PHENOTYPIC AND GENOTYPIC CHARACTERISATION OF VANCOMYCIN RESISTANCE DETERMINANTS IN *Enterococcus faecalis* ISOLATED FROM GROUNDWATER IN MAFIKENG NORTH WEST PROVINCE, SOUTH AFRICA

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CHAPTER 1

INTRODUCTION AND RATIONALE FOR THE STUDY

1. INTRODUCTION AND PROBLEM STATEMENT

1.1. Introduction

Enterococcus species are Gram-positive, catalase-negative, non-spore-forming, facultative anaerobic bacteria, which usually inhabit the gastrointestinal tract of humans and animals (Fisher and Phillips, 2009; Péréz-Fontán *et al.,* 2011). The presence of enterococci in the environment usually results from human and animal dejections; hence these species are used as indicators of faecal pollution (Ahmed *et al.,* 2005). Initially, enterococci were classified as faecal streptococci that belong to the Lancefield group D-streptococci in the genus *Streptococcus.*However, its taxonomy and ecology were reviewed and the organisms were reclassified under the genus *Enterococcus.*The genus *Enterococcus* currently comprises 26 species and differentiation of these species using serologically based grouping may no longer constitute the best definition for these bacteria.Moreover, the term "faecal streptococci" is not a very reliable characteristic for describing these bacteria species. Therefore to look for enterococci it is important to utilise techniques that are accurate and specific (Reuter and Klein, 2003).

Identification of enterococci is of primary importance to differentiate them from other Gram-positive, catalase-negative cocci especially in situations of disease (Klein, 2003). Serological group D antiserum can be used for the differentiation of enterococci from the genus *Streptococcus*. However, a few *Streptococcus* species such as *Sc. bovis*, *Sc. alactolyticus* and *Sc. equinus* also belong to serogroup D serotype. Against this backdrop, the ability of enterococci to grow in 6.5% NaCl when compared to streptococci is currently considered an important distinguishing characteristic that facilitates indentification (Klein, 2003). However,

more reliable results are obtained when this test is combined with other phenotypic, PCR based and proteomic techniques.

Given the fact that enterococci may colonise humans, domestic and farm animals (Fisher and Phillips, 2009; Péréz-Fontán *et al.,* 2011), colonised individuals could be at risk of developing severe infections if proper hygiene and farm management techniques are not implemented. The majority of infections caused by enterococci are nosocomial with a higher prevalence being reported from the ICU, haemodialysis and oncology wards. Enterococci are therefore among the most common nosocomial pathogens and have been implicated as an important cause of endocarditis, bacteraemia, central nervous system infections, intra-abdominal and pelvic infections (Lopez *et al.,* 2009; Péréz-Fontán *et al.,* 2011). These organisms are also the leading cause of surgical site infections and the third most common cause of both bloodstream and urinary tract infections (Shepard and Gilmore, 2002).

Initially vancomycin was used as the last line of therapy for treating infections caused by enterococci. Vancomycin and teicoplanin are high-molecular-weight molecules that inhibit cell-wall synthesis of gram-positive bacteria by interacting with the C-terminal D-alanyl-D-alanine (D-Ala-D-Ala) of the pentapeptide of the peptidoglycan precursors (Cremniter *et al.,* 2006; Hartmann *et al.,* 2010; Ramaswamy *et al.,* 2013). The interaction between the DAla-D-Ala terminus and glycopeptide prevents transglycosylation and transpeptidation reactions needed for the normal polymerisation of peptidoglycan (Péréz-Fontán *et al.,* 2011). Vancomycin-resistant enterococci (VRE) were first reported in human medicine at the end of the 1980's in France and the United Kingdom (Lopez *et al.*, 2009). The development and emergence of strains resistant to vancomycin posed severe health consequences on susceptible hosts worldwide (Lopez *et al.,* 2009; Hartmann *et al.,* 2010).

Moreover, the ability of *Enterococcus* species to cause infections is amplified by their potential to exhibit multiple antibiotic resistance patterns. Despite the fact that multiple antibiotic resistance strains have been identified in nearly all pathogenic bacteria (Williams and Hergenrother, 2008), there is increasing evidence of the presence of multiple antibiotic resistant VRE strains in environmental samples (Foulquie′ *et al.,* 2006; Fisher and Phillips, 2009; Ateba *et al.,* 2013). This explains the need to constantly monitor the occurrence of these pathogens in both food and water that is intended for human consumption.

Transmission of VRE to humans is usually through faecal-oral route and contaminated food and water have been implicated in most reported outbreaks worldwide (Evers and Courvalin, 1996; Reynolds and Courvalin 2005). According to Mutnick *et al.*, (2003), resistance rates of *Enterococcus* species have reached endemic or epidemic proportions in North America, with Europe having lower, but increasing levels (Fisher and Phillips, 2009). One of the main reasons for the rapid rise in resistance is the capacity of enterococci to acquire and disseminate antimicrobial-resistant determinants, including those that confer resistance to aminoglycosides and glycopeptides (Simjee *et al.*, 2002).

Vancomycin-resistant phenotypes in enterococci are usually encoded by resistant determinates that portray different genotypic combinations (Talebi *et al.*, 2008). In enterococci, six vancomycin-resistant genotypes have been described and these include *vanA*, *vanB*, *vanC*, *vanD*, *vanE* and *vanG* (Talebi *et al.*, 2008). However, there is also the *vanF* glycopeptide resistant genotype that has been described, but it has not yet been detected in enterococci. The *vanA* and *vanB* resistant genes remain the genes of great concern due to the ease with which they are horizontally transmitted from one bacteria cell to the other (Zirakzadeh and Patel, 2006; Williams and Hergenrother, 2008; Fisher and Phillips, 2009;

Sujatha and Praharaj, 2012). Moreover, these genes are known to be associated with plasmids and transposable elements which make it easy for them to be transferred from host *Enterococcus* species to other gram-positive bacteria (Patel *et al.,* 1997; Zirakzadeh and Patel, 2006; Fisher and Phillips, 2009; Lopez *et al*., 2009). It was reported that in VRE, there were a set of genes, harboured on a transposon and these genes were involved in an inducible mechanism that resulted in resistance to glycopeptide antibiotics (Michel and Gutmann, 1997).

VanA and *vanB* are widespread globally and are responsible for the most prevalent glycopeptide resistance phenotypes (Reynold and Courvalin, 2005). The *vanA* phenotype is characterised by inducible, high-level resistance to vancomycin and teicoplanin (Park *et. al.* 2008). On the contrary, strains that exhibit the *vanB* phenotype also portray inducible resistance to vancomycin, but they are susceptible to teicoplanin (Lefort *et al*., 2003; Park *et al.,* 2008). Resistant phenotypes in *Enterococcus* species have frequently been associated with the acquisition of two gene clusters, *VanA* and *VanB* (Henrique *et al*., 2008). The *vanA* gene cluster is usually located within the transposon *Tn1546* and comprises of seven genes (*vanH, vanA, vanX, vanR, vanS, vanY* and *vanZ*), while the *vanB* gene cluster has mostly been associated with *Tn1547* and *Tn5382*-like transposons (including *Tn1549*) (Gilmore, 2002, Merquior *et al.,* 2012). The products of *vanA* gene cluster include transposase and resolvase (ORF 1 and ORF 2), *vanS* and *vanR* proteins, which are a response regulator and histidine kinase sensor, respectively. *VanH* and *vanA* synthesise the depsipeptide D-alanyl-D-lactacte, *vanX* hydrolyses D-alanine-D-alanine, and *vanY* hydrolyses the terminal D-alanine residue from the peptidoglycan precursor protein (Henrique *et al.,* 2008). *VanY* encodes a carboxypeptidase while the *vanZ* has an unknown function, although its expression is

associated with resistance to the antibiotic teicoplanin. This machinery ensures that resistance against vancomycin and teicoplanin is achieved (Clark *et al.,* 2005).

Detailed molecular analysis of *Tn1546*-like elements in enterococci isolated from human and animal sources have revealed the presence of different *Tn1546* subtypes. These differences are based on point mutations, presence of insertion sequence (IS) elements and some deletions (Schouten *et al.*, 2001). Various factors account for virulence of an *Enterococcus* species, particularly *E. faecalis*. Outstanding among those are the ability to produce cellular surface characters such as aggregation substance (*Agg*), extracellular surface protein (*Esp*) and some substances that are excreted out of the bacteria cell, known as cytolysin and hyaluronidase (Padilla and Lobos, 2013).

The *VanB* phenotype originally described in 1989 is characterised by high-level resistance to vancomycin and susceptibility to teicoplanin. It is determined by a cluster of genes, *vanRB*, *vanSB*, *vanYB*, *vanW*, *vanHB*, *vanB*, and *vanXB*. This gene cluster is highly associated with transposon Tn*1547*. In addition, the *VanB* genotype and resistance to vancomycin in isolates that harbour this transposon is regulated by a two component regulatory system, *VanRB-VanSB*. However, the acquisition of resistance to teicoplanin results from mutations in the *vanSB* glycopeptide sensor gene that leads to constitutive or teicoplanin-inducible expression of the resistant genes (Kawalec *et al.,* 2001; Lauderdale *et al.,* 2002; Lefort *et al.,* 2004).

The gene cluster operates in such a way that the genes *vanYB*, *vanW*, *vanHB*, *vanB*, and *vanXB* are transcribed together from promoter *PYB*, which is located upstream of *vanYB*, and this process is activated by *VanRB* in its phosphorylated form. *VanRB* phosphorylation is catalysed by the *VanSB* sensor histidine kinase in response to its stimulation by Vancomycin

Switching off the resistance genes on the other hand is due to phosphatase activity of *VanSB*, which dephosphorylates *VanRB* in the absence of vancomycin. Since teicoplanin fails to interact with *VanSB*, VRE strains with the phenotype *VanB* demonstrate susceptibility to this glycopeptides (Kawalec *et al.*, 2001).

Recently, baseline studies conducted in the Mafikeng area of the North-West Province – South Africa, indicated the presence of VRE in groundwater based on phenotypic and PCR assays (Ateba and Maribeng, 2011; Ateba *et al.*, 2013). Vancomycin and Teicoplanin are not used in both human and animal medicine in the area and therefore, the presence of these resistant determinants was a cause for concern. In the present study, the investigation is expanded to provide an overview of the occurrence of VRE in *Enterococcus faecalis* isolated from groundwater that is intended for human consumption in the North West Province. Emphasis is also placed on the identification of transposable elements *Tn1546* and *Tn1547* that are associated with the *vanA* and *vanB* genes clusters.

1.2. Problem statement

Vancomycin resistant enterococci are said to be one of the leading causes of nosocomial infections, especially in immunocompromised individuals (Naas *et al.*, 2005, Sood *et al.*, 2008). The virulence of VRE is also associated with their ability to thrive under a wide range of conditions and the ease with which these strains acquire antibiotic resistance determinants. It is very difficult to treat infections caused by VRE, especially if the causative strains harbour multiple antibiotic resistant genes for other antimicrobial agents (Hayes *et al.*, 2003). Despite the fact that vancomycin and other related glycopeptides such as teicoplanin are not used as treatment options in both human and animal medicine worldwide, recent baseline

studies conducted in Mafikeng have revealed the presence of VRE (Ateba and Maribeng, 2011; Ateba *et al.*, 2013).

The acquisition of glycopeptide, aminoglycoside and ampicillin-resistant phenotypes in enterococci, coupled with their ability to acquire both virulence and colonisation determinants, has shown that they can enhance the potential of these strains in causing disease and to disseminate (Rice *et al.,* 2001; Willems *et al.,* 2001). Given the fact that it is very difficult to manage infections caused by multi-drug-resistant *Enterococcus* strains coupled with the ability of vancomycin resistance determinants to spread rapidly among a population and cause severe health implications on individuals, there is need for continuous surveillance and control measures to be implemented to limit their dissemination.

Moreover, to better understand the epidemiology of VRE infections in humans that visit hospitals in the North West Province, there is need to further investigate the molecular characteristics of VRE isolates obtained in the area. Results obtained may be very useful in the management of VRE contamination and facilitate the urgent detection of point source contamination during outbreaks.

1.3. Aims and objectives of the research

1.3.1. Aim

The aim of the study was to determine the occurrence of VRE in groundwater samples and identify the presence of Vancomycin resistant determinants associated with transposable elements *Tn1546* and *Tn1547* in *vanA* and *vanB*-resistant *Enterococcus faecalis* isolated in the North West Province, South Africa.

1.3.2. Objectives

The objectives of the study were to:

- isolate of *Enterococcus* species from ground water samples;
- identify the isolates using conventional microbiological methods;
- confirm the identities of the *Enterococcus faecalis* isolates using bacterial 16S rRNA and species specific PCR analysis;
- determine the identities of randomly selected *E. faecalis* Matrix Assisted Lasser Desorption-Ionization –Time-Of-Flight Mass Spectrometry;
- screen isolates that are phenotypically resistant to vancomycin for the presence of the *vanA* and *vanB* resistant gene using specific PCR analysis;
- determine the occurrence of resistant determinants;
- screen *vanA* resistant *E. faecalis* for the presence of the transposon *Tn1546*;
- determine the presence of transposon *Tn1547* in *vanB* resistant *E. faecalis*.

CHAPTER 2

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Background

The history of enterococci began when Thiercelin (1899) first used the term "entérocoque" to describe *Enterococcus*, which referred to its intestinal origin and its spherical shape. The new genus *Enterococcus* was therefore, proposed by Thiercelin and Jouhaud in 1903. Later on, and according to Andrews and Horder, the isolates were renamed *Streptococcus faecalis* in 1906 (Lopez *et al.,* 2009). But it was not until 1984 when enterococci were sub grouped as a different group of *Streptococcus* species. The name was revised again when *Streptococcus faecalis* and *Streptococcus faecium* were transferred from the genus *Streptococcus* to *Enterococcus* due to new molecular information (Kahlmeter *et al.,* 2003; Klein, 2003).

Since then, entrococci have undergone important taxonomical changes in the last few years. These changes involved taking into consideration, the classification of serological group D streptococci (Devriese *et al.,* 1987). Nucleic acid studies have shown that *Streptococcus faecalis* and *Streptococcus faecium* are distantly related to *S. bovis* and *S. equinus*. These studies resulted in the proposal to transfer *S. faecalis* and *S. faecium* to a new genus *Enterococcus,* as *E. faecalis* and *E. faecium* (Devriese *et al.,* 1987). Other group D streptococci which belong to the enterococcal group have since been transferred to this genus, and new species have been added (Devriese *et al.,* 1987; Schlegel *et al.,* 2003; Carvalho *et al.,* 2004). *Enterococcus faecalis* and *Enterococcus faecium* then became clinically significant species known to cause majority of enterococcal infections (Naas *et al.*, 2005, Sood *et al.*, 2008). When clinical isolates of these enterococcal species with acquired vancomycin resistance began to appear in the late 1980s, it prompted significant changes in

testing, identification strategies employed against enterococci in clinical microbiology laboratories, infection control measures and the treatment of enterococcal infections (Cetinkaya *et al.*, 2000; Gold, 2001; Sood *et al.,* 2008).

The first vancomycin-resistant enterococcal strain was isolated in 1986 in Europe and since then, vancomycin resistance has spread amongst other enterococci species (Marques *et al.,* 2011). During that period, vancomycin was considered an effective drug for treating enterococci infections and other drugs such as gentamycin were also effective against enterococci. However, the appearance of high-level gentamicin resistance in clinical medicine had a substantial negative effect on the treatment of severe enterococcal infections. The only available antibiotic left thereafter, in the mid-1990s for the successful treatment of enterococcal infections, was vancomycin (Simjee *et al.,* 2002; Boneca and Chiosis, 2003).

Over the past decade, there has been a rapid rise in the prevalence of vancomycin resistance among enterococci, and this is predominantly caused by the *vanA* and *vanB* phenotypes (Simjee *et al.,* 2002). The *VanB* phenotype was first described in 1989 and was found to encode an inducible resistance protein. In 1990, the *vanA* gene was characterised and it was discovered to have d-Ala-d-Lac ligase activity (Marques *et al.,* 2011). The two phenotypes, *VanA* and *VanB*, which are responsible for either high-level or low-level glycopeptide resistance, have been described mainly in *Enterococcus faecium* and *Enterococcus faecalis* and are the most frequently encountered phenotypes (Michel and Gutmann, 1997; Jensen *et al.,*1998). Therefore, the acquisition of transposons containing the vancomycin resistance clusters that harbour the *vanA* or *vanB* genes are generally considered as the basis for emerging virulent enterococcal strains that are responsible for life threatening diseases in humans and animals worldwide (Willems and Bonten, 2007).

Enterococcus species are frequently isolated from the soil, plants, surface water and other environmental sources that are exposed to human and/ or animal faecal matter. *E. faecalis* has been used as an indicator of contamination with faecal matter from human or animal origin and is therefore considered an important agent for microbial source tracking (Aarestrup *et al.*, 2002; Domingo *et al.*, 2003; Kuhn *et al.*,2003; Fisher and Phillips, 2009). Moreover, *E. faecalis,* together with *E. faecium* have been linked to the increases in vancomycin-resistant enterococci (VRE) strains isolated from clinical samples (Domingo *et al.*, 2003; Kuhn *et al.*, 2003; Fisher and Phillips, 2009).

From 1989 to 1998, the National Nosocomial Infections Surveillance System was involved in collecting data on infections in patients in intensive care units (Gold, 2001). The data showed that enterococci were the third most common isolates from the bloodstream and urinary samples of infected individuals, the most common from surgical site infections and the fourth most common from all other infected sites (Gold, 2001). In another report by the National Healthcare Safety Network at the Centres for Disease Control and Prevention, released in 2006-2007, it was stipulated that *Enterococcus* species were the second most common pathogen in hospitals in the United States (Donabedian *et al.*, 2010). It has been suggested by another study that, in the United States, antimicrobial agents are used widely as food additives to improve growth and feed conversion in many types of animal operations, including poultry, swine and cattle (Macovei and Zurek, 2006; Clark *et al.*, 2012). As a result, antibiotic resistance in the bacterial communities in the intestinal tracts of domestic animals has become common (Macovei and Zurek, 2006; Clark *et al.*, 2012). Moreover, the widespread use of vancomycin and extended-spectrum cephalosporins in U.S. hospitals is likely to have contributed to the emergence and dramatic increase of vancomycin-resistant enterococci (VRE) over the past 20 years (Donabedian *et al.*, 2010). Given the fact that these

isolates cause problems in countries with more advanced public health and health care facilities, it is very important to implement strategies to limit their occurrence in the environment and hence reduce human contamination.

2.2. Epidemiology

2.2.1. Source tracking of resistant determinants

The discovery of antibiotics was the turning point in human history. Antibiotics have revolutionised medicine in many respects and countless lives have been saved (Davies and Davies, 2010). Over the years, selective pressure by different drugs has resulted in the evolution of bacterial pathogens associated with human diseases bearing additional types of resistance mechanisms that led to multidrug resistant strains. Two important aspects of significance have emerged from the studies of natural resistome. Firstly, the environmental microbiota contains a much larger number of resistant genes than those seen to be acquired by bacterial pathogens. Secondly, many drug-resistant determinants currently known, especially for glycopeptide resistance are probably the most complex (Alekshun and Levy, 2007; Davies and Davies, 2010; Martinez, 2012). Moreover, antibiotic resistance in human pathogens is primarily derived from mutation or horizontal gene transfer (Alekshun and Levy, 2007) and the major driving force has been the selective pressure of antibiotics used in medical therapy, veterinary practice, agriculture and animal farming. This correlates with the fact that in the United States, antimicrobial agents were used widely as food additives to improve growth and feed conversion in many types of animal operations, including poultry, swine and cattle operations. As a result, antibiotic resistance in the intestinal tracts of domestic animals has become common and risen to higher levels. It is therefore suggested that faecal matter from the gastrointestinal tracts of wild and domestic animals serve as a vehicle for horizontal transfer of antibiotic resistant genes to humans (Macovei and Zurek,

2006). However, another study conducted by Donabedian *et al.* (2010) presented contradicting facts that pointed out that glycopeptides have never been permitted for use in farms in the United States and VRE have not been isolated from food animals or retail meat in the United States. Moreover, VRE have only rarely been found in companion animals, the environment and humans without hospital exposure in the community (Donabedian *et al.*, 2010). In Europe a glycopeptide, avoparcin, was used as a growth promoter in animal feed and its use has been shown to create a reservoir for vancomycin-resistant *E. faecium* in animals (Jensen *et al.*, 1998). After the discovery that the glycopeptide avoparcin was commonly used as a growth promoter in agriculture, the hypothesis that VRE could spread from animals to humans *via* the food chain was raised (Jensen *et al.*, 1998). Since then, the European Union banned the use of several antibiotics including avoparcin, bacitracin, spiramycin, tylosin and virginiamycin as growth promoters in the animal industry However, avoparcin is not used as a growth promoter in the United States and no *VanA*-positive isolates at that time were found in animals or in healthy volunteers in the United States (Jensen *et al.*, 1998; Macovei and Zurek, 2006).

It is imperative to remember that, often, the genes that code for resistance phenotypes may have a role in inhibiting growth of bacterial competitors in the soil and the species responsible for infections are not producers of antibiotics themselves (Martinez, 2008). This then raises the question as to where the selective pressure really comes from. Could the problem reside with the farmers and the use of growth promoters like avoparcin or do these resistant genes originate from environmental factors such as the soil and water? Martinez (2012), has suggested that the resistant genes, especially those acquired through horizontal gene transfer by human pathogens, might have evolved in their original host. It is therefore

important to constantly monitor the occurrence of antibiotic resistance determinants in the environment, food and water sources and correlate data with the usage of drugs in the area.

2.2.2. Clinical significance of enterococci

Vancomycin-resistant enterococci species have been isolated as nosocomial pathogens and during the past 15 to 20 years, an increasing number of strains of *E. faecium*, which is much less common than *E. faecalis* in clinical material, became resistant to ampicillin and other penicillins and acquired high-level resistance to aminoglycosides. Vancomycin-resistant *E. faecium* has been found increasingly not only in hospitalized patients, but also in the healthy human population, in animals and sewage plants. After the detection of vancomycin- resistant *E. faecium* in sewage plants, animals, healthy humans and pet animals, it was hypothesised that the hospital environment was creating vancomycin-resistant *E. faecium*, which subsequently spread to the environment (Michel and Gutmann, 1997; Jensen *et al.*, 1998; Svec and Sedlacek, 1999; Harwood *et al.*, 2001).

So far, nine types (*vanA, vanB, vanC, vanD, vanE, vanG, vanL, vanM* and *vanN*) of glycopeptides (vancomycin and or teicoplanin) resistant classes have been characterised in enterococci based on both phenotypic and genotypic data (Hegstad, 2010; Xu *et al.*, 2010). Six of these classes (*vanA, vanB, vanC, vanD, vanE,* and *vanG*) correspond to acquired resistance; one type (*vanC*) is an intrinsic property of *E. gallinarum* and *E. casseliflavus* and it is frequently identified in human patients. *VanD* has been found in a few strains of *E. faecium*, *vanE* has been found in *E. faecalis* and *vanG* has been found in a few *E. faecalis* strains from Australia and Canada (Noskin, 1997; Jensen *et al.*, 1998; Courvalin, 2006). Tec *et al.* (2011) and Lebreton *et al.* (2011) have presented evidence that there are new types of resistant determinants named *vanM* and *vanN*. Thus far, *vanM* phenotype has only been

reported in China and it was discovered that the *vanM* DNA sequence displays some similarity to that of *vanA*. However, the organisation of its gene cluster is most similar to that of *vanD* (Xu *et al.*, 2010; Teo *et al.,* 2011, Lebreton *et al.,* 2011). The study conducted by Lebreton *et al.* (2011) was very significant because the determinant *VanN* was not just discovered but phenotypic and genotypic traits of *VanN* resistance were identified and their transferability assessed.

Of all the nine recognised vancomycin-resistant phenotypes, *vanA* and *vanB* are the most acquired and isolated (Jing *et al.,* 2013). However, *vanA* is the most common acquired glycopeptide resistance found among the enterococci. This is also the only type detected in *S. aureus* so far (Schouten *et al.,* 2001, Courvalin, 2006). The *vanA* resistance locus, which is most prevalent in *Enterococcus* species, consists of a cluster of seven genes (*vanS*, *vanR*, *vanH*, *vanA*, *vanX*, *vanY* and *vanZ*) present in the *Tn1546* transposon which is, 10.8-kb in size (Simjee *et al.,* 2002). The structure of the *Tn1546* transposon was studied and the predicted gene pattern on the *vanA* cluster published (Demertzi *et al.*, 1998). The same *Tn1546* profile was identified by (Biavasco *et al.*, 2007) in samples from pork and healthy humans suggesting a relationship between human and food VRE (Lopez *et al.,* 2009). *Tn1546* is often associated with plasmids and several reports have emphasised the fact that there are polymorphisms between *Tn1546* elements due to insertions, deletions and point mutations (Simjee *et al.,* 2002).

Although the use of vancomycin has played a role in the emergence of VRE, a transposon (*Tn1546*) that carries the Vancomycin-resistant genes, may be an adaptive response regardless of prior glycopeptide exposure (Noskin, 1997). Since the hospital is not the only source of VRE, many authors have raised the hypothesis of an animal reservoir for VRE

given that enterococci are part of the normal bowel flora of many animals (Michel and Gutmann, 1997, Cetinkaya *et al.*, 2000). The findings by Michel and Gutmann (1997) for VRE with indistinguishable ribotypes isolated from both human and non-human sources are in keeping with such a hypothesis. In addition, the changing epidemiology of nosocomial infections suggests that the number of infections caused by enterococci will continue to increase. The nature of the challenge rests in both the mechanisms by which enterococci cause disease and in the increasing resistance of enterococci to most, and in some cases, all antimicrobial agents currently approved to treat infections (Noskin, 1997; Shepard and Gilmore, 2002).

At the University Hospital Eppendorf, VRE were isolated from 38 patients between August 1993 and April 1997, of whom 32 were hospitalised at the Paediatrics Department. Pulsed-field gel electrophoresis revealed that 26 *E. faecium* isolates from patients of the Department of Paediatrics were identical or closely related, and that isolates from three additional patients of the same department were possibly related. All these isolates were of *vanA* phenotype and resistant to glycopeptides, ampicillin, ciprofloxacin, clindamycin and erythromycin. Most isolates displayed high-level resistance to gentamicin, but were all susceptible to quinupristin/dalfopristin. It is note-worthy that, vancomycin-resistant enterococcal infections and colonisation are usually seen in the most debilitated patients who require prolonged hospitalisation (Noskin, 1997; Elsner *et al.*, 2000).

2.3. Pathogenicity

2.3.1. Route of transmission

Enterococci are opportunistic pathogens which are harmless in healthy individuals and mainly cause infections in patients who are in intensive care units, who have severe

underlying disease, or who are immunocompromised (Rathnayake *et al.*, 2012). It was established by Noskin (1997) that proximity to another patient with VRE or exposure to a nurse caring for an infected patient were the most important risk factors for acquisition of the organism. This suggested that nosocomial transmission from patient to patient was done by nurses. Additional evidence supporting nosocomial transmission was reported in an outbreak of VRE bacteraemia among oncology patients. Using molecular typing of bacterial DNA brought about the conclusion that, the majority of their patients became colonized or infected with VRE *via* nosocomial transmission (Noskin, 1997; Weinstein, 2001). The spread of infectious enterococci from the hospital environment or other sources to environmental water bodies through sewage discharge or other means, could increase the prevalence of the enterococci strains in the human population and become a potential risk to human health (Rathnayake *et al.*, 2012).

Many studies have addressed the importance of enterococci as a reservoir of antibiotic resistant genes in the environment. However, less information is available about enterococci from the food safety perspective, particularly for ready-to-eat food (Macovei and Zurek, 2006). Enterococci of food-borne origin have not been conclusively identified as direct causes of clinical infections but, the consumption of meat carrying antibiotic-resistant bacterial populations is a possible route of transmission and could result in either colonisation or transfer of resistant determinants to host-adapted strains (Hayes *et al.*, 2003). Macovei and Zurek (2006) depicted the significance of insects, particularly houseflies and the ecology of antibiotic resistant and virulent genes of enterococci from houseflies collected in food settings. Their findings were that, insects such as houseflies (*Musca domestica* L.), that develop in animal manure and other decaying organic materials can play an important role in the ecology and dissemination of bacteria in agricultural and urban environments.

Houseflies are also a significant factor in the transmission of this pathogen. It has been revealed that houseflies can carry potential pathogens, such as *Yersinia pseudo tuberculosis*, *Helicobacter pylori*, *Campylobacter jejuni*, *Escherichia coli* O157:H7 and *Salmonella* species. Several studies have shown that there was a positive correlation between the incidence of food-borne diarrhoea and the density of fly populations. For example, suppression of flies in military camps in the Persian Gulf resulted in an 85% decrease in shigellosis and a 42% reduction in the incidence of other diarrheal diseases (Macovei and Zurek, 2006). Moreover, the high prevalence of VRE in the gastrointestinal tracts of many food animals is often unavoidable, that these organisms may enter the human food chain *via* contamination of raw milk and meat (Huys *et al.*, 2004).

2.3.2. Antimicrobial resistance

Resistance to glycopeptides was not reported for approximately 30 years after the introduction of vancomycin into clinical practice. This was due to the limited use of the antibiotic until the mid-1970s. The lengthy period was also due to the difficulties experienced by bacteria in developing mechanisms of resistance to an antibiotic which binds to an essential substrate in a biosynthetic pathway rather than to a protein or nucleic acid (Reynold and Courvalin, 2005).

The basic mechanism of vancomycin resistance in enterococci is the formation of peptidoglycan receptors with reduced glycopeptide affinity. This results in decreased binding of vancomycin and decreased inhibition of cell wall synthesis (Sujatha and Praharaj, 2012; Jing *et al.*, 2013). Both the *vanA* and the *vanB* phenotypes share a common molecular basis of resistance (Michel and Gutmann, 1997). In both *VanA*- and *VanB*-type resistances,

vancomycin resistance is due to synthesis of peptidoglycan precursors ending in the depsipeptide d-Ala-d-Lac instead of d-Ala-d-Ala (Marques *et al.*, 2011).

VanA and *vanB* vancomycin resistant genes can be transferred by conjugation among enterococci and to other gram-positive bacteria such as *Streptococcus pyogenes, S. sanguis, Listeria monocytogenes* and *S. bovis*. More alarming, is the report of an in vitro transfer of vancomycin resistance from *E. faecalis* into *S. aureus,* with MICs of vancomycin for transconjugant *S. aureus* clones reaching 1000 μg/ml (Mouthon *et al.*, 1997).

Studies on vancomycin-resistant enterococci have shown high clonal diversity, indicating that horizontal gene transfer to some extent plays a part in the dissemination of vancomycin-resistance (Jensen *et al.*, 1998). The *vanA* and the *vanB* gene clusters encoding high-level glycopeptide resistance are located on the mobile DNA elements, *Tn1546* and *Tn1547,* respectively.

The *vanA* phenotype or rather its cluster (*vanR*, *vanS*, *vanH*, *vanX*, *vanY*, and *vanZ*) is involved in the regulation and expression of vancomycin resistance and the cluster is harboured within the transposon *Tn1546* and can be part of the chromosome, on non-conjugative or conjugative plasmids. The *VanB* phenotype, mediated by the *vanB* gene cluster, is characterised by inducible resistance to various levels of vancomycin and susceptibility to teicoplanin. The *vanB* gene cluster can reside on a composite transposon, Tn*1547*, but is not always linked to IS*16*- or IS*256*-like elements, which characterise Tn*1547* (Dahl *et al.*, 1996; Cetinkaya *et al.*, 2000). Both clusters possess accessory proteins that do not contribute detectably to vancomycin resistance (Marques *et al.*, 2011). By investigation of selected vancomycin-resistant *E. faecium* isolates, variations in this element have been

found. Molecular characterisation of the *vanA* gene cluster could therefore, provide additional information regarding the variation or identities of isolates of different origins and could allow for epidemiological studies of the dissemination of vancomycin resistance due to horizontal gene transfer (Jensen *et al.*, 1998).

Following the publication in 1988 of reports of the first vancomycin-resistant enterococci, it was predicted that resistance might arise by one of four possible routes, which were inactivation of the antibiotic, sequestration of the antibiotic in the outer cell wall layers by specific and nonspecific binding, by an increased production of those intermediates to which Vancomycin binds, or by a change in the target site. It was later recognised that the last mechanism would involve not only a new pathway to achieve a change of target, but also, elimination of at least part of the normal susceptible pathway (Reynold and Courvalin, 2005).

The *vanA* phenotype is determined by seven *van* genes present on Tn*1546*-type transposons, located immediately downstream of genes designated *orf1* and *orf2*, which are associated with transposition functions (Naas *et al.*, 2005). There are three *van* genes that are essential for expression of the vancomycin resistant phenotype, and they are: *vanA, vanH*, and *vanX* (Naas *et al.*, 2005). Expression of all these genes together is required for resistance. *VanA* alone cannot confer resistance to Vancomycin, probably because D-hydroxy acids such as D-Lac are neither natural products present in the environment of enterococci nor normally produced by enterococci. Thus, to synthesise D-lactate, enterococci must acquire the gene(s) within the *vanA* operon required to produce the substrate for *vanA*. *VanH* is responsible for the synthesis of D-lactate. In strains with the *vanA* gene cluster, the action of a signal molecule on the extracellular domain of the *vanS* protein is believed to lead to the activation of the *VanR* response regulator protein, increasing its activity as a transcriptional activator of

structural genes encoding enzymes for peptidoglycan precursor synthesis. However, the exact identity of the signal molecule acting on the *vanS* membrane sensor has not been determined (Lai and Kirsch, 1996; Cetinkaya *et al.*, 2000).

Another explanation of how *vanS* and *vanR* operates comes from a study conducted by Cetinkaya *et al.*, 2000. It was elucidated in the study that, *vanR* and *vanS* proteins constitute a two-component regulatory system that regulates the transcription of the *vanHAX* gene cluster. *VanS* apparently functions as a sensor to detect the presence of Vancomycin or, more likely, some early effects of vancomycin on cell wall synthesis. *VanS* then signals *vanR*, the response regulator, which results in activation, or turning on, of the synthesis of some other proteins (*vanH*, *vanA*, and *vanX*) involved in resistance (Cetinkaya *et al.*, 2000).

The *vanB* gene cluster is usually carried by large conjugative elements (90-250 kb) which are transferable from chromosome to chromosome between *Enterococcus* species, suggesting that *vanB* resistant genes are carried by conjugative transposons (Quintiliani and Courvalin, 1996). Reports have shown DNA sequence heterogeneity suggesting three subtypes of the *vanB* ligase gene: *vanB1*, *vanB2* and *vanB3*. The *vanB1* gene has previously been designated *vanB*. However, the potential differences in the organisation and structure of the *vanB* gene clusters in genomically diverse vancomycin-resistant enterococcus (VRE) strains have not been examined extensively (Dahl *et al.*, 1996). The *vanB* cluster is organised and functions in a manner similar to *vanA*, differing mainly in that, unlike the *vanA* operon, it is induced by vancomycin but not teicoplanin. The *vanB* proteins, *VanHB*, *VanB* and *VanXB*, exhibit a high level of sequence identity (67–76%) with corresponding proteins of the *vanA* operon (Marques *et al.*, 2011).

Another difference between *VanA*- and *VanB*-type resistances is that, *VanA* is more widely distributed and is by far the predominant type of resistance reported in Europe. While *VanB* strains are fairly common in the United States, with some hospitals reporting *VanB* exclusively, *VanA* still predominates (Cetinkaya, *et. al* 2000). The difference in the dissemination of these resistance traits may be related to the observation that the *vanA* gene cluster is often located in a transposon similar to Tn*1546*, which, in turn, can be part of a conjugative (transferable) plasmid. Such a genetic arrangement is an excellent avenue for the dissemination of these genes. The *vanB* cluster is often located in the host chromosome and initially was thought not to be transferable to other bacteria. However, it can also occur on plasmids and even when it is chromosomal (Ligozzi *et. al* 1998; Cetinkaya, *et. al* 2000; Sood *et al.,* 2008).

2.3.3. Microbiological characterization of the species

Enterococcus species are facultative anaerobic, gram positive ovoid cocci shaped bacteria (Murray, *et al* 1990; Fisher and Phillips, 2009; Hollenbeck and Rice, 2012). They grow at a temperatures ranging between 5°C and 50°C and that includes the average human temperature of 37°C (Fisher and Phillips, 2009). *Enterococcus* species are catalase negative. However, it has been established that there may be variations between *E. faecium* and *E. faecalis.* There have been times when *E. faecium* isolates were found to be catalase negative and *E. faecalis* were catalase positive (Devriese and Pot, 1995; Moy *et al.,* 2004). *Enterococcus* species have the ability to grow at a pH 9.6 and in saline conditions (6.5% NaCl) (Messer and Dufour, 1998). The tolerance of enterococci to bile and its ability to breakdown is an indication of the presence of *Enterococcus* species, hence bile esculin agar (BAE) is used for growth of enterococci (Chuard and Reller, 1998; Messer and Dufour, 1998; Domig *et al.,* 2003).

However, Levin *et al.* (1975) suggested that Esculin Iron (EI) agar be used as an alternative for the cultivation and identification of enterococci (Domig *et al.,* 2003).

In a study, the researchers tried to find correlation among the taxonomic position of enterococcal strains and the colour, size or form of their colonies on Slanetz-Bartley agar after identification. The results obtained showed there was no relation. From a total of 523 identified enterococci, 345 strains formed purple coloured colonies, 136 red colonies, 37 pink colonies and only 5 were cream coloured colonies. The exception was *E. faecium* morphological structure of the colony that was somewhat different from the colonies formed by the other isolated species. The *E. faecium* had a very wide spectrum of various combinations of colours, sizes or forms of colonies formed by those species on Slanetz-Bartley agar. It was concluded that, identification of isolates as *E. faecium* may not be as reliable because of phenotypic similarities among *E. faecium* and other species (Leclerc *et al.* 1996; Devriese *et al.* 1993; Merquior *et al.,* 1994). This was an indication that the taxonomic position and description of *E. faecium* is not strict and that isolates which are determined as *E. faecium* could include other enterococci (Svec and Sedlacek, 1999).

Enterococci are important indicators of faecal pollution of waters and their presence is commonly monitored during the microbiological testing of water. The detection of enterococci (and other "faecal streptococci") in drinking water is carried out by membrane filtration, which is a suitable method for the examination of drinking and bottled water. Another method for detection is by enrichment in a liquid medium (Svec and Sedlacek, 1999; Noble *et al.,* 2003). The membrane filtration technique is conventionally used to enumerate faecal enterococci and is one of the reliable techniques in the identification of species including enterococci (Domingo *et al.* 2003; Domig *et al.,* 2003).

Polymerase chain reaction (PCR) assay is another method used to detect vancomycin resistant species and or genes including *vanA* and *vanB*. Primer pairs and amplification conditions previously defined by Clark *et al.* (1993) and Klein, (2003) are used for the amplification and characterisation of species and genes. Template DNA needs to be prepared with the illustrate kits like Bacteria genomic Prep Mini Spin kit (GE Healthcare Bio-Sciences Corp, Piscataway, NJ). The boiling method and/ the CTAB method are also effective in DNA extraction (Merquior *et al.*, 2012).

Molecular genetics techniques, such as randomly amplified polymorphic DNA analysis, intergenic ribosomal PCR, or other PCR-based methods targeting various genes, have been successfully used to identify enterococci at the species level. Although these techniques are specific and sensitive, it is difficult to adapt them for use in routine laboratories due to their high costs and the requirement for highly skilled personnel. Infection control and epidemiological studies primarily require rapid and simple means of identifying and typing clinical isolates (Kirschner *et al.*, 2001). Many studies have used the disk diffusion test to establish the resistance of species or genes to antimicrobial agents. Disk diffusion test is a method that is conventionally used and is performed on Müeller-Hinton agar according to Clinical and Laboratory Standards Institute guidelines (CLSI 2010). The minimum inhibitory concentrations (MICs) for Vancomycin, Streptomycin, Gentamycin and other antibiotics are determined using the Etest (AB Biodisk, Solna, Sweden) (Merquior *et al.*, 2012).

Taking into consideration every aspect, there is a great need for rapid and accurate identification of enterococci at the species and subspecies level as a means of effectively assisting infection control and epidemiological studies. For most clinical microbiological laboratories, the primary method of identifying *Enterococcus* strains relies on phenotypic

characterisation. However, various studies have shown that an unequivocal species identification of enterococci by phenotypic means is a challenging procedure that can take several days to accomplish because of the phenotypic and biochemical similarities between many enterococci. In addition, the automated systems currently in use often fail to accurately identify rare species (Kirschner *et al.*, 2001). Although culture-based methods in bacteriology are widely used, they are time-consuming and provide little clinical information regarding the pathogen's genotype, including antibiotic resistance genes and virulence factors. Molecular methods using DNA microarrays show great potential in research, food safety, medical, agricultural, regulatory, public health and industrial settings. Other molecular typing methods, such as pulsed-field gel electrophoresis (PFGE), arbitrarily primed PCR (AP-PCR), and multilocus sequence typing (MLST), have been used to compare pathogens (Diarra, 2010). Moreover, the application of techniques such as vibrational spectroscopic (Fourier transform-infrared [FT-IR] and near-IR Raman spectroscopies) are techniques used as an alternative to conventional methods (Kirschner *et al.*, 2001; Lam *et al.*, 2012).

Among all the typing methods for examining relatedness of bacterial genetic backgrounds, multilocus sequence typing (MLST) is frequently employed in molecular epidemiological analyses of *E. faecium* strains. Analysis by eBURST suggests the emergence of a lineage, termed clonal complex 17 (CC17), that appears to represent a hospital adapted subpopulation of *E. faecium* strains, as they have been associated with clinical infections and *E. faecium* outbreaks on five continents. Strains belonging to CC17 are proposed to possess particular traits for enhancing persistence in the health care environment, including acquiring ampicillin and quinolone resistance, and a pathogenicity island that commonly harbours the *esp* gene encoding the putative virulence factor enterococcal surface protein (Nallapareddy *et al.*, 2002; Homan *et al.*, 2002; Nallapareddy *et al.*, 2005; Lam *et al.*, 2012).

Vibrational spectroscopies are also easy to use and may become very cost-effective, because they enable considerable reduction in sample handling and use of reagents and do not require highly skilled personnel. These methods allow the discrimination of intact microbial cells without their destruction and produce complex biochemical fingerprint-like spectra which are reproducible and distinct for different microorganisms. Various studies have shown that vibrational spectroscopy provides sufficient resolution power to distinguish microbial cells at different taxonomic levels, even at the strain level. These techniques are rapid because little biomass is needed and they reduce culturing time significantly (Kirschner *et al.*, 2001; Ehling-Schulz and Messelhäusser, 2012).

Recent molecular methods for microbial identification, such as real-time PCR, sequence analysis, or microarray analysis, have found significant application in bacteriology. However, these methods do not provide the complete solution in routine bacterial identifications. A new revolution in the identification of bacteria and fungi is ongoing with the introduction of mass spectrometry (MS) in the form of matrix-assisted laser desorption ionisation–time of flight (MALDI-TOF) MS. The application of microbial identification based on species-specific spectra of peptides and protein masses by mass spectrometry was first reported about 30 years ago. Through further improvement of the technique, a rapid, accurate, easy-to-use and inexpensive method has become available for identification of microorganisms. This technique has been used for identification of various gram-negative and gram-positive microorganisms, including *Enterobacteriaceae*, non-fermenting bacteria, mycobacteria, anaerobes and yeasts identification. It has been found to be very accurate and rapid in its identification. The effectiveness of this technique depends on the recognition of peak patterns characteristic of and mostly constant for different taxa and the reference strains included in the MALDI Biotyper database. MALDI-TOF MS is a promising technique for the

identification of strains and is indeed a major contribution to clinical microbiology (van Veen *et al.,* 2010; Christensen *et al.,* 2012; Edouard *et al.,* 2012; Samadi, 2013; da Silva Paim, 2013).

2.4. Treatment

Enterococci are either intrinsically resistant when the resistance genes are located in the chromosome, or they possess acquired resistance determinants located on plasmids or transposons. This suggests that the treatment of enterococcal infections could be difficult to treat as they possess intrinsic resistance determinants to many antibiotics. (Kirschner *et al.*, 2001; Rathnayake *et al.*, 2012).

The emergence of glycopeptide resistant enterococci is a major problem because it leaves few options for treatment. The vancomycin resistant genes are transferable to other species, including *S. aureus*, and selection pressure for the VRE may give rapid expansion of resistant populations. In addition, once the problem with VRE has been established, it is difficult to treat (Mundy and Gilmore, 2000). It is even harder to treat when isolates harbouring *vanA, vanB*, and *vanM* are resistant to high levels of vancomycin (>128 mg/liter). Moreover, *vanA* and *vanM* isolates are resistant to high levels of teicoplanin, although exceptions have been noted. The *vanB* gene cluster, on the other hand, produces little or no resistance to teicoplanin (MIC, <1 mg/liter). *VanD* strains are resistant to moderate levels of vancomycin (MIC, 16 to 128 mg/liter) and susceptible to teicoplanin, while *vanC, vanE, vanL,* and *vanG* strains exhibit low-level resistance to vancomycin (Teo *et al.,* 2011). Despite the VRE endemic being global problem it is important to emphasise that vancomycin resistance is not the only challenge the world faces. Enterococci are becoming gradually resistant to macrolides,

amphenicols, fluoroquinolones, aminoglycosides and even new antibiotics such as oritavancin (Lefort *et al.*, 2000; Arias *et al.*, 2010; Guskey and Tsuji, 2010).

Progress in medical technology and treatment, such as the use of various intravascular access devices, implanted prosthetic devices, cytotoxic chemotherapy and immunosuppression, have magnified the impact of organisms of relatively low virulence, such as enterococci. Of critical importance is the intensive use of relatively broad-spectrum antibiotics in the hospital, which provides selective pressure favouring the growth of intrinsically drug-resistant commensal organisms such as enterococci (Gold, 2001). It is generally admitted that treatment of systemic enterococcal infections is based on the synergistic bactericidal combination of a cell-wall-active antibiotic (such as amoxicillin or glycopeptides) plus an aminoglycoside, usually gentamicin or streptomycin (Michel and Gutmann, 1997).

The virulence of enterococci is associated with several genes, including *ace* (collagen binding cell wall protein), *acm* (surface-exposed antigen), *agg* (aggregative pheromone-inducing adherence to extra-matrix protein), *agrBEfs* (AgrB protein of *E. faecalis*), *esp* (enterococcal surface protein), *hyl* (hyaluronidase), *cad1* (pheromone cAD1 precursor lipoprotein), the cAM373 gene (sex pheromone cAM373precursor), the cCF10 gene (pheromone cCF10 precursor lipoprotein), *cob* (pheromone cOB1 precursor/lipoprotein, YaeC family), *cpd1* (pheromone cPD1 lipoprotein), *cylABLM* (hemolysin), *efaAEfs* (endocarditis-specific antigen), *sagA* (secreted antigen) and *gelE* (gelatinase). These virulence factors have been reported in enterococci isolated from food of animal origin (Diarra *et al.*, 2010). Moreover, the virulence factor, Enterococcal surface protein (*Esp*) plays an important role in the pathogenicity of enterococci, produced by *E. faecalis* and *E. faecium*. The presence of the *esp* gene contributes to colonisation and persistence of *E. faecalis* and *E. faecium* in host tissue

(Rathnayake *et al.*, 2012). A greater challenge to therapeutic measures and public health is the ability of enterococci to acquire resistance to antimicrobial agents through transfer of plasmids and transposons, chromosomal exchange, or mutation. Through such mechanisms, enterococci have acquired additional resistances to high concentrations of β-lactams and aminoglycosides, as well as teicoplanin (Shepard and Gilmore, 2002). The situation is further complicated by the fact that, enterococci have also developed a number of mechanisms for the transfer of resistance genes. Therefore, perhaps the greatest threat posed by VRE comes not from these organisms themselves but from the potential that they could transfer their resistant genes to other more pathogenic gram-positive bacteria, thus creating a highly dangerous pathogen difficult to treat with currently available antibiotics (Kirschner *et al.*, 2001). The presence of virulence factors associated with enterococci enhances their pathogenicity and triggers the pathogenicity of the infecting strains by allowing the colonisation of host tissue, invasion of host tissue, translocation through epithelial cells and evading the host's immune response. In addition, virulent strains produce pathological changes either directly by toxin production or indirectly by inflammation (Rathnayake *et al.*, 2012).

Previous studies have shown that a combination therapy with a cell-wall active agent plus an aminoglycoside improves the outcome of enterococcal endocarditis but may not improve the outcome in bacteraemia (Mouthon *et al.,* 1997). It is worth acknowledging that high-level resistance to β-lactam antibiotics is, in this species, an intrinsic mechanism due either to the overproduction of the essential target, the low-affinity PBP5, or mutations of different residues at its active site (Michel and Gutmann, 1997).

In addition, enterococci carry a chromosomal gene encoding an aminoglycoside-modifying enzyme that prevents synergy between cell wall-active agents and the aminoglycosides tobramycin, kanamycin and netilmicin. Although the combination of trimethoprim and sulfamethoxazole may appear to be active against enterococci in vitro, the microorganisms are presumed to be clinically resistant by virtue of their ability to use exogenous folate, thus circumventing the mechanism of action of those drugs (Gold, 2001). Due to the intrinsic glycopeptide resistance and the development of multidrug resistance, therapy for patients is limited. In fact, many of the glycopeptides-resistant strains are untreatable. Therefore, vancomycin resistance represents new challenges in the diagnosis, treatment and control of enterococcal infections (Noskin, 1997).

2.5. Prevention

Antibiotic resistance is a worldwide problem that threatens the successful treatment of hospitalised patients. Multiple strategies to limit the spread of antibiotic-resistant pathogens, either as single or bundled interventions have emerged. Some proposed strategies include: the need to recognise the problem and develop strong public health policies which include surveillance nationally, regionally and at the hospital level (Hayden *et al.*, 2006; Savarda and Perla, 2010). Other methods include active surveillance to identify and isolate colonised patients, efforts to increase hand hygiene adherence, modifications of antibiotic policies and the routine use of gloves for patient care. Interpretation of many studies has been hampered because, the studies were performed during outbreaks, involved implementation of multiple interventions simultaneously or failed to determine relevant variables and to account for interdependency of observations in statistical analyses (Weinstein, 2001; Hayden *et al.*, 2006; Savarda and Perla, 2010).

In response to the dramatic increase in vancomycin-resistance in enterococci, the CDC Hospital Infection Control Practices Advisory Committee (HICPAC) proposed some recommendations (Sood *et al.,* 2008). It was compelling for these recommendations to be made because of the data that demonstrated widespread and persistent environmental contamination by enterococci of surfaces and medical instruments, despite rigorous protocols for cleaning and sterilisation (Shepard and Gilmore, 2002). The recommendations included prudent use of vancomycin; the appropriate use of oral and parenteral vancomycin policies; discouraging the use of third-generation cephalosporins; and agents most likely to cause *C. difficile* colitis were encouraged. Moreover, it was recommended that hospital staff should be educated and that there should be effective use of the microbiology laboratory (Sood *et al.,* 2008). Clinicians also have a responsibility to prevent the selective growth of resistant mutants: pharmacokinetic/ pharmacodynamic *in vitro* model experiments show that when the antibiotic concentration is maintained in the vicinity of the MIC, resistance occurs more readily. Thus, antimicrobial drug selection and dosing regimens should be based on the drug's pharmacokinetic/ pharmacodynamic (PK/PD) parameters (Jing *et al.,* 2013).

Another factor to consider is the role of environmental contamination in the epidemiology of antibiotic-resistant pathogens, which has received media and political attention, but relatively little scientific attention. Several studies have demonstrated contamination of the inanimate environment of colonised patients, the direction of spread between patients and fomites that are mostly unresolved (Sood *et al.,* 2008; Hayden *et al.,* 2006). Vancomycin resistance in enterococci has not come under the microscope just because of its medical importance, but also because of the frequent multiple-antibiotic resistance and the seemingly limitless capacity for horizontal gene transfer *via* numerous mobile genetic elements (Macovei and Zurek, 2006). It is therefore important to conduct studies that will assist in finding new methods to address the effectiveness of the specific components of these recommendations

which remain controversial (Weinstein *et al.*, 1999) and to prevent the ongoing spread of multi-drug resistant genes which cannot be over emphasized.

CHAPTER 3

MATERIALS AND METHODS

3. METHODS OF INVESTIGATION

3.1. Area of the study

The research was conducted at the North-West University-Mafikeng Campus, South Africa. Groundwater samples were collected from areas around the North West.

3.2. Collection of samples

Sixty water samples were collected from borehole taps using sterile 500ml Duran Schott bottles and transported on ice to the laboratory for analysis. The number of samples collected from the different areas are shown in Table 3.1.

3.3. Analysis of water samples

3.3.1. Selective isolation of Enterococcus species

Upon arrival in the laboratory, water samples were analysed within 2 hours according to standard methods (APHA, 1998). An aliquot of 100ml from each water sample was filtered through a 0.45µm filter paper (Whatman®Glass Microfiber GS Filter paper) on a water pump machine (model, Sartorius 16824). Sterile forceps were used to remove the membrane filters from the machine and the filter papers were placed on bile esculin agar (BEA) plates (Biolab, South Africa). The plates were incubated aerobically at 45°C for 24 hours and typical grey to black colonies were considered as potential *Enterococcus* species. The isolates were purified by sub-culturing on bile esculin agar (BEA) and the plates incubated aerobically at 45°C for 24hours. Pure colonies were used for bacteria identification.

3.3.2. Control strains

Enterococcus faecalis (ATCC 6569) was used as a positive control strain while *S. aureus* (ATCC 25923) was used as a negative control strain in all experiments.

Table 3.1: Areas from which groundwater samples were collected:

District	Sampling Area	Number of samples
Dr Ruth Mompati	Delareyville	3
	Stella	3
	Vryburg	4
	Taung	3
Dr Modiri Molema	Disaneng	3
	Mabule	3
	Masamane	2
	Tshidilamolomo	2
	Dingateng	5
	Logagane	3
	Deelpan	6
	Makgobistad	2
	Ramosadi	5
	Leporung	2
	Motlhabeng	5
	Dibate	5
	Maeyaeyane	2
	Phitsane	2
TOTAL		**60**

3.4. Bacterial identification

Presumptive isolates were identified using the following criteria:

3.4.1. Cellular morphology

The isolates were Gram stained using standard protocols (Cruikshank *et al.*, 1975). Gram-positive cocci were subjected to both preliminary biochemical tests and confirmatory identification tests for characters of *Enterococcus* species.

3.5. Preliminary biochemical identification tests for enterococci

3.5.1. 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 system. Enterococci and streptococci are exceptions and do not possess the enzyme. Catalase enzymes decompose poisonous hydrogen peroxide to water and oxygen. In performing the test, a sterile wire loop was used to transfer a single bacterial colony onto a clean microscope slide and a drop of 2% hydrogen peroxide (H_2O_2) was poured onto the isolate. Catalase positive organisms produce bubbles while negative organisms do not produce any bubbles. Enterococci are catalase negative and all isolates that satisfied this preliminary identification criterion were subjected to further confirmatory tests.

3.5.2. Growth on Sodium chloride (NaCl) broth

The ability of enterococci to grow in 6.5% NaCl broth is currently considered an important distinguishing characteristic that facilitates identification of these species (Klein, 2003). Consequently, presumptive isolates were cultured aerobically at 45°C for 24 hours in 10ml of 6.5% sodium chloride to differentiate them from streptococci.(APHA, 1998; Klein, 2003). A MacCartney bottle that contained a culture of *Enterococcus faecalis* in 6.5% NaCl and one without a culture were used as positive and negative controls respectively. Bacteria growth was determined by measuring the optical density at 600nm using a Heλiosε Thermo Spectronic spectrophotometer (model Helios Epsilon) obtained from Merck, South Africa.

3.5.3. Haemolysis on blood agar

Haemolytic activity of the isolates was determined by culturing on blood agar base (Merck, S.A) supplemented with 5% ox-blood (Kilian, 2002). Plates were incubated aerobically at

45°C for 24 hours and later observed for the presence of haemolytic patterns. Enterococci are gamma haemolytic indicating they do not breakdown erythrocytes. Results were recorded.

3.6. Confirmatory identification tests for enterococci

3.6.1. Serotyping

A SLIDEX® Strepto Plus Latex agglutination test kit obtained from BioMérieux South Africa was used for serological identification of *Enterococcus* species based on the Lancefield grouping. The test is based on the principle that, a specific *Enterococcus* antigen is identified using latex particles sensitised with group D specific anti-streptococcal antibody. The test was performed by reacting a cell wall specific carbohydrate extract of isolates with a suspension of polystyrene microparticles sensitised with rabbit anti-group D streptococcal antibodies. Results were recorded as positive when a visible agglutination pattern was identified on the slide resulting from the clumping of latex particles in approximately two minutes. To reduce errors during the interpretation of data, a group D enzyme extract mix from strains of enterococci were provided in the kit and used as a positive control during the analysis. This positive control anti-serum was reacted against extracts from both the *Enterococcus faecalis* positive control and *S. aureus* negative control strains respectively.

3.6.2. Genomic DNA Extraction

Total genomic DNA was extracted from purified fresh cultures using the boiling method (Tunung *et al.*, 2007). Bacterial suspensions were prepared in 100µl of sterile distilled water using 1.5ml eppendorf tubes. The tubes were placed in a dry bath (Biorad, Digital dry bath) at 100°C for 15 minutes and later centrifuged at 13500 rpm for 2 minutes. The tubes were then placed on ice for 5 minutes. The supernatants were transferred into new tubes and 5µl

aliquots were used for PCR analysis. The DNA was stored at -20°C and used for amplification.

3.7. PCR for amplification of 16SrRNA gene fragments

Designated primer pair that appear in Table 3.2 were used to amplify the bacterial 16S rRNA gene fragments (Butterworth *et al.,* 2002). Standard 25µl PCR reaction mixtures were prepared. The reactions contained 1X PCR master mix, 5µl of template DNA, 50 pmol of each primer and nuclease free water. All the reagents used for PCR were obtained from Fermentas, USA and supplied by Inqaba biotec – Pretoria, South Africa. Amplifications were performed on a DNA thermal cycler (C1000 Touch™, BIO-RAD; South Africa). The cycling conditions used were as follows: initial denaturation at 94°C for 10 minutes and 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute. A final elongation step was performed at 72°C for 10 minutes. PCR products were held at 4°C until electrophoresis.

Table 3.2: Primers sequences used for amplification of 16SrRNA specific PCR

Primer	Sequence (5 –3)'	Target gene / organism	Amplicon size (bp)
E16SF[a]	GGATTAGATACCCTGGTAGTCC	16SrRNA	320
E16SR[a]	TCGTTGCGGGACTTAACCCAAC		

[a]Butterworth *et al.,* 2002

3.8. PCR analysis for specific identification of *Enterococcus faecalis*

Species specific identification of *Enterococcus faecalis* was performed using specific primers that are shown in Table 3.3 (Jackson *et al.,* 2004). Standard 25µl PCR reaction mixtures containing 1X PCR master mix, 5µl of template DNA, 50 pmol of each primer and nuclease free water were prepared. All the reagents used for PCR were obtained from Fermentas,

USA. Amplifications were performed on a DNA thermal cycler (C1000 Touch™, BIO-RAD; South Africa). The cycling conditions utilised were as follows: initial denaturation of 94°C for 10 minutes and 30 cycles of 94°C for 1 minute, 54°C for 1 minute and 72°C for 1 minute. A final elongation step was performed at 72°C for 10 minutes and samples were held at 4°C.

Table 3.3: Specific primers sequences used for amplification of *Enterococcus faecalis*

Primer	Sequence (5-3)	Target gene / organisms	Amplicon size (bp)
EF1F[b]	ATCAAGTACAGTTAGTCTTTATTAG	*ddlE / E. faecalis*	941
EF1R[b]	ACGATTCAAAGCTAACTGAATCAGT		

[b]Sharifi *et al.*, 2012

3.9. Matrix-assisted laser desorption ionisation, time-of-flight Mass Spectrometry (MALDI-TOF) as a tool for confirming the identities of *E. faecalis*

The identities of randomly selected isolates that were positively identified by PCR analysis as belonging to the species *E. faecalis* were subjected to MALDI-TOF mass spectrometry. Pure isolates were sub-cultured on bile esculin agar and plates were incubated at 45°C for 24 hours. The plates were transported to the Microbiology Laboratory at the University of Pretoria, South Africa for MALDI-TOF MS analysis using the Biotyper. The isolates were subjected to mass spectrometry by detecting the mass-to-charge ration (m/z) of bioanalytes and providing specific spectra. Highly accurate and reproducible spectral fingerprints of total bacterial protein extracts were generated. The protein profiles were compared with data present in an extensive database to facilitate identification to species level.

3.10. Antibiotic susceptibility test

An antibiotic susceptibility test was performed using the Kirby-Bauer disc diffusion method (Bauer *et al.*, 1996). The susceptibility profiles of the isolates were determined against a panel of 13 different antimicrobial agents obtained from Mast Diagnostics, South Africa. Details of the antibiotics tested are shown in Table 3.5. The tests were performed according to the guidelines of the Clinical Laboratory Standards Institute (CLSI, 2007) and standard reference values were used to classify isolates as susceptible, intermediate resistant or resistant to a particular antibiotic.

3.11. Detection of high-level aminoglycosides resistance (HLAR) among enterococci

All isolates that were resistant to vancomycin and exhibited the VRE phenotype were screened for HLAR. Aminoglycoside antibiotics gentamycin and streptomycin at concentrations of 120µg and 300µg respectively, were used (CLSI, 2007). The test was performed using standard guidelines and results interpreted using standard reference values as shown in Table 3.5 (CLSI, 2007).

Table 3.4: Antibiotics used in the study including their details. The superscripts [a] to [d] indicate the generally accepted concentrations of antibiotics in the discs according to the standard method stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom

Group	Antibiotic	Abbrev	Disc conc. (µg)	Inhibition zone (mm)		
				R	I	S
Penicillins	Penicillin	PG	10[b]	≤20	21-28	≥29
	Ampicillin	AP	10[b]	≤11	12-14	≥15
	Amoxicillin	A	10[d]	≤19	-	≥20
Aminoglycosides	Streptomycin	S	10[b]	≤11	12-14	≥15
	Kanamycin	K	30[d]	≤13	14-17	≥18
	Gentamycin	GM	10[d]	≤12	13-14	≥15
Macrolides	Erythromycin	E	15[c]	≤13	14-22	≥23
Tetracyclines	Tetracycline	T	10[b]	≤14	15-18	≥19
Glycopeptides	Vancomycin	VA	30[d]	≤9	10-11	≥12
	Teicoplanin	TEC	30[d]	≤10	11-13	≥14
Quinolones	Ciprofloxacin	CIP	5[d]	≤15	16-20	≥21
	Norfloxacin	Nor	10[d]	≤12	13-15	≥17
β-lactamase	Chloramphenicol	C	30[d]	≤12	13-17	≥18

PG (Penicillin), AP (Ampicillin), A (Amoxicillin), S (Streptomycin), K (Kanamycin), GM (Gentamycin), E (Erythromycin), OT (Oxytetracycline), VA (Vancomycin), CIP (Ciprofloxacin), Nor (Norfloxacin), C (Chloramphenicol), TEC (Teicoplanin)

Table 3.5: Antibiotics used in the study, for high level resistance including their details. The superscripts [e] to [f] indicate the generally accepted concentrations of antibiotics in the discs according to the standard method stipulated by the manufacturer, Mast Diagnostics, Merseyside, United Kingdom

Group	Antibiotic	Abbreviation	Disc conc. (µg)	Inhibition zone (mm)		
				Resistant	Intermediate	Susceptible
Aminoglycosides	Gentamycin	GM	120[e]	6	7-9	≥10
	Streptomycin	S	300[f]	6	7-9	≥10

G (Gentamycin), S(Streptomycin)

3.12. Multiple Antibiotic-Resistant (MAR) phenotypes determination

MAR phenotypes were generated for isolates resistant to 3 and more antibiotics using the abbreviations that appear on antibiotic discs (Rota *et al.,* 1996).

3.13. Cluster analysis of *Enterococcus faecalis* isolates using antibiotic inhibition zone diameter data (IZD)

A total of 70 *Enterococcus faecalis* isolates were subjected to cluster analysis using the antibiotic inhibition zone diameter data. Cluster analysis of antibiotic susceptibility data for *E. faecalis* isolated from the different stations was used to determine the relationships of isolates obtained in the study.

3.14. Detection of *vanA* genes in *Enterococcus faecalis by PCR*

All *E. faecalis* isolates that were positively identified by PCR analysis and phenotypically resistant to vancomycin were screened for the presence of the *vanA* and *vanB* gene using specific primer sequences that appear in Table 3.6.

Table 3.6: Specific primer sequences used for amplification of *VanA* and *VanB* phenotypes

Primer	Sequence (5 –3)'	Target gene / organism	Amplicon size (bp)
*VanA*F[a]	CATGAATAGAATAAAAGTTGCAATA	*VanA*	1030
*VanA*R[a]	TCCCCTTTAACGCTAATACGATCAA		
*VanB*F[b]	CAAAGCTCCGCAGCTTGCATG	*VanB*	484
*VanB*R[b]	TGCATCCAAGCACCCGATATAC		

[a]Butterworth *et al.*, 2002, [b]Dahl *et al.*, 2000

3.14.1 Detection of the vanA cluster in *Enterococcus faecalis by PCR*

The *vanA* cluster is frequently responsible for glycopeptides resistance in enterococci and this gene cluster is carried on transposon *Tn1546* or closely related elements that contain *vanR*, *vanS*, *vanA*, *vanX* and *vanZ* (Arthur *et al.,* 1993). All isolates that revealed phenotypic resistance to vancomycin were screened for the presence of the *vanA* gene cluster. A Peltier

Thermal Cycler (model-PTC-220 DYAD™ DNA ENGINE) was used for PCR amplification. Amplifications were performed in a final volume of 25µl consisting of 12.5µl of master mix and 5µl of DNA template, 0.5µl primer sets and free-nuclease water. Cycling conditions used were as follows: 95°C for 4 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 1 minute and 72°C for 1 minute and a final extension at 72°C for 7 minutes (Jackson *et al.*, 2004). PCR products were stored at 4°C until they were resolved by electrophoresis. The oligonucleotide primer sequences that were used to detect the *vanA* cluster are shown in Table 3.7.

Table 3.7: Primer sequences used for the amplification of gene fragments present in the *vanA* gene cluster

Primer	Sequence (5 –3)'	Target gene in the *vanA* cluster	Amplicon size (bp)
VanRF[a]	AGCGATAAAATACTTATTGTGGA	*VanR*	645
VanRR[a]	CGGATTATCAATGGTGTCGTT		
VanSF[a]	TTGGTTATAAAATTGAAAAATAA	*VanS*	1155
VanSR[a]	TTAGGACCTCCTTTTATC		
VanHF[a]	ATCGGCATTACTGTTTATGGAT	*VanH*	943
VanHR[a]	TCCTTTCAAAATCCAAACAGTTT		

[a]Dezfulian *et al.*, 2011

3.14.2. Amplification of insertion points flanking the Tn1546 by PCR

All *vanA* resistant isolates were screened for insertion points that flank the *Tn1546* transposon both up and downstream (Garcia-Migura *et al.*, 2008). The primer sequences used are indicated in Table 3.8.

Table 3.8: Primer combinations used to screen for insertion points to the left and right of *Tn1546*

Primer	Sequence (5 –3)'	Annealing temperature (°C)	Amplicon size (bp)
Upstream Tn1546			
P1727[a]	GTCTTTCTATATCCATTCATCTC	58	331
P745[a]	AAAGCTTACCTAACACTATAG		
Downstream Tn1546			
P1728[a]	ATTTGGAGAATTATTGTTTATACAG	55	514
P1715[a]	GGAAAACCGGTGATAAAG		
P1745[a]	CTTTTGATTTGGTACCTCTCATC	58	484

[a]Garcia-Migura *et al.*,2008

3.15. Detection of *vanB* genes in *Enterococcus faecalis by PCR*

All *E. faecalis* that were positively identified by PCR analysis and phenotypically resistant to vancomycin were screened for the presence of the *vanB* gene using specific primer sequences. The *vanB* /*vanB* subtypes (*vanB1*, *vanB2* and *vanB3*) were genotypically identified using specific primers as indicated in Table 3.6.

3.15.1. Detection of the transposon *Tn1547* in *vanB* resistant *Enterococcus faecalis*

VanB resistant gene clusters are found in composite transposon *Tn1547* bounded by 2 insertion-like sequences (ISs), that are designated respectively as IS16 and IS256 (Quintiliani and Courvalin, 1996). PCR was performed using specific oligonucleotide primer sequences to amplify these target regions flanking the transposon *Tn1547* in *vanB* resistant isolates. Primer sequences used are shown in Table 3.9.

Table 3.9: Specific primer sequences used for amplification of *vanB* insertion points

Primer	Sequence (5-3)	Target gene / organisms	Amplicon size (bp)
IS16F[a]	GCCATTGATCTCAGTTAGGAG	*Tn1547/E. faecalis*	1183
IS16R[a]	AAAGTTTCCAATTATCCGAG	*Tn1547/ E. faecalis*	
IS256F[a]	AAAAACATACCCAGGAGGAC	*Tn1547/ E. faecalis*	1115
IS256R[a]	GGCTGATGTTTGATTGGGGA	*Tn1547/ E. faecalis*	

[a]Dahl *et al.*, 2000

3.16. *Agarose gel electrophoresis of PCR products*

The PCR products were resolved by electrophoresis on a 2% (w/v) agarose gel in 1X TAE buffer. A 100bp DNA molecular weight marker (Fermentas, USA) was used during each run to confirm the sizes of the amplicons. Gels were separated at 100V for 10 minutes and later 60V for 1 hour. Electrophoretic patterns were analysed by a Gene Genius Bio imaging system (Syngene, Synoptics; UK) equipped with Gene Tools (version 3.07.01) software (Syngene, Synoptics; UK).

3.17. *Statistical analysis*

The percentage antibiotic resistance of isolates from a particular sampling station was determined by dividing the number of resistant isolates for a given antibiotic by the total number of isolates tested. Furthermore, cluster analysis of antibiotic susceptibility data for *E. faecalis* isolated from the different stations was determined using Wards algorithm and Euclidean distances on Statistica version 10 (Statsoft, US).

CHAPTER 4

RESULTS AND INTERPRETATION

4.1. Occurrence of *Enterococcus faecalis* in groundwater using preliminary identification tests

4.1.1. Morphological analysis

Sixty groundwater samples were collected from borehole taps in some communities in the North West Province and analysed for the presence of enterococci. Selective isolation was achieved with bile esculin agar which facilitates growth of organisms belonging to the genus. *Enterococcus* species were presumptively identified by the presence of black colonies that hydrolysed the esculin incorporated into the media. In the laboratory, enterococci are distinguished by their morphological appearance after/ following Gram staining (Gram-positive cocci that grow in chains) and their ability to hydrolyze esculin in the presence of bile when cultured. When subjected to Gram staining, a total of 253 isolates satisfied the morphological identification characteristics (Gram positive cocci) for enterococci. These isolates were selected and preserved for further analysis.

4.1.2. Catalase test

All the 253 Gram positive cocci were subjected to the catalase test and results are shown in Table 4.1. A large proportion 212 (83.8%) of the isolates were able to breakdown hydrogen peroxide despite the fact that a few catalase negative strains were observed. This observation was in agreement with the finding that some *E. faecium* isolates are usually catalase negative whereas *E. faecalis* are frequently catalase positive (Moy *et al.*, 2004).

Table 4.1: Results for catalase and haemolysis tests

Sampling Area	No. Tested	Catalase (No positive)	Haemolysis (Gamma)
Delareyville	NT	9	3
	NP	3(33.3%)	3(100%)
Vryburg	NT	9	2
	NP	9(100%)	2(100%)
Taung	NT	11	7
	NP	9(81.8%)	1(14.3%)
Disaneng	NT	12	3
	NP	8(66.6%)	0(0%)
Mabule	NT	11	2
	NP	10(90.9%)	0(0%)
Masamane	NT	8	2
	NP	8(100%)	2(100%)
Tshidilamolomo	NT	8	2
	NP	8(100%)	0(0%)
Dingateng	NT	17	2
	NP	17(100%)	2(100%)
Logagane	NT	9	6
	NP	7(77.8%)	0(0%)
Deelpan	NT	16	5
	NP	15(93.8%)	1(20%)
Makgobistad	NT	6	1
	NP	6(100%)	1(100%)
Ramosadi	NT	40	20
	NP	27(67.5%)	3(15%)
Loporung	NT	8	2
	NP	8(100%)	1(50%)
Motlhabeng	NT	28	3
	NP	26(92.9%)	1(33.3%)
Dibate	NT	37	15
	NP	27(72.9%)	2(13.3%)
Mayaeyaene	NT	8	2
	NP	8(100%)	2(100%)
Phitsane	NT	8	0
	NP	8(100%)	(%)
Stella	NT	8	1
	NP	8(100%)	0(0%)
TOTAL	NT	253	78
	NP	212 (83.8%)	21(26.9%)

NT= Number Tested, NP= Number Positive

4.1.3. Presumptive identification of Enterococcus species based on ability to grow on 6.5% Sodium Chloride (NaCl) broth

The identities of presumptive isolates were also screened to determine their abilities to grow in 6.5% NaCl and the optical density (O.D) was measured at 600 nm. A total of 78 randomly selected isolates were screened and they were all able to grow in the presence of 6.5% NaCl. However, there was a difference in the ability of isolates from different sampling areas to resistant the NaCl concentration and O.D values obtained ranged from 0.006 to 0.743. Detailed results for the isolates are indicated in Appendix Table 61. The results revealed that isolates may presumptively belong to the genus *Enterococcus*.

4.1.4. Haemolytic patterns on blood agar

The same 78 isolates that were tested for their ability to grow in 6.5% NaCl, were cultured on 5% ox-blood agar to determine their haemolytic patterns and the results obtained are shown in Table 4.1. Only a small proportion (26.9%) of isolates were gamma haemolytic and therefore, were not able to breakdown erythrocytes in the agar while 73.1% were haemolytic. Despite the fact that *E. faecalis* are known to display gamma haemolytic patterns, it is also suggested that the presence of the haemolysin A gene (*hlyA*) greatly determines the expression of this phenotypic trait in bacterial species. Hemolysin is a cytolytic protein capable of lysing human, horse and rabbit erythrocytes. Hemolysin producing strains are found to be associated with increased severity of infections. However, even the isolates that presented haemolytic patterns were subjected to further identification tests.

4.2. Occurrence of *Enterococcus faecalis* in groundwater using confirmatory identification tests

4.2.1. SLIDEX Strepto Plus slide agglutination test

A total of 78 randomly selected isolates were subjected to streptococcal group D serotyping to identify enterococci and a similarly small proportion (28.2%) of the isolates agglutinated and were identified as enterococci (Table 4.2). Antigens from alpha-hemolytic streptococci that blacken bile esculin medium but that do not grow in 6.5% sodium chloride broth may produce weak reactions with specially produced group D antiserum (Watson *et al.*, 1975). Moreover, some non-enterococcal antigens are found that cause weak cross reactions and these should not interfere with accurate identification of enterococci. In addition, weak agglutination reactions portrayed by *Enterococcus* species can be missed and therefore falsely considered negative (Levinson, 1980). It is suggested that serological assays should be supplemented with molecular tools to ensure correct identification of bacteria isolates.

Table 4.2: Proportion of isolates that were positive for serotyping, 16S rRNA PCR analysis and *E. faecalis* PCR assay

Sampling Area	No. Tested	Serotyping(No. positive)	16S rRNA	*E. faecalis*
Delareyville	NT	3	9	9
	NP	3(100%)	6(66.7%)	9(100%)
Vryburg	NT	2	9	9
	NP	0(0%)	6(66.7%)	5(55.6%)
Taung	NT	7	11	11
	NP	2(28.6%)	8(72.7%)	10(90.9%)
Disaneng	NT	3	12	12
	NP	1(33.3%)	6(50%)	10(83.3%)
Mabule	NT	2	11	11
	NP	0(0%)	10(90.9%)	3(27.3%)
Masamane	NT	2	8	8
	NP	0(0%)	5(62.5%)	1(12.5%)
Tshidilamolomo	NT	2	8	8
	NP	1(50%)	6(75%)	0(0%)
Dingateng	NT	2	17	17
	NP	0(0%)	10(58.8%)	4(23.5%)
Logagane	NT	6	9	9
	NP	3(50%)	7(77.8%)	1(11.1%)
Deelpan	NT	5	16	16
	NP	0(0%)	8(50%)	7(43.8%)
Makgobistad	NT	1	6	6
	NP	0(0%)	4(66.7%)	1(16.7%)
Ramosadi	NT	20	40	40
	NP	6(30%)	27(67.5%)	27(67.5%)
Loporung	NT	2	8	8
	NP	0(0%)	6(75%)	5(62.5%)
Motlhabeng	NT	3	28	28
	NP	2(66.7%)	20(71.4%)	13(46.4%)
Dibate	NT	15	37	37
	NP	3(20%)	22(59.5%)	20(54.1%)
Mayaeyaene	NT	2	8	8
	NP	1(50%)	6(75%)	1(12.5%)
Phitsane	NT	0	8	8
	NP	(%)	6(75%)	0(0%)
Stella	NT	1	8	8
	NP	0(0%)	6(75%)	5(62.5%)
TOTAL	NT	78	253	253
	NP	22(28.2%)	169 (66.8%)	122 (48.2%)

NT= Number Tested, NP= Number Positive

4.2.2. PCR for the amplification of 16S rRNA gene in enterococci

All the 253 presumptive enterococci isolated from water samples were subjected to PCR analysis for the amplification of 16S rRNA gene fragments. A large proportion 169 (66.8%) of the isolates were positively identified as enterococci and detailed results are shown in Table 4.2. Amplicons of the expected size (320 bp) were obtained and Figure 4.1 shows a representation of a 2% agarose (w/v) gel depicting the 16S rRNA gene fragments amplified from isolates obtained from the different sampling sites. A large proportion (66.7% to 90.9%) of the isolates from Taung, Mabule, Logagane, Motlhabeng, Tshidilamolomo, Loporung, Mayaeyane, Phitsane and Stella were positively identified as enterococci. All the isolates including those that tested positive for the identity of enterococci in smaller proportions (50% to 58.8%), portrayed multiple antibiotic resistant phenotypes especially to vancomycin and other related antibiotics. This might suggest that, the consumption of untreated groundwater may have severe health implications on individuals.

Figure 4.1: Agarose gel electrophoresis of 16S rRNA gene fragments amplified from isolates obtained during the study. Lanes 1 and 16 = 100 bp DNA marker; Lanes 2-15=16S rRNA genes from isolates obtained from the different sample sites

4.2.3. Enterococcus faecalis specific PCR analysis

All the 253 presumptive enterococci isolates that were screened for the 16S rRNA gene fragment were also subjected to specific PCR analysis for the detection of a *ddlE/ E. faecalis* gene fragment. Results indicated that a total of 122 (48.2%) *E. faecalis* isolates from the different areas were positively identified. Moreover, *E. faecalis* was frequently detected in samples obtained from Taung, Delareyville and Disaneng with percentage occurrence ranging from 83.3% to 100%. Despite the fact that none of the isolates from Tshidilamolomo were positive for *E. faecalis*, a small proportion (11.1% to 16.7%) of the isolates from Logagane, Makgobistad and Dingateng were positive for *E. faecalis* by PCR assay. Figure 4.2 shows an image of a 2% (w/v) agarose gel depicting the *E. faecalis* species specific PCR amplicons that were amplified. The amplicons were of the expected size (941bp).

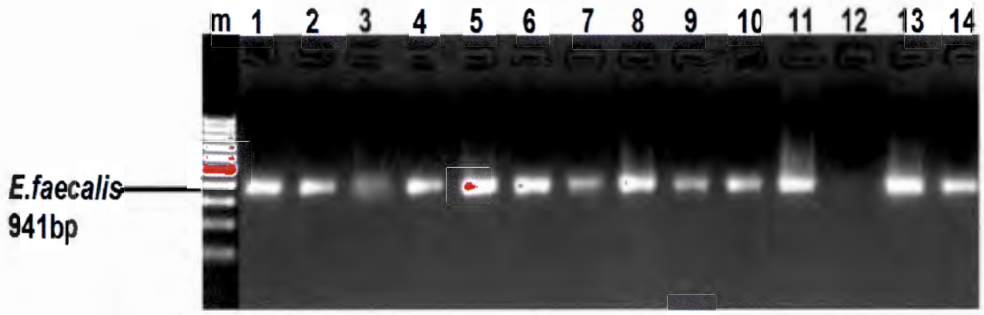

Figure 4.2: Agarose gel electrophoresis depicting *E. faecalis* species specific gene fragments amplified from isolates obtained during the study. Lanes M = 1kb DNA marker; Lanes 1-11 and Lanes 13-14 = *E. faecalis* species specific gene fragments amplified from isolates in the study; Lane 12= Negative control reaction with no amplified band.

4.2.4. Identification of E. faecalis isolates using the MALDI-TOF Mass Spectrometry

A total of ten representative isolates that had been identified by PCR analysis as *ddlE/ E. faecalis* genes were subjected to a rapid analytic identification tool known as the matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry. *Salmonella* species was used as a negative control in the experiment. A total of 5 isolates were positively identified and the identities of both the first and second best matches for all the isolates are shown in Table 4.3. A representation of the spectral protein fingerprints obtained for whole cell colonies is shown in Figure 4.3 while detailed results of both the *Enterococcus faecalis* and the *Salmonella* species appear in Appendix Tables 60A, 60B, 60C, 60D, 60E and 60F. As shown in Figure 4.3, spectra were recorded at 2000 to 18000Da but a majority of the protein peaks were observed between 2000 to 10000Da. The similarities in the protein profiles indicated that the peaks recorded encode for proteins that are specific to *E. faecalis* in all the isolates.

Table 4.3: Identities of isolates obtained through MALDI-TOF Mass spectrometry of total cell protein profiles

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
DEEL4-2	2.244	*Enterococcus faecalis*	2.12	*Enterococcus faecalis*
MP3-1	1.736	*Enterococcus faecalis*	1.471	*Enterococcus faecalis*
V2-2	2.303	*Enterococcus faecalis*	2.204	*Enterococcus faecalis*

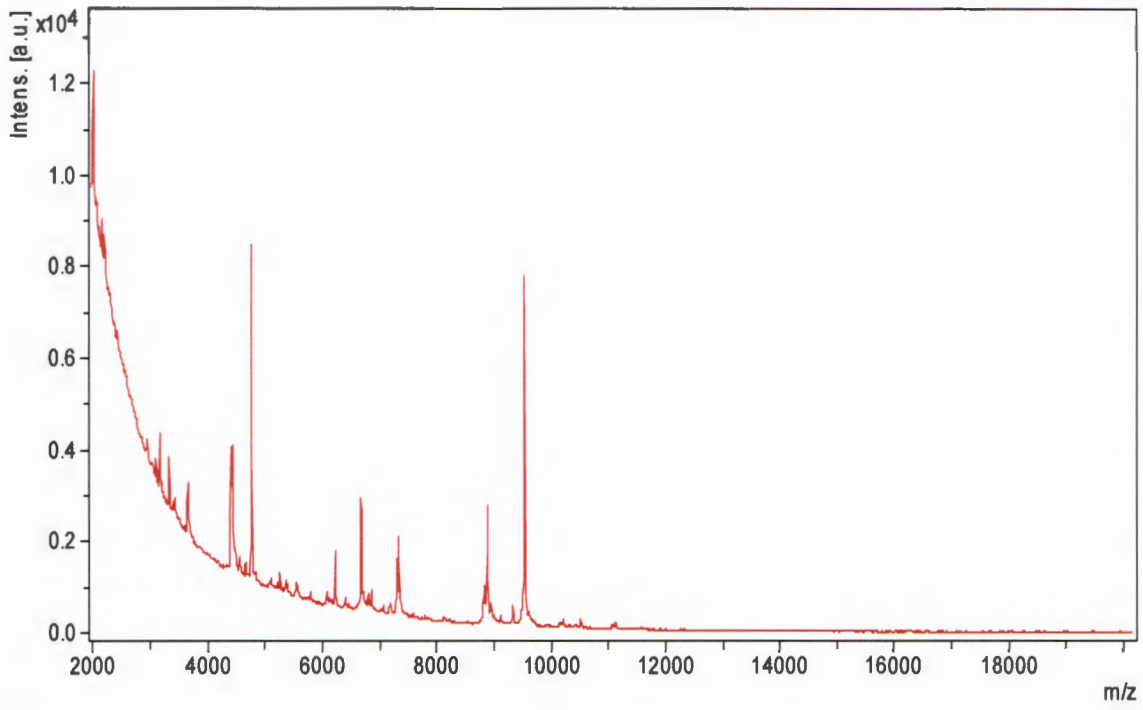

Figure 4.3: A representation of the spectral fingerprints for *Enterococcus faecalis* isolated from groundwater in the North West Province.

4.3. Antibiotic resistance profiles of isolates

4.3.1. Antibiotic disc susceptibility test

All the 122 *E. faecalis* isolates that were positively identified using the specific PCR analysis were subjected to antibiotic susceptibility tests using a panel of 13 antibiotics. Results are shown in Tables 4.4A, 4.4B, 4.4C and 4.4D. Generally, large proportions (57.4% to 100%) of isolates from most of the stations sampled were resistant to vancomycin, penicillin, amoxicillin, ampicillin, tetracycline and erythromycin. Despite this, *E. faecalis* isolates showed little resistance (23% to 49.2%) against streptomycin, kanamycin, gentamycin, ciprofloxacin, norfloxacin and teicoplanin. Interestingly, all *E. faecalis* isolates tested in this study were resistant to penicillin while 86 (70.5%) and 108 (88.5%) of these isolates were also resistant to ampicillin and amoxicillin. It is suggested that susceptibility to penicillin may indicate susceptibility to both ampicillin and amoxicillin for enterococci that are not able to

produce β-lactamase enzyme and vice versa (CLSI, 2007). This may account for the trend observed. Moreover, resistance to ampicillin and penicillin among enterococci resulting from the production of β-lactamase enzyme is not easily detected using conventional disc diffusion tests (CLSI, 2007). Consequently, it is recommended that the β-lactamase test still remains a gold standard method for determining resistance to penicillin (CLSI, 2007). However, the results obtained in this study revealed the presence of multiple antibiotic resistant *E. faecalis* isolates that portray phenotypic resistance to antimicrobial agents belonging to the class beta-lactams. Of all the drugs tested, chloramphenicol showed the least resistance again isolates and only a small proportion (20.5%) of resistant isolates was detected among isolates from the different areas sampled.

Generally, a large proportion 81(66.4%) of the isolates was resistant to vancomycin (Tables 4.4A and 4.4B) despite the fact that the drug is not used in both human and animal medicine in the area. However, it is suggested that enterococci that display high level aminoglycoside resistant (HLAR) phenotypes to gentamycin and streptomycin usually show synergistic resistance to ampicillin, penicillin and vancomycin. Therefore, HLAR screening test was used as a tool to confirm the presence of vancomycin resistance among *E. faecalis* isolates. An interesting factor was that a smaller proportion 60 (49.2%) of the isolates was resistant to teicoplanin, even though it is a glycopeptide antibiotic whose mode of action is similar to that of vancomycin.

Little resistance (30.3% to 41%) was observed against quinolones, ciprofloxacin and norfloxacin. The data obtained is not surprising since these antibiotics are not used routinely in both human and animal medicine in the area.

4.3.2. Detection of High Level Aminoglycoside Resistance (HLAR) of E. faecalis using streptomycin and gentamycin

Two antibiotics, streptomycin (300 μg) and gentamycin (120 μg) were used to determine HLAR among isolates obtained in the study. Results obtained are shown in Table 4.5. Generally, a large proportion of the isolates (76% to 100%) revealed HLAR traits. Moreover, a large proportion of these HLAR isolates were also resistant to vancomycin based on the phenotypic disc diffusion assay. These results were in agreement with the observation that HLAR in enterococci may serve as a confirmation for the presence of the VRE phenotypes observed when isolates are subjected to the antibiotic disc diffusion test (CLSI, 2007).

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

Sampling Area	No. Tested	VA(30)	PG(10)	AP(10)	A(10)	S(10)	K(30)	GM(10)	E(15)
Delarey Ville	NT				9			9	9
	NR	6(66.7%)	9(100%)	0(0%)	2(22.2%)	2(22.2%)	0(0%)	5(55.6%)	7(77.8%)
Vryburg	NT							5	5
	NR	4(80%)	5(100%)	4(80%)	4(80%)	1(20%)	2(40%)	1(20%)	3(60%)
Taung	NT							10	10
	NR	5(50%)	10(100%)	9(90%)	8(80%)	1(10%)	3(30%)	1(10%)	8(80%)
Disaneng	NT							10	10
	NR	7(70%)	10(100%)	2(20%)	9(90%)	5(50%)	1(10%)	0(0%)	7(70%)
Mabule	NT							3	3
	NR	3(100%)	3(100%)	3(100%)	3(100%)	1(33.3%)	0(0%)	1(33.3%)	2(66.7%)
Masanlane	NT							1	1
	NR	1(100%)	1(100%)	1(100%)	1(100%)	1(100%)	0(0%)	0(0%)	1(100%)
Dingateng	NT							4	4
	NR	2(50%)	4(100%)	2(50%)	4(100%)	2(50%)	1(25%)	4(100%)	0(0%)
Logagane	NT							1	1
	NR	1(100%)	1(100%)	1(100%)	1(100%)	0(0%)	0(0%)	0(0%)	1(100%)
Deelpan	NT							7	7
	NR	4(57.1%)	7(100%)	7(100%)	6(85.7%)	2(28.6%)	5(71.4%)	0(0%)	4(57.1%)
Makgobistad	NT							1	1
	NR	1(100%)	1(100%)	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
Ramosadi	NT							27	27
	NR	14(51.8%)	27(100%)	23(85.2%)	27(100%)	3(11.1%)	4(14.8%)	15(55.6%)	17(63%)

VA (Vancomycin), PG (Penicillin), AP (Ampicillin), A (Amoxicillin), S (Streptomycin), K (Kanamycin), GM (Gentamycin), E (Erythromycin), NT= Number Tested, NP= Number Resistant

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

ampling Area	No. Tested	VA(30)	PG(10)	AP(10)	A(10)	S(10)	K(30)	GM(10)	E(15)
Loporung	NT	5	5	5	5	5	5	5	5
	NR	3(60%)	5(100%)	3(60%)	3(60%)	5(100%)	2(40%)	0(0%)	1(20%)
Moilhat(G)									13
	NR	5(38.4%)	13(100%)	9(69.2%)	13(100%)	3(23.1%)	3(23.1%)	0(0%)	8(61.5%)
Dibai									20
	NR	20(100%)	20(100%)	17(85%)	20(100%)	0(0%)	8(40%)	9(45%)	16(69.6%)
Mayalevalen									1
	NR	1(100%)	1(100%)	0(0%)	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
Lowsiel									5
	NR	4(80%)	5(20%)	5(20%)	5(20%)	2(40%)	0(0%)	0(0%)	4(80%)
TOTAL									122
	NR	81(66.4%)	122(100%)	86(70.5%)	108(88.5%)	28(23%)	29(23.8%)	36(29.5%)	79(64.8%)

VA (Vancomycin), PG (Penicillin), AP (Ampicillin), A (Amoxicillin), S (Streptomycin), K (Kanamycin), GM (Gentamycin), E (Erythromycin)
NT= Number Tested, NP= Number Resistant

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

Sampling Area	No. Tested	OT(10)	CIP(5)	Nor(10)	C(30)	TEC(30)
Delaney Hill						
	NR	3(33.3%)	1(11.1%)	5(55.6%)	2(22.2%)	4(44.4%)
Vivpury						
	NR	1(20%)	2(40%)	2(40%)	1(20%)	3(60%)
Tauly						
	NR	4(40%)	3(30%)	4(40%)	2(20%)	9(100%)
Disaneng						
	NR	6(60%)	6(60%)	7(70%)	1(10%)	6(60%)
Mbale						
	NR	3(100%)	1(33.3%)	3(100%)	0(0%)	2(66.7%)
Masamane						
	NR	0(0%)	1(100%)	1(100%)	0(0%)	1(100%)
Dingatebe						
	NR	2(50%)	1(25%)	0(0%)	0(0%)	1(25%)
Lopagan						
	NR	1(100%)	0(0%)	0(0%)	0(0%)	0(0%)
Deelpan						
	NR	3(42.9%)	4(57.1%)	4(57.1%)	0(0%)	2(28.6%)
Molopobisani						
	NR	0(0%)	0(0%)	1(100%)	0(0%)	1(100%)
Ramosadi						
	NR	21(77.8%)	6(22.2%)	10(37%)	11(40.7%)	13(48.1%)

OT (Oxytetracycline), CIP (Ciprofloxacin), Nor (Norfloxacin), C (Chloramphenicol), TEC (Teicoplanin), NT= Number Tested, NP= Number Resistant

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

Sampling Area	No. Tested	OT(10)	CIP(5)	Nor(10)	C(30)	TEC(30)
Lopatung	NT					5
	NR	0(0%)	3(60%)	0(0%)	2(40%)	0(0%)
Motlhabeng	NT					13
	NR	9(69.2%)	7(53.8%)	7(53.8%)	2(15.4%)	8(61.5%)
Dibate	NT					20
	NR	15(75%)	0(0%)	6(30%)	1(5%)	7(35%)
Mayaeyaeng	NT					1
	NR	0(0%)	0(0%)	0(0%)	0(0%)	1(100%)
Stella	NT					5
	NR	2(40%)	2(40%)	1(20%)	3(60%)	2(40%)
TOTAL	NT					122
	NR	70(57.4%)	37(30.3%)	50(41%)	25(20.5%)	60(49.2%)

OT (Oxytetracycline), CIP (Ciprofloxacin), Nor (Norfloxacin), C (Chloramphenicol), TEC (Teicoplanin)
NT= Number Tested, NP= Number Resistant

Table 4.5: Proportion of *Enterococcus faecalis* isolates from the different stations that portrayed High Level Aminoglycoside Resistant phenotypes

Sample area	No tested and Proportion with HLAR	S (300)	GM(120)	Sample area	No tested and Proportion with HLAR	S (300)	GM(120)
Delareyville	NT	7	7	Mothabeng	NT	7	7
	% with HLAR	100	100		% with HLAR	100	100
Vryburg	NT	5	5	Makgobistad	NT	1	1
	% with HLAR	100	100		% with HLAR	100	100
Taung	NT	10	10	Ramotsadi	NT	27	27
	% with HLAR	100	100		% with HLAR	100	100
Disaneng	NT	10	10	Loporung	NT	5	5
	% with HLAR	100	100		% with HLAR	100	100
Mabule	NT	3	3	Molhabeng	NT	13	13
	% with HLAR	100	100		% with HLAR	76.9	100
Masamane	NT	1	1	Dibate	NT	20	20
	% with HLAR	100	100		% with HLAR	100	100
Dingateng	NT	4	4	Mmatau	NT	1	1
	% with HLAR	100	100		% with HLAR	0	100
Logagane	NT	1	1	Stella	NT	5	5
	% with HLAR	100	100		% with HLAR	100	100
TOTAL	NT	43	43	TOTAL	NT	79	79
	NR	42(97.7%)	43(100%)		NR	75(94.9%)	79(100%)

S =Streptomycin, GM=Gentamycin, NT= Number Tested, NR= Number Resistant

4.4. Phenotypic relationship between multiple antibiotic resistant *Enterococcus faecalis* isolated from groundwater based on clustering patterns using the antibiotic inhibition zone diameter data

A total of 68 isolates obtained from groundwater in the various sampling stations were randomly selected and subjected to cluster analysis based on their antibiotic inhibition zone diameter data. This was designed to generate a dendogram that could be used to determine the relationship between the isolates from the different stations. Results are shown in Figure 4.4. The data in Figure 4.4 was analysed for patterns of association between isolates in the different clusters and results presented as cluster Tables (Table 4.6). As shown in Figure 4.3 and Table 4.6, four major clusters were observed. Cluster 2A was the largest cluster with 22 (32.4%) of the isolates tested. This was followed by cluster 2B that had 18 (24.5%) of the *E. faecalis* isolates. Moreover, these two clusters (clusters 2A and 2B) were considered mixed clusters when compared to the others since they had isolates from most of the sampling areas that were randomly selected for the antibiotic cluster analysis.

Generally, isolates from Ramosadi and Dibate showed similarities in the antibiotic resistance patterns for most of the areas sampled and this was revealed by the association shown in Figure 4.4 and Table 4.6. An interesting observation was made for cluster 1B, where 4 and 5 of the 10 isolates present in that cluster were from Dibate and Ramosadi, respectively. These two villages are close to each other and residents do not have access to both proper sanitary facilities and potable water. Despite this, a small proportion 9 (13.2%) of isolates tested in the present study did not cluster with either isolates from the same or different sampling stations (refer to Figure 4.4 in page 64). These isolates were arbitrarily grouped in cluster C and contained isolates from Deelpan, Dibate, Ramosadi, Mabule and Taung. Although these sample sites are far apart, the data presented suggests that the isolates might have similar antibiotic histories. Considering the fact that the *E. faecalis* subjected to cluster analysis in

the present study are environmental isolates, it amplifies the need to implement strategies aimed at controlling the use of antibiotics among humans and exposing animals to them in their feed.

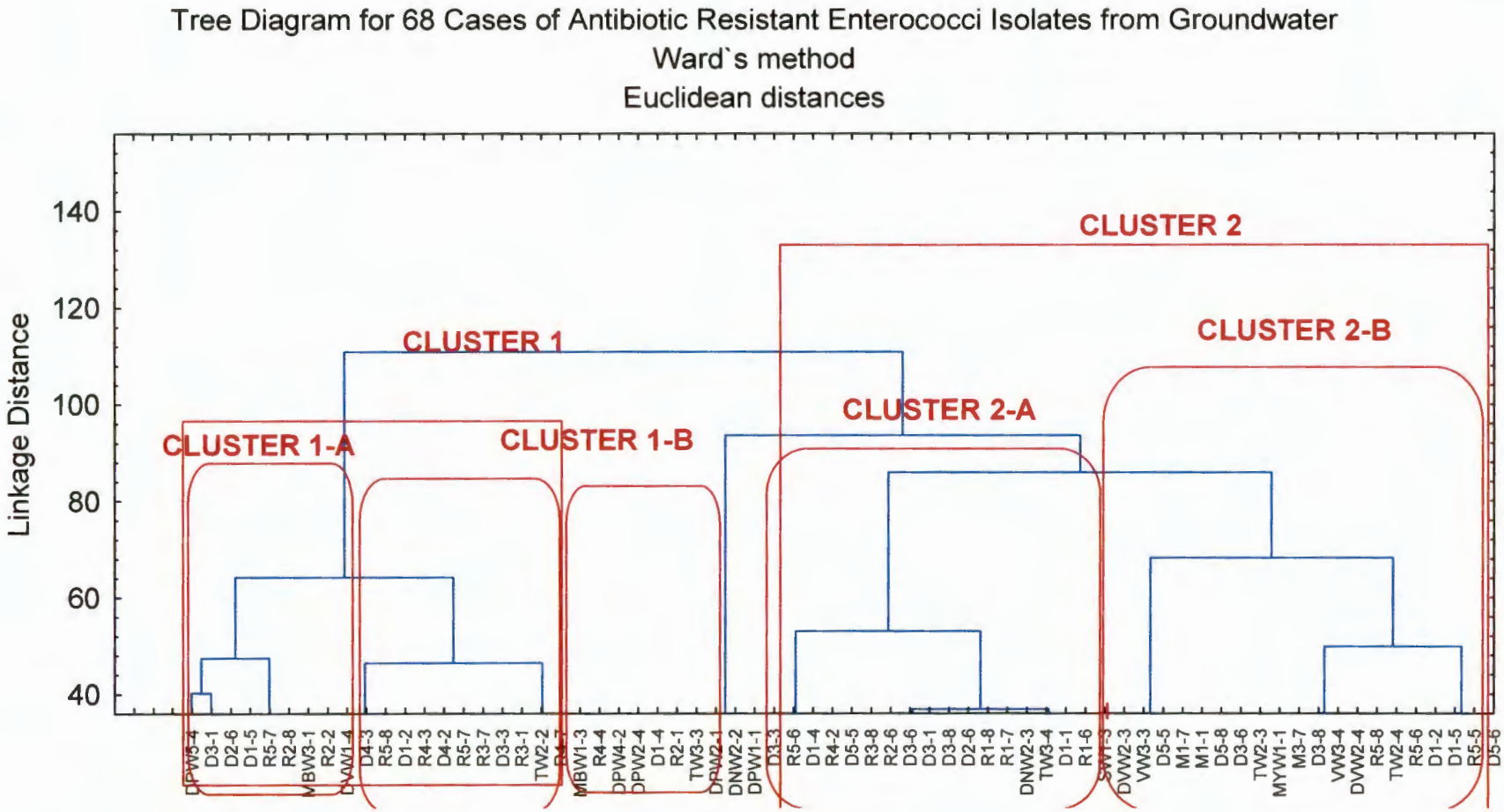

Figure 4.4: Dendogram showing the relationship between enterococci isolates from groundwater in the different stations. Bacterial designation prefixes are based on sampling station origin and sample type. The tree was constructed using Ward's method and Euclidean distances in Statistica, version 10 (Statsoft, US). Designation: DPW=Deelpan, D=Dibate, R=Ramosadi, MBW=Mabule, DVW=Delareyville, PW=Phitsane, TW=Taung, DNW=Dingateng, SW=Stella, VW=Vryburg, MW=Motlhabeng, MYW=Mayaeyane

Table 4.6: The percentage representation of *E. faecalis* from different sample stations within the various clusters

Sample station	Cluster 1A N=9	Cluster 1B N=10	Cluster 2A N=22	Cluster 2B N=18	Cluster C N=9
Deelpan	1 (11.1%)	0 (0%)	1 (4.5%)	0 (0%)	3 (33.3%)
Dibate	3 (33.3%)	4 (40%)	8 (36.4%)	7 (38.9%)	1 (11.1%)
Mabule	1 (11.1%)	0 (0%)	0 (0%)	0 (0%)	1 (11.1%)
Phitsane	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Dingateng	0 (0%)	0 (0%)	2 (9.1%)	0 (0%)	0 (0%)
Vryburg	0 (0%)	0 (0%)	1 (4.5%)	1 (5.6%)	0 (0%)
Mayaeyane	0 (0%)	0 (0%)	0 (0%)	1 (5.6%)	0 (0%)

4.5. Multiple Antibiotic-Resistant (MAR) Phenotypes of *E. faecalis* isolated from groundwater

A total of 122 *E. faecalis* were screened for the presence of multiple antibiotic-resistant phenotypes and 120 isolates were resistant to three or more antibiotics. The predominant multiple antibiotic-resistant phenotypes observed for the *E. faecalis* isolated from groundwater samples obtained from the various areas are shown in Table 4.7 while detailed results are indicated in Appendix (Table 62A-62C). The phenotypes VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC, VA-PG-AP-A-K-GM-E-OT-Nor-C and VA-PG-AP-A-S-GM-E-OT-C-TEC were predominant among isolates obtained from Ramosadi while the phenotype VA-PG-AP-A-GM-E-OT was dominant for those obtained from Dibate.

On the contrary, there was no dominant phenotype for MAR isolates obtained in all the other stations sampled. However, a cause for concern was the number of antibiotics to which individual MAR *E. faecalis* isolates from the different areas were resistant to. This was evident among isolates obtained from Ramosadi, Dibate, Motlhabeng, Deelpan, Vryburg and

Taung, where isolates presented with phenotypes VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC, VA-PG-AP-A-K-GM-E-OT-CIP-Nor-C-TEC, VA-PG-AP-A-S-K-E-OT-CIP-Nor-C-TEC, VA-PG-AP-A-S-K-GM-E-OT-CIP-Nor-C, VA-PG-AP-A-S-K-GM-E-CIP-Nor-C-TEC and VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC respectively. These phenotypes indicate that, isolates were resistant to twelve of the thirteen antibiotics tested. The results therefore indicate that these isolates could serve as reservoirs for antibiotic resistant determinants in the environment and could pose severe health implications to humans.

Table 4.7: Predominant multiple antibiotic-resistant phenotypes for *E. faecalis* isolated from groundwater in different sampling stations in the North West Province. Phenotypes were generated using abbreviations that occur on the antibiotic discs

Sample Area	Phenotype	No. Observed	% Observed
Ramosadi (NO=27)	VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC	2	7.4%
	VA-PG-AP-A-K-GM-E-OT-Nor-C	2	7.4%
	VA-PG-AP-A-S-GM-E-OT-C-TEC	2	7.4%
Dibate (NO=20)	VA-PG-AP-A-GM-E-OT	3	15.0%
Motlhabeng (NO=11)	VA-PG-AP-A-S-K-E-OT-CIP-Nor-C-TEC	1	9.1%
Deelpan (NO=7)	VA-PG-AP-A-S-K-GM-E-OT-CIP-Nor-C	1	14.3%
Taung (NO=10)	VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC	1	10.0%
Vryburg (NO=5)	VA-PG-AP-A-S-K-GM-E-CIP-Nor-C-TEC	1	20%

NO= Number of MAR isolates obtained in the sample area

4.6. Detection of *vanA* and *vanB* antibiotic-resistant genes in *E. faecalis* using PCR analysis

All the 122 *E. faecalis* isolates that were positively identified based on species-specific PCR analysis were subjected to another PCR assay to detect the presence of *vanA* and *vanB* resistant genes. A motivation was the fact that some of these isolates portrayed phenotypic resistance to the antimicrobial agent vancomycin when subjected to the antibiotic disc susceptibility test. Moreover, a preceding study by Ateba and Maribeng, (2011) also detected the presence of phenotypic vancomycin resistant strains. Given that the antibiotic vancomycin is not used in both human and animal medicine, it was recommended that the study be expanded to screen vancomycin resistant enterococci for the occurrence of vancomycin resistant determinants by PCR analysis. This is important to confirm if the phenotypic vancomycin resistant profiles observed amongst isolates is due to the presence of vancomycin resistant genes.

Out of the 122 isolates, a total of 63 (51.6%) tested positive for *vanA* gene while a much larger proportion 83 (68%) harboured the *vanB* genotype (Table 4.8). Figure 4.4 shows an image of a 2% (w/v) agarose gel depicting the *vanA* and *vanB* genes that were detected by PCR with the expected amplicon sizes of 1030bp (*vanA*) and 484bp (*vanB*).

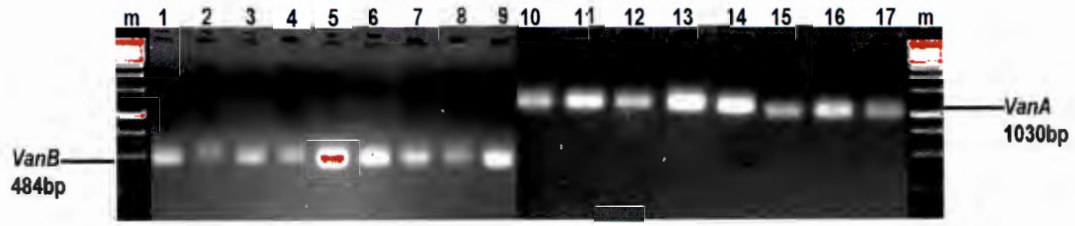

Figure 4.5: Agarose gel electrophoresis depicting *vanA* and *vanB* genes amplified using DNA from obtained from vancomycin resistant *E. faecalis* isolated during the study. Lanes M = 100 bp DNA marker; Lanes 1-9 = *vanB* gene fragments amplified from isolates in the study; Lanes 10-17= *vanA* gene fragments amplified from isolates in the study

Table 4.8: Proportion of *E. faecalis* isolates from the different stations that possessed the *vanA* and *vanB* gene fragments by PCR analysis

Sampling Area	No. Tested	*VanA*	*VanB*
Delareyville	NT	9	9
	NP	2(22.2%)	6(66.7%)
Vryburg	NT	5	5
	NP	2(40%)	2(40%)
Taung	NT	10	10
	NP	6(60%)	9(90%)
Disaneng	NT	10	10
	NP	4(40%)	9(90%)
Mabule	NT	3	3
	NP	3(100%)	3(100%)
Masamane	NT	1	1
	NP	0(0%)	1(100%)
Tshidilamolomo	NT	0	0
	NP	0(0%)	0(0%)
Dingateng	NT	4	4
	NP	1(25%)	3(75%)
Logagane	NT	1	1
	NP	0(0%)	0(0%)
Deelpan	NT	7	7
	NP	5(71.4%)	6(85.7%)
Makgobistad	NT	1	1
	NP	1(100%)	1(100%)
Ramosadi	NT	27	27
	NP	15(55.6%)	16(59.3%)
Loporung	NT	5	5
	NP	5(100%)	4(8075%)
Motlhabeng	NT	13	13
	NP	8(61.5%)	6(46.2%)
Dibate	NT	20	20
	NP	9(45%)	13(65%)
Mayaeyaene	NT	1	1
	NP	0(0%)	1(100%)
Phitsane	NT	0	0
	NP	0(0%)	0(0%)
Stella	NT	5	5
	NP	2(40%)	3(60%)
TOTAL	NT	122	122
	NP	63(51.6%)	83(68%)

NT== Number Tested, NP= Number Positive

4.7. Detection of transposons *Tn1546* and *Tn1547* from *vanA* and *vanB* positive isolates using PCR analysis

All the 63 isolates that tested positive for the presence of *vanA*, were screened for the presence of the *Tn1546* transposon and 27 (42.9%) possessed this resistance determinant. The *vanB* genotype which was originally described in 1989 is characterised by its high-level resistance to vancomycin and susceptibility to teicoplanin. These traits are determined by a cluster of genes which reside within transposons *Tn1547* or *Tn5382* (Gold, 2001; Kawalec *et al.*, 2001). In this study, isolates were screened for transposon *Tn1547* and none of the isolates harboured this transposon. The characterisation of this transposon is of great importance given the correlation between vancomycin resistance phenotypes in enterococci and *Tn1546* transpososn (Simjee *et al.*, 2002). Moreover, there is great concern regarding the ability of transposon *Tn1546* to be expressed in a variety of bacteria hosts such as *Staphylococcus aureus* and cause high levels resistance to both vancomycin and teicoplanin (Gold, 2001; Courvalin, 2006). Detailed results indicating the number of isolates from the different areas that were positive for the *Tn1546* transposon are shown in Table 4.9. Figure 4.6 is an image of a 2% (w/v) agarose gel depicting 514bp amplicons of the *Tn1546* transposon that were amplified from isolates.

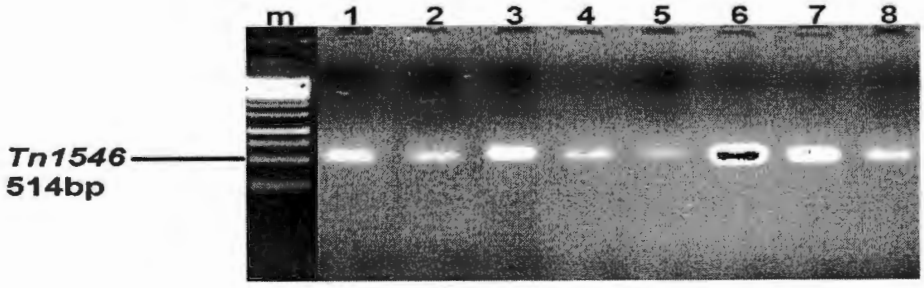

Figure 4.6: Agarose gel electrophoresis depicting 514bp amplicons of the *Tn1546* transposon that were amplified using DNA obtained from vancomycin resistant *E. faecalis* isolated during the study. Lanes M = 100 bp DNA marker; Lanes 1-8 = amplicons of the *Tn1546* transposon amplified from isolates in the study.

Table 4.9: Results for the detection of *Tn1546* and *Tn1547* gene fragments by PCR

Sampling Area	No. Tested	*Tn1546*	*Tn1547*
Delareyville	NT	2	6
	NP	0(0%)	0(0%)
Vryburg	NT	2	2
	NP	0(0%)	0(0%)
Taung	NT	6	9
	NP	0(0%)	0(0%)
Disaneng	NT	4	9
	NP	3(75%)	0(0%)
Mabule	NT	3	3
	NP	2(66.7%)	0(0%)
Masamane	NT	0	1
	NP	0(0%)	0(0%)
Tshidilamolomo	NT	0	0
	NP	0(0%)	0(0%)
Dingateng	NT	1	3
	NP	0(0%)	0(0%)
Logagane	NT	0	0
	NP	0(0%)	0(0%)
Deelpan	NT	5	6
	NP	1(20%)	0(0%)
Makgobistad	NT	1	1
	NP	0(0%)	0(0%)
Ramosadi	NT	15	16
	NP	10(66.7%)	0(0%)
Loporung	NT	5	4
	NP	5(100%)	0(0%)
Motlhabeng	NT	8	6
	NP	3(37.5%)	0(0%)
Dibate	NT	9	13
	NP	3(33.3%)	0(0%)
Mayaeyaene	NT	0	1
	NP	0(0%)	0(0%)
Phitsane	NT	0	0
	NP	0(0%)	0(0%)
Stella	NT	2	3
	NP	0(60%)	0(0%)
TOTAL	NT	63	83
	NP	27(42.9%)	0(0%)

NT= Number Tested, NP= Number Positive

4.8. Detection of the *vanR*, *vanS* and *vanH* genes that are components of the *vanA* cluster using PCR analysis

A total of sixty-one isolates that were identified to possess the *vanA* genotype by PCR analysis were subjected to genotypic screening for detecting three genotypes (*vanR*, *vanS*, and *vanH*) that are components of the *vanA* cluster. The selection of the phenotypes was due to the fact that *vanR* and *vanS* genotypes operate as a two-component regulatory system that regulates the transcription of the *vanHAX* gene cluster (Cetinkaya *et al.*, 2000). *VanH* was selected as a representative phenotype of the *van* genes (*vanA, vanH* and *vanX)* that are said to work together to confer resistance to vancomycin in VRE and vancomycin resistant *S. aureus* (VRSA). *VanS* is a membrane-associated sensor of vancomycin that controls the level of phosphorylation of *VanR* while *VanH* is a dehydrogenase that reduces pyruvate to D-Lactose. Results obtained are shown in Table 4.10. As shown in Table 4.10, a small proportion, of 8 (29.6%) of the isolates as positive for both the *vanR* and *vanS* gene fragments while only a smaller proportion 1(3.7%) as positive for *vanH* gene. Figure 4.7 indicates a 2% (w/v) agarose gel image showing the *vanR* and *vanH* gene fragments that amplified. Gene fragments with the expected amplicon sizes of 645bp and 943bp for the *vanR* and the *vanH* respectively were obtained (Figure 4.7).

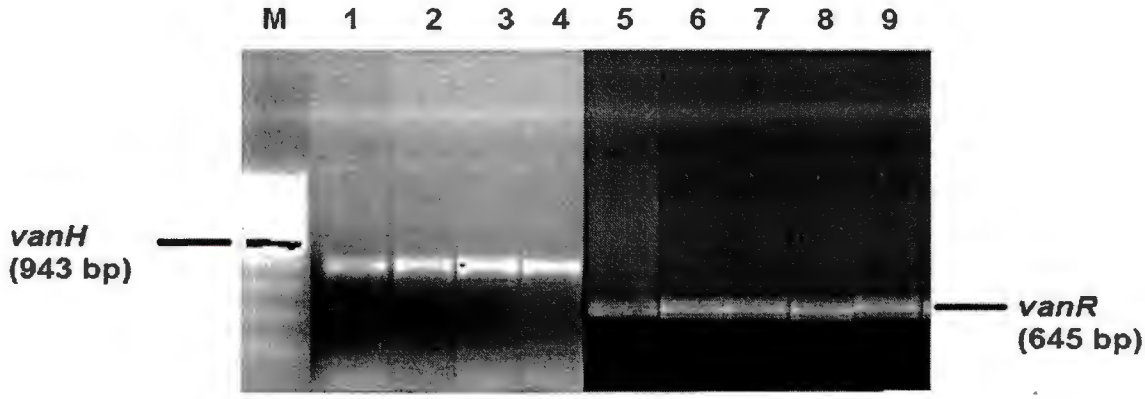

Figure 4.7: A composite agarose gel electrophoresis depicting the *vanH* and *vanR* genes amplified using DNA obtained from vancomycin resistant *E. faecalis* isolated during the study. Lanes M = 1 kb DNA marker; Lanes 1-4 = *vanH* amplified from isolates in the study; Lanes 5-9 = *vanH* amplified from isolates in the study.

Table 4.10: Results for the detection of *vanA* cluster gene fragments by PCR

Sampling Area	No. Tested	*VanS*	*VanR*	*VanH*
Delareyville	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Vryburg	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Taung	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Disaneng	NT	3	3	3
	NP	0(0%)	0(0%)	0(0%)
Mabule	NT	2	2	2
	NP	0(0%)	0(0%)	0(0%)
Masamane	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Tshidilamolomo	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Dingateng	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Logagane	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Deelpan	NT	1	1	1
	NP	0(0%)	0(0%)	0(0%)
Makgobistad	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Ramosadi	NT	10	10	10
	NP	2(20%)	2(20%)	0(0%)
Loporung	NT	5	5	5
	NP	0(0%)	0(0%)	0(0%)
Motlhabeng	NT	3	3	3
	NP	3(100%)	3(100%)	0(0%)
Dibate	NT	3	3	3
	NP	3(100%)	3(100%)	1(33.3%)
Mayaeyaene	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Phitsane	NT	0	0	0
	NP	0(0%)	0(0%)	0(0%)
Stella	NT	2	2	2
	NP	0(0%)	0(0%)	0(0%)
TOTAL	NT	27	27	27
	NP	8(29.6%)	8(29.6%)	1(3.7%)

NT= Number Tested, NP= Number Positive

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1. General Discussion

The primary aim of this study was to determine the occurrence of enterococci and *E. faecalis* in particular in groundwater samples intended for consumption by individuals in some communities in the North West Province, South Africa. In the present study, a total of 253 isolates satisfied the morphological identification characteristics for *Enterococcus* species and a large proportion 212 (83.8%) of these isolates possessed the catalase enzyme (van Veen *et al.,* 2010). The presumptive finding presented herein, supports a previous report in which, *E. faecalis* rather than *E. faecium* isolates were usually catalase positive (Moy *et al.,* 2004). Moreover, a large proportion 78 (30.8%) of the isolates were able to grow in the presence of 6.5% NaCl while 26.9% of the isolates were gamma haemolytic. The ability of enterococci to grow in 6.5% NaCl broth is currently considered an important distinguishing characteristic that facilitates identification of these species (Klein, 2003). *E. faecalis* are known to display gamma haemolytic patterns and it is also suggested that the presence of the haemolysin A gene (*hlyA*) greatly determines the expression of this phenotypic trait in bacterial species.

Despite the fact there are no specific preliminary cultural and phenotypic criteria that may be used to conclusively distinguish *Enterococcus* species from other bacteria, the preliminary identification characteristics obtained for isolates in the present study are similar to those of a previous report (Sherman, 1937). In addition, the identification of typical enterococci is accurately achieved by detecting a combination of genus and species characteristics (Devriese and Pot, 1995). Owing to the fact that enterococci have been isolated in different food products, clinical samples, water intended for consumption and from environmental sources, coupled with the fact that they are able to resist heat treatment, makes them a central

issue of concern in safety regulating procedures (Stiles and Holzapfel, 1997; Giraffa, 2002). Enterococci are also known to play important roles in various fermented products. However, due to the fact that they are dominant in the soil and the gastrointestinal tract of animals and subsequently in the faeces, they may serve as indicators of faecal contamination, especially in areas that do not have access to proper sanitary and health care facilities (Aarestrup *et al.*, 2002; Domingo *et al.*, 2003; Kuhn *et al.*,2003; Fisher and Phillips, 2009). During the last two decades, enterococci obtained increasing medical attention as agents of nosocomial infections and are easily acquired in hospitals where they pose severe challenges among immuno-compromised patients. A recent healthcare report lists *Enterococcus* spp. as the second leading cause of nosocomially acquired urinary tract and bloodstream infections among patients in intensive care units (ECDC, 2011). Moreover, enterococci are not primarily recognized as pathogens in animals. This therefore would mean it is necessary to constantly monitor the occurrence of enterococci species in the environment since the potential to carry pathogenic determinants may be prevalent and affects in consumers.

Another objective of the study was to confirm the identities of presumptive enterococci obtained through preliminary cultural and phenotypic tests using bacterial 16S rRNA specific PCR, *E. faecalis* specific PCR assay and the MALDI-TOF Mass spectrometry. A large proportion 169 (66.8%) of the isolates were positively identified through PCR amplification using a previously reported bacterial 16S rRNA oligonucleotide sequences (Creti *et al.*, 2004). Similarly a large proportion 122 (48.2%) of these isolates were positively identified as *Enterococcus faecalis*. It is therefore suggested that despite the high prevalence of *E. faecalis* among the isolates that were subjected to PCR analysis the samples that were collected may have been contaminated with a diverse group of *Enterococcus* species. The results obtained in this study showed some significant similarities to the study conducted by Trivedi *et al.*

(2011), where the majority of species isolated were *E. faecalis,* followed by *E. faecium* and other *Enterococcus* species.

Despite the complications caused by *Enterococcus* species and the need to detect in water, food and environmental samples it has been reported that a number of enterococci may be misidentified by biochemical and phenotypic assays (Wyder *et al.,* 2011) and therefore there is the need to combine these identifications criteria with DNA and proteomic tests. Against this backdrop new diagnostic techniques for a valid species identification are currently been developed and the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) analysis appear to be a powerful and promising alternative that facilitates correct identification of bacteria species including enterococci (Seng *et al.,* 2010). Moreover, the routine use of MALDI-TOF MS has revolutionized the identification of microorganisms especially in clinical settings (DeMarco and Ford, 2013). In addition, the identification efficiency for bacteria, particularly *E. faecalis* and *E. faecium,* was greatly improved when MALDI-TOF MS was combined with serotyping and PCR analysis (van Veen *et al.,* 2010; Christensen *et al.,* 2012; Werner *et al.,* 2012 Samadi, 2013; da Silva Paim, 2013).

Despite the fact that only a small proportion of the isolates were subjected to the MALDI-TOF MS, the results of obtained in the present study indicated that *E. faecalis* was correctly identified based on total cell proteins analysis. Based on the MALDI-TOF MS, the results obtained in this study are in line with one conducted in the USA, (Giebel *et al.,* 2008). The spectra peaks for *Enterococcus* species isolates in a previously mentioned study ranged from 900 to 7150 *m/z* but when the cells were treated with lysozyme for 20 hours the peaks were found between 1046 and 9546 *m/z* (Vargha *et al.,* 2006). However, in the present study

spectra were recorded at 2000 to 18000Da but a majority of the protein peaks were observed between 2000 to 10000 Da. The similarities in the protein profiles indicated that the peaks recorded encode for proteins that are specific to *E. faecalis* in all the isolates.

Existing bacteria source tracking tools (BST) are time consuming, labour-intensive and often ineffective in the identification and characterisation of bacteria species. Consequently, the development of more rapid and accurate methods of characterizing sources of bacterial contamination of water bodies especially those that are used for immediate consumption is of paramount importance. The MALDI-TOF MS is one method that shows promise in the characterization of bacteria (Lay, 2001). Similar to the results presented in the current study the MALDI-TOF MS has been able to identify bacteria to the genus (Pribil and Fenselau, 2005) and species (Vargha *et al.,* 2006; Pignone *et al.,* 2006) levels.

Despite the significant success made towards the correct identification and characterization of bacteria using MALDI-TOF-MS in recent years (Pribil and Fenselau, 2005; Vargha *et al.,* 2006; Pignone *et al.,* 2006), the application of this approach to characterizing indicator bacteria commonly used in BST, including *Escherichia coli* and *Enterococcus,* has been limited (Giebel *et al.,* 2008). Although *Enterococcus* species are often used as indicator organisms (Kinzelman *et al.,* 2003), no efforts have been made to characterize these microorganisms with MALDI-TOF-MS for purposes of BST. Thus, available data suggest that application of traditional DNA fingerprinting techniques to environmental isolates of *Enterococcus* may fail to group *Enterococcus* isolates by source, making source tracking with this indicator organism difficult. However, in a previous study, the overall ability of MALDI-TOF MS to group enterococci based on the source, particularly those originating from gull, chicken, and human sources, supports the potential of using this technique as a reliable BST

tool (Giebel *et al.,* 2008). Unfortunately, the current study was not designed to determine to genetic similarities of isolates using MALDI-TOF MS. However, it is suggested that further studies be conducted that would employ different genotypic methods including MALDI-TOF MS for characterization of *Enterococcus* species, particularly *E. faecalis*.

A new wave of concern is the detection of enterococci that are resistant to vancomycin in many parts of the world (Williems *et al.,* 2005; Werner, *et al.,* 2012). Moreover, the detection of VRE in environmental samples collected in the Mafikeng area served as a motivation for this study (Ateba and Maribeng, 2011). Consequently a further objective of the study was to determine the antibiotic resistant profiles and the presence of vancomycin resistant determinants in isolates obtained from groundwater. Vancomycin demonstrates a time-dependent killing of susceptible bacteria cells (Liu *et al.,* 2011; Schweizer *et al.,* 2011), and it is suggested that the decision to use the antibiotic depends largely on the type of bacteria that needs to be eradicated (Liu *et al.,* 2011; Schweizer *et al.,* 2011). On the contrary, the development and persistence of vancomycin resistant determinants in the environment has been reported to have severe health implications to individuals since the presence of these genetic traits complicates clinical treatment of infections (Rubinstein and Keynan, 2013).

Untreated drinking water is frequently overlooked as a source of antibiotic resistance. Therefore, to ascertain if groundwater obtained from some communities in the North West Province could possess antibiotic resistant *E. faecalis* isolates, the antimicrobial susceptibility and high-level aminoglycoside resistance (HLAR) tests were performed using the Kirby-Bauer disc diffusion techniques (Bauer *et al.*, 1996; Junco *et al.,* 2001; Macedo *et al.,* 2011). The results obtained revealed that a large proportion of the isolates were resistant to vancomycin, ampicillin, amoxicillin, erythromycin, tetracycline and penicillin. These findings were significantly different from those of a study conducted in Spain where *E.*

faecalis isolates were susceptible to all the antibiotics to which isolates in the present study were resistant to (Junco *et al.,* 2001). However, some isolates were susceptible to gentamycin, streptomycin, norfloxacin, kanamycin, ciprofloxacin, chloramphenicol and teicoplanin. Multiple antibiotic resistance strains defined as strains resistant to three or more antibiotics, was evident in almost all the isolates that were screened. However, a large proportion of the isolates displayed distinguished phenotypes, hence, there was no dominant phenotype for MAR isolates obtained for most stations sampled. Similar observations have been reported (Aarestrup *et al.,* 2000; Johnston and Jaykus, 2004; Macedo *et al.,* 2011).

In the present study a large proportion (96% to 100%) of the isolates from the different areas revealed HLAR traits and this high level resistance to gentamycin and streptomycin was a concern. Similar observations have been reported for enterococci isolated from water from wells in Chile (Padilla and Lobos, 2013). These antibiotics are used for the treatment of enterococcal infections. Moreover, the isolates that presented with the HLAR traits were also resistant to vancomycin. VRE are difficult to treat; therefore, the implementation of infection control measures in the communities and hospitals are of prime importance in preventing the establishment of these pathogens. In addition, most severe VRE infections will need combination therapy because many of the effective antimicrobial agents, when used alone, have only a bacteriostatic effect.

Given the fact that other antimicrobials targeted at killing VRE are currently under development, quinupristin-dalfopristin and linezolid are among the drugs that have recently become available to treat infection caused by VRE (Zirakzadeh and Patel, 2006). It is therefore reported that severe enterococcal infections require a bactericidal therapy, which is usually a combination of a cell wall active agent including gentamycin or streptomycin. The

increasing resistance to high levels of aminoglycosides and to other antimicrobials may lead to the loss of effective synergisms and thus, bactericidal activity (Pasticci *et al.,* 2008; Arias *et al.*, 2010). This pattern of resistance might significantly influence the use of wide spectrum antibiotic treatment of infections.

Since the emergence of vancomycin-resistant enterococci, several studies have been conducted to investigate the dissemination of resistant genes in these strains. It was revealed that *vanA* and *vanB* genes were responsible for resistance to vancomycin. However, bacteria that harbour the *vanA* and *vanB* genotypes were found to contain transposonsable elements *Tn1546, Tn1547* and other closely related transferable genetic elements, which confer resistance to vancomycin (Jensen *et al.,* 1998; Lauderdale *et al.,* 2002). The results obtained in the present study indicated that the *vanA* gene was present in a large proportion (51.6%) of the isolates. However, there was even a larger proportion 83 (68%) of the isolates that tested positive for the *vanB* genotype. These results are consistent with those obtained by Malani *et al.* (2002). Similar to the present findings, in their study, 89.4% of the isolates were positive for the *vanB* genotype. Trivedi *et al.* (2011) also demonstrated that the presence of either *E. faecalis* or *E. faecium* in environmental samples does not automatically imply that there is the presence of *vanA* or *vanB* genes in the isolates. It is therefore suggested that the occurrence of vancomycin-resistant genes cannot be predicted even in isolates that portray phenotypic resistance patterns. This explains the need to constantly monitor the presence of both vancomycin resistance phenotypes and genotypes among antibiotic resistant entorococci from food and water sources.

In an attempt to screen for the presence of transposable elements, results revealed that the transposon *Tn1546* was more prevalent in *vanA* isolates while the *Tn1547* transposon that is often associated with the *vanB* genotype was not detected in any of the isolates. Of the two transferable elements, *vanA* confers high level resistance to vancomycin and is carried on the *Tn1546* transposon (Sung *et al.,* 2008). Despite the fact that resistance to vancomycin is expressed through the action of various genes, it has been shown that the genes are present together on the *Tn1546* and are collectively known as the *vanA* structural elements (Arthur *et al.,* 1992). In the present study, a large proportion of the *vanA E. faecalis* isolates possessed the *Tn1546* transposon. Similar to a previous report (Sung *et al.,* 2008) the isolates *vanA E. faecalis* isolates in the present study were highly resistant to both vancomycin and teicoplanin. Moreover, a cause for concern was the fact that none of the *E. faecalis* isolates that portrayed the vanB phenotype that is encoded by the *vanB* gene harboured the *Tn1547* transposon. In addition isolates with the vanB genotype are known to exhibit low-level resistance to vancomycin but susceptibility to teicoplanin (Cetinkaya *et al.,* 2000). This was evident in some isolates although this trend was uniform for all the isolates.

In recent years, studies conducted on vancomycin resistant *E. faecium* have revealed the emergence of strains that display high level incompatibility between phenotypes and genotypes (Hashimoto *et al.,* 2000; Eom *et al.,* 2004; Lee *et al.,* 2004; Ko *et al.,* 2005). Similar, to the observations made in the current study, some enterococci possess the vanA phenotype with the *vanB* gene and vice versa (Hashimoto *et al.,* 2000; Eom *et al.,* 2004; Lee *et al.,* 2004; Ko *et al.,* 2005). It has been reported that the arrangement of genes in the *Tn1546* transposon can be altered through the addition of insertion sequences and this leads to genetic heterogeneity (Arthur *et al.,* 1992). Genetic heterogeneity of the *Tn1546* transposon most often occurs through mutations in the *vanR* and *vanS* genes, deletions and

movement of insertion sequences within the *Tn1546* (Handwerger *et al.,* 1995). Moreover, it has also been reported the point mutations in the sensor domain of *vanS* gene (Hashimoto *et al.,* 2000; Eom *et al.,* 2004) or impairment of the accessory proteins VanY and vanZ (Lee *et al.,* 2004; Ko *et al.,* 2005) may account for the loss of teicoplanin resistance in enterococci. These may account for the low levels of resistance to teicoplanin observed in the study. Given the fact that there is still a lot of controversy regarding the exact mechanism through which VanB phenotype – vanA genotype and vice versa develop in VRE, it is suggested that the epidemiology or molecular characteristics of such strains is not yet known.

The present study has successfully documented the presence of vancomycin resistance *E. faecalis* from groundwater that is intended for human consumption. In addition it was revealed that these isolates harbour multiple antibiotic resistant phenotypes especially to vancomycin and this is a cause for concern. The isolates that were found to possess *vanA* and *vanB* resistant genes and some of the isolates with the *vanA* gene also possessed the *Tn1546* transposon even though the *Tn1547* transposon was not detected. The antibiotic vancomycin, is generally considered the first drug of choice in the treatment of nosocomial infections caused by methicillin resistant *Staphylococcus aureus* and other Gram-positive bacteria such as ampicillin resistant enterococci (Lauderdale *et al.,* 2002; Courvalin, 2006; Covajes *et al.,* 2013). However, the misuse of vancomycin in both human and animal medicine resulted in the development and spread of vancomycin resistant bacteria strains and particularly VRE in many parts of the world (Rice, 2006; Donabedian *et al.,* 2010). Consequently, the drug was banned and its use in both human and animal medicine was discontinued (Salemi *et al.,* 1998). The results presented in here indicate that, there is a need to limit contamination. This will then assist to reduce the burden of disease in humans.

Given the level of heterogeneity among the vancomycin genotypes and their associated phenotypes, it is suggested the present investigation be expanded to determine the exact genotype harboured by isolates that portray either the vanA or vanB phenotypes. Results obtained may be of great importance from an epidemiological point of view.

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APPENDIX

Table 1: Preliminary identification tests and antibiogram raw data

Sample number: **RW1**

Sampling site: **Ramosadi**

Isolate number	RW1-1	RW1-2	RW1-3	RW1-4	RW1-5	RW1-6	RW1-7	RW1-8
Biochemical Tests								
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	-	-	+	+	+	+	-	+
Specific PCR analysis								
16SrRNA	-	+	+	-	+	+	+	+
E. faecalis	+	+	+	+	+	+	+	+
VanA	+	-	-	+	+	-	+	+
VanB	+	-	-	-	+	+	+	-
Tn1546	-	-	-	+	+	+	-	+
Antibiotic IZD data (mm)								
VA(30)	6	6	6	6	10	15	6	10
AP(10)	6	6	6	6	6	6	6	6
S(10)	17	10	10	26	22	27	18	17
GM(10)	20	12	12	12	11	15	11	12
OT(10)	12	13	14	13	15	15	12	15
Nor(10)	18	16	15	10	15	17	22	21
TEC(30)	14	10	12	24	14	16	16	13
HLAR [Antibiotic IZD data (mm)]								
S(300)	27	30	30	45	23	29	25	26

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 2: Preliminary identification tests and antibiogram raw data

Sample number: **RW2**

Sampling site: **Ramosadi**

Isolate number	RW2-1	RW2-2	RW2-3	RW2-4	RW2-5	RW2-6	RW2-7	RW2-8
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	-	+	+	+	-	-	-	-
Specific PCR analysis								
16SrRNA	+	+	-	-	-	+	+	+
VanA	-	+	-	-	-	-	+	+
Tn1546	-	+	-	+	-	-	+	+
Antibiotic IZD data (mm)								
VA(30)	6	20	12	NT	10	6	NT	12
AP(10)	6	18	9	NT	12	6	NT	12
S(10)	12	16	18	NT	12	14	NT	10
GM(10)	17	17	16	NT	15	16	NT	16
OT(10)	9	9	11	NT	9	18	NT	12
Nor(10)	22	18	15	NT	6	18	NT	6
TEC(30)	14	16	10	NT	30	20	NT	11
HLAR [Antibiotic IZD data (mm)]								
S(300)	20	20	30	NT	16	17	NT	20
GM(120)	20	15	20	NT	15	20	NT	20

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 3: Preliminary identification tests and antibiogram raw data

Sample number: **RW3**

Sampling site: **Ramosadi**

Isolate number	RW3-1	RW3-2	RW3-3	RW3-4	RW3-5	RW3-6	RW3-7	RW3-8
Biochemical Tests								
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	-	+	-	+	-	-	-	+
Specific PCR analysis								
16SrRNA	+	-	+	+	-	-	+	+
E. faecalis	+	+	-	-	+	-	+	+
VanA	+	+	-	+	-	+	+	+
VanB	-	+	-	+	+	+	+	+
Tn1546	-	+	-	-	-	-	+	+
Antibiotic IZD data (mm)								
VA(30)	8	12	NT	NT	10	NT	6	8
AP(10)	6	8	NT	NT	6	NT	9	6
S(10)	16	15	NT	NT	20	NT	20	21
GM(10)	16	14	NT	NT	13	NT	14	16
OT(10)	10	9	NT	NT	10	NT	8	10
Nor(10)	18	16	NT	NT	18	NT	12	15
TEC(30)	23	6	NT	NT	17	NT	25	20
HLAR [Antibiotic IZD data (mm)]								
S(300)	25	24	NT	NT	15	NT	17	30
G(120)	22	20	NT	NT	26	NT	17	32

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 4: Preliminary identification tests and antibiogram raw data

Sample number: **RW4**

Sampling site: **Ramosadi**

Isolate number	RW4-1	RW4-2	RW4-3	RW4-4	RW4-5	RW4-6	RW4-7	RW4-8
Biochemical Tests								
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	+	+	+	+
Specific PCR analysis								
16SrRNA	-	+	+	+	+	-	+	-
E. faecalis	-	+	+	-	-	-	+	+
VanA	+	+	+	-	+	-	-	+
VanB	+	+	+	+	-	+	+	+
Tn1546	+	-	-	+	-	-	-	+
Antibiotic IZD data (mm)								
VA(30)	NT	10	13	NT	NT	NT	6	9
AP(10)	NT	12	8	NT	NT	NT	6	10
S(10)	NT	24	20	NT	NT	NT	14	16
GM(10)	NT	16	16	NT	NT	NT	12	12
OT(10)	NT	20	11	NT	NT	NT	15	10
Nor(10)	NT	12	14	NT	NT	NT	11	15
TEC(30)	NT	26	10	NT	NT	NT	12	6
HLAR [Antibiotic IZD data (mm)]								
S(300)	NT	30	25	NT	NT	NT	25	25
GM(120)	NT	40	31	NT	NT	NT	26	26

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 5: Preliminary identification tests and antibiogram raw data

Sample number: **RW5**

Sampling site: **Ramosadi**

Isolate number	RW5-1	RW5-2	RW5-3	RW5-4	RW5-5	RW5-6	RW5-7	RW5-8
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase test	+	+	+	+	+	+	+	+
Specific PCR analysis								
16SrRNA	-	+	-	+	+	+	+	+
VanA	+	-	-	-	+	+	+	+
Tn1546	-	-	-	-	-	+	-	-
Antibiotic IZD data (mm)								
VA(30)	NT	10	12	NT	NT	9	NT	15
AP(10)	NT	10	10	NT	NT	10	NT	10
S(10)	NT	14	12	NT	NT	14	NT	12
GM(10)	NT	14	12	NT	NT	10	NT	20
OT(10)	NT	10	10	NT	NT	9	NT	16
Nor(10)	NT	20	19	NT	NT	20	NT	16
TEC(30)	NT	6	6	NT	NT	6	NT	10
HLAR [Antibiotic IZD data (mm)]								
S(300)	NT	22	21	NT	NT	26	NT	30

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 6: Preliminary identification tests and antibiogram raw data

Sample number: **DW1**

Sampling site: **Dibate**

Isolate number	DW1-1	DW1-2	DW1-3	DW1-4	DW1-5	DW1-6	DW1-7	DW1-8
Gram staining	+	+	+	+	+	+	+	+
Catalase test	-	+	+	-	-	-	+	+
Specific PCR analysis								
16SrRNA	+	+	-	+	+	-	-	+
VanA	+	-	+	+	-	+	+	+
Tn1546	+	-	-	-	-	+	-	+
Antibiotic IZD data (mm)								
VA(30)	6	6	NT	6	6	NT	NT	NT
AP(10)	6	6	NT	12	11	NT	NT	NT
S(10)	25	20	NT	15	20	NT	NT	NT
GM(10)	12	10	NT	12	11	NT	NT	NT
OT(10)	10	10	NT	15	9	NT	NT	NT
Nor(10)	18	16	NT	17	17	NT	NT	NT
C(30)	18	20	NT	20	16	NT	NT	NT
TEC(30)	16	6	NT	20	10	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]								
S(300)	27	25	NT	25	22	NT	NT	NT
GM(120)	35	30	NT	30	25	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 7: Preliminary identification tests and antibiogram raw data

Sample number: **DW2**

Sampling site: **Dibate**

Isolate number	DW2-2	DW2-3	DW2-4	DW2-5	DW2-6	DW2-7	DW2-8
Biochemical Tests							
Gram staining	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	-	+	+	+	+	-
Specific PCR analysis							
16SrRNA	+	-	-	+	+	-	+
E. faecalis	-	-	-	-	+	+	-
VanA	+	+	-	-	-	-	+
VanB	-	+	+	+	-	+	-
Tn1546	+	-	+	-	-	-	-
Antibiotic IZD data (mm)							
VA(30)	NT	NT	NT	NT	6	6	NT
AP(10)	NT	NT	NT	NT	9	6	NT
S(10)	NT	NT	NT	NT	20	12	NT
GM(10)	NT	NT	NT	NT	16	16	NT
OT(10)	NT	NT	NT	NT	14	14	NT
Nor(10)	NT	NT	NT	NT	22	21	NT
TEC(30)	NT	NT	NT	NT	16	10	NT
HLAR [Antibiotic IZD data (mm)]							
S(300)	NT	NT	NT	NT	25	24	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 8: Preliminary identification tests and antibiogram raw data

Sample number: **DW3**

Sampling site: **Dibate**

Isolate number	DW3-1	DW3-2	DW3-3	DW3-4	DW3-5	DW3-6	DW3-8
Biochemical tests							
Gram staining	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	-	+	+	+	+	-
Specific PCR analysis							
16SrRNA	+	-	+	+	+	+	+
E. faecalis	+	-	-	+	-	-	+
VanA	+	-	+	+	+	+	-
VanB	-	-	+	-	-	+	-
Tn1546	+	-	-	-	-	-	-
Antibiotic IZD data (mm)							
VA(30)	6	NT	NT	6	NT	NT	6
AP(10)	6	NT	NT	6	NT	NT	12
S(10)	14	NT	NT	26	NT	NT	21
GM(10)	14	NT	NT	12	NT	NT	14
OT(10)	12	NT	NT	13	NT	NT	15
Nor(10)	17	NT	NT	20	NT	NT	20
TEC(30)	10	NT	NT	15	NT	NT	14
HLAR [Antibiotic IZD data (mm)]							
S(300)	27	NT	NT	45	NT	NT	27
GM(120)	24	NT	NT	31	NT	NT	24

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 9: Preliminary identification tests and antibiogram raw data

Sample number: **DW4**

Sampling site: **Dibate**

Isolate number	DW4-1	DW4-2	DW4-3	DW4-4	DW4-5	DW4-6	DW4-7	DW4-8
Biochemical Tests								
Gram staining	+	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	+	+	+	+
Specific PCR analysis								
16SrRNA	-	+	+	+	-	-	-	-
VanA	-	-	+	-	-	-	+	+
Tn1546	-	-	+	-	-	-	-	+
Antibiotic IZD data (mm)								
VA(30)	6	6	6	NT	6	6	6	NT
AP(10)	6	6	6	NT	6	6	6	NT
S(10)	20	12	20	NT	14	16	14	NT
GM(10)	18	12	11	NT	14	20	12	NT
OT(10)	15	9	9	NT	11	11	10	NT
Nor(10)	20	12	16	NT	16	20	16	NT
TEC(30)	28	10	6	NT	14	14	12	NT
HLAR [Antibiotic IZD data (mm)]								
S(300)	24	23	23	NT	21	22	24	NT
GM(120)	35	24	28	NT	24	22	25	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 10: Preliminary identification tests and antibiogram raw data

Sample number: **DW5**

Sampling site: **Dibate**

Isolate number	DW5-1	DW5-2	DW5-3	DW5-4	DW5-5	DW5-6	DW5-7	DW5-8
Gram staining	+	+	+	+	+	+	+	+
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Catalase test	-	+	+	+	+	-	-	+
Specific PCR analysis								
16SrRNA	-	-	-	-	+	+	+	+
VanA	-	+	-	-	+	+	+	+
Tn1546	-	-	-	-	-	-	-	-
Antibiotic IZD data (mm)								
VA(30)	NT	6	6	6	6	6	NT	NT
AP(10)	NT	6	6	6	6	6	NT	NT
S(10)	NT	12	20	20	18	22	NT	NT
GM(10)	NT	18	18	12	14	10	NT	NT
OT(10)	NT	17	6	17	12	6	NT	NT
Nor(10)	NT	16	20	18	20	18	NT	NT
TEC(30)	NT	18	30	20	19	24	NT	NT
HLAR [Antibiotic IZD data (mm)]								
S(300)	NT	25	25	24	26	26	NT	NT
GM(120)	NT	30	40	37	28	41	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 11: Preliminary identification tests and antibiogram raw data

Sample number: **MW1**

Sampling site: **Motlhabeng**

Isolate number	MW1-1	MW1-4	MW1-5	MW1-7	MW1-8
Gram staining	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	+
Specific PCR analysis					
16SrRNA	+	-	+	+	+
VanA	+	+	+	+	-
Tn1546	-	+	-	-	-
Antibiotic IZD data (mm)					
VA(30)	16	18	NT	NT	NT
AP(10)	6	8	NT	NT	NT
S(10)	20	22	NT	NT	NT
GM(10)	20	16	NT	NT	NT
OT(10)	14	20	NT	NT	NT
Nor(10)	18	18	NT	NT	NT
TEC(30)	6	14	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]					
S(300)	26	6	NT	NT	NT
GM(120)	26	26	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 12: Preliminary identification tests and antibiogram raw data

Sample number: **MW2**

Sampling site: **Motlhabeng**

Isolate number	MW2-1	MW2-2	MW2-3	MW2-4	MW2-6	MW2-7
Gram staining	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	+	+
Specific PCR analysis						
16SrRNA	+	-	+	+	+	-
VanA	-	+	+	+	+	-
Tn1546	-	-	-	-	-	-
Antibiotic IZD data (mm)						
VA(30)	NT	10	NT	6	6	NT
AP(10)	NT	6	NT	6	6	NT
S(10)	NT	12	NT	14	6	NT
GM(10)	NT	20	NT	16	16	NT
OT(10)	NT	6	NT	14	6	NT
Nor(10)	NT	22	NT	25	14	NT
TEC(30)	NT	16	NT	6	6	NT
HLAR [Antibiotic IZD data (mm)]						
S(300)	NT	28	NT	25	18	NT
GM(120)	NT	25	NT	28	26	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 13: Preliminary identification tests and antibiogram raw data

Sample number: **MW3**

Sampling site: **Motlhabeng**

Isolate number	MW3-1	MW3-2	MW3-3	MW3-4	MW3-6	MW3-7
Gram staining	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	+	+
Specific PCR analysis						
16SrRNA	+	+	-	+	-	+
VanA	+	+	-	-	-	+
Tn1546	-	-	-	-	-	+
Antibiotic IZD data (mm)						
VA(30)	NT	16	NT	NT	6	14
AP(10)	NT	16	NT	NT	6	6
S(10)	NT	22	NT	NT	17	21
GM(10)	NT	24	NT	NT	18	14
OT(10)	NT	26	NT	NT	6	6
Nor(10)	NT	26	NT	NT	14	15
TEC(30)	NT	18	NT	NT	6	6
HLAR [Antibiotic IZD data (mm)]						
S(300)	NT	30	NT	NT	20	23
GM(120)	NT	31	NT	NT	28	27

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 14: Preliminary identification tests and antibiogram raw data

Sample number: **MW4**

Sampling site: **Motlhabeng**

Isolate number	MW4-1	MW4-2	MW4-3	MW4-4	MW4-6	MW4-7	MW4-8
Biochemical Tests							
Gram staining	+	+	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	-	+	+	+	-
Specific PCR analysis							
16SrRNA	-	-	+	+	-	+	+
E. faecalis	-	-	-	+	+	-	-
VanA	+	-	-	+	-	+	-
VanB	-	-	-	-	-	-	-
Tn1546	-	-	-	-	-	-	-
Antibiotic IZD data (mm)							
VA(30)	NT	NT	NT	6	15	NT	NT
AP(10)	NT	NT	NT	6	6	NT	NT
S(10)	NT	NT	NT	6	18	NT	NT
GM(10)	NT	NT	NT	20	18	NT	NT
OT(10)	NT	NT	NT	6	6	NT	NT
Nor(10)	NT	NT	NT	16	22	NT	NT
TEC(30)	NT	NT	NT	6	16	NT	NT
HLAR [Antibiotic IZD data (mm)]							
S(300)	NT	NT	NT	14	16	NT	NT
GM(120)	NT	NT	NT	21	26	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 15: Preliminary identification tests and antibiogram raw data

Sample number: **MW5**

Sampling site: **Motlhabeng**

Isolate number	MW5-1	MW5-2	MW5-3	MW5-7	MW5-8
Biochemical Tests					
Gram staining	+	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+	-
Specific PCR analysis					
16SrRNA	-	+	+	+	+
E. faecalis	+	+	+	-	-
VanA	+	-	-	-	-
VanB	-	-	+	+	+
Tn1546	+	-	-	-	-
Antibiotic IZD data (mm)					
VA(30)	6	14	6	NT	NT
AP(10)	6	6	6	NT	NT
S(10)	18	13	6	NT	NT
GM(10)	18	15	18	NT	NT
OT(10)	21	12	21	NT	NT
Nor(10)	12	16	30	NT	NT
TEC(30)	6	14	6	NT	NT
HLAR [Antibiotic IZD data (mm)]					
S(300)	18	24	24	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 16: Preliminary identification tests and antibiogram raw data

Sample number: **DVW1**

Sampling site: **Delareyville**

Isolate number	DVW1-2	DVW1-3	DVW1-4
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	+	-	-
Specific PCR analysis			
16SrRNA	+	-	+
VanA	-	-	-
VanB	-	-	+
Tn1546	-	+	+
Antibiotic IZD data (mm)			
VA(30)	8	6	6
AP(10)	18	20	12
S(10)	6	10	12
GM(10)	12	17	18
OT(10)	20	9	15
Nor(10)	19	16	21
TEC(30)	22	17	18
S(300)	30	25	22

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 17: Preliminary identification tests and antibiogram raw data

Sample number: **DVW2**

Sampling site: **Delareyville**

Isolate number	DVW2-1	DVW2-2	DVW2-3	DVW2-4
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	-	-	-	-
Specific PCR analysis				
16SrRNA	-	-	+	+
E. faecalis	+	+	+	+
VanA	-	+	+	-
VanB	+	+	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	6	16	6	6
AP(10)	17	18	20	20
S(10)	20	24	20	20
GM(10)	10	10	12	10
OT(10)	18	20	6	20
Nor(10)	15	15	12	12
TEC(30)	6	6	16	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	30	28	30	30
GM(120)	16	17	18	16

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 18: Preliminary identification tests and antibiogram raw data

Sample number: **DVW3**

Sampling site: **Delareyville**

Isolate number	DVW3-2	DVW3-4
Biochemical Tests		
Gram staining	+	+
Cell morphology	Coccus	Coccus
Catalase test	+	+
Specific PCR analysis		
16SrRNA	+	+
E. faecalis	+	+
VanA	-	-
VanB	-	+
Tn1546	-	-
Antibiotic IZD data (mm)		
VA(30)	16	16
AP(10)	6	6
S(10)	13	17
GM(10)	16	20
OT(10)	12	18
Nor(10)	12	16
TEC(30)	6	16
HLAR [Antibiotic IZD data (mm)]		
S(300)	30	28

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 19: Preliminary identification tests and antibiogram raw data

Sample number: **DPW1**

Sampling site: **Deelpan**

Isolate number	DPW1-1	DPW1-2	DPW1-4
Gram staining	+	+	+
Catalase test	-	+	+
Specific PCR analysis			
16SrRNA	+	-	+
VanA	-	-	-
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	30	NT	NT
AP(10)	6	NT	NT
S(10)	14	NT	NT
GM(10)	18	NT	NT
OT(10)	12	NT	NT
Nor(10)	10	NT	NT
TEC(30)	20	NT	NT
HLAR [Antibiotic IZD data (mm)]			
S(300)	28	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 20: Preliminary identification tests and antibiogram raw data

Sample number: **DPW2**

Sampling site: **Deelpan**

Isolate number	DPW2-1
Biochemical tests	
Gram staining	+
Cell morphology	Coccus
Catalase test	+
Specific PCR analysis	
16SrRNA	+
E. faecalis	+
VanA	+
VanB	-
Tn1546	+
Antibiotic IZD data (mm)	
VA(30)	6
AP(10)	6
S(10)	14
GM(10)	12
OT(10)	12
Nor(10)	14
TEC(30)	18
HLAR [Antibiotic IZD data (mm)]	
S(300)	28

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 21: Preliminary identification tests and antibiogram raw data

Sample number: **DPW3**

Sampling site: **Deelpan**

Isolate number	DPW3-1	DPW3-2	DPW3-3	DPW3-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
E. faecalis	-	-	-	-
VanB	+	+	+	+
Antibiotic IZD data (mm)				
PG(10)	NT	NT	NT	NT
A(10)	NT	NT	NT	NT
K(30)	NT	NT	NT	NT
E(15)	NT	NT	NT	NT
CIP(5)	NT	NT	NT	NT
C(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 22: Preliminary identification tests and antibiogram raw data

Sample number: **DPW4**

Sampling site: **Deelpan**

Isolate number	DPW4-2	DPW4-3	DPW4-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	+	+	+
Specific PCR analysis			
16SrRNA	+	+	-
E. faecalis	+	-	+
VanA	+	-	+
VanB	+	+	-
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	6	NT	6
AP(10)	6	NT	6
S(10)	18	NT	14
GM(10)	18	NT	18
OT(10)	17	NT	6
Nor(10)	30	NT	30
TEC(30)	6	NT	22
HLAR [Antibiotic IZD data (mm)]			
S(300)	25	NT	22
GM(120)	21	NT	26

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 23: Preliminary identification tests and antibiogram raw data

Sample number: **DPW5**

Sampling site: **Deelpan**

Isolate number	DPW5-1	DPW5-2	DPW5-3	DPW5-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	-	-	+
E. faecalis	+	-	+	+
VanA	-	-	+	+
VanB	-	+	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	8	NT	14	16
AP(10)	6	NT	6	6
A(10)	8	NT	20	18
S(10)	18	NT	6	17
GM(10)	20	NT	18	14
OT(10)	21	NT	21	18
Nor(10)	16	NT	12	12
TEC(30)	6	NT	18	16
HLAR [Antibiotic IZD data (mm)]				
S(300)	22	NT	24	20

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 23: Preliminary identification tests and antibiogram raw data

Sample number: **DPW6**

Sampling site: **Deelpan**

Isolate number	DPW6-1	DPW6-4
Biochemical Tests		
Gram staining	+	+
Cell morphology	Coccus	Coccus
Catalase test	+	+
Specific PCR analysis		
16SrRNA	-	-
E. faecalis	-	-
VanA	-	-
VanB	+	+
Tn1546	-	-
Antibiotic IZD data (mm)		
VA(30)	NT	NT
AP(10)	NT	NT
S(10)	NT	NT
GM(10)	NT	NT
OT(10)	NT	NT
Nor(10)	NT	NT
TEC(30)	NT	NT
HLAR [Antibiotic IZD data (mm)]		
S(300)	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 24: Preliminary identification tests and antibiogram raw data

Sample number: **DNW1**

Sampling site: **Dingateng**

Isolate number	DNW1-2	DNW1-3	DNW1-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	+	+	+
Specific PCR analysis			
16SrRNA	-	+	+
E. faecalis	-	-	+
VanA	-	-	-
VanB	+	+	+
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	NT	NT	10
P(10)	NT	NT	10
AP(10)	NT	NT	16
AU(10)	NT	NT	8
S(10)	NT	NT	16
K(10)	NT	NT	20
GM(10)	NT	NT	20
B(10)	NT	NT	10
OT(10)	NT	NT	18
C(10)	NT	NT	10
Nor(10)	NT	NT	18
CN(10)	NT	NT	20
TEC(30)	NT	NT	6
HLAR [Antibiotic IZD data (mm)]			
S(300)	NT	NT	26
CN(120)	NT	NT	10

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 25: Preliminary identification tests and antibiogram raw data

Sample number: **DNW2**

Sampling site: **Dingateng**

Isolate number	DNW2-1	DNW2-2	DNW2-3	DNW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	+	-	+
Van gene				
VanA	-	+	-	-
Van gene				
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	6	NT	NT
P(10)	NT	6	NT	NT
AP(10)	NT	6	NT	NT
S(10)	NT	6	NT	NT
GM(10)	NT	10	NT	NT
OT(10)	NT	6	NT	NT
Nor(10)	NT	22	NT	NT
TEC(30)	NT	14	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	22	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 26: Preliminary identification tests and antibiogram raw data

Sample number: **DNW3**

Sampling site: **Dingateng**

Isolate number	DNW3-1	DNW3-2	DNW3-3	DNW3-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
E. faecalis	-	-	-	-
VanA	+	-	-	+
VanB	+	+	+	-
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 27: Preliminary identification tests and antibiogram raw data

Sample number: **DNW4**

Sampling site: **Dingateng**

Isolate number	DNW4-1	DNW4-2	DNW4-3	DNW4-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	+	+	-
E. faecalis	-	+	-	+
VanA	-	-	+	-
VanB	-	+	-	-
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	6	NT	25
AP(10)	NT	22	NT	6
S(10)	NT	6	NT	17
GM(10)	NT	15	NT	20
OT(10)	NT	25	NT	18
Nor(10)	NT	24	NT	20
TEC(30)	NT	16	NT	18
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	24	NT	28

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 28: Preliminary identification tests and antibiogram raw data

Sample number: **DNW5**

Sampling site: **Dingateng**

Isolate number	DNW5-1	DNW5-4
Biochemical Tests		
Gram staining	+	+
Cell morphology	Coccus	Coccus
Catalase test	+	+
Specific PCR analysis		
16SrRNA	-	-
E. faecalis		
VanA	-	+
VanB		
Tn1546	-	+
Antibiotic IZD data (mm)		
VA(30)	NT	NT
AP(10)	NT	NT
S(10)	NT	NT
GM(10)	NT	NT
OT(10)	NT	NT
Nor(10)	NT	NT
TEC(30)	NT	NT
HLAR [Antibiotic IZD data (mm)]		
S(300)	NT	NT
GM(120)	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 29: Preliminary identification tests and antibiogram raw data

Sample number: **MBW1**

Sampling site: **Mabule**

Isolate number	MBW1-1	MBW1-3	MBW1-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	+	+	+
Specific PCR analysis			
16SrRNA	+	+	+
E. faecalis	-	-	-
VanA	+	+	-
VanB	+	-	-
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	NT	NT	NT
AP(10)	NT	NT	NT
S(10)	NT	NT	NT
GM(10)	NT	NT	NT
OT(10)	NT	NT	NT
Nor(10)	NT	NT	NT
TEC(30)	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]			
S(300)	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 30: Preliminary identification tests and antibiogram raw data

Sample number: **MBW2**

Sampling site: **Mabule**

Isolate number	MBW2-1	MBW2-2	MBW2-3	MBW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
E. faecalis	-	-	-	-
VanA	-	+	+	+
VanB	+	+	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 31: Preliminary identification tests and antibiogram raw data

Sample number: **MBW3**

Sampling site: **Mabule**

Isolate number	MBW3-1	MBW3-2	MBW3-3	MBW3-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	-	+	+
E. faecalis	+	-	+	+
VanA	+	+	+	+
VanB	+	+	+	+
Tn1546	+	+	+	-
Antibiotic IZD data (mm)				
VA(30)	6	NT	6	6
AP(10)	6	NT	6	6
S(10)	14	NT	6	16
GM(10)	14	NT	14	6
OT(10)	10	NT	21	10
Nor(10)	22	NT	21	25
TEC(30)	6	NT	6	11
HLAR [Antibiotic IZD data (mm)]				
S(300)	18	NT	14	16

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 32: Preliminary identification tests and antibiogram raw data

Sample number: **LW1**

Sampling site: **Logagane**

Isolate number	LW1-1	LW1-2	LW1-3	LW1-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
E. faecalis				
VanA	-	-	-	-
VanB				
Tn1546	-	-	+	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
P(10)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
C(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
Ke(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
Ch(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Cip(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
Cef(30)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
Gen(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 33: Preliminary identification tests and antibiogram raw data

Sample number: **LW2**

Sampling site: **Logagane**

Isolate number	LW2-1	LW2-2	LW2-3	LW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	-	+	+
E. faecalis	-	-	-	-
VanA	+	+	-	+
VanB	+	-	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 34: Preliminary identification tests and antibiogram raw data

Sample number: **LW3**

Sampling site: **Logagane**

Isolate number	LW3-1	LW3-2	LW3-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	-	-	-
Specific PCR analysis			
16SrRNA	-	-	+
E. faecalis	-	+	-
VanA	-	-	+
VanB	-	-	-
Tn1546	-	-	+
Antibiotic IZD data (mm)			
VA(30)	NT	6	NT
AP(10)	NT	6	NT
S(10)	NT	13	NT
GM(10)	NT	20	NT
OT(10)	NT	12	NT
Nor(10)	NT	20	NT
TEC(30)	NT	16	NT
HLAR [Antibiotic IZD data (mm)]			
S(300)	NT	20	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 35: Preliminary identification tests and antibiogram raw data

Sample number: **SW1**

Sampling site: **Stella**

Isolate number	SW1-2	SW1-3
Biochemical Tests		
Gram staining	+	+
Cell morphology	Coccus	Coccus
Catalase test	+	+
Specific PCR analysis		
16SrRNA	+	+
E. faecalis	-	+
VanA	+	+
VanB	+	-
Tn1546	-	-
Antibiotic IZD data (mm)		
VA(30)	NT	10
AP(10)	NT	10
S(10)	NT	6
GM(10)	NT	18
OT(10)	NT	17
Nor(10)	NT	30
TEC(30)	NT	6
HLAR [Antibiotic IZD data (mm)]		
S(300)	NT	25

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 36: Preliminary identification tests and antibiogram raw data

Sample number: **Sample number: SW2**

Sampling site: **Stella**

Isolate number	SW2-1	SW2-2	SW2-3	SW2-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	+	+	-
E. faecalis				
VanA	-	+	-	-
VanB				
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	8	6	6
P(10)	NT	6	6	6
AP(10)	NT	6	6	6
S(10)	NT	13	6	17
K(30)	NT	13	6	20
GM(10)	NT	16	20	18
E(15)	NT	12	21	18
OT(10)	NT	12	21	18
C(30)	NT	15	14	16
Nor(10)	NT	15	14	16
P(30)	NT	18	19	20
TEC(30)	NT	6	17	20
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	24	26	18
GM(120)	NT	31	27	40

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 37: Preliminary identification tests and antibiogram raw data

Sample number: **SW3**

Sampling site: **Stella**

Isolate number	SW3-2	SW3-3
Biochemical Tests		
Gram staining	+	+
Bacteriology	Cocci	Cocci
Catalase test	+	+
Specific PCR analysis		
16SrRNA	+	+
E. faecalis		
VanA	-	-
Tn1546	-	-
Antibiotic IZD data (mm)		
VA(30)	6	NT
P(10)		
AP(10)	6	NT
CN(10)		
S(10)	13	NT
C(30)		
GM(10)	16	NT
PG(10)		
OT(10)	12	NT
CIP(5)		
Nor(10)	18	NT
CD(30)		
TEC(30)	18	NT
HLAR [Antibiotic IZD data (mm)]		
S(300)	14	NT
CN(300)		

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 38: Preliminary identification tests and antibiogram raw data

Sample number: **VW1**

Sampling site: **Vryburg**

Isolate number	VW1-1	VW1-2	VW1-3
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	+	+	+
Specific PCR analysis			
16SrRNA	-	-	-
E. faecalis			
VanA	+	-	+
Van B			
Tn1546	-	-	+
Antibiotic IZD data (mm)			
VA(30)	NT	NT	NT
AP(10)	NT	NT	NT
S(10)	NT	NT	NT
GM(10)	NT	NT	NT
OT(10)	NT	NT	NT
Nor(10)	NT	NT	NT
TEC(30)	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]			
S(300)	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 39: Preliminary identification tests and antibiogram raw data

Sample number: **VW2**

Sampling site: **Vryburg**

Isolate number	VW2-1	VW2-2	VW2-3	VW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	-	+
E. faecalis				
VanA	-	+	+	-
VanB				
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	6	NT
P(10)	NT	NT	6	NT
AP(10)	NT	NT	6	NT
CIP(5)	NT	NT	18	NT
S(10)	NT	NT	18	NT
SXT(25)	NT	NT	18	NT
GM(10)	NT	NT	18	NT
E(15)	NT	NT	22	NT
OT(10)	NT	NT	22	NT
CTX(5)	NT	NT	20	NT
Nor(10)	NT	NT	20	NT
CIP(10)	NT	NT	6	NT
TEC(30)	NT	NT	30	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	25	NT
GM(120)	NT	NT		

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 40: Preliminary identification tests and antibiogram raw data

Sample number: **VW3**

Sampling site: **Vryburg**

Isolate number	VW3-1	VW3-3	VW3-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	cocci	cocci	cocci
Catalase test	+	+	+
Specific PCR analysis			
16SrRNA	+	+	+
Vancomycin			
VanA	-	-	+
Tetracycline			
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	16	6	NT
P(10)	24	6	NT
S(10)	21	6	NT
K(10)	25	12	NT
OT(10)	25	21	NT
C(10)	20	10	NT
TEC(30)	6	6	NT
HLAR [Antibiotic IZD data (mm)]			
S(300)	30	14	NT
GM(120)	28	27	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 41: Preliminary identification tests and antibiogram raw data

Sample number: **VW4**

Sampling site: **Vryburg**

Isolate number	VW4-1	VW4-4
Biochemical Tests		
Gram staining	+	+
Cell morphology	Coccus	Coccus
Catalase test	+	+
Specific PCR analysis		
16SrRNA	-	-
E. faecalis	+	+
VanA	-	+
VanB	-	+
Tn1546	-	-
Antibiotic IZD data (mm)		
VA(30)	6	6
AP(10)	6	6
S(10)	18	17m m
GM(10)	20	20
OT(10)	21	18
Nor(10)	24	12
TEC(30)	24	24
HLAR [Antibiotic IZD data (mm)]		
S(300)	26	25

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 42: Preliminary identification tests and antibiogram raw data

Sample number: **DSW1**

Sampling site: **Disaneng**

Isolate number	DSW1-1	DSW1-2	DSW1-3	DSW1-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	-
Specific PCR analysis				
16SrRNA	+	-	-	-
VanA	-	+	+	+
Tn1546	-	+	+	-
Antibiotic IZD data (mm)				
VA(30)	10	14	6	6
AP(10)	10	12	10	16
S(10)	10	16	15	24
GM(10)	20	24	18	24
OT(10)	6	18	14	30
Nor(10)	16	22	24	30
TEC(30)	14	22	6	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	30	30	20	30

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 43: Preliminary identification tests and antibiogram raw data

Sample number: **DSW2**

Sampling site: **Disaneng**

Isolate number	DSW2-1	DSW2-2	DSW2-3	DSW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	-	-	+	-
Specific PCR analysis				
16SrRNA	-	-	-	+
E. faecalis	+	+	+	+
VanA	-	-	-	+
VanB	+	+	+	+
Tn1546	-	-	+	-
Antibiotic IZD data (mm)				
VA(30)	6	6	6	6
AP(10)	14	6	12	16
S(10)	10	10	10	14
GM(10)	18	20	18	26
OT(10)	6	6	6	6
Nor(10)	20	24	22	24
TEC(30)	6	6	6	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	15	14	15	28

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 44: Preliminary identification tests and antibiogram raw data

Sample number: **DSW3**

Sampling site: **Disaneng**

Isolate number	DSW3-1	DSW3-2	DSW3-3	DSW3-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
VanA	-	+	-	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	6	10	NT	NT
AP(10)	20	20	NT	NT
S(10)	16	6	NT	NT
GM(10)	18	21	NT	NT
OT(10)	15	25	NT	NT
Nor(10)	10	14	NT	NT
TEC(30)	22	16	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	12	16	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 45: Preliminary identification tests and antibiogram raw data

Sample number: **TW1**

Sampling site: **Taung**

Isolate number	TW1-1	TW1-2	TW1-4
Biochemical Tests			
Gram staining	+	+	+
Cell morphology	Coccus	Coccus	Coccus
Catalase test	-	-	+
Specific PCR analysis			
16SrRNA	-	-	-
E. faecalis	+	+	+
VanA	-	-	-
VanB	+	-	+
Tn1546	-	-	-
Antibiotic IZD data (mm)			
VA(30)	6	14	6
AP(10)	6	12	6
S(10)	14	18	14
GM(10)	18	18	14
OT(10)	6	6	6
Nor(10)	14	16	10
TEC(30)	6	14	6
HLAR [Antibiotic IZD data (mm)]			
S(300)	20	26	20
GM(120)	22	28	22

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 46: Preliminary identification tests and antibiogram raw data

Sample number: **TW2**

Sampling site: **Taung**

Isolate number	TW2-1	TW2-2	TW2-3	TW2-4
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
E. faecalis	+	+	+	+
VanA	+	+	+	+
VanB	+	+	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	6	6	6	12
AP(10)	6	6	10	6
S(10)	14	13	12	17
GM(10)	20	22	28	18
OT(10)	21	12	21	18
Nor(10)	22	21	14	22
TEC(30)	6	6	6	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	20	30	24	16
GM(120)	25	31	20	40

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 47: Preliminary identification tests and antibiogram raw data

Sample number: **TW3**

Sampling site: **Taung**

Isolate number	TW3-1	TW3-2	TW3-3	TW3-4
Gram staining	+	+	+	+
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
VanA	-	-	+	+
Tn1546	-	-	-	-
VA(30)	14	NT	12	16
AP(10)	6	NT	6	16
S(10)	18	NT	6	18
GM(10)	14	NT	18	18
OT(10)	18	NT	21	23
Nor(10)	18	NT	22	16
TEC(30)	6	NT	6	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	24	NT	24	24
GM(120)	31	NT	27	20

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 48: Preliminary identification tests and antibiogram raw data

Sample number: **MKW1**

Sampling site: **Makgobistad**

Isolate number	MKW1-1	MKW1-2	MKW1-3	MKW1-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Cocci	Cocci	Cocci	Cocci
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	-	+	-
VanA	-	+	+	-
Tn1546	-	-	+	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	8
AP(10)	NT	NT	NT	18
S(10)	NT	NT	NT	28
GM(10)	NT	NT	NT	18
OT(10)	NT	NT	NT	16
Nor(10)	NT	NT	NT	12
TEC(30)	NT	NT	NT	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	25

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 49: Preliminary identification tests and antibiogram raw data

Sample number: **MKW2**

Sampling site: **Makgobistad**

Isolate number	MKW2-1	MKW2-2	MKW2-3	MKW2-4
Gram staining	+	+	+	+
Catalase test	+	+	+	+
16SrRNA	-	+	+	+
VanA	-	-	-	+
Tn1546	-	-	-	+
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 50: Preliminary identification tests and antibiogram raw data

Sample number: **MYW1**

Sampling site: **Mayaeyane**

Isolate number	MYW1-1	MYW1-2	MYW1-3	MYW1-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	-	+	+
VanA	+	+	-	-
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	6	6
AP(10)	NT	NT	16	18
S(10)	NT	NT	21	22
GM(10)	NT	NT	25	16
OT(10)	NT	NT	18	18
Nor(10)	NT	NT	25	14
TEC(30)	NT	NT	10	6
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	30	25

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 51: Preliminary identification tests and antibiogram raw data

Sample number: **MYW2**

Sampling site: **Mayaeyane**

Isolate number	MYW2-1	MYW2-2	MYW2-3	MYW2-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	-	+	+
E. faecalis	-	-	-	-
VanA	+	+	+	-
VanB	-	+	+	-
Tn1546	+	-	-	+
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
PG(10)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
A(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
K(30)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
E(15)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
CIP(5)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
C(30)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 52: Preliminary identification tests and antibiogram raw data

Sample number: **PW1**

Sampling site: **Phitsane**

Isolate number	PW1-1	PW1-2	PW1-3	PW1-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
E. faecalis	-	-	-	-
VanA	+	-	+	+
VanB	-	+	+	+
Tn1546	+	+	+	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
PG(10)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
A(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
K(30)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
E(15)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
CIP(5)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
C(30)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 53: Preliminary identification tests and antibiogram raw data

Sample number: **PW2**

Sampling site: **Phitsane**

Isolate number	PW2-1	PW2-2	PW2-3	PW2-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	+	-	+
VanA	+	-	+	-
Tn1546	-	-	+	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 54: Preliminary identification tests and antibiogram raw data

Sample number: **LPW1**

Sampling site: **Loporung**

Isolate number	LPW1-1	LPW1-2	LPW1-3	LPW1-4
Biochemical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	-	+	-
E. faecalis	+	-	+	-
VanA	+	+	-	+
VanB	+	+	+	+
Tn1546	+	+	+	+
Antibiotic IZD data (mm)				
VA(30)	18	NT	10	NT
PG(10)	6	NT	6	NT
AP(10)	22	NT	20	NT
A(10)	22	NT	20	NT
S(10)	18	NT	24	NT
K(30)	15	NT	6	NT
GM(10)	16	NT	30	NT
E(15)	18	NT	22	NT
OT(10)	21	NT	24	NT
CIP(5)	12	NT	25	NT
Nor(10)	20	NT	25	NT
C(30)	20	NT	24	NT
TEC(30)	6	NT	10	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	26	NT	28	NT
GM(120)	26	NT	32	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 55: Preliminary identification tests and antibiogram raw data

Sample number: **LPW2**

Sampling site: **Loporung**

Isolate number	LPW2-1	LPW2-2	LPW2-3	LPW2-4
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	+
VanA	+	+	+	-
Tn1546	+	+	+	+
Antibiotic IZD data (mm)				
VA(30)	6	6	6	NT
AP(10)	6	6	6	NT
S(10)	18	22	24	NT
GM(10)	28	18	14	NT
OT(10)	20	20	26	NT
Nor(10)	30	30	28	NT
TEC(30)	6	6	6	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	18	15	24	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 56: Preliminary identification tests and antibiogram raw data

Sample number: **MSW1**

Sampling site: **Masamane**

Isolate number	MSW1-1	MSW1-2	MSW1-3	MSW1-4
ical Tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	-
VanA	+	-	-	+
Tn1546	+	-	-	+
Antibiotic IZD data (mm)				
VA(30)	NT	NT	6	NT
AP(10)	NT	NT	6	NT
S(10)	NT	NT	6	NT
GM(10)	NT	NT	18	NT
OT(10)	NT	NT	21	NT
Nor(10)	NT	NT	16	NT
TEC(30)	NT	NT	6	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	24	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 57: Preliminary identification tests and antibiogram raw data

Sample number: **MSW2**

Sampling site: **Masamane**

Isolate number	MSW2-1	MSW2-2	MSW2-3	MSW2-4
Gram staining	+	+	+	+
Morphology	Cocci	Cocci	Cocci	Cocci
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	-	+	-	+
VanA	+	-	-	+
Tn1546	+	-	-	+
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 58: Preliminary identification tests and antibiogram raw data

Sample number: **TMW1**

Sampling site: **Tshidilamolomo**

Isolate number	TMW1-1	TMW1-2	TMW1-3	TMW1-4
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	-	+	+
E. faecalis	-	-	-	-
VanA	+	-	+	-
VanB	+	-	+	+
Tn1546	-	-	-	-
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 59: Preliminary identification tests and antibiogram raw data

Sample number: **TMW2**

Sampling site: **Tshidilamolomo**

Isolate number	TMW2-1	TMW2-2	TMW2-3	TMW2-4
Biochemical tests				
Gram staining	+	+	+	+
Cell morphology	Coccus	Coccus	Coccus	Coccus
Catalase test	+	+	+	+
Specific PCR analysis				
16SrRNA	+	+	+	-
E. faecalis	-	-	-	-
VanA	+	-	+	-
Tn1546	-	-	+	+
Antibiotic IZD data (mm)				
VA(30)	NT	NT	NT	NT
AP(10)	NT	NT	NT	NT
S(10)	NT	NT	NT	NT
GM(10)	NT	NT	NT	NT
OT(10)	NT	NT	NT	NT
Nor(10)	NT	NT	NT	NT
TEC(30)	NT	NT	NT	NT
HLAR [Antibiotic IZD data (mm)]				
S(300)	NT	NT	NT	NT
GM(120)	NT	NT	NT	NT

NT=Not tested; isolates were negative for *E. faecalis*; IZD=Inhibition Zone Diameter

Table 60A: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
DEEL4-2	2.244	*Enterococcus faecalis*	2.12	*Enterococcus faecalis*

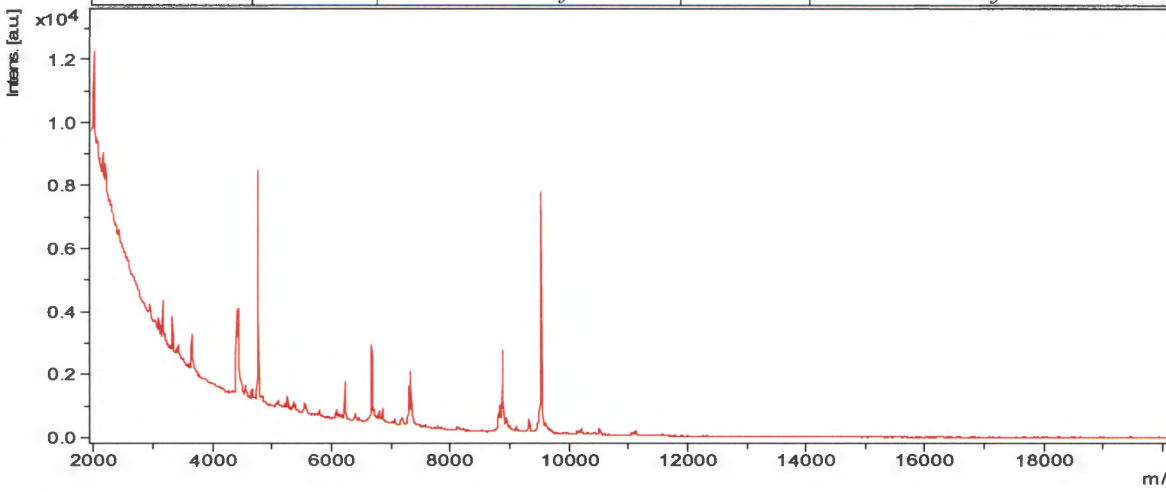

Table 60B: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
STL1-3	2.176	*Enterococcus faecalis*	2.121	*Enterococcus faecalis*

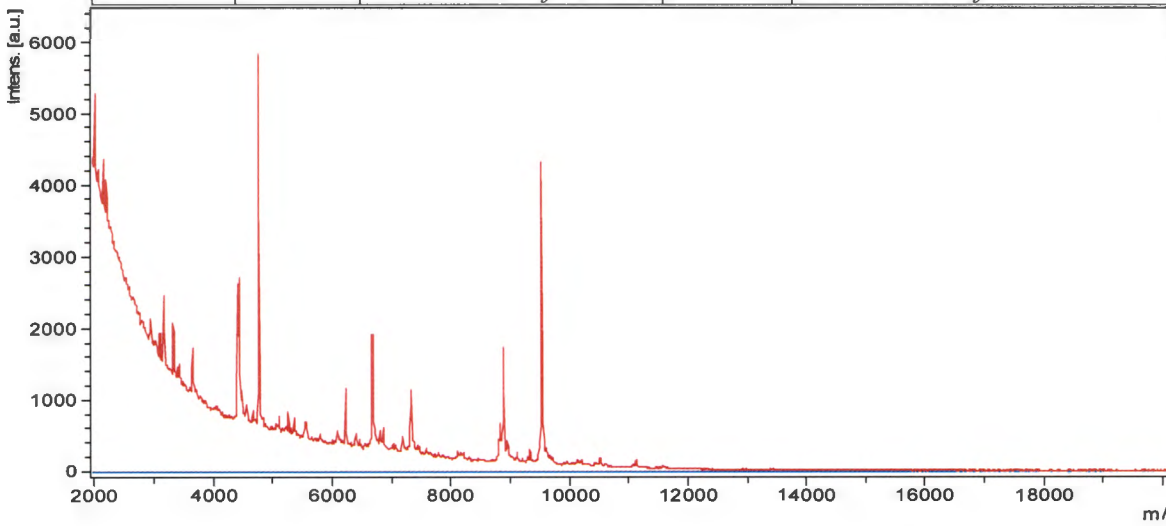

Table 60C: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
MP3-1	1.736	*Enterococcus faecalis*	1.471	*Enterococcus faecalis*

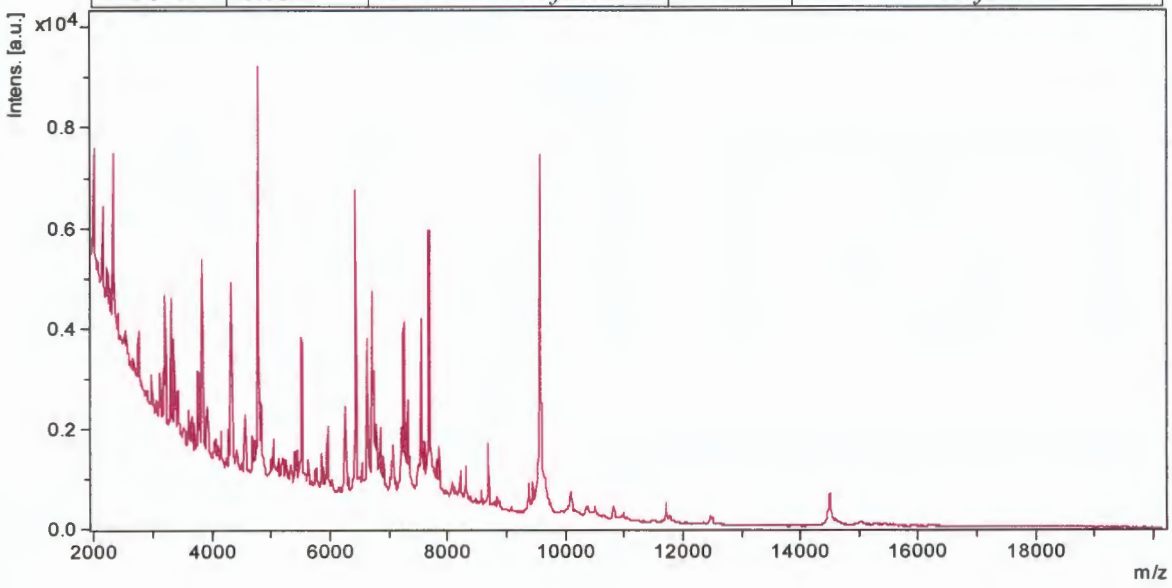

Table 60D: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
V2-2	2.303	*Enterococcus faecalis*	2.204	*Enterococcus faecalis*

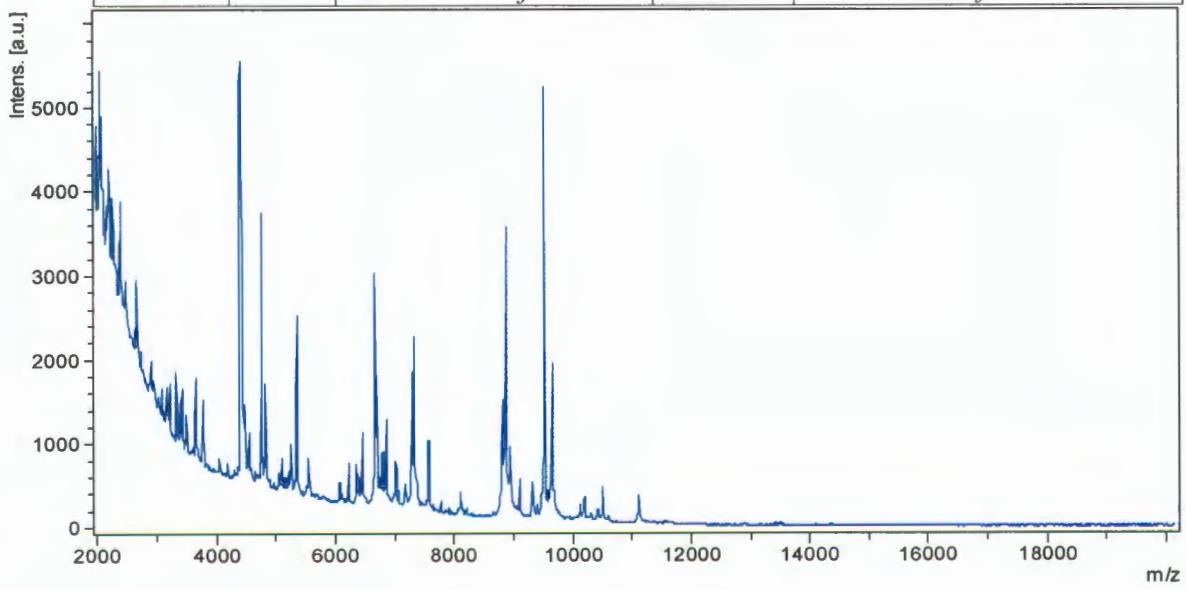

Table 60E: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
V2-4	2.223	*Enterococcus faecalis*	2.163	*Enterococcus faecalis*

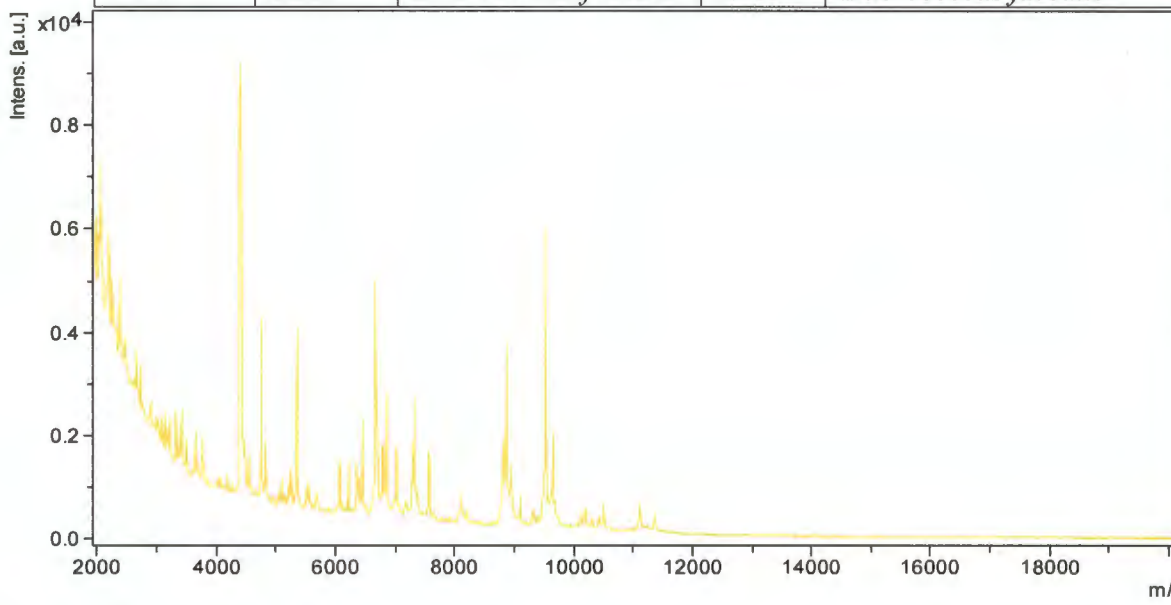

Table 60F: MALDI-TOF MS results for *Enterococcus* species isolated from ground water

Isolate Number	Score Value	Identity based on MALDI-TOF MS analysis	Score Value	Identity based on MALDI-TOF MS analysis
		Best Match		**Second Best Match**
1RUS1	2.421	*Salmonella* species	2.375	*Salmonella* species

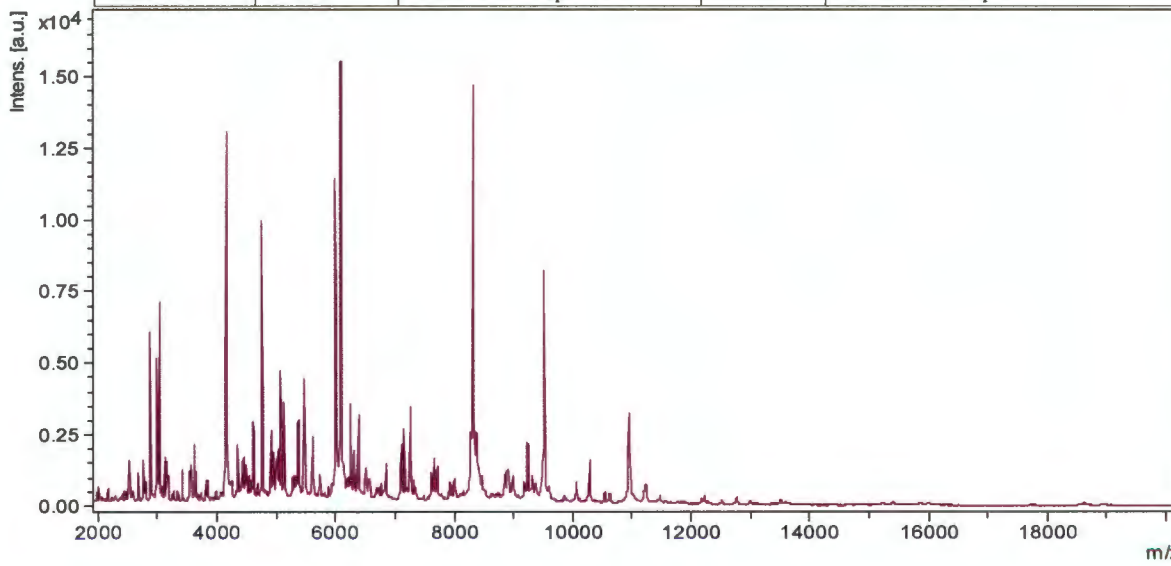

Table 61: Results for *Enterococcus* species growth in 6.5% Sodium Chloride (NaCl) broth

Sample area	Isolate No.	OD Reading(nm)	Sample area	Isolate No.	OD Reading(nm)	Sample area	Isolate No.	OD Reading(nm)
Delaryville								0.377
	2-3	0.338		1-3	0.135		1-2	0.235
								0.044
				1-7	0.105		1-5	0.085
Vryburg								0.663
	3-4	0.192		2-1	0.242		3-1	0.743
								0.136
Taung	2-1	0.050		2-6	0.330		3-4	0.079
								0.047
	2-3	0.166		3-1	0.145		3-8	0.253
								0.256
	3-1	0.245		3-8	0.025		4-3	0.117
								0.109
	3-4	0.112		4-3	0.280		5-6	0.100
								0.122
Disaneng	1-1	0.514		4-7	0.181			
								0.116
	2-1	0.176		5-6	0.585		1-3	0.455
	2-4	0.692		5-8	0.099	Logagane	1-1	0.139
Mabule								0.071
	1-3	0.493	Loporung	1-4	0.708		2-1	0.348
								0.599
Tshidilamolomo	1-1	0.035					3-1	0.350
								0.169
				1-7	0.398		3-4	0.609
Dingateng								0.015
	2-3	0.123					2-4	0.050
								0.136
Makgobistad	1-3	0.092					5-4	0.006

Table 62A: Multi Antibiotic Resistance (MAR) Phenotypes

Sample area	Phenotype	Sample area	Phenotype
Ramosadi			
	VA-PG-AP-A-S-K-GM-E-OT-Nor-C-TEC		VA-PG-AP-A-GM-E-OT-Nor-TEC
	VA-PG-AP-A-K-GM-E-OT-Nor-C		VA-PG-AP-A-GM-E-OT-C-TEC
	PG-AP-A-E-OT-C		VA-PG-AP-A-S-E-OT-TEC
	VA-PG-AP-A-GM-E-OT-C-TEC		VA-PG-AP-A-GM-E-OT-C
	PG-A-E-OT-C		VA-PG-AP-A-K-E-OT
	VA-PG-AP-A-S-K-E-OT-Nor-C		VA-PG-AP-A-K-GM-E-OT-CIP-Nor-C-TEC
	PG-AP-A-S-K-E-OT-Nor-C-TEC		VA-PG-AP-A-K-E-OT-CIP-C
	PG-AP-A-GM-E-OT-Nor-TEC		VA-PG-AP-A-S-K-E-OT-CIP-Nor
	VA-PG-AP-A-K-GM-E-OT-CIP-Nor-C		VA-PG-AP-A-K-GM-E-OT
	VA-PG-AP-A-E-Nor		VA-PG-AP-A-GM-E-OT-C
	VA-PG-AP-A-S-GM-E-OT-CIP-Nor-C-EC		
	VA-PG-AP-A-S-GM-E-OT-C-TEC		
	VA-PG-AP-A-S-GM-E-OT-C-TEC		

Table 62B: Multi Antibiotic Resistance (MAR) Phenotypes

Sample area	Phenotype	Sample area	Phenotype
Motlhabeng			
	PG-AP-A-E-CIP		VA-PG-AP-A-S-K-GM-E-OT-CIP-Nor-C
	VA-PG-AP-A-S-K-E-OT-C-TEC		VA-PG-AP-A-S-K-E-OT
	PG-A-E-C-TEC		PG-AP-S-K-E-CIP-Nor-C
	PG-AP-A-K-GM-E-OT-Nor-TEC		
	PG-AP-A-K-OT-CIP		VA-PG-AP-A-S-K-GM-E-OT-CIP
			PG-AP-A-K-E-OT-CIP-C
Delaryville			
	VA-PG-S-E-OT-CIP-Nor	Logagane	VA-PG-AP-A-S-E-OT-C
	VA-PG-GM-E-OT-CIP-Nor-C-TEC		
	VA-PG-GM-E-CIP-Nor-C		
	PG-AP-A-S-E-OT-Nor-C-TEC		

Table 62C: Multi Antibiotic Resistance (MAR) Phenotypes

Sample area	Phenotype	Sample area	Phenotype
Mabule	[illegible]	[illegible]	[illegible]
	VA-PG-AP-A-S-K-GM-E-CIP-C-TEC		PG-AP-E-OT-Nor
	[illegible]		[illegible]
			VA-PG-AP-A-S-E-CIP-TEC
Stella	[illegible]		[illegible]
	VA-PG-AP-A-S-E-OT-Nor-C-TEC		VA-PG-AP-A-S-K-E-CIP-Nor-C-TEC
	[illegible]		[illegible]
	VA-PG-AP-A-E-OT-Nor-C		PG-AP-A-K-GM-E-OT-CIP-C-TEC
	[illegible]		[illegible]
			PG-AP-A-E-Nor-C-TEC
Viryburg	[illegible]		[illegible]
	PG-A-E-TEC	**Makgobistad**	VA-PG-A-K-E-OT-CIP-Nor-TEC
	[illegible]		[illegible]
	VA-PG-AP-A-E-CIP	**Mayaeyane**	VA-PG-A-E-OT-TEC
	[illegible]		[illegible]
		Loporung	PG-K-E-CIP-TEC
Disaneng	[illegible]		[illegible]
	VA-PG-AP-A-E-OT		VA-PG-AP-A-E-CIP-C-TEC
	[illegible]		[illegible]
	VA-PG-A-E-CIP-TEC		VA-PG-AP-A-K-GM-E-CIP-TEC
	[illegible]		[illegible]
	VA-PG-AP-A-S-E-OT-CIP-TEC	**Masamane**	VA-PG-AP-A-S-E-CIP-Nor-TEC
	[illegible]		[illegible]
	VA-PG-S-E-OT-CIP-TEC		
	[illegible]		[illegible]
	VA-PG-A-K-E-OT-CIP-Nor-C		
	[illegible]		[illegible]