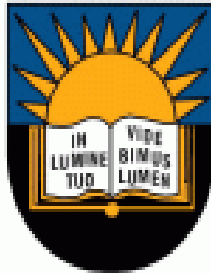


**ENTEROCOCCUS PATHOTYPES AS RESERVOIRS OF ANTIBIOTIC RESISTANCE  
DETERMINANTS IN THE KAT RIVER AND FORT BEAUFORT ABSTRACTION  
WATERS**



**University of Fort Hare**  
*Together in Excellence*

**A DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR  
THE AWARD OF A MASTER OF SCIENCE (MSc) DEGREE IN MICROBIOLOGY**

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## DECLARATION

I, the undersigned, declare that this dissertation submitted to the University of Fort Hare for the degree of Masters of Science in Microbiology in the Faculty of Science and Agriculture, School of Biological and Environmental Sciences, and the work contained herein is my original work with exception of the citations and that this work has not been submitted at any other university, either in part or in its entirety, for the award of any degree.

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Date: \_\_\_\_\_

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## **DEDICATION**

This work is dedicated to my lovely parents, my dad Lungile Ntloko and my mom Fanelwa Nosiseko Ntloko.

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## ABSTRACT

In this study, 400 presumptive *Enterococcus* isolates previously recovered from Kat River and Fort Beaufort Abstraction water dam were subjected to molecular confirmation and pathotyping. Two hundred and seventy-four (68%) of these isolates were confirmed to be enterococci species. Confirmations studies were polymerase chain reaction (PCR) based, using enterococci specific primers targeting the *tuf* gene. The confirmed enterococci isolates were further differentiated into their pathotypes, the targets of which were: *E. faecalis*, *E. avium*, *E. hirae*, *E. casseliflavus* and *E. gallinarum* using well documented species specific primer sequences. *E. faecalis* accounted for 20% of the isolates, followed by *E. avium* (16%), *E. hirae* (13%), *E. casseliflavus* (5%) and *E. gallinarum* (3%). Furthermore, all the confirmed isolates were analysed for antibiotic susceptibilities using a panel of nine different antibiotics, namely vancomycin, linezolid, ciprofloxacin, ampicillin, gentamicin, chloramphenicol, tetracycline, erythromycin, penicillin, and those that were resistant were assayed for the presence of relevant antibiotic resistance genes. All the 274 isolates were found to harbour *vanA* resistance gene confirming their phenotypic resistance to the vancomycin. Similarly, 60% (109/180) of the isolates showed phenotypic resistance to erythromycin which was further confirmed by the presence of *ermA* genes in these isolates. The presence of antibiotic resistant bacteria in surface waters poses a risk to public health.

# CHAPTER ONE

## 1.0 BACKGROUND OF STUDY

For many years *Enterococcus* species were not detrimental to humans and considered insignificant medically, for the reason that they produce bacteriocins. *Enterococcus* species have been used widely over the very last decade in the food industry as probiotics and at the same time as starter cultures (Foulquie Moreno *et al.*, 2006). Of late, enterococci have turned out to be one of the most widespread nosocomial pathogens, with patients having a high mortality rate of up to 61% (De Fa'tima *et al.*, 2005).

Additionally, it is used worldwide to monitor water quality (Toranzos and Mcfeters, 1997; APHA, 1998; Anderson *et al.*, 2005; Harwood *et al.*, 2005; Gerberg *et al.*, 2006). Faecal contamination of water is well thought-out as a human health risk, and has since the inception of water microbiology been assessed using coliform bacteria (Djuikom *et al.*, 2006). Human faeces can harbour pathogenic bacteria that can contaminate water and shellfish (Shielh *et al.*, 2003; Rueekret *et al.*, 2004; Loderr and de Roger human, 2005; Mvunies *et al.*, 2006). Exposure to microbiologically contaminated surface water may have adverse health effects and may result in gastroenteritis (GE), fever, skin, ear, and eye complaints or more severe illnesses such as hepatitis and meningitis (WHO, 2003). The public health hazard is linked with faecal pollution which indicates the possibility of microbial pathogens being present in water (APHA, 1998; WHO, 2000; Pruss *et al.*, 2002; Okoh *et al.*, 2007). Also, another emerging public health risk is the proliferation of antibiotic resistant strains of bacteria in surface water sources. Such bacteria has the possibility of transferring resistance to previously susceptible strains, especially those of public health concern, in aquatic environments (Ash *et al.*, 2002; Zang *et al.*, 2009; Chigor *et al.*, 2010).

In 2005, the Health Protection Agency (2007) reported 7066 cases of bacteremia caused by *Enterococcus* species in the UK, an 8% increase from 2004. Twenty eight per cent of the entire cases were due to antibiotic resistant strains (Health Protection Agency, 2007). The risk of death from vancomycin-resistant enterococci (VRE) is 75%, compared with 45% for those infected with a susceptible strain (Bearman and Wenzel, 2005). These figures are mirrored in the USA. Over a 15-year period there was a 20-fold increase in VRE associated with nosocomial infections reported to CDC's National Nosocomial Infections Surveillance (NNIS) (National Nosocomial Infections Surveillance, 2004).

Potgieter *et al.* (2007) reported poor quality ground water consumption by people in some rural areas of Limpopo Province in South Africa while Samie *et al.* (2011) also reported similar results in their study of borehole water used by schools in Greater Giyani Municipality, Mopani District, South Africa. Boreholes equipped with hand pumps are ordinary technology adopted by deprived rural communities, and there are currently about 250,000 hand pumps in Africa (HTN, 2003). In 1994, it was estimated that 40-50% of hand pumps in sub-Saharan Africa were not functional (Diwi and Bureau, 1994). This is backed up by more current data from Uganda (HTN, 2003) and South Africa (Hazelton and WRC, 2000) which pointed out comparable operational failure rates. A huge proportion of the world's population do not have access to improved or microbiologically safe sources of water for drinking and other essential purposes (IDRC, 2007). Utilization of unsafe water for drinking causes about 2.2 million diarrheal disease related deaths annually, mostly in children (Samie *et al.*, 2001; IDRC, 2007). The millennium declaration was established with a goal to halve the proportion of the worldwide population lacking access to safe water by 2015.

This was motivated by the fact that faecal contamination of source and treated water is a persistent worldwide problem (WHO, 2005). In a number of cities, the water systems abstract unsafe water from unprotected or contaminated sources and distribute it to consumers with no or inadequate treatment (WHO, 2005). An additional difficulty contributing to the underestimation of the population served by unsafe water is contamination of water during distribution whether water is piped or carried into the home (WHO, 2005). The surface waters in populous countries have become reservoirs of antimicrobial-resistant pathogenic microbes due to indiscriminate use of antimicrobials in human and veterinary medicine and faecal contamination (Ahmed *et al.*, 2005). Members of the genus *Enterococcus* are recommended as an indicator organism for both freshwater and saltwater (Anderson *et al.*, 2005). Enterococci are Gram-positive facultative anaerobes and commensal residents of the gastrointestinal tract of vertebrates (Aarestrup *et al.*, 2002). Enterococci are potentially pathogenic and have become one of the leading causes of urinary tract infections and endocarditis in humans (Kayser, 2003).

The increasing use of antimicrobial agents in both human medicine and animal agriculture has resulted in many pathogens developing drug resistance. Certain organisms like *Escherichia coli* and enterococci play an important role in many existing surveillance systems for antibiotic resistance in the bacterial flora of livestock and food of animal origin (Riou *et al.*, 1985; Wright *et al.*, 1999). Vancomycin resistance among Gram-positive cocci is rare but there have been recent reports of vancomycin resistance in strains of *E. faecium*, *E. gallinarum* (characterized by a low-level resistance to vancomycin, with a minimal inhibitory concentration (MIC) of 16 mg/L). There are also reports of enterococci with MICs of vancomycin > 100 mg/L (Leclereq *et al.*, 1988).

Nosocomial infections caused by enterococci have increased, with these pathogens now ranked the third most common in hospitals after *Escherichia coli* and *Staphylococcus aureus* (Brooks *et al.*, 2008).

The sources of enterococcal infections in humans are not clear, but animal reservoirs have been suggested. A study comparing enterococcal isolates from 4 European countries and the United States demonstrated that *E. faecalis* isolated from pigs in Portugal had pulsed-field gel electrophoresis (PFGE) patterns identical to those of multidrug-resistant isolates (Donabedian *et al.*, 2003, Chow and Bartlett, 2003).

## **1.1 PROBLEM STATEMENT**

Fresh water makes up only 0.01% of the global water and approximately 0.8 % of the Earth's surface, yet this tiny fraction of global water supports at least 100 000 species. Inland waters and freshwater biodiversity constitute a valuable natural resource, in economic, cultural, aesthetic, scientific and educational terms for any nation. The conservation and management of these resources is critical to the interests of all humans, nations and governments. Yet this precious heritage is in crisis (Dudgeon *et al.*, 2006). Surface and underground water resources are experiencing declines in quality and biodiversity particularly in developing countries due to rapid industrialisation and population growth, inadequate sanitation, mismanagement of animal wastes and surface run-off from agricultural runoff (Schaper *et al.*, 2002;WHO, 2003). Faecal contamination of surface water resources is a problem of growing concern.

Information on the human or non-human origin of faecal pollution may contribute to the assessment of health risks and to water resource pollution control (Joklik, 1992; Grabow, 1996; Sinton *et al.*, 1998; Schaper *et al.*, 2002).

The microbiological quality of the Kat River, a life-sustaining surface water resource for Fort Beaufort Town and surrounding rural communities who rely heavily on the river water mostly for irrigated agriculture (Farolfi *et al.*, 2008), is affected by non-point sources of pollution such as runoff from the river banks with animal and human excreta. Although an increasing number of villages around the river are being supplied with tap water, many people in the area still rely on the river as their main water source (Farolfi *et al.*, 2008). *Enterococcus* has been used as an indicator of water quality assessment and correlates best with the incidence of gastrointestinal diseases as well as prevalence of pathogenic microorganisms (Ryan and Ray, 2004). *Enterococcus* has also been known as an important nosocomial bacterium. In order to forestall possible *Enterococcus* related out breaks in the communities that depend on the river, it is imperative to assess the microbiological qualities of the river in terms of the prevalence of enterococci spp.

## **1.2 HYPOTHESIS**

This study was premised on the hypothesis that *Enterococcus* pathotypes in the Kat River are reservoirs of antibiotic resistance determinants in the Nkonkobe District Municipality.

### **1.3 AIM AND OBJECTIVES**

The broad aim of this study was to assess the prevalence of *Enterococcus* pathotypes in Kat River and Fort Beaufort abstraction water and their antibiogram characteristics. The specific objectives of the study were to:

1. Confirm the identities of presumptive enterococci isolates obtained from Kat River and Fort Beaufort abstraction water.
2. Characterise the confirmed isolates into five selected *Enterococcus* pathotypes.
3. Determine the antibiogram characteristics of the *Enterococcus* pathotypes.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Water Situation

South Africa is known as water scarce country as 60% is off the record as semi-arid to arid (Nomqophu *et al.*, 2007). About 77% of water resources are obtained from surface water, groundwater (9%) and from return flows (14%) such as sewage and effluent purification waters (DWAF, 2009; Haldenwang, 2009). During the year 2010, South Africa had an predicted population of 49.99 million, at the same time the total population of the Western Cape was probable about 5.22 million (Stats SA., 2010). In 2004, 42% of the population was by then living in rural areas (FAO, 2005). As estimated 14 million rural and sub-urban South Africans do not have access to running water in their homes (Perret, 2002). In 2008, it was predicted that about 5 million people lack adequate and safe water supplies, at the same time 15 million still lacked essential sanitation (Haldenwang, 2009).

#### 2.2 River and surface waters

The accessibility of water, climate, sewage spills, runoff from animal production, storm-related contamination of surface waters as well as illegal discharge of waste have impacted negatively to the quality of rivers and groundwater (Suslow *et al.*, 2006). Surface waters also function as the receptors of wastewater (Tarver, 2008). Runoff water more often drains into streams, rivers or any other water basis as it not is not necessary absorbed by soil (DWAF, 2005). As a result, there can be contamination of the surface water, since there is a direct contact between surface runoff water along with pollutants (Tarver, 2008).

According to Arnone and Walling (2007), storm water emerges from pervious and impervious areas during rainfall periods and can have pollutants as well as pathogens. DWAF (2005) reported that waste that is thrown away into streets usually ends up in storm water systems, which finally end up in the rivers.

### **2.3 Microbial Pollution of Rivers**

Over the previous years, it was discovered that there is increase of pollution on the quality of the water of many of South Africa's rivers (DWAF, 2000; Bezuidenhout *et al.*, 2002; DWAF, 2002; Griesel and Jagals, 2002; Barnes and Taylor, 2004; Dalvie *et al.*, 2004; DWAF, 2004; Germs *et al.*, 2004; DWAF, 2005). There were also findings on the microbiological pollution levels that reached undesirable and risky levels (Barnes and Taylor, 2004). As stated by Arnone and Walling, (2007), the water can get directly or indirectly (sub-surface water flow) contaminated with pathogens that are initially present in animal and human faeces. The storm water runoff, sanitary sewer overflows, wastewater treatment plant effluents, poorly treated wastewater or collapsed sewers; all have an input to the pollution of surface waters (DWAF, 2005; Arnone and Walling, 2007).

### **2.4 Indicator Organisms**

It is a challenge to keep an eye on each and every pathogen in water or food as the presence of certain pathogens is uncommon, difficult to culture, have a patchy distribution and is highly infectious even at low doses (Field and Samadpour, 2007). In certain situation large assays are needed to monitor all organisms available, which can be exclusive and technically complicated (Field and Samadpour, 2007). As a result, indicator organisms are used to assess the quality and safety of raw or processed food products and water as well as authorizing the usefulness of microbial control measures (Busta *et al.*, 2006).

An indicator organism is used as a water quality parameter (Leclerc *et al.*, 2001). Enterococci have been successfully used as an indicator of faecal pollution in water (Scott *et al.*, 2002).

## **2.5 Ecology and Epidemiology of Enterococci**

As stated by Klein (2003), *Enterococcus* species varies as of environmental to animal and human sources. Seeing that enterococci are an important part of the microflora of humans and animals, their distribution is much associated in these sources. *E. faecium* and *E. faecalis* are more frequent in the human gastrointestinal tract while *E. faecium* is mostly isolated from animals and *E. mundtii* and *E. casseliflavus* in plant sources (Klein, 2003). According to the findings, the numbers of *E. faecalis* in human faeces range from  $10^5$  to  $10^7$  per gram, and those of *E. faecium* from  $10^4$  to  $10^5$  per gram. The isolation of *E. faecium* and *E. faecalis* is less prevalent from livestock than from human faeces (Franz *et al.*, 1999).

The distribution of *Enterococcus* species (Kuhn *et al.*, 2003) varies throughout Europe. In Spain and the UK, *E. faecalis* and *E. faecium* are predominately isolated species from both clinical and environmental sources. As for Sweden, there is not much prevalence of *E. faecium* and a higher isolation rate of *E. hirae*, while in Denmark *E. hirae* is the foremost species and is isolated mainly from slaughtered animals (Kuhn *et al.*, 2003). Clinical isolates of enterococci verify for a minor range than those obtained from the environment and other human sources, with *E. faecalis* being the ruling species (Kuhn *et al.*, 2003). The motive meant for the deficiency of diversity can be associated with the virulence factors according to the species. *Enterococcus* species are well known as opportunistic pathogens as highlighted by a study in Denmark which exhibits that hospitalised patients have a 57% isolation rate of *E. faecalis* whereas healthy individuals show only a 39–40% prevalence (Mutnick *et al.*, 2003).

Hospitalised patients may have a substantial occurrence of enterococcal infection not just because of virulence, but because the hospital itself is a hub of infection. A report for the Department of Health in the UK underlined enterococci may infect and continues to live around the patient for several days (Brown *et al.*, 2006).

## **2.6 *Enterococcus* Pathotypes**

In spite of the fact that several *Enterococcus* species have been identified, only two species (*E. faecalis* and *E. faecium*) have commonly been associated with infection in humans (Gordon *et al.*, 1992; Lewis and Zervos, 1990; Moerllering, 1992; Patterson *et al.*, 1995; Ruoff *et al.*, 1990). These two species are known to be Gram-positive, facultatively anaerobic, coccus occurring singly, in pairs or short chains (Ryan and Ray, 2004) which are normal microflora of human intestinal tract and commonly the female genital tract. Murray *et al* (1999) discovered 38 species within the *Enterococcus* genus with merely those from humans and animals having been studied in detail. The most significant species are probable human pathogens *Enterococcus faecalis* and *Enterococcus faecium*, nonetheless, *Enterococcus gallinarum* and *Enterococcus casseliflavus* have also been studied because they are inherently vancomycin-resistant and inhabit the intestinal tract (Reid *et al.*, 2001; Murray *et al.*, 1999). *Enterococcus* infection by means of bacteriophages is a different method of genetic material exchange used by enterococci to allow for the spread of virulence factors or antibiotic resistance (Mazaheri *et al.*, 2011; Vinodkumar *et al.*, 2011).

Enterococci are also capable to flourish in a broad pH range—growing optimally at pH 7.5, but their surviving pH may range from 4.8 to 9.6, although some strains can withstand pH 10.0

( Fisher and Phillips, 2009; Teixeira *et al.*, 2011). As a final point, they can resist 40% (w/v) bile salts that can devastate other bacteria like *Streptococcus pneumoniae* (Devriese *et al.*, 2006; Murray *et al.*, 1999; Teixeira *et al.*, 2011). The polymerase chain reaction (PCR) has become a powerful molecular tool for bacterial identification, and scientists have developed a rapid PCR assay for the detection of the enterococci to the genus level (Ke *et al.*, 1999).

### **2.6.1 *Enterococcus faecalis***

Scientists have identified a virulence region never seen before in the genome of *Enterococcus faecalis* - a leading cause of bacterial infection among hospital patients (Shankar *et al.*, 2002). This bacterium is a commensal in the human gut, but it also flourishes on wounds and burns (Collier, 2003). There has long been concern about the dangers of antibiotic resistance and its implications for the return of infectious diseases that cannot be effectively treated (Aarestrup *et al.*, 2011; Tannock and Cook, 2002). However, Science (2003) argued that hospitals are not only spreading bacteria from patient to patient, but are also harbouring a tougher breed of bugs that are resistant to antibiotics. The sequencing of the genome reinforces knowledge of the remarkable fluidity of the bacterial gene pool. This fluidity allows bacteria to exchange DNA to enhance their ability to cause disease or their resistance to antibiotics (Science, 2003)

### **2.6.2 *Enterococcus faecium***

*Enterococcus faecium* is a human pathogen that causes nosocomial bacteremia, surgical wound infection, endocarditis and urinary tract infections (Emori and Gaynes, 1993; Jarvis *et al.*, 1996). This species had received attention in the scientific literature.

Increasing reports had surfaced describing an increase in the incidence of nosocomial infections in humans due to some strains of *Enterococcus faecium* that have become resistant to the antibiotic vancomycin (Devries and Pot, 1995). Nosocomial infections are those acquired in medical setting during treatment of a prior complaint (Achakar, 2010). *E. faecium* normal inhabitant of the gastrointestinal tract of a multitude of animals but it can also be established in the oral cavity and vaginal tract (Mark *et al.*, 1998). This microbe can survive for long periods of time in soil, sewage, and inside hospitals on a range of surfaces. It can grow in temperatures ranging from 10 to 45°C, in basic or acidic environments, and in environments which are isotonic or hypertonic (Barbara and Murray, 1998).

*E. faecium* is a Gram-positive, spherical cell that can occur in pairs or chains. The colonies formed are 1-2 mm in length and appear wet. The cells are non-motile (De Perio *et al.*, 2006). *E. faecium* can be highly drug resistant and acquires its drug resistance by plasmids and conjugative transposons as well as chromosomal genes that encode resistance. A few strains have become resistant to vancomycin, penicillin, gentamicin, tetracycline, erythromycin and teicoplanin (Ryan and Ray, 2004). The outspread of the disease occurs between patients in hospitals due to transfer of the pathogen by hands or medical instruments (Mody, 2007). Also, antibiotic use can decrease the number of other intestinal bacteria that are susceptible to the antibiotic and decrease competition for the drug resistant *E. faecium* (Ryan and Ray, 2004).

*E. faecium* can obtain drug resistance through three types of conjugation: pheromone-responsive plasmids, broad host-range plasmids, and conjugative transposons. Pheromone response plasmid occurs when the cell secretes a sex pheromone for a specific plasmid (Mark *et al.*, 1998). When a donor cell comes into contact with the pheromone, transcription of the relevant portion of the

plasmid is turned on and it also secretes a sticky substance. The sticky or aggregation substance facilitates the transfer of the plasmid to the recipient cell by helping them to stick together. Transfer of other plasmids can also occur between different genera of bacteria including staphylococci, and streptococci. The consequence of the ability of *E. faecium* to acquire broad host-range plasmids is that drug resistance can be widely and more easily spread (Fisher *et al.*, 2009) Conjugative transposons can also transfer antibiotic resistance between genera as well as between Gram-positive and Gram-negative bacteria because they do not need to co-operate with host machinery in order to insert themselves into a plasmid or chromosome of the bacterium. *E. faecium* can interact with other bacteria to spread drug resistance through conjugation (Mark *et al.*, 1998).

### **2.6.3 *Enterococcus hirae***

According to Farrow and Collins (1985) this species is not often isolated from humans. In older literature, *Enterococcus hirae* is recognised as a minority constituent of the human intestinal flora (Noble, 1973; Watanabe *et al.*, 1981; Farrow and Collins, 1985). *E. hirae* infections in humans are apparently uncommon. Only a few well described clinical cases of *E. hirae* infection have come out in literature (Gilad *et al.*, 1998) although *E. hirae* regularly accounts for a small minority of strains present in collections of clinical enterococci isolates in numerous surveys of isolation results in hospitals. *E. hirae* is a frequently occurring component of the intestinal flora of several domestic animals' species (Derviese *et al.*, 1987). The bacteria have, furthermore, been found in food of animal origin and in water.

#### **2.6.4 *Enterococcus gallinarum***

*Enterococcus gallinarum* has been identified as the causative agent of a wide variety of infections in humans, especially immunocompromised persons (Van *et al.*, 1998). This species of enterococci have also been shown to populate the intestinal tracts of both hospitalised and non-hospitalised individuals, with overall rates of colonisation that range from 5.7% (Toye *et al.*, 1997) to 12.1% (Van *et al.*, 1998). *E. gallinarum* is not frequently isolated from clinical specimens but can cause serious invasive infection. A review of the literature reveals that *E. gallinarum* may be isolated from a variety of clinical specimens and from patients who are either chronically ill or immunosuppressed (Facklam and Collin, 1989; Ruoff *et al.*, 1990; Pompei *et al.*, 1991; Gordon *et al.*, 1992; McNamara *et al.*, 1995; Toye *et al.*, 1997). The majority of cases of bacteremia due to this organism take into account patients with underlying conditions such as renal failure (Kaplan *et al.*, 1988; Pompei *et al.*, 1991).

#### **2.6.5 *Enterococcus avium***

*Enterococcus avium* is most commonly found in birds (Mohanty, 2005). On rare occasions, it is also a cause of infection in humans, and in such cases, may be vancomycin-resistant, and is referred to as vancomycin resistant enterococci avium (VREA) (EA, 2010).

### **2.7 Infection caused by enterococci.**

Enterococci are regarded as aetiological agents of urinary tract infections (UTIs), bacteremia, endocarditis, meningitis, wound infections and intraabdominal and pelvic infections (Moellering, 1992; Teixeira *et al.*, 2011). Enterococci are also important causative agents of the most common infections acquired in hospitals (Emori and Gaynes, 1993; Teixeira *et al.*, 2011).

These infections are problematic in developing world countries (Budavari *et al.*, 1997; Cohen, 1997; Khudaier *et al.*, 2007; Singh 2009; Panesso *et al.*, 2010),

but are also established in wealthier nations (Emori and Gaynes, 1993). Death related with enterococci is normally due to endocarditis (Megran, 1992). Of all the species, *E. faecalis* and *E. faecium* are the two most important pathogens, with *E. faecalis* accounting for 80–90% of infection cases and *E. faecium* constituting the majority of the remaining cases (Jett *et al.*, 1994). A small number of clinical isolates include *E. casseliflavus* and *E. gallinarum* (Reid *et al.*, 2001). A large number of enterococcal infections are UTIs, which are habitually of nosocomial origin and are largely derived from patients using urinary catheters (Moellering, 1992; Teixeira *et al.*, 2011). Worldwide, *Escherichia coli* is considered to be the number one cause of UTIs (Forbes *et al.*, 2007), but *E. faecalis* has overtaken *E. coli* as the number one cause of UTIs in many hospitals and other health facilities. Another enterococcal infection, a polymicrobial infection, is a result of intraabdominal and pelvic infections in which the enterococci are usually associated with other organisms (Moellering, 1992). Many times these infections are from wounds (Moellering, 1992). Bacteremia is regarded as the third common infection caused by *Enterococci* species (Caballero-Granado *et al.*, 2001; Song *et al.*, 2003; Teixeira *et al.*, 2011). Patients that experience enterococcal bacteremia have an increased likelihood of developing endocarditis (Caballero-Granado *et al.*, 2001; Song *et al.*, 2003; Teixeira *et al.*, 2011). Enterococcal meningitis is uncommon and apparent mostly in neonates and in patients who have undergone complex neurosurgical measures. It is hardly ever a consequence of enterococci bacterium (Moellering and Robert, 1992).

## **2.8 Emergence of antimicrobial resistance**

An imperative point in the emergence of the enterococci as a cause of nosocomial infection is their increasing resistance to a wide range of antibiotics (Jamison, 2006). They exhibit both intrinsic and acquired resistance (Hunt, 1998). Intrinsically, *Enterococci* are able to tolerate or

resist beta-lactam antibiotics since they have low affinity penicillin-binding proteins (PBPs); which enable them to synthesize cell wall components even in the presence of modest concentration of most beta-lactam antibiotics (Murray, 1990). Enterococci show intrinsic resistance to penicillinase-susceptible penicillin, penicillinase-resistant penicillin, cephalosporin, nalidixic acid aminoglycoside and clindamycin (Murray, 1990). Enterococci also have acquired resistance, which includes resistance to penicillin like beta-lactamases, chloramphenicol, tetracyclines, rifampin, fluoroquinolones, aminoglycosides (high levels), and vancomycin (Fraser 2003; Maroth, 2008; Sood, 2008). The genes that encode intrinsic or acquired vancomycin resistance result in a peptide to which vancomycin cannot bind; therefore, cell-wall synthesis is still possible (Brooks *et al.*, 2007).

Acquired resistance in enterococci can occur either by means of mutations in existing DNA or through acquisition of new DNA (Isenberg, 1992). VRE, particularly *E. faecium* strains, are frequently resistant to all antibiotics that are effective treatment for vancomycin-susceptible enterococci, which leaves clinicians treating VRE infections with limited therapeutic options (Udo *et al.*, 2002). Some enterococcus strains have the potential to be reservoirs of glycopeptide resistance genes and transfer them to more virulent pathogens such as methicillin-resistant *Staphylococcus aureus* (Noble *et al.*, 1992; Morrison *et al.*, 1997; Sung and Lindsay, 2007).

There are known strains of enterococci that produce  $\beta$ -lactamases that are able of inactivating penicillin, ampicillin, and related drugs from transferable plasmids (Herman and Gerding, 1991; Teixeira *et al.*, 2011).

## **2.9 Sources of Antibiotic Resistance in the Environment**

### **2.9.1. Hospital Effluent**

Antibiotics used in medicine for the treatment of infections are mostly released non-metabolized into the aquatic environment via waste water. Unused therapeutic drugs are from time to time disposed off down drains. Amongst other active compounds used, antibiotics and disinfectants are present in the effluent of hospitals (Kummerer, 2000). Ampicillin was found in concentrations of between 20 and 80 mg/L in the effluent of a large German hospital (Kummerer and Henninger, 2004). There have been reports that the extensive use of biocides used in hospitals and homes may perhaps select for antibiotic-resistant bacteria (Russell, 2000).

### **2.9.2 Municipal sewage and activated sludge sewage treatment plant**

Resistant bacteria are present in municipal sewage as well as in aeration tanks and the anaerobic digestion process of sewage treatment plant (Romling *et al.*, 1994; Feuerpfell *et al.*, 1999; Heur *et al.*, 2000; Schwartz *et al.*, 2003). Bacteria carrying the *vanA* gene have been detected in waste water in Europe as well as in the USA (Feuerpfell *et al.* 1999; Romling *et al.*, 1994; Heur *et al.*, 2003). Resistant and multi-resistant pathogenic bacteria such as *Acinetobacter* spp. (Davison *et al.*, 1999) have been detected in waste water and STPs also the transfer of resistance (Harwood *et al.*, 2001). Exchange of genes encoding for resistance between *Pseudomonads* and *E. coli* in sewage sludge (Iversen *et al.*, 2002) has been reported. Up to 99% of *Campylobacter* spp. was removed from sewage water by treatment in an (Heur *et al.*, 2003).

A alike elimination rate was established for *P.aeruginosa*, ciprofloxacin-resistant *E. coli* and VRE. Elimination rates were 95–99% for *E. coli*, *Pseudomonas* spp. and *Enterococcus* spp (Wiethan *et al.*, 2001).

### **2.9.3 Surface water**

Bacteria resistant to antibiotics are present in surface water (Muela *et al.*, 1994; Guardabass *et al.*, 1999; Schwartz *et al.*, 2003). Goni-Urizza *et al.*(2000) discovered a correlation between resistant bacteria in rivers and urban water input. Schwartz *et al.* (2003) were able to amplify AmpC b-lactamase gene sequences by PCR in surface water.

### **2.9.4 Drinking water**

Antibiotic-resistant bacteria were detected in drinking water as early as the 1980s and later in the 1990s (Amstrong *et al.*, 1981; Kolwazan *et al.*, 1991). The authors established that resistant bacteria identified using classical microbiological methods, i.e. standard plate counting, occurred within the distribution network of drinking water supply systems. It was concluded that the treatment of raw water and its subsequent distribution selects for antibiotic-resistant bacteria. According to the findings, increased phenotypic resistance rates were also detected at drinking water sampling points Schwartz *et al.* (2003).

## **2.10 Antibiotic resistance and public health risks**

A bacterium that is resistant to one or more antibiotics may perhaps present direct and indirect risks to human health (CDC, 2013). Direct risks are associated with contact with pathogens that are resistant to antibiotics applicable for treatment of infections, as this may directly result in disease that is complicated to treat (Salyers *et al.*, 2004; Kelly *et al.*, 2009; Trobos *et al.*, 2009).

Indirect risks are associated with exposure to relatively harmless bacteria, such as commensals, that carry antibiotic resistance. These bacteria can colonise intestines or skin (without causing disease), and pass their resistance genes on to other bacteria that inhabit these tissues (Trobos *et al.*, 2009). Since enterococci can grow in the intestinal tract of humans and other animals, it can

be found in hospitals and communities, and are even present in the waters and sands of the beaches, where sewage contamination and river runoff have increased their numbers (Yamahara *et al.*, 2007). Recognised examples of antibiotic resistant commensal bacteria that can be harmful to man are those causing nosocomial infections, such as methicilline-resistant *Staphylococcus aureus* (MRSA) (Falcone *et al.*, 2009). Although this bacteria cause problems in hospital patients and institutionalised people, it is also common in healthy people, animals and the environment (Falcone *et al.*, 2009).

It is understandable that in the hospital environment, antibiotics may cause selection of antibiotic resistant pathogenic enterococci, which may show the way to infections or super infections (Murray, 1990). The most problematic part is strains that contain acquired multiple antibiotic resistances, especially resistance to vancomycin and to the synergistic action of  $\beta$ -lactams and aminoglycosides (Murray, 1990).

### **2.11 Antibiotic resistance genes in the environment**

Bacterial antibiotic resistance genes are excreted into the environment mostly with faeces of humans and animals that are treated with antibiotics (Cooke, 1976). These bacteria end up in soil and surface water all the way through the discharge of untreated or incompletely treated sewage application of activated sludge from wastewater treatment plants as fertiliser on agricultural soil, and runoff of animal manure or faeces of pasture animals (Armstrong *et al.*, 1981; Kolwzan *et al.*, 1991).

The public is at risk of being exposed to these bacteria when they come into contact with contaminated environmental compartments, for instance during recreation in contaminated surface water, when consuming water produced from unprotected drinking water sources or

when eating food that has been irrigated with faecally contaminated surface or groundwater, fertilised using contaminated manure or grown in contaminated soil (Marcinek *et al.*, 1998). Once in the environment, bacteria of diverse source come into physical contact and may swap resistance genes with the endogenous bacterial population (Genthner *et al.*, 1988; Coughter and Stewart, 1989; Xu *et al.*, 2007; Cattoir *et al.*, 2008). Despite the fact that the resistant bacteria of human and animal origin may die off in the environment, the endogenous environmental bacteria may pass the acquired resistance genes on to their offspring.

Regardless of the generally believed negative impact of acquired antibiotic resistance on fitness of the bacteria (Andersson and Levin, 1999; Andersson and Hughes, 2010), these genes can possibly stay behind for a long time (Andersson, 2003). When these bacteria are subsequently re-introduced in humans or animals that are treated with antibiotics, this may contribute to the rapid development of resistance. Antibiotic resistant genes (ARGs) can enter into aquatic environments by direct discharging of untreated wastewater or into STPs through wastewater collection systems and subsequently into the environments with effluents and discharged sludge (Auerbach *et al.*, 2007). ARGs can be transferred into soils by amending farm land with animal manure and processed biosludge from STPs and then can leach to groundwater or be carried by runoff and erosion to surface water (Yang and Carlson 2003).

Surface water and shallow groundwater are commonly used as source of drinking water; thus, ARGs can go through drinking water treatment services and go into water delivery systems (Schwartz *et al.* 2003).

Even though antibiotics have been useful in large quantities for some decades, an anticipation of the existence of these substances in the environment was given very little attention. Studies conducted in various countries have detected a number of antibiotics in different environmental compartments, i.e. hospital effluent, municipal waste water, effluent from sewage treatment plants, surface water and in some cases ground water (Richardson *et al.*, 1985; Kolpin *et al.*, 2002; Ku'mmerer,2003; Kummer, 2004). The compounds detected are from different important antibiotic classes such as macrolides, tetracyclines, sulphonamides, quinolones and others as far as analytical methods are available.  $\beta$ -Lactams have not been detected yet despite the fact that  $\beta$ -lactams are used in the highest amounts (Moslstad *et al.*, 2002). There has been growing concern about antimicrobial resistance for some years now. In a report by the UK House of Lords, it is stated: 'Resistance to antibiotics and other anti-infective agents constitutes a major threat to public health and ought to be recognised as such more widely than it is at present (House of Lords, 1998) There is lot of literature that is associated to use of antimicrobials and resistance against them in medicine, veterinary medicine and animal husbandry (Witte *et al.*, 1999; Bjorkman *et al.*, 2000). Resistance genes as well as resistant bacteria in the environment are increasingly seen as an ecological crisis (Davison, 1999; Bjorkman *et al.*, 2000).

## **2.12 Treatment**

Resistance makes enterococci infection difficult to treat; however, there is advantage of using specialised techniques to demonstrate their susceptibilities in the clinical laboratory (Reller *et al.*, 2009).

This can be achieved by looking for high-level aminoglycoside resistance in the laboratory.

Likewise, standard testing can fail to demonstrate penicillin/ampicillin resistance in many beta-lactamases producing strains (Mendeleman *et al.*, 1986). Uncomplicated enterococcal infections

are usually treated with single-drug therapy such as ampicillin, penicillin and vancomycin. Serious enterococcal infections e.g. bacteremia and endocarditis require treatment with bactericidal combination antibiotics that should include penicillin (ampicillin or penicillin G) to which the isolate is susceptible and an aminoglycoside (gentamicin or streptomycin) to which it does not exhibit high-level resistance (Bartoloni *et al.*,1990)

## **CHAPTER THREE**

### **3.0 METHODOLOGY**

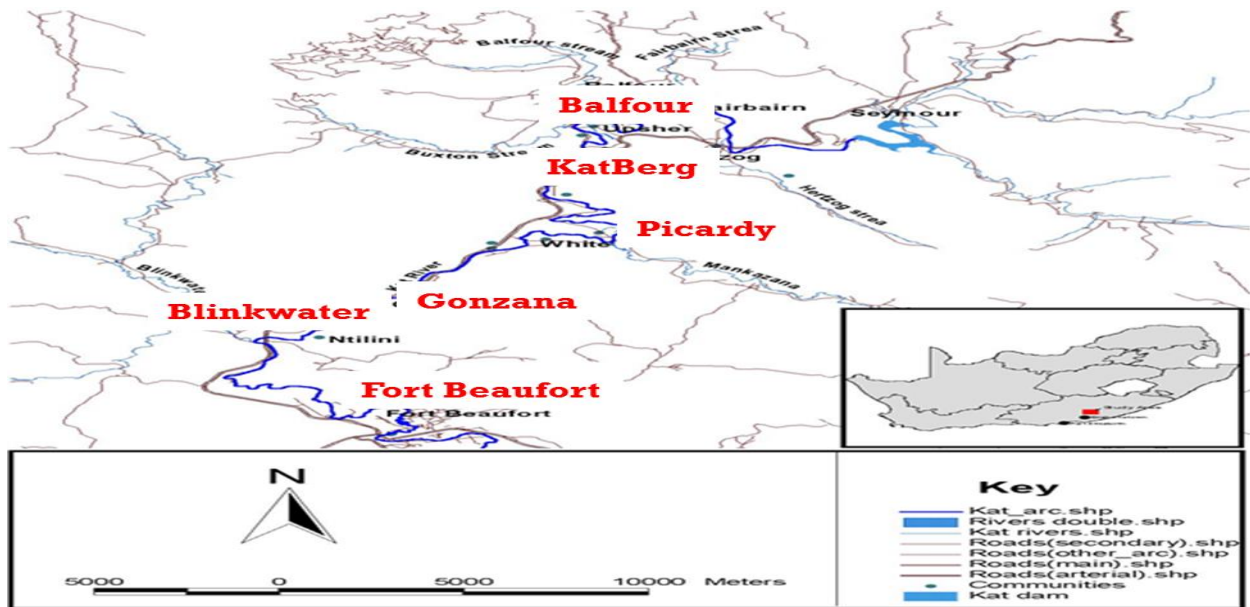
#### **3.1 Description of study area**

The study was undertaken in the upper Kat River area (Fig. 3.1). The Kat River falls within the primary catchment of the Great Fish River. Climate in the study area is mild with temperatures varying between 0°C and 20°C in winter and 20°C and 35°C in summer. The area falls in the summer rainfall region with average rainfall varying between 400 mm in winter and 1600 mm in summer. The altitude in the region varies between 600 m at the confluence with Great Fish River

and 1600 m at the source in the escarpment. Kat River and lies in the in town of Seymour, under Amathole District Municipality in the Eastern Cape province of South Africa.

### 3.1.1 Study Area for Kat River

The figure below shows the six sites where water samples were collected namely, Balfour, Gonzana, Blink Water, KatBerg, Picardy and Fort Beaufort.



**Figure 3.1: Illustration of the sampling sites on Kat River.**

Fort Beaufort is a town situated in the Eastern Cape Province of South Africa and lies on geographical co-ordinates 32°47'0"S, 26°38'0"E. Figure 3.2 shows the Fort Beaufort abstraction water used as source of raw water at the Fort Beaufort municipal water plant. Currently raw

water is extracted from the Kat River at a barrage and transferred to this raw water storage dam situated near the water treatment works.



**Figure 3.2: Photographs of the Fort Beaufort abstraction water, one of the sampling sites.**

### **3.2 Culture collection**

A total of 400 presumptive *Enterococcus* isolates previously recovered from the Kat River were selected for identity confirmation using *Enterococcus* specific primers targeting SodA gene. This

was all done using conventional Polymerase Chain Reaction Technique. ATCC 19433 strain of *Enterococci* was used as the positive control and water (H<sub>2</sub>O) as the negative control.

### **3.3 DNA extraction**

DNA extraction was done following the description of Torres *et al.* (2003) and Maugeri *et al.* (2004) with little modifications. Single colonies of overnight growth culture were picked using sterile toothpick to avoid agar contamination (an important cause of erratic PCR amplification) and suspended in 200µl of sterile nuclease free water. The suspension was vortexed and the cells were lysed by heating for 10 min at 100°C using a MS2 a Dri-Block DB.2A (Techne, SA). The suspension was then centrifuged at 13 000 rpm for 5 min to pellet the cell debris. Thereafter, the lysate supernatant was incubated on ice for 5min after which 1 µl aliquots of it were used as template in 25 µl PCR assays immediately after the extraction.

### **3.4 Protocol for PCR**

In a single PCR reaction tube the following reagents were added for each isolate: 12.5µl of master mix, 5µl of sample (template) DNA, 0.5µl each of the forward and reverse primer, and 6.5µl of nuclease free water to make a 25µl reaction, which was amplified using the PCR conditions below as described by Moyo *et al.* (2007), shown in Table 3.3. Target specific primers that were used are shown in Tables 3.1 and 3.2 below.

**Table 1: Primers for confirmation of presumptive *Enterococcus* isolates**

PRIMER	NUCLEOTIDE SEQUENCE (5'-3')	AMPLICON SIZE	REFERENCE
ENTEROCOCCUS GENUS <b>ATCC 19433</b>			
Forward	TACTGACAAACCATTCATGATG	112	Ke et al.(1999)
Reverse	AACTTCGTCACCAACGCGAAC		

**Table 3.2: Primers for *Enterococcus* pathotypes characterisation**

Strain	SodA accession number	Sequence (5-3')	Amplicon size (bp)	Reference
<i>E. faecalis</i> ATCC 19433	AJ387912	ACTTATGTGACTAACTTAACC  TAATGGTGAATCTTGGTTTGG	360	Aarestrup et al.(2005)
<i>E. gallinarum</i> ATCC 49673	AJ387915	TTACTTGCTGATTTTGATTCG  TGAATTCTTCTTTGAAATCAG	173	Aarestrup et al.(2005)
<i>E. casseliflavus</i> ATCC 25788	AJ387907	TCCTGAATTAGGTGAAAAAAC  GCTAGTTTACCGTCTTTAACG	288	Aarestrup et al.(2005)
<i>E. avium</i> ATCC 14025	AJ387906	GCTGCGATTGAAAAATATCCG  AAGCCAATGATCGGTGTTTTT	368	Facklam et al.(2000)

**Table 3.3: PCR cycling conditions for *Enterococcus* strains characterisation**

<b>Step</b>	<b>Temperature</b>	<b>Time</b>
Denaturing	95°C	30 seconds
Annealing and Extension	72°C	2 minutes
Final Extension	72°C	5 minutes 30 Cycles
Hold	4°C	Until removed

### **Preparing McFarland Standard**

McFarland standards are suspensions of either barium sulfate or latex particles that allow visual comparison of bacterial density. A 0.5 McFarland is equivalent to a bacterial suspension containing between  $1 \times 10^8$  and  $2 \times 10^8$  CFU. 0.5 ml aliquot of 0.048 mol/litre  $\text{BaCl}_2$  (1.175% wt/vol  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ ) was added to 99.5ml of 0.18mol/litre  $\text{H}_2\text{SO}_4$  (1% v/v) with constant stirring to maintain a suspension. The correct density of turbidity standard was verified by measuring absorbance using a spectrometer with 1-cm light path and matched of cuvette. The absorbance at 625nm should be 0.08 to 0.13 for the standard. The barium sulfate suspension in 4-6ml aliquots was transferred into screw-cap tubes of the same size as those used in standardizing the bacterial inoculums. Seal tubes tightly and store in the dark at room temperature. Prior to use, vigorously shaking was done for a uniform turbid appearance. McFarland standard was used to compare the turbidity of the culture to that of the McFarland before the suspension to the Miller Hounton Agar plate.

### **Preparation of inoculums**

Using a sterile inoculating loop or needle, three to four isolated colonies of an 18 h old culture were picked and suspended sterile saline. The saline tube was vortexed to create a homogenous suspension. The turbidity of this suspension was adjusted to 0.5 McFarland standards by adding more organisms or diluting with sterile saline. The suspension was used within 15 minutes of preparation.

### **3.6 Antibiotic susceptibility test**

Antimicrobial susceptibility testing was done on Mueller-Hinton agar (MH) by the standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2011). Briefly, fresh cultures (about 22 hours old) were transferred into test tubes containing 5 ml sterile normal saline and the turbidity of the suspension adjusted to 0.5 McFarland standard. Sterile swabs were then dipped into the bacterial suspensions and used to inoculate the MH agar plates by spreading uniformly on the surface of the agar, after which the antibiotic discs were placed on the bacterial lawn and the plates incubated at  $35 \pm 20^{\circ}\text{C}$  for 18 to 24 h.

The antibiotics that were used in this study are shown in Table 3.4 below. All confirmed *Enterococcus* isolates were used for antimicrobial susceptibility testing against the panel of 9 antibiotics. After incubation, the plates were examined for zones of inhibition which were then measured and interpreted using the minimal inhibitory concentration (MIC) breakpoints for *Enterobacteriaceae* and *Enterococcus* (CLSI, 2011). Thereafter, the prevalence of relevant antibiotic resistance genes was determined using PCR.

**Table 3.4:** Zone Diameter Interpretative Standards and Equivalent Minimal Inhibitory Concentration (MIC) Breakpoints for *Enterococcus*. Source: (CLSI/NCCLS, 2011)

Test/ Report group	Antimicrobial agent	Disk Content ( $\mu\text{g}$ )	Zone diameter Breakpoints, nearest whole mm			MIC Interpretive standard ( $\mu\text{g/mL}$ )		
			S	I	R	S	I	R
A	Ampicillin	10	$\geq 17$	-	$\leq 16$	$\leq 8$	-	$\geq 16$
A	Penicillin	10	$\geq 15$	-	$\leq 14$	$\leq 8$	-	$\geq 16$
B	Vancomycin	30	$\geq 17$	15-16	$\leq 14$	$\leq 4$	8-16	$\geq 32$
C	Tetracycline	30	$\geq 19$	15-18	$\leq 14$	$\leq 4$	8	$\geq 16$
B	Erythromycin	15	$\geq 23$	14-22	$\leq 13$	$\leq 0.5$	1-4	$\geq 8$
U	Ciproflaxacin	5	$\geq 21$	16-20	$\leq 15$	$\leq 1$	2	$\geq 4$
B	Fosfomycin	200	$\geq 16$	13-15	$\leq 12$	$\leq 64$	128	$\geq 258$
C	Linezolid	30	$\geq 23$	21-22	$\leq 20$	$\leq 2$	4	$\geq 8$

### 3.5 Evaluation of antibiotic resistance genes

Following susceptibility testing, the relevant antibiotic resistance determinants were evaluated using PCR. The target genes for the antibiotics that the organisms were resistant to are shown in the following table:

Table shows the sequence of the target genes, molecular size (bp), resistance genes and the annealing temperature.

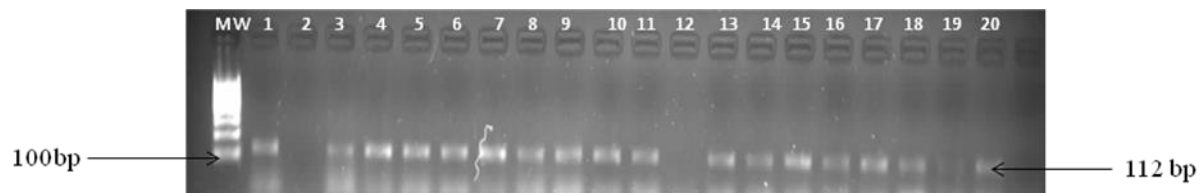
<b>Antibacterial agent</b>	<b>Resistance gene</b>	<b>Sequence</b>	<b>Size (bp)</b>	<b>Annealing Temp. (°C)</b>	<b>References</b>
Ampicillin	bla	<b>F</b> -TCGCCTGTGTATTATCTCCC <b>R</b> -CGCAGATAAATCACCACAATG	52	55	Van et al. (2008)
Vancomycin	vanA	<b>F</b> -CATGAATAGAATAAAAAGTTGCAATA <b>R</b> - CCCCTTTAACGCTAATACTACGATCAA	1090	50	Roth et al. (2001)
Erythromycin	ermA	<b>F</b> -TATCTTATCGTTGAGAGGGGATT <b>R</b> -CTACACTTGGCTTAGGATGAAA	134	64	Roth et al. (2001)

## CHAPTER FOUR

### 4.0 RESULTS

#### 4.1 Molecular confirmation with PCR

The result of the molecular confirmation by (PCR) showed that 68.5% (274/400) isolates were positive for *tuf* gene. Figure 4.2 below shows a gel picture for some of the isolates that were confirmed to be *Enterococci*.

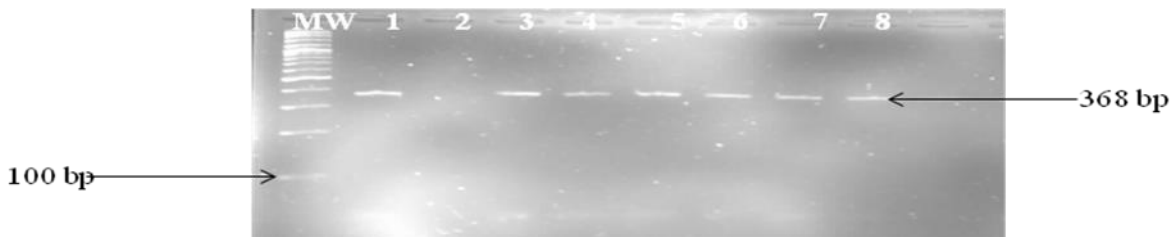


Lane 1 (100 bp MW ladder); Lane 2 positive control (ATCC 19433); Lane 3 (negative control); and Lanes 3-20 (test samples).

**Figure 4.1: Agarose gel electrophoresis of PCR products of *tuf* gene amplification. 4.2 *Enterococcus* spp. pathotyping**

#### *E. avium* pathotyping:

Sixteen percent (16%) of the confirmed isolates (46/274) were further confirmed as *E. avium* with *SodA* accession number **AJ387906** target at locus. The confirmation agarose gel picture is shown in Figure4.

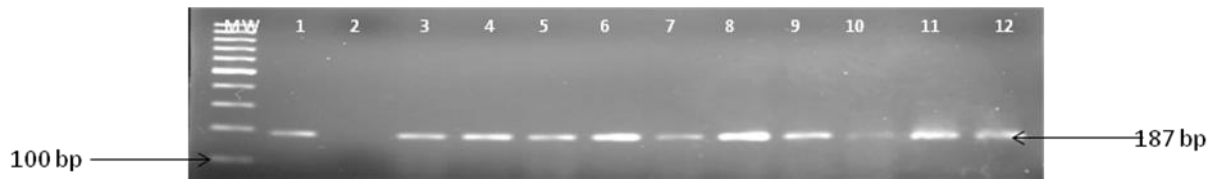


Lane 1 (100bp MW ladder); Lane 2 positive control (ATCC 14025); Lane 3 (negative control); and Lanes 3-8 (test samples).

**Figure 4.2: Agarose gel electrophoresis of PCR products of some *Enterococcus avium* amplification.**

***E. hirae* pathotyping**

Thirteen percent (13%) of the confirmed (38/274) were further confirmed as *E. hirae* with *SodA* accession number **AJ387916** target at locus. A representative gel picture is shown in Figure 4.3 below

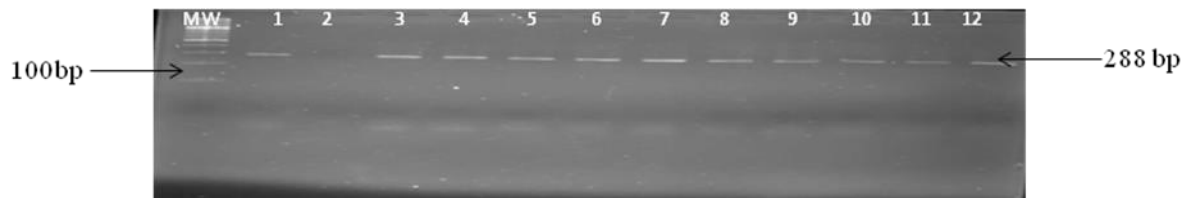


Lane 2 positive control (ATCC 8043); Lane 3 (negative control); and Lanes 3-13 (test samples).

**Figure 4.3: Agarose gel electrophoresis of PCR products of *Enterococcus hirae* amplification. Lane 1 (100 bp MW ladder);**

***E. casseliflavarus* pathotyping:**

Five percent (5%) of the confirmed (16/274) were further confirmed as *E. casseliflavarus* with *SodA* accession number **AJ387907** target at locus. Figure 4.4 below shows some of the confirmed isolates bands.



Lane 1 (100 bp MW ladder); Lane 2 positive control (ATCC 25788); Lane 3 (negative control); and Lanes 3-13 (test samples).

**Figure 4.4: Agarose gel electrophoresis of PCR products of *Enterococcus casseliflavarus* amplification.**

***E. faecalis* pathotyping**

Twenty percent (20%) of the confirmed (55/274) were further confirmed to be *E. faecalis* with *SodA* accession number **AJ387912** target at locus. Figure 4.5 shows some of the confirmed isolates bands.

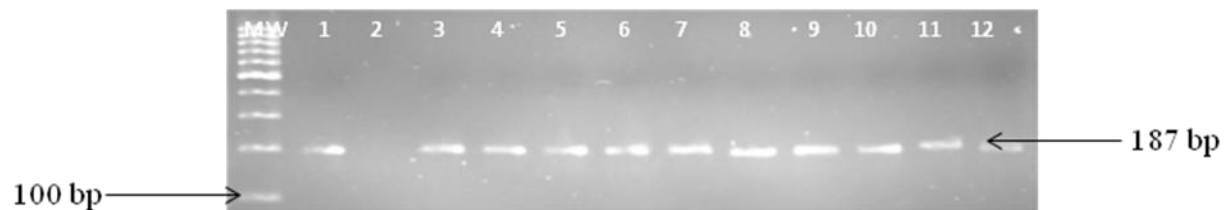


Lane 1 (100 bp MW ladder); Lane 2 positive control (ATCC 19433); Lane 3 (negative control); and Lanes 3-13 (test samples).

**Figure 4.5: Agarose gel electrophoresis of PCR products of *Enterococcus faecalis* amplification.**

***E.gallinarium* pathotyping:**

Four percent (4%) of the confirmed (12/274) were confirmed to be *E.gallinarium* with *SodA* accession number **AJ387915** target at locus. Figure 4.6 below shows an agarose gel picture confirmation of some of the isolates.



Lane 1 (100 bp MW ladder); Lane 2 positive control (ATCC 49673); Lane 3 (negative control); and Lanes 3-13 (test samples).

**Figure 4.6: Agarose gel electrophoresis of PCR products of *Enterococcus gallinarum* amplification.**

### **4.3 Antibiotic susceptibility test:**

Disk diffusion method was used for the response of *Enterococcus* pathotypes against a panel of 9 antibiotics. The table below shows the frequency of resistant, intermediate and susceptible of the pathotypes against each of listed antimicrobial agents.

**Table 5: Antibiotic susceptibility pattern of *Enterococcus* spp. pathotypes recovered from Kat River and Fort Beaufort abstraction water.**

	Antibiogram percentage (%) response of <i>Enterococcus</i> pathotypes obtained from (n=274) isolates.														
	<i>E. faecalis</i> n=55			<i>E. avium</i> n=46			<i>E. hirae</i> n=38			<i>E. gallinarum</i> n=13			<i>E. casseliflavus</i> n=16		
Antimicrobial agents	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Vancomycin (30 µg)	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0
Ciprofloxacin (15 µg)	0	24	76	0	10	90	0	0	100	0	0	100	0	3	97
Ampicillin (10 µg)	17	20	63	3	12	85	7	3	90	0	12	88	2	0	98
Gentamycin(10 µg)	0	4	96	0	2	98	0	2	98	0	0	100	0	0	100
Chloramphenicol(10 µg)	0	3	96	0	0	100	0	1	99	0	0	99	0	0	100
Erythromycin (15 µg)	78	2	10	9	3	88	2	0	98	3	14	83	4	0	96
Penicillin (10 µg)	62	8	30	5	11	84	16	2	82	30	12	58	1	0	99
Tetracycline (30 µg)	2	5	93	1	18	81	0	13	87	0	16	84	0	0	100
Linezolid (30 µg)	100	0	0	100	0	0	100	0	0	100	0	0	100	0	0

**Legend:**

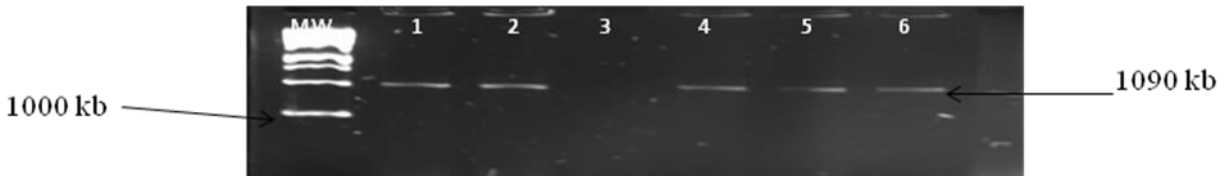
**R-Resistant,**

**I-Intermediate,**

**S-Susceptibility**

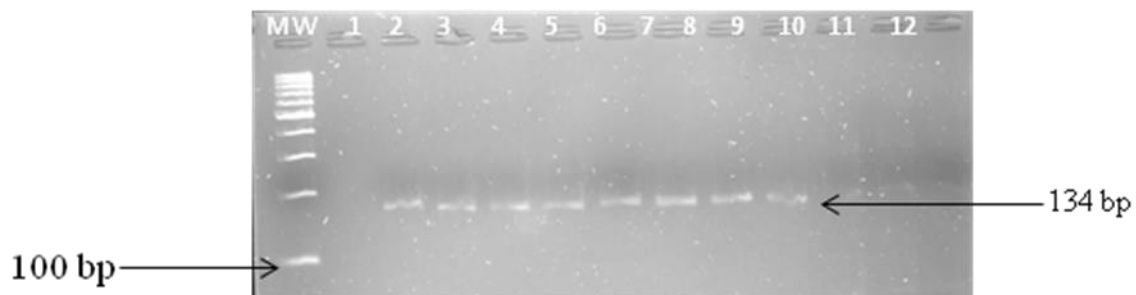
#### 4.4 Resistance genes detection

Polymerase chain reaction was used for the detection of antibiotic resistant genes *vanA* and *ermA* target genes for vancomycin and erythromycin respectively, to which most of the *Enterococcus* species pathotypes showed high frequencies of resistance. Figure 4.6 below is the illustration of *vanA* with a molecular size of 1090kb and figure 4.7 shows the *ermA* bands size of 134bp.



Lane 1 (1000 kb MW ladder); control); and Lanes 2-6 (test samples).

**Figure 4.7: Agarose gel electrophoresis of PCR products of VanA amplification.**



Lane 1 (1000 kb MW ladder); control); and Lanes 2-6 (test samples).

**Figure 4.8: Agarose gel electrophoresis of PCR products of ermA amplification.**

## CHAPTER FIVE

### 5.0 Discussion

The microbiological quality of the Fort Beaufort abstraction water and Kat River, which are life sustaining surface waters surrounding rural communities (Farolfi *et al.*, 2008), are affected by non-point sources of pollution such as runoff from the river banks with animal and human excreta. Because of lack of proper sanitary infrastructure, human feces are not an uncommon sight in the bushes and along the streams throughout the Kat River catchment and Fort Beaufort abstraction dam. Domestic and wild animals have often been spotted either feeding along or drinking from the rivers. Although an increasing number of villages around the river are being supplied with tap water, many people in the area still rely on the rivers as their main water source. Concentrations of *Enterococcus* spp. in surface water samples collected in this study were consistently in excess of the bacterial water quality standard for drinking water as they had exceeded the recommended limit of 0 CFU/100ml (DWAF, 1996: WRC, 1998). Throughout the sampling period for this study, human and animal excreta were spotted along the shore of the river banks. This could expose both human and animals around the Kat River and Fort Beaufort abstraction water to elevated concentrations of *Enterococcus* spp., and other more harmful microorganisms that may have been present.

This study provides a scheme for the rapid identification of enterococci using a molecular technique (PCR). The specificity of the genus was determined by testing four hundred (400) isolates from Kat River and Fort Beaufort abstraction water that were presumptively identified as enterococci. Sixty eight percent (274/400) of the isolates were amplified with the *Enterococcus* genus specific primers targeting the *tuf* gene, producing an amplicon size of 112bp.

These genotypic characteristics indicate that the isolated strains belong to the genus *Enterococcus* with the exception of hundred and twenty six (126) isolates. Although isolates obtained in the study were confirmed as *Enterococcus* spp. by molecular characterisation, their immediate sources were not determined. Studies have shown that *Enterococcus* spp survive in the environment. Ahmed and Katouli (2008) found from an Australian study that approximately 5% of surface water enterococci isolates could not be associated with a known faecal source and were suspected to be of environmental origin. In addition Badgley *et al* (2010a); Balzer *et al.*, (2010) reported that possible environmental sources of enterococci in the South Nation River may include sediments, biofilms, plants and submerged aquatic vegetation.

The two hundred and seventy four isolates confirmed with the *tuf* gene were further characterised into five different species, namely *E. faecalis*, *E. hirae*, *E. avium*, *E. casseliflavus* and *E. gallinarum*. The target gene for the characterisation of the *Enterococcus* spp used in this study is the *SodA* gene targeting manganese-dependent superoxide dismutase which has been reported to have species-specific variable regions and have been used for the characterisation of the genus into different species due to variations in sequences of *SodA* genes which appeared to be greater between species and less within species (Ke *et al.*, 1999; Goh *et al.*, 2000; Ozawa *et al.*, 2000 Poyart *et al.*, 2000). The species that showed high frequency of identification were *E. faecalis* (20%), *E. avium* (16%), *E. hirae* (13%) while *E. casseliflavus* (5%) and *E. gallinarum* (4%) were identified to a lesser extent as shown in Figure 4.2 to 4.6. In another study, Moto *et al* (2001) reported the detection of enterococcal species in environmental waters, but their overall prevalence varied considerably. The study reported the frequencies of different species as follows: *E. faecalis* (31%), *E. munditti* (31%), *E. casseliflavus* (16%) while *E. faecium* and *E. gallinarum* were identified with less frequencies (10% and 4% respectively).

In spite of these differences, these results indicate that multiple enterococcal species can be present in the same water sample. The differences in the occurrence of enterococcal species may be in the original place of the species growth and environmental survival rates (Lleo *et al.*, 2001). In this study, *Enterococcus faecalis* in both sampling areas was the dominant pathotype recovered. This is in agreement with recent findings of Getachew *et al.* (2012), who reported this pathotype to be predominant in human faeces.

The results of antibiotic susceptibility profiling showed high frequency of resistance against vancomycin and erythromycin (100% and 60% respectively) while ampicillin (16%) had low frequency of resistance. None of the isolates were resistant to ciprofloxacin; gentamicin and chloramphenicol, penicillin and tetracycline as shown in Table 4.5. The wide variability observed in the antimicrobial susceptibility profiles suggests that the origin of water contamination comes from a variety of sources which may include runoff from agricultural soils, storm water systems and streams, birds, sediments and aquatic vegetation

Further analysis of the isolates that were resistant to Vancomycin and Erythromycin using PCR, which detect *vanA* and *ermA* genes respectively, showed that some of the isolates harbor these genes which may have mediated resistance to these antibiotics. One of the few reports on the detection of *vanA* and *vanB* in recreational waters results suggest that several bacterial species in tropical environments may harbor *vanA* (Zhu *et al.*, 2008). The source of *vanA* in surface water may possibly be fecal matter since vancomycin has been detected in sewage effluent (Novais *et al.*, 2005). Antibiotic resistance genes can be transferred into soils by amending farmland with animal manure which can be carried by runoff and soil erosion to surface water (Yang and Carlson 2003).

## Conclusion and Recommendations

This study confirms that *tuf* and *SodA* genes are good molecular markers for the identification of *Enterococcus* genus and *Enterococcus* species, respectively. The high prevalence of *Enterococcus* species may pose a health risk mainly to person who use Kat River and Fort Beaufort abstraction water. The results from this study demonstrate that animals frequently implicated in the fecal contamination of environmental waters shed different enterococcal species in their feces. It also clearly demonstrates that surface water is an important reservoir of antibiotic resistant genes, *VanA* a glycopeptide and a macrolide (*ermA*), in both Kat River and Fort Beaufort abstraction water. The present study also emphasizes that human health risks associated with exposures to surface water could be exacerbated by the presence of antibiotic resistant bacteria. Overall findings indicate that *Enterococcus* spp. recovered from the Kat River and Fort Beaufort abstraction water samples express higher levels of resistance to antibiotics that are commonly used in human clinical medicine (erythromycin, ampicillin and vancomycin).

- Further studies are needed to determine whether *Enterococcus* species specific correlate better with risk than genus-specific assays and how they can be of value in public health and environmental monitoring studies.
- The prompt and early detection of the pathogenic enterococci in Kat River and Fort Beaufort abstraction water is crucial for preemptive strategy and better management of these water resources.
- The high prevalence of enterococci exhibiting resistance to *vanA* and *ermA*, needs to be linked to epidemiological studies to establish the role played by these pathogens especially in swimmers and bathers.

- The rural communities together with the municipal governments have to participate positively in efforts to protect and manage the quality of their water resources by regularly monitoring the Kat River and Fort Beaufort abstraction water to ensure satisfaction of set guidelines and protect public health.

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