Prior to 1996 there were a lot of discrepancies in the access to safe drinking water in South Africa. In response to this the constitution, drawn up in 1996, prescribes that every individual has the right to safe drinking water (CRSA, Act 108 of 1996). This mandate was handed over to the Department of Water Affairs and Forestry (DWAF), and Department of Provincial and Local Government (DPLG) and it was based on the premise that contaminated water poses severe public health consequences on consumers (Keswick *et al.*, 1984; Sood and Perl, 2016). Unfortunately, South Africa does not only have very low rainfall resulting in shortages of water (Ateba and Maribeng, 2011) but the increase in industrialization has been found to significantly affect the quality of water supplied to communities also in rural places. With this in mind, most individuals who do not have access to portable water rely on water from other alternative sources such as dams, boreholes as well as rivers for survival and for use in household activities (Chamaille-Jammes *et al.*, 2007).

Untreated water from most of these unprotected sources is usually exposed to microbial contamination through contact with faeces of animal and humans origin (Reeves *et al.*, 2004). Pathogenic strains of *Salmonella* and *Enterococcus* species as well as *Escherichia coli* have been isolated from water that is intended for human consumption including in the study area (Reeves *et al.*, 2004; Kinge *et al.*, 2010; Ateba and Maribeng, 2011; Phokela *et al.*, 2011). Therefore, these bacterial species have been used as indicators of faecal pollution and *Enterococcus* species are one of the microbial indicators, which when found in water indicate that there is a possibility of having other pathogenic bacteria of faecal origin in the water (Franz *et al.*, 2001; He and Jiang, 2005; Valenzuela *et al.*, 2008). This therefore means contamination of water bodies with these pathogens is usually associated with poor hygiene practices of any sort and this explains the need to improve water treatment, purification and distribution practices to ensure that they adhere to generally recommended levels.

Antibiotic resistant bacteria particularly vancomycin-resistant enterococci (VRE) pose a significant challenge to the medical profession even in advanced countries with appropriately designed public health policies and proper health care facilities especially when they harbour multiple antibiotic resistant determinants (Takeuchi *et al.*, 2005). Despite the fact that some limited information exists on the occurrence of enterococci in environmental water in the North-West Province of South Africa (Ateba and

Maribeng, 2011; Molale and Bezuidenhout, 2016) the present study was designed to expand on these previous investigations by isolating VRE from ground and surface water and also determine their virulence capabilities using phenotypic and genotypic assays. This was designed to assess the public health implications that these isolates may have on consumers in the area and therefore generate data that may be of great epidemiological significance.

1.2 PROBLEM STATEMENT

Recently, bacterial species are increasingly becoming resistant to a number of commonly recommended antibiotics. As such, the treatment of many microbial infections in humans has been compromised by the presence of these resistant strains (Cetinkaya *et al.*, 2000; Sood and Perl, 2016). Moreover, the difficulty in managing infections caused by antibiotic resistant bacteria particularly VRE has resulted in extensive surveillance studies with this pathogen in different geographical areas worldwide (Ramsey and Zilberberg, 2009; Ateba and Maribeng, 2011; Fraser, 2012).

The high interest in VRE surveillance studies is also due to the fact that the pathogen is most often isolated from untreated water that is intended for consumption in communities that do not have access to potable water as well as from patients suffering from nosocomial infections in wards in hospitals (Cetinkaya *et al.*, 2000). In addition, VRE have also been isolated from animal species and food products such as meat and vegetables and this therefore indicates that the presence of these pathogens in water and the food chain may provide opportunities for transmission to consumers (Phillips *et al.*, 2004). Once in the gastrointestinal tract of humans these organisms are able to transfer their resistance determinants to other commensal bacterial species through conjugation (Wheeler *et al.*, 2002). This therefore means the GIT may serve as a potential reservoir for antibiotic resistant genes resulting in severe clinical implications in a given area. It is therefore important to implement proper water treatment procedures, appropriate hygiene practices, surveillance strategies of VRE's and standard sanitary standards to ensure that transmission with these pathogens is highly minimized.

1.3 RESEARCH AIM AND OBJECTIVES

1.3.1 Aim

The aim of this study was to isolate VRE from ground and surface water as well as determine their virulence capabilities using phenotypic and genotypic assays.

1.3.2 Objectives

The specific objectives of the study were to:

i. isolate the *Enterococcus* species from ground and surface water samples
ii. confirm the identities of the isolates using conventional microbiological techniques
iii. determine the presence of VRE by phenotypic assays using the agar diffusion technique
iv. screen for the presence of VRE genes using multiplex PCR analysis
v. determine the virulence gene factors in VRE isolates using phenotypic and genotypic assays.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER 2

LITERATURE REVIEW

2.1 Background

Enterococcus belongs to the family *Enterococcaceae* that are lactic acid producing bacteria of the phylum Firmicutes. The classification of organisms in the family *Enterococcaceae* was based on 16S rRNA gene sequences to accommodate the phylogenetically closely related genera *Enterococcus*, *Melissococcus*, *Tetragenococcus* and *Vagococcus* (Vos *et al.*, 2011). *Enterococcus* species are Gram-positive, coccus-shaped bacteria that are usually present in the gastro-intestinal and genito-urinary tracts of humans and warm-blooded animals (Zirakzadeh and Patel, 2006).

Previously *Enterococcus* species have been considered to be harmless to humans and since they produce bacteriocins, these organisms have been used widely in the food industry as probiotics or as starter cultures (Moreno *et al.*, 2006). However, a number of species in the genus *Enterococcus* are now known to be the most common cause of nosocomial infections worldwide resulting in high mortality rates among patients (Fisher and Phillips, 2009). In addition, these organisms have been reported to also cause life threatening infections in other vital parts of the human body (Zirakzadeh and Patel, 2006; Valenzuela *et al.*, 2008). Their pathogenecity is also enhanced by the fact that enterococci have significantly acquired resistance determinants to several commonly used antimicrobial agents, including vancomycin that was previously used as an effective drug for the treatment of multi-drug resistant Enterococcal infections (Phillips *et al.*, 2004; Mohapi and Ateba, 2013).

The genus *Enterococcus* consists of more than 50 species but *Enterococcus faecium* and *Enterococcus faecalis* are the most frequently isolated species in most disease cases and therefore are known to cause most of the pathological complications in humans worldwide, especially in hospital environments (Zirakzadeh and Patel, 2006; Valenzuela *et al.*, 2008). Despite this, *E. gallinarum*, *E. casseliflavus*, and *E. raffinosus* have also been associated with disease in humans although the frequency of infections caused by these species in very low (Valenzuela *et al.*, 2008).

Among the enterococci, the presence of strains that are resistant to vancomycin generally termed Vancomycin-Resistant *Enterococcus* (VRE) usually limits treatment options in patients suffering from Enterococcal infections (Ramsey and Zilberberg, 2009). The natural hosts of *Enterococcus* species is humans and animals but these organisms may be present in water bodies as well as the environment as a result of improper deposition of faeces (Busani *et al.*, 2004). Against this background, *Enterococcus* species have been isolated from environmental sources such as soil, surface waters, agricultural plants and animal products (Harwood *et al.*, 2000; Johnston and Jaykus, 2004). Contaminated unprotected water bodies are the main reservoirs of most environmental *Enterococcus* species (Ateba *et al.*, 2013) and this therefore justifies the need to constantly monitor the occurrence of these pathogens in water that is intended for direct and indirect consumption by humans as well as for use in both recreational and household activities.

Vancomycin resistance particularly in enterococci may only occur through one of two mechanisms and these include acquired or intrinsic resistance (Levine, 2006). Acquired resistance occurs when sensitive *Enterococcus* strains pick up a genetic determinant usually a plasmid that contains vancomycin resistance gene from resistant strains or the environment and the presence of this gene confers the resistance to vancomycin in the recipient cells (Wright and Berghuis, 1999). This generally limits the therapeutic options for human if the infecting strain harbours the resistant determinant. Acquired resistance in enterococci is most common among *E. faecium* and *E. faecalis*, although it has been also recognized in *E. avium, E.*

durans, *E. raffinosus*, and other Enterococcal species (Borgen *et al.*, 2001; CDC, 2010). Moreover, VRE strains are also capable of transferring resistance determinants to unrelated bacterial species such as *Staphylococcus aureus* and this has accounted for the development of methicillin-resistant *Staphylococcus aureus* (MRSA) strains that are currently a serious challenge to the medical profession (Borgen *et al.*, 2001).

Intrinsic resistance results from the innate ability of enterococci to show resistance to antimicrobial agents through its inherent structural or functional characteristics (Harwood *et al.*, 2001). Isolates belonging to *Enterococcus gallinarum*, *Enterococcus casseliflavus* and *Enterococcus flavescens*, are known to demonstrate an inherent, low-level resistance to vancomycin (Borgen *et al.*, 2001; CDC, 2010). In addition to vancomycin, intrinsic low level resistance is also shown to some other antibiotics including the beta-lactams (Wright and Berghuis, 1999), aminoglycosides (Wright and Berghuis, 1999) and lincosamides (Stranden *et al.*, 2003). Several genes, including *van*A, *van*B, *van*C, *van*D, and *van*E, respectively contribute to the resistance to vancomycin in Enterococcal isolates (Wright and Berghuis, 1999). Therefore, virulent VRE isolates may carry one or more of these vancomycin resistant genes.

The *vanA* and *vanB* genes are most frequently associated with *E. faecium* and *E. faecalis* respectively (Harwood *et al.*, 2001). VRE species that harbour the *vanA* gene determinant showed high level resistance to vancomycin with minimum inhibitory concentrations (MICs) of (>128 µg/mL to vancomycin) and (≥16 µg/mL to teicoplanin) (Borgen *et al.*, 2001; Harwood *et al.*, 2001). However, VRE isolates that contain the *vanB* genotype typically display a much lower level of resistance to vancomycin with MIC ranging from 16 – 64 µg/mL and are also susceptible to teicoplanin with MICs of ≤1 µg/ml (Borgen *et al.*, 2001; Harwood *et al.*, 2001; CDC, 2010).

E. faecium isolates harbouring *vanD and vanE* were reported to show moderate levels of resistance to vancomycin with MICs ranging from 64 – 128 µg/mL for vancomycin and MICs of 4 – 8 µg/mL for teicoplanin. In addition, the *vanC* genotype has been associated with *E. gallinarum*, *E. casseliflavus* and *E. flavescens* and the presence of this determinant results in intrinsic resistant to vancomycin with MIC values of 2 – 16 µg/mL (Wright and Berghuis, 1999; Harwood *et al.*, 2001).

In addition to these antibiotic resistance determinants a number of virulence factors in *Enterococcus* species including gelatinase, aggregation substance, Enterococcal surface protein, cytolysin and hyaluronidase (Mundy *et al.*, 2000; Eaton and Gasson, 2001; Vankerckhoven *et al.*, 2004; Molale and Bezuidenhout, 2016) have also been known to increase the pathogenecity of the isolates. The majority of studies documented so far have screened *E. faecalis* and *E. faecium* for these virulence factors mainly due to the fact that these strains are mostly implicated in disease conditions in clinical settings (Molale and Bezuidenhout, 2016).

Despite these documented associations between antibiotic resistance determinants and specific *Enterococcus* species as well as the guidelines for MIC values that are known to be displayed by both high and low level resistance to vancomycin and teicoplanin, the resistance profiles of vancomycin resistant enterococci in a given area must be based on data obtained through laboratory findings. In addition, baseline studies conducted in the area have revealed the presence of VRE using basic microbiology detection techniques (Ateba and Maribeng, 2011; Ateba *et al.*, 2013; Mohapi and Ateba, 2013). The present study was designed to expand on previous investigations with the aim of isolating *Enterococcus* species, especially *E. faecalis* and *E. faecium* strains that are resistant to vancomycin and also characterize the isolates for the presence of both vancomycin resistance and virulence genes. The main purpose was to assess the public health implications of these isolates in the environment, particularly in water bodies.

2.2 CLASSIFICATION AND ECOLOGY OF *ENTEROCOCCUS* SPECIES

Enterococcus species are classified under the family *Enterococcaceae* which were previously considered as part of the Group D *Streptococcus* species (Rose *et al.*, 2001; Mascini *et al.*, 2006). Individual cells are usually 0.5 µm in diameter (Rose *et al.*, 2001; Mascini *et al.*, 2006) and more than 50 species that include *E. faecium*, *E. faecalis*, *E. gallinarum*, *E. avium*, *E. casseliflavus*, *and E. mundtii* have been identified and classified (Murray *et al.*, 1981). Enterococci are gamma haemolytic due to the fact that they normally do not breakdown human and animal erythrocytes (Mascini *et al.*, 2006). *Enterococcus* species are also generally non-motile, facultative anaerobic organisms which ferment glucose without gas production, and are negative for both the catalase and oxidase tests (Murray *et al.*, 1981; Rose *et al.*, 2001). These organisms normally grow on Bile Esculin Agar and produce characteristic black colonies due to their ability to break down azide that is incorporated in the medium (Murray *et al.*, 1981; Rivera *et al.*, 1988). *Enterococcus* species are also capable of metabolising a wide variety of energy sources including lactate, carbohydrates, malate, glycerol, citrate, agmatine, arginine, and many keto acids (Willey, 2008).

The ability of these isolates to survive in a wide variety of environments is also enhanced by the fact that cells can grow in extreme alkaline pH (9.6) as well as high salt concentrations (6.5% NaCl) (Rivera *et al.*, 1988; Teixeira and Merquior, 2013). These organisms also resist bile salts, heavy metals, detergents, ethanol and desiccation (Rivera *et al.*, 1988; Mascini *et al.*, 2006). According to a recent report from the National Nosocomial Infectious Surveillance (NNIS) surveys, enterococci prevail as the top 3 most common microbial pathogens that cause nosocomial infections worldwide and this is, mainly due to the fact that these organisms thrive in varied and extreme environments.

2.3 EPIDEMIOLOGY

2.3.1 Geographic distribution

VRE have been detected in different ecological niches within a number of geographical locations and this has severe setbacks in fully understanding their epidemiology. Despite the fact that it has been suggested that host specificity and overrepresentation of certain clones may account for the patterns of and distribution of diseases associated with VRE in a given area, these fail to provide explanations of the patterns of evolutionary descent among VRE (Willems *et al.*, 2000; Homan *et al.*, 2002). VRE were first discovered in France and the United Kingdom especially in farm animals and this resulted from the use of avoparcin in animals (Bonten *et al.*, 2001). On the contrary, VRE strains were not isolated from humans and farm animals in the USA since the drug avoparcin was never used in that country (CDC, 2004).

However, VRE strains from France and the United Kingdom spread in other European countries, including Belgium, Denmark, Australia, Italy, Germany, Sweden, Spain and the Netherlands (Woodford *et al.*, 1995; Klein, 2003) while recent studies in the United States (Coque *et al.*, 1996; Deshpande *et al.*, 2007), Canada (Zoutman *et al.*, 2003), Malaysia (Sood *et al.*, 2008) have indicated that nosocomial VRE infections and transmission have occurred much more frequently and this might be due to migration. In addition, VRE have been detected among farm animals, food products and environmental samples in South Africa despite the fact that the antibiotic vancomycin and its derivatives are not used in the country (Ateba and Maribeng, 2011; Ateba *et al.*, 2013; Mohapi and Ateba, 2013) which justifies the need for constant surveillance studies to be conducted.

2.3.2 VRE in the community

In order to determine the epidemiology of enterococci particularly VRE in a given area, there is a need to understand their modes of transmission. Despite the fact that *Enterococcus* species have increasingly been recognized as bacteria that infect severely debilitated hospitalized patients who have received antibiotic treatment for long periods (Cetinkaya *et al.*, 2000) thereby causing as nosocomial infections, community-acquired VRE infections have also been reported in some countries. Generally, a lot of attention has been focused on the epidemiology and occurrence of VRE mainly in hospitals, and there are few reports that reveal the transmission of VRE to healthy individuals within the community (Murray, 1997; Cetinkaya *et al.*, 2000; Teixeira and Merquior, 2013). The transmission of VRE among individuals in a community plays a significant role in the epidemiology of VRE infections in a given area and this is even worse in rural communities where proper hygiene is not usually practiced.

Community acquired VRE has been detected even in the USA (Cetinkaya *et al.*, 2000; WHO, 2004) when a husband caregiver of an elderly woman colonized with VRE presented with urinary retention and severe urinary tract infection in which molecular typing of the infecting strains revealed high genetic similarities (Shekar *et al.*, 1995; Murray, 1997). Given that VRE have been isolated from animals, groundwater (intended for human consumption) and food products in the North West Province of South Africa (Moneoang and Bezuidenhout, 2009; Ateba and Maribeng, 2011; Mohapi and Ateba, 2013), further studies on the community prevalence of VRE among environmental samples and individuals may indicate the extent of VRE colonisation in the community and also assess the risks associated with these organisms in the area.

2.3.3 Contamination of water and water sources

Water and water bodies are easily contaminated with various waterborne pathogens at different stages and this results in considerable challenges to consumers (Beuchat, 1996; Dallal, 2009). The microbial quality of surface and ground water bodies have declined over the years especially in developing countries as a result of industrialization and rapid growth in population (Lata *et al.*, 2009). Increase in water pollution does not only result in the deterioration of water quality but it also compromises human health, affects the stability of aquatic ecosystems and economic growth (Moe *et al.*, 2007). Against this background, waterborne contamination must be addressed collectively among communities and countries worldwide to ensure success (Yang *et al.*, 2012). Proper and effective indicators must be used and reliable as well as highly reproducible water quality assessment schemes must be used to detect microbial contaminants in water especially if the water is used for household and recreational purposes since contaminated water is known to contribute significantly to the dissemination of waterborne pathogens worldwide (Aw and Rose, 2012).

Guidelines for Drinking Water Quality have been set by the World Health Organisation (2004) from which the South African Water Quality Guidelines (2004) have also been developed. These guidelines are used as a basis for developing materials to inform water users about the physical, chemical, biological and aesthetic properties of water that is intended for use by humans. Unfortunately individuals who reside in most rural communities in developing countries, including South Africa usually do not have access to potable water and therefore obtain water from alternative sources such as boreholes, lakes, rivers and dams to support their livelihood (WHO, 2003; Reeves *et al.*, 2004; Chamaille-Jammes *et al.*, 2007). However, these water bodies are usually unprotected and thus are subjected to microbial contamination of faecal origin including VRE (Reeves *et al.*, 2004; Chamaille-Jammes *et al.*, 2007). In addition, VRE have been isolated from water from a municipal treatment plant (Goldstein *et al.*, 2014). This amplifies the need to ensure that water quality assessment standards and regulations are strictly adhered to in all water treatment plants and also accelerate the provision of portable water to all rural communities as stipulated in the South African constitution.

2.3.4 Clinical significance of VRE species

Despite the fact that *Enterococcus* species reside in the gastro-intestinal tract of humans and various animals, it still remains one of the most important pathogen that is responsible for a wide variety of nosocomial infections worldwide (Willey, 2008). VRE species like most pathogenic microbes frequently infect young children, elderly individuals as well as hospitalized and immune-compromised individuals especially those with underlying diseases such as diabetes as well as HIV and AIDS (Borgen *et al.*, 2001; Fraser, 2012). This explains why VRE pose a serious challenge to human medicine worldwide especially in countries where the incidences of these co- infectious complications are very high (Frieden *et al.*, 1993; Nannini *et al.*, 2005).

VRE may in a few cases exist in the body without causing infection due to the fact that these organisms have a very high infectious dose (Borgen *et al.*, 2001; Fraser, 2012). However, when the number of cells in the body increases the organisms are able to invade the bloodstream or spread locally to cause abdominal abscess or urinary tract infections.

The symptoms of VRE infection generally vary according to the site of infection and typical symptoms range from fever, a fast heart rate, to low blood pressure that leads to shock (Lin and Hayden, 2010). Predominantly, individuals with urinary infections may have burning with urination, back pain, meningitis, headache and confusion (Fraser, 2012). Moreover, endocarditis leads to prolonged sepsis and this may in turn cause valves in the heart to leak and eventually fail (Lin and Hayden, 2010). Pneumonia subsequently causes cough, difficulty in breathing and fever (Lin and Hayden, 2010). Given the elevating prevalence of multiple antibiotic resistant *Enterococcus* strains particular VRE coupled with the difficulties surrounding the management of their associated complications in humans there is a need for increased efforts and a rising need to constantly determine the resistance profiles of circulating strains.

2.4 PATHOGENECITY

2.4.1 Route of transmission

VRE have been recovered from between 7 to 30% of environmental sources (Cetinkaya *et al.*, 2000) and since cells of VRE remain viable for days to weeks on dry surfaces, contaminated surfaces may also serve as potential sources for the transmission of these pathogens to healthy individuals (Boyce *et al.*, 1994). Environmental sources such as ground and surface water contaminated with faeces from humans and animals have also been linked to human VRE infections (Klein, 2003; Ateba and Maribeng, 2011; CDC, 2011). It is evident that proper management and a reduction of contamination through the fecal-oral route may significantly reduce the impact of these pathogens on consumers (Fraser, 2012). Other routes of transmission includes contaminated equipment used in wastewater treatment plants, farms and in food production facilities thereby brings about the spread of this pathogen to humans and animals residing in the same geographical location (Varela *et al.*, 2013).

2.4.2 Antimicrobial resistance

The misuse of antimicrobial agents especially in hospitals largely contributes to the development and expression of antibiotic resistant bacteria including VRE in the environment (Clark *et al.*, 1993; Ramsey and Zilberberg, 2009). Antimicrobial resistant strains from the environment can be easily transmitted to humans through the consumption of contaminated water and food products, particularly animal food products like meat (Ramsey and Zilberberg, 2009). A number of studies have revealed that *Enterococcus* isolates were resistant to two or more antimicrobial agents, the detection of multiple antibiotic resistant strains has severe health implications in humans and such strains are of great clinical importance (Murray, 1997; Ramsey and Zilberberg, 2009; Ranotkar *et al.*, 2014; Yang *et al.*, 2015; Molale and Bezuidenhout, 2016). Given the challenges presented by the ever-increasing antibiotic resistance among bacterial pathogens complicated by the fact that new resistant strains are evolving daily, it is therefore appropriate to determine antibiotic resistance profiles of circulating *Enterococcus* strains. In addition, the progress in medical technology and treatment, which involves the usage of implanted prosthetic devices, diverse intravascular access devices, cytotoxic chemotherapy, and also immune-suppression has significantly intensified the impact of these antibiotic resistant microbes (Eliopoulos, 1997) Significantly, the widespread use of broad-spectrum antibiotics in hospitals also promotes selective pressure favouring the growth of multidrug-resistant organisms such as enterococci that have intrinsic resistance to these antibiotics. Table 2.1 indicates the different antibiotics to which *Enterococcus* species may display both intrinsic and acquired resistance mechanisms (Eliopoulos and Gold, 2001).

2.4.3 Mechanisms of resistance

The most important motive of using antimicrobial agents is to inhibit the growth of pathogens that are harmful to humans and prevent debilitating effects to humans and animals (Ramatlhape, 2006). Thus the antibiotic, with its chemical antagonistic composition should be able to bind to a specific target-binding spot on the microorganism to disrupt the biochemical reaction that is required for it to exert its effect. Data in Table 2.2 indicates the different antimicrobial agents, specific targets in the cell for them to effectively destroy the pathogen and the mechanism used by the cell to evade destruction. Despite, the resistance mechanisms, the concentration of the antibiotic must also be sufficient and adequate to stop microbial growth.

Table 2.1: Intrinsic and acquired antimicrobial drug resistance in enterococci

Type of resistance and antimicrobial drugs	
Acquired	**Intrinsic**
• High concentrations of β-lactams (via penicillin-binding proteins or β-lactamase)	• β-Lactams (particularly cephalosporins and penicillinase resistant penicillins)
• High concentrations of aminoglycosides	• Low concentrations of aminoglycosides
• Glycopeptides (vancomycin and teicoplanin)	• Clindamycin
• Tetracycline	• Fluoroquinolones
• Erythromycin	• Trimethoprim-sulfamethoxazole (in vivo)
• Fluoroquinolones	
• Rifampin	
• Chloramphenicol	
• Fusidic acid	
• Nitrofurantoin	

(Eliopoulos and Gold, 2001)

Table 2.2: Antibiotics, mechanisms of action and mechanisms though which bacteria evade destruction

Antibiotic Group	Examples	Target	Active against G+	Resistance mechanisms
Phenicols	Chloramphenicols	Bind to 50S subunit of ribosomes – inhibit protein synthesis	✓	Efflux mechanisms Inactivation by enzymes
Beta-Lactams	Ampicillin	Cell wall synthesis – Interfering with seven penicillin binding proteins (PBP)	✓	Penicillin –G impermeable to G Mutation in PBPs. Produce β-Lactamase
Penicillins	Penicillin	Cell wall synthesis – Interfering with seven penicillin binding proteins (PBP)	✓	Penicillin –G impermeable to G Mutation in PBPs. Produce β-Lactamase
Glycopeptides	Vancomycin	Inhibit cell wall synthesis	✓	Bind to D-alnyl-Dalanine, inhibit transfer of linear glycan acceptor, to the N-acetylmuramyl pentapeptide-N-acetyl-glucosamine
Aminoglycosides	Gentamicin Kanamycin Streptomycin	Bind to 16S rRNA subunit of 30S ribosomal - lead to misreading and translation inhibition	✓	Aminoglycosides modifying enzymes and ribosomal modification
Tetracyclines	Tetracycline	Bind to 30S subunit of ribosomes – Inhibit protein synthesis	✓	Efflux mechanisms 16S mutations
Quinolones	Nalidixic acid	Inhibit DNA gyrase synthesis	✓	Inhibit the microbial enzyme. DNA gyrase and block chromosomal replication
Macrolide	Erythromycin	Bind to 50S subunit of ribosomal – Inhibit protein synthesis	X	Inhibition of extracellular signal regulated kinase 1/2 (ERK1/2) Activate nuclear factor kappa (NF-κB)
Fluoroquinolones	Ciprofloxacin Norfloxacin	Inhibit synthesis of DNA gyrase and topoisomerase IV enzymes	X	Inhibit the microbial enzymes DNA gyrase and topoisomerase IV Block chromosomal replication

G+ = Gram positive (Cetinkaya *et al.*, 2000; Rice, 2001; Ramatlhape, 2006; Ranotkar *et al.*, 2014).

2.4.3.1 *Glycopeptides (Vancomycin resistance)*

The antibiotic vancomycin belongs to the class glycopeptides. Generally glycopeptide antibiotics, for example vancomycin, teicoplanin, ristocetin, and avoparcin are effective in the treatment of severe infections that are caused by Gram-positive pathogens (Miele *et al.*, 1995; Paganelli *et al.*, 2012; Zhao *et al.*, 2016). Vancomycin was initially used in the treatment of infections caused by penicillin-resistant *S. aureus* (Miele *et al.*, 1995). The bactericidal activity of vancomycin in Gram-positive bacteria results from the fact that the antibiotic is able to interrupt the polymerization of peptidoglycan by simply binding to peptides containing the D-alanyl-D-alanine which is a substrate of peptidoglycan synthesis (Levine, 2006). Therefore, in effect it blocks the transpeptidation stage of peptidoglycan synthesis. Vancomycin is therefore an amphoteric glycopeptide that is highly effective against *Enterococcus* species, penicillin-resistant *Corynebacterium*, methicillin-resistant staphylococci and *Clostridium difficile* (Ranotkar *et al.*, 2014).

Under ordinary conditions of peptidoglycan synthesis, two molecules of D-alanine are linked by a ligase enzyme to create D-Ala–D-Ala, which is further added to UDP-N-acetylmuramyl-tripeptide to structure the UDP-N-acetylmuramyl-pentapeptide (Figure 2.1). When consolidated into the developing peptidoglycan (transglycosylation), the UDP-N-acetylmuramyl-pentapeptide allows the arrangement of cross-bridges (transpeptidation) and adds to the strength of the peptidoglycan layer (Levine, 2006) Vancomycin ties-up with high affinity to the D-Ala–D-Ala ends of the pentapeptide precursor units, hindering their addition to the developing peptidoglycan chain and thus preventing resulting cross-linkage.

Recently, Cattoir and Leclercq, (2013) reported that there are eight types of acquired Enterococcal resistance determinants that confer resistance to glycopeptides that prevail on the basis of genotypic and phenotypic criteria and these include *vanA*, *vanB*, *vanD*, *vanE*, *vanG*, *vanL*, *vanM*, and *vanN*. They further identified another type of resistance (*vanC*) which is associated with intrinsic resistance in *Enterococcus* *gallinarum* and *Enterococcus casseliflavus* strains (Cattoir and Leclercq, 2013). In addition, *vanC* exhibit natural, low-level resistance to vancomycin and are relatively susceptible to teicoplanin (Table 2.3).

VanA and *vanB* resistance determinants are displayed by *E. faecium* and *E. faecalis*. Among these, *vanA*-resistant strains carry inducible, relatively high-level of insensitivity to vancomycin (MICs, ≥64 µg/ml) and teicoplanin (MICs, ≥16 µg/ml) as shown in Table 2.3; (Cetinkaya *et al.*, 2000). These resistance genotypes may be induced by glycopeptides (vancomycin, teicoplanin, ristocetin, and avoparcin) as well as other non-glycopeptide agents, such as bacitracin, polymyxin B, and robenidine (Lai and Kirsch, 1996). The expression of vancomycin resistance has been frequently associated with the *vanA* gene cluster which is found on the transposon also called jumping hereditary component, *Tn1546* (Lai and Kirsch, 1996).

Most isolates that possess the *vanB* genotype display moderate levels of resistance to vancomycin (MICs, 32 – 64 µg/ml) despite their susceptibility to teicoplanin (Cetinkaya *et al.*, 2000; Lin and Hayden, 2010). Furthermore characteristics of phenotypes of glycopeptide-resistant enterococci are expanded on Table 2.3, which includes all the eight types of acquired and one intrinsic resistance.

Table 2.3: **Characteristics of phenotypes of glycopeptide-resistant enterococci**

Characteristic	**Phenotype**				
	VanA* and *VanM	***VanB***	***VanD***	***VanE*, *VanG*, *VanL* and *VanN***	***VanC***
Genetic determinant	Acquired				Intrinsic
Vancomycin MIC (µg/ml)	64–>1,000	4–1,024	128	16	2–32
Teicoplanin MIC (µg/ml)	16–512	≤0.5	4	0.5	≤0.5
Most frequently associated *Enterococcus* species	*E. faecium*, *E. faecalis*	*E. faecium*, *E. faecalis*	*E. faecium*	*E. faecalis*	*E. gallinarum*, *E. casseliflavus*, *E. flavescens*
Transferable	Yes	Yes	No	No	No

Adapted in 2016 from Cetinkaya *et al*., 2000 and; Cattoir and Leclercq, 2013; original version Copyright, Massachusetts Medical Society

Considering this at the molecular level, microbes that are vancomycin-resistant bring only one and only change, that is the amide connection of the two peptidoglycan alanines is supplanted by a ketone group, in this way dispensing with one of the hydrogen bonds and presenting solitary pair repulsion between the carbonyl groups (Ranotkar *et al.*, 2014). Unfortunately, *vanA* genes cannot carry out its job alone; it therefore requires the help of two other different gene determinants, *vanH* and *vanX*. *VanH* codes for ketoacid dehydrogenase that is needed for the synthesis of D-lactate as it neither exists in the environment nor is created by *Enterococcus* species (Nam *et al.*, 2013).

The progressive enzymatic pair of *VanA* and *VanH* function together resulting in the formation of new peptidoglycans as shown in Figure 2.1 (Cohen *et al.*, 1992). Moreover, Ranotkar et al., 2014 recently reported that *vanX*, a Zn [II]-containing metalloenzyme, acts on produced peptidoglycan molecules by splitting the C-terminal D-Ala-D-Ala bond, along these lines expelling the terminal D-Ala and making room for *vanA* to ligate D-lactate onto the peptidoglycan (Figure 2.1) (Cohen *et al.*, 1992; Klare *et al.*, 1995; Courvalin, 2009). Thus, resistance displayed by *vanA* requires in any event these three compounds or their homologs to ensure that resistance to vancomycin is attained by the cell. Significantly, the *vanA* gene is customarily connected with a transposable component, *Tn1546*, which likewise contains alternate determinant genes required for regulation, direction and articulation of vancomycin resistance (*vanH*, *vanS*, *vanR*, *vanX* and *vanZ*) (Figure 2.1). *Tn1546* is relatively 10,581 bp long and is regularly situated on a plasmid (Foucault *et al.*, 2010).

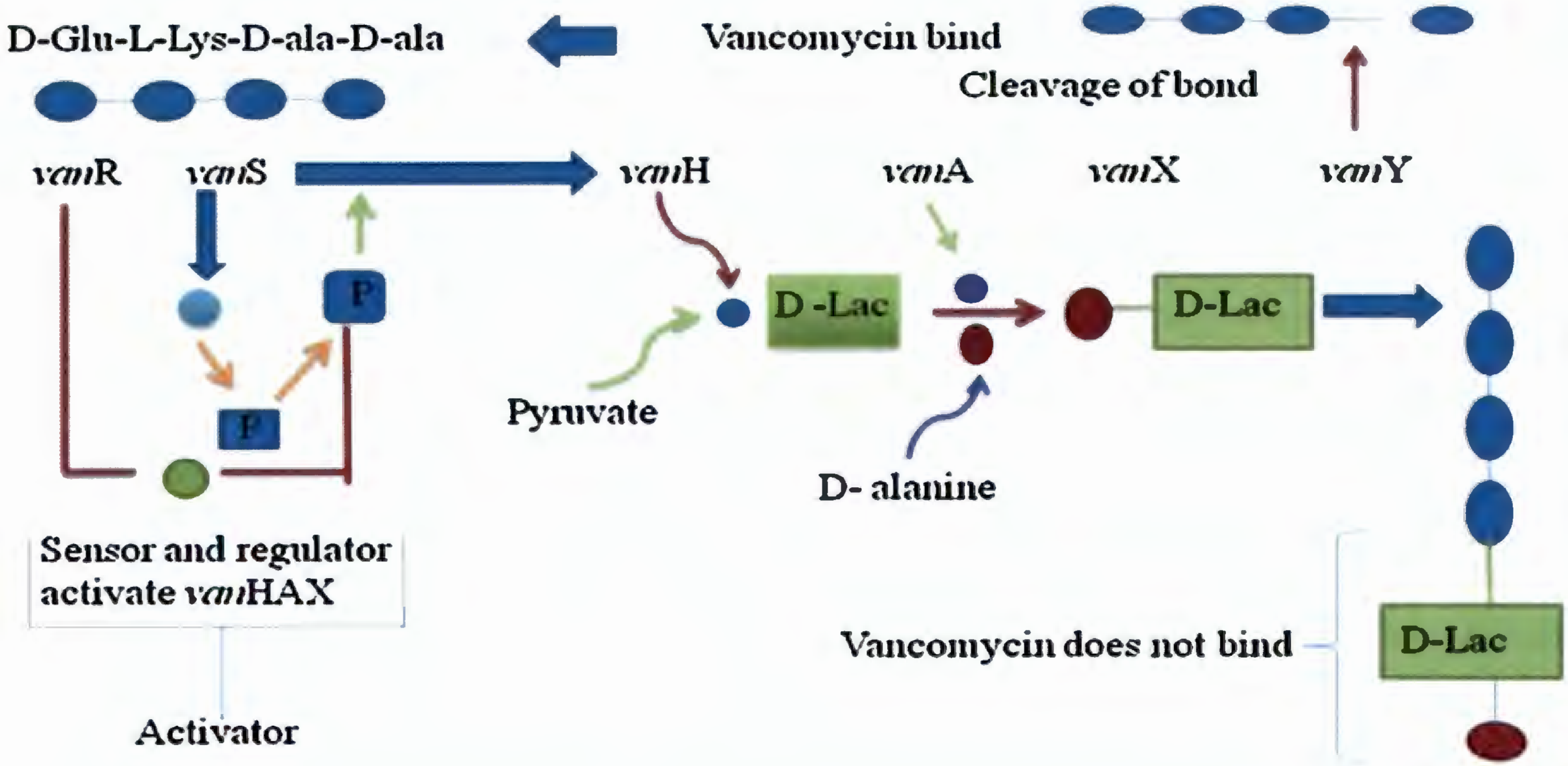

Figure 2.1: Representation of the development of vancomycin resistance in enterococci (Ranotkar *et al.*, 2014).

2.4.4 Microbiological Identification of VRE species

To define specific characteristics of the object under investigation and analysis, thorough identification and typing systems are commonly utilized (Vos *et al.*, 2011). Usually the procedures are specific for various phenotypic or genetic parameters. Moreover these procedures can also be utilized and applied to any microbial species, which can also be genus and strain specific. Typing procedures are the basis for the integration of epidemiology, taxonomy and evolution genetics (Ramatlhape, 2006).

Enterococci is Gram positive, coccus shaped bacteria which can either occur in short chains, diplococcic or single cocci and these organisms are also oxidase and catalase negative (Klein, 2003; Mascini *et al.*, 2006). In addition, *Enterococcus* species are not able to ferment lactose and therefore grow optimally in different selective media that include; Bile Esculin Agar (BEA), Membrane *Enterococcus* Indole-β-D-Glucoside (mEI) agar and Nutrient Agar (NA) (Mascini *et al.*, 2006; Fraser, 2012). Although a number of selective growth media have been used to isolate *Enterococcus* species from different types of samples, BEA has produced more reliable and reproducible results in isolating these organisms from water samples (Ateba and Maribeng, 2011), and this explains why it is frequently utilized in most laboratory analysis.

Standard biochemical tests that are used for presumptive identification of *Enterococcus* species include; the ability or inability to break down red blood cells, growth on NaCl and degradation of substrates in the API 20 STREP (Ramatlhape, 2006). In addition, serological assays have been used to confirm the identities of *Enterococcus* species and agglutination with the polyvalent group D antiserum is also a valuable identification assay. However, the preliminary identification assays of these VRE are usually time consuming which also gives room for high human error and the sensitivity depend on a number of factors. Having this in mind it is suggested that preliminary identification assays should be coupled with more sensitive PCR or DNA based techniques to ensure the generation of true positive results. In addition there are a number of PCR assays designed to amplify the vancomycin resistance and some virulence genes that are widely utilized as targets for VREs (Depardieu *et al.*, 2004; Vankerckhoven *et al.*, 2004) are useful for detection and identification.

2.5 TREATMENT

Generally, Enterococcal infections such as bacteraemia and endocarditis are treated with a combination of an aminoglycoside (streptomycin or gentamicin) to which the *Enterococcus* isolates do not display relatively high resistance and a penicillin (penicillin G or ampicillin) (Herman and Gerding, 1991). Significantly, when vancomycin is combined with an aminoglycoside these antibiotics exhibit synergistic actions against enterococci both *in vitro* and *in vivo* (Cetinkaya *et al.*, 2000), and therefore it was strongly suggested as potential treatment for Enterococcal infections in patients with genuine penicillin sensitivity or in the treatment of ampicillin and penicillin resistant strains (Cattoir and Leclercq, 2013). However, due to the development of VRE and the subsequent use of vancomycin, the treatment of infections caused by VRE is extremely problematic especially if the strains harbour multiple antibiotic resistance determinants and virulent traits. However, knowledge of the antibiotic profiles of VRE is very important in providing guidance on the selection of treatment protocols.

2.6 PREVENTION AND CONTROL

The most effective strategic approach to preventing VRE infections in humans is to limit transmission and usually this is achieved through the implementation of proper hygiene and sanitary practices especially in the farms where animals are kept and also in hospital settings. In addition, water bodies from which water is distributed to consumers must be free of pathogenic microorganisms. Since enterococci have been highly implicated in nosocomial infections, hospitals and health care facilities must implement infection-control mechanisms that are derived from standard guidelines set out by regulatory authorities in order to reduce the spread of VRE among patients (Lin and Hayden, 2010; Fraser, 2012)

In response to the dramatic rise in vancomycin resistant enterococci, the Subcommittee on Prevention and Control of Antimicrobial-Resistant Microorganisms in Hospitals of the CDC Hospital Infection Control Practices Advisory Committee (HICPAC) had several meetings in 1993 and 1994. This was based on an effort to prevent and control the nosocomial transmission of VRE and HICPAC published recommendations in February 1995 (Cetinkaya *et al.*, 2000). Mainly, these recommendations focused on:

(i) prudent use of vancomycin,
(ii) education of people,
(iii) effective use of the microbiology laboratory, and
(iv) implementation of infection control and prevention measures.

To minimize nosocomial transmission of VRE, hospitals ought to use a multi-disciplinary approach that requires participation by various departments and personnel. Individuals in most households can reduce their risk by drinking clean water, washing hands after using the bathroom as well as before and after touching the mouth or nose. Other risks are closely related with developing rural areas where ground and surface water is utilized for almost all the household activities. Therefore, it is strongly recommended that these individuals be provided with clean potable water that is constantly monitored for microbial contamination of faecal origin. Most importantly, antibiotics should be used only for appropriate indications.

2.7 ENTEROCOCCI AS POTENTIAL INDICATORS OF MICROBIAL CONTAMINATION IN WATER

Enterococci can grow at a wide temperature range (10 to 45 °C), appropriate optimal pH (9.6) and high salinities (6.5 % NaCl). They are considered as indicators of faecal contamination due to the fact that they are normally found in the gastro intestinal tract of warm blooded animals, as such their presence in water implies faecal contamination (Santiago-Rodriguez *et al.*, 2013). In addition, the concentration of enterococci in faecal samples is proportional to that of numerous other indicator bacterial organisms (Wheeler *et al.*, 2002). This explains why enterococci are constantly utilized to determine faecal microbial contamination in water as well as to track the source of the fecal pollution particularly in environmental water bodies (Toranzos *et al.*, 2007).

CHAPTER THREE

METHODS AND MATERIALS

CHAPTER 3

MATERIALS AND METHODS

3.1 STUDY DESIGN, AREA AND SAMPLE SIZE

This study was conducted at North West University-Mafikeng Campus in the North West Province, South Africa and water samples were collected from the different districts of the Province. The number of samples collected during the current study was determined using the formula outlined below:

$$\text{Sample (N)} = \frac{(Z_{1-\alpha/2})^2 P\ (1-P)}{d^2}$$

$Z_{1-\alpha/2}$ = is standard normal variate at 5% type I error ($P< 0.05$) and it is 0.05

P = Expected prevalence in population based on a previous study

d = Absolute error or precision (which is 5%)

$$\text{Sample (n)} = \frac{(1.96)^2(0.11)\ (1-0.11)}{(0.05)^2}$$

$$= 150.4370556 \text{ (Charan and Biswas, 2013)}$$

For estimation of the prevalence of VRE, the sample size for this study was determined using the prevalence of 11 % obtained in the study by Ramatlhape, (MSc Dissertation, 2006) in South Africa to be the expected prevalence with the 95% confidence level and desired precision of 5% using the formula described by Charan and Biswas (2013). Accordingly, the minimum sample size required was 150 water samples. Table 3.1 shows the number and nature of samples collected from different areas.

3.1.1 Permission

Before collection of ground water (borehole) and surface water (dams, rivers, and springs) samples in certain areas permission was obtained from representatives of households as well as community leaders in most villages.

3.2 SAMPLE COLLECTION

A total of 170 water samples comprising of 119 ground and 51 surface water samples were randomly collected from different villages, rural and urban communities in different areas in the North West Province between August 2015 and April 2016 (Table 3.1). The water samples were collected based on the availability and the presence of the water sources that included boreholes, dams, rivers and springs but also ensured that samples collected reflected a clear representation of the different geographical locations consisting of the distinctive districts in the North West Province. Thus the sampling was based on a random convenient approach.

Groundwater samples were collected from borehole taps and surface water samples were collected from river, dam and spring sources (Table 3.1), using sterile 500 mL Duran Schott bottles. Samples were properly labeled and transported on ice to the laboratory and were immediately analyzed for the presence of *Enterococcus* species.

3.3 ANALYSIS OF WATER SAMPLES

3.3.1 Selective isolation of VRE species

An aliquot of 100 mL from each water sample was filtered through a 0.45 µM filter paper (Whatman® Glass Microfiber GS Filter paper) on a vacuum water pump machine (Model, Sartorius 16824). Using a sterile forceps the membrane filters were placed on Bile Esculin Agar (BEA) (Biolab, South Africa) supplemented with vancomycin (16 µg/mL) to select for VRE. The plates were then incubated aerobically at 37°C for 24 hours and typical VRE with characteristic black colonies were considered as potential presumptive species. The presumptive isolates were purified by sub-culturing on BEA plates and the plates incubated aerobically at 37°C for 24 hours. Pure colonies were preserved and retained for further biochemical identification tests.

3.3.2 Control strains

In the present study *Enterococcus faecium* (ATCC 700221) was used as positive control strains while *Staphylococcus aureus* (ATCC 43322) as a negative control strain.

Table 3.1: Areas from which water samples were collected

District	Sampling area	Number of groundwater samples	Number of surface water samples
Bojanala Platinum District	Rustenburg	Borehole = 16	Dam = 10
	Swarstruggens	Borehole = 3	0
Ngaka Modiri Molema District	Mafikeng	Borehole = 55	Dam = 12
	Zeerust	Borehole = 7	Dam = 3 Spring = 3
	Coligny	Borehole = 5	Dam = 5
	Lichtenburg	Borehole = 3	0
Dr Ruth Segomotsi Mompati	Taung	Borehole = 14	Dam = 2 River = 2
Dr Kenneth Kaunda	Potchefstroom	Borehole = 10	Dam = 7 River = 7
	Ventersdorp	Borehole = 6	0
		119	**51**
Total			

3.4 BACTERIAL IDENTIFICATION

Presumptive VRE isolates were identified using the following criteria:

3.4.1 Cellular morphology

The isolates were Gram stained using standard protocols (Cruickshank *et al.*, 1975; Murray *et al.*, 1981).

3.5 PRELIMINARY BIOCHEMICAL IDENTIFICATION TESTS FOR ENTEROCOCCI

All the isolates were subjected to the following biochemical identification tests:

3.5.1 Oxidase

The oxidase test was performed using oxidase paper strips obtained from Whatman International Ltd, Maidstone, England. With the help of a sterile tooth pick a single pure colony was picked from BEA agar plate and rubbed on oxidase paper strip. The strip was observed for the formation of a purple colour within 30 seconds in which case such an isolate was considered oxidase positive and vice versa. Generally, *Enterococcus* species are oxidase negative (Ateba *et al.*, 2013) hence all the isolates that satisfy this preliminary identification criterion were considered and subjected to the catalase test.

3.5.2 Catalase test

The catalase test is designed to detect the presence of the catalase enzyme in most aerobic and facultative anaerobic bacteria that contain the cytochrome compounds. Enterococci and streptococci are exceptions and these organisms do not possess the catalase enzyme (Ateba *et al.*, 2013). Catalase enzymes decompose hydrogen peroxide which is poisonous to the cell, to water and oxygen. A pure bacterial colony was mixed with a drop of 2% (v/v) hydrogen peroxide (H_2O_2) onto a clean microscope slide and observed for effervescence. Catalase positive organisms produce effervescence and vice versa. Enterococci are catalase negative (Ateba and Maribeng, 2011; Ateba *et al.*, 2013) and all isolates that satisfied this preliminary identification criterion were subjected to further preliminary identification tests.

3.5.3 Growth in Sodium chloride (NaCl) broth

The ability of enterococci to grow in 6.5% (w/v) NaCl broth is considered an important distinguishing characteristic that facilitates identification of organisms belonging to these genus (Klein, 2003). All the presumptive enterococci were cultured aerobically at 37°C for 24 hours in Falcon tubes containing 10 mL of 6.5% (w/v) NaCl broth to differentiate them from streptococci (APHA, 1998; Klein, 2003). *Enterococcus faecium* (ATCC 700221) was also used as a positive control while an un-inoculated NaCl broth was used as a negative control. Bacterial growth was determined by measuring the optical density at 600 nm using a Heλiosε Thermo Spectronic spectrophotometer (model Helios Epsilon) obtained from Merck, South Africa.

3.6 CONFIRMATORY IDENTIFICATION FOR VRE

3.6.1 Serotyping

The isolates were screened for serological identification of *Enterococcus* species based on the Lancefield grouping of A, B, C, D, F and G streptococci (Ingram *et al.*, 1983) using A SLIDEX® Strepto Plus Latex agglutination test kit obtained from BioMériux South Africa. All the isolates which showed positive result (agglutination) were subjected to molecular identification test.

3.6.2 Molecular identification of enterococci using PCR analysis

3.6.2.1 Extraction of DNA from potential enterococci

Genomic DNA was extracted from all presumptive VRE isolates using the hot (65°C) Cetyltrimethyl Ammonium Bromide (CTAB), polyvinyl pyrrolidone (PVP) extraction protocol (Doyle, 1990). An overnight culture of the pure isolate was prepared by picking one pure colony from the BEA agar and inoculated into

5 mL Nutrient Broth (NB). The cultures were incubated overnight at 37°C without shaking. An aliquot of 1 mL of the overnight culture was dispensed into 2 mL micro-centrifuge tube and centrifuged at 13,000 rpm for 4 minutes and the supernatant was discarded without disturbing the pellet. Forty micro-liters of lysozyme (10 mg/mL) was added to the pellet, mixed by vortexing and incubated at 37°C for 45 minutes. An aliquot of 500 µL hot (65°C) CTAB (Sigma H-6269) isolation buffer [2% (w/v) hexadecyltrimethylammonium bromide, 1.4 M NaCl, 0.1% (v/v) of *β*-mercaptoethanol] was added to the contents in the micro-centrifuge tube and then incubated at 65°C on a water bath for 40 minutes. The tubes were vortexed every 10 minutes to mix the contents. This mixture was extracted at room temperature with an equal volume (500 µL) of chloroform: isoamyl alcohol at ratios of 96:4. The sample was centrifuged at 13000 rpm for 4 minutes and the aqueous phase was transferred to a new sterile micro-centrifuge tube. The previous step involving the addition of chloroform: isoamyl alcohol (96:4) was repeated and the aqueous phase was transferred to a new sterile microfuge tube. To the aqueous phase, 3 M sodium acetate (NaOAc) and iso-propanol 0.1: 0.7 volumes were added and the samples were incubated on ice for 10 minutes to precipitate the DNA. Precipitated DNA in the sample was collected by centrifuging at 13000 rpm for 10 minutes and the supernatant was discarded. The pellet was washed in 500 µL of ice cold 70% (v/v) ethanol to remove excess NaOAc. The sample was centrifuged at 13000 rpm for 5 minutes to secure the pellet and the supernatant was again discarded. Lastly, the pellet was dried by placing the tube in an inverted position with the cap open on a clean paper towel on the work bench for 1 hour. The pellet was reconstituted by dissolving in 50 µL TE buffer and the DNA was stored at -20°C.

3.6.2.2 Quantification of genomic DNA extracted

The genomic DNA was quantified using a Nano-drop lite spectrophotometer (Model 1558) obtained from Thermo Scientific, USA.

3.6.3 PCR for the amplification of bacterial 16S rRNA gene

Presumptive *Enterococcus* isolates that satisfied both preliminary and confirmatory biochemical tests were subjected to bacterial 16S rRNA gene PCR (Mohapi and Ateba, 2013). The 16S rRNA PCR was performed using oligonucleotide primer combinations and cycling conditions that appear in Table 3.2. Amplifications were performed using a DNA thermal cycler (C1000 Touch™, BIO-RAD, South Africa) and the PCR reactions were performed in 25 µL standard volumes that comprised 12.5 µL of 1X Master mix, 0.25 µL each primer, 2µL template DNA and 10 µL nuclease free water. PCR amplicons were held at 4°C until electrophoresis.

3.6.4 Multiplex PCR for the identification of *Enterococcus* species specific *ddl* gene

The identities of the presumptive *E. faecalis* and *E. faecium* isolates were determined using a multiplex PCR assay designed to amplify the *ddl* gene species specific sequences that code for D-alanine:D-alanine ligase (*ddl*) in *Enterococcus faecalis* and *Enterococcus faecium* respectively (Depardieu *et al.*, 2004). Amplifications were performed using a DNA thermal cycler (C1000 Touch™, BIO-RAD, Hercules, California, United States of America) and PCR reactions were performed in standard 25 µL volumes that comprised 12 µL of 1X Master mix, 0.25 µL each primer, 2 µL template DNA. The primer pairs and PCR conditions that were used in the multiplex PCR assay are shown in Table 3.3.

3.7 ANTIBIOTIC RESISTANCE SUSCEPTIBILITY TEST

3.7.1 The disc diffusion technique

The antibiotic resistant profiles of the isolates were determined using the Kirby-Bauer disc diffusion technique (Kirby *et al.*, 1966). In order to achieve this, isolates were screened against a panel of nine antimicrobial agents that appear in Table 3.4 obtained from Mast Diagnostics, UK. A single well-isolated pure colony was suspended into 5 mL of sterile distilled water to prepare homogenous bacterial suspension with the concentration of a McFarland standard. An aliquot of 100 µL from each suspension was spread-plated onto Mueller Hinton agar that contained 5% (v/v) defibrinated sheep blood and left to dry at room temperature for about 30 minutes. The different antibiotic discs were placed on the surface of the agar at equal distances and the plates were incubated at 37°C for 24 hours. After incubation the plates were observed for the presence of zones of growth inhibition and the diameters of these zones were measured in mm. The growth inhibition diameter data for the different antibiotics were compared with standard reference values and used to classify isolates as being resistant, intermediately resistant or sensitive to an antimicrobial agent (Table 3.4).

Table 3.2: Oligonucleotide primers sequences and PCR conditions used during this study for the identification of bacterial gene by 16S rRNA

Targeted Genes	Sequence (5'- 3')	Primer Name	Amplicon size (bp)	PCR cycling conditions
16S rRNA	GGATTAGATACCCTGGTAGTCC	E16SF	322 bp	95°C for 4 min, 30 cycles of 95°C for 30 sec, 58°C for 60 sec, and 72°C for 60 sec, followed by 72°C for 7 min
	TCGTTGCGGGACTTAACCCAAC	E16SR		

(Mohapi and Ateba, 2013)

Table 3.3: Oligonucleotide primers sequences and PCR conditions used during this study for the identification of specific VRE species

Targeted Genes	Sequence (5'- 3')	Primer Name	Amplicon size (bp)	PCR cycling conditions
***ddl* gene (*E. faecalis*)**	CACCTGAAGAAACAGGC	DD13 (F)	475 bp	94°C for 3 min, 35 cycles of 94°C for 60 sec, 56°C for 60 sec, and 72°C for 60 sec, followed by 72°C for 7 min
	ATGGCTACTTCAATTTCACG	DD3-2(R)		
***ddl* gene (*E. faecium*)**	GAGTAAATCACTGAACGA	FAC1-1(F)	1091 bp	
	CGCTGATGGTATCGATTCAT	FAC2-1(R)		

(Depardieu *et al.*, 2004)

3.7.1.1 Multiple antibiotic resistances (MAR) Index and cluster analysis

Furthermore, multiple antibiotic resistances (MAR) indices were determined for the isolates that were resistant to three or more antibiotics as described by Kaspar *et al.*, (1990). MAR indices were calculated by adding the numbers of drugs to which each isolate was resistant and dividing the resulting number by the product of the total number of antibiotics as well as the number of isolates that were tested and the formula is as shown below (Kaspar *et al.*, 1990).

$$\text{MAR Index} = \frac{\text{Number of isolates resistant to all antibiotics in a specific sampple population}}{(\text{Number of antibiotics tested})\,X\,(\text{Total number of organsms in the sample})}$$

3.7.1.2 Cluster analysis of isolates based antibiotic resistance data

Cluster analysis of antibiotic susceptibility data for *Enterococcus* species isolated from water samples from the different sampling sites was determined by using Ward's algorithm and Euclidean distances on Statistica version 7.0 software (Statsoft, US) and the result was efficiently expressed as dendrogram.

3.8 MULTIPLEX PCR FOR SCREENING AND CONFIRMATION OF VRE USING VAN GENES

The presence of vancomycin resistance determinants (*vanA*, *vanB*, *vanC*) in VRE strains were determined using a multiplex PCR analysis with specific primer sets that appear in Table 3.5 (Clark *et al.*, 1993; Harwood *et al.*, 2001; Depardieu *et al.*, 2004).

Table 3.4: The details of antibiotics used in this study

Class	Antibiotic used	Abbreviations	Concentration	Resistant	Intermediate	Susceptible
Penicillin/β-Lactamase	Ampicillin	AMP	10 µg	≤16	-	≥17
Aminoglycoside	Streptomycin	STR	10 µg	≤6	7-9	≥10
	Gentamycin	GEN	10 µg	≤12	13-14	≥15
Nitrofurans	Nitrofurantoin	NIT	100 µg	≤14	15-16	≥17
Tetracycline	Oxytetracycline	OXY-TET	30 µg	≤14	15-18	≥19
Quinolone	Nalidixic acid	NAL	30 µg	≤13	14-18	≥19
Sulphonamides	Sulphamethoxazole	SMX	25 µg	≤10	11-15	≥16
Chloramphenicol	Chloramphenicol	CHIL	30 µg	≤12	11-17	≥18
Glycopeptides	Vancomycin	VAN	30 µg	≤14	15-16	≥17

Concentrations used as well as the inhibition zone measurements (in mm) that were considered resistance, intermediate and susceptible are shown.

The abbreviations were adopted from Ramatlhape, (2006).

3.9 DETERMINATION OF VIRULENCE FACTORS OF ENTEROCOCCI

3.9.1 Cytolysin

Cytolysin production was evaluated by examining *β*-haemolysis on blood agar plates and the haemolysis test was performed according to a standard protocol (Frobisher and Denny, 1928). Pure fresh cultures were spot inoculated on 5% (v/v) bovine blood agar plates (Institutional Farm, SA) and incubated at 37°C for 24 hours. Beta haemolytic isolates produced clear zones around the colonies resulting from complete hydrolysis of red cells in the medium.

3.10 PCR FOR THE DETECTION OF VIRULENCE GENES IN ENTEROCOCCI

3.10.1 Multiplex PCR for screening and detecting virulence genes

The virulence determinants of VRE isolates was determined through the amplification of the *asa1, cylA, esp, gel and hyl* gene sequences using chromosomal DNA extracted from the isolates (Vankerckhoven *et al.*, 2004; Molale and Bezuidenhout, 2016). Oligonucleotide primer combinations and cycling conditions that were used for the PCR analysis are shown in Table 3.6.

Table 3.5: Oligonucleotide primers sequences and PCR conditions used during this study for determining the *Van* genes by multiplex PCR

Targeted Genes	**Sequence (5'- 3')**	**Primer Name**	**Amplicon size (bp)**	**PCR cycling conditions**
***vanA* gene**	GGGAAAACGACAATTGC	EA1(F)	732 bp	94 °C for 3 min, 35 cycles of 94 °C for 60 sec, 54 °C for 60 sec, and 72° for 60 sec, followed by 72 °C for 7 min
	GTACAATGCGGCCGTTA	EA2(R)		
***vanB* gene**	ACGGAATGGGAAGCCGA	EB3(F)	647 bp	
	TGCACCCGATTTCGTTC	EB4(R)		
***vanC21/2* gene**	ATGGATTGGTAYTKGTAT	EC5(F)	815/827 bp	
	TAGCGGGAGTGMCYMGTAA	EC8(R)		

(Clark *et al.*, 1993; Harwood *et al.*, 2001; Depardieu *et al.*, 2004).

Table 3.6: Oligonucleotide primers sequences and PCR conditions used in this study to determine the virulence determinants

Target Genes	Primer sequence (5' - 3')	Primer name	Virulence factor	Amplicon size (bp)	PCR cycling conditions
asa1	GCACGCTATTACGAACTATGA	ASA 11F	Aggregation substance	375	95 °C for 15 min, 35 cycles of 94 °C for 60 sec, 56 °C for 60 sec, and 72° for 60 sec, followed by 72 °C for 10 min
	TAAGAAAGAACATCACCACGA	ASA 12R			
gel	TATGACAATGCTTTTTGGGAT	GEL 11F	Gelatinase	213	
	AGATGCACCCGAAATAATATA	GEL 12R			
cylA	ACTCGGGGATTGATAGGC	CYT IF	Cytolysin	688	
	GCTGCTAAAGCTGCGCTT	CYT IIbR			
esp	AGATTTCATCTTTGATTCTTGG	ESP 14F	Enterococcal surface protein	510	
	AATTGATTCTTTAGCATCTGG	ESP 12R			
hyl	ACAGAAGAGCTGCAGGAAATG	HYL n1F	Hyaluronidase	276	
	GACTGACGTCCAAGTTTCCAA	HYL n2R			

(Vankerckhoven *et al.*, 2004; Molale and Bezuidenhout, 2016).

3.11 ELECTROPHORESIS OF PCR PRODUCTS

Four microliters of the amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel (containing ethidium bromide 0.001µg/ml) gel using 1 X TAE (40 mM Tris (pH 7.6), 20 mM acetic acid, 1 mM EDTA) at 80V for 15 minutes and later at 60V for 4 hours. A ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, Hercules, California, USA) was used to capture the image using Gene Snap (version 6.00.22) software. Each gel contained a 100 bp or 1 kb molecular weight marker (BioLab, New England).

3.12 GENOTYPIC CHARACTERISATION OF *ENTEROCOCCUS* SPECIES USING ERIC-PCR ANALYSIS

The genetic relatedness of all the isolates was determined using ERIC-PCR analysis based on a previously described protocol (Ateba and Mbewe, 2014). The PCR reactions were prepared using a non-specific oligonucleotide primer sequence ERIC2 (5'-AAGTAAGTGACTGGGGTGAGCG-3'). Amplifications were performed using a DNA thermal cycler (model- Bio-Rad C1000 Touch ™ Thermal Cycler, Hercules, California, United States of America). The reactions were prepared in 25 µL volumes that constituted 12.5 µL of 2X DreamTaq Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM $MgCl_2$ and loading buffer), 11 µL nuclease free distilled water, 0.5 µL of the primer and 1 µL of template DNA. All the PCR reagents were from Thermo-Fischer, USA products supplied by Inqaba Biotechnical Industry Ltd, Sunnyside, South Africa. PCR cycling conditions comprised 95°C for 2 minutes, 30 cycles of 94°C for 3 seconds, 50°C for 1 minute, 65°C for 8 minutes and final elongation at 65°C for 8 minutes. The amplicons were separated by electrophoresis on a 1.5% (w/v) agarose gel and a ChemiDoc Imaging System (Bio-RAD ChemiDoc™ MP Imaging System, Hercules, California, USA) was used to capture the image using Gene Snap (version 6.00.22) software and the relative sizes of the amplicons was determined using a mixture of 100 bp and 1 kb DNA gene ruler (BioLab, New England).

3.13 SEQUENCE ANALYSIS OF PCR AMPLICONS

The amplified 16S rRNA gene fragments were sequenced by Inqaba Biotech, South Africa. Sequence data was subjected to BLAST search on the NCBI WebTool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the identities of the amplified sequences as well as isolates.

CHAPTER FOUR

RESULTS AND INTERPRETATION

CHAPTER 4

RESULTS AND INTERPRETATION

4.1 OCCURRENCES OF *ENTEROCOCCUS SPECIES* IN GROUND AND SURFACE WATER USING PRELIMINARY IDENTIFICATION TESTS

4.1.1 Cellular morphology

A total of one hundred and seventy samples (ground and surface water) were collected from villages and rural communities within the North West Province. Eighty one potential *Enterococcus* isolates were obtained based on differences in their colonial morphologies (dark to black colonies with black centers that produces dark areas on Bile Esculin Agar). The cellular morphologies of the isolates were determined using standard techniques as described in Materials and Methods (Section 3.4.1) and detailed results are shown in Table 4.1. All the 81 isolates were Gram positive cocci, therefore satisfied the preliminary identification for *Enterococcus* species. The isolates were subjected to further preliminary and confirmatory biochemical identification tests that are specific for organisms that belong to the genus *Enterococcus*.

Table 4.1: Results for Preliminary Biochemical tests

Sampling Area	**No. Isolates Tested**	**Gram-stain (+ve cocci)**	**Oxidase (-)**	**Catalase (-)**	**Growth on 6.5 NaCl**
Mafikeng	NT	32	32	32	32
	NP	32 (100%)	29 (90.6%)	30 (93.8%)	31 (96.9%)
Rustenburg	NT	25	25	25	25
	NP	25 (100%)	25 (100%)	23 (92%)	25 (100%)
Potchefstroom	NT	5	5	5	5
	NP	5 (100%)	4 (80%)	4 (80%)	5 (100%)
Taung	NT	2	2	2	2
	NP	2 (100%)	2 (100%)	2 (100%)	2 (100%)
Zeerust	NT	10	10	10	10
	NP	10 (100%)	10 (100%)	10 (100%)	10 (100%)
Coligny	NT	7	7	7	7
	NP	7 (100%)	7 (100%)	6 (85.7%)	7 (100%)
Ventersdorp	NT	0	0	0	0
	NP	0	0	0	0
Swatruggens	NT	0	0	0	0
	NP	0	0	0	0
Lichtenberg	NT	0	0	0	0
	NP	0	0	0	0
TOTAL	**NT**	**81**	**81**	**81**	**81**
	NP	**81 (100%)**	**77 (95.1 %)**	**75 (92.5%)**	**80 (98.8%)**

NT = Number Tested, NP = Number Positive

4.1.2 Preliminary Biochemical Identification tests

All 81 isolates that were Gram positive cocci were subjected to the oxidase and catalase tests and results are presented in Table 4.1. A large proportion 77 (95.1%) of the isolates were oxidase negative, thus did not possess the cytochrome oxidase which satisfied their presumptive identification as *Enterococcus* species. In addition, a large proportion 75 (92.6%) of these isolates were catalase negative. The presumptive *Enterococcus* isolates were inoculated on 6.5% (w/v) NaCl broth to assess their ability to grow in the presence of high salt conditions. A total of 80 (98.8%) isolates produced significant growth on the broth which was indicated by the presence of turbidity and the results are shown in Table 4.1.

4.2 SEROLOGICAL ASSAY

All 81 presumptive *Enterococcus* isolates were serotyped using the SLIDEX® Strepto Plus Latex agglutination test kit obtained from BioMérieux (France). Sixty (74.1%) isolates were positively identified as *Enterococcus* species based on agglutination with the group D polyvalent antiserum and detailed results are shown in Table 4.2.

Table 4.2: Results for confirmatory biochemical test by serological essay

Sampling Area	**No. Tested**	**Serology**
		Group D Latex Antigen
Mafikeng	NT	32
	NP	23 (71.9%)
Rustenburg	NT	25
	NP	18 (72%)
Potchefstroom	NT	5
	NP	3 (60%)
Taung	NT	2
	NP	2 (100%)
Zeerust	NT	10
	NP	8 (80%)
Coligny	NT	7
	NP	6 (85.7%)
TOTAL	**NT**	**81**
	NP	**60 (74.1%)**

NT = Number Tested, NP = Number Positive

4.3 BACTERIAL DNA EXTRACTED FROM ISOLATES

Chromosomal DNA was extracted from all the 81 presumptive isolates and control strains as described in Section 3.8.1. The presence of DNA was confirmed by electrophoresis on a 1.5% (w/v) agarose gel and a representative image of genomic DNA from the isolates is depicted in Figure 4.1. The DNA was of good quality and with no fragmentation.

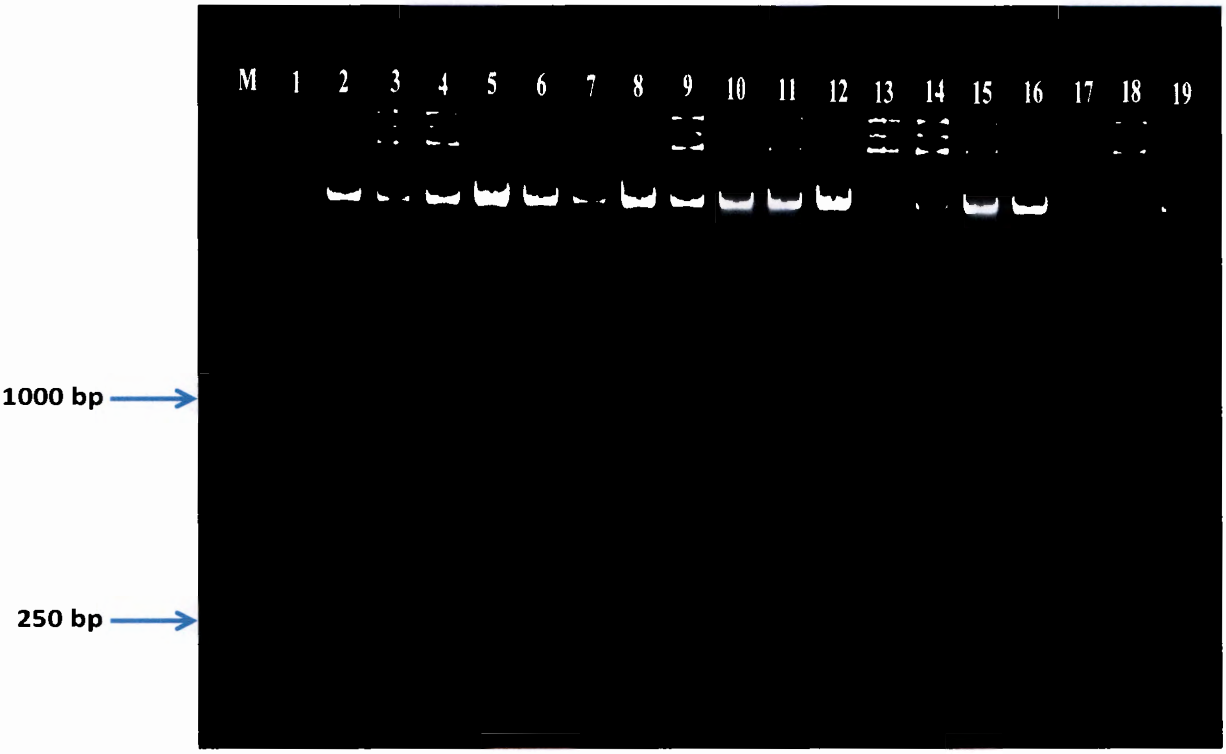

Figure 4.1: Agarose gel (1.5% w/v) image depicting DNA extracted from presumptive *Enterococcus* isolates and control strains. Lane M= DNA marker (O'GeneRuler 1kb DNA Ladder), Lane 1 = Negative Control, Lane 2 = DNA extracted from *Enterococcus faecium* (ATCC 700221), Lane 3-19 = DNA extracted from Presumptive *Enterococcus* isolates

4.4 BACTERIAL 16S rRNA GENE PCR ANALYSIS

In order to avoid any bias all the 81 presumptive *Enterococcus* isolates obtained from the ground and surface water samples, based on colony morphologies, were taken through 16S rRNA PCR analysis. The results indicated that all 81 (100%) of the isolates were positive for the 16S rRNA PCR analysis. Figure 4.2 shows a representation of a 2% (w/v) agarose gel depicting 16S rRNA gene fragments amplified from isolates and the PCR amplicons possessed the expected size (322 bp).

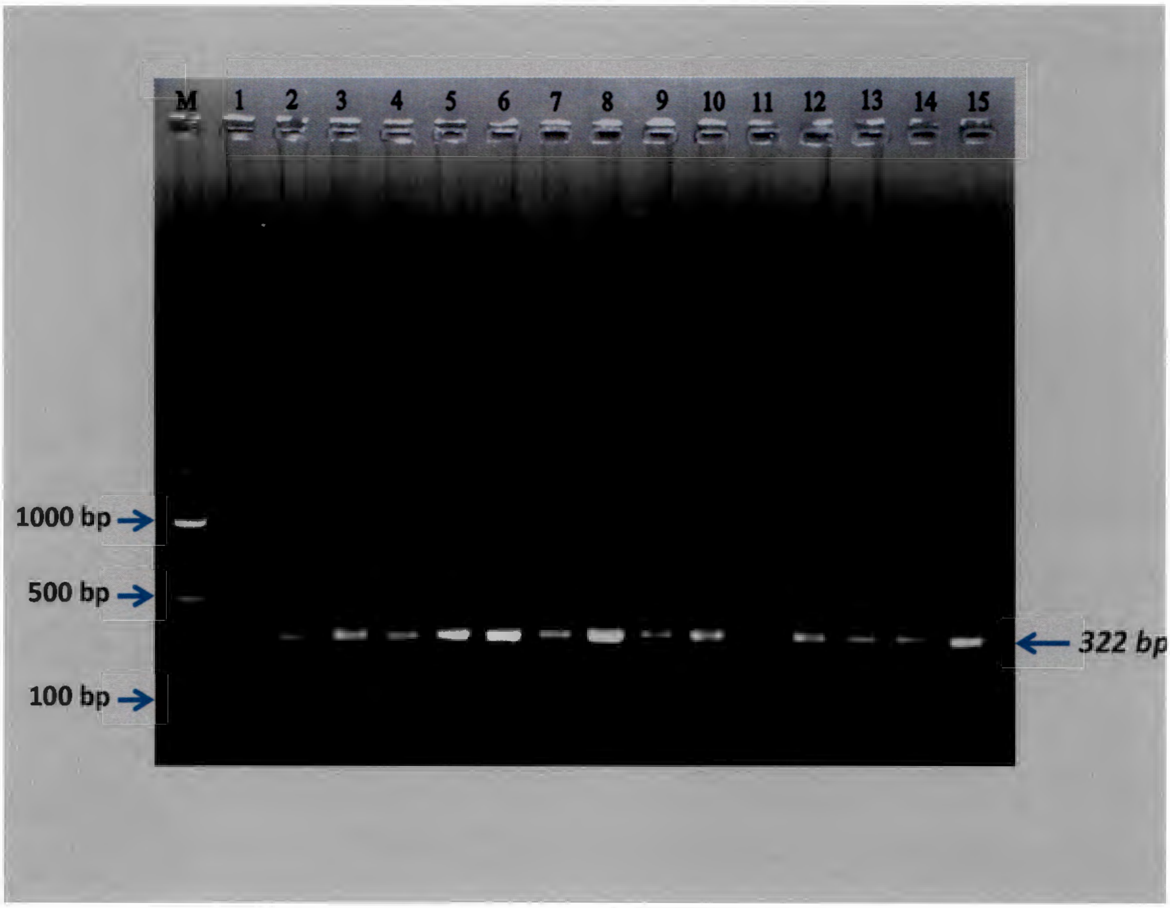

Figure 4.2: Agarose gel electrophoresis of *Enterococcaceae* specific 16S rRNA gene fragments amplified from isolates obtained during the study. Lane M = 100 bp DNA marker; Lane 1 = Negative Control; Lane 2 = 16S rRNA gene fragment amplified from *Enterococcus faecium* (ATCC 700221); Lanes 3-15 = 16S rRNA gene fragments amplified from isolates obtained from the different sampling sites.

4.5 CONFIRMATORY IDENTIFICATION TESTS FOR *ENTEROCOCCUS* SPECIES

4.5.1 Multiplex PCR for confirming the identities of *E. faecium* and *E. faecalis*

Enterococcus species specific PCR for *E. faecium* and *E. faecalis* was performed on all isolates that were positive for 16S rRNA gene. To avoid any significant bias, all the isolates were subjected to *Enterococcus* species specific PCR analysis regardless of their presumptive macroscopic and biochemical identification test results. A large proportion 55 (67.9%) of these isolates were positive for the *ddl* gene of *E. faecium* and *E. faecalis* (Figure 4.3; Figure 4.4). Among the isolates that were positive for *Enterococcus* species a large proportion 38 (46.9%) were *E. faecium* and these possessed the expected amplicon size of 1091 bp, while 17 (21%) were *E. faecalis* and also produced the expected amplicon size (475 bp) as described in Table 3.3. Figure 4.3 shows a 1.5% (w/v) agarose gel of the *E. faecium ddl* (1091 bp) and the *E. faecalis ddl* (475 bp) genes fragments amplified from representative isolates respectively. Data in Table 4.3 indicates the total number of isolates that were positively identified as *Enterococcus* species by PCR analysis and sequencing while Figure 4.3 outlines the distribution of *Enterococcus* and non-*Enterococcus* species isolated during the study.

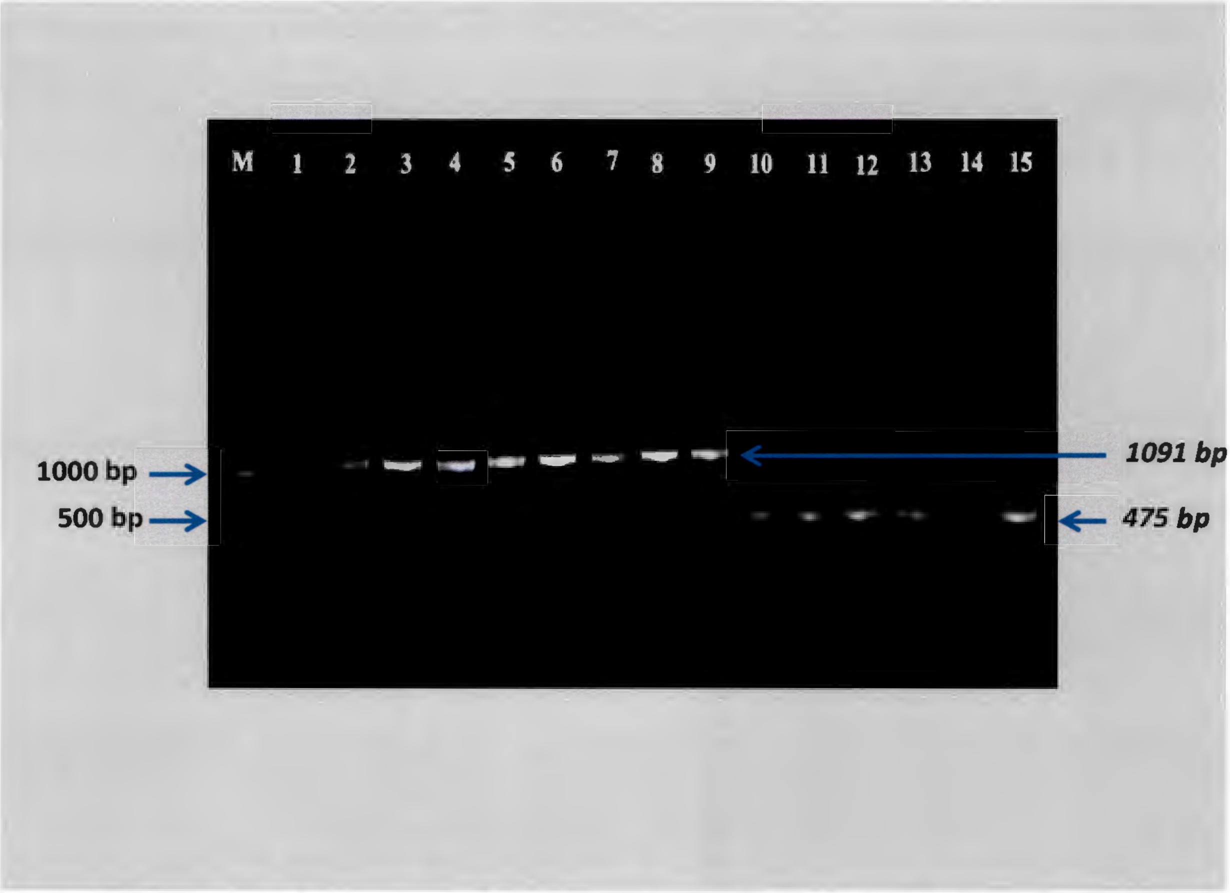

Figure 4.3: Agarose gel electrophoresis of *Enterococcus* species specific a gene fragments amplified from isolates obtained during the study by multiplex PCR analysis. Lane M = 100 bp DNA marker; Lane 1 = Negative Control; Lane 2 = *ddl* gene fragment amplified from *E. faecium* (ATCC 700221); Lane 3-9 = *E. faecium ddl* gene fragments amplified from isolates and Lane 10-15 = *E. faecalis ddl* gene fragments amplified from isolates obtained from the different sampling sites.

4.5.2 Confirming the identities of isolates using bacterial 16S rRNA sequence data

Bacterial 16S rRNA gene fragments of *E. faecium*, *E. faecalis* as well as other distinctive isolates were subjected to sequence analysis to confirm their identities. Sequence data of *E. faecium* isolates obtained in the present study revealed 97% similarities to *E. faecium* strain CNM139 (Accession No: KC699049.1), *E. faecium* strain WYD09 (Accession No: KX583576.1) and *E. faecium* strain 200573 (Accession No: KU892956.1) 16S ribosomal RNA gene, partial sequence previously deposited in gene bank. In addition, the results for *E. faecalis* revealed 98% similarities to *E. faecalis* strain SGM4 (Accession No: KX830839.1) and an *E. faecalis* strain SNNU0267 (Accession No: KX752888.1) 16S ribosomal RNA gene, partial sequence. In addition, an isolate that was not positively identified as either *E. faecium* or *E. faecalis* was included in the analysis and it's sequence data revealed 98% similarity to *E. saccharolyticus* strain SU383 (Accession No: KX880974.1) 16S ribosomal RNA gene, partial sequence.

Table 4.3: The number of samples collected from various sources in villages and rural communities, the total number of *Enterococcus* isolates, total number tested and the names of the *Enterococcus* species obtained.

Area	Sample source	No. samples collected	No. samples positive	No. Isolates	*Enterococcus* species indentified	No. Tested Positive	%
Mafikeng	GW – Boreholes	55	18	25	*E. faecium*	12	48
					E. faecalis	5	20
					E. saccharolyticus	1	4
	SW – Dams	12	4	7	*E. faecium*	1	14.3
					E. faecalis	3	42.9
Rustenburg	GW – Boreholes	16	4	8	*E. faecium*	6	75
	SW – Dams	10	7	17	*E. faecium*	6	35
					E. faecalis	4	23.5
Potchefstroom	GW – Boreholes	10	1	1		0	0
	SW – Dams	7	1	1	*E. faecium*	1	100
	SW – Rivers	7	2	3	*E. faecium*	2	66.7
Taung	GW – Boreholes	14	0	0			
	SW – Dams	2	0	0			
	SW – Rivers	2	1	2	*E. faecium*	1	50
					E. faecalis	1	50
Zeerust	GW – Boreholes	7	1	2	*E. faecium*	2	100
	SW – Dams	3	0	0			
	SW – Spring	3	3	8	*E. faecium*	4	50
					E. faecalis	2	25
Coligny	GW – Boreholes	5	0	0			
	SW – Dams	5	3	7	*E. faecium*	3	42.9
					E. faecalis	2	28.6
Ventersdorp	GW – Boreholes	6	0	0			
Swartruggens	GW – Boreholes	3	0	0			
Lichtenberg	GW – Boreholes	3	0	0			
TOTAL		170	45	81	*E. faecium*	38	46.9
					E. faecalis	17	21
					E. saccharolyticus	1	1.2
					TOTAL	**56**	

GW = Ground water; SW = Surface water

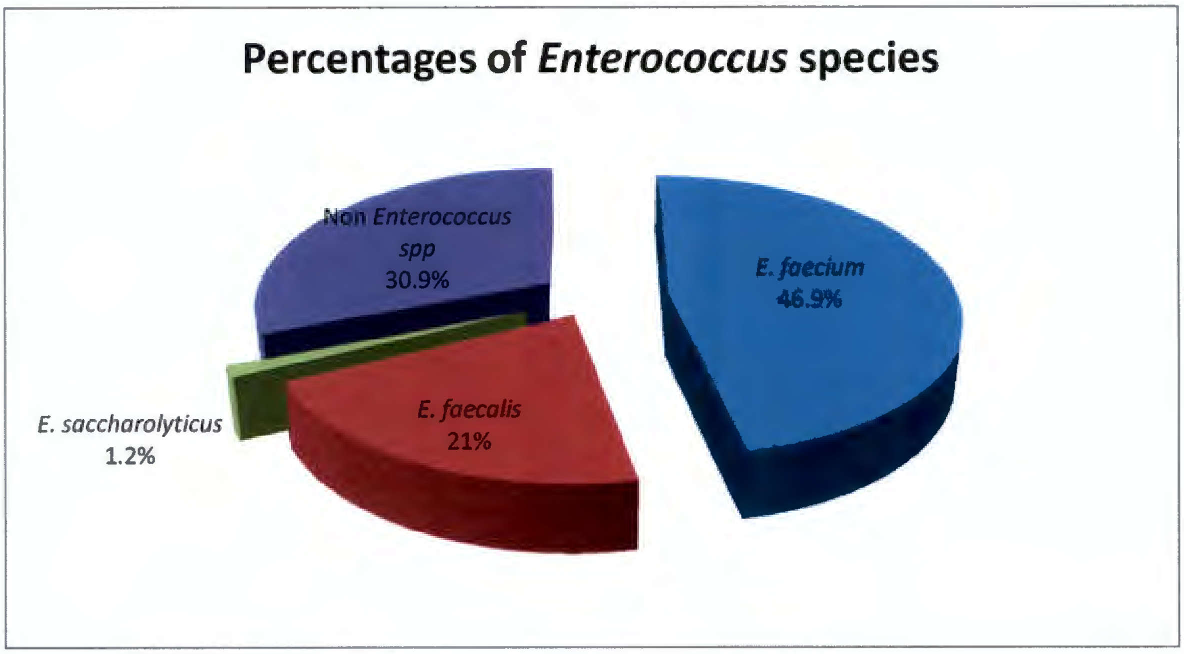

Figure 4.4: Proportion of *Enterococcus* and non-*Enterococcus* isolates that were positively identified in the study.

4.6 ANTIBIOTIC SUSCEPTIBILITY PROFILES OF ISOLATES

The antibiotic susceptibility profiles of isolates were achieved by performing the disc diffusion test as previously described by Kirby *et al.*, (1966). A total of 56 isolates that were phenotypically detected as Vancomycin Resistant Enterococci (based on growth on Bile Esculin Agar supplemented with vancomycin) were subjected to the disc diffusion antimicrobial susceptibility test. This test was done to evaluate the resistance patterns of isolates from the different sampling sites. The data in Table 4.4 indicates the percentage resistance of isolates against a panel of nine antimicrobial agents. The resistance of the isolates to each antibiotic is depicted as percentages (Figure 4.5). Detailed data on the diameters of the growth inhibition zones for these isolates against the different antibiotics are shown in APPENDIX 1B.

As indicated in Figure 4.5, large proportions of the isolates were resistant to Nalidixic acid (83.9%) and Vancomycin (78.6%). In addition, the majority (68.8-87.5%) of the isolates from Mafikeng and Rustenburg were resistant to VAN, NAL and AMP. Similarly, a majority (60-100%) of the isolates from Potchefstroom and Coligny were resistant to VAN, NAL and NIT. Despite this resistance, it was observed that a small proportions of the isolates from some of these sampling sites were resistance to Gentamycin (0-40%), Chloramphenicol (27-50%) and Sulphamethoxazole (0-66.7%) (Figure 4.5). Although isolates from other areas showed moderate resistance to these antibiotics it is also important to highlight that small proportions (0% - 25%) of the isolates from Mafikeng, Rustenburg and Zeerust were highly susceptible to SMX, GEN, STR and CHIL.

Table 4.4: Proportion of isolates from the different stations that were resistant to the antibiotics tested.

Sampling Area	**No. Tested**	**VAN(30)**	**NA(30)**	**STR(10)**	**CHIL (30)**	**AMP(10)**	**OXY-TET (30)**	**GEN(10)**	**NIT(100)**	**SMX(25)**
Mafikeng	NT	22	22	22	22	22	22	22	22	22
	NR	16 (72.7%)	17 (77.3%)	5 (22.7%)	6 (27.3%)	8 (36.4%)	8 (36.4%)	4 (18.2%)	11 (50%)	0 (0%)
Rustenburg	NT	16	16	16	16	16	16	16	16	16
	NR	13 (81.3%)	14 (87.5%)	4 (25%)	4 (25%)	11 (68.8%)	8 (50%)	0 (0%)	8 (50%)	3 (18.6%)
Potchefstroom	NT	3	3	3	3	3	3	3	3	3
	NR	2 (66.7%)	2 (66.7%)	1 (33.3%)	1 (33.3%)	2 (66.7%)	2 (66.7%)	1 (33.3%)	2 (66.7%)	2 (66.7%)
Taung	NT	2	2	2	2	2	2	2	2	2
	NR	2 (100%)	2 (100%)	1 (50%)	1 (50%)	1 (50%)	1 (50%)	2 (100%)	2 (100%)	1 (50%)
Zeerust	NT	8	8	8	8	8	8	8	8	8
	NR	7 (87.5%)	7 (87.5%)	2 (25%)	2 (25%)	2 (25%)	5 (62.5%)	0 (0%)	5 (62.5%)	0 (0%)
Coligny	NT	5	5	5	5	5	5	5	5	5
	NR	4 (80%)	5 (100%)	4 (80%)	3 (60%)	1 (20%)	4 (80%)	2 (40%)	3 (60%)	1 (20%)
TOTAL	**NT**	**56**	**56**	**56**	**56**	**56**	**56**	**56**	**56**	**56**
	NP	**44 (78.6%)**	**47 (83.9%)**	**17 (30.4%)**	**17 (30.4%)**	**25 (44.6%)**	**28 (50%)**	**9 (16.1%)**	**31 (55.4%)**	**7 (12.5%)**

VAN (Vancomycin), NA (Nalidixic Acid), STR (Streptomycin), CHIL (Chloramphenicol) APM (Ampicillin), OXY-TET (Oxytetracycline), GEN (Gentamicin), NIT (Nitrofurantoin), SMX (Sulphamethoxazole) NT= Number Tested, NR= Number Resistant

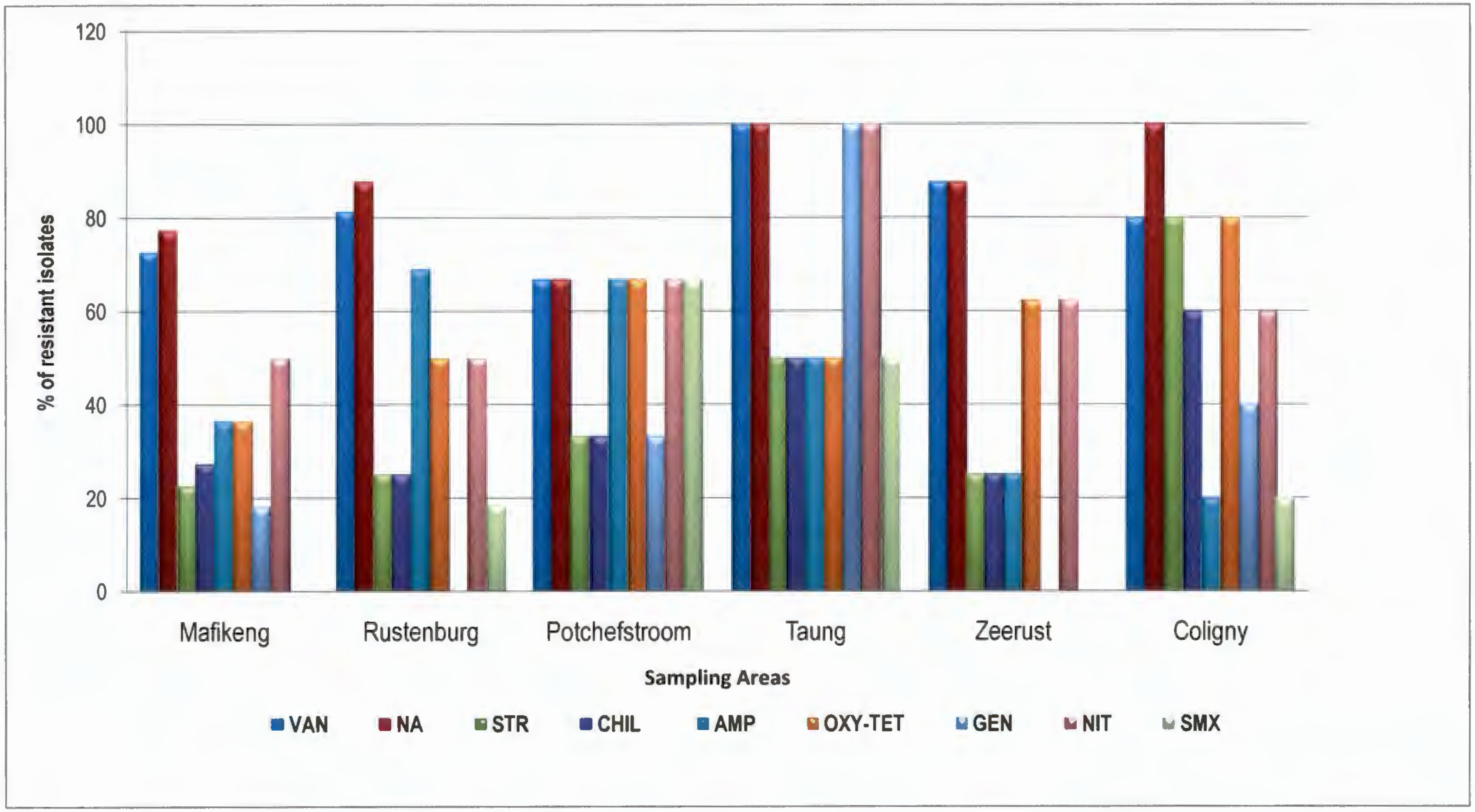

Figure 4.5 Proportion of isolates from the different stations that were resistant to the antibiotics tested were: VAN (Vancomycin), NA (Nalidixic acid) STR (Streptomycin) CHIL (Chloramphenicol) APM (Ampicillin), OXY-TET (Oxytetracycline), GEN (Gentamycin), NIT (Nitrofurantoin) SMX (Sulphamethoxazole).

4.7 MULTIPLE ANTIBIOTIC-RESISTANT (MAR) PHENOTYPES OF VRE SPECIES ISOLATED FROM SURFACE AND GROUND WATER

The multiple antibiotic resistant phenotypes of 56 confirmed *Enterococcus* isolates were generated for isolates showing resistance to three or more antibiotics using abbreviations that appear on the discs. The predominant multiple antibiotic-resistant phenotypes observed in isolates from surface and ground water samples obtained from the various areas in the North West Province are shown in Tables 4.5.

The multiple antibiotic resistant indices obtained this study was 1.63. A large proportion 45 (80.4%) of the isolates obtained in this study showed MAR phenotypes and amongst these MAR isolates a total of 38 (84.4%) were resistant to vancomycin (APPENDIX 2A). Despite the fact that several phenotypes were observed among isolates from Mafikeng MAR phenotypes VAN-NAL-AMP-OXYTET-NIT and VAN-NAL-AMP were predominant among these isolates. Phenotypes VAN-NAL-AMP and VAN-AMP-NIT were dominant among isolates from Mafikeng and Rustenburg (Table 4.5). A cause for concern was the fact that 1 isolate each from Potchefstroom, Taung and Coligny was resistant to all the 9 antibiotics that were tested.

Despite the fact that a large proportion of the isolates possessed MAR phenotypes, the majority of these isolates were resistant to only three or four of the antibiotics tested, indicating why the phenotypes VAN-NAL-AMP and VAN-NAL-OXYTET-NIT were predominant among isolates in the study. In general, the detection of multiple antibiotic resistant *Enterococcus* isolates in this study indicates that these isolates may not only have severe effects on the treatment of human infections but may also serve as reservoirs for the transmission of antibiotic resistant determinants to other related bacterial strains with whom they share a common ecological niche.

Table 4.5 Predominant multiple antibiotic-resistant phenotypes for Vancomycin Resistant *Enterococcus* (VRE) isolated from different sampling areas.

Sample Area	Phenotype	No. Observed	% Observed
Mafikeng NT = 22, NO = 18	VAN-CHIL-NIT	1	4.5%
	VAN-NAL-STR-AMP-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-AMP-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-AMP-OXYTET-NIT	1	4.5%
	VAN-STR-NIT	1	4.5%
	NAL-STR-NIT	1	4.5%
	VAN-NAL-AMP	3	13.6%
	VAN-NAL-CHIL-OXYTET	1	4.5%
	VAN-NAL-STR-CHIL-OXYTET-NIT	1	4.5%
	VAN-NAL-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-OXYTET	2	9.1%
	VAN-NAL-CHIL-NIT	1	4.5%
	VAN-NAL-STR-CHIL-AMP-GEN-NIT	1	4.5%
	VAN-NAL-CHIL-AMP-OXYTET	1	4.5%
	STR-CHIL-GEN-NIT	1	4.5%
Rustenburg NT = 16, NO = 11	VAN-NAL-AMP	1	6.3%
	VAN-STR-CHIL-AMP-OCYTET-NIT-SMX	1	6.3%
	VAN-NAL-STR-CHIL-NIT	1	6.3%
	VAN-NAL-AMP-OXYTET-NIT	2	12.5%
	NAL-STR-CHIL-AMP-OYTET-NIT-SMX	1	6.3%
	NAL-CHIL-AMP-NIT	1	6.3%
	VAN-NAL-AMP-OXYTET	2	12.5
	VAN-NAL-STR-AMP-OXYTET-NIT-SMX	1	6.3%
	VAN-NAL-STR-AMP-OXYTET-NIT	1	6.3%
Potchefstroom NT = 3, NO = 2	VAN-NAL-AMP-OXYTET-NIT-SMX	1	33.3%
	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	33.3%
Taung NT = 2, NO = 2	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	50%
	VAN-NAL-GEN-NIT	1	50%
Zeerust NT = 8, NO = 8	NAL-STR-CHIL-AMP	1	12.5%
	VAN-NAL-CHIL	1	12.5%
	VAN-NAL-OXYTET-NIT	3	37.5%
	VAN-NAL-OXYTET	1	12.5%
	VAN-STR-OXYTET-NIT	1	12.5%
	VAN-NAL-AMP-NIT	1	12.5%
Coligny NT = 5, NO = 4	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	20%
	VAN-NAL-STR-CHIL-OXYTET-NIT	1	20%
	NAL-NAL-STR-CHIL-NIT-OXYTET	1	20%
	NAL-STR-OXYTET-GEN-NIT	1	20%
TOTAL NT = 56, NO = 45		**45**	**80.4%**

NT = Number Tested, NO = Number Observed

4.8 PHENOTYPIC RELATIONSHIP BETWEEN VANCOMYCIN RESISTANT *ENTEROCOCCUS* ISOLATES OBTAINED FROM SURFACE AND GROUND WATER BASED ON CLUSTERING PATTERNS USING THE ANTIBIOTIC INHIBITION ZONE DIAMETER DATA

Significantly, as indicated in Table 4.4 the results obtained in this study revealed that there were 44 (78.6%) isolates that showed phenotypic to vancomycin resistance and these were considered for clusterring patterns. Based on their inhibition zone diameters, a total of 44 Vancomycin Resistant Enterococci strains, isolated from different locations within the North West Province, were characterized using cluster analysis. A dendrogram was generated and detailed results are shown in Figure 4.5. The dendrogram was analysed for associations of isolates from the different sampling sites and results are shown in Table 4.6. The Statistica software (version 12) was used to determine the relatedness of the VRE isolates based on clustering of the antibiotic inhibition zone diameter data. The data was exported from a Microsoft excel office file into Statistica software (version 12) and a dendrogram constructed using Ward's method and Euclidean distances. The dendrogram was used as a tool to determine similarities and resolving differences between the phenotypes of isolates from different sampling areas (Ateba and Bezuidenhout, 2008). As indicated in Figure 4.5, two clusters (Cluster 1 and Cluster 2) were generated. Cluster 1 was subdivided into five sub-clusters (Cluster 1A – 1E), while Cluster 2 was subdivided into only one sub-cluster and 3 non-clustered isolates. Furthermore, all the sub-clusters were analysed for patterns of association of isolates from different sources and/or locations (Table 4.6). As indicated in Figure 4.8, the largest sub-cluster was sub-cluster 1C, which was mostly comprised of isolates derived from ground water samples. The second largest sub-cluster (sub-cluster 2A) contained isolates from all the sampling areas, except Taung. In addition, large proportions (75%) of isolates in this sub-cluster (Cluster 2A) were derived from surface water samples and most of the isolates observed were *E. faecium* species. On the contrary, the results revealed 3 isolates that were not sub divided according to sub-clusters (in Cluster 2). Moreover, only isolates from Mafikeng and Rustenburg were represented across all the clusters and large proportions (40%) of isolates present in sub-cluster 1C were from Mafikeng. The smaller non-clustered isolates in Cluster 2 were from Potchefstroom, Taung as well as Coligny and they were all derived from surface water samples.

The great similarities in the antibiotic resistance profiles of VRE isolates from the different locations may have resulted from the indiscriminate and frequent use of these antibiotics in humans and animals. It is therefore suggested that studies designed to determine relatedness of different isolates based on clustering of their antibiogram data may provide an understanding of the evolution of newer antibiotic resistant profiles. Considering these facts, such data may be of great epidemiological significant and therefore be very useful in identifying the source of contamination.

Table 4.6: The percentage representation of VREs isolates obtained from different areas within the various clusters

Sampling Area	**Source**	**Cluster 1A N=5**	**Cluster 1B N=8**	**Cluster 1C N=9**	**Cluster 1D N=6**	**Cluster 1E N=5**	**Cluster 2A N=8**	**Non-Cluster 2 N=3**
Mafikeng	GW	2 (40%)	2 (25%)	4 (40%)	4 (66.7%)	1 (20%)	1 (12.5%)	0 (0%)
	SW	0 (0%)	1 (12.5%)	0 (0%)	1 (16.7%)	0 (0%)	0 (0%)	0 (0%)
Rustenburg	GW	0 (0%)	0 (0%)	1 (11.1%)	0 (0%)	1 (20%)	1 (12.5%)	0 (0%)
	SW	1 (20%)	2 (25%)	3 (33.3%)	1 (16.7%)	1 (20%)	2 (25%)	0 (0%)
Potchefstroom	GW	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	SW	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)	1 (33.3%)
Taung	GW	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	SW	1 (20%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (33.3%)
Zeerust	GW	0 (0%)	0 (0%)	1 (11.1%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)
	SW	1 (20%)	3 (37.5%)	0 (0%)	0 (0%)	0 (0%)	1 (12.5%)	0 (0%)
Coligny	GW	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
	SW	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	2 (25%)	1 (33.3%)

GW = Ground water; SW = Surface water; N = Number

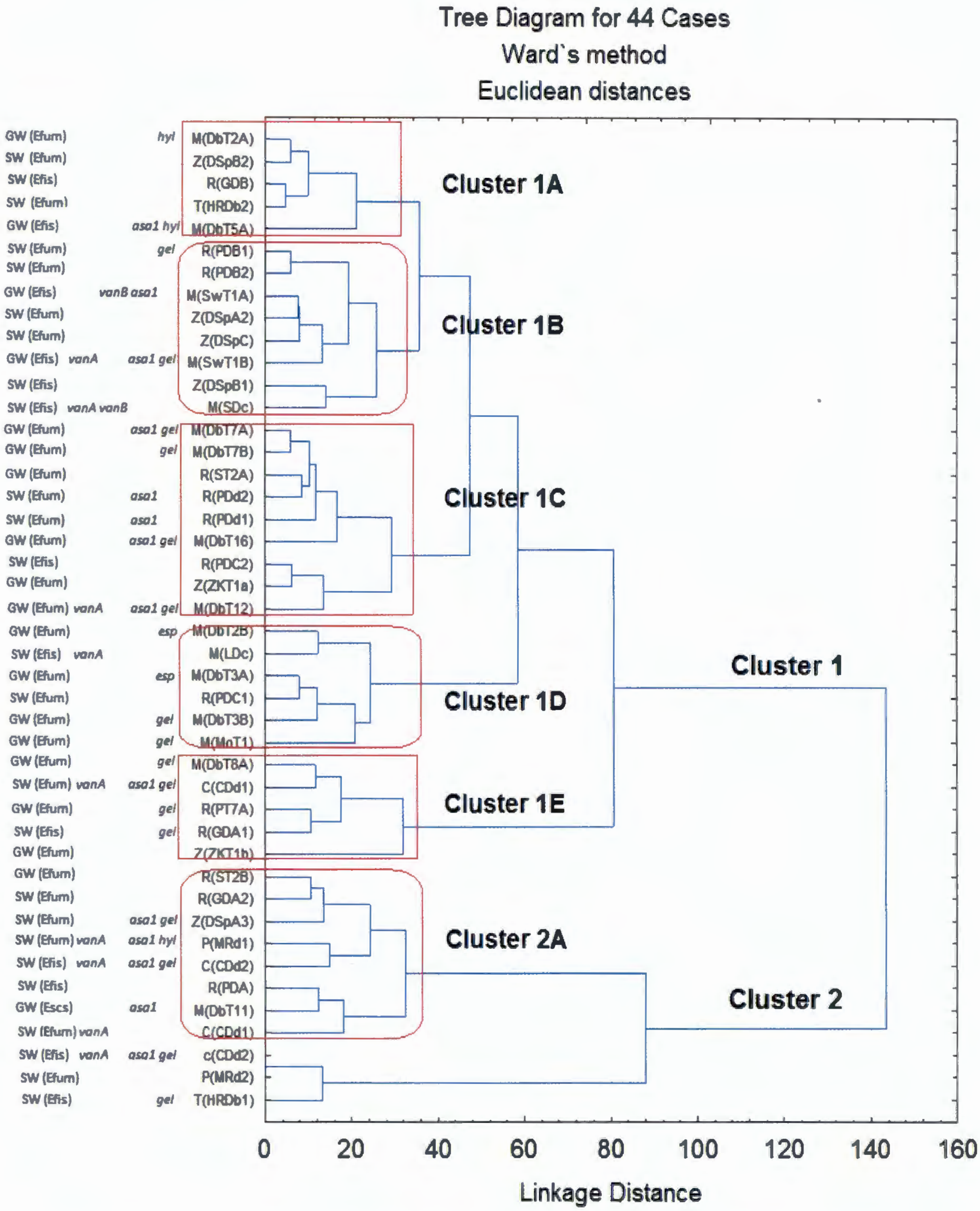

Figure 4.6: Dendrogram showing the relationship between Vancomycin Resistant *Enterococcus* isolates from ground and surface water samples obtained in the different locations. Bacterial designation prefixes are based on sampling station origin and sample type. The tree was constructed using Ward's method and Euclidean distances in Statistica, version 13.2 (Statsoft, US). Designation: M=Mafikeng, R=Rustenburg, P=Potchefstroom, T=Taung, Z=Zeerust, C=Coligny; GW=Groundwater, SW=Surfacewater; Efum=*E. faecium*; Efis=*E. faecalis*; Escs=*E. saccharolyticus*.

4.9 MULTIPLEX PCR ASSAY TO DETECT VANCOMYCIN RESISTANT GENES IN ENTEROCOCCUS ISOLATES

To avoid any bias, a total of 56 confirmed *Enterococcus* isolates that were isolated from BEA supplemented with vancomycin were subjected to a multiplex PCR analysis in order to amplify *vanA, vanB* and *vanC21/2* respectively that code for resistance to vancomycin in enterococci (Clark *et al.*, 1993; Harwood *et al.*, 2001; Depardieu *et al.*, 2004). Results indicated that a small proportion 9 (16%) of the isolates possessed the *vanA* resistant gene while 2 (4%) possessed the *E-vanB* resistance gene. In addition, the results showed that all the isolates possessed vancomycin resistant genes they showed phenotypic resistance to vancomycin. On the contrary, none of the isolates possessed the *vanC21/2* resistance gene. It was also identified that an isolate from Mafikeng possessed both the *vanA* and *vanB* resistance genes. The composition of isolates from the different sampling stations that were positive for these genes are shown in Table 4.7 while Figure 4.6 shows a 1.5% (w/v) agarose gel depicting the *vanA* (732 bp) *and vanB* (647 bp) gene fragments amplified from isolates.

Figure 4.7: Agarose gel electrophoresis of Vancomycin Resistant (*vanA* and *vanB*) gene fragments amplified from *E. faecium* and *E. faecalis* isolates obtained from different sampling sites using multiplex PCR analysis. Lane M = 100 bp DNA marker; Lane 1 = Negative control; Lane 2 = *E. faecalis*, with both

the *vanA* (732 bp) and *vanB* (647 bp) gene fragments; Lane 3-10 = *vanA* gene fragments; Lane 11 = *E-vanB* gene fragment.

Table 4.7: Proportion of isolates that were positive for Vancomycin Resistant *Enterococcus* genes multiplex PCR analysis

Sampling Area	**No. Tested**	***vanA***	***vanB***
Mafikeng	NT	22	22
	NP	4 (18%)	2 (9%)
Rustenburg	NT	16	16
	NP	0 (0%)	0 (0%)
Potchefstroom	NT	3	3
	NP	1 (33%)	0 (0%)
Taung	NT	2	2
	NP	0 (0%)	0 (0%)
Zeerust	NT	8	8
	NP	0 (0%)	0 (0%)
Coligny	NT	5	5
	NP	4 (80%)	0 (0%)
TOTAL	**NT**	**56**	**56**
	NP	**9 (16%)**	**2 (4%)**

NT = Number Tested, NP = Number Positive

4.10. PHENOTYPIC ASSAYS TO DETERMINE VIRULENCE CAPABILITIES

4.10.1 Cytolysin

Cytolysin production was evaluated by examining Beta (*β*)-haemolysis on blood agar plates, supplemented with 5% (w/v) ox-blood (Kilian and Inoue, 2002). Generally, most enterococci are gamma haemolytic indicating they do not breakdown erythrocytes on blood agar. However, there are some virulent *Enterococcus* strains that possess cytolysin as a virulence factor and therefore display a haemolytic pattern on blood agar. Out of the 44 VRE isolates that were tested for haemolysis on blood agar, a large proportion 49 (88.6%) were non haemolytic while only 5 (11.4%) isolates showed incomplete or *β*-haemolysis. Detailed results are shown in Table 4.8.

Table 4.8: Proportion of isolates that were positive for cytolysin production

Sampling Area	**No. Isolates Tested**	**Haemolysis on Blood Agar**
Mafikeng	NT	16
	NP	5 (31.3%)
Rustenburg	NT	13
	NP	0 (0%)
Potchefstroom	NT	2
	NP	0 (0%)
Taung	NT	2
	NP	0 (0%)
Zeerust	NT	7
	NP	0 (0%)
Coligny	NT	4
	NP	0 (0%)
TOTAL	**NT**	**44**
	NP	**5 (11.4%)**

NT = Number Tested, NP = Number Positive

4.11 DETECTION OF VIRULENCE GENES DETERMINANTS IN ENTEROCOCCI ISOLATES BY MULTIPLEX PCR ASSAY

A total of 44 Vancomycin Resistant *Enterococcus* isolates whose identities had been confirmed by PCR and sequencing analysis, were subjected to a multiplex PCR designed to amplify virulence gene determinants that included aggregation substance (*asa1*), cytolysin (*cylA*), Enterococcal surface protein (*esp*), gelatinase (*gel*) and Hyalutonidase (*hyl*) (Vankerckhoven *et al.*, 2004; Molale and Bezuidenhout, 2016). The results obtained indicated that 16 (36.4%) of the isolates possessed the *gel* virulence gene, 14 (27.3%) isolates possessed *asa1* virulent gene, 3 (6.8%) were positive for *hyl* the gene while a small proportion(4.5%) of 2 isolates harboured the *esp* gene (Table 4.9). On the contrary, none of the isolates possessed *cylA,* which is responsible for cytolysin production. The composition and the percentages of isolates from the different sampling stations that were positive for these genes are shown in Table 4.9. Figure 4.8 shows a 1.5% (w/v) agarose gel depicting the *asa1* (375 bp), *esp* (510 bp) *gel* (213 bp) and *hyl* (276 bp) virulence gene fragments amplified from the isolates. Despite the fact that the expected amplicon size of *hyl* was 276 base pairs, fragments obtained from this study were slightly larger (300 bp). In addition, there are some isolates that possessed more than one of the virulence genes and these are shown on Figure 4.9.

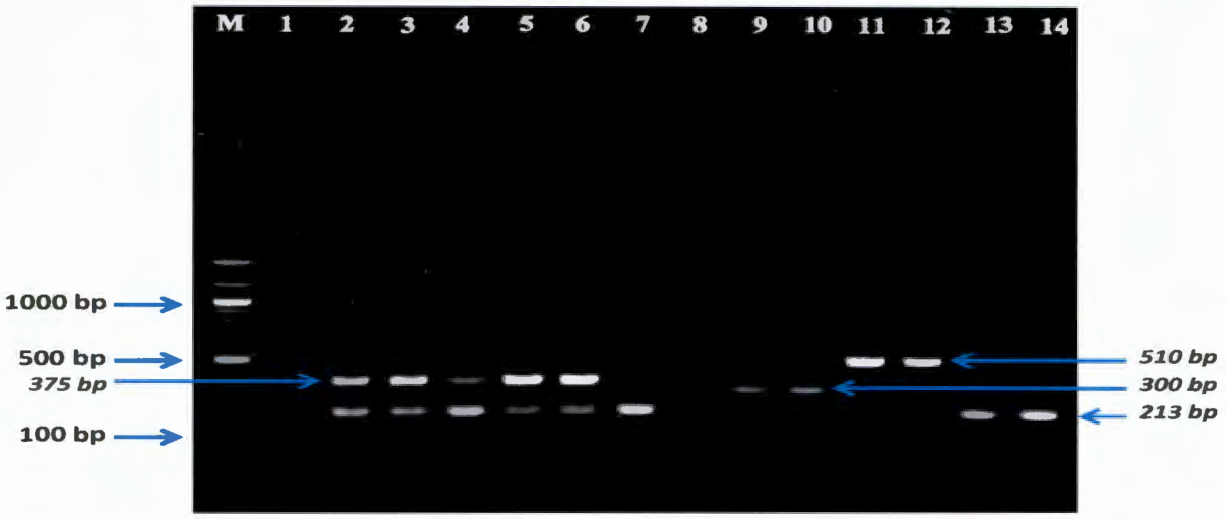

Figure 4.8: Agarose gel electrophoresis of *Enterococcus* virulence gene fragments amplified from isolates obtained during the study by multiplex PCR analysis. Lane M = 100 bp DNA marker; Lane 1 = Negative Control; Lanes 2-6 = *asa1* (375 bp) and *gel* (213 bp) gene fragment; Lanes 7-8 and 13-14 = *gel* (213 bp) gene fragment; Lanes 9-10 = *hyl* (300 bp) gene fragment; Lanes 11-12 = *esp* (510 bp) gene fragments amplified from isolates obtained from the different sampling sites.

Table 4.9: Proportion of isolates that were positive for *Enterococcus* virulence genes multiplex PCR analysis

Sampling Area	**No. Tested**	***asa1***	***esp***	***gel***	***hyl***
Mafikeng	NT	16	16	16	16
	NP	7 (43.8%)	2 (12.5%)	7 (43.8%)	2 (12.5%)
Rustenburg	NT	13	13	13	13
	NP	2 (15.4%)	0 (0%)	4 (30.8%)	0 (0%)
Potchefstroom	NT	2	2	2	2
	NP	1 (50%)	0 (0%)	0 (0%)	1 (50%)
Taung	NT	2	2	2	2
	NP	1 (50%)	0 (0%)	1 (50%)	0 (0%)
Zeerust	NT	7	7	7	7
	NP	0 (0%)	0 (0%)	1 (14.3%)	0 (0%)
Coligny	NT	4	4	4	4
	NP	3 (75%)	0 (0%)	3 (75%)	0 (0%)
TOTAL	**NT**	**44**	**44**	**44**	**44**
	NP	**14 (31.8%)**	**2 (4.5%)**	**16 (36.4%)**	**3 (6.8%)**

NT = Number Tested, NP = Number Positive

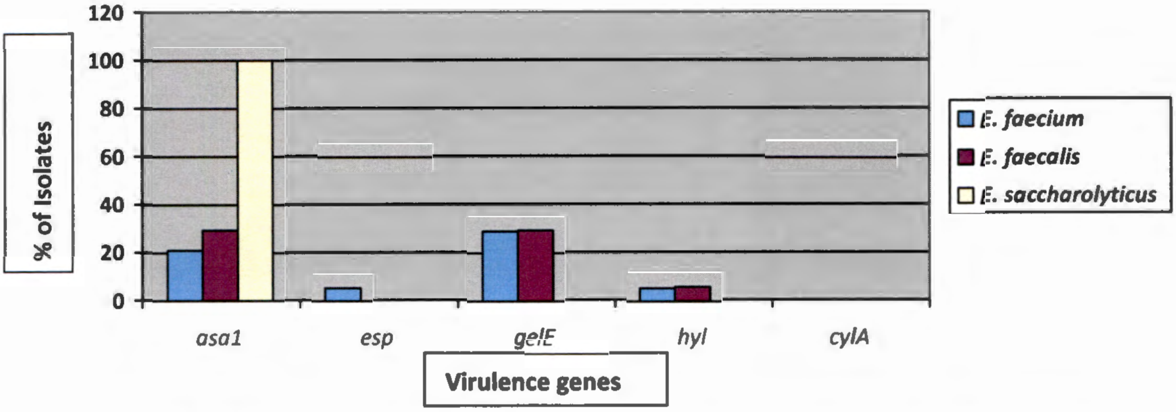

Figure 4.9: Prevalence of the five virulence genes distributed in 44 Vancomycin-Resistant *Enterococcus* strains. For 30 *E. faecium*, eighteen isolates showed a more diverse and multiple virulence genes; including *asa1* for 26.7% (8/30), *esp* for 6.7% (2/30), *gel* for 36.7% (11/30), and *hyl* for 6.7% (2/30) virulence genes. For 17 *E. faecalis*, only *asa1*, *gel* as well as *hyl* genes were detected with the frequencies of 38.5% (5/13), 38.5% (5/13) and 7.7% (1/13), respectively. Moreover, the *E. saccharolyticus* isolate only showed *asa1* 100% (1/1) virulence gene. Generally, there were no *cylA* virulence genes that were detected.

4.12 ENTEROBACTERIAL REPETITIVE INTERGENIC CONSENSUS (ERIC) SEQUENCE PCR ANALYSIS

The genetic relatedness of 44 VRE isolates comprising of 30 *E. faecium*, 13 *E. faecalis* and 1 *E. saccharolyticus*) were subjected to an ERIC PCR fingerprinting technique. Amplification was carried out using ERIC2 single non-specific primer to amplify various repetitive intergenic regions of DNA that are flanked by conserved sequences in order to generate isolate specific genetic fingerprints. The DNA fragments generated fingerprints that ranged between 3 to 11 bands per isolate (Figure 4.9) large proportion of isolates produced banding patterns that comprised of five bands per isolate. Moreover, the band sizes ranged from 0.4 kb to more than 8 000 kb. Based on the DNA banding patterns there were great similarities in the DNA fingerprints between some isolates from ground and surface water. These results suggest that VRE from both ground and surface water bodies have similar fingerprints and may have originated from a common ancestral strain. It is also suggested that these isolates may pose severe health risks to consumers especially those that harbour both virulence and antibiotic resistance gene determinants.

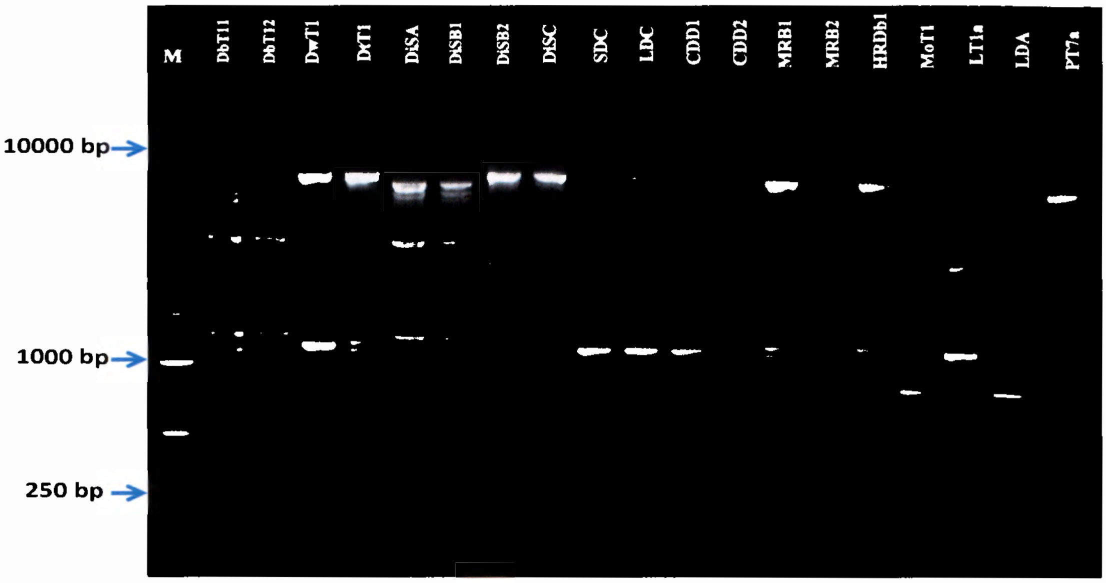

Figure 4.10 Agarose gel (1.5% w/v) image depicting Enterobacterial Repetitive Intergenic Consensus (ERIC) amplified from representative *Enterococcus* isolates. Lane M = 1kb DNA ladder (O'GeneRuler 1kilo base pairs DNA Ladder) and 100bp DNA Ladder. Lanes 1-19 *Enterococcus* isolates from ground and surface samples.

CHAPTER FIVE

DISCUSSION

CHAPTER 5

DISCUSSION

5.1 GENERAL DISCUSSION

The purpose of this study was to isolate Vancomycin-Resistant Enterococci (VRE) from ground and surface water, determine their virulence capabilities as well as their antibiotic resistance profiles. Generally, *Enterococcus* species exist as normal flora in the gastro – intestinal tract of humans and animals (Zirakzadeh and Patel, 2006). Despite the fact that enterococci possess intrinsic resistance to a number of antimicrobial agents, they can also acquire very high levels of resistance to some commonly used antibiotics as well as their derivatives (Ramatlhape, 2006). These organisms easily acquire antimicrobial resistance determinants by horizontal gene transfer of motile genetic determinants contained in the different bacterial strains (Cattoir and Leclercq, 2013).

Water is an important resource for life and is used in a number of household activities. However, enterococci may be released into water bodies through human and animal faeces, therein faecal matter of human origin is currently thought to be a potential reservoir for pathogenic enterococci, especially those that are resistant to vancomycin (Chamaille-Jammes *et al.*, 2007; Ateba and Maribeng, 2011). In addition, a few previous studies conducted in South Africa, in the North West Province in particular, have revealed the presence of VRE in animal stools, water sources, food and in vegetables (Ramatlhape, 2006; Ateba and Maribeng, 2011; Ateba *et al.*, 2013). Considering the health risks that are associated with VRE isolates as well as the difficulties in managing infections caused by these organisms, the importance of this study cannot be over–emphasized. The present study was designed to expand on previous investigations and assist in the generation of data from the first comprehensive study involving the characterization of *Enterococcus* species for both antibiotic and virulence gene determinants. In addition, this is the first study in the area in which the genetic relatedness of enterococci especially VRE strains have been determined. Data obtained from this study may be of great epidemiological importance especially in bacteria source tracking.

In this study, *Enterococcus* species were isolated from both the ground and surface water sources (boreholes, rivers, dams and spring) in some parts of the North West province. Despite the fact that the study focused on the identification of *Enterococcus* species, some samples may have also contained other pathogenic enteric bacteria species that are known to cause severe complications in humans and animals. A total of 81 isolates were obtained from BEA supplemented with vancomycin and 56 were confirmed as either *E. faecium* or *E. faecalis*. A number of culture media have been assessed for their potential to isolate and presumptively differentiate enterococci from other genera based on macroscopic colonial morphologies. Bile esculin azide agar has been reported to have very good selectivity and pronounced growth of most Enterococcal strains producing results that are highly reproducible and with good electivity that is associated with their ability to hydrolyze esculin (Weiss *et al.*, 2005). Fifty six *Enterococcus* species were obtained in this study and *E. faecium* (67.9%) and *E. faecalis* (30.4%) which are most often associated with human diseases and contamination were dominant in the samples (Zirakzadeh and Patel, 2006; Valenzuela *et al.*, 2008). In addition, an *E. saccharolyticus* isolate was also detected in the water samples. Despite the fact that *E. saccharolyticus* is generally not associated with virulence in their hosts, a cause for concern is that this isolate was resistant to vancomycin. Similar observations have been reported in which *E. faecium* and *E. faecalis* were the two predominant species isolated from both ground and surface water bodies (Boyce *et al.*, 1994; Cattoir and Leclercq, 2013). Similar to the current findings *E. faecium* was also more frequently isolated when compared to *E. faecalis* in both ground (35.7%) and surface (32.1%) water sources (Boyce *et al.*, 1994; Harwood *et al.* 2001 Da Silva *et al.* 2006; Ramatlhape, 2006 Goldstein *et al.*, 2014) which then justifies the need for continuous environmental surveillance studies.

Another objective of this study was to determine the antibiotic resistance profiles of enterococci isolated with particular focus on VRE using both phenotypic and genotypic assays. Initially enterococci were isolated on BEA supplemented with Vancomycin. The isolates from the BEA were subjected to phenotypic antibiotic characterization based on their resistance patterns observed against the antibiotics tested. A total of 81 characteristic isolates were obtained on BEA supplemented with vancomycin and 56 isolates that were positively identified as enterococci. When subjected to the disc diffusion antibiotic assay a total of 45 (80.4%) isolates were resistant to three or more antibiotics. Amongst these MAR isolates a total of 38 (84.4%) were resistant to vancomycin.

Microbial resistance to glycopeptide antibiotics has recently increased and VRE traits are frequently detected among *Enterococcus* species even though vancomycin antibiotic is rarely used in human and veterinary medicine (Courvalin, 2009; CDC, 2011; Roberts *et al.*, 2016). Generally, antibiotics are used for treatment, chemotherapy and prophylaxis (Cattoir and Leclercq, 2013). Glycopeptides, particularly vancomycin was used as the treatment of choice against infections caused by multiple antibiotic resistant *Enterococcus* species as well as Methicillin Resistant *Staphylococcus aureus* (Patel and Gallagher, 2014; Arias and Murray, 2015; Rehaiem *et al.*, 2016; Thomson *et al.*, 2016). However, the application of glycopeptide antibiotics especially vancomycin led to the development of vancomycin resistance determinants in pathogenic, non-pathogenic and normal commensal bacteria stains as well as environmental isolates (Courvalin, 2009; Ateba *et al.*, 2013). In addition, vancomycin resistant determinants have the ability to be transferred among various related and unrelated bacterial strains (Borgen *et al.*, 2001; Cattoir and Leclercq, 2013). Motile genetic elements such as plasmids and transposons have been implicated in the transmission of vancomycin resistance determinants among bacteria strains (Cetinkaya *et al.*, 2000). It is therefore suggested that strains harbouring these as well as other resistance genes may serve as reservoirs for the dissemination of antibiotic resistance traits within a given area.

The impact or contribution of vancomycin resistance determinants in enhancing severity of Enterococcal infections in humans still remains a controversial issue. This is based on the premise that findings of a particular study indicated that bacteraemia caused by *Enterococcus* species that possess vancomycin resistance determinants was associated with refractory infection, serious morbidity and ultimately death in patients (Fowler *et al.*, 2015). In addition, bacterial isolates that possess MAR traits, especially those that code for resistance to vancomycin posed severe health infectious complications in humans resulting from the consumption of contaminated or improperly treated water (Micallef *et al.*, 2013; Carey *et al.*, 2016; Molale and Bezuidenhout, 2016). On the contrary, the findings of another study revealed that it is difficult to determine the direct impact of vancomycin resistance determinants on the outcome of Enterococcal infections in humans since there are a number of underlying factors in patients colonised by VRE strains that must be taken into consideration (Patel and Gallagher, 2014; Lisboa *et al.*, 2015). However, since antimicrobials are very valuable agents that are used for therapeutic purposes in humans and animals, their efficacy is seriously compromised by the emergence and spread of antimicrobial resistance determinants (Marshall and Levy, 2011; Cattoir and Leclercq, 2013). Given that there is the substantial and expanding volume of evidence on the continuous spread of resistant bacteria from animals and water to humans, there is a need to perform routine monitoring and surveillance studies in order to help reduce the growing environmental load of resistance genes.

In the present study, 45 (80.4%) isolates were resistant to three or more antibiotics. Amongst these MAR isolates large proportions (66.7-87.5%) of these isolates from different sampling areas were resistant to nalidixic acid. In addition, large proportions (66.7-81.3%) of the isolates from the different sampling sites were resistant to vancomycin. However, the proportion of VRE isolates obtained on BEA supplemented with at a concentration of 16 µg/mL was larger than those detected by agar disc diffusion antimicrobial susceptibility test in which the concentration of the antibiotic was 30µg/mL. Some previously conducted studies including the present one assessed the antimicrobial resistance profiles of enterococci against a panel of drugs that belong to different classes but are known to be highly effective against Gram positive bacteria (Ramatlhape, 2006). Despite the fact that the proportion of isolates that were resistant to these antibiotics varied among the different areas, detection of isolates that were resistant to vancomycin was a cause for concern. In previous studies, *Enterococcus* strains from water sources were reported to pose severe public health concerns in humans (Santiago-Rodriguez *et al.*, 2013; Molale and Bezuidenhout, 2016; Taučer-Kapteijn *et al.*, 2016). Vancomycin resistant *E. faecium*, *E. faecalis* and *E. saccharolyticus* isolated in the study especially from groundwater may have resulted through contamination with human faecal matter since boreholes are constructed very close to pit toilets and similar observations had been reported (Ramatlhape, 2006; Ateba and Maribeng, 2011).

The use of antibiotics in humans and in the production of animals is known to be the main contributor to the spread of antimicrobial resistant genes, through contamination of water sources (Ateba *et al.*, 2013; Goldstein *et al.*, 2014). Microorganisms that harbour antibiotic resistance determinants that possess intrinsic or acquired resistance to commonly utilized antimicrobial agents significantly contribute towards the transmission of antibiotic resistance genes to humans, related bacterial strains and animals (Borgen *et al.*, 2001; Klein, 2003; Moe *et al.*, 2007). In the present study, a large proportion (80.4%) of enterococci possessed multiple antibiotic resistances (MAR) traits. The MAR phenotypes VAN-NAL-AMP and VAN-NAL-AMP-OXYTET were among the *E. faecium* and *E. faecalis* isolated from the different sampling areas. It is therefore suggested that these resistance phenotypes may have resulted from previous exposure to these antimicrobial agents or related drugs as well as their derivatives. Similar justifications were made for isolates with MAR phenotypes from previous studies (Ramatlhape, 2006; Moneoang and Bezuidenhout, 2009).

Based on cluster analysis of antibiotic resistance data, the sub-cluster 2A contained isolates from all the different sampling areas except from Taung, and was considered a mixed cluster. This was a cause for concern because the linkage between VRE isolates from all the sampling areas and/or sources where samples were collected indicated that isolates shared similar antibiotic resistance phenotypes. Furthermore, this link is an indication that isolates from the different areas may have been exposed to the comparable antimicrobial agents.

A further objective of the study was to screen enterococci for the presence of selected vancomycin resistance genes (*vanA*, *vanB* and *vanC21/C2*) using multiplex PCR analysis (Harwood *et al.*, 2001; Depardieu *et al.*, 2004). The motivation for selecting these was based on the fact that both *vanA* and *vanB* genes are acquired and therefore easily transferred among bacterial strains while *vanC* gene codes for intrinsic resistance (Levine, 2006; Ranotkar *et al.*, 2014). In the presence study, 10 (17%) isolates possessed vancomycin resistance determinants based on genotypic analysis. These comprised three and seven isolates from ground and surface water samples respectively. The *vanA* resistance gene was dominant 9 (90%) in both *E. faecalis* and *E. faecium* isolates when compared to the other resistance determinants. In addition, an *E. faecalis* isolate from Mafikeng possessed both *vanA* and *vanB* resistance genes. These findings are in accordance with those of a previous study conducted in USA, in which high levels of vancomycin resistance associated with the presence of *vanA* was obtained among *E. faecalis* strains (Carey *et al.*, 2016). However, a recent study conducted in China also revealed that the *vanA* gene was dominant among *E. faecium* isolates (Yang *et al.*, 2015). It is therefore suggested that since both the *vanA* and *vanB* genes are acquired (Cetinkaya *et al.*, 2000; Ranotkar *et al.*, 2014) their presence in enterococci as well as other related bacterial strains largely depends on the availability of these determinants in a given ecological niche (Harwood *et al.*, 2000; Harwood *et al.*, 2001; Nam *et al.*, 2013; Teixeira and Merquior, 2013; Taučer-Kapteijn *et al.*, 2016) as well as the host bacteria susceptibility to these resistance genes. In addition, the *vanA* genotype confers high-levels of resistance to glycopeptides among *E. faecalis* and *E. faecium* (Levine, 2006; Ranotkar *et al.*, 2014). Against this background, constant monitoring and the implementation of strategies to help reduce contamination with these resistance determinants especially in water sources must be given serious attention. None of the isolates in the present study possessed the *vanC* resistance genes and this is due to the fact that *Enterococcus* species such as *E. gallinarum* and *E casseliflavus* that are associated with the *vanC1/C2* (Cetinkaya *et al.*, 2000; Ramatlhape, 2006; Ranotkar *et al.*, 2014; Sood and Perl, 2016) were not isolated in the present study.

An assessment of the occurrence of virulent gene determinants among the enterococci that were resistant to vancomycin was performed. The presence of five clinically relevant virulence gene determinants (*asa1*, *cylA*, *esp*, *hyl* and *gel*) was detected through phenotypic and genotypic characterization. Cytolysin production was detected in only five (11.4%) *E. faecalis* isolates phenotypically. In addition, the *gel* (36.4%) was dominant among the VRE isolates when compared to the *asa1* (31.8%), *hyl* (6.8%), and *esp* (4.5%) genes respectively. These findings are similar to those of a previous study in which the *asa1* gene was dominant and detected in 100% of *E. faecalis* isolated (Yang *et al.*, 2015). In addition, a study conducted by Molale and Bezuidenhout, (2016) revealed that the *cylA* gene was dominant and detected in 59% of the *Enterococcus* species isolated. However, in the present study, the *cylA* virulence gene was not detected in any of the *Enterococcus* species and these findings contradict previous reports (Vankerckhoven *et al.*, 2004; Yang *et al.*, 2015; Molale and Bezuidenhout, 2016). The presence of these virulence genes in a large proportion of the VRE strains isolated from ground and surface water in the current study was a cause for concern. These isolates, if they infect humans they may cause severe infections especially in rural communities.

An additional objective of this study was to determine the genetic relatedness of vancomycin resistant *Enterococcus* isolated from ground and surface water samples, using ERIC genetic fingerprints.

Despite the fact that there are very few studies (Ramatlhape, 2006) conducted determining the genetic relatedness of VRE strains, the great similarities between VRE isolates from the different water sources and geographical locations revealed that ERIC PCR typing can be a reliable tool for comparing genetic relatedness of the isolates and hence may be of great epidemiological importance. Band patterns of ERIC PCR analysis for ground and surface water indicated that the VRE strains originated from a common ancestral strain that might have been spread through cross contamination. These findings suggest the need to strictly monitor and also a need for the implementation of standard water safety measures in rural communities as well as the wastewater and water treatment facilities.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

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6.1 CONCLUSION AND RECOMMENDATION

This study analyzed the occurrence and prevalence of Vancomycin Resistant *Enterococcus* from ground and surface water samples. Few related studies from some parts of the province have been documented. However, to the best of our knowledge this is the first study that has been conducted in the North West Province, whereby *Enterococcus* species were identified, screened for VRE determinants, for virulence elements and their genetic relatedness was determined. Among the VRE strains analyzed, only *E. faecium*, *E. faecalis* as well as *E. saccharolyticus* species were confirmed through specific PCR and sequence data analysis. Due to the fact that *E. faecium* and *E. faecalis* are commonly recognized to be of clinical importance, theirs presence in water sources is a cause for concern. Although some other *Enterococcus* species might have been identified by morphological characterization, their identities were not confirmed by conventional PCR and sequence analysis. Analysis of antibiotic resistance patterns of *Enterococcus* isolates evaluated in this study revealed that a large proportion of the isolates are highly resistant to glycopeptides and amiglycosides antibiotics. Moreover, it was observed that VRE isolates derived from distinctive sources and districts shared related MAR phenotypes. Therefore it was concluded that there is high possibilities that VRE isolates obtained in this study may have previously been substantially exposed to these antimicrobial agents.

The evaluation of isolates for the presence of VRE genes revealed that both *E. faecium* and *E. faecalis* possess vancomycin resistance determinants. High levels of *vanA* and *vanB* acquired resistance genes were detected in these isolates. Nevertheless, *vanA* genes were prevalent among *E. faecalis* strains. The findings of this study showed that *Enterococcus* species isolated in the province possess VRE genes. Therefore, surface and ground water sources in the province are reservoirs of VRE strains and this is considered as a cause of concern. Despite the fact that four of the virulence genes were also observed, dissemination of carriers of both resistance and virulence determinants in environmental water exposes individuals to severe health risks, especially in rural communities and hospital settings. In addition, VRE are one of the primary causes of social and economic loss in humans globally. Considering the distribution of MAR VRE, a wise use of antimicrobial agents is therefore vital in human medicine, animal husbandry as well as in veterinary medicine. It is also recommended that ground and surface water intended for recreational and agricultural use be frequently tested for the existence of VRE determinants. Furthermore, the enforcement of strategic and effective intervention methods will also improve water quality and human health.

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APPENDICES

APPENDIX A

APPENDIX 1A: Details of materials; chemicals enzymes, reagents, and culture media used in this study.

1.1 CULTURE MEDIA

1.1.1 Bile Esculin Agar g/L

Peptone 14.0

Bile salts 15.0

Ferric citrate 0.5

Esculin 1.0

Agar 15.0

Forty five point five grams (45.5g) of the above components were dissolved in 1L of distilled water. The media was sterilized by autoclaving at 121°C for 15 minutes. Vancomycin (16µg/ml) antibiotic was added to the media after autoclaving for supplementing. The media was then distributed into90 mm Petri dishes. Bile Esculin Agar (OXOID, CM0888; South Africa) was used as a selective medium for VRE isolates.

1.1.2 Nutrient Broth g/L

Meat extracts 1.0

Yeast extract 2.0

Peptone 5.0

Sodium Chloride 8.0

Sixteen grams (16 g) of the above components were dissolved in 1L of distilled water. The media was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. Nutrient broth (Biolab, Merck Diagnostic, South Africa) was used as pre-enrichment medium for overnight cultures during DNA extraction.

1.1.3 Blood Agar g/L

Proteose peptone 15.0

Liver digest 1.5

Yeast extract 5.0

Sodium Chloride 5.0

Agar 12.0

Thirty seven point five (37.5 g) of the above components were dissolved in 1L of distilled water. The media was autoclaved at 121°C for 15 minutes. The media was then supplemented with 5% bovine blood. Blood agar (TMast, DM100D; South Africa) was used as a differential medium for evaluating Cytolysin production.

1.1.4 Mueller-Hinton Agar g/L

Meat Infusion 5.0
Casein Hydrolysate 17.5
Soluble Starch 1.5
Agar 14.0

Thirty eight grams (38 g) of the above components were dissolved in 1L of distilled water. The medium was distributed into McCartney bottles and autoclaved at 121°C for 15 minutes. The media was poured into plates and used to determine the antimicrobial susceptibility profiles of the isolates.

2.1 GENERAL CHEMICALS

2.1.1 Buffers (50 X TAE)

Thermo Scientific 50X TAE Electrophoresis Buffer (40mM Tris, 20mM Acetic Acid and 1mM EDTA) stock solution was supplied by Thermo Scientific, Johannesburg, South Africa. A 1X TAE buffer working solution was prepared and used for resolving either DNA or amplified PCR products by agarose gel electrophoresis.

2.1.2 Ethanol (70%) Alcohol

Absolute ethanol (99% v/v) was supplied by Merck, Diagnostics, South Africa. A 70% (v/v) working solution was prepared by aliquoting 750 ml of absolute ethanol into 1L Duran bottle and the volume adjusted to 1L by adding 300 ml of distilled water. The solution was stored at room temperature and used for sterilizing the working area.

2.1.3 Sodium Hypochlorite

A 10% (v/v) sodium hypochlorite (working solution) was prepared by aliquoting 10ml of sodium hypochlorite (stock solution) into 1L Duran bottle and the volume adjusted to 1L by adding 900ml of distilled water. The solution was stored at room temperature and used for disinfecting the working area.

3.1 DNA LOADING DYE (6X)

0.25% (w/v) bromophenol blue

0.25% (w/v) xylene cyanol FF

30% (w/v) glycerol

A working solution was prepared by mixing all the above agents into 50 ml Duran bottle. The solution was filter sterilized using 0.45 µm filter and stored at room temperature. The solution was used for agarose gel electrophoresis of extracted DNA or amplified PRC products.

3.2 ETHIDIUM BROMIDE

A stock solution of 10mg/ml was prepared in 5 ml Duran bottle by dissolving the powder in distilled water and the solution was protected by wrapping the bottle with a masking tape and stored at 4°C. A final concentration of 0.1 µl was used for visualising DNA and PCR products in electrophoresis gel.

3.3 ENZYMES AND CHEMICALS FOR DNA ISOLATION

3.3.1 Lysozyme (10 mg/mL)

3.3.2 CTAB (Sigma H-6269) isolation buffer

3.3.3 2% (w/v) hexadecyltrimethylammonium bromide, 1.4 M, NaCl, 0.1% (v/v) of β-mercaptoethanol

3.3.4 Chloroform: isoamyl alcohol at ratios of 96:4.

3.3.5 0.1 volume of 3 M sodium acetate (NaOAc) and 0.7 volumes of iso-propanol

3.3.6 Cold 70% (v/v) ethanol

3.3.7 TE buffer

3.4 PCR MASTER MIX (2X DREAMTAQ GREEN)

Thermo Scientific 2X DreamTaq Green Master Mix (0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP and 0.4 mM dTTP, 4mM MgCl2 and loading buffer) was used for PCR amplification of target genes. This was supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside; Pretoria, South Africa. The Master Mix was stored at -20oC.

3.5 OLIGONUCLEOTIDE PRIMERS

Primer sets used in this present study to amplify various genes encoding several determinants were synthesised and supplied by Inqaba Biotechnical Industries (Pty) Ltd, Sunnyside; Pretoria, South Africa. The primer sets (Forward and Reverse) were stored in separate tubes at -4 °C for future use. The working solution was prepared by aliquoting the required volume of forward and reverse primer set from the stock solution into a 1.5 µl sterile eppendorf tube.

3.6 DNA LADDER OR DNA MARKER

The standard DNA markers, O'GeneRuler 1 Kilo base pairs and GeneRuler 100 base pairs ranging from 250-10000 bp to 100-1000 bp fragments were supplied by Thermo Scientific Company and used to determine the relative sizes of all amplicons immediately during agarose gel electrophoresis

APPENDICES

APPENDIX B

APPENDIX 1B: Antibiogram data of isolates derived from the inhibition zone data

Sample ID	**Isolate ID**	**VAN**	**NAL**	**STR**	**CHIL**	**AMP**	**OXY-TET**	**GEN**	**NIT**	**SMX**
P(PDB)	T1	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
M(DbT1)	T3	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
M(DbT2A)	T4	Intermediate	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible
M(DbT2B)	T5	Resistant	Resistant	Intermediate	Susceptible	Resistant	Resistant	Intermediate	Resistant	Susceptible
M(DbT3A)	T6	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Intermediate	Resistant	Susceptible
M(DbT3B)	T7	Resistant	Resistant	Susceptible	Susceptible	Resistant	Resistant	Susceptible	Resistant	Susceptible
M(DbT5A)	T10	Resistant	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible
M(DbT5B)	T11	Susceptible	Intermediate	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible
M(DbT7A)	T14	Resistant	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
M(DbT7B)	T15	Resistant	Resistant	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible

M(DbT8A)	T16	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
M(DbT7B)	T17	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
R(ST2A)	T18	Resistant	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
R(ST2B)	T20	Resistant	Susceptible	Resistant	Intermediate	Resistant	Intermediate	Susceptible	Resistant	Intermediate
R(PDA)	T21	Resistant	Resistant	Resistant	Intermediate	Susceptible	Susceptible	Susceptible	Resistant	Susceptible
R(PDB1)	T24	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
R(PDB2)	T25	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
R(PDC1)	T27	Resistant	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Intermediate	Susceptible
R(PDC2)	T28	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Intermediate	Susceptible
M(DbT11)	T29	Resistant	Resistant	Intermediate	Intermediate	Susceptible	Resistant	Susceptible	Intermediate	Susceptible
M(DbT12)	T30	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Resistant	Resistant	Resistant	Susceptible
M(SwT1A)	T31	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
M(SwT1B)	T32	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
Z(DSpA1)	T33	Susceptible	Resistant	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible

Z(DSpB1)	T35	Resistant	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
Z(DSpB2)	T36	Intermediate	Intermediate	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Intermediate	Susceptible
Z(DSpC)	T37	Intermediate	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Intermediate	Susceptible
M(SDc)	T39	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible
M(LDc)	T40	Resistant	Resistant	Resistant	Intermediate	Resistant	Susceptible	Intermediate	Intermediate	Susceptible
C(CDd1)	T41	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
C(CDd2)	T42	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
P(MRd1)	T43	Resistant	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Resistant	Resistant
P(MRd2)	T44	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
T(HRDb1)	T45	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant	Resistant
M(MoT1)	T46	Intermediate	Resistant	Susceptible	Resistant	Resistant	Resistant	Susceptible	Susceptible	Susceptible
M(LT1A)	T48	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
M(LDA1)	T49	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible

R(PT7A)	T50	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
R(ST2b)	T52	Susceptible	Intermediate	Resistant	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Intermediate
R(ST2c)	T53	Susceptible	Resistant	Susceptible	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Susceptible
Z(ZKT1b)	T54	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Susceptible	Susceptible
R(PDd1)	T55	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Susceptible	Susceptible
R(PDd2)	T56	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Susceptible	Susceptible
R(GDA1)	T59	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible	Susceptible
R(GDA2)	T60	Resistant	Intermediate	Intermediate	Susceptible	Resistant	Intermediate	Susceptible	Resistant	Intermediate
R(GDB)	T61	Resistant	Intermediate	Susceptible	Intermediate	Resistant	Intermediate	Susceptible	Resistant	Susceptible
Z(DSpA2)	T63	Resistant	Resistant	Susceptible	Susceptible	Susceptible	Intermediate	Susceptible	Intermediate	Susceptible
Z(DSpA3)	T64	Resistant	Susceptible	Resistant	Susceptible	Susceptible	Intermediate	Susceptible	Resistant	Susceptible
M(DbT16)	T65	ResistanT	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
C(CDd1)	T67	Resistant	Resistant	Resistant	Resistant	Susceptible	Intermediate	Susceptible	Resistant	Susceptible
C(CDd2)	T68	Intermediate	Resistant	Resistant	Intermediate	Susceptible	Intermediate	Susceptible	Resistant	Resistant

C(CDE)	T70	Susceptible	Resistant	Resistant	Susceptible	Susceptible	Resistant	Intermediate	Intermediate	Susceptible
R(ST2d)	T73	Susceptible	Susceptible	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Susceptible	Susceptible
Z(ZKT1a)	T74	Intermediate	Resistant	Susceptible	Susceptible	Resistant	Susceptible	Susceptible	Intermediate	Susceptible
T(HRDb2)	T75	Intermediate	Intermediate	Susceptible	Susceptible	Susceptible	Susceptible	Intermediate	Intermediate	Susceptible
M(LDA2)	T77	Susceptible	Susceptible	Resistant	Intermediate	Susceptible	Susceptible	Intermediate	Intermediate	Susceptible

APPENDIX 2B: Predominant multiple antibiotic-resistant phenotypes for presumptive Vancomycin Resistant *Enterococcus* (VRE) isolated from surface and ground water in different sampling areas in the North West Province. Phenotypes were generated using abbreviations that occur in the antibiotic discs

Sample Area	Phenotype	No. Observed	% Observed
Mafikeng NT = 22, NO = 20	VAN-CHIL-NIT	1	4.5%
	VAN-NAL-STR-AMP-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-AMP-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-AMP-OXYTET-NIT	1	4.5%
	VAN-STR-NIT	1	4.5%
	NAL-STR-NIT	1	4.5%
	VAN-NAL-AMP	3	13.6%
	VAN-NAL-CHIL-OXYTET	1	4.5%
	VAN-NAL-STR-CHIL-OXYTET-NIT	1	4.5%
	VAN-NAL-OXYTET-GEN-NIT	1	4.5%
	VAN-NAL-OXYTET	2	9.1%
	VAN-NAL-CHIL-NIT	1	4.5%
	VAN-NAL-STR-CHIL-AMP-GEN-NIT	1	4.5%
	VAN-NAL-CHIL-AMP-OXYTET	1	4.5%
	NAL	2	9.1%
	STR-CHIL-GEN-NIT	1	4.5%
Rustenburg NT = 16, NO = 16	VAN-NAL-AMP	1	6.3%
	VAN-STR-CHIL-AMP-OCYTET-NIT-SMX	1	6.3%
	VAN-NAL-STR-CHIL-NIT	1	6.3%
	VAN-NAL	4	25%
	VAN-NAL-AMP-OXYTET-NIT	2	12.5%
	NAL-STR-CHIL-AMP-OYTET-NIT-SMX	1	6.3%
	NAL-CHIL-AMP-NIT	1	6.3%
	VAN-NAL-AMP-OXYTET	2	12.5
	VAN-NAL-STR-AMP-OXYTET-NIT-SMX	1	6.3%
	VAN-NAL-STR-AMP-OXYTET-NIT	1	6.3%
	AMP	1	6.3%
Potchefstroom NT = 3, NO = 2	VAN-NAL-AMP-OXYTET-NIT-SMX	1	33.3%
	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	33.3%
Taung NT = 2, NO = 2	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	50%
	VAN-NAL-GEN-NIT	1	50%
Zeerust NT = 8, NO = 8	NAL-STR-CHIL-AMP	1	12.5%
	VAN-NAL-CHIL	1	12.5%
	VAN-NAL-OXYTET-NIT	3	37.5%
	VAN-NAL-OXYTET	1	12.5%
	VAN-STR-OXYTET-NIT	1	12.5%
	VAN-NAL-AMP-NIT	1	12.5%
Coligny NT = 5, NO = 5	VAN-NAL	1	20%
	VAN-NAL-STR-CHIL-AMP-OXYTET-GEN-NIT-SMX	1	20%
	VAN-NAL-STR-CHIL-OXYTET-NIT	1	20%
	NAL-NAL-STR-CHIL-NIT-OXYTET	1	20%
	NAL-STR-OXYTET-GEN-NIT	1	20%
TOTAL **NT = 56, NO = 53**		**53**	**94.6%**

NT = Number Tested, NO = Number Observed

APPENDIX 3B: Phenotypic resistance of *Enterococcus* isolates for Vancomycin antibiotic from ground and surface water sources

Sample source	***Enterococcus* species**	**No. Isolated**	**No. Resistant to Vancomycin**	**% Observed**
Ground Water	*E. faecium*	20	15	75%
	E. faecalis	5	3	60%
	E. saccharolyticus	1	1	100%
TOTAL		**26**	**19**	**73.1%**
Surface Water	*E. faecium*	18	15	83.3%
	E. faecalis	12	10	83.3%
TOTAL		**30**	**25**	**83.3%**
OVERALL TOTAL		**56**	**44**	**78.6%**

APPENDIX 4B: Phenotypic resistance of *Enterococcus* isolates for Vancomycin antibiotic from different sampling area

Sampling Area	**No. Tested**	**No. Resistant to Vancomycin**
Mafikeng	NT	22
	NP	16 (72.7%)
Rustenburg	NT	16
	NP	13 (81.3%)
Potchefstroom	NT	3
	NP	2 (66.7%)
Taung	NT	2
	NP	2 (100%)
Zeerust	NT	8
	NP	7 (87.5%)
Coligny	NT	5
	NP	4 (80%)
TOTAL	**NT**	**56**
	NP	**44 (78.6%)**

NT= Number Tested, NR= Number Resistant