DETECTION OF VANCOMYCIN RESISTANT ENTEROCOCCI FROM CLINICAL SPECIMENS AT UNIVERSITY TEACHING HOSPITALS

BY

SIMPOKOLWE KAZIWE

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DECLARATION

I, **Simpokolwe Kaziwe**, do solemnly declare that this dissertation is my own original work. I further declare that this work has not previously been submitted for the award of any diploma, degree or other qualification at this or any other University.

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I, **DR GINA MULUNDU**, having supervised and read this thesis is satisfied that this is the original work of the author under whose name it is being presented. I confirm that the work has been completed satisfactorily and is ready for presentation to the examiners.

Supervisor signature…… Date……

Head of Department:

…….

Signature……

Date…….....

Department of Pathology and Microbiology, School of Medicine, the University of Zambia

APPROVAL

This dissertation of Simpokolwe Kaziwe has been approved as fulfilling the requirements or partial fulfillment of the requirements for the award of Master of Science in Medical Microbiology by the University of Zambia.

Examiner I …… Signature ……..….. Date ……

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Examiner III …….Signature …….....…… Date …….

Chairperson,

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ABSTRACT

Enterococci are commensal gram-positive bacteria in the gastrointestinal tract of humans. They have emerged as important nosocomial pathogens due to their ability to acquire and confer antimicrobial resistance genes, hence making management of infections due to *Enterococcus* species difficult. Resistance to glycopeptide antibiotics, especially Vancomycin is of special concern as Vancomycin resistant genes are encoded on plasmids which can be transferred to other organisms. Currently, there is no documented information relating to the occurrence and resistance genes of VRE at the University Teaching Hospitals (UTH's) in Lusaka Zambia.

To isolate and determine the occurrence of Vancomycin resistant *Enterococcus* (VRE), and the genes responsible for VRE resistance from blood, urine and pus specimens received in the Microbiology Laboratory at the UTH's, Lusaka.

This was a cross-sectional study. *Enterococci* were isolated from urine, pus and blood specimens submitted to UTH's Microbiology Laboratory from July to August 2017. Enterococci isolation was done by culture of specimens on blood agar, cystine lactose electrolyte deficient medium (CLED), and Bile Esculin Azide (BEA) agar. Presumptive identification of VRE isolates was carried out using Brilliance VRE chromogenic media. Identification to species level, antibiotic susceptibility testing and minimum inhibitory concentrations (MIC) was determined with the Vitek 2 compact. Polymerase chain reaction (PCR) was used to confirm presence of resistance genes.

Out of 817 specimens, 25 (3%) *Enterococcus* isolates, comprising 22 *Enterococcus faecalis* and 3 *Enterococcus faecium,* were Vancomycin resistant as shown by chromogenic media and Vitek 2 compact. *VanB* genes were confirmed by PCR with most isolates coming from urine, followed by pus then blood. Some isolates were resistant to Penicillin, Ampicillin and Clindamycin, with none being resistant to nitrofurantoin.

More enterococci were isolated from urine compared to pus and blood, with most patients affected being aged between 28 and 46. More women were affected as compared to men. Brilliance chromogenic media was accurate in detection of VRE, as was PCR in confirming presence of resistance genes from the isolates.

Vancomycin resistant *Enterococci* were isolated, with the main isolate being *E. faecalis* as compared to *E. faecium*. *VanB* genes were detected by PCR. The study showed presence of multidrug resistant VRE. There is need for determination of risk factors and regular surveillance of antimicrobial susceptibilities for VRE.

Keywords: Vancomycin resistant enterococci, Chromogenic media, Glycopeptide antibiotics, Polymerase chain reaction

DEDICATION

To God almighty for the abundant favor. My dedication also extends to my parents and siblings and most of all my wife, for the moral support, prayers and encouragement throughout my studies.

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ABBREVIATIONS AND ACRONYMS

CNS	Central nervous system
DISA	Data intensive systems and applications
HGT	Horizontal gene transfer
MIC	Minimum inhibitory concentration
MOA	Mechanism of action
PCR	Polymerase chain reaction
UNZA	University of Zambia
USA	United states of America
UTH's	University teaching hospitals
UTI	Urinary tract infection
VRE	Vancomycin resistant enterococci
VSE	Vancomycin susceptible enterococci

CHAPTER 1

INTRODUCTION

1.1 Background

Enterococci have emerged as important nosocomial pathogens over the last 20 years (Rice, 2001). They are predominantly opportunistic pathogens (Byappanahalli et al. 2012; Camargo et al. 2008; Olawale et al. 2011; Van Tyne & Gilmore 2014; Nilsson 2012). The increase in severity of illness in hospitalized patients has contributed to the ascendance of enterococci as nosocomial pathogens (Olawale et al. 2011; Miller et al. 2014; Ott et al. 2013; Melaku 2012). Of critical importance is the intensive use of broad-spectrum antibiotics, which provides selective pressure favoring the growth of intrinsically drug-resistant enterococci (Eliopoulos and Gold, 2001). As a genus, *Enterococcus* contains some of the most multidrug resistant species, resulting in clinicians having reduced options for drugs to treat and manage infections caused by these microorganisms (Sujatha and Praharaj, 2012). These microbes tend to mostly infect debilitated and prolonged hospitalized patients (Arias and Murray, 2012). These gram positive bacteria not only pose a challenge to the clinicians but also result in treatment failure, selection pressure and spread of resistant strains in health care institutes (Phukan et al., 2016).

The Enterococci are a dominant bacterial group commonly found in the intestinal tract of humans and are a common cause of urinary tract infections (UTI), endocarditis and septicaemia (Chakraborty et al., 2015). The intrinsic nature of enterococci to acquire and share extra chromosomal elements encoding virulence traits or antibiotic resistance genes explains their increasing importance as nosocomial pathogens (Olawale et al., 2011). This characteristic has led to the limitation of drugs available for treatment and management of infections caused by these organisms (Guzman Prieto et al., 2016). Of particular importance is the resistance of these

microorganisms to Vancomycin, which is the last line drug used in management of infections caused by *Enterococcus* species (O'Driscoll and Crank, 2015).

Vancomycin is a glycopeptide used in the treatment of infections caused by organisms belonging to the genus Enterococci (Miller et al., 2014). Vancomycin's mechanism of action (MOA) is by binding to the terminal d-alanine-d-alanine (d-Ala-d-Ala) moiety of peptidoglycan precursors (Eliopoulos, 1997), thus preventing cross-linking of peptidoglycan chains and inhibiting synthesis of the cell wall (Eliopoulos and Gold, 2001). According to Miller et al., (2014), resistance to Vancomycin can be described as high-level (Minimum Inhibitory Concentration (MIC) >64 µg/ml) or low-level (MIC between 4 and 32 µg/ml). This can be said to be due to the change in the terminal amino acids of the peptidoglycan precursor from d-Ala-d-Ala to d-alanine-d-lactate (d-Ala-d-Lac) or to a much less degree due to d-alanine-d-serine (d-Ala-d-Ser) (Chang et al., 2017). The type of amino acid change determines the level of resistance (Reynolds and Courvalin, 2005). Thus, low-level resistance is conferred by the d-Ala-d-Ser-ending precursors (Lebreton et al., 2011), which decreases the binding affinity of the antibiotic to resistant organisms. On the other hand, high-level resistance relies on the change of the terminal penta-peptide to d-Ala-d-Lac (Chang et al., 2017), a substitution that eliminates one of the five hydrogen bonds required for the binding of Vancomycin to the peptidoglycan chain, reducing its affinity by about 1000-fold (Reynolds and Courvalin, 2005).

Resistance to Vancomycin is conferred by genes present in the plasmids of *Enterococcus* species (Rezvani et al., 2016). Some of these genes make some species intrinsically resistant while other genes are transferrable to susceptible enterococci (Hollenbeck and Rice, 2012). Currently, eight genotypes of glycopeptide resistance, which differ in the level and range of resistance and in transferability to glycopeptides, have been described for enterococci (Patel et al., 1997). Five of the van genes are acquired (*van A, B, D, E and G*) and three (*van C1, C2 and C3*) are intrinsic (Praharaj et al., 2013). Of these, *vanA* is the most prevalent and is

predominantly found in *E. faecium* and *E. faecalis*, the enterococcal species responsible for most infections in humans (Phukan et al., 2016). These genotypes determine the resistance pattern of Vancomycin resistant organisms (Kolar et al., 2006). This type of information with regards to the Vancomycin resistant enterococci occurrence, antimicrobial susceptibility profiles and the genes responsible for resistance is lacking at the University Teaching Hospitals (UTH's).

Undertaking a study to identify species of enterococci from clinical samples to determine antibiotic susceptibility profiles to Vancomycin and to detect the genes responsible for resistance in the isolated species would help in treatment and management of infections caused by these microorganisms. At the moment, there is no documented information in the country, and in particular, at the UTH's, Lusaka, relating to these aspects of enterococci and Vancomycin.

1.2 Statement of the problem

Health care facilities at all levels have patients admitted in wards for treatment and observation and this has potential for transmission of nosocomial pathogens from one patient to another (Puchter et al. 2018). Enterococci are a good example of such opportunistic microorganisms that have emerged as a cause of nosocomial infections, usually in long-term hospitalized patients (Moemen et al., 2015). Vancomycin resistant enterococci (VRE) have been an important subject of investigation in several studies (Nilsson 2012; Arias et al. 2010; Yilema et al. 2017; Kee et al. 2012) due to their increased prevalence precipitated by their ability to transfer Vancomycin resistance to other bacterial species (including methicillin-resistant *Staphylococcus aureus*) (Faron et al., 2016). The treatment and eradication of VRE is of paramount importance because these organisms could further increase the number of

infections, the costs and the length of hospital stay (Butler et al., 2010), thus becoming a major concern in medical practice (Rezvani et al., 2016).

Data obtained from the Data Intensive Systems and Applications (DISA) for Microbiology Laboratory at the UTH's lacked susceptibility of enterococci to Vancomycin as it is not routinely performed hence necessitating the need for research in this microorganism, as there is no documented study so far which has been undertaken at UTH's with regards to the Vancomycin resistant enterococci occurrence, antimicrobial susceptibility profiles and the genes responsible for resistance.

1.3 Justification of study

Enterococci are part of the normal intestinal flora (Abebe et al., 2014; van den Braak et al. 1998; Miller et al. 2014; Cetinkaya et al. 2000). In hospital environments, enterococci resistant to Vancomycin are able to survive for a long time and can be transmitted from patient to patient through fomites and health workers - thus spreading resistance (Sood et al., 2008). Infection with VRE depends largely on the immune status of the patient and therefore the risk of transmission in hospitalized patients is heightened (Schmidt-Hieber et al., 2007).

The importance of detection of VRE at the UTH's Lusaka cannot be overemphasized as Vancomycin, a glycopeptide, is usually administered as a last line drug in management of infections with enterococci and other pathogens which may be resistant to other glycopeptides.

Retrospective data obtained from the DISA database at the UTH's microbiology lab showed that one case of Vancomycin resistant *E. faecalis* and one of *E. faecium* were obtained between January and December 2016. These cases were from Main Intensive Care Unit (ICU) and from Neonatal Intensive Care Unit. From the high cost ward, one specimen was identified to be resistant to Vancomycin in June 2017. From the pediatrics ward, Vancomycin resistant *E.*

faecalis was isolated in January 2016. The antimicrobial susceptibility to Vancomycin is not routinely performed hence the gap in the available information in relation to VRE.

The study has given an understanding of the prevalence of VRE and the resistant genes in these microorganisms. Information obtained from the study may be used to institute preventive measures to ensure minimization and/or stoppage of transmission of these microorganisms in hospitalized patients.

1.4 Research question

What is the occurrence of Vancomycin resistant *Enterococcus* and the genes responsible for the resistance in blood, urine and pus isolates from clinical specimens at University teaching Hospitals Lusaka, Zambia?

1.5 OBJECTIVES

1.5.1 Main Objective

To isolate and determine the occurrence of Vancomycin resistant *Enterococcus*, and to detect genes responsible for resistance from blood, urine and pus specimens received in the Microbiology Laboratory at the UTH's, Zambia.

1.5.2 Specific Objectives

a) To isolate and identify Vancomycin resistant *Enterococci* species from UTH's Lusaka.

b) To determine antimicrobial susceptibility patterns of *Enterococci* isolates to Vancomycin and other commonly used antimicrobials.

c) To determine the genes encoding for resistance in the isolated Vancomycin resistant enterococci species from UTH's Lusaka.

CHAPTER 2

LITERATURE REVIEW

2.1 History of Vancomycin-resistant Enterococci

Although they were initially thought of as merely harmless commensal microorganisms, enterococci have emerged as significant human pathogens and are currently the third most common nosocomial bloodstream pathogen in USA (Mascini & Bonten, 2016). These enterococci can acquire and confer antimicrobial resistance, and ultimately lead to Vancomycin resistant enterococci (Hollenbeck & Rice, 2012).

Vancomycin-resistant enterococci (VRE) were first encountered in clinical isolates in England and France in 1986, and 1987 in the United States of America (USA) (O'Driscoll and Crank, 2015; Cetinkaya et al. 2000). In Europe, the rise of VRE was principally in the community setting, due to transmission from animal food products to humans, whereas in the USA the predominance of VRE was in the hospital setting, probably due to the increased use of Vancomycin (Nilsson, 2012). To date, 54 different species and two subspecies of enterococci have been described, with the most clinically relevant species being *E. faecalis* and *E. faecium* (O'Driscoll and Crank, 2015). These microorgamisms, which are able to acquire resistance to the last line drug used in their management, Vancomycin, are responsible for up to 12% of cases of nosocomial infections worldwide and lead to life-threatening infections among immunocompromised patients (Ekuma et al., 2016; Abebe et al. 2014; Tavadze et al. 2014; Miller et al. 2014; Schmidt-Hieber et al. 2007). The most common clinical impact of VRE is

intestinal colonization, which does not result in symptoms, and may serve as a reservoir for transmission of VRE to other patients (Munita & Arias 2016; Linden 2002).

In studies conducted in Europe, Ireland had the highest rate of Vancomycin resistance among enterococcal bloodstream isolates from humans, with 43.1% of *E. faecium* isolated from blood being resistant to Vancomycin in 2013 (Ryan et al., 2015).

In studies carried out in Australia, VRE isolated showed considerable diversity in their phenotypes, genotypes, and geographic locations (Bell et al., 1998). All four combinations of genotype and species were found, with the commonest being *E. faecium vanB* (Eliopoulos and Gold, 2001). While the clinical profiles of VRE affected patients appeared to be similar to those recorded in the USA and elsewhere, the predominance of *E. faecium vanB* rather than *E. faecium vanA* genes suggested an epidemiologic difference from that in either Europe or the United States (Bell et al., 1998).

In other studies conducted in Korea in patients undergoing hemodialysis between August and September 2005, VRE was found to have a prevalence of 4.5% (Kee et al., 2012). In Iran, between February 2012 and February 2013, VRE prevalence was found to be 23.7% in a study conducted at the education hospital of Iran with the most isolated organisms being *E. faecalis* (Kafil and Asgharzadeh, 2014).

In Saudi Arabia, a study conducted during the period from September 2014 to November 2015 at King Khalid University Hospital showed that the prevalence of VRE was 17.1% (Alotaibi & Bukhari, 2015)The VRE in this study was mostly from blood samples.

A study conducted in Nigeria between February and August 2013 showed that the prevalence of VRE among patients admitted was 4.03% (Ekuma et al., 2016) with the isolated VRE being *E. faecalis.*

In South Africa, *E. faecium* carrying *vanB* gene on its plasmid was detected as early as 1993 in Cape Town, followed by four *E. faecalis* with *vanA* gene isolates from Bloemfontein in 1995 (Mahabeer et al., 2016). The first cases reported in 1997 had *E. faecalis* and *E. faecium* of the *vanA* phenotype isolated from blood culture and pus swab from patients previously treated with Vancomycin (Cetinkaya et al., 2000). A prevalence study which was done at four hospitals in Johannesburg that screened high-risk patients using rectal swabs found that almost 11% of those screened harboured VRE (Lochan et al., 2016).

From the literature review, it is evident that this organism has caused serious nosocomial infections from all over the world hence necessitating its importance in the hospital setup. The research findings from studies carried out in different parts of the world were significant enough to prompt and support similar research in Zambia about these microorganisms.

2.2 Clinical significance of Enterococci

Vancomycin resistant Enterococci (VRE) arise due to interaction of species without resistance genes with those that harbor VRE genes (Nasaj et al. 2016; Hollenbeck & Rice 2012; Dutka-Malen et al. 1995; Jovanović et al. 2015). Enterococci are mostly associated with urinary tract and wound infections, most often caused by *E. faecalis* species (Miller et al., 2004). In infected wounds like diabetic foot wounds, *E. faecalis* is commonly isolated as part of polymicrobial flora (Edmonds, 2009). It was very early recognized that enterococci were able to cause bacteremia in about 5-20% of endocarditis cases (Hollenbeck and Rice, 2012). In a point-prevalence study on nosocomial urinary tract infection (UTI) in 228 European hospitals during 1999, enterococci were the second most commonly isolated microorganisms (15.8%) (Ott et al. 2013). Other infections included bacteremia, surgical site and intra-abdominal infections, and more rarely CNS, neonatal and pulmonary infections (Sartelli et al., 2017). Later on, it was

observed that some enterococcal infections would resolve without specific therapy (Linden, 2002).

A study by Miller et al., (2014) showed that the underlying condition of the patient seemed to play an important role in the outcome of enterococcal infections. Patients with haematological malignancies, a history of transplantation or severe burns had also been observed to be more readily colonized with multidrug-resistant strains and had been more likely to experience bacteremia and subsequent serious outcome than non-immunocompromised patients according to Bodro et al., (2013).

2.3 General characteristics of Enterococci

Enterococci are gram-positive, catalase negative, pyrrolidonyl peptidase (PYRase) positive, facultative anaerobic microorganisms (Fisher and Phillips, 2009). They occur singly, in pairs, or as short chains (Ramsey et al., 2014). More than 20 enterococcal species are now recognized (Byappanahalli et al., 2012). They are ubiquitous and can be found free-living in the soil, on plants, or in dairy products (Van Tyne and Gilmore, 2014). They can survive hostile conditions and a range of environmental stresses, including extreme temperature (5-65ºC), as well as high (6.5%) Sodium Chloride (NaCl) concentration (Arias and Murray, 2012; Ramsey et al. 2014) and pH 4.5-10.0 (Rezvani et al., 2016; Ramsey et al. 2014; Fisher & Phillips 2009). These microorganisms can also grow in the presence of a high concentration of bile (Gearhart et al. 2005; Suwantarat et al. 2014).

2.4 Non-Glycopeptide antimicrobial resistance in Enterococci

Enterococci have emerged as important nosocomial pathogens in the past decade (Noskin, 1997). This importance is attributed primarily to the high degree of antimicrobial resistance that is exhibited by most enterococci (Miller et al., 2014). The species responsible for most

infections in the community, long-term care, and hospital settings is *E. faecalis* (O'Driscoll and Crank, 2015). Overall, *E. faecium*, intrinsically more resistant than *E. faecalis*, accounts for approximately 10% of enterococcal infections (Huycke et al., 1998). Enterococci are associated with a variety of different clinical infections, such as urinary tract infections, intra- abdominal, pelvic, and soft tissue infections, bacteremia and endocarditis (Cetinkaya et al., 2000). Some uncommon infections, such as meningitis, haematogenous osteomyelitis, septic arthritis and pneumonia, have also been diagnosed in clinical settings (Bell et al., 1998).

Enterococci have intrinsic resistance to a range of antibiotics (Kuriyama et al., 2003). Among the enterococci, *E. faecalis* is intrinsically resistant to macrolides, lincosamides, and streptogramin antibiotics (Hollenbeck and Rice, 2012). Some species of Enterococci can continue to synthezise their cell wall in the presence of β-lactam antibiotics due to the presence of penicillin-binding proteins (pbp), making them intrinsically resistant to Penicillins, Cephalosporins, and Carbapenems (Munita and Arias, 2016). In the early 1940s, it was observed that penicillin treatment for enterococcal endocarditis produced worse outcomes than penicillin treatment for streptococcal endocarditis (Nigo et al., 2014), thus agreeing with the observation that enterococci were considerably less susceptible to Penicillins than streptococci (Murray, 2000).

E. faecium's possession of low-affinity penicillin-binding proteins makes this enterococci species highly resistant to Penicillin and Ampicillin (Munita and Arias, 2016). Single mutations can lead to high-level resistance to streptomycin and increased intrinsic resistance to the Penicillins (Fair and Tor, 2014)

Some species of enterococci can acquire resistance to certain antimicrobials through horizontal gene transfer (HGT) or conjugation, transformation, or transduction between and among bacteria (Huddleston, 2014). Genes can also be exchanged through plasmids, transposons, or

bacteriophages (van Schaik and Willems, 2010). Evidence of gene exchange has been found between Enterococci and Staphylococci, Streptococci, Listeria, *E. coli*, *Campylobacter coli* and other gram-positive bacteria (Verraes et al., 2013). The close contact in the gastrointestinal tract biofilm of enterococci with gram-negative and other gram- positive bacteria allows for the exchange of genes by conjugation (Beceiro et al., 2013). Rapid horizontal gene transfer occurs through a pheromone-induced conjugation system (Huddleston, 2014). Plasmid-free recipient cells secrete a specific sex pheromone peptide to initiate plasmid transference with the plasmid-sharing bacteria (Grohmann et al., 2003). Antibiotic resistance, as well as virulence factors, can be exchanged on transposons via plasmids through this process (van Schaik and Willems, 2010).

2.5 Vancomycin Resistance in Enterococci

Vancomycin is a glycopeptide antimicrobial drug which was introduced in the 1950s and is produced by soil bacteria *Streptomyces orientalis* (Levine, 2006). It is active against most gram-positive bacteria, whereas the majority of gram negatives are resistant (Rezvani et al., 2016). It is used primarily to treat drug-resistant bacteria when other antibiotics fail (Ventola, 2015).

Vancomycin was first clinically used as an antimicrobial to treat enterococci infections in 1972 (Werner, 2013). The rampant use of Vancomycin most often led to the promotion of colonization by VRE (Ryan et al., 2015) and only 15 years later, VRE was isolated in the United Kingdom and the United States (Corso et al., 2007). High-level Vancomycin resistance in enterococci (due to Van A or Van B genes) is associated with the acquisition of ~10 kb of DNA encoding polypeptides (Courvalin, 2006). The use of essential drugs such as third-generation cephalosporins, clindamycin, imipenem, and metronidazole (Nasaj et al., 2016) which have potent activity against anaerobes, lead to VRE colonization of gastrointestinal tract

(GIT) by competitive eradication of sensitive species (Rice, 2001). This colonization often leads to cross-infection, dissemination, and endogenous infection (Cetinkaya et al., 2000) by VRE. VRE have caused hospital outbreaks worldwide, and these have been on the rise in recent years mainly due to widespread abuse and misuse of antibiotics (Biswas et al., 2016). Cases of human infections associated with VRE were detected in the late 1980s in Europe and in the United States. Since then, VRE isolation has been reported from different parts of the world (Olawale et al., 2011).

VRE infections have led to an increase in clinical treatment failure and mortality when compared to Vancomycin-susceptible enterococci (VSE) infections (Werner, 2013). Mortality occurs in 75% of those with VRE bacteremia infections but in only 45% of those with VSE infections (Courvalin, 2006).

Although seven known genes (*vanA*-*vanG*) confer Vancomycin resistance, the three most prevalent genes are *van A, van B,* and *van C* (McKessar et al., 2000). These genes alter the binding target for Vancomycin in resistant enterococci through the repression and activation of certain bacterial cell wall precursors (Courvalin, 2006). The *vanA* gene confers high-level resistance to Vancomycin and teicoplanin; however, *vanB* confers moderate to high-level resistance to only Vancomycin (Arthur and Quintiliani, 2001). Both *vanA* and *vanB* are associated with acquired resistance to Vancomycin, while *vanC* is an intrinsic resistance gene that is most commonly found in *E. gallinarum, E. casseliflavus,* and *E. Flavescens* (Abebe et al., 2014). Since *vanC* is chromosomally located, this gene is non-transferable; however, *vanA* and *vanB* genes may be transferred to other gram-positive bacteria on plasmids during horizontal gene transfer (Huddleston, 2014).

Due to resistance genes, the composition of the VRE's cell wall is altered to resist Vancomycin (Ryan et al., 2015). The peptidoglycan precursor D-Ala-D-Ala, which is

Vancomycin-susceptible, is changed to D-Ala-D-Lactate (D-Lac), which has 1,000 times less affinity for Vancomycin (Xie et al., 2011). Another precursor, D-Ala-D-Ser (D-Ser), has a 7-fold decrease in affinity for Vancomycin (Miller et al., 2014). These two peptidoglycan precursors essentially remove the susceptible target of Vancomycin (Munita and Arias, 2016). Two genes, van S/van R, are involved in the repression of the binding site of Vancomycin (Miller et al., 2014). With the presence of Vancomycin, the vanS sensor kinase is activated, initiating the production of either the D-Lac or D-Ser peptidoglycan precursor and the repression of D-Ala-D-Ala (Willems et al., 2005).

Vancomycin has been used successfully to manage infections caused by penicillin-resistant strains of enterococci alone or in combination with aminoglycosides (Rice, 2001) and thus acquired Vancomycin resistance by this organism greatly reduces the treatment options (Willems et al., 2005). The emergence of enterococci with high-level resistance to glycopeptides has further narrowed therapeutic options available (Lochan et al., 2016). This problem is further made intricate by the fact that resistance genes can potentially be transferred to other pathogenic organisms such as *Staphylococcus aureus* (Biendo et al., 2010) and this has been confirmed as clinical cases of infection with a Vancomycin- resistant *S. aureus* strain carrying a *vanA* gene that originated in VRE have been described (Armeanu et al., 2005).

In management of VRE bacteremia, chloramphenicol and tetracycline has been used successfully but the development of resistance to these drugs during treatment, however, has also been documented (Biendo et al., 2010).

Another drug in use is Nitrofurantoin which is effective against many strains of VRE, but it's use is limited to urinary tract infections (Lochan et al., 2016).

Fluoroquinolones like Ciprofloxacin have been found to be bacteriostatic for enterococci and, in combination with ampicillin or gentamicin, are *bactericidal* in vitro (Kuriyama et al., 2003). The newer quinolones, such as moxifloxacin, clinafloxacin, and sparfloxacin, possess better activity than ciprofloxacin against enterococci and have offered better outcomes in the management of VRE infections (Mascini and Bonten,2005). New antibiotics, such as linezolid, daptomycin, and quinupristin/dalfopristin are active against VRE (Linden, 2002). However, they are expensive, and resistant VRE strains to these antibiotics have already been reported (Butler et al., 2010).

2.6 Habitat, distribution, and colonization of VRE

Enterococci makeup part of the normal flora of the human gastrointestinal tract and are also found in other anatomical sites including the vagina and oral cavity (Fisher and Phillips, 2009) and although the oral cavity and vaginal tract can become colonized, enterococci are recovered from these sites in fewer than 20% of cases (Linden, 2002).

Of the 14 or more enterococcal species, only *E. faecalis* and *E. faecium* commonly colonize and infect humans in detectable numbers (Arias et al., 2010) and are the leading cause of enterococcal infections.

E. faecalis is isolated from approximately 80% of human infections, while 20% from mostly *E. faecium* (Nigo et al., 2014 ; Huycke et al. 1998). Infections due to other enterococcal species are rare (Bell et al., 1998). Infections due to these organisms occur mostly in patients with immunodeficiencies and those with a breach in normal defensive barriers, which may either be due to an underlying illness or immunosuppressive therapy or intravascular lines and urinary catheters, respectively (Mascini et al., 2005).

The risk of VRE colonization depends first on being exposed to VRE, and second on being a 'susceptible' host (Ryan et al., 2015). With regard to being exposed to VRE, the most significant factors are proximity to other patients who are colonized with VRE-especially those with diarrhoea and carriers of VRE (e.g. as a result of antimicrobial therapy)-and length of hospital stay (Rice, 2001). When the proportion of patients colonized with VRE is low, those most at risk of becoming colonized include immunocompromised patients and those receiving prolonged courses of antimicrobial therapy (O'Driscoll and Crank, 2015). Even healthcare workers are at risk of becoming colonized with VRE, leading to spread of these microorganisms (Ryan et al., 2015). This may occur if these workers unintentionally act as vectors in the nosocomial spread of VRE, through acts such as touching a VRE-colonized patient's intact skin or resting a hand on a bed rail in the patient's room (Duckro et al. 2005).

The colonization of the skin may increase the risk of intravascular catheter-related sepsis (Ryan et al., 2015). Patients colonized with VRE have a high prevalence of skin colonization (Karahan et al., 2006) while liver transplant recipients have a high incidence of biliary VRE colonization (Bodro et al., 2013). The most common source of VRE bacteremia in these recipients is the abdomen, typically the peritoneal space and biliary tract; precipitating co-factors in such cases include biliary leaks, stenosis or obstruction; perforated viscus; and stenosis or thrombosis of the hepatic artery (Gearhart et al., 2005).

High-density stool colonization is associated with contamination of the environment with VRE when patients have faecal incontinence (Arias and Murray, 2012) and diarrhoea (Miller et al., 2004). Patients in long-term care facilities have been shown to be a major reservoir of VRE (Ryan et al., 2015). Other patients like those with solid (especially abdominal) organ transplant beneficiaries are at increased risk of VRE colonization (Kuriyama et al., 2003). Among hemodialysis patients, a history of injection drug use may be a risk factor for colonization (Iwen et al.,1997).

Antibiotics may promote colonization and infection with VRE by at least two mechanisms (Fair and Tor, 2014). First, many broad-spectrum antibiotics have little anti-enterococcal activity, and administration commonly leads to overgrowth of susceptible (or resistant) enterococci (Munita and Arias, 2016). Second, most antibiotics significantly reduce the resistance of the intestinal tract to colonization by exogenous organisms (Arias and Murray, 2012). Colonization resistance results primarily from the "limiting action" of the normal anaerobic flora, and to a lesser extent from an intact mucosa, gastric acid secretion, intestinal motility, and intestinal-associated immunity (Cetinkaya et al., 2000). Antibiotic-induced alterations in the protective flora of the intestine provide large footholds for colonization with exogenous pathogens such as VRE (Miller et al., 2014).

2.7 Pathogenicity and mechanisms of infection of VRE

Enterococci are commensal organisms well suited to survival in intestinal and vaginal tracts and in the oral cavity (Nilsson, 2012). Portals of entry for VRE include the urinary tract, intra-abdominal (e.g., the gastrointestinal tract) sources, wounds (e.g., surgical wounds) and intravascular catheters (Linden, 2002).

Several factors contribute to the disease causing power (virulence) of Enterococci (Jett et al., 1994). They have ability to colonize the human intestinal tract, adhere to multiple extracellular matrix proteins, and to urinary tract and oral cavity epithelia, and human embryo kidney cells (Faron et al., 2016). They are invasive and can cause abscess formation, resistance and modulation of host defense mechanisms, secretion of cytolysins and other toxic products and production of plasmid-encoded pheromones (Camargo et al., 2008).

Infections due to Enterococci can present both endogenously (arising from within patient) and exogenously (due to external sources such as fomites) (Fisher and Phillips, 2009; Byappanahalli et al. 2012). Enterococci can translocate from the intestinal tract to the

bloodstream (Ryan et al., 2015), resulting in an endogenous infection initiating in the lymph nodes (Camargo et al., 2008). Also, exposure to contaminated objects, hands, food, or water may give rise to an exogenous Enterococci infection (O'Driscoll and Crank, 2015). This can be attributed to the fact that VRE are capable of prolonged survival in the healthcare environment, and can be found on fomites such as monitoring devices like call bells, electrocardiography monitors and pulse oximeters (Ott and Wirick, 2008). This can lead to nosocomial infections including urinary tract infections, bacteremia, endocarditis, CNS infections, and surgical wound infections (Biendo et al., 2010).

The natural ability of Enterococci to readily acquire, accumulate, and share extrachromosomal elements encoding virulence traits or antibiotic resistance explains their increasing importance as nosocomial pathogens which lead to VRE (O'Driscoll and Crank, 2015).

2.8 Risk factors and transmission of VRE

Enterococci can survive in the environment (Miller et al., 2014) on fomites, leading to their transmission through direct contact by health care workers (Courvalin, 2006).

Some risk factors for bacteremia with VRE include hemodialysis, surgery, the severity of illness, antimicrobial administration, indwelling bladder catheters, and mucositis (Zaas et al., 2002). According to Rice (2001), use of antimicrobial agents with anti-anaerobic activity (metronidazole, clindamycin, piperacillin/tazobactam, ampicillin/sulbactam, and Vancomycin) were also associated with high-density VRE colonization, whereas antimicrobial agents with minimal anti-anaerobic activity (e.g., fluoroquinolone and trimoxazoles) were not. This agreed with other studies (Tavadze et al. 2014; Abebe et al. 2014; Isikgoz Tasbakan et al. 2013; Zaas et al. 2002; Bodro et al. 2013; Kee et al. 2012) that suggested that administration of antimicrobial agents with anti-anaerobic activity is a risk factor for VRE infection in colonized patients.

Other risk factors such as patients with fecal incontinence, discharging wounds, and patients incapable of maintaining own personal hygiene may lead to spread of VRE in the hospital environment (Abebe et al., 2014).

2.9 Treatment and control of VRE

Most infections due to VRE can be managed with antibiotics other than Vancomycin, such as cephalexin, clindamycin and metronidazole (Lai, 1996; Ott and Wirick, 2008). Some of these infections include UTIs, intra-abdominal and uncomplicated wound infections (Huycke et al., 1998). In the clinical setup, combination therapy with a cell wall active agent and a synergistic aminoglycoside should be considered when managing enterococcal infections in debilitated patients and those with evidence of sepsis, endocarditis, meningitis, or joint infections (Biendo et al., 2010).

For VRE strains resistant to ampicillin because of beta-lactamase production, a combination of ampicillin and sulbactam may be employed (Arias et al., 2010; Ceci et al. 2015). Other drugs like Linezolid, daptomycin, and tigecycline including combination therapy with cell wall–active agents (e.g., ampicillin) and an aminoglycoside (eg, gentamicin) may also be used (Biendo et al., 2010). Combination of various beta-lactam antibiotics with daptomycin may result in synergy against VRE (Arias et al., 2010).For gentamicin-resistant VRE strains, the only alternative is streptomycin, as tobramycin and amikacin are not effective (Fair and Tor, 2014 ; Moemen et al. 2015; Salem-Bekhit 2011).

Infections due to *E. faecalis* can also be managed by prolonged therapy with high doses of a combination of ampicillin and imipenem-cilastatin, or ampicillin and ceftriaxone (Nigo et al., 2014). For *E. faecium* infection, either linezolid or daptomycin may be effective, including quinupristin- dalfopristin or tigecycline (O'Driscoll and Crank, 2015).

VRE infections due to isolates susceptible to penicillin or ampicillin (MICs of 0.5-2 μg/ml) may be treated with high doses of these agents (Rice, 2001). Doxycycline, chloramphenicol, and rifampin in various combinations can also be used (Linden, 2002).

Control methods for VRE include routine screening for Vancomycin resistance among clinical isolates (Beceiro et al., 2013), active surveillance in intensive care units (Mukhopadhyay 2018), contact isolation to minimize person-to-person transmission (Mutters et al. 2013), rigorous decontamination of patient-contact areas (Duckro et al. 2005) and judicious restriction of Vancomycin and other broad-spectrum antibiotics (Miller et al., 2014).

There is a continued need for the development of new antimicrobial agents for treating VRE infection, as well as a regimen that would eradicate VRE colonization (without selection of further antimicrobial resistance), and potentially a role for a regimen for suppressing VRE colonization during periods of high risk for enterococcal infection (O'Driscoll and Crank,2015).

These measures to limit VRE spread, however, have had a few challenges (Arias et al., 2010). Firstly not all hospitals are willing to perform active surveillance (Ekuma et al. 2016). Secondly, more patients are typically colonized with VRE (3% to 47%) than are infected hence passive surveillance by routine cultures allows colonized inpatients to go unidentified and serve as point sources for continued spread of VRE (Nigo et al., 2014).

It has been noted that even if all colonized inpatients were successfully identified, VRE may be spread by health-care workers through either inadequate hand washing or contact with items such as bed rails, sinks, faucets, and doorknobs (Kampf et al., 2009).

A study undertaken by Faron et al., (2016) suggested that the endemic prevalence of VRE may be reduced by decreasing the duration of VRE colonization, limiting hospital acquisition of VRE and improving compliance with hand hygiene. Further, according to a study by Kampf et

al., 2009, increasing the frequency of hand washing was associated with a decrease in nosocomial VRE infections.

It was suggested by Abdel Rahman et al., (2010) that hospitals detecting their first cases of VRE colonization should particularly be aggressive in implementing appropriate infection control measures. According to Mukhopadhyay (2018) the challenges to implementation were identified to be due to patient inconvenience, increased workload for healthcare workers and increased costs.

From this literature review, there is clear evidence that there is inadequate documented information on the epidemiology of Vancomycin-resistant enterococci species in Zambia and particularly at the University Teaching Hospitals Lusaka. To date, no study has been undertaken to ascertain which species are predominant at this hospital.

CHAPTER 3

STUDY DESIGN AND METHODOLOGY

3.1 Study design

This was a Laboratory-based cross-sectional study.

3.2 Study Site

The study was carried out in the Microbiology Laboratory at the University Teaching Hospitals Lusaka Zambia. The Laboratory was chosen due to its central location in Lusaka and the high level of clinical specimens it receives. This Laboratory also serves as a reference Laboratory for various parts of the city and the country as a whole. The Vancomycin resistant Enterococci isolated from specimens were processed at this laboratory for confirmation as well as antibiotic disc sensitivity and detection of VRE genes by PCR.

3.3 Sampling Frame

The study used different clinical samples (blood, urine, and pus) submitted to the Bacteriology Laboratory of the Department of Pathology and Microbiology at the University Teaching Hospitals during the study period from July 2017 to August 2017. An isolate from the archive which was from a blood specimen received from Neonatal intensive care unit was included in the study for confirmation of our isolates. Only urine, pus and blood specimens received during the study period were included in the study.

3.4 Sample Size Estimation

The prevalence of VRE in Zambia is not known and thus the prevalence which was used was 50%.

$$N = \frac{pqz^2}{d^2}$$

$$= \frac{0.50(1-0.50)(1.96)^2}{(0.05)^2}$$

= 384 Total Sample size

n= minimum sample size required

p = proportion of the target population estimated to have a particular problem

q = 1 – p

z = level of precision (1.96) which corresponds to 95 % confidence level

d = degree of accuracy desired set at 0.05

3.5 Sampling method

Stratified random sampling method was used.

3.6 Specimen sampling

Specimens that were used in this study were those submitted to UTH's Microbiology Laboratory by patients from different wards at UTH's. These included 621 urine, 117 pus and 79 blood specimens.

Microbiological examination of specimens was done from the University Teaching Hospitals Bacteriology Laboratory while PCR was carried out from the Centre for Zoonosis Control, University of Zambia, School of Veterinary Medicine, with the help of Laboratory specialists in those departments.

3.7 Laboratory procedures for detection of VRE

3.7.1 Culture and isolation of Enterococci

Initial screening was carried out by inoculating pus and blood samples on blood agar (Oxoid Altrincham, UK), while urine samples were inoculated on Cysteine Lactose Electrolyte Deficient (CLED) agar (Oxoid Altrincham, UK) and incubated at 37ºC for 24 hours. CLED is differential media which allows growth of Enterococci as small yellow colonies, about 0.5mm in diameter. After 24 hours, growth was observed and gram stain was carried out to identify gram positive organisms for further testing.

All gram positive cultures from urine, pus and blood specimens were further inoculated on blood agar for making pure cultures and then incubated at 37ºC for 24 hours.

Blood agar is a general purpose enriched medium used to grow fastidious organisms. Bile esculin agar (BEA) (Oxoid Altrincham, UK) is a differential and selective medium and is mainly used to differentiate group D Streptococci and *Enterococci* based on the organism's potential to hydrolyze esculin. The BEA comprises of oxygall (bile salts, first selective ingredients) and azide (second selective ingredients), the former inhibits the growth of gram positive organisms and latter inhibits the growth of gram negative organisms. BEA also comprises of nutrients ferric citrate and esculin, where esculin is a differential ingredient and is also a fluorescent compound, upon hydrolyzation the fluorescence is lost. BEA is used for bile esculin test which is based on the hydrolysis of esculin into esculetin (6, 7-dihydroxy-coumarin) and glucose by a micro-organism that produce an enzyme esculinase, which in this case is *Enterococcus*.

In this study, gram positive colonies were collected from blood, urine and pus cultures and made into pure cultures by inoculating onto blood agar and incubated at 37ºC for 24 hours. After 24 hours, pure colonies were collected and inoculated on BEA agar and incubated for a

further 24 hours. Growth of black colonies on the BEA agar were presumed to be enterococci. All pure cultures presumed to be enterococci inoculated on Brilliance VRE agar (Oxoid Altrincham UK) plates and incubated at 37ºC for 24 hours to obtain VRE.

3.7.2 Confirmation of VRE

Brilliance VRE Agar is a chromogenic screening plate for the detection of Vancomycin Resistant Enterococci (VRE). The medium provides presumptive identification of *Enterococcus faecium* and *Enterococcus faecalis* in 24 hours when incubated at 37ºC.

All growth on Brilliance VRE agar were recorded and assumed to be specific for VRE. Original pure cultures for those organisms that came out positive for VRE were then collected and inoculated on Müller-Hinton agar to obtain pure cultures for use in the antimicrobial susceptibility testing to Vancomycin and other antibiotics. These plates were then incubated at 37ºC for 24 hours.

Table 1. Primers sequences used in this study

Gene	Primer	Sequence	Expected amplicon size	Reference
Van A	*Van A–F*	CATGAATAGAATAAAAGTTGCAATA	1030	(Clark et al., 1993)
	Van A–R	CCCCTTTAACGCTAATACGATCAA		
Van B	*Van B –F*	GTCACAAACCGGAGGCGAGGA	433	(Clark et al., 1993)
	Van B –R	CCGCCATCCTCCTGCAAAAAA		

Primer sequences according to a study done by Clark et al., 1993)

For genetic detection, DNA extraction for enterococci was done by first culturing VRE isolates in Brain Heart Infusion (BHI) broth (Oxoid Altrincham UK) at 37ºC for 24 hours. After incubation, 1ml of bacterial suspension was centrifuged at *5800 x g* for 5 minutes. After

centrifuging, the supernatant was discarded. The remaining cell pellet was washed with 500µl of normal saline, centrifuged at *13000 x g* for 5 minutes and the supernatant discarded. After washing with normal saline, 500µl of TE buffer (pH 8.0) was added to the cell pellet and then heat treated until boiling, then immediately transferred to ice for 10 minutes. A further centrifugation at *13000 x g* for 5 minutes was carried out to remove any cell debris and the supernatant was transferred into a new microfuge tube and stored at -20ºC until use (Yuan et al., 2012). PCR was performed to detect the glycopeptide resistance genes *vanA* and *vanB* in the Enterococci isolates using specific primers (Table 1).

The amplification reactions were prepared in a final volume of 25 µl, as follows: 12.5 µl of amplification mix (22 mM Tris/HCl, pH 8.4; 55 mM KCl; 1.65 mM $MgCl_2$; 25 µM each dNTP; 0.6 U recombinant Taq DNA polymerase/ml), 50 ng/µl of bacterial DNA, 5 µl of H_2O and 2.5 µl of primer solution (10 pg/ µl). A thermocycler was programmed to run for 30 cycles with the following parameters: denaturing at 94°C for 3min, annealing at 55°C for 45s and extension at 72°C for 1min, with a final extension at 72°C for 2min. The PCR products were analyzed via electrophoresis in 1% agarose gels (Agarose LE, Promega) using a 100 bp DNA ladder (Gibco/BRL Life Technologies, Breda, The Netherlands). *E. faecium* strain ATCC 51559 (*vanA*+) and *E. faecalis* strain ATCC 51299 (*vanB*+) were used as controls in the PCR experiments (Ochoa et al., 2013).

3.7.3 Antimicrobial susceptibility testing

After growth, colonies were collected and standardized to 0.5 marcfarlands. These were then were then put into the VITEK 2 Compact automated system for identification and resistance profiling using Gram positive (GP) 67 Identification and GP 67 Sensitivity Vitek cards. The antibiotics used in this study included Ampicillin, Benzylpenicillin, Gentamicin High Level, Streptomycin High Level,Ciprofloxacin, Levofloxacin, Moxifloxacin, Ertythromycin, Clindamycin, Quinipristin/Dalfopristin, Vancomycin, Tetracycline and Tigecycline.

The VITEK 2 compact is an automated microbial identification system that provides highly accurate and reproducible results as shown in multiple independent studies (Liassine et al., 1998, Hegstad et al., 2014). With its colorimetric reagent cards, and associated hardware and software advances, the VITEK 2 compact offers a state-of-the-art technology platform for phenotypic identification methods (Kassim et al., 2016).

3.8 Ethical considerations and permissions

The study was Laboratory-based, and there was no direct contact with patients. Study numbers were used to identify isolates instead of patients' names or hospital numbers. Permission was obtained from the Management at University Teaching Hospitals Lusaka. The study proposal was submitted to and approved by the University of Zambia Biomedical and Research Ethics Committee (UNZABREC).

3.9 Data analysis

The Study data was descriptive in nature. Data analysis was performed using Microsoft Excel 2007 and Statistical package for social sciences (SPSS) version 20 for cross tabulation.

Results from the phenotypic detection of VRE as outlined in objective 1 were used to determine the prevalence of VRE. Special media called VRE Brilliance chromogenic media was used to presumptively determine the isolates of Enterococci that were Vancomycin resistant.

For the second objective, VITEK 2 Compact automated system was employed to determine antimicrobial susceptibility profiles of the Vancomycin resistant organisms to Vancomycin and other antimicrobials. The VITEK 2 Compact system was able to use the drugs in Table 6 for analysis. PCR was employed to ascertain which of the Van genes were being carried in the VRE organisms that were isolated at the Laboratory during the study.

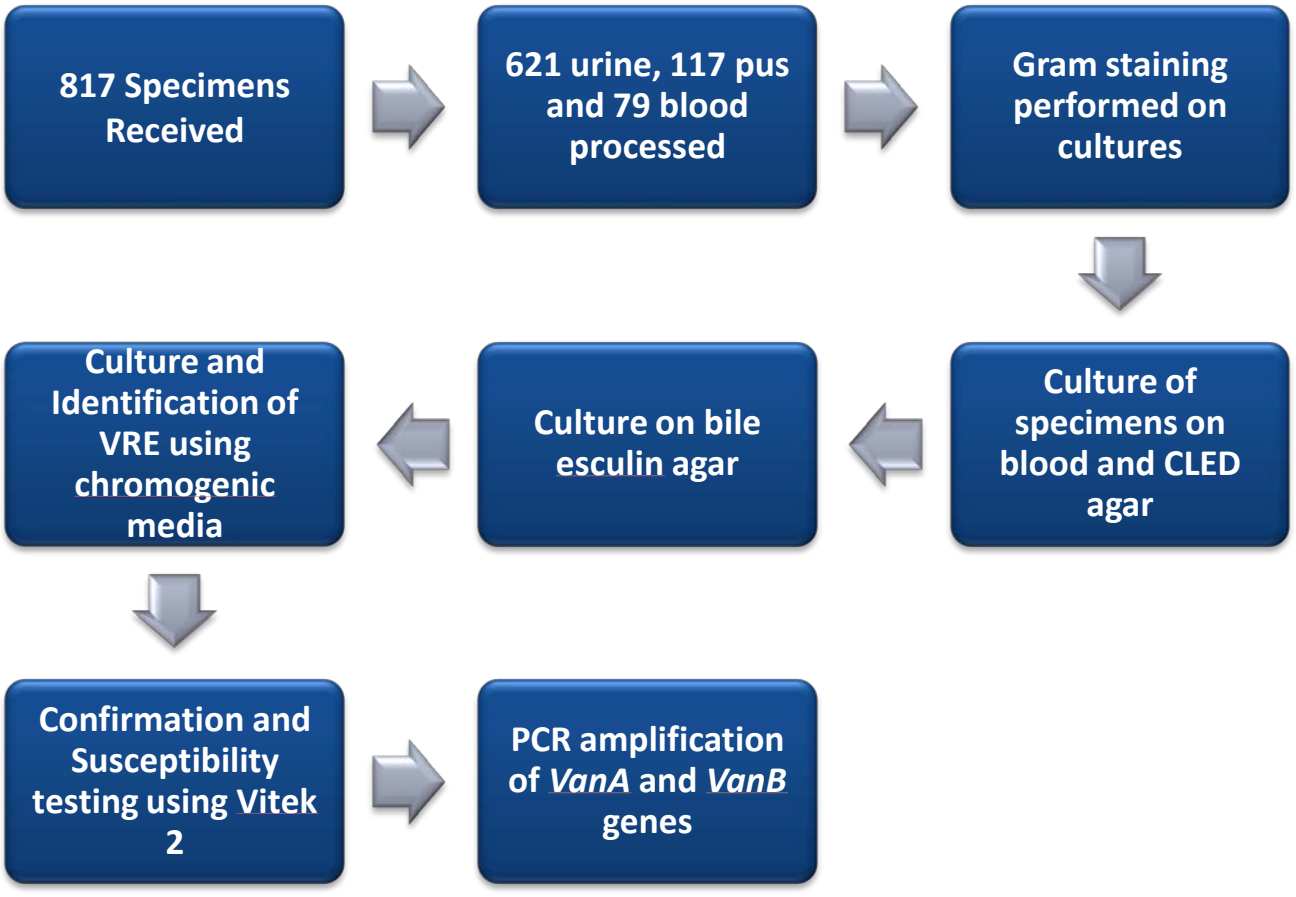


Figure 1. Work flow showing how results were obtained for VRE genes in this study.

CHAPTER 4

RESULTS

4.1 Demographics

This study had a total of 817 specimens which were processed through the Microbiology Laboratory between July and August 2017. Of these specimens, 25 were found positive for VRE. Of these, 22 were *E. faecalis* while 3 were *E. faecium*. In this study, 18 females and 7 males were affected. *E. faecium* only affected females. This is shown in Table 2.

Table 2. Gender vs organism isolated

		Organism Isolated		Total
		E. faecalis	***E. faecium***	
GENDER	**Female**	15	3	**18**
	Male	7	0	**7**
	Total	**22**	**3**	**25**

Different age groups were affected by the VRE organisms in different ways in this study. The age groups between 28 to 46 years old were most affected compared to those above 46 years. This is shown in Table 3.

Table 3. Ages of patient's vs organism isolated in this study.

	Years	Organism Isolated		Total
		E. faecalis	***E. faecium***	
AGE CATEGORY	**<28**	6	3	**9**
	29 to <46	10	0	**10**
	47 to <67	6	0	**6**
	Total	**22**	**3**	**25**

Specimens from different wards showed different isolation rates of Enterococci in this study. It was observed that from inpatient and outpatient departments, the specimens from general ward had more *E. faecalis* than *E. faecium* being isolated as compared to other wards. Only *E. faecalis* was isolated in the high cost and paediatrics wards. A total of 25 VRE were isolated. This is shown in Table 4.

Table 4. Wards vs Organism isolated in our study.

		Organism Isolated		Total
		E. faecalis	***E. faecium***	
WARD	**General**	10	3	**13**
	High cost	7	0	**7**
	Paediatrics	5	0	**5**
	Total	**22**	**3**	**25**

A total of 817 specimens were received during our study. These included 621 Urine, 117 Pus and 79 Blood specimens. From these, 25 VRE were isolated. In our study, most organisms were isolated from urine, followed by pus and finally blood. *E. faecalis* was more prevalent than *E. faecium.* These are summarized in Table 5.

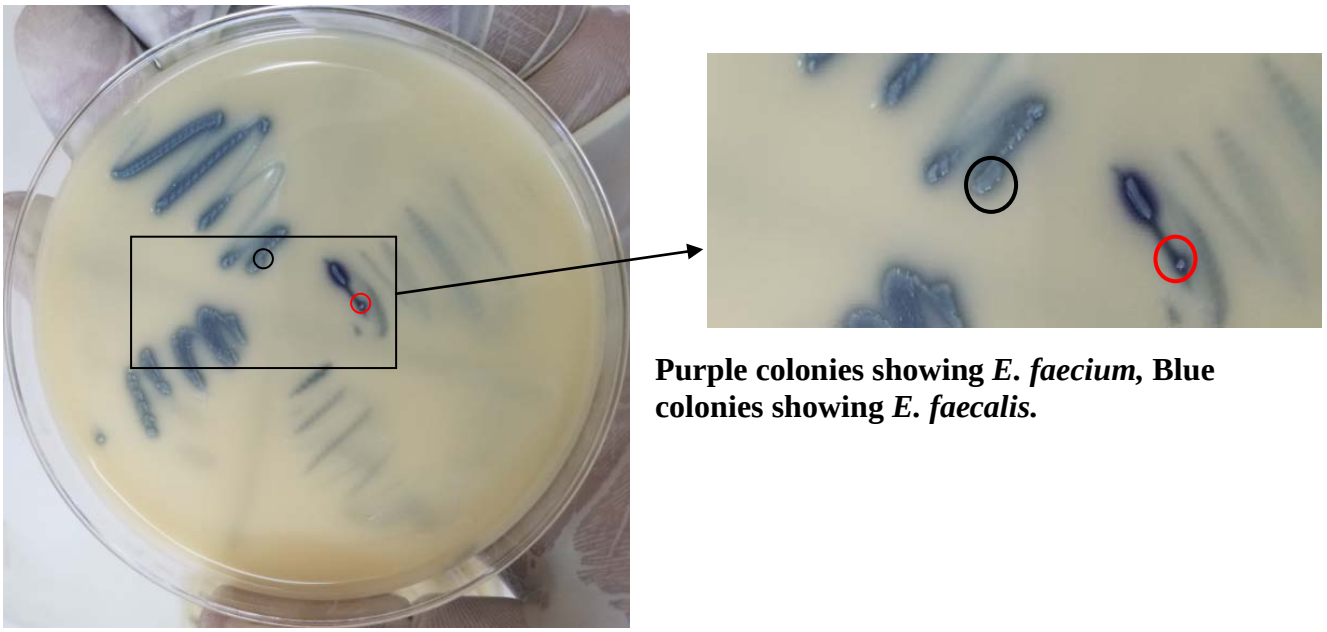
Table 5. Specimens vs Organism isolated in our study.

		Organism Isolated		**Total**
		E. faecalis	***E. faecium***	
SPECIMEN	**Urine**	13	3	**16**
	Pus	6	0	**6**
	Blood	3	0	**3**
	Total	**22**	**3**	**25**

4.2 Culture results

A total of 817 specimens were processed through the Microbiology Laboratory, with 25 Specimens being positive for VRE on culture. In this study, Brilliance VRE chromogenic media was used to accurately identify Vancomycin resistant *E. faecalis* and *E. faecium.*

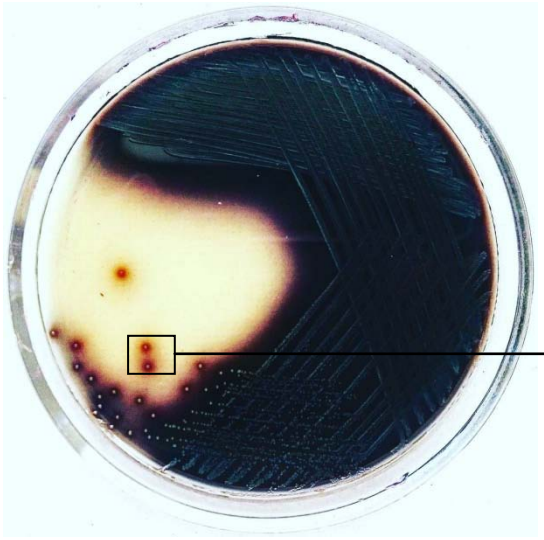
The results are shown in Figure 2.



Purple colonies showing *E. faecium*, Blue colonies showing *E. faecalis.*

Figure 2. Brilliance VRE Chromogenic media results- Brilliance VRE Chromogenic media showing growth of *E. faecalis* (black circle) and *E. faecium* (red circle) after 24 hours of incubation.

Bile esculin Agar was also used to presumptively identify Enterococci. Results show growth of black colonies. This is shown in Figure 3.



Black colonies showing enterococci

Figure 3. Bile esculin agar results-Bile esculin agar showing growth of black colonies of *Enterococci* after 24 hours of incubation.

Figure 4. Blood agar results- Blood agar was used to enrich the specimens that tested positive for enterococci. All positive isolates were cultured on blood agar. This is growth after 24 hours of incubation of plates.

4.3 Genotypic characteristics

A total of 817 specimens were processed and out of these 25 (3%) samples were confirmed to be VRE after being cultured on Brilliance VRE chromogenic media and by use of Vitek 2 compact. All the 25 (100%) VRE organisms were positive for *VanB* gene. None were positive for *VanA* gene. This is show in Figures 5 and 6.

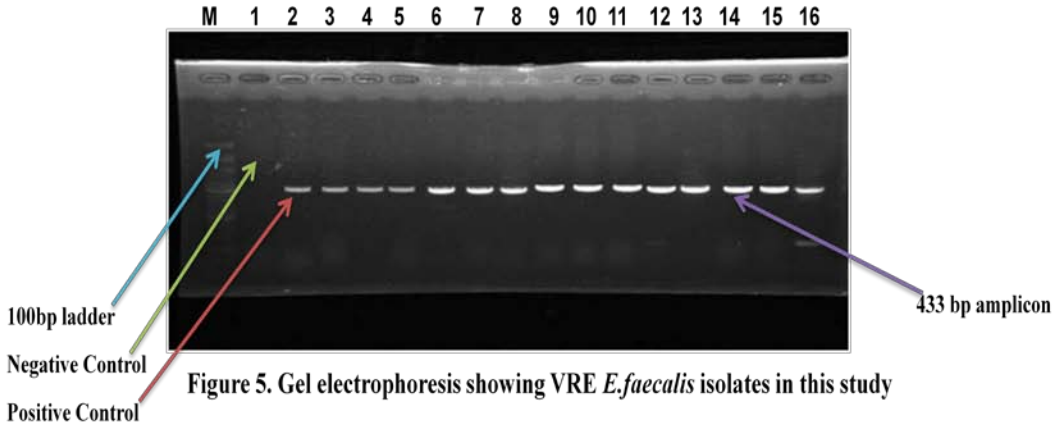


Figure 5. Gel electrophoresis showing VRE *E.faecalis* isolates in this study

Figure 5. Results of the PCR for *E. faecalis* on gel electrophoresis. PCR results for *VanB* gene from specimens positive for *E. faecalis*. M is the DNA ladder, Lane 1 is negative control. Lane 2 is positive control. 3 to 16 are samples.

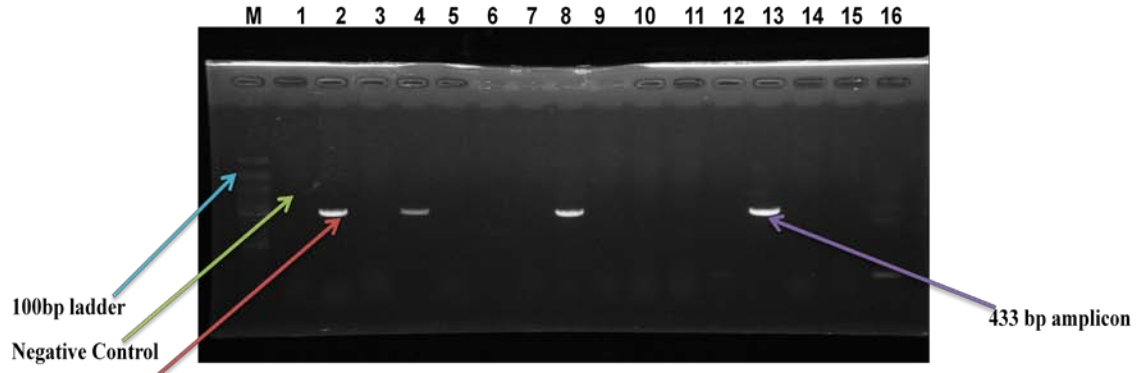


Figure 6. Gel electrophoresis showing VRE *E.faecium* isolates

Figure 6. Results of the PCR for *E. faecium* on gel electrophoresis- PCR results for *VanB* gene from specimens positive for *E. faecium*. M is the DNA ladder, Lane 1 is negative control. Lane 2 is positive control. 3 to 16 are samples.

The resistant genes identified in this study were all *VanB* representing 100 percent of the isolates. There were no *VanA* genes.

4.4 Antimicrobial susceptibility results

All the 25 VRE organisms that were detected by the Chromogenic VRE media were subjected to the VITEK 2 compact for antimicrobial susceptibility testing. A total of 15 antibiotics were used by the VITEK 2 compact. All the organisms were resistant to Vancomycin. They were all susceptible to nitrofurantoin. Some species exhibited resistance to high level Gentamicin and streptomycin. All the 25 isolates had multi drug resistant phenotypes i.e. they were resistant to at-least 3 (20%) of the 15 antibiotics that were used in the study.

This was a cross sectional study which involved processing urine, pus and blood specimens brought to the Bacteriology Laboratory from patients attending the inpatient and outpatient departments, in the general, high cost and paediatric wards. The research sought to identify and ascertain the prevalence of VRE from these samples. The antibiotic resistance patterns of the isolated *Enterococci* was determined and the research further sought to detect the resistance genes that were encoded by the VRE bacterial isolates.

Table 6. Antimicrobial susceptibility results in VRE isolates (n=25) by VITEK 2 for *E. faecalis* and *E. faecium* in this study.

Antibiotic	Organism			
	E. faecalis		***E. faecium***	
	Resistant	Sensitive	Resistant	Sensitive
	No. of isolates (Percentage)	No. of isolates (Percentage)	No. of isolates (Percentage)	No. of isolates (Percentage)
Ampicillin	3(12)	19(76)	3(12)	0(0)
Benzylpenicillin	3(12)	19(76)	3(12)	0(0)
Gentamicin High level	17(68)	5(20)	1(4)	2(8)
Streptomycin High Level	6(24)	16(64)	2(8)	1(4)
Ciprofloxacin	12(48)	10(40)	1(4)	2(8)
Levofloxacin	12(48)	10(40)	1(4)	2(8)

Moxifloxacin	12(48)	10(40)	1(4)	2(8)
Erythromycin	22(88)	0(0)	3(12)	0(0)
Clindamycin	22(88)	0(0)	3(12)	0(0)
Quinipristin/Dalfopristin	19(76)	3(12)	3(12)	0(0)
Linezolid	0(0)	22(88)	0	3(12)
Vancomycin	22(88)	0(0)	3(12)	0(0)
Tetracycline	19(76)	3(12)	3(12)	0(0)
Tigecycline	2(8)	20(80)	0(0)	3(12)
Nitrofurantoin	0(0)	22(88)	0(0)	3(12)

Table 7. Antimicrobial susceptibility pattern in VRE *E. faecalis* and *E. faecium* isolates according to VITEK 2 system (n=25) in this study.

Resistance Combination	Number of isolates
EM,CLI,VAN	25
EM,CLI,VAN,TET	4
EM,CLI,VAN,TET,GM	8
EM,CLI,VAN,TET,GM,SM	5
EM,CLI,VAN,TET,GM,SM,QD	7
EM,CLI,VAN,TET,GM,SM,QD,AMP	2
EM,CLI,VAN,TET,GM,SM,QD,AMP,BPC	3
EM,CLI,VAN,TET,GM,SM,QD,AMP,BPC,CIP,LVX,MXF	1
EM,CLI,VAN,TET,GM,SM,QD,AMP,BPC,CIP,LVX,MXF,TGC	1

Key: Ampicillin(Amp), Benzylpenicillin(BPC), Gentamicin High Level(GM), Streptomycin High Level(SM),Ciprofloxacin(CIP), Levofloxacin(LVX), Moxifloxacin(MXF), Ertythromycin(EM), Clindamycin(CLI), Quinipristin/Dalfopristin(QD), Vancomycin(VAN), Tetracycline(TET), Tigecycline(TGC).

CHAPTER 5

DISCUSSION

5.1 Demographic data

There were three types of specimens that were included in this study. These were urine, blood and pus specimens from patients attending the inpatient and outpatient departments from the general, high cost and paediatrics wards. More VRE were isolated from urine as compared to blood and pus. This would have probably been as a result of the commensal nature of enterocci in the GIT, which probably translocated to the urinary system causing infection. Another possible explanation is that samples may have been contaminated on collection by the patients prior to submission to the Microbiology Laboratory. This agreed with Gaido & Wilson 2004, in which their study did ascertain sample contamination by patients.

Information obtained from DISA at UTH's to identify the patients from specimen numbers showed that VRE was present more in women (total 18) as compared to men (total 7). This also was in agreement with literature in which females were most affected according to (Parameswarappa et al., 2013). Our study also showed *E. faecalis* was more isolated compared to *E. faecium,* thus agreeing with another study by Sreeja et al., (2012). *E. faecalis* is more prevalent than *E. faecium,* as observed in other studies (Ekuma et al. 2016; van den Braak et al. 1998; Nelson et al. 2000; Biswas et al. 2016; Schmidt-Hieber et al. 2007; Jovanović et al. 2015).

In this study, we were not able to determine the sources of infection as specimens were received after being collected from the patients by health care workers. However, from studies conducted by Biswas et al., (2016) and Sievert et al., (2013), it was established that for UTI, factors including catheterization and immuno-compromisation may lead to acquisition of VRE. The catheters provide a surface for bacterial adhesion, further leading to colonization by VRE from the bowels (Linden, 2002; Sievert et al., 2013). Immunocompromisation on the other hand

encourages these VRE to proliferate (Miller et al. 2014; Abebe et al. 2014; Tavadze et al. 2014; Lochan et al. 2016; Schmidt-Hieber et al. 2007). The immune system being compromised leads to opportunistic infections by VRE and other organisms.

In this study, 817 specimens were processed and of these, 621 were urine, 117 pus and 79 blood. VRE was isolated from these specimens. Urine had 16, followed by pus which had 6 and finally blood with 3 isolates. It was clear from the results urine had more isolates compared to blood and pus. These results agreed with another study by Chakraborty et al. (2015) where it was evident that Enterococci are most likely to be isolated from urine , compared to blood and pus.

In this study, we had bacteremia due to Enterococci. There was no patient history as to when the bacteremia was diagnosed and how long patients were hospitalized. However, according to research carried out in the last few years (Tavadze et al. 2014; Ceci et al. 2015), it has been established that the source of a bacteremia due to VRE is usually the genitourinary tract, although bacteremia can also be due to indwelling central lines or soft tissue infections (Tavadze et al., 2014 ,Murray; 1990). It has also been established that Enterococcal bacteremias often lead to endocarditis, the treatment of which can be more problematic according to Sood et al. 2008 and Nigo et al. 2014. However, even when a specific source is found, the overall mortality rate from enterococcal bacteremia is between 26% and 46% (Jett et al., 1994). In some studies, *E. faecium* bacteremia was associated with a higher mortality rate than *E. faecalis* and patients with rapidly fatal underlying diseases had mortality rates as high as 75% (Armeanu et al., 2005). These high rates likely reflect patients who are at risk for developing enterococcal bacteremia and these include older adults with conditions such as diabetes mellitus, heart disease, and those that previously had surgery (Tavadze et al., 2014).

Enterococci were isolated from Pus specimens in this study. There was no history as to how long the patients were hospitalized prior to the pus forming in the wounds from whom specimens were collected. However, this isolation from pus was an important finding as indwelling catheters or even wounds exposed to the environment can eventually be colonized by *Enterococcus* exogenously (Liassine et al., 1998). The skin is exposed to different organisms, Enterococci inclusive, agreeing with a study by (O'Driscoll & Crank 2015; Duckro et al. 2005) in which it was shown that different organisms can colonise wounds and cause pus.

Our study showed that age played a role in the acquisition of VRE. Ages most affected with VRE were between 29 to 46 years. This could have probably been because patients admitted were in these age groups at the time of our study. This agreed literature according to Ekuma et al., 2016 and Kolar et al., 2006 in which it was observed that hospitalization played a major role in acquisition of VRE. However, it was observed that *E. faecalis* was isolated from all the age groups as compared to *E. faecium* which was only from ages 0 to 28 and 4 to 67. This also agreed with literature that *E. faecium* was not as prevalent as *E. faecalis* (Moemen et al. 2015; Olawale et al. 2011).

Most of the patients attended to as shown from the DISA were from the inpatient departments as compared to the outpatient department. Inpatients have a higher chance of acquiring VRE as these patients tend to stay longer in hospital and can easily be exposed to carriers of VRE (Nourse et al., 2000). Carriers for these organisms may include patient care givers and members of staff who do not adhere to strict hygiene protocols (Mutters et al., 2013).

Different specimens from different wards were affected by these microorganisms. Specimens from General ward had both *E. faecalis* and *E. faecium*. *E. faecalis* was isolated from high cost and paediatrics wards. In this study, a total of 22 *E. faecalis* was isolated as compared to 3 *E. faecium* in general, paediatric and high cost wards. This showed that *E. faecalis* was more

prevalent than *E. faecium*. These microorganisms may have arisen either from the hospital environment or from the patients themselves (Moemen et al., 2015). Most of these infections from the wards were UTI, which could have come about because these microorganisms being part of the normal flora would easily gain entry to the urinary tract through catheterization, thus agreeing with a study done by Sievert et al., (2013).

5.2 Prevalence

This study involved 817 specimens received and processed through the Microbiology Laboratory for routine diagnosis of infections. Out of this total number, 621 were urine, 117 pus and 79 blood specimens. From these, 25 specimens tested positive for VRE, representing a prevalence of 3.06% in this study. *E. faecalis* amounted to 22 (88%) isolates and *E. faecium* to 3 (12%) isolates. The maximum number of *Enterococcus* isolates were obtained from urine-16 (64%), followed by 6 from pus (24%) and 3 from blood (12%). These specimens were from both the inpatient and outpatient departments from general, high cost and paediatrics wards. Urine specimens had a higher number of isolates compared to blood and pus, probably because of the commensal nature of Enterococci being part of the GIT.

The *Enterococcus* species have emerged as nosocomial pathogens (Rice, 2001) and this has necessitated the need for knowing the changing patterns of the *Enterococcal* infections and the antimicrobial susceptibility patterns of the isolates which are resistant to especially Vancomycin (Mutters et al., 2013).

The prevalence for enterococci in this study was in line with similar studies (Salem-Bekhit et al., 2012; Moemen et al. 2015; Olawale et al. 2011; Lu et al. 2001; Werner et al. 2012). In a study done by Olawale et al., (2011) in Nigeria, it was established that the prevalence of VRE was 5.9% in urine specimens from patients that were hospitalized for a period longer than 1 week. In another study conducted in Ethiopia in immuno-compromised patients, a prevalence of

VRE from urine of 5.5% was established (Abebe et al., 2014). In South Africa, the prevalence from blood ranged from 2.8% to 7.1% (Mahabeer et al., 2016). According to a study by Tavadze et al., (2014) it was established that immuno-compromisation could be a major player in contributing to the observed pattern in resource-constrained countries as immune compromised patients are more affected with VRE agreeing with Abebe et al., (2014) in a study which compared healthy and immuno-compromised patients. These immunocompromised patients are more prone to these infections due to lowered immunity, overuse of antibiotics which are used to treat infections by other organisms (opportunistic infections). However, in our study we were not able to determine immune status of patients as specimens were received in the Laboratory without such confidential information.

Enterococci, which are part of the normal flora, tend to lead to serious infections in patients that are unable to fight off infection due to these microorganisms (Camargo et al., 2008). Their increasing importance is largely due to their resistance to antimicrobials (Phukan et al., 2016). According to Miller et al., (2012) the therapeutic failures in enterococcal infections are mainly due to the intrinsic as well as transferable drug resistance. Once debilitated patients have been colonized with *Enterococci*, antibiotic use is one of the factors that lead to an increase in the prevalence of VRE (Kothari et al., 2014) because these antibiotics destroy other microorganisms in the body hence allowing VRE to proliferate. The prevalence in our study agreed with several studies conducted in other parts Africa (Salem-Bekhit et al., 2012, Olayinka Olawale et al., 2011 Abebe et al., 2014).

5.3 Genes involved in antibiotic resistance

The types of genes conferring resistance in VRE that were identified in this study were *VanB* genes. Enterococci carrying these genes have caused nosocomial infections in hospitalized

patients and those residing in long-term-care facilities according to a study by Ekuma et al., (2016).

In this study, VRE were isolated from all three types of specimens that were included in the study. *E. faecalis* and *E. faecium* isolates all had the *VanB* resistant gene as shown from the gel electrophoresis in figures 5 and 6.

The VRE carrying *VanB* genes exhibited various levels of resistance to Vancomycin, which is the last line treatment drug in most gram positive infections (Sood et al., 2008). Therefore, this study showed that isolates were multidrug resistant. The rapid emergence of VRE places great emphasis on the use of accurate and convenient testing methods for routine detection of VRE to reduce spread of these microorganisms at the UTH's.

Results from the PCR amplification of *VanA* and *VanB* genes showed all the VRE isolates contained *VanB* and not *VanA* genes. *VanB* confers low to moderate resistance of the antibiotic Vancomycin (Biswas et al. 2016). This is the first study in Zambia to have detected at *VanA* and *VanB* genes in VRE in UTIs, blood stream infections including pus at UTH's. Since there is no baseline data for comparison, the significance of these figures cannot be established at present.

The presence of other genes which include *VanC* and *VanD* were also investigated in other studies by Praharaj et al., (2013). These *VanC* and *VanD* genes also posess genes that encode for intrinsic resistance of Vancomycin in Enterococci (Bell et al., 1998). This study did not however check for the presence of *VanC* and *VanD* genes, since they do not confer Vancomycin resistance to other organisms, as compared to *VanA* and *VanB* .

The *VanB* genes were responsible for the observed antibiotic resistant phenotypes. This gene was detected in 16 urine, 6 pus and 3 blood specimens. Some of the VRE *VanB* positive isolates

exhibited multidrug antibiotic resistant patterns. They were resistant to Vancomycin, Erythromycin and Clindamycin. This agreed with Sievert et al., (2013) and Salem-Bekhit et al., (2012) in studies conducted which showed VRE to be multidrug resistant.

This study shows that different isolates of VRE carrying the same VanB resistant gene have different resistance phenotypes.

5.4 Antibiotic susceptibility pattern

A total of 817 Specimens were received and processed through the Microbiology Laboratory of which 25 of these, after culture and gram stain, showed presence of Enterococci by growth of black colonies on bile esculin agar. Although this agar is not definitive for enterococci, it enables presumptive differentiation of Group D streptococci and enterococci in the hospital setup (Brown et al., 1983).

Identification and sensitivity were carried out using Vitek 2 compact. The highest resistant rate at 100% was observed in Vancomycin, a glycopeptide antibiotic, Clindamycin a Lincosamide, and Erythromycin a Macrolide. All the 25 isolates found in this study were resistant to these 3 antibiotics. There was multi drug resistance (as shown in tables 6 and 7) including resistance to high level gentamicin and high level streptomycin. Resistance to Tetracycline and Ciprofloxacin was equally observed in this study. The implications for this pattern of resistance are that this further narrows the drugs available to treat infections due to enterococci (Linden, 2002). These findings agree with those of a study by Sievert et al., (2013) in which it was observed that VRE were multidrug-resistant opportunistic pathogens in the hospital environment. This must probably be due to selective pressure and widespread use and abuse of broad-spectrum antimicrobial drugs. Enterococci are resilient organisms that survive on the hands of health care workers and on inanimate objects (Austin et al., 1999).

According to a study carried out by Shawa et al, (not yet published) at UTH's, cephalosporin's and penicillin's account for about 79% of antibiotics used at UTH's. The high resistant rates to the glycopeptide antibiotics is of concern as these are the last line drugs in management of infections due to gram positive organisms (Xie et al. 2011). This has potential to complicate the already limited treatment options for UTI, blood stream and pus infections which cause pus even more at UTH's. Macrolides, Penicillin's, Lincosamides and tetracyclines are the most readily available drugs in use at UTH's and in this study, resistance was observed to these antimicrobials. This observation of drug resistance could be due to failure to follow dosage regimens by patients, and administration of drugs by health care workers.

In this study, the high resistance rate observed in Vancomycin (100%) was probably due to the ability of Enterococci to transmit resistance amongst themselves. They can also transmit this resistance to other species of organisms using *VanB* genes which are found on their plasmid through HGT (Grohmann et al. 2003; Hollenbeck & Rice 2012; Huddleston 2014).

Nitrofurantoin was the only drug to which all study isolates were sensitive. However, it must be noted that this drug is only effective for UTI's and thus it is rendered ineffective to infections which are not from the urinary tract (Zhanel et al., 2001). These results suggest that Nitrofurantoin may be an alternative in the treatment of VRE affecting the urinary tract. More studies need to be undertaken to determine other drugs which may be effective in managing infections due to VRE at the UTH's.

Most of the antibiotics used in this study had a high resistance rate possibly due to selective pressures of antimicrobial usage in the treatment of infections due to Enterococci since these antimicrobials can readily be accessed over the counter in Zambia without need for a prescription. Gupta et al., 2015 in the study on response of Enterococci to different antimicrobials, emphasized that oral administration of antimicrobials can increase antimicrobial

resistance in Enterococci. They found that drugs like tetracycline, aminoglycosides and quinolones had a strong effect on the development of antimicrobial resistance.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusions

Multidrug resistant VRE containing *VanB* genes were isolated in this study. These microorganisms were 22 *E. faecalis* and 3 *E. faecium*, giving a total of 25 isolates with VRE, which amounted to a occurrence of 3%. These isolates were completely resistant to Vancomycin, Clindamycin and Erythromycin.

6.2 Recommendations

i. Patients being admitted for prolonged periods should be screened for VRE owing to this organism's capability of resistant gene transfer to other susceptible species within the hospital.

ii. Lincosamides, Macrolides and Glycopeptides should not be recommended for the treatment of UTI's, bloodstream infections and skin infections characterized by pus as a result of infection with VRE at UTH's because of their high resistance rates observed in our study.

iii. There is need for continued antimicrobial resistance surveillance to monitor the VRE resistance patterns found in other hospitals within Zambia apart the UTH's.

iv. Brilliance chromogenic VRE media can be used for faster diagnosis of VRE infections at the UTH's.

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