TITLE: DETERMINING VIRULENCE FACTORS AND GENES CODING FOR ANTIBIOTIC RESISTANCE IN ENTEROCOCCI SPECIES ISOLATED FROM PREGNANT WOMEN IN WINDHOEK, NAMIBIA

By

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October 2019
DECLARATION

I, Daniella Chantelle Mouton hereby declare that the work contained in the thesis entitled “Determining virulence factors and genes coding for antibiotic resistance in Enterococci species isolated from pregnant women in Windhoek, Namibia” is my own original work and that I have not previously, in its entirety or in part, submitted it at any university or other higher education institution for the award of a degree.

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▪ Maxi Medical Laboratory for their understanding and patience during this study process.
DEDICATION

This thesis is dedicated to my grandfather, Albert (Boetkas) Mouton. May your soul rest in peace.
ABSTRACT

Purpose of the research: Research into Enterococci is increasingly important as Enterococci was classified as high priority bacteria regarding antimicrobial resistance. Improvements in medical care have also increased the chances of survival of infants with low birth weight. These infants have an increased chance of infection by antibiotic resistant Enterococcus species due to their low birth weight which is further increased by increased hospital stays. In developing countries neonatal meningitis occurs in 0.8-6.1 per 1000 live births. Enterococcus species are one of the causative organisms of late onset neonatal meningitis. Therefore, it is important to monitor colonization of the maternal genitourinary tract to prevent mother to child transmission of these bacteria.

Objectives: To determine the frequency distribution of Enterococcus species found among pregnant women between 35 and 37 weeks gestation in Windhoek. To establish the antibiotic resistance patterns of the Enterococcus species. To screen for resistance and virulence genes found in Enterococcus isolates.

Methodology: A descriptive cross-sectional study done at the antenatal clinic at the Windhoek central hospital in Windhoek. Study population: Pregnant women at 35 – 37 weeks of gestation attending the antenatal clinic at the Windhoek central hospital. Samples were collected from November 2018 to May 2019, in which a total of 193 participants that fit the inclusion criteria participated in the study. An estimated 45 participants refused to participate in the study during this period. Most of the patients (39.9 %) fell with the age range of 25-30 years of age. The average gravidity was 2.31 and the average parity was 2.09. The urine samples amounted to 101 and from a separate group of participants the vaginal swabs and rectal swabs amounted to 92 samples each.

Results: The total number of isolates collected from the 193 participants was 20. In rectal swabs 30.8% were Enterococcus faecalis, 7.7% were E. faecium and 61.5% were other Enterococci. In vaginal swabs, 33.3% were E. faecalis and 66.6% were other Enterococcus. Half the isolates found in urine were E. faecalis and the rest was other Enterococci. In these isolates 83.3% were resistant to at least 1 antibiotic. In E. faecalis, 16.7% were positive for vanA, 50.0% were positive for vanB, blaZ were positive for 33.3% and blaR1 were positive for 33.3% isolates. In the E. faecalis isolates 16.7% were positive for agg and 16.7% were positive for gelE. E. faecium did not test positive for any virulence or antibiotic resistance genes. The other Enterococcus species were not confirmed with molecular methods, with 9.1% being positive for vanA, 9.1% positive for vanB, 9.1% positive for tetM, 60.0% positive for blaZ and 9.1% positive for blaR1.

Conclusions & Recommendations: The majority of the isolates collected in this study were not the most common species found in human disease namely E. faecalis and E. faecium. This then becomes important to speciate, in order to better treat patients when faced with species with intrinsic resistance. Virulence genes were only found in 3 isolates. Although there are resistance genes present in these isolates, empirical antibiotics would still be effective. This is good considering the global trend of resistance.
Key Words: E. faecalis, E. faecium, resistance genes, virulence genes, antibiotics
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<tr>
<td>AIPS</td>
<td>Autoinducing Peptide</td>
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<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
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<td>CDC</td>
<td>Centre for Disease Control</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>DTR</td>
<td>DNA Transfer Proteins</td>
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<tr>
<td>Ebp</td>
<td>Endocarditis and Biofilm-associated pili</td>
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<tr>
<td>EBS</td>
<td><em>Enterococcal</em> binding substance</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene Diaminetetraacetic acid</td>
</tr>
<tr>
<td>EUCAST</td>
<td>European Committee on Antimicrobial Susceptibility testing</td>
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<tr>
<td>FISH</td>
<td>Fluorescence in situ Hybridization</td>
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<tr>
<td>GBS</td>
<td>Group B <em>Streptococcus</em></td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HSV2</td>
<td>Herpes Simplex Virus type 2</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>MALDI-TOF MS</td>
<td>Matrix Assisted Laser Desorption/Ionization Time of Mass Spectrometry</td>
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<tr>
<td>MIC</td>
<td>Minimum Inhibitory Concentration</td>
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<tr>
<td>MOHSS</td>
<td>Ministry of Health and Social Services</td>
</tr>
<tr>
<td>NUST</td>
<td>Namibia University of Science and Technology</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen associated Molecular Patterns</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
</tr>
<tr>
<td>PNP</td>
<td>Penicillin-binding protein</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>STIs</td>
<td>Sexually Transmitted Infections</td>
</tr>
<tr>
<td>TB</td>
<td>Tuberculosis</td>
</tr>
<tr>
<td>TBE</td>
<td>Tris/Borate/EDTA</td>
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<tr>
<td>TLR</td>
<td>Toll-like Receptors</td>
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<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
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<tr>
<td>UNICEF</td>
<td>United Nations Children’s Emergency Fund</td>
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<td>WHO</td>
<td>World Health Organization</td>
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CHAPTER 1

INTRODUCTION
**Enterococcus** species are normal commensals of the gastrointestinal system. They are considered opportunistic pathogens and have gained importance as nosocomial infections (Sharma et al., 2012). In the United States of America, **Enterococcus** has a prevalence of 14% in the total hospital acquired infections (Goh et al., 2017). **Enterococcus faecalis** and **Enterococcus faecium** are the species most commonly associated with infection in humans (Lebretor et al., 2014). Antimicrobial resistance is most commonly found in *E. faecium* whereas virulence traits are more commonly found in *E. faecalis* (Banerjee et al., 2015). Both antimicrobial resistance and virulence are transferred to bacteria via plasmids. Bacteria would lose antibiotic resistance after gaining a plasmid with virulence factors due to incompatibility (Banerjee et al., 2015). Improvements in medical care have also increased the chances of survival of infants with low birth weight. These infants have an increased chance of infection by **Enterococcus species** due to their low birth weight which is further increased by increased hospital stays (Ghasemi et al., 2016). In developing countries neonatal meningitis occurs in 0.8-6.1 per 1000 live births. **Enterococcus** species are one of the causative organisms of late onset neonatal meningitis (Lawrence et al., 2015). Therefore, it is important to monitor colonization of the maternal genitourinary tract to prevent mother to child transmission of these bacteria (Khalessi et al., 2014).

Neonatal sepsis is ranked as fifth in the main killers of children under 5 (United Nations Children’s Fund 2015). A study in Germany showed there is a correlation between colonization of a potentially pathogenic organism and subsequent infection (Bohme et al., 2012). Furthermore, *E. faecalis* and *E. faecium* are some of the first bacteria to colonize infant intestines and are an important part of healthy intestinal flora (Bohme et al., 2012). Throughout the world, the average rate of neonatal mortality is 24 deaths per 1000 live births (UNICEF et al., 2015). In Africa and Sub-Saharan Africa, it is 27 and 29 deaths per 1000 live births respectively (UNICEF et al., 2015). These are much lower than Namibia with 45 deaths per 1000 live births (UNICEF et al., 2015).

**Enterococcus** is responsible for 30% of the bacterial isolates from urinary tract infections collected in hospitals in the United Kingdom (Lin et al., 2012). Asymptomatic urinary tract infections are treated in pregnant women because they have suppressed immune systems and as such urinary tract infections can progress to pyelonephritis and kidney failure unnoticed making it dangerous for both the mother and the unborn child (Lin et al., 2012).

The antibiotic that is most important considering resistance to **Enterococci** is vancomycin (Banerjee et al., 2015). A study on Nigeria showed a prevalence rate of 5.9% in infection with **Enterococcus** related to nosocomial infections (Olawale et al., 2011). All **Enterococcus** species were resistant to
at least one antibiotic. Forty-three percent were vancomycin resistant (Olawale et al., 2011). A study in Iran showed that 8.14% of patients between 35-37 weeks of gestation had vaginal colonization by Enterococci species (Ghasemi et al., 2016). All Enterococci isolates were sensitive to gentamicin (2-8µg/ml) and amoxicillin (0.5-8µg/ml). All isolates were resistant to ciprofloxacin and were identified as E. faecalis (Ghasemi et al., 2016). In a study done in Brazil on the blood culture specimens from new-borns, 8% of isolates were E. faecium and 44% were E. faecalis (Furtado et al., 2014).

Largely the virulence factors of Enterococci are related to their ability to attach to host cells (Comerlato et al., 2013). These virulence factors include gelatinase activity and aggregation substance. Aggregation substance is however also said to mediate the aggregation of bacterial cells during the transfer of plasmids (Comerlato et al., 2013). Gelatinase is an enzyme that facilitates hydrolytic activity. In a study conducted in Brazil showed that 60% of Enterococcus isolates carried the gelE gene which encodes for gelatinase (Comerlato et al., 2013). In the same study 76% carried the esp gene encoding for Enterococcus surface protein and 74% carried the asal gene which encodes for aggregation substance (Comerlato et al., 2013). In a study conducted in North India 9.6% of E. faecalis and 8.3% of E. faecium isolates carried the gelE gene (Banerjee et al., 2015). Sixty-two of E. faecalis isolates and 36% of E. faecium isolates showed phenotypical haemolysis. In this Indian study only 5% of these isolates encoded for cylA, however this is only one of 5 genes which encode for haemolysis (Banerjee et al, 2015). Haemagglutination was seen in 33% of E. faecalis and 35% of E. faecium isolates (Banerjee et al, 2015).

1.1 Statement of the problem

Research into Enterococci is increasingly important as Enterococci was classified as high priority bacteria regarding antimicrobial resistance (World Health Organisation, 2017). This is a highly resistant organism, especially in a hospital settings and treatment of Enterococci now involves use of highly toxic drugs such as vancomycin. A cause for more concern is that Enterococcal species have shown resistance to vancomycin in some studies, decreasing the options for treatment even more. This means that treatment is very limited in cases where neonates are infected with this bacteria and neonatal mortality is high. Furthermore, patients who have neonatal meningitis are 10 times more likely to suffer neurologic impairment compared to healthy patients (Lawrence et al., 2015). Enterococci are not routinely screened in pregnant women in Namibia despite the risk of vertical transmission from colonized mothers to their new-born babies who can subsequently develop meningitis and other neurologic disorders as noted by WHO. This study therefore aims to determine maternal colonization by Enterococci, the genetic factors spurring virulence and drug resistance in these bacterial isolates in pregnant women in Windhoek.
1.2 Research questions

1.2.1. What is the frequency distribution of *Enterococcus* species found in pregnant women in Windhoek?
1.2.2. Which antibiotics are effective against *Enterococcus* species?
1.2.3. What genes code for antibiotic resistance and virulence in *Enterococcus species*

1.3 Aim of study

To determine virulence and gene-based resistance in *Enterococci* species isolated from pregnant women between 35 and 37 weeks gestation in Windhoek.

1.4 Objectives of the study

1.4.1. To determine the frequency distribution of *Enterococcus* species found among pregnant women between 35 and 37 weeks gestation in Windhoek.
1.4.2. To establish the antibiotic resistance patterns of the *Enterococcus* species.
1.4.3. To screen for the resistance and virulence genes found in the *Enterococcus* isolates.
CHAPTER 2

LITERATURE REVIEW
2.1. Introduction

*Enterococci* is part of the Enterococcaceae family (Lebretor *et al*., 2014). *Enterococcus* is a commensal of the human and animal gastrointestinal tract and can multiply in the gastrointestinal tract without causing disease (Ibrahim *et al*., 2015). It is also found in the biliary tract, vagina and male urethra as a commensal (Winn *et al*., 2006). In patients with compromised immune systems *Enterococcus* becomes an opportunistic causative organism (Ibrahim *et al*., 2015). The most important species of *Enterococci* in human disease are *Enterococci faecalis* and *Enterococci faecium* (Lebretor *et al*., 2014). Other *Enterococcus* species involved in human disease, though less commonly, include: *E. avium*, *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. disper*, *E. raffinosus*, *E. malodaratus*, *E. mundtii* and *E. flaveseens* (Ibrahim *et al*., 2015). The ability to exchange genes, particularly genes responsible for antibiotic resistance and virulence factors, is what makes *Enterococcus* an important disease-causing organism (Ibrahim *et al*., 2015). *Enterococci* are the second most common nosocomial urinary tract and wound infections, and the third most common nosocomial causative organism (Lebretor *et al*., 2014). *Enterococcus* can share genes for virulence and antibiotic resistance that are expressed through plasmids. Plasmid free *Enterococci* produce sex pheromone peptides, eliciting a response from the *Enterococci* bacteria which have plasmids, leading to conjugation and transfer of plasmids (Van Tyne *et al*., 2013). *Enterococci* further communicates through quorum sensing allowing different strains to function in synchronicity in their expression of virulence factors (Rutherford *et al*., 2012).

2.2. Classification of *Enterococci*

*Enterococcus* are living celled organisms under the kingdom bacteria, the subkingdom Posibacteria, the phylum Firmicutes, the class Bacilli, the Order Lactobacillales, and the Family Enterococcaceae (Intergrated Taxanomic Information System, 2012). The genus is *Enterococcus* and the species involved in human disease include *E. faecalis*, *E. faecium*, *E. avium*, *E. gallinarum*, *E. casseliflavus*, *E. durans*, *E. disper*, *E. raffinosus*, *E. malodaratus*, *E. mundtii* and *E. flaveseens*.

The term *Streptococcus faecalis* was first used in 1906. In 1937 distinctions were made in the genus of *Streptococcus* through groups known as *Enterococcal* and non-Enterococcal streptococci. It was also during the early 1930’s that the Lancefield group of classification came out. *Enterococcal streptococci* were classified as bacteria that could grow at 10-45°C at 6.5% sodium chloride and at a pH of 9.6, that survived at 60°C for 30 min, furthermore bacteria that could split esculin (Murray, 1990). *Enterococcal* bacteria also had group D Lancefield antigens. In 1984 DNA-DNA and DNA-rRNA hybridization was used to show that *S. faecalis* and *S. faecium* were so distant from
Streptococcus genetically that they should be transferred to a new order namely Lactococcus. Thereafter, Enterococcus was introduced as the genus name (Murray, 1990).

2.3. Pathogenicity of Enterococci

The most common infection by Enterococci is a urinary tract infection (UTI). It causes largely lower urinary tract infections, however in older men these infections progress to cystitis, prostatitis and infection of the epididymis (Lebretor et al., 2014). Urinary tract infections caused by Enterococcus are more commonly found in patients with prolonged hospital stays. Fifteen percent of intensive care unit (ICU) and healthcare associated UTI’s are cause by Enterococcus species (Lebretor et al., 2014).

Enterococcal bacteraemia usually result from an infection elsewhere in the body, for example; urinary tract infection, biliary tract infection. They may also result from Enterococci found in the GIT or the genitourinary tract (Winn et al., 2006). Bacteraemia and endocarditis are common manifestations of Enterococcal infections. Enterococcus are intrinsically resistant to bactericidal activity and as such antibiotics of two different mechanisms of action often required (Lebretor et al., 2014). When the infection is cause by vancomycin resistant Enterococcus (VRE), treatment often fails and surgery is required to remove the infected valve (Lebretor et al., 2014). Enterococcus faecalis is more likely to cause endocarditis than E. faecium with the source being the GIT (Lebretor et al., 2014).

Enterococci are found throughout the GIT, the oral cavity as well as the genital tract (Lebretor et al., 2014). Often patients whose GIT is colonized by VRE have the same bacteria colonizing their skin (Lebretor et al., 2014).

Enterococcal infections often cause nosocomial infections, spread through the hands of healthcare workers to intravenous or urinary catheters (Lebretor et al., 2014). They are also known to be spread through thermometers, stethoscopes and other medical devices (Ghasemi et al., 2016). In healthcare centres, the use of broad-spectrum antibiotics is common, which means antibiotic resistant Enterococci are more commonly found in these places (Lebretor et al., 2014). Furthermore, patients on dialysis, with liver transplants, haematological malignancies and other chronic diseases are at a higher risk of contracting VRE (Lebretor et al., 2014).

Most common infections in neonates include meningitis, sepsis and bacteraemia (Olawale et al., 2011). Neonatal sepsis is the most common nosocomial infection in neonatal intensive care wards (Furtado et al., 2014). Neonatal sepsis can be early onset or late onset. Early onset occurring within the first 72 hours of birth and late onset occurring 72 after birth (Furtado et al., 2014). Due to progress in medicine the chances of low birth weight neonate’s survival has increased. However,
this is a risk factor for infection and subsequent sepsis by *Enterococci* (Furtado et al., 2014). Other risk factors for late onset sepsis in neonates are; increased hospital stays, prematurity and invasive procedures (Furtado et al., 2014). The most common routes of infection include central venous catheters (54%), mechanical ventilation (32%) and parental nutrition (32%) (Furtado et al., 2014). Neonatal Enterococcal sepsis presents with fever, lethargy, respiratory distress and bacteraemia (Winn et al., 2006).

Virulence occurs through either or both of the following methods: toxins produced by the pathogen or activation of the host immune response and inflammation (Sharifi et al., 2013).

The virulence of *Enterococcus* is determined by its ability to colonize the gastrointestinal tract, the ability to adhere to substances such as thrombospondin, lactoferrin and vitronectin that form part of the extracellular matrix and the ability to adhere to the epithelial cells of the urinary tract (Fisher et al., 2009). *E. faecalis* is more commonly associated with having the following virulence factors: Aggregation substance, gelatinase, cytolysin and *Enterococcal* surface protein. *E. faecium* is more commonly associated with having *Enterococcal* surface protein and hyaluronidase (Vankerkhoven et al., 2004). Extracellular signalling in gram positive bacteria was first demonstrated through oligopeptides such as acyl-homoserine-lactone signals. Extracellular communication forms an important part of *Enterococcal* virulence which occurs through: Quorum sensing and sex pheromones (Dunny et al., 2016). They fall in two categories of communication: secreted as unmodified peptides that are then processed from precursors or peptides that are processed and post translationally modified (Dunny et al., 2016). Furthermore, signals can either be transduced across the cell surface membrane or imported and bound to a cytoplasmic receptor protein. Quorum sensing is a means of communication between bacteria produced in response to increased cell density. This form of communication involves the production, detection and response to extracellular signalling molecules called autoinducers (Rutherford & Bassler, 2012). Autoinducers have low molecular weight and belong to the following chemical classes: acyl-homoserine-lactones, furanosyl borate diesters, cis-unsaturated fatty acids family signals and peptides (Solano et al., 2014). Quorum sensing is used by bacteria for virulence, sporulation, bioluminescence, virulence factor secretion, symbiosis, competence, conjugation, antibiotic production, motility, sporulation and biofilm production (Miller & Bassler, 2001; Rutherford & Bassler, 2012). These are activities that are performed better at higher cell densities of bacteria acting together (Rutherford & Bassler, 2012). Gram positive bacteria use Autoinducing peptides (AIPs) as signalling molecules, that are secreted once produced. When the cell density of that particular organism is high, the extracellular concentration of AIPs becomes high, and binds to similar membrane bound two component histidine kinase receptor. The binding activates autophosphorylation of the receptor’s kinase,
passing the phosphate to a similar cytoplasmic response receptor, which spurs transcription of genes in the quorum sensing regulon. In human pathogens such as *Staphylococcus aureus, Listeria monocytogenes, Clostridium perfringes* and *E. faecalis* quorum signalling controls virulence production (Rutherford & Bassler, 2012).

### 2.3.1. Virulence factors:

#### 2.3.1.1. Cytolysin

Cytolysin/haemolysin are virulence factors that lyse cells. *E. faecalis* strains that are highly virulent produce cytolysin that lyses both eukaryotic and prokaryotic cells (Van Tyne *et al.*, 2013). This is the virulence factor responsible for the presence of haemolysis of some strains of *E. faecalis* on blood agar. Different to the family of thiol-activated, cholesterol dependent cytolysins produced by other Gram-positive bacteria, *Enterococcal* cytolysin is heat labile and oxygen stable (Van Tyne *et al.*, 2013). “The presence of *Enterococci* in the complex ecology of the gastrointestinal tract provides an ample reservoir where genetic exchange and selection can occur” (Van Tyne *et al.*, 2013). Due to the bacteriocin activity of the *Enterococcal* cytolysin, this plays an important role in the colonization of the intestine prior to the infection of other areas of the body that are normally sterile (Van Tyne *et al.*, 2013). It was found that 45% of *E. faecalis* isolates taken from patients (190) that participated in a study by Huycke *et al.*, (1991) in the United States of America were cytolysin positive (Huycke *et al.*, 1991). In this study it was found that patients with isolates that were cytolysin positive had a 5-fold increased risk for death compared to patients with isolates that were cytolysin negative (Van Tyne *et al.*, 2013).

The cytolysin operon is encoded in one of two ways: chromosomally or on a conjugative, pheromone responsive plasmid. (Van Tyne *et al.*, 2013). There are six genes which make up the cytolysin operon and two genes that are responsible for regulation. When not activated the cytolysin repressor protein binds to the promotor region. However, a low level of transcription still takes place in producing the cytolysin subunits. Through autoinduction the presence of target cells is sensed and the cytolysin repressor protein on the promoter region is released resulting in the expression of the cytolysin operon (Van Tyne *et al.*, 2013). The L component is made up of *cylL1*, *cylL2*, *cylM* and *cylB*. Component A is encoded by *cylA* which is more commonly studied in research (Vankerckhoven *et al.*, 2004). Cytolysin has β-haemolytic properties and expresses bactericidal properties against other gram-positive bacteria (Kafil *et al.*, 2013). Cytolysin acts on red cells and has exhibited its toxicity on rabbit endophthalmitis and endocarditis models. Coccolysin is an extracellular metallopeptidase produced by 50-65% of *E faecalis* and may inactivate endothelin vasoactive peptide.
The cytolysin toxin consists of two oligopeptides encoded by cylLL and cylL5, which undergo post translation modification (Van Tyne et al., 2013). These subunits are classified as type A lantibiotics. Through catalysation of cylM, the serine and threonine residues of these subunits are dehydrated. Thereafter intramolecular lanthionine and methyllanthionine bridges are formed between the residues and cysteine thiol groups (Van Tyne et al., 2013). The ATP-binding cassette (ABC) transporter cylB secretes and trims the peptides cylLL and cylL5 modified by cylM. Thereafter these subunits are processed by cylA serine protease and thereby become active subunits (Van Tyne et al., 2013). The last gene that forms part of the cytolysin operon encodes for the immunity protein cylI which is not completely understood but is thought to protect cytolysin producing cells. When no target cells are present cylLL and cylL5 form insoluble complexes. When target cells are present the complex the subunits work together to form a pore in the cell membrane of the target cells (Van Tyne et al., 2013). The larger cylLL subunit has a higher affinity for the target cell than it does to cylL5, resulting in an accumulation of free cylL5 which then acts as a quorum sensor resulting in the release of cylR2 and consequently a higher rate of cytolysin operon expression (Van Tyne et al., 2013).

2.3.1.2. Biofilm formation

A biofilm is a community of bacteria in high density attached to the surface at the site of infection and to each other in an extracellular matrix (Solano et al., 2014). Biofilms may attach to organic or non-organic material and are encased in proteins, polysaccharides and nucleic acids (Hashem et al., 2017). Biofilm formation occurs through quorum sensing and it protects the bacteria from environmental stresses, desiccation, attack by the immune system, protozoan ingestion and antimicrobials (Solano et al., 2014). A biofilm is built through the following steps 1. Irreversible binding to the surface, 2. Bacterial division and production of the extracellular matrix and finally the disassembly of the extracellular matrix resulting in the dispersion of bacteria (Solano et al., 2014). Biofilm formation is possible through communication via: quorum sensing and peptide pheromones (Hashem et al., 2017). Step 1 of forming a biofilm also allows the bacteria to attach to urinary and intravascular catheters forming infections in these sites (Hasheem et al., 2017). Plasmids carry the virulence genes that promote or regulate biofilm production and an example of this aggregation substance. There is little evidence from research that provides a direct correlation from genes to biofilm production yet. Aggregation substance and gelatinase production are good indicators of biofilm formation (Hashem et al., 2017).

In formed and mature biofilms the normal minimum inhibitory concentrations of antibiotics do not work because in these matrices the bacteria can handle 10-1000 times higher concentrations of bacterial cells that are not in biofilms (Hasheem et al., 2017). The use of sub-inhibitory antibiotics
is used to remove and prevent biofilm formation (Hashem et al., 2017). This was shown to change the outer surface of Enterococcal cells as well as the physiochemical properties, thereby reducing the virulence (Hashem et al., 2017). These lower levels of antibiotics also prevent the initial adhesion to artificial devices such as catheters. In a study Hashem et al. (2017) in Egypt on biofilm formation only 4.5% of the 90 Enterococcal isolates were not biofilm formers. Even though gelatinase production is thought to be a good indicator of biofilm formation only 27% of biofilm formers showed gelatinase activity in vitro (Hashem et al., 2017). The strength of the biofilm may be associated with aggregation substance, as the strength increased with the presence thereof. Furthermore, all biofilm formers had the geIE gene although only 27% had the phenotypic expression in vivo, and all non-formers did not have the gene (Hashem et al., 2017). GeIE is thought to participate in the formation of biofilms through either: 1. The proteolytic action of the enzymes which are thought to act as an initiator to the attachment to surfaces or 2. Through the physical presence of the enzyme rather than the enzymatic activity (Hashem et al., 2017). Another factor thought to be necessary for biofilm formation is pili, encoded by the gene cluster ebp (Fisher et al., 2009). Epb stands for endocarditis and biofilm-associated pili (Pinkston et al., 2014). This gene cluster consists of ebpA, ebpB, ebpC and a sortase C gene (Fisher et al., 2009). Enterococcal pili consist of a pilus shaft made up of 2 smaller pilins and have a specific sortase devoted to their assembly. They are covalently bound to the Enterococcal cell wall (Fisher et al., 2009). Pili assists with the attachment of Enterococcus to medical devices such as catheters and artificial heart valves, as well as the inner endothelial lining of the heart or heart valve (Pinkston et al., 2014). Infective endocarditis is rarely found in healthy hearts, but rather when artificial devices are used such as artificial heart valves, pacemakers and implantable defibrillators (Goh et al., 2017).

2.3.1.3. Aggregation substance

Aggregation substance facilitates exchange of genetic information through plasmids, contributing to the pathogenesis of Enterococcus (Soares et al., 2014). In vitro testing showed that aggregation substance also leads to the adherence of the bacteria to intestinal, renal epithelial and cardiac cells (Winn et al., 2006). Aggregation substance facilitates the adherence of bacteria to each other to exchange plasmids, promotes the growth of cardiac vegetation in rabbit endocarditis and adheres to cultured intestinal and renal epithelial cells. Aggregation substance are surface bound proteins that are encoded by pheromone-inducible conjugative plasmids (Sava et al., 2010). The pheromones are excreted by recipient cells without plasmids (Sava et al., 2010). The following proteins are all different forms of aggregation substance: Asa1, Asp1, Acs16 and Agg (Sava et al., 2010). In E. faecalis, isolates with Asa1 showed increased adherence to renal tubular cells and macrophages as well as survival within human macrophages (Sava et al., 2010). Asc10 increased
the internalization of Enterococcal cells into polymorphonuclear leucocytes as well as the increased survival therein (Sava et al., 2010). Ace in E. faecalis assists the adhesion of the bacteria to collagen I and IV as well as laminin (Medeiros et al., 2014). This assists Enterococcus in its pathogenicity in endocarditis. Studies suggest that aggregation substance is involved in the binding and intracellular survival of Enterococci in neutrophils and cultured intestinal cells. Aggregation substance mediates plasmid transfer between bacteria (Schieverf et al., 2010). Agg is pheromone inducible and necessary for the donor and recipient cell contact during plasmid transfer during bacterial conjugation (Medeiros et al., 2014). Agg has increases the hydrophobicity of the Enterococcal cell wall. Therefore, once the bacterial cells have been engulfed by phagosomes, the fusion with vesicles is delayed (Fisher et al., 2009). Aggregation substance assists in forming a clotted mass of platelets, fibrin, large numbers of bacteria and leucocytes (Chuang-Smith et al., 2010). Though the formation of this mass is not necessary for endocarditis, it contributes significantly (Schieverf et al., 2010). This vegetation can occur in two different cases; with previous valvular damage or scarring and in undamaged, healthy cells. In cases with previous valvular damage, blood flow is irregular, damage to the cells recruits platelets and fibrin (Chuang-Smith et al., 2010). Once bacteria enter the bloodstream through either surgically or translocation through the intestinal trace the bacteria are trapped in the platelet and fibrin mass causing endocarditis. In cases with no previous damage when E. faecalis enters the bloodstream and lyses endothelial cell layer with initiates and amplifies the coagulation cascade (Chuang-Smith et al., 2010). The gene cpd encodes for sex-pheromone peptides which along with aggregation substance are necessary for the acquisition and transfer of genetic information through plasmids (Sharifi et al., 2013).

2.3.1.4. Hyaluronidase

Hyaluronidase which is encoded by hyl, was only identified in recent years and is commonly associated with nasopharynx infections by Enterococci and Pneumococcal pneumonia (Vankerckhoven et al., 2004). Hyl encodes for enzymes that incite tissue damage and facilitate the spread of Enterococci (Kafil et al., 2013). The depolymerization of mucopolysaccaridees leads to the spread of Enterococcus and the Enterococcal toxins through the infected host (Fisher et al., 2009).

2.3.1.5. Extracellular surface protein

Extracellular surface protein is a protein which serves as an adhesion of the bacterial cell to the host, located on the bacterial cell wall (Medeiros et al., 2014). Forty percent of Enterococci isolates from blood, and patients with endocarditis contain extracellular surface protein (Esp). Esp is located on the pathogenicity island on the genome of both E. faecalis and E. faecium (Sava et al., 2010).
This helps the bacteria evade the immune system (Winn et al., 2006). This virulence factor is unique as it has a central core, from which an arm can extend through to the cell wall and subsequently be withdrawn from the cell wall to evade antibodies. This is associated with colonization and persistence in the urinary tract (Vankerckhoven et al., 2004). The esp gene has been identified through research to more common strains of vancomycin resistant E. faecium among hospitalized patients (Vankerckhoven et al., 2004). Forty percent of Enterococci isolates from blood and endocarditis produce extracellular surface protein. This protein is said to be involved in the initial adherence of the bacterial cell to polystyrene and to biofilm formation (Sava et al., 2010). Isolates that have Esp show increased resistance to ampicillin, ciprofloxacin and imipenem (Fisher et al., 2009).

2.3.1.6. Gelatinase

GelE is regulated by the Fsr-quorum sensing in E. faecalis and assists in bacterial adherence and biofilm formation (Stinemetz et al., 2017). GelE expression is induced at high cell density through the quorum sensing locus Fsr, which consists of FsrA; FsrB; and FsrC (Waters et al., 2003). These genes regulate expression of both GelE and a downstream serine protease gene sprE (Waters et al., 2003). The Fsr locus is expressed when FsrD accumulates extracellularly, sensed by histidine kinase (FsrC) and subsequently activating the regulation and transcription factor (Sava et al., 2010). When FsrA is phosphorylated it activates the expression of GelE and sprE (Sava et al., 2010). GelE encodes for the zinc metalloprotease called gelatinase, similar to the aureolysin in S. aureus and elastase in P aeruginosa (Thurlow et al., 2010). GelE is produced through translation and cleaved into the active gelatinase in the 192-amino acid N’region (Waters et al., 2003). Gelatinase assists in the formation of which is a clotted mass of platelets, fibrin, large numbers of bacteria and leucocytes (Chuang-Smith et al., 2010). It is difficult for antibodies and antibiotics to penetrate the platelet-fibrin mass extending damage and prolonging the infection.

C5a which forms part of complement, is an important part of humoral immunity as it causes the activation and recruitment of neutrophils. Gelatinase cleaves C5a reducing neutrophil migration, prolonging infection. Gelatinase furthermore degrades collagen, fibrinogen, fibrin endothelin-1 spurring the coagulation cascade (Thurlow et al., 2010). Gelatinase also hydrolyses casein and haemoglobin (Galloway-Pena et al., 2011). Moreover, the virulence of GelE is also credited to the part it plays in regulating AtlA, which is an autolysin which hydrolyses peptidoglycan. AtlA plays an important role in separation of cells during the last step of replication (Stinemetz et al., 2017). GelE promotes virulence through its role in adherence and colonization of the bacteria to the host. GelE cleaves AtlA in the post translational process between the T/E rich domain 1 and the catalytic region.
(domain 2) resulting in a functional autolysin (Stinemetz et al., 2017). Domain 3 is responsible for the attachment of the autolysin to the peptidoglycan cell wall. GelE also functions in order to clear the bacterial cell of misfolded proteins and is therefore responsible for the instability of substrates such as Asc10 (aggregation substance) (Waters et al., 2003). The production of gelatinase in clinical samples assists dissemination of the bacteria in biofilms as the cleavage decreases the chain length of Enterococcal cells (Waters et al., 2003). Gelatinase is produced in approximately 60% of clinical E. faecalis isolates (Galloway-Pena et al., 2011).

Most E. faecalis and E. faecium isolated from bacteraemia produce a large extracellular superoxide. This may enhance virulence in mixed-flora abscesses and promote chromosomal instability in mammalian cells. (Wang and Huycke, 2007). Group D antigen is made of lipoteichoic acids and induces production of tumour necrosis factor (TNF) and interferon.

2.3.2. Enterococcal sensory systems

Sex pheromones assist in the exchange of genetic material such as virulence factors and antibiotic resistance through plasmids (Dunny et al., 2016). Sex pheromones are single peptide pheromones encoded by chromosomes and are then imported into the donor cell. The pheromone response is induced in the donor by importation of conjugation peptide pheromones. The conjugation/clumping factor regulates the transcription of the operon that contains the conjugation genes, which is further affected by peptide binding (Dunny et al., 2016).

There are additional plasmid encoded peptides that compete with conjugation/clumping peptide for binding to the same receptor in Enterococcal sex pheromones (Dunny et al., 2016). There are several post transcriptional regulatory steps that affect the expression of conjugation genes. The donor cells are the bacterial cells with the plasmid and therefore the genetic material which can be shared. The donor and recipient cells have similar chromosomal material, however the donor cell has the addition of pCF10. Through pCF10, the plasmid translates a response to the conjugation/clumping peptide inducing conjugation (Dunny et al., 2016). Along with pCF10, the plasmid encodes for peptide I, which has an antagonistic relationship with conjugation/clumping peptide. Prg Z and Prg Y are expressed by pCF10, import and reduce the number of active conjugation peptides by donor cells. Once the conjugation peptides are imported, they interact with Prg X in the cytoplasm which then induces a conjugation response. Pheromone induction of donor cells results in the synthesis of peptides involved in conjugation such as surface adhesion proteins and type 4 secretion proteins (T4SS) and DNA transfer proteins (DTR) (Dunny et al., 2016). The production of pheromones by recipient cells allows conjugative/clumping pheromone
responsive plasmids to transfer genetic information much higher rates per donor cells (Van Tyne et al., 2013).

2.4. Disease in women including pregnant women

During pregnancy there are several physiological changes that take place that increase the susceptibility to infection such as the decrease in respiratory volumes and urinary stasis due to an increasing pressure from the uterus (Kourtis et al., 2014).

The most common genital tract infection in women of childbearing age is bacterial vaginosis (Machado et al., 2015). Bacterial vaginosis is associated with preterm delivery, spontaneous abortion, pelvic inflammatory disease and endometritis and increased risk of sexually transmitted infections (STIs). Bacterial vaginosis is largely characterized by a decrease in the lactobacilli colonies which are beneficial, and an increase in non-beneficial and inadvertently harmful bacteria such as Gardnerella vaginalis, Atopobium vaginae, Bacteriodes species and Providencia species. Healthy numbers of Lactobacilli in the female genital tract assists in maintaining an acidic pH, production of hydrogen peroxide and through colonization prevents genitourinary infections (Machado et al., 2015). Vaginosis is largely caused by bacteria, Candida species and Trichomonas (Bitew et al., 2017)

Urinary tract infections are common in women with around one third of women experiencing at least one symptomatic episode in their lifetime. Women are more likely to contract urinary tract infections due to their anatomical structure. Bacteria colonizing the intestinal tract can ascend the urinary tract due to the shorter length of the urethra and a proximity to the anus in women. In addition, in pregnant women from the second trimester, the enlarged uterus places pressure on the urinary bladder. Urinary tract infections can lead to pyelonephritis and kidney failure if not adequately treated (Minardi et al., 2011). The most common uropathogens are Escherichia coli and Staphylococcus saprophyticus in immune competent patients. The most common causative bacteria in nosocomial urinary tract infections are E. coli, followed by Klebsiella, Enterobacter, Pseudomonas, Serratia, Citrobacter, Proteus, Providencia species and Corynebacterium urealyticum. These bacteria are commonly involved in nosocomial infections since they make up the intestinal microbiota and become opportunistic infectious agents in immune compromised patients. Furthermore, these bacteria form biofilm which makes it difficult for antibiotics to affect the causative agents (Minardi et al., 2011). Due to selective pressure from prescribed antibiotics in hospital settings gram positive bacteria such as Staphylococcus and Enterococcus species become causative agents in nosocomial infections. Fungal urinary tract infections are largely caused by Candida species, and to a lesser extent Aspergillus and Cryptococcus neoformans in immune
compromised or hospitalized patients. Hospitalized patients, and in particular those with urinary devices (catheters, urinary stents and percutaneous nephrostomy) are at a larger risk for nosocomial urinary tract infections.

In urinary tract infections the innate immune system responds. In order for the immune system to respond, the pathogen – associated molecular patterns (PAMPs) should be recognized by the receptors of leucocytes such as monocytes and macrophages. These pathogen-recognition receptors include Toll-like receptors; retinoic acid inducible gene-1-like receptors, nucleotide-binding oligomerization domain-like receptors and Deoxyribose nucleic acid (DNA) receptors. These receptors there after stimulate the production of cytokines such as Interleukin (IL) -6, Tumour Necrosis factor (TNF) – alpha; IL-1β (Tanaka et al, 2014). Interleukin 6 has broad expression in the urinary bladder and releases a signal indicating tissue damage causing inflammation and therefore a symptomatic UTI. Patients with low expression of Toll-like receptor (TLR)-4 are more likely to contract asymptomatic UTIs, indicating its importance in urinary tract infections (Minardi et al, 2011). After IL8 is produced it binds to the receptor CXCR-1 which facilitates the movement of neutrophils leading to pyuria. First episodes of asymptomatic UTIs are more often caused by 

*Klebsiella* and *E. faecalis*.

The inflammatory response plays an important role in pregnancy. In early pregnancy a mild systemic inflammatory response is activated with pro-inflammatory mediators such as prostaglandin E2, TNF, IL-1 and IL-6. These inflammatory markers promote the remodelling of vessels and placental invasion to ensure adequate foetal growth throughout the pregnancy. When the placenta reaches its full size, vascular remodelling is complete and in uncomplicated pregnancies at this point the inflammation remains suppressed until labour (Lain et al, 2011). Paternal-foetal antigens from semen are processed by dentritic cells and presented to T cells in the uterine draining lymph nodes. The process is facilitated by seminal plasma factors (Gomez-Lopez et al., 2014). Regulatory T cells that are antigen specific proliferate and create foetal-maternal tolerance allowing for implantation. The numbers are maintained throughout pregnancy reducing inflammatory response towards paternal antigens until late in gestation (Gomez-Lopez et al., 2014). Once the patient is due, proinflammatory cytokines and PGE2 facilitate the ripening of the cervix, the membranes are ruptured and proinflammatory cytokines assist in the myometrial contractibility. Subsequently, pregnant women are more likely to have asymptomatic UTIs which can lead to more dangerous complications. Furthermore, acute infections become that much more important in pregnancy ensuring the safety of the mother and the foetus. Leucocytes that form part of both innate and adaptive immune systems such as natural killer T cells and dendritic cells are involved in the pathophysiology of preterm labour (Gomez-Lopez et al., 2014).
Other infections which affect pregnant women more severely include; influenza, hepatitis E, herpes simplex type 2 and malaria. Influenza affects pregnant women more severely due to decreased lung volume and reduced residual lung capacity. Furthermore, the increased heart rate and stroke volume due to pregnancy contributes to the increased severity of the disease (Kourtis et al., 2014). Hepatitis E is more severe in pregnant women than in immune competent patients. The mortality of pregnant women in the first trimester is high and it is a major cause of maternal death and foetal loss. This is thought to occur through acute liver failure (Kourtis et al., 2014). In 2018 there were 237 cases of Hepatitis E in Namibia with one pregnant woman dying a few days before she was due to give birth (WHO, 2018). When pregnant women are infected acutely with herpes simplex type 2 the risk of dissemination and hepatitis is increased (Kourtis et al., 2014). Pregnant women are the largest group of patients with disseminated HSV2 after immune compromised patients. The severity of infection by *P. falciparum* is largely determined by the patient’s immune system. The risk of a severe malarial infection among pregnant women is three times higher than that of non-pregnant women. The median mortality of pregnant women with malaria was reported at 39% in the Asia-Pacific region (Kourtis et al., 2014). *P. falciparum* is the most common causative species for malaria in Namibia at almost 100% followed by *P. vivax* (WHO, 2018). The infection of *P. falciparum* is increased because the parasite accumulates in the placenta selectively particularly the variants with the antigen syncytiotrophoblastic chondroitin sulfate A. Women who are not pregnant and live in endemic areas are not likely to show symptoms when infected with *P. falciparum* due to a selective immunity. When these women become pregnant and infected with chondroitin sulfate A binding *P. falciparum* the infection becomes symptomatic because these patients usually do not have immunity to these antigenic variants (Kourtis et al., 2014). *L. monocytogenes* has tropism for the placenta and during infection this species may lead to the loss of pregnancy, still birth, preterm birth or serious neonatal infection (Kourtis et al., 2014).
2.5. Disease in the general population

In 2016 there were 56.9 million deaths world-wide (WHO, 2018). The top 10 causes of death made up 54% of these deaths. Figure 2.1 below presents the top 10 global causes of death in 2016.

**Top 10 global causes of deaths, 2016**

As shown in the diagram above, ischaemic heart disease and stroke caused 15.2 million deaths in 2016. Three million deaths were caused by chronic obstructive pulmonary disease, with lower respiratory infections also claiming 3 million lives and remaining the deadliest communicable disease (WHO, 2018). Lung, trachea and bronchus cancer caused 1.7 million deaths in 2016 (WHO, 2018). Diabetes lead to around 1.6 million deaths world-wide (WHO, 2018). This is followed by road injuries in the list of top 10 global causes of deaths (WHO, 2018). Diarrhoeal diseases lead to 1.4 million deaths in 2016, followed by tuberculosis on the list with 1.3 million deaths (WHO, 2018). HIV/AIDS caused 1.0 million deaths in 2016, no longer forming part of the top 10 global causes of death.

In 2016 Africa’s population was 1.2 billion with 8.8 million deaths recorded (Jansen van Vuuren, 2017). There were 9,130 deaths in 2016 due to yellow fever across Africa. The WHO categorises the causes of death into 3 groups. Group1 is comprised of communicable diseases such as infections, maternal, new-born and nutritional conditions (Jansen van Vuuren, 2017). This group comprises 56% (5 million) of the total deaths in Africa. In this group lower respiratory tract infections made up 10.4% of total deaths, HIV/AIDS related deaths made up 8.1% and malaria made
up 4.6%. Lastly Diarrhoeal diseases and TB made up 7.4% and 4.6% of total deaths in Africa respectively (Jansen van Vuuren, 2017). Group 2 is made up of non-communicable disease most commonly associated with urbanisation and lifestyle diseases. Ischaemic heart disease made up 5.8% of total deaths in Africa in 2016. Stroke and liver cirrhosis made up 4.2% and 2% of total deaths in Africa respectively (Jansen van Vuuren, 2017). The last group is made up of deaths due to injuries such as road injuries (3.2%), interpersonal violence (1.2%) and self-harm (0.9%) (Jansen van Vuuren, 2017). The top 5 causes of death in Africa are; 1. Lower respiratory infections, 2. HIV, 3. Diarrhoeal diseases, 4. Ischaemic heart disease and 5. Parasitic and vector-borne diseases. Deaths due to HIV were 718800 in 2016, down from 1 million in 2000 (Jansen van Vuuren, 2017). Diarrhoeal deaths, due to viruses, bacteria and parasites are largely due to poor sanitation, insufficient hygiene and inadequate access to safe water. Diarrhoeal disease is the leading cause of deaths in children under the age of 5. The category parasites and vector-borne diseases resulted in 473,000 deaths, with 408,000 deaths being caused by malaria (Jansen van Vuuren, 2017).

Namibia is classified as an upper-middle class country according to the WHO (WHO, 2017). In 2015 Namibia had 265 maternal deaths per 100 000 compared the rest of Africa at 210 (WHO, 2017). The infant mortality rate in Namibia was 39/1000 live births compared to the rest of Africa at 63/1000 live births. Tuberculosis prevalence was 394/100 000 people in Namibia, compared with 303/100 000 people in the population in Africa, however the success rate for treatment of TB in Namibia was 86% (WHO, 2017). Prevention of mother to child transmission of HIV was 95% in Namibia in 2015, and 81% in the rest of Africa (WHO, 2017). The top 10 causes of deaths in Namibia in 2015 are ranked as follows 1. HIV/AIDS, 2. Lower respiratory infections, 3. Ischaemic heart disease 4. TB, 5. Cerebral vascular disease, 6. Diarrhoeal diseases, 7. Road injuries, 8. Diabetes, 9. Chronic obstructive pulmonary disease, 10. Neonatal preterm (CDC, 2017).

### 2.6. Disease in neonates

Of all childhood deaths, neonatal deaths account for 44% which is 2.9 million deaths worldwide (Molyneux, 2014). Of all neonatal deaths, about a third is caused by infection. More than 6.9 million cases of possible severe infections in neonates were reported in 2012 (Molyneux, 2014). Latin America had the highest incidence at 9.2% with the lowest death rate, whereas Sub Saharan Africa had the lowest incidence rate (6.2%) with the highest fatality rate (12.2) (Molyneux, 2014). The most common infections in neonates include meningitis, sepsis and bacteraemia (Olawale et al., 2011). Neonatal sepsis is the most common nosocomial infection in neonatal intensive care wards (Furtado et al., 2014). Neonatal sepsis can be early onset or late onset, with early onset occurring...
within the first 72 hours of birth and late onset occurring 72 after birth (Furtado et al., 2014). Due to progress in medicine, the chances of low birth rate neonate’s survival have increased. However, this is a risk factor for infection and subsequent sepsis by Enterococci (Furtado et al., 2014). Other risk factors for late onset sepsis in neonates are; increased hospital stays, prematurity and invasive procedures (Furtado et al., 2014). The most common routes of infection include central venous catheters (54%), mechanical ventilation (32%) and parental nutrition (32%) (Furtado et al., 2014). Neonatal Enterococcal sepsis presents with fever, lethargy, respiratory distress and bacteraemia (Winn et al., 2006).

Infections can be prevented firstly through antenatal care; immunization against tetanus, prophylaxis against group B Streptococcus, prevention of prematurity and HIV transmission (Molyneux, 2014). Deliveries should be done by skilled health care professionals and midwives. A study in Asia showed that when the umbilical cord was washed with chlorhexidine it led to a 23% decrease in neonatal mortality (Molyneux, 2014). Early and exclusive breast feeding is best, and neonates should be kept warm always observing the warning signs: abnormal temperature, poor feeding, difficulty breathing, convulsions and reduced spontaneous movement (Molyneux, 2014). The World Health Organization recommends empirical treatment of neonates with infection with ampicillin or penicillin and gentamicin (Molyneux, 2014). Almost half of infant deaths in Pakistan occur within the first 28 days (Ali et al., 2013). In a study done in Pakistan, prematurity and infection were the main reasons for neonate admission into hospitals followed by birth asphyxia and neonatal jaundice (Ali et al., 2013). Prematurity is the cause of 27.9% of the deaths while infection makes up 20.33%. The most common infections were sepsis (70.8%), pneumonia (12.6%) and acute gastroenteritis (8.22%) (Ali et al., 2013). The most common nosocomial infection in neonatal units is neonatal sepsis with the frequency increasing due to the increased survival of infants (Furtado et al., 2014). Infants born prematurely, with low birth weight, that have had invasive procedures done and infants with prolonged periods of hospitalization (Furtado et al., 2014). The neonatal gastrointestinal tract is colonized from oral ingestion of breast milk, or through the vaginal and gastrointestinal flora of the mother during birth (Shantala et al., 2014). At birth the infant gastrointestinal environment is aerobic, gradually becoming anaerobic over a few days. Enterococcus and E. coli colonize the gastrointestinal tract first which establish the anaerobic environment allowing obligate anaerobes such as Firmicutes such as Clostridia, Bacteriodes and Bifidobacteria to colonize too (Houghteling et al., 2016). Intestinal colonization can be disrupted due to caesarean section, premature delivery and administration of antibiotics before or immediately after birth (Walker, 2016). This disruption can hamper the development of the host.
immune system and predispose the neonate to inflammation (Walker, 2016). Early onset sepsis is more likely to have been acquired from the mother, unless the baby was delivered via caesarean section. Late sepsis presents as septicaemia and pneumonia 72 hours after birth (Furtado et al., 2014). Infants are at risk of infection with Enterococcus due to its long periods of survival on the hands of health care professionals and health care instruments such as thermometers and stethoscopes (Furtado et al., 2014). In neonatal bacteraemia and septicaemia 10% are caused by Enterococci (Shantala et al., 2014). These serious infections are difficult to treat due to the intrinsic resistance to cephalosporins, lincosamides, nalidixic acid, aminoglycosides (low level), clindamycin (low level) and penicillinase resistant penicillins (low level) (Shanala et al., 2014). Enterococcus has the ability to acquire resistance through plasmids and transfer resistance to other bacteria such as S. aureus (Shatala et al., 2014). Studies have shown that after hospitalization ampicillin resistant E. faecalis strains replace ampicillin susceptible E. faecium strains that form part of the commensals (Montealegre et al., 2016).

2.7. Prevalence of Enterococci

Eighty to eighty seven percent of health care-associated infections worldwide are caused by around 17 microorganisms including Enterococci (McKimm et al., 2018). Over a year clinical isolates were collected from a tertiary care hospital in Bangalore, India, and 2.3% of them were Enterococcal species (Sreeja et al., 2012). Seventy six percent of these isolates were E. faecalis and 24% were E. faecium (Sreeja et al., 2012). Another study in India compared the prevalence of VRE colonization of the gastrointestinal tract in the medical intensive unit (MCU) and in the Paediatric intensive care unit (PICU) (Amberpet et al., 2016). The results showed that in the MCU, 29% of patients were colonized with VRE with only 19% in the PICU (Amberpet et al., 2016). E. faecalis made up 62.6% of these VRE whereas E. faecium only made up 38.4% (Amberpet et al., 2016). Furthermore, amongst patients who were colonized with VRE, 4.7% developed infections with VRE (Amberpet et al., 2016). In Nepal compared gastrointestinal colonization with Enterococci with the vanA gene which encodes glycopeptide resistance (Subramanya et al., 2018). Out of all rectal swabs screened, 52.59% of the isolates were Enterococci, with 33.1% of them being multidrug resistant (MDR) (Subramanya et al., 2018). All isolates collected had the vanA gene, with 16.2% of the community participants with MDR isolates, and 43 of the hospitalized patients (Subramanya et al., 2018). A study looked at the prevalence of Enterococci in the oral cavity in a healthy Brazilian population and found that 40 (16.6%) participants had commensal oral Enterococci (Komiyama et al., 2016).
In Istanbul a study was conducted to determine the prevalence of VRE in patients with haematological malignancies and consequently febrile neutropenia. In the patients with VRE, 81% of the isolates were *E. faecium* and 19 were *E. faecalis* (Habip et al., 2014). Among the 50 patients with VRE, 4% developed bacteraemia with VRE (Habip et al., 2014).

In a study using clinical samples of paediatric patients in a specialized hospital in Ethiopia the prevalence of *Enterococcal* species was 5.5% (Toru et al., 2018). Twenty two percent of these isolates were vancomycin resistant with the overall antibiotic resistance at 95.5% (21 out of 22 clinical isolates) (Toru et al., 2018). These isolates also had various virulence factors such as haemolysin (45.5%), gelatinase production (68.2%) and biofilm formation (77.3%) (Toru et al., 2018). Another study in Ethiopia observed the correlation between *Enterococci* colonization in the gastrointestinal tract in HIV positive and HIV negative patients and found no statistically significant difference (Abebe et al., 2014). The overall colonization with *Enterococcus* amongst these participants was 88.9% of which 5.5% were vancomycin resistant strains (Toru et al., 2014). A study in Jimma Ethiopia looked at the prevalence of *Enterococcal* colonization in hospitalized patients and found that 76% gastrointestinal colonization (Abamecha et al., 2015). *E. faecium* was the most common *Enterococcal* species making up 35.1%, followed by *E. faecalis* (29.8%), *E. gallinarum* (17.5%), *E. casseflavus* (8.8%) and *E. durans* (8.8%) (Abamecha et al., 2015). In this study multiple drug resistance was found in *E. faecium*, as exhibited by 89.5% of these isolates (Abamecha et al., 2015). In another study in Ethiopia looking at the prevalence of *Enterococcus* species in clinical isolates in a hospital, 15 out of 422 (3.6%) samples were *Enterococci* (Ferede et al., 2018). In a study conducted at a South African tertiary care hospital the total prevalence during the study period of VRE in patients residing there was 14.5% (Lochan et al., 2016).

Treatment of *Enterococcal* infections depends on the species and subsequent intrinsic resistance patterns, the resistance patterns of the clinical isolate, and the location and severity of infection (Hollenbeck et al., 2012). Uncomplicated infections with isolates without acquired resistance usually only require monotherapy, usually with ampicillin. Complicated infections such as endocarditis require two antibiotics exhibiting synergistic properties (Hollenbeck et al., 2012).

### 2.8. Antimicrobial susceptibility of *Enterococci*

Antibiotic resistance can be intrinsic and common throughout the species of bacteria or it can be acquired through exchange of resistance genes through plasmids and transposons (Bertelloni et al.,
The excessive use of antibiotics in both medicine and agriculture is what has led to the widespread antibiotic resistance. In agriculture, antibiotics are used as growth promoters and for preventing disease in livestock, however the antibiotics leak into the environment through the stool and urine of the livestock leading to the development of resistant environmental strains of bacteria (Lobanovska et al., 2017). Due to the high rates of resistance alternative methods of infection treatment should be used and researched. Examples include the use of metal nanoparticles, genetically engineered bacteria, antimicrobial peptides and phage therapy (Lobanovska et al., 2017). In addition, Enterococci genomes are capable of the exchange of large fragments of chromosomal DNA and lack the clustered regularly spaced short palindromic repeats (CRISPR) elements aiding in the formation of resistance seen in Enterococci involved in nosocomial infections (Arias et al., 2012). Enterococcus further spreads resistance through genetic exchange of information through plasmids. Pheromone-responsive plasmids are found largely in E. faecalis. Pheromones that are made up of lipoprotein fragments and are released by the recipient cells and sensed by donor cells which the results in the stimulation of aggregation substance (Asa 1 and Prg B) encoded by the plasmid (Hollenbeck et al., 2012). Aggregation substance stimulates recipient-donor contact when it interacts with the Enterococcal binding substance (EBS) on the surface of the recipient cell. Plasmids then transmit genetic information between the E. faecalis strains (Hollenbeck et al., 2012). The plasmids in E. faecalis have a narrow host range through which they can transmit genetic information at a higher rate ($10^{-3}$/ donor cell in 4 hours of mating) than broader host range plasmids. E. faecium has a similar plasmid, pRUM, which also transfer genetic information at a high frequency in a narrow range (Hollenbeck et al., 2012). Broad range plasmids transfer genetic information at a lower frequency, however they can also transfer information to gram positive and gram negative bacteria than pheromone responsive plasmids. Broad range plasmids are responsible for the transfer of vancomycin resistance genes to S. aureus (Hollenbeck et al., 2012). The following 3 family transposons are important for most gene mobility in Enterococci: 1. Tn3 family transposons, 2. Composite transposons and 3. Conjugative transposons (Hollenbeck et al., 2012). The prototypical Tn3 family transposons are Tn917 and confer resistance to macrolides, lincosamides and streptogramin and Tn1546 confers resistance to glycopeptides (Hollenbeck et al., 2012). The prototypical conjugative transposon is Tn916 which confers resistance to, minocycline and tetracycline (Hollenbeck et al., 2012). Composite transposons are readily formed by interaction with related IS elements (Hollenbeck et al., 2012). The movement of IS element allow resistance genes to become mobile and further promotes integration of plasmids with other plasmids and with bacterial chromosomal material (Hollenbeck et al., 2012). There are 3 mechanisms of resistance that are most common in environmental samples: 1. Acetylation of the
antibiotic, 2. Efflux of the antibiotic, 3. Dimethylation of the 23S rRNA targets (Hollenbeck et al., 2012).

*E. faecalis* is intrinsically resistant to the following antibiotics and antibiotic classes: fusidic acid, ceftazidime, aminoglycosides (low level resistance), erythromycin, clindamycin, quinupristin/dalfopristin and sulphonamides (EUCAST, 2019). *E. faecium* is intrinsically resistant to the following antibiotics and antibiotic classes: fusidic acid, ceftazidime, cephalosporins, aminoglycosides (low level resistance), erythromycin, vancomycin and sulphonamides (EUCAST, 2019). *E. gallinarum* and *E. casseliflavus* are intrinsically resistant to fusidic acid, ceftazidime, cephalosporins, aminoglycosides (low level resistance), erythromycin, clindamycin, quinupristin/dalfopristin, vancomycin and sulphonamides (EUCAST, 2019).

2.9. Antibiotics and drug classes

2.9.1. Penicillins

Penicillin is composed of a 4 membered β-lactam ring which binds irreversibly to the active site of transpeptidase, preventing the crosslinking of PGN. The structural integrity of the cell wall is then compromised, leaving the bacterial cell vulnerable to lysis due to osmotic stress (Lobanovska et al., 2017). In some bacterial species the incomplete formation of the cell wall leads to the creation of reactive oxygen species which also leads to the death of the cell (Hollenbeck et al., 2012). Penicillin was originally extracted from *Penicillium notatum* as benzylpenicillin or penicillin G. It was more effective against gram positive bacteria, as gram negative bacteria have outer membrane that acts as a selective barrier from what can affect the cell wall. Additionally, gram negative bacteria have genes which encode for β-lactamases (Lobanovska et al., 2017).

β-lactamases are enzymes which cleave the β-lactam ring of penicillin, thereby inactivating the antibiotic (Miller et al., 2015). With the increased use of penicillin, resistance started developing which lead to the production of second generation penicillins such as oxacillin; methicillin and dicloxacillin and third generation penicillins such as amoxicillin and ampicillin. Amoxicillin and ampicillin have proved to be more effective against gram negative bacteria such as *Haemophilus influenzae; E. coli; Salmonella* and *Shigella* species (Lobanovska et al., 2017). The last generation of penicillins include carboxypenicillins which have a broader spectrum of coverage including *P. aeruginosa* (Lobanovska et al., 2017). The first record of antibiotic resistance was in 1940 by *E. coli* through production of β-lactamases, and by 1960 more than 80% of hospital acquired *S. aureus* was penicillin resistant. Methicillin resistant is conferred through the presence of PBP-2a which
have reduced penicillin affinity (Lobanovska et al., 2017). In addition to resistance the prevalence of penicillin allergies also plays a role in treatment. In the USA up to 8% of patients using the health care system have a penicillin allergy, less than 1% have cephalosporin allergies and the other antibiotic classes are even rarer (Macy, 2014). When a patient starts experiencing an allergic reaction it is recommended that they stop treatment, which can spur resistance, this then leaves fewer treatment options.

Enterococci express low affinity PBP’s which bind weakly to β-lactam antibiotics. Enterococcus faecium expresses PBP5 and E. faecalis expresses PBP4 (Hollenbeck et al., 2012). Penicillin binding protein-4 is identical to the resistance gene against penicillin in S. aureus encoded Staphylococcal β-lacatamase transposon Tn552 (Hollenbeck et al., 2012). The minimum inhibitory concentration for penicillin in E. faecalis is 2-8µg/ml and 8-16µg/ml in E. faecium which is higher than in other gram positive bacteria (Hollenbeck et al., 2012). Once Enterococcus is exposed to the minimum inhibitory concentrations of penicillin it removes the reactive oxygen species that are produced when the cell wall is not fully formed (Hollenbeck et al., 2012). This is referred to as tolerance. As such penicillin should not be prescribed for Enterococcus infections alone even when it exhibits susceptibility in vitro (Hollenbeck et al., 2012). Enterococcus faecium additionally gains resistance to penicillin through point mutation through selective pressure from antibiotics. Moreover, chromosome to chromosome transfer of PBP-5 genes has been documented and contributes to the emergence of high-level penicillin resistance (Hollenbeck et al., 2012).

The use of fifth generation cephalosporins is a risk factor for the acquisition of an Enterococcal infection due to the intrinsic resistance of both E. faecalis and E. faecium (Kristich et al., 2014). The following proteins are necessary for intrinsic cephalosporin resistance: 1. The low affinity penicillin-binding protein PBP5, 2. A 2-component signal transduction system CroRS, 3. Transmembrane Ser/Thr kinase, IreK and 4. One of the enzymes involved in peptidoglycan precursor synthesis, MurAA (Kristich et al., 2014). Like S. aureus, Enterococcus encodes for the genes blaZ, blaR1 and blal (Miller et al., 2015). The blaR1 gene is involved in signal transducing, blal is a repressor gene and blaZ encodes for the β-lactamase enzyme (Miller et al., 2015). This resistance operon is expressed in much lower levels in Enterococcus than in Staphylococcus and as such, at lower concentrations of the bacteria, β-lactams can still be effective antibiotics. However, at a larger inoculum, the concentration of the β-lactamases increases linearly, and treatment will not be effective (Miller et al., 2015). Penicillins, specifically amoxicillin, are used with clavulanic acid which is a β-lactamase inhibitor increasing the chances of treatment success.
2.9.2. Aminoglycosides

*Enterococcus faecium* and *E. faecalis* express intrinsic resistance to aminoglycosides due to their inability to enter the cell (Hollenbeck *et al.*, 2012). When *Enterococci* are exposed to aminoglycosides with a cell wall inhibitor such as penicillin higher intracellular concentrations are possible (Hollenbeck *et al.*, 2012). The combination of penicillins and aminoglycosides results in synergism and is recommended for treatment. Certain *Enterococcal* species express enzymes chromosomally which increase the MIC for aminoglycosides preventing synergism (Hollenbeck *et al.*, 2012). An example of this is the aminoglycoside 6’acetyltransferase [ACC(6’)-I] which causes resistance to tobramycin and kanamycin in *E. faecium* (Hollenbeck *et al.*, 2012). Low level resistance to dibekacin, tobramycin and kanamycin is caused by efmM- encoded m5C methyltransferase in *E. faecium* (Hollenbeck *et al.*, 2012). The methylation of 16S rRNA by efmM results in a ribosomal target site which is altered and no longer effective. The synergistic activity between cell wall inhibitors and aminoglycosides remains the choice of treatment for severe *E. faecalis* and *E. faecium* (Hollenbeck *et al.*, 2012).

Due to the acquisition bifunctional enzymes encoded by APH(2’’)-la-ACC(6’)-le the MICs of aminoglycosides have increased to the range of 2,000µg/ml and 128,000µg/ml (Hollenbeck *et al.*, 2012). These enzymes inactivate gentamicin and similar aminoglycosides by phosphorylation and acetylation respectfully. The gene aph(2’’)-la-acc(6’)-le is commonly flanked by IS256 in a composite transposon designated Tn4001 in *S. aureus* and Tn5281 in *E. faecalis* (Hollenbeck *et al.*, 2012). Other genes such as aph(2’’)-lc, aph(2’’)-ld and aph(2’’)-lb do not confer resistance to gentamicin as much as the afore mentioned gene. These genes however, still show resistance to the synergism with cell wall inhibitors (Hollenbeck *et al.*, 2012). Gentamicin and streptomycin are relied on for their synergism with penicillins in serious *Enterococcal* infections and can then no longer be used (Hollenbeck *et al.*, 2012). High level resistance to streptomycin is caused by adenylyltransferases such as ant(6’)-la and ant(3’)-la which are formed from single point mutations (Hollenbeck *et al.*, 2012). These enzymes increase the MIC of streptomycin to a range of 4,000 to 6,000µg/ml, whereas ribosomal mutations result in MICs as high as 128,000µg/ml.

2.9.3. Glycopeptides

Thirty percent of clinical isolates in the United States are resistant to glycopeptides (Hollenbeck *et al.*, 2012). Inc18 type plasmids are broad host range plasmids which are known to transfer vancomycin resistance to *S. aureus*, particularly vanA (Hollenbeck *et al.*, 2014). Vancomycin inhibits
cell wall synthesis by binding to the D-ala-D-ala terminus of the pentapeptide precursor. By replacing the D-ala-D-ala with D-ser or D-lac the cell biosynthetic enzymes cannot use these substrates for transglycosylation and transpeptidation (Kristich et al., 2014) (Hollenbeck et al., 2012). At least 7 enzymes are needed to confer resistance, furthermore full resistance requires the elimination of normal precursors.

The vanA cassette requires the following enzymes: vanA, vanH, vanX, vanY, vanZ, vanS and vanR. These genes are regulated by a 2-component sensor-transducer system made up of vanS and vanR which are part of the vanA operon on Tn1546 (Hollenbeck et al., 2012). VanS is a sensory kinase and vanR is a response regulator. Together they are responsible for sensing the presence of glycopeptides and are transcribed from a mutual promoter (Kristich et al., 2014). The remaining genes from the vanA cassette are transcribed from a separate promoter (Kristich et al., 2014). Activated vanS and vanR increase the transcription of vanA, vanH, vanX, vanY and vanZ through interaction with specific promoter region (Hollenbeck et al., 2012). VanA confers resistance to vancomycin and teicoplanin whereas VanB confers resistance to vancomycin only, although resistance to teicoplanin can be induced after exposure (Hollenbeck et al., 2012). VanC conveys intrinsic resistance glycopeptides in E. gallinarum and E. casseliflavus. Operons that encode D-lac ligases confer high level aminoglycoside resistance (MICs of >256µg/ml) such as vanA, vanB, vanD and vanM. Operons that encode D-ser ligases result in low level resistance such as vanC, vanE, vanG, vanL and vanN (Hollenbeck et al., 2012). VanA is transferred horizontally on the transposon Tn1546 which is part of the prototypical Tn3 family. Figure 2.2 below shows how the van genes act to confer resistance to aminoglycosides in Enterococcus.

![Figure 2.2: Glycopeptide resistance demonstrated through the van locus.](image)

The diagram above illustrates the resistance mechanism of Enterococcus to aminoglycosides

(Adapted from Hollenbeck et al., 2012)
When \textit{vanS} senses the presence of vancomycin it autophosphorylates transferring the phosphoryl group to an aspartate residue on \textit{vanR}. This leads to \textit{vanR} dimerization (Kristich \textit{et al.}, 2014). This enhances the \textit{vanR} binding to the 2 promoters in the van locus leading to the transcription of all genes in the van locus. \textit{VanS} also acts as a phosphatase preventing the expression of \textit{vanR} through the phosphorylation by other kinases in the cell in the absence of glycopeptides (Kristich \textit{et al.}, 2014). \textit{VanH} dehydrogenase converts cellular pyruvate to d-lactate as viewed in Figure 2.2. (Hollenbeck \textit{et al.}, 2012). \textit{VanA} ligates D-ala to D-lac. Host enzymes ligate D-ala-D-lac to the tripeptide precursor, yielding low affinity pentapeptide precursor (Hollenbeck \textit{et al.}, 2012). \textit{VanX} hydrolyses D-ala-D-ala to its constituent amino acids making D-ala-D-lac the only substrate available for cell wall synthesis. \textit{VanY} D,D carboxypeptidase removes any normal pentapeptide precursor, rendering it useless for cell wall construction(Kristich \textit{et al.}, 2014; Hollenbeck \textit{et al.}, 2012). When \textit{VanZ} is present it reduces susceptibility to teicoplanin, however the exact mechanism by which it induces resistance is unknown.

The presence of \textit{vanB} results in moderate to high level resistance with teicoplanin susceptibility (Kristich \textit{et al.}, 2014). \textit{VanB} is typically acquired on the transposons Tn5382 and Tn1549 which can be present on plasmids or chromosomes of the host cell (Kristich \textit{et al.}, 2014). \textit{VanB} also encodes a two component sensor-transducer system, however it is different from the \textit{vanS} and \textit{vanR} of \textit{vanA}, and therefore designated by \textit{vanR}' and \textit{vanS}'(Kristich \textit{et al.}, 2014). \textit{VanS} has a short amino acid sequence, connecting 2 transmembrane helices outside the cell surface membrane (Kristich \textit{et al.}, 2014). Alternatively, \textit{vanS}' is larger, and is folded in the extracellularly where it serves to recognise signals (Kristich \textit{et al.}, 2014). These two sensor kinases recognise and respond to different signals in the presence of vancomycin to cause resistance (Kristich \textit{et al.}, 2014). \textit{VanR}' can be induced in the absence of \textit{vanS}' (Kristich \textit{et al.}, 2014). The \textit{vanB} cassette encodes for \textit{vanH}, \textit{vanB} (the D-ala-D-ala ligase), \textit{vanZ} and \textit{vanY}. \textit{VanB} however has \textit{vanW} instead of \textit{vanZ} (Kristich \textit{et al.}, 2014).

### 2.9.4 Quinolones

Quinolone resistance presents through 1. Mutation which prevent the binding of the antibiotic and 2. Efflux of the antibiotic out of the bacterial cell (Kristich \textit{et al.}, 2014). These efflux pumps are usually non-specific, and their presence usually leads to multidrug resistance. The genome of \textit{E. faecalis} encodes around 34 of these multi drug resistant efflux pumps encoded on VS83 (Kristich \textit{et al.}, 2014).
2.9.5. Lincosamides, streptogramins and Macrolides

*E. faecalis* is intrinsically resistant to clindamycin (lincosamide), quinupristin (streptogramin B class) and dalfopristins (streptogramin A class) (Hollenbeck et al., 2012). This resistance is expressed from the *Isa* gene which encodes for an efflux pump which is structurally similar to the ATP-binding cassette efflux pumps (Hollenbeck et al., 2012). For phenotypic resistance to quinupristin-dalfopristin to occur, bacteria should be resistant to both streptogramin A and streptogramin B (Hollenbeck et al., 2012). Quinupristin-dalfopristin is FDA approved for the treatment of VRE (Hollenbeck et al., 2012). The most common resistance mechanism against macrolides is the methylation of an adenine on 23S rRNA of the 50S ribosomal subunit (Kristich et al., 2014). Clindamycin, a commonly used antibiotic against *Enterococcus* forms part of this group of antibiotics.

*E. faecium* has a different efflux pump encoded by *MrC* gene and confers low level resistance to streptogramin and is also involved in the horizontal transfer of resistance genes (Hollenbeck et al., 2012). The prototypical family transposon Tn917 confer resistance to macrolides, lincosamides and streptogramins (Hollenbeck et al., 2012).

2.9.6 Tetracyclines

The transposon Tn916 is a prototypical conjugative transposon which confers resistance to minocycline and tetracycline (Hollenbeck et al., 2012). The genes involved in conferring resistance to tetracycline and minocycline include *tetK* and *tetL* amongst many others (Miller et al., 2015). These genes act through the efflux of the antibiotic out of the bacterial cell and through the prevention of the ribosomes from binding. The efflux pump is synthesized in the presence of tetracycline as in this condition the ribosome complex is unable to synthesize a normal peptide, and as a result forms an alternative loop structure in the mRNA and the second binding site becomes accessible (Miller et al., 2015). The genes that confer resistance further include *tetM*, *tetO* and *tetS* which are chromosomal resistance determinants (Miller et al., 2015)

2.9.7 Trimethoprim-sulfamethoxazole

*Enterococcus* is able to absorb folate from the environment therefore even if the antibiotic sensitivity test shows that *Enterococcus* is susceptible to Trimethoprim-sulfamethoxazole, in the presence of environmental folate it will bypass this mechanism (Hollenbeck et al., 2012).
2.9.8 Oxacolidnones

Oxacolidnones (including linezolid) are completely synthetic and bind to the initiation complex and inhibit protein synthesis (Hollenbeck et al., 2012). Specifically, linezolid inhibits the formation of the 70S initiation complex (Kumar et al., 2014). Enterococcus faecalis has 4 genes encoding for 23S rRNA whereas E. faecium has 6. This, however, does not prevent resistance sporadic mutations as recombination between resistant and susceptible genes occurs leading to resistance (Hollebeck et al., 2012). The mutation of one rRNA gene in E. faecium leads to MICs of 8-16µg/ml, while mutations of more than one rRNA gene leads to MICs of 64-128µg/ml (Hollenbeck et al., 2012). The most important gene involved in linezolid mutation is G2576 in the domain V of the 12S ribosomal genes of Enterococcus (Kumar et al., 2014). The gene cfr encodes rRNA methyltransferase which changes the adenosine in the linezolid-binding region on 23S rRNA (Hollenbeck et al., 2012). First identified in S. aureus, cfr is a source of resistance in linezolid, lincosamides and streptogramins (Hollenbeck et al., 2012).

2.9.9 Nitrofurantoin

Nitrofurantoin is a synthetic antibiotic that was formed from furan and had a nitro group added with a side chain containing hydrantion (Squadrito et al., 2019). Nitrofurantoin is used solely in uncomplicated UTIs as the concentrations in plasma do not become high enough for bactericidal activity in other infections. This however is considered a good thing as consequently nitrofurantoin does not affect the normal flora of the bowel (Squadrito et al., 2019). The mechanism of nitrofurantoin is not well understood however; it is said that it binds to ribosomes and inhibits bacterial enzymes. The inhibition of bacterial enzymes leads to the inhibition of RNA, DNA and cell wall protein synthesis (Squadrito et al., 2019).

2.9.10 Daptomycin

Daptomycin is used in cases of multi resistant gram positive bacterial infections (Kristich et al., 2014).
2.10. Disk diffusion method

Table 2.10.1 Zone of inhibitions in Antibiotics

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive ≥ (mm)</th>
<th>Resistance &lt; (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amipicillin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Penicillin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Augmentin</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Quinupristin-dalfopristin (E. faecium)</td>
<td>22</td>
<td>20</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Linezolid</td>
<td>19</td>
<td>19</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Vancomycin</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Teicoplanin</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>Nitrofurantoin (only used in uncomplicated UTIs)</td>
<td>15</td>
<td>15</td>
</tr>
</tbody>
</table>

(Adapted from EUCAST, 2019)

In the table above the antibiotic susceptibility is detailed according to the zones of inhibition (EUCAST, 2019). Susceptibility to ampicillin, amoxycillin and piperacillin can be inferred from ampicillin susceptibility (EUCAST, 2019). Enterococcus faecium isolates resistant to penicillin can be considered resistant to all other Beta lactam antibiotics (EUCAST, 2019).

Enterococci are intrinsically resistant to aminoglycosides and therefore two antibiotics that are synergistic should be prescribed. For example, aminoglycosides and penicillins or glycopeptides. Furthermore, gentamicin can be used to screen for high level aminoglycoside resistance (HLAR) (EUCAST, 2019). A negative test with low level aminoglycoside resistance should be ≥8 mm and positive test would be <8 mm. Streptomycin resistance should be tested independently from other aminoglycosides (EUCAST, 2019). While E. faecium has low level intrinsic resistance aminoglycosides it also produces an enzyme that affects the synergism of aminoglycosides and penicillins or glycopeptides (EUCAST, 2019). Susceptibility to vancomycin should exhibit sharp zone edges with no colonies in the inhibition zone. If the zone edge is not clear and sharp confirmatory testing should be done even if the inhibition zone is ≥12mm (EUCAST, 2019).

Vancomycin resistant Enterococci that have vanA gene grow up to the vancomycin disc. These isolates are also resistant to teicoplanin (The CDS reference laboratory, 2018). E. faecalis that contain the vanB gene presents with an annular radius of less than the 2mm, and a hazy edge. E. faecium with the vanB phenotype have an inhibitory annular radius of up to 3mm light growth near
the vancomycin 5 µg (The CDS reference laboratory, 2018). *E. gallinarum* and *E. casseliflavus* naturally possess *vanC* phenotype (The CDS reference laboratory, 2018).

### 2.11. Genes coding for resistance

*E. gallinarum* has intrinsic resistance to vancomycin whereas *E. casseliflavus* has inherent low-level resistance (Ligitha et al., 2018). These species however are not very common in human disease. *E. faecalis* and *E. faecium* acquire their resistance from other organisms through plasmids (Ligitha et al., 2018). The genes involved in vancomycin resistance include *vanA*, *vanB*, *vanC*, *vanD* and *vanE* with the most common being *vanA* (Ligitha et al., 2018). In a study conducted in India it was found that *E. faecium* is most commonly isolated with vancomycin resistance (Ligitha et al., 2018). The minimum inhibitory concentrations from these isolates were 64-1000µg/ml for vancomycin and 16-512 µg/ml in teicoplanin. These highly resistant isolates contain *vanA* and *vanB* genes and varied resistance to vancomycin (Ligitha et al., 2018).
CHAPTER 3

METHODOLOGY
3.1 Study population:
It consisted of pregnant women at 35 – 37 weeks of gestation that attended the antenatal clinic at the Windhoek central hospital, Windhoek, Namibia. It is estimated that there were around 60 000 live births in Namibia in 2013 (WHO, 2015).

3.2 Sampling technique:
A convenience sampling technique was used. Patients were identified as they entered the clinic for their antenatal care. Patients between 35-37 weeks of gestation, who fell within the inclusion criteria were asked to participate in the study. Patients who consented to participation were requested to fill in a consent form and then a sample was taken. Furthermore, all specimens of participants who fell in the inclusion criteria and consented during the specimen collection period were used in the study. Patients were sampled consecutively.

3.3 Sample size
The sample size was calculated using a formula proposed by (Charan et al., 2013) at 95% confidence level and 0.05 degree of accuracy. The prevalence used in this calculation was established using the following formula proposed by WHO:

\[
\frac{\text{Number of new cases during a specified period}}{\text{Size of population at start of period}} = \text{Prevalence}
\]

Equation 3. The equation used to calculate Prevalence (Adapted from WHO, 2015).

- 60 000 live births during 2013 (WHO, 2015)
- 2 303 000 population size during 2013 (WHO, 2015)

Prevalence was 2.61

The sample size was calculated at 39.1 pregnant women between 35 and 37 weeks gestation.

3.4 Inclusion criteria:
Pregnant women between 35 – 37 weeks of gestation who attended the antenatal clinic at the Windhoek central hospital, Windhoek, Namibia.
3.5 Exclusion criteria:

Pregnant women who were either on antibiotic therapy or who received antibiotic therapy up to two weeks prior to the study were excluded from the study. Patients who were below 35 weeks of gestation or above 37 weeks of gestation were excluded including patients under the age of 18, as they could not consent to the study.

3.6 Sample collection

Vaginal, rectal and urine samples were collected from participants who fit the inclusion criteria and attended the Windhoek Central Hospital Antenatal clinic. The samples were collected in accordance with the CDC guidelines for GBS sample collection. There were no guidelines for Enterococcal specimens from pregnant women. Essentially, these guidelines aim to ensure the safety of the mother and foetus during sample collection. A lower vaginal swab was collected by a registered nurse. This means the swab was first inserted 2 cm into the vagina, then the external genitalia were swabbed including the labia (Khan et al., 2016).

The rectal specimen was collected by inserting the swab through the anal sphincter and swabbing the walls of the rectum (Lerner et al., 2013). A midstream urine specimen was collected regardless of urinary tract infections symptoms from a separate group of participants that fit the acceptance criteria. This means that the participant first urinated into the toilet, thereby ‘washing away’ all normal flora, then urinated into the specimen cup and voided her bladder into the toilet. The procedure was explained to the participants by the clinician before the sample collection. Samples were transported to the laboratory using maintaining the cold chain.

3.6.1 Testing for Enterococcus

The vaginal and rectal swabs were cultured within four hours from collection, samples that were cultured the next day were kept in the refrigerator at 2-8°C. Urine samples were all cultured the same day and stored at 2-8°C.

Samples were inoculated on 5% sheep blood agar and MacConkey agar and incubated at 37°C for 24 hours. The cultures were checked for gamma or beta haemolysis with small silver-like/translucent colonies on blood agar and small deep pink (magenta) colonies on MacConkey agar (Shanmukhappa et al., 2015). The colonies were then inoculated on bile esculin. Colonies that turned the agar black were considered bile esculin positive, and this was considered as the presumptive confirmation of Enterococcus species. Presumptive colonies were stored in 10% glycerol at -20°C. Speciation was not done by biochemical methods.
3.6.2 Antibiotic sensitivity testing

The isolates that were stored in 10% glycerol were resuscitated by streaking on blood agar and incubated at 37°C for 24 hours. A 0.5 MacFarland solution was made for each isolate and inoculated conflually on Muller Hilton agar using a sterile swab. The antibiotic discs were placed on the inoculated agar and incubated at 37°C for 24 hours. The zone of inhibition of each antibiotic was measured with a ruler after incubation and recorded.

Antibiotic sensitivity was done using the Kirby-Bauer disc diffusion method with the following antibiotic discs: Penicillin (30iu), clindamycin (2mcg), vancomycin (30mcg), amoxicillin (10 mcg), amoxicillin-clavulanic acid (30mcg), imipenem (10mcg), ciprofloxacin (5 mcg), cephalothin (30mcg), gentamicin (10 mcg) and nitrofurantoin (100ug). Nitrofurantoin was only tested for in urine samples as the plasma concentration does reach a high enough plasma concentration to be used for the treatment of other infections (Banjeree et al., 2015). Results were interpreted using CLSI guidelines, 2017

3.6.3 Molecular testing:

3.6.3.1 DNA extraction

A colony was taken from each isolate from the Muller Hilton agar and emulsified in separate 2ml Eppendorf tubes with 200µl RNAse and DNAse free water, then vortexed. These tubes were placed on a heating block at 100°C for 10-15minutes. Boiling causes the bacterial cells to lyse, releasing the DNA. Thereafter the tubes were spun for 6 minutes at 1000rpm and the supernatant was aspirated and separated from the pellet. The supernatant contained the extracted Enterococcal DNA material. The DNA samples were stored at -80°C (Mukesi et al., 2019).

3.6.3.2 PCR:

Each primer was reconstituted as per manufacturers guidelines. A working solution from each of the primer was made consisting of 30µl of the primer and 270µl DNAse and RNAse free water. Both these solutions were stored at -80°C. The reaction mixture consisted of 1µl each of forward and reverse primers (as seen in Table 3.2.) respectively, 12µL Master mix (Inqaba biotec, Pretoria, South Africa) and 1µL of DNAse and RNAse free water. The primers used are tabulated below in Table 3.1.
Table 3. 1: Oligonucleotide primers for species confirmation, virulence factors and antibiotic resistance

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>bp</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>CATGAATAGAATAAAAGTTGCAATA</td>
<td>1,030</td>
<td>(Kariyama et al., 2000)</td>
</tr>
<tr>
<td>vanA</td>
<td>CCCCTTAAACGCTAATACGATCAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>vanB</td>
<td>GTGACAAACCGGAGGCGAGGA</td>
<td>433</td>
<td>(Kariyama et al., 2000)</td>
</tr>
<tr>
<td>vanB</td>
<td>CCGCCTACCTCTGTGCAAAAAAA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tetM</td>
<td>GGAAAATACGAAGGTAACA</td>
<td>289</td>
<td>(Santiago-Rodriguez et al., 2013)</td>
</tr>
<tr>
<td>tetM</td>
<td>GAATCCCCATTTTCTTAAGT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaZ</td>
<td>ACTCTTTGCGATGTAAGCT</td>
<td>173</td>
<td>(Gulzar et al., 2018)</td>
</tr>
<tr>
<td>blaZ</td>
<td>TGA CCA TTA TTA CCA ACC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>blaR1</td>
<td>AATCTGCAAGAAGAGTTAG</td>
<td>537</td>
<td>(Ida et al., 2002)</td>
</tr>
<tr>
<td>blaR1</td>
<td>TTCCTTCATTACACTCTTGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecalis</td>
<td>ATCAAGTACAGTTAGTCTTTATAG</td>
<td>941</td>
<td>(Kariyama et al., 2000)</td>
</tr>
<tr>
<td>E. faecalis</td>
<td>ACGATTTAAGCTAACTGAATG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. faecium</td>
<td>TTGAGGCAGACCAGATTTGACG</td>
<td>658</td>
<td>(Kariyama et al., 2000)</td>
</tr>
<tr>
<td>E. faecium</td>
<td>TATGACAGGACGCTCCATCCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gelE</td>
<td>ACCCGGTATCATTTGGT</td>
<td>419</td>
<td>(Comerlato et al., 2013)</td>
</tr>
<tr>
<td>esp</td>
<td>TTGCTTAATGCTAGTGCCGGAC</td>
<td>933</td>
<td>(Comerlato et al., 2013)</td>
</tr>
<tr>
<td>agg</td>
<td>AAGAAAAAGAAGTAGACCA</td>
<td>1,553</td>
<td>(Comerlato et al., 2013)</td>
</tr>
<tr>
<td>agg</td>
<td>AAACGGGCAAGATAGGAATAA</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Nineteen PCR tubes were labelled 0-18, 0 being the negative control and 1-18 represented the isolates. In each of these PCR tubes, 20µl of reaction mixture was pipetted, and then 5µl of Enterococcal DNA template was added. The PCR conditions were as follows, 94°C for 4 minutes for 1 cycle, 93°C for 1 minute, the annealing temperatures as detailed in the Table 3.2 below; elongation at 72°C for 1 minute for 35 cycles followed by a cycle of 72°C for 7 minutes and finally held at 4°C for storage until further testing.

ATCC reference strains E. faecalis ATCC 29212 and E. faecium ATCC 2317 were used as positive controls while S. pyogenes was used as a negative control in all PCR assays.
Table 3. 2: PCR annealing temperatures of the different genes used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annealing temperature used</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>50°C</td>
</tr>
<tr>
<td>vanB</td>
<td>56°C</td>
</tr>
<tr>
<td>agg</td>
<td>46°C</td>
</tr>
<tr>
<td>gelE</td>
<td>46°C</td>
</tr>
<tr>
<td><em>E. faecalis</em> and <em>E. faecium</em> confirmation</td>
<td>54°C</td>
</tr>
<tr>
<td>esp</td>
<td>56°C</td>
</tr>
<tr>
<td><em>blaZ, blaR</em></td>
<td>47°C</td>
</tr>
<tr>
<td><em>tetM</em></td>
<td>47°C</td>
</tr>
</tbody>
</table>

3.6.3.3 Gel electrophoresis

A 2% agarose gel was prepared using 0.5 TBE (Tris borate ethylenediaminetetraacetic acid) buffer. Four grams of agarose gel was placed into a conical flask in 200ml of TBE buffer. The flask was microwaved until the agarose gel dissolved and swirled every few seconds to avoid the solution from boiling over. Once the solution was clear and the agarose gel had completely dissolved, the conical flask was set aside to cool down. Once the solution cooled down to around 50°C, 5µl of ethidium bromide was pipetted and mixed into the solution. The solution was then poured into a setting tray with two sets of combs to set. After around 30 minutes the gel was ready for use and gently placed in an electrophoresis chamber with the same TBE buffer used in the making of the agarose gel. The agarose gel was slightly covered with the TBE buffer.

One microlitre of loading buffer was added to 5µl of the respective amplicons and dispensed into the wells in the prepared gel, including the negative control. Three microliters of 100bp ladder was added on each row of sample wells. The negative control ensured no non-specific amplification of DNA, and the ladder assisted in the measurement and subsequent identification of the genes amplified. The gel electrophoresis was run at 110 volts for 45 minutes. The agarose gels were viewed on an UV transilluminator and photographed.

3.7. ETHICAL CONSIDERATIONS

Permission to carry out the study was received from the NUST and MoHSS ethical committees. Thereafter, written consent was received from each participant. No patient names or results were disclosed as a unique number was allocated to each participant, and all data was kept completely confidential in a password protected computer.
Samples were collected from November 2018 to May 2019, in which a total of 193 participants that fit the inclusion criteria participated in the study. An estimated 45 participants refused to participate in the study during this period. The age range was 18 to 50 years and the majority of the patients (39.9 %) fell with the age range of 25-30 years of age with an average of 29 years of age. The average gravidity was 2.31 and the average parity was 2.09. Urine samples amounted to 101 and from a separate group of participants the vaginal swabs and rectal swabs amounted to 92 samples each.

Table 4.1: Frequency distribution of Enterococci isolates according to sample type

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Frequency (n)</th>
<th>Prevalence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal swabs</td>
<td>15</td>
<td>7.8</td>
</tr>
<tr>
<td>Vaginal swabs</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>Random urine</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>Cumulative total</td>
<td>20</td>
<td>10.3</td>
</tr>
</tbody>
</table>

The total number of Enterococcus isolates recovered from the 193 participants was 20, giving a prevalence of 10.3%. Table 4.2 below illustrates the molecular confirmation of the Enterococcus isolates collected.

Table 4.2: Molecular confirmation of Enterococci using species specific primers

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>E. faecalis Frequency (%)</th>
<th>E. faecium Frequency (%)</th>
<th>Other Enterococci Frequency (%)</th>
<th>Total Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal swabs</td>
<td>4 (30.8)</td>
<td>1 (7.7)</td>
<td>8 (61.5)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Vaginal swabs</td>
<td>1 (33.3)</td>
<td>0 (0.0)</td>
<td>2 (66.7)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Urine swabs</td>
<td>1 (50.0)</td>
<td>0 (0.0)</td>
<td>1 (50.0)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

Two of the 20 presumptive isolates could not be recovered after freezing in glycerol, and as such only 18 isolates were confirmed by molecular testing. The E. faecalis isolates made up 30.8% of the isolates collected from the rectal swabs, with other Enterococcal species making up 61.5%. The only E. faecium isolates was recovered from the rectal swabs. At least one E. faecalis isolate was identified from each specimen type. The antibiotic sensitivity patterns of the isolates are presented in Table 4.3 below.
Table 4. 3: Frequency distribution of antibiotic susceptibility in *Enterococcus* isolates

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Sensitive Frequency (%)</th>
<th>Intermediate Frequency (%)</th>
<th>Resistant Frequency (%)</th>
<th>Total Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillin (30 iu)</td>
<td>18 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Amoxicillin (2 mcg)</td>
<td>18 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Augmentin (30 mcg)</td>
<td>18 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Ciprofloxacin (5 mcg)</td>
<td>16 (88.8)</td>
<td>1 (5.6)</td>
<td>1 (5.6)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Clindamycin (2 mcg)</td>
<td>5 (28.0)</td>
<td>0 (0.0)</td>
<td>13 (72.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Vancomycin (30 mcg)</td>
<td>18 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Gentamicin (10 mcg)</td>
<td>8 (44.5)</td>
<td>4 (22.2)</td>
<td>6 (33.3)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Cephalothin (30 mcg)</td>
<td>7 (38.9)</td>
<td>4 (22.2)</td>
<td>7 (38.9)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Imipenem (10 mcg)</td>
<td>16 (88.9)</td>
<td>2 (11.1)</td>
<td>0 (0.0)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>Nitrofurantoin (100 ug)</td>
<td>2 (100.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

Most of the isolates were resistant to clindamycin and sensitive to penicillin, amoxicillin, augmentin and vancomycin. In these isolates 83.3% were resistant to at least 1 antibiotic. The frequency distribution of genes encoding for drug resistance in the *Enterococcus* isolates is presented in Table 4.4. The percentages presented in the table are calculated from the number of isolates that are sensitive/intermediate/resistant and the total number of isolates.

Table 4. 4: Frequency distribution of antibiotic resistance genes screened in *Enterococci* isolates

<table>
<thead>
<tr>
<th>Enterococcal species</th>
<th>Total frequency of isolates (n)</th>
<th>vanA Frequency (%)</th>
<th>vanB Frequency (%)</th>
<th>tetM Frequency (%)</th>
<th>blaZ Frequency (%)</th>
<th>blaR1 Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>6</td>
<td>1 (16.7)</td>
<td>3 (50.0)</td>
<td>0 (0.0)</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>1</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other <em>Enterococci</em></td>
<td>11</td>
<td>1 (9.1)</td>
<td>1 (9.1)</td>
<td>1 (9.1)</td>
<td>3 (60.0)</td>
<td>1 (9.1)</td>
</tr>
</tbody>
</table>

No antibiotic resistance genes were found in *E. faecium*, however it should be considered that only one isolate was recovered. The *vanB* gene was found mostly in *E. faecalis*. The most common
The most common gene which tested positive was *gelE* while *esp* was not identified in any of the isolates. One isolated had the *agg* gene. *gelE* and *agg* were each found in 16.75% of *E. faecalis* isolates. *gelE* was found in 9.1% of the other *Enterococcal* species.
CHAPTER 5

DISCUSSION
In this study samples from a total of 193 patients attending the Antenatal clinic at Windhoek Central hospital were tested, with 10.3% prevalence of *Enterococci* as shown in Table 4.1. Furthermore, *Enterococcus* was recovered from 30.8% of the rectal swabs. This was less than the study done in Ethiopia by Abamecha *et al.*, (2015) in which 76% of *Enterococcal* isolates were isolated from stool samples in hospitalized patients (Abamecha *et al.*, 2015). It should be noted that the study done in Ethiopia included patients that were hospitalized, and literature shows that patients are more likely to be colonized with *Enterococcus* in hospital settings. A study by Miller *et al.*, (2004) in the United States of America reported a prevalence of 68% of *Enterococcus species* in rectal and vaginal swabs which is higher than the current study at 10.3% as shown in Table 4.1 (Miller *et al.*, 2004). However, the current study has a prevalence of *Enterococcus* which is similar to a study done by Ghasemi *et al.*, (2016) in Iran on pregnant women which reported a prevalence of *Enterococcus* species at 8.6% (Ghasemi *et al.*, 2016). *Enterococcus* had a prevalence of 8% in a study in Uganda done by Ngonzi *et al.*, (2018) which is similar to the current study (Ngonzi *et al.*, 2018). However, a study conducted in Denmark by Stockholm *et al.*, (2013) showed a prevalence of *Enterococci* of 24% which is higher than the current study (Stockholm *et al.*, 2013). A study done in India by Sibi *et al.*, (2014), showed a prevalence of 6.7% in urine in pregnant women (Sibi *et al.*, 2014). This is relatively similar to the current study with 1.5% of *Enterococcal* isolates found in urine. In a university hospital in Egypt Toru *et al.*, (2018) established that the overall prevalence of *Enterococcus* in clinical isolates from paediatric patients was 5.5% which is less than this current study with a prevalence of 10.3% (Toru *et al.*, 2018). This difference could be attributed to the difference in age groups of the studies however no direct correlation between *Enterococcal* colonization and age has been made in previous studies. In a study in Iran by Saffari *et al.*, (2017) the faecal colonization with *Enterococcus* was 37% compared to a frequency of 16.3% of *Enterococcus* in rectal swabs in the current study (Saffari *et al.*, 2017). Another different study conducted in Egypt by Hashem *et al.*, (2017), reported 64% *Enterococcal* isolates with 72.2% being *E. faecalis* and 24.4% being *E. faecium* (Hashem *et al.*, 2017). The remaining isolates were *E. casseflavus* and *E. galinarum* as tested using VITEK 2. The study conducted in Ethiopia by Wondwossen *et al.*, (2014) investigated whether there was a relationship between *Enterococcal* infection and colonization in patients living with HIV (Wondwossen *et al.*, 2014). The results did not show a statistically significant connection between *Enterococcal* infection or colonization and HIV infection. A study done in Brazil by Soares *et al.*, (2014), showed that 92.1% of the clinical isolates obtained were *E. faecalis* and 7.9% were *E. faecium* (Soares *et al.*, 2014). A study done in Iran by Kafil *et al.*, (2013), the *Enterococcal* isolates were made up of 56.9% *E. faecalis* and 43.1% *E. faecium* (Kafil *et al.*, 2013). Research conducted in Istanbul by Habip *et al.*, (2014) looked for *Enterococcal* colonization in patients with haematological
malignancies and showed a prevalence of 44% of patients were colonized with Enterococcus (Habip et al., 2014). This is more than this study with 16.3% GIT colonization with Enterococcus in pregnant women. This could be due to the fact that patients with haematological malignancies have immune systems which are impaired more so than in pregnant women. In a study in India by Amberpet et al., (2016) gut colonization studied for the presence of Enterococcus in patients in the ICU, the prevalence was 29% (Amberpet et al., 2016). In the Enterococcal isolates 62.6% were *E. faecalis* and 38.4% were *E. faecium* compared to 30.8% *E. faecalis* and 7.7% which were *E. faecium* in the rectal swabs (Habip et al., 2016). In a study done in Nepal, 52.59% of the rectal swabs screened were positive for Enterococcus (Subramanya et al., 2018). In a study done in Namibia by Jitaleni et al., (2015), the prevalence of Enterococcus in urine was 3.5% which compares well with the prevalence of 1.5% found in this study. This was the only Namibian study in literature that was available for comparison to this study.

As seen in Table 4.1, only 1.0% of isolates were found in urine while there were higher rates of prevalence in the swabs. This could be attributed to the fact that in urine is sterile, and not meant to have any bacteria, unless the patient has a urinary tract infection. Only urine samples with a colony count more than 10 000 were considered in this study. Women are more likely to have urinary tract infections due to the smaller distance between the urethra and the anus. The source of the infection therefore is largely the bacteria colonizing the gastrointestinal tract (Minardi et al., 2011). A larger percentage of Enterococcal isolates were found in the rectal swabs seen as 7.8% in Table 4.1. because Enterococcus is known to colonize the gastrointestinal tract (Goh et al., 2017). Consequently, the presence of Enterococcus in clinical isolates is due to its abundance in the GIT (Goh et al., 2017). This is also thought to be the source of Enterococci found colonising the vagina at a prevalence of 1.5% shown in Table 4.1. In screening for Enterococcus, the preferred method would be testing for rectal swabs or stool samples. However, in hospitalised patients the source of infection is usually not the patient’s own gastrointestinal flora, but from the hospital equipment and the hands of hospital staff (Agudelo-Higuita et al., 2014).

One isolate was confirmed as *E. faecium* and 6 isolates were confirmed as *E. faecalis* by molecular techniques as presented in Table 4.2. The remaining isolates were considered non-*faecalis*, non-*faecium* Enterococcal species as molecular confirmation was not done for the other species of Enterococci. The results of this current study are different from the study done in Iran by Ghasemi et al., (2016) where the prevalence of *E. faecalis* was 89.8% (Ghasemi et al., 2016). *E. faecalis* made up 33.3% of the isolates in this study, while *E. faecium* made up 5.6% of isolates. The remaining (61.1%) were other Enterococci and were not confirmed through molecular testing in this study. In a study done in Ethiopia by Abamecha et al., (2015), *E. faecalis* made up 29.8% of the isolates which
is similar to this current study, but conversely *E. faecium* made up 35.1% of the clinical isolates (Abamecha *et al*., 2015). In a study done in India by Sreeja *et al*., (2012) in which stool specimens were collected, 76% of the bacterial isolates recovered were *E. faecalis* and 24% were *E. faecium* and none of the other species under *Enterococcus* were isolated (Sreeja *et al*., 2012). This is different from the current study in which the prevalence of *E. faecalis* and *E. faecium* was 30.8% and 7.7% respectively in rectal swabs as seen in Table 4.2. It should be noted that in the study in India, speciation was done through biochemical testing and not molecular testing. In a study in India by Padmasini *et al*., (2014), 157 *Enterococcus* isolates were recovered from various tertiary care hospitals, 53% of which were *E. faecalis* and *E. faecium* made up 46.5% (Padmasini *et al*., 2014). Differences in prevalence and recovery rates of *Enterococcus* could be due to different populations being used with some studies using the general population while others use selected groups like pregnant women as is the case with this current study. Other factors which can contribute to differences in prevalence rates could be because of type of sample used (urine, stool, low vaginal swab, rectal swab); sampling technique; methods for culture and identification of bacteria. In this study most of the isolates were other *Enterococci* which did not form part of the species that are considered the most important in human disease. *E. faecalis* has been proven to have virulence factors while *E. faecium* has shown higher rates of resistance in previous studies. The cause of the lack of these two species in the study could be attributed to the fact that this study looked at colonization and not clinical isolates.

Table 4.3 show that the isolates in this study were all sensitive for penicillin, amoxicillin, augmentin (amoxicillin-clavulanic acid), nitrofurantoin and vancomycin. A study done in Iran by Ghasemi *et al*., (2016) showed similar results as all the isolates that were recovered in that study were also sensitive to amoxicillin and vancomycin (Ghasemi *et al*., 2016). The proportion of gentamicin sensitivity in the study done on urinary tract infections in India by Sibi *et al*., (2014), was 33.3%, which is similar to the one reported in this current study as shown in Table 4.3. However, the frequency of gentamicin resistance in this study is different to the study done in Uganda by Ngonzi *et al*., (2018) in which none of the isolates were resistant to gentamicin. In the same study all isolates were sensitive to vancomycin which is the same as the current study (Ngonzi *et al*., 2018). In this current study isolates were sensitive to imipenem and ciprofloxacin (88.9%) and the highest frequency of resistance was reported against clindamycin (72.0%) which is different from the study done in India by Sibi *et al*.,(2014) in which clindamycin sensitivity was reported as 75% (Sibi *et al*., 2014). Resistance to cephalothin was 38.9% in this current study as shown in Table 4.3. The study in Iran done by Saffari *et al*., (2017), the *Enterococcal* isolates had gentamicin resistance at 38% which compared to this study with gentamicin resistance at 33.3%. In the study in Iran, 93% of
isolates were resistant for ciprofloxacin and conversely 88.9% of isolates sensitive for ciprofloxacin in this study (Saffari et al., 2017). The current study compared well to the study done in Egypt by Hasheem et al., (2017) with 96.7% sensitivity to vancomycin and 100% sensitivity to vancomycin in this study (Hasheem et al., 2017). In the same study sensitivity to ciprofloxacin was 60.8%, compared to 88.9% in this study. Isolates were 46.7% sensitive to gentamicin compared to 44.4% in this study (Hashem et al., 2017). Research done in an Indian tertiary hospital by Biswas et al., (2016), Enterococcal isolates from clinical samples 7.4 % showed vancomycin resistance and 5.6 showed reduced susceptibility (Biswas et al., 2016). This does not compare to the current study in which there was no vancomycin resistance. In the isolates in the current study, 83.3% were resistant to at least 1 antibiotic, which is less than the 90% of isolates observed in a study done in Ethiopia by Wondwossen et al., (2014) (Wondwossen et al., 2014). All the isolates were sensitive for penicillin, amoxicillin, amoxicillin-clavulanic acid and vancomycin. In the same study in Ethiopia, 81.8% and 54.5% of isolates were resistant to amoxicillin and amoxicillin-clavulanic acid respectively (Wondwossen et al., 2014). In a study in China by Jia et al., (2014), all the Enterococcal isolates were sensitive to vancomycin which is the same as this study as seen in table 4.4 (Jia et al., 2014). In a study done in Ethiopia by Abamecha et al., (2015) looking at the colonization of the GIT by Enterococci, 5% exhibited resistance to vancomycin similar to the current study which showed no resistance to vancomycin in rectal swabs (Abamecha et al., 2015). In a study in a teaching hospital in Ethiopia done by Ferede et al., (2018), 53.3% of Enterococcal isolates were resistant to ciprofloxacin compared to 5.6% of resistance in this study, and 5.6% of isolates that tested intermediate (Ferede et al., 2018). In the same study 60% of isolates were resistant to gentamicin compared to 33.3% of isolates in this study (Ferede et al., 2018). The study done in Brazil by Soares et al., (2014) compared well in nitrofurantoin with 97.04% of urine isolates sensitive, and in the current study 100% of urine isolates were sensitive. Similarly, vancomycin sensitivity was 91.7% compared to this study with vancomycin sensitivity of 100% (Soares et al., 2014). The vancomycin and gentamicin resistance were 33.8% and 83.9% respectively in E. faecium, and 16.3% and 88.1% in E. faecalis in a study done in Iran by Kafil et al., (2013) (Kafil et al., 2013). In the study in Istanbul, E. faecalis made up 81% of the vancomycin resistant Enterococcal isolates, while the remaining were E. faecium (Habip et al., 2014). In the study done in Nepal by Subramanya et al., (2018) 33% of the isolates were multi- drug resistant compared to this study where 83% of isolates were resistant to at least one antibiotic. In a research done in a university hospital in China by Wei et al., (2014), the vancomycin resistance was 66% and the penicillin resistance was 88% (Wei et al., 2014). This did not compare well to the current study were there was no vancomycin and penicillin resistance as seen in Table 4.3. In a study done in Namibia by Jitaleni et al., (2015),
the sensitivity of vancomycin was 99.3% which compares well to this study with a 100% phenotypic sensitivity to vancomycin (Jitaleni et al., 2015). Conversely the 97% of the isolates found in that study were resistant to amoxicillin, in comparison to this study with 100% sensitivity. This could be due to the fact that the study conducted by Jitaleni et al., (2015) was done from data from the Namibian Institute of Pathology (NIP), in which a large percentage of the patients are hospitalised. Furthermore, amoxicillin is the empirical choice of antibiotic for a large host of infections and as such the prevalence of resistance is much higher in hospital settings than in community settings.

In Table 4.4 it is observed that the *E. faecium* isolate recovered did not test positive for any resistance genes. All isolates were vancomycin susceptible even though 2 isolates tested positive for *vanA*, and 4 tested positive for *vanB*. *vanA* encodes for high level resistance however, if the full *vanA* locus is not present high-level resistance will not be conveyed phenotypically. Four isolates tested positive for *vanB* which conveys a lower level of resistance than *vanA*. The gene *vanC* would have been significant in this study considering that isolates that are non-*E. faecalis* and non-*E. faecium* can harbour *vanC* chromosomally conferring intrinsic resistance. One isolate tested positive for *tetM* and was found in the other *Enterococcus* species along with 60% of the *blaZ* gene. *E. faecalis* isolates tested positive of the isolates tested positive for *blaZ* and *blaR1*, at 40% and 33.1% respectively. In a study done in India by Phukan et al., (2016), 24% of the *Enterococcal* isolates were vancomycin resistant and 56.25% of these isolates had the *vanA* gene (Phukan et al., 2016). This does not compare well to the current study in which all the isolates were sensitive to vancomycin and *vanA* was recovered from 2 isolates as seen in table 4.4. In a study in Nepal conducted by Subramanya et al., (2018), in which all of the *Enterococcal* isolates that were multi-drug resistant had the *vanA* gene (Subramanya et al., 2018). This does not compare well to the current study as *vanA* was only recovered from 2 isolates, neither of which exhibited phenotypic vancomycin resistance. A study done in Germany by Werner et al., (2012) showed that 79% of clinical *E. faecium* isolates from neonates had the *vanA* gene (Werner et al., 2012). In this study no *vanB* genes were found in *E. faecium*. However, *vanB* was found in 50% of the *E. faecalis* isolates as seem in Table 4.4. In a study done in India by Amberpet et al., (2016) All the vancomycin resistant isolates in this study had *vanA* (Amberpet et al., 2016). In the study in China conducted by Wei et al., (2014) 5.0% of isolates harbouried the *vanA* gene and 31.0% harboured the *tetM* gene. In comparison to this study in which 16.7% of *E. faecalis* isolates and 9.1% of *E. faecium* isolates had the gene *vanA* and 9.1% of the other *Enterococci* had the gene *tetM* gene. In a study done in the United states of America by McBride et al., (2007) of clinical isolates of *Enterococcus*, the prevalence of *vanA* was 7.1%, *vanB* was 22%, *blaZ* was 16.0% and *tetM* was 61% (McBride et al., 2007). In this study the prevalence of *vanA* was 16.7% in *E. faecalis* and 9.1% in other *Enterococci,
the vanB was 50.0% on *E. faecalis* and 9.1% in other *Enterococci*, tetM was 9.1% in other *Enterococci*, blaZ was 33.3% in *E. faecalis* and 60.0% in other *Enterococcus* and blaR1 was present in 33.3% of *E. faecalis* isolates and 9.1% in other *Enterococci*.

Both agg and gelE were found in *E. faecalis*, with one of the other *Enterococcal* species also harbouring gelE. The esp gene was not identified in any of the isolates as shown in Table 4.5. In a study done in Egypt by Toru *et al.* (2018) 31.8% of the identified *Enterococcus* isolates were positive for biofilm production, haemolysin and gelatinase (Toru *et al.*, 2018). In the same study overall prevalence of biofilm formation was 77.3%, 45.5% for haemolysin production and 68.2% was positive for gelatinase production (Toru *et al.*, 2018). A study done in Egypt by Toru *et al.* (2018), the phenotypical gelatinase prevalence was 68.2%, which is higher than this study with a prevalence of 16.7% in *E. faecalis* and 9.1% in other *Enterococcus* species (Toru *et al.*, 2018). No genotyping was done in this study and as such it could result in a higher prevalence of gelE. A study in India done by Padmasini *et al.* (2014) reported that 10.19% of isolates showed phenotypic gelatinase activity, but 51.59% were positive for gelE (Padmasini *et al.*, 2014). In the study conducted in Iran by Saffari *et al.*, (2017), 71.8% of positive isolates tested positive for gelE which did not compare well to this study with 16.7% prevalence in *E. faecalis* and 9.1% in the other *Enterococci* species (Saffari *et al.*, 2017). This could be attributed to the fact that the study in Iran tested clinical isolates which are therefore already proven to be virulent as opposed to isolates that are colonising the vaginal and gastrointestinal tract. Unlike this current study where no esp genes were found, the study in Iran done by Saffari *et al.*, (2017) had a prevalence of 76.7% of the esp gene (Saffari *et al.*, 2017). In a study done in India by Biswas *et al.*, (2016) the most common virulence gene was gelE with 44.4% in the vancomycin resistant isolates and 16.4% in vancomycin sensitive isolates (Biswas *et al.*, 2016). In a study done in Brazil by Soares *et al.*, (2014) 73.3% of the isolates were positive for gelE, 70% were positive for esp and 58.3% were positive for agg. This does not compare well to this study with only 16.7% positive for agg in the *E. faecalis* isolates and 16.7% and 9.1% of *E. faecalis* and other *Enterococci* positive for gelE respectively. A study done in Brazil by Comerlato *et al.*, (2013) showed a gelE prevalence of 60% in clinical isolates, different from the 11.1% in this study (Comerlato *et al.*, 2013). In a study in Brazil by Soares *et al.*, (2014), 58.3% of the clinical isolates harboured the agg gene (Soares *et al.*, 2014). And only 16.7% of *E. faecalis* had agg in the current study as shown in table 4.5. In a study done in India, 30.43% of *E. faecalis* and 27.77% of *E. faecium* tested positive for the gelE gene, compared to 16.7% of *E. faecalis* isolates and no *E. faecium* isolates testing positive in this study (Banerjee *et al.*, 2015). In the same study 17.39% of *E. faecalis* and 11.11% of *E. faecium* isolates tested positive for the esp gene (Banerjee...
et al., 2015). The esp factor was not found in this study. A study in Brazil showed that 70% of the clinical isolates had the esp gene (Soares et al., 2014). In a study done in Bulgaria by Strateva et al., (2016) the prevalence of esp was 44.3%, agg at 38.4% and gelE at 64.3% in clinical Enterococcus isolates (Strateva et al., 2016). The virulence factors in that research were the same that were done in this research however the overall prevalence of agg and gelE were 16.7% each in E. faecalis isolates, with 9.1% of other Enterococci testing positive for gelE and no esp genes.
In this study samples from a total of 193 patients attending the Antenatal clinic at Windhoek Central hospital were tested, with 10.3% prevalence of *Enterococci*. In rectal swabs 30.8% of the isolates were *E. faecalis*, 7.7% were *E. faecium* and 61.5% were other *Enterococci*. In vaginal swabs, 33.3% were *E. faecalis* and 66.6% were other *Enterococci*. Half the isolates found in urine were *E. faecalis* and the rest was other *Enterococci*. In *E. faecalis*, 16.7% were positive for *vanA*, 50.0% were positive for *vanB*, *blaZ* were positive for 33.3% and *blaR1* were positive for 33.3% isolates. In the *E. faecalis* isolates 16.7% were positive for *agg* and 16.7% were positive for *gelE*. *E. faecium* did not test positive for any virulence or antibiotic resistance genes. The other *Enterococcus* species were not confirmed with molecular methods, with 9.1% being positive for *vanA*, 9.1% positive for *vanB*, 9.1% positive for *tetM*, 60.0% positive for *blaZ* and 9.1% positive for *blaR1*. The other *Enterococci* were also positive for *gelE* with a prevalence of 9.1%. The majority of the isolates collected in this study were not the most common species found in human disease namely *E. faecalis* and *E. faecium*. This then becomes important to speciate, in order to better treat patients when faced with species with intrinsic resistance. Furthermore, virulence genes were only found in 3 isolates. Although there are resistance genes present in these isolates, empirical antibiotics would still be effective. This is good considering the global trend of resistance.

**RECOMMENDATIONS**

- A study looking at the virulence factors and antibiotic sensitivities of *Enterococcus* in pregnant women should be carried out using a larger sample size and collected from different areas in Namibia.
- More studies should be conducted around *Enterococcus* in general, for example in clinical isolates due to the lack of statistics available.
- In future studies on *Enterococcus* the other species of *Enterococcus* should be identified through molecular techniques as well. The other *Enterococcal* species have intrinsic resistance harbouring genes such as *vanC*, making speciation important for treatment.
- With a prevalence of 10.3% of *Enterococcus* in pregnant women, screening pregnant patients can be of importance considering the high rates of maternal and neonatal deaths as reported in Namibian newspapers.
- Future studies can include the screening of neonates for *Enterococcus* to determine prevalence.
LIMITATIONS OF THE STUDY

- This study was limited due to the small sample size and the collection of samples from only one Antenatal clinic.
- All the species of *Enterococcus* weren’t identified and confirmed through molecular methods.
- Two isolates in this study could not be revived after freezing and as such no further testing could be done on these isolates.
- In future studies the phenotypic virulence of isolates can be tested as well as exploring more virulence and resistance genes.
- The quality and concentration of DNA extracted could not be determined.
REFERENCES


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Scheiverff, P., Chuang-Smith, O., Peterson, M., Cook, L., & Dunny, G. (2010). Enterococcus faecalis Endocarditis Severity in Rabbits is Reduced by IgG Fabs Interfering with Aggregation substance. *PLOS One*, 5(10), e13194.


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APPENDIX A: Consent form

I, Daniella Chantelle Mouton, am a Masters student at the Namibia University of Science (NUST) and Technology. To complete my studies, I need to carry out research entitled “Determining the virulence factors and genes coding for antibiotic resistance in Enterococci species isolated from pregnant women in Windhoek, Namibia”.

Introduction

You are invited to participate in a study on the bacteria Enterococcus. This bacteria forms part of the ‘good’ bacteria or normal flora but may have negative effects on a mother and/or her newborn baby. You are invited to participate in this study. At any point, should you feel unsafe or are no longer willing, you may opt out of the study. If there are any questions, feel free to ask.

Purpose of the research

This study is being conducted to see how many pregnant women have these bacteria, and to see whether further actions should be taken to ensure the safety of mothers and their babies.

Type of research intervention

Should you choose to participate in this study, the registered nurse will take a lower vaginal swab, a rectal swab or a urine sample from you. This will be safe for you and the baby, however, if you feel unsafe, you may choose to discontinue in this study. Furthermore, there is a short questionnaire to be filled in.

Participant selection

You have been selected for this study because you are between 35 – 37 weeks of gestation and attend the antenatal clinic at the Windhoek central hospital. You are over 18 and have not been on antibiotics for the last 2 weeks. You will still receive your Antenatal care even if you do not participate in this study. It is completely your choice to participate in this study. You will only have to participate in this study once.

Confidentiality

Your and your baby’s name will not be used in this study. You will be completely anonymous. We will assign a number to your sample to ensure that no one has access to your details.

Research results

The results from this research will be shared with NUST and the ministry of health and social services (MOHSS). The results will be shared in the following format, for example, “it was found that 24% of the patients at the Antenatal clinic are colonised with Enterococcus species.” No names will be revealed.

Right to withdraw
You can choose to withdraw from the study at any point. You should only sign the consent form, and consent to the study if you agree to it. If you have any doubt, you are free to withdraw.

Who to contact

If you have any questions you can contact me, Daniella Mouton at 0812211698 or at my email address daniellacmouton@gmail.com.

This study has been granted ethical approval by the NUST ethical committee and will be granted ethical approval by the MOHSS.

If you wish to partake in the study, please fill in all the fields in the bottom of this page.

Certificate of consent:

I have read the previous information, or it has been read to me. I have had the opportunity to ask questions and my questions were answered to my satisfaction. I consent voluntarily to be a participant in this study.

Print name of participant___________________________________________________

Signature of participant________________________

Date____________________

Statement by the researcher/person taking consent

I accurately read out the previous information to the participant, and to the best of my capability made sure that the participant understands that the following will be done:
1. A rectal/lower vaginal swab or urine specimen will be taken
2. The results of the study will be used as to complete a Masters, and will be shared with NUST, MOHSS and may potentially be published.

I confirm that I encouraged the participant to ask questions and ensured to the best of my capability that her questions were answered correctly. I have in no way forced the patient to participate in this study.
Name of researcher/person taking consent______________________________

Signature of researcher/person taking consent_________________________

Date______________
**APPENDIX B: Questionnaire**

Please answer all questions, ask if you need any help.

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<td>5. Location/area you live</td>
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APPENDIX C: Ethical Approval from the MoHSS

[Image of a letterhead with contact information]

OFFICE OF THE PERMANENT SECRETARY

Ref: 17/3/3 DM
Enquiries: Mr. J. Nghipangelwa

Date: 08 December 2017

Ms. Daniella Mouton
Namibia University of Science and Technology
Windhoek

Dear Ms. Mouton,

Re: Determining virulence factors and genes coding for antibiotic resistance in Enterococci species isolated from pregnant women in Windhoek, Namibia.

1. Reference is made to your application to conduct the above-mentioned study.

2. The proposal has been evaluated and found to have merit.

3. Kindly be informed that permission to conduct the study has been granted under the following conditions:

3.1 The data to be collected must only be used for academic purposes;
3.2 No other data should be collected other than the data stated in the proposal;
3.3 Stipulated ethical considerations in the protocol related to the protection of Human Subjects should be observed and adhered to, any violation thereof will lead to termination of the study at any stage;
3.4 A quarterly report to be submitted to the Ministry's Research Unit;
3.5 Preliminary findings to be submitted upon completion of the study;

[Signature]
APPENDIX D: Approval from the Chief Hospital superintendent at Windhoek Central Hospital

MINISTRY OF HEALTH AND SOCIAL SERVICES

Private Bag 13215
Windhoek Namibia

Harvey Street
Windhoek Central Hospital

Enquiries: Ms. S.Jipinge
Ref. Date: 27 March 2018

OFFICE OF THE CHIEF MEDICAL SUPERINTENDENT

Ms. Danielle Mouton
Namibia University of Science and Technology
0812201698
Email: danielleacmouton@gmail.com

Dear Ms. Mouton

RE: PERMISSION TO RESEARCH ON THE DETERMINING VIRULENCE FACTORS AND GENES CODING FOR ANTIBIOTIC RESISTANCE IN ENTEROCOCCI SPECIES ISOLATED FROM PREGNANT WOMEN IN WINDHOEK, NAMIBIA.

This letter serves to inform you that permission has been granted for you to conduct a study at Windhoek Central Hospital on the above mentioned subject as you have requested and does not include any remuneration.

The patients/Clients information should be kept confidential at all times.

Yours sincerely

[Signature]

DR. LOUIRAB
CHIEF MEDICAL SUPERINTENDENT

[Stamp: 27-03-2018]
APPENDIX E: Ethical approval from NUST

Dear Prof/Dr/Mr/Ms/Other(s): Ms Daniella Chantelle Mouton

Student No (if applicable): 213105942

Research Topic: Determination of virulence factors and gene coding for antibiotic resistance in Entorococi species isolated from pregnant women in Windhoek, Namibia

Supervisor (if applicable): Mr Munyaradzi Mukesi

Co-supervisor(s), if applicable: Prof Sylvester R Moyo

Qualification registered for (if applicable): Master of Health Sciences

Re: Ethical screening application No: FHAS-REC: 000024

The Research Ethics Screening Committee has reviewed your application for the above-mentioned research project. Based on the recommendation of the expert reviewer, the research as set out in the application is hereby:

| Approved: i.e. may proceed with the project | X |
| Approved provisionally: i.e. may proceed but subject to compliance with recommendation(s) listed below | |
| Not approved: Not to proceed with the project until compliance with recommendation(s) listed below and resubmit ethics application for consideration | |

Is Ministry of Health & Social Services (MoHSS) Approval required? Yes: X | No: |

It is important to note that as a researcher, you are expected to maintain ethical integrity of your research, strictly adhere to the ethical policy of NUST, and remain within the scope of your research proposal and supporting evidence as submitted to the REC. Should any aspect of your research change from the information as presented, which could have an impact or effect on any research participants/subjects/environment, you are to report this immediately to your supervisor or REC as applicable in writing. Failure to do so may result in withdrawal of approval. Kindly consult your supervisor or HoD if you need further clarification.

We wish you success in your research endeavour and are of the belief that it will have positive impact on your career as well as the development of NUST and the society in general.

Ethical issues that require compliance/must be addressed

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<td>Prior approval from the Faculty of Health and Applied Sciences at NUST and the Ministry of Health and Social Services required</td>
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NB: May attach additional page as required

Full Name (reviewer): Prof Habauka M. Kwaambwa Signature: Date: 7/09/2017

Full Name: Prof Omotayo Awofolu Signature: Date: 7/09/2017

Chair: Ethics Screening Committee
APPENDIX F: Manuscript submitted to African Journal of Laboratory Medicine

Virulence factors and genes coding for antibiotic resistance in Enterococci species isolated from pregnant women in Windhoek, Namibia

Daniella C Mouton¹, Munyaradzi Mukesi¹

¹Department of Health Sciences, Faculty of Health and Applied Sciences, Namibia University of Science and Technology, Private Bag 13388, Windhoek, Namibia

Background: Research into Enterococci is increasingly important as Enterococci was classified as high priority bacteria regarding antimicrobial resistance. Improvements in medical care have increased the chances of survival of infants with low birth weight, therefore increased duration of hospital stays and consequently increased risk of infection by Enterococcus. Therefore, it is important to monitor colonization of the maternal genitourinary tract to prevent mother to child transmission of these bacteria.

Objectives: To determine virulence and gene-based resistance in Enterococci species isolated from pregnant women between 35 and 37 weeks gestation in Windhoek.

Methodology: Rectal, vaginal swabs and urine were collected and cultured for presumptive identification. The isolates species were confirmed through molecular techniques and virulence and antibiotic resistance genes were tested and reported.

Results: The total number of isolates collected from the 193 participants was 20. The total prevalence was 10.3%. E. faecalis was positive for vanA (16.7%), vanB (50.0%), blaZ (33.3%), blaR₁(33.3%), agg (16.7%), gelE (16.7). E. faecium did not test positive for any virulence or antibiotic resistance genes. The other Enterococcus species were not speciated with molecular methods and were positive for vanA (9.1%), vanB (9.1%), tetM (9.1%), blaZ (60.0%) and blaR₁(9.1%)

Conclusion: The majority of the isolates collected in this study were not the most common species found in human disease namely E. faecalis and E. faecium. Virulence genes were only found in 3 isolates. Although there are resistance genes present in these isolates, empirical antibiotics would still be effective. This is good considering the global trend of resistance.
INTRODUCTION

Research into Enterococci is increasingly important as Enterococci was classified as high priority bacteria regarding antimicrobial resistance\(^1\). This is a highly resistant organism, especially in a hospital settings and treatment of Enterococci now involves use of highly toxic drugs such as vancomycin. A cause for more concern is that Enterococci species have been shown in some researches to be resistant to vancomycin, which was one of the few antibiotic options left for treatment of this organism. This means that treatment is very limited in cases where neonates are infected with this bacteria and neonatal mortality is high. Furthermore, patients who have neonatal meningitis are 10 times more likely to suffer neurologic impairment compared to healthy patients\(^2\). Enterococci are not routinely screened in pregnant women in Namibia despite the risk of vertical transmission from colonized mothers to their new-born babies who can subsequently develop meningitis and other neurologic disorders as noted by WHO. This study therefore aims to determine maternal colonization by Enterococci, the genetic factors spurring virulence and drug resistance in these bacterial isolates in pregnant women in Windhoek.

Aims and objectives: The study aimed to determine virulence and gene-based resistance in Enterococci species isolated from pregnant women between 35 and 37 weeks gestation in Windhoek. To determine the frequency distribution of Enterococcus species found among pregnant women between 35 and 37 weeks gestation in Windhoek, to establish the antibiotic resistance patterns of the Enterococcus species and to screen for the resistance and virulence genes found in the Enterococcus isolates.

Study design: It consisted of pregnant women at 35 – 37 weeks of gestation that attended the antenatal clinic at the Windhoek central hospital, Windhoek, Namibia. It is estimated that there were around 60 000 live births in Namibia in 2013\(^3\).

Study population and sampling strategy: A convenience sampling technique was used, patients who fell within the inclusion criteria and consented were be asked to participate in the study. Patients who consented to participation were requested to fill in a consent form and then a sample was taken. Furthermore, all specimens of participants who fell in the inclusion criteria and consented during the specimen collection period were used in the study. Patients were sampled consecutively.

Inclusion criteria: Pregnant women between 35 – 37 weeks of gestation who attended the antenatal clinic at the Windhoek central hospital, Windhoek, Namibia.

Exclusion criteria: Pregnant women who were either on antibiotic therapy or who received antibiotic therapy up to two weeks prior to the study were excluded from the study. Patients who are below 35 weeks of gestation or above 37 weeks of gestation were excluded including patients under the age of 18, as they are underage and could not consent to the study.
The sample size was calculated using a formula proposed by Charan et al., 2013 at 95% confidence level and 0.05 degree of accuracy. The prevalence used in this calculation was established using the following formula proposed by WHO:

\[
\frac{\text{Number of new cases during a specified period}}{\text{Size of population at start of period}} = \text{Prevalence}
\]

Equation 3. The equation to calculated Prevalence

\[
\begin{align*}
\text{60 000 live births during 2013}^3 \\
\text{2 303 000 population size during 2013}^3 \\
\text{Prevalence is 2.61}
\end{align*}
\]

The sample size was calculated at 39.1 pregnant women between 35 and 37 weeks gestation.

Sample collection: Vaginal, rectal and urine samples were collected from participants who fit the inclusion criteria and attended the Windhoek Central hospital Antenatal clinic. The samples were collected in accordance with the CDC guidelines for GBS sample collection. There are no guidelines for Enterococcus specimens from pregnant women. Essentially, these guidelines aim to ensure the safety of the mother and foetus during sample collection. A lower vaginal swab was collected by a registered nurse. This means the swab will first be inserted 2 cm into the vagina, then the external genitalia were swabbed including the labia.

The rectal specimen was collected by inserting the swab through the anal sphincter and swabbing the walls of the rectum. A midstream urine specimen was collected regardless of urinary tract infections symptoms from a separate group of participants that fit the acceptance criteria. This means that the participant first urinated into the toilet, thereby ‘washing away’ all normal flora, the urinated into the specimen cup and voided her bladder into the toilet. The procedure was explained to the participants by the clinician before the sample collection. Samples were transported to the laboratory using a cold chain method.

Testing process: The vaginal and rectal swabs were culture within four hours from collection, samples that were cultured the next day were kept in the refrigerator at 2-8°C. Urine samples were all cultured the same day and stored at 2-8°C.

Samples were inoculated on 5% sheep blood agar and MacConkey agar and incubated at 37°C for 24 hours. The cultures were checked for gamma or beta haemolysis with small silver-like colonies on blood agar and small deep pink colonies on MacConkey agar. The colonies were then inoculated on bile esculin. Colonies that turn the agar black were considered bile esculin positive, and this was
considered as the presumptive confirmation of Enterococcus species. Presumptive colonies were stored in 10% glycerol at -20°C. Speciation was not done by biochemical methods.

Antibiotic sensitivity testing: The isolates that were stored in 10% glycerol were resuscitated by streaking on blood agar and incubated at 37°C for 24 hours. A 0.5 MacFarland solution was made for each isolate and inoculated confluently on Muller Hilton agar using a sterile swab. The antibiotic discs were placed on the inoculated agar and incubated for at 37°C for 24 hours. The zone of inhibition of each antibiotic was measured with a ruler after incubation and recorded.

Antibiotic sensitivity was done using the Kirby-Bauer disc diffusion method with the following antibiotic discs: Penicillin, clindamycin, vancomycin, amoxicillin, amoxicillin-clavulanic acid, imipenem, ciprofloxacin, cephalothin, gentamicin and nitrofurantoin. Nitrofurantoin was only tested for in urine samples as the plasma concentration does reach a high enough plasma concentration to be used for the treatment of other infections. Results were interpreted using CLSI guidelines, 2017

Molecular testing

DNA extraction: A colony was taken from each isolate from the Muller Hilton agar and emulsified in separate 2ml Eppendorf tubes with 200µl RNase and DNase free water, then vortexed. These tubes were placed on a heating block at 100°C for 10-15minutes. Thereafter the tubes were spun for 6 minutes at 1000rpm and the supernatant was aspirated and pipetted into another labelled PCR tube. The supernatant contained the extracted Enterococcal DNA material. The DNA samples were stored at -80°C.

Polymerase chain reaction (PCR): Each primer was reconstituted as per manufacturers guidelines. A working solution from each of the primer was made consisting of 30µl of the primer and 270µl DNase and RNase free water. Both these solutions were stored at -80°C. The reaction mixture consisted of 1µl each of forward and reverse primers (as seen in Table 3.1.) respectively, 12µL Master mix (Inqaba biotec, Pretoria, South Africa) and 1µL of DNase and RNase free water. The primers used are tabulated below in Table 1.
Table 3: Oligonucleotide primers for species confirmation, virulence factors and antibiotic resistance

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA -F</td>
<td>CATGAATAGAATAAAGTTGCAATA</td>
<td>1,030&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>vanA -R</td>
<td>CCCCTTTAACGCTAATACGATCAA</td>
<td></td>
</tr>
<tr>
<td>vanB -F</td>
<td>GTGACAAACCGGAGGCGGAGGA</td>
<td>433&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>vanB -R</td>
<td>CCGCCATCCTCCTGCAAAAAA</td>
<td></td>
</tr>
<tr>
<td>tetM -F</td>
<td>GGAAATATACGAAGGTGAACA</td>
<td>289&lt;sup&gt;11&lt;/sup&gt;</td>
</tr>
<tr>
<td>tetM -R</td>
<td>GAATCCCCCATTTTCCTAAGT</td>
<td></td>
</tr>
<tr>
<td>blaZ -F</td>
<td>ACTCTTTGGCATGTGACTG</td>
<td>173&lt;sup&gt;12&lt;/sup&gt;</td>
</tr>
<tr>
<td>blaZ -R</td>
<td>TGA CCA CTT TTA TCA GCA ACC</td>
<td></td>
</tr>
<tr>
<td>blaR1 -F</td>
<td>AATCTCTGCAAGAAGAGTTAG</td>
<td>537&lt;sup&gt;13&lt;/sup&gt;</td>
</tr>
<tr>
<td>blaR1 -R</td>
<td>TTTCTTCATTACACTCTTGG</td>
<td></td>
</tr>
<tr>
<td>E. faecalis -F</td>
<td>ATCAAGTACAGTATGTCTTATTAG</td>
<td>941&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. faecalis -R</td>
<td>ACAGTTCAAAGCTAACTGAATCAGT</td>
<td></td>
</tr>
<tr>
<td>E. faecium -F</td>
<td>TTGAGGCCAGACCAGATTGACG</td>
<td>658&lt;sup&gt;10&lt;/sup&gt;</td>
</tr>
<tr>
<td>E. faecium -R</td>
<td>TATGACAGCGACTCCGATTCCC</td>
<td></td>
</tr>
<tr>
<td>gelE</td>
<td>ACCCGGTATCATTTGGTTT</td>
<td>419&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>ACGCATTTGCTTTTCCATC</td>
<td></td>
</tr>
<tr>
<td>esp</td>
<td>TTGCTAATGCTAGTCCACGACC</td>
<td>933&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>GCGTCAACACTTGCATTGCCGAA</td>
<td></td>
</tr>
<tr>
<td>agg</td>
<td>AAGAAAAAGAAGTAGACCAAC</td>
<td>1,553&lt;sup&gt;14&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>AAACGGAAGACAAGTAAATA</td>
<td></td>
</tr>
</tbody>
</table>

Nineteen PCR tubes were labelled 0-19, 0 being the negative control and 1-18 represented the isolates and 19 represented the positive control. In each of these PCR tubes, 20µl of reaction mixture was pipetted, and then 5µl of Enterococcal DNA template was added. The PCR conditions were as follows, 94°C for 4 minutes for 1 cycle, 93°C for 1 minute, the annealing temperatures as detailed in the Table 2 below; elongation at 72°C for 1 minute for 35 cycles followed by a cycle of 72°C for 7 minutes and finally held at 4°C for storage until further testing.

ATCC reference strains E. faecalis ATCC 29212 and E. faecium ATCC 2317 were used as positive controls while S. pyogenes was used as a negative control in all PCR assays.
Table 4: PCR annealing temperatures of the different genes used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Annealing temperature used</th>
</tr>
</thead>
<tbody>
<tr>
<td>vanA</td>
<td>50°C</td>
</tr>
<tr>
<td>vanB</td>
<td>56°C</td>
</tr>
<tr>
<td>agg</td>
<td>46°C</td>
</tr>
<tr>
<td>gelE</td>
<td>46°C</td>
</tr>
<tr>
<td><em>E. faecalis</em> and <em>E. faecium</em> confirmation</td>
<td>54°C</td>
</tr>
<tr>
<td>esp</td>
<td>56°C</td>
</tr>
<tr>
<td><em>blaZ, blaR</em></td>
<td>47°C</td>
</tr>
<tr>
<td><em>tetM</em></td>
<td>47°C</td>
</tr>
</tbody>
</table>

Gel electrophoresis: A 2% agarose gel was prepared using 0.5 TBE (Tris borate ethylenediaminetetraacetic acid) buffer. Four grams of agarose gel was placed into a conical flask in 200ml of TBE buffer. The flask was microwaved until the agarose gel dissolved and swirled every few seconds to avoid the solution from boiling over. Once the solution was clear and the agarose gel had completely dissolved, the conical flask was set aside to cool down. Once the solution cooled down to around 50°C, 5µl of ethidium bromide was pipetted and mixed into the solution. The solution was then poured into a setting tray with two sets of combs to set. After around 30 minutes the gel was ready for use and gently placed in an electrophoresis chamber with the same TBE buffer used in the making of the agarose gel. The agarose gel was slightly covered with the TBE buffer.

One microlitre of loading buffer was added to 5µl of the respective amplicons and dispensed into the wells in the prepared gel, including the negative control. Three microliters of 100bp ladder was added on each row of sample wells. The negative control ensured no non-specific amplification of DNA, and the ladder assisted in the measurement and subsequent identification of the genes amplified. The gel electrophoresis was run at 110 volts for 45 minutes. The agarose gels were viewed on an UV transilluminator and photographed.

Ethical permission: Permission to carry out the study was sought from the Namibia University of Science and Technology and the Namibian Ministry of Health and Social Services ethical committees. Thereafter, written consent was sought from each participant. No patient names or results were disclosed as a unique number was allocated to each participant, and all data was kept completely confidential in a password protected computer.
RESULTS

Samples were collected from November 2018 to May 2019, in which a total of 193 participants that fit the inclusion criteria participated in the study. An estimated 45 participants refused to participate in the study during this period. The age range was 18 to 50 years and the majority of the patients (39.9%) fell with the age range of 25-30 years of age with an average of 29 years of age. The average gravity was 2.31 and the average parity was 2.09. Urine samples amounted to 101 and from a separate group of participants the vaginal swabs and rectal swabs amounted to 92 samples each.

Table 3: Frequency distribution of Enterococci isolates according to sample type

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Frequency (n)</th>
<th>Prevalence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal swabs</td>
<td>15</td>
<td>7.8</td>
</tr>
<tr>
<td>Vaginal swabs</td>
<td>3</td>
<td>1.5</td>
</tr>
<tr>
<td>Random urine</td>
<td>2</td>
<td>1.0</td>
</tr>
<tr>
<td>Cumulative total</td>
<td>20</td>
<td>10.3</td>
</tr>
</tbody>
</table>

The total number of Enterococcus isolates recovered from the 193 participants was 20, giving a prevalence of 10.3%. Table 4 below illustrates the molecular confirmation of the Enterococcus isolates collected.

Table 4: Molecular confirmation of Enterococci using species specific primers

<table>
<thead>
<tr>
<th>Specimen type</th>
<th>E. faecalis</th>
<th>E. faecium</th>
<th>Other Enterococci</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
<td>Frequency (%)</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>4 (30.8)</td>
<td>1 (7.7)</td>
<td>8 (61.5)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Vaginal swabs</td>
<td>1 (33.3)</td>
<td>0 (0.0)</td>
<td>2 (66.7)</td>
<td>3 (100)</td>
</tr>
<tr>
<td>Urine swabs</td>
<td>1 (50.0)</td>
<td>0 (0.0)</td>
<td>1 (50.0)</td>
<td>2 (100)</td>
</tr>
</tbody>
</table>

Two of the 20 presumptive isolates could not be recovered after freezing in glycerol, and as such only 18 isolates were confirmed by molecular testing. The E. faecalis isolates made up 30.8% of the isolates collected from the rectal swabs, with other Enterococcal species making up 61.5%. The only E. faecium isolates was recovered from the rectal swabs. At least one E. faecalis isolate was identified from each specimen type. The antibiotic sensitivity patterns of the isolates are presented in Table 5 below.
Most of the isolates were resistant to clindamycin and sensitive to penicillin, amoxicillin, augmentin and vancomycin. In these isolates 83.3% were resistant to at least 1 antibiotic. The frequency distribution of genes encoding for drug resistance in the *Enterococcus* isolates is presented in Table 4.4. The percentages presented in the table are calculated from the number of isolates that are sensitive/intermediate/resistant and the total number of isolates.

Table 6: Frequency distribution of antibiotic resistance genes screened in *Enterococci* isolates

<table>
<thead>
<tr>
<th>Enterococcal species</th>
<th>Total frequency of Isolates (n)</th>
<th>vanA (Frequency %)</th>
<th>vanB (Frequency %)</th>
<th>tetM (Frequency %)</th>
<th>blaZ (Frequency %)</th>
<th>blaR1 (Frequency %)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. faecalis</em></td>
<td>6</td>
<td>1 (16.7)</td>
<td>3 (50.0)</td>
<td>0 (0.0)</td>
<td>2 (33.3)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>1</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other</td>
<td>11</td>
<td>1 (9.1)</td>
<td>1 (9.1)</td>
<td>1 (9.1)</td>
<td>3 (60.0)</td>
<td>1 (9.1)</td>
</tr>
</tbody>
</table>
No antibiotic resistance genes were found in *E. faecium*, however it should be considered that only one isolate was recovered. The *vanB* gene was found mostly in *E. faecalis*. The most common virulence gene was *blaZ*, found in 5 isolates, 60% of which were recovered from other *Enterococci*. The percentages presented in the table are calculated from the number of isolates with a resistance gene and the isolates of a specific species of *Enterococcus*. Some of the isolates have more than one resistance gene. The genes frequency distribution of virulence genes is shown in the Table 7 below.

Table 7: Frequency distribution of positive virulence factor genes screened from *Enterococci* isolates

<table>
<thead>
<tr>
<th>Enterococcal Species</th>
<th>Total Frequency of isolates (n)</th>
<th>Virulence genes</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>agg</em> Frequency (%)</td>
<td><em>gelE</em> Frequency (%)</td>
</tr>
<tr>
<td><em>E. faecalis</em></td>
<td>6</td>
<td>1 (16.7)</td>
<td>1 (16.7)</td>
</tr>
<tr>
<td><em>E. faecium</em></td>
<td>1</td>
<td>0 (0.0)</td>
<td>0 (0.0)</td>
</tr>
<tr>
<td>Other <em>Enterococci</em></td>
<td>11</td>
<td>0 (0.0)</td>
<td>1 (9.1)</td>
</tr>
</tbody>
</table>

The most common gene which tested positive was *gelE* while *esp* was not identified in any of the isolates. One isolated had the *agg* gene. *gelE* and *agg* were each found in 16.75% of *E. faecalis* isolates. *gelE* was found in 9.1% of the other *Enterococcal* species.

**DISCUSSION**

In this study samples from a total of 193 patients attending the Antenatal clinic at Windhoek Central hospital were tested, with 10.3% prevalence of *Enterococci* as shown in Table 4.1. Furthermore, *Enterococcus* was recovered from 30.8% of the rectal swabs. This was less than the study done in Ethiopia by Abamecha *et al.*, 2015 in which 76% of *Enterococcal* isolates were isolated from stool samples in hospitalized patients. A study by Miller *et al.*, 2004 in the United States of America reported a prevalence of 68% of *Enterococcus species* in rectal and vaginal swabs which is higher than the current study at 10.3% as shown in Table3. However, the current study has a prevalence of *Enterococcus* which is similar to a study done by Ghasemi *et al.*, 2016 in Iran on pregnant women which reported a prevalence of *Enterococcus* species at 8.6%. *Enterococcus* had a prevalence of 8% in a study in Uganda done by Ngonzi *et al.*, 2018 which is similar to the current study. However, a study conducted in Denmark by Stockholm *et al.*, 2013 showed a prevalence of *Enterococci* of 24% which is higher than the current study. A study done in India by Sibi *et al.*, 2014, showed a prevalence of 6.7% in urine in pregnant women. This is relatively similar to the current study with
1.5% of Enterococcal isolates found in urine. In a university hospital in Egypt Toru et al., 2018 established that the overall prevalence of Enterococcus in clinical isolates from paediatric patients was 5.5% which is less than this current study with a prevalence of 10.3% 37. This difference could be attributed to the difference in age groups of the studies however no direct correlation between Enterococcal colonization and age has been made in previous studies. In a study in Iran by Saffari et al., 2017 the faecal colonization with Enterococcus was 37% compared to a frequency of 16.3% of Enterococcus in rectal swabs in the current study 32. Another different study conducted in Egypt by Hashem et al., 2017, reported 64% Enterococcal isolates with 72.2% being E. faecalis and 24.4% being E. faecium 33. The remaining isolates were E. casei and E. gallinarum as tested using VITEK 2. The study conducted in Ethiopia by Wondwossen et al., 2014 investigated whether there was a relationship between Enterococcal infection and colonization in patients living with HIV 34. The results did not show a statistically significant connection between Enterococcal infection or colonization and HIV infection. A study done in Brazil by Soares et al., 2014, showed that 92.1% of the clinical isolates obtained were E. faecalis and 7.9% were E. faecium 35. A study done in Iran by Kafil et al., 2013, the Enterococcal isolates were made up of 56.9% E. faecalis and 43.1% E. faecium 36. Research conducted in Istanbul by Habip et al., 2014 looked for Enterococcal colonization in patients with haematological malignancies and showed a prevalence of 44% of patients were colonized with Enterococcus 37. This is more than this study with 16.3% GIT colonization with Enterococcus in pregnant women. This could be due to the fact that patients with haematological malignancies have immune systems which are impaired more so than in pregnant women. In a study done in Nepal by Subramanya et al., 2018, 52.59% of the rectal swabs screened were positive for Enterococcus 38.

As seen in Table 3, only 1.0% of isolates were found in urine while there were higher rates of prevalence in the swabs. This could be attributed to the fact that in urine is sterile, and not meant to have any bacteria, unless the patient has a urinary tract infection. Only urine samples with a colony count more than 10 000 were considered in this study. Women are more likely to have urinary tract infections due to the smaller distance between the urethra and the anus. The source of the infection therefore is largely the bacteria colonizing the gastrointestinal tract 29. A larger percentage of Enterococcal isolates were found in the rectal swabs seen as 7.8% in Table 3, because Enterococcus is known to colonize the gastrointestinal tract 30. Consequently, the presence of Enterococcus in clinical isolates is due to its abundance in the GIT 30. This is also thought to be the source of Enterococci found colonising the vagina at a prevalence of 1.5% shown in Table 3. In screening for Enterococcus, the preferred method would be testing for rectal swabs or stool samples. However, in hospitalised patients the source of infection is usually not the patient’s own gastrointestinal flora, but from the hospital equipment and the hands of hospital staff 31.
The results of this current study are different from the study done in Iran by Ghasemi et al., 2016 where the prevalence of *E. faecalis* was 89.8%\(^7\). *E. faecalis* made up 33.3% of the isolates in this study, while *E. faecium* made up 5.6% of isolates. The remaining (61.1%) were other *Enterococci* and were not confirmed through molecular testing in this study. In a study done in Ethiopia by Abamecha et al., 2015, *E. faecalis* made up 29.8% of the isolates which is similar to this current study, but conversely *E. faecium* made up 35.1% of the clinical isolates\(^5\). In a study done in India by Sreeja et al., 2012 in which stool specimens were collected, 76% of the bacteria isolates recovered were *E. faecalis* and 24% were *E. faecium* and none of the other species under *Enterococcus* were isolated\(^3\). This is different from the current study in which the prevalence of *E. faecalis* and *E. faecium* was 30.8% and 7.7% respectively in rectal swabs as seen in Table 4. It should be noted that in the study in India, speciation was done through biochemical testing and not molecular testing. In a study in India by Padmasini et al., 2014, 157 *Enterococcus* isolates were recovered from various tertiary care hospitals, 53% of which were *E. faecalis* and *E. faecium* made up 46.5%\(^3\). Differences in prevalence and recovery rates of *Enterococcus* could be due to different populations being used with some studies using the general population while others use selected groups like pregnant women as is the case with this current study. Other factors which can contribute to differences in prevalence rates can because of the type of sample used (urine, stool, low vaginal swab, rectal swab); sampling technique; methods for culture and identification of bacteria.

Table 5 show that the isolates in this study were all sensitive for penicillin, amoxicillin, augmentin (amoxicillin-clavulanic acid), nitrofurantoin and vancomycin. A study done in Iran by Ghasemi et al., 2016 showed similar results as the all the isolates that were recovered in that study were also sensitive to amoxicillin and vancomycin\(^9\). The proportion of gentamicin sensitivity in the study done on urinary tract infections in India by Sibi et al., 2014, was 33.3%, which is similar to the one reported in this current study as shown in Table 5\(^20\). However, the frequency of gentamicin resistance in this study is different to the study done in India by Ngonzi et al., 2018 in which none of the isolates were resistant to gentamicin\(^19\). In the same study all isolates were sensitive to vancomycin which is the same as the current study\(^18\). In this current study Isolates were sensitive to imipenem and ciprofloxacin (88.9%) and the highest frequency of resistance was reported against clindamycin (72.0%) which is different from the study done in India by Sibi et al., 2014 in which clindamycin sensitivity was reported as 75%\(^20\). Resistance to cephalothin was 38.9% in this current study as shown in Table 5. Although there are some differences in the sensitivity patterns from this study to those reported in other studies, the results are comparable to those of other studies. The study in Iran done by Saffari et al., 2017, the *Enterococcal* isolates had gentamicin resistance at 38% which compared to this study with gentamicin resistance at 33.3%\(^22\). In the study in Iran, 93% of isolates were resistant for ciprofloxacin and conversely 88.9% of isolates sensitive for ciprofloxacin in this study\(^22\). The current study compared well to the study done in Egypt by Hasheem et al., 2017 with
96.7% sensitivity to vancomycin and 100% sensitivity to vancomycin in this study. In the same study sensitivity to ciprofloxacin was 60.8%, compared to 88.9% in this study. Isolates were 46.7% sensitive to gentamicin compared to 44.4% in this study. Research done in an Indian tertiary hospital by Biswas et al., 2016, Enterococcal isolates from clinical samples 7.4 % showed vancomycin resistance and 5.6 showed reduced susceptibility. This does not compare to the current study in which there was no vancomycin resistance. In the isolates in the current study, 83.3% were resistant to at least 1 antibiotic, which is less than the 90% of isolates observed in a study done in Ethiopia by Wondwossen et al., 2014. All the isolates were sensitive for penicillin, amoxicillin, amoxicillin-clavulanic acid and vancomycin. In the same study in Ethiopia, 81.8% and 56% of isolates were resistant to amoxicillin and amoxicillin-clavulanic acid respectively. In a study done in Ethiopia by Abamecha et al., 2015 looking at the colonization of the GIT by Enterococci, 5% exhibited resistance to vancomycin similar to the current study which showed no resistance to vancomycin in rectal swabs. In a study in a teaching hospital in Ethiopia done by Ferede et al., 2018, 53.3% of Enterococcal isolates were resistant to ciprofloxacin compared to 5.6% of resistance in this study, and 5.6% of isolates that tested intermediate. In the same study 60% of isolates were resistant to gentamicin compared to 33.3% of isolates in this study. The vancomycin and gentamicin resistance were 33.8% and 83.9% respectively in E. faecium, and 16.3% and 88.1% in E. faecalis in a study done in Iran by Kafil et al., 2013. In the study in Istanbul, E. faecalis made up 81% of the vancomycin resistant Enterococcal isolates, while the remaining were E. faecium. In the study done in Nepal by Subramanya et al., 2018 33% of the isolates were multi-drug resistant compared to this study where 83% of isolates were resistant to at least one antibiotic. In a research done in a university hospital in China by Wei et al., 2014, the vancomycin resistance was 66% and the penicillin resistance was 88%. This did not compare well to the current study were there was no vancomycin and penicillin resistance as seen in Table 5. This did not compare well to the current study were there was no vancomycin and penicillin resistance as seen in Table 4.3. In a study done in Namibia by Jitaleni et al., 2015, the sensitivity of vancomycin was 99.3% which compares well to this study with a 100% phenotypic sensitivity to vancomycin. Conversely the 97% of the isolates found in that study were resistant to amoxicillin, in comparison to this study with 100% sensitivity. This could be due to the fact that the study conducted by Jitaleni et al., 2015 was done from data from the Namibian Institute of Pathology (NIP), in which a large percentage of the patients are hospitalised. Furthermore, amoxicillin is the empirical choice of antibiotic for a large host of infections and as such the prevalence of resistance is much higher in hospital settings than in community settings.

In Table 6 it is observed that the E. faecium isolate recovered did not test positive for any resistance genes. All isolates were vancomycin susceptible even though 2 isolates tested positive for vanA, and 4 tested positive for vanB. vanA encodes for high level resistance however, if the full vanA locus is
not present high-level resistance will not be conveyed phenotypically. Four isolates tested positive for vanB which conveys a lower level of resistance than vanA. The gene vanC would have been significant in this study considering that isolates that are non-E. faecalis and non-E. faecium can harbour vanC chromosomally conferring intrinsic resistance. One isolate tested positive for tetM and was found in the other Enterococcal species along with 60% of the blaZ gene. E. faecalis isolates tested positive of the isolates tested positive for blaZ and blaR1, at 40% and 33.1% respectively. In a study done in India by Phukan et al., 2016, 24% of the Enterococcal isolates were vancomycin resistant and 56.25% of these isolates had the vanA gene. This does not compare well to the current study in which all the isolates were sensitive to vancomycin and vanA was recovered from 2 isolates as seen in table 4.4. In a study in Nepal conducted by Subramanya et al., 2018, in which all of the Enterococcal isolates that were multi-drug resistant had the vanA gene. This does not compare well to the current study as vanA was only recovered from 2 isolates, neither of which exhibited phenotypic vancomycin resistance. A study done in Germany by Werner et al., 2012 showed that 79% of clinical E. faecium isolates from neonates had the vanB gene. In this study no vanB genes were found in E. faecium. However, vanB was found in 50% of the E. faecalis isolates as seen in Table 6. In a study done in India by Amberpet et al., 2016 All the vancomycin resistant isolates in this study had vanA. In the study in China conducted by Wei et al., 2014 5.0% of isolates harboured the vanA gene and 31.0% harboured the tetM gene. In comparison to this study in which 16.7% of E. faecalis isolates and 9.1% of E. faecium isolates had the gene vanA and 9.1% of the other Enterococci had the gene tetM gene. In a study done in the United states of America by McBride et al., 2007 on clinical isolates of Enterococcus, the prevalence of vanA was 7.1%, vanB was 22%, blaZ was 16.0% and tetM was 61%. In this study the prevalence of vanA was 16.7% in E. faecalis and 9.1% in other Enterococci, the vanB was 50.0% on E. faecalis and 9.1% in other Enterococci, tetM was 9.1% in other Enterococci, blaZ was 33.3% in E. faecalis and 60.0% in other Enterococcus and blaR1 was present in 33.3% of E. faecalis isolates and 9.1% in other Enterococci.

Both agg and gelE were found in E. faecalis, with one of the other Enterococcal species also harbouring gelE as seen in Table 7. The esp gene was not identified in any of the isolates as shown in Table 6. In a study done in Egypt by Toru et al., 2018 31.8% of the identified Enterococcus isolates were positive for biofilm production, haemolysin and gelatinase. In the same study overall prevalence of biofilm formation in this study was 77.3%, 45.5% for haemolysin production and 68.2% was positive for gelatinase production. A study done in Egypt by Toru et al., 2018, the phenotypical gelatinase prevalence was 68.2%, which is higher than this study with a prevalence of 16.7% in E. faecalis and 9.1% in other Enterococcus species. No genotyping was done in this study and as such it could result in a higher prevalence of gelE. A study in India done by Padmasini et al., 2014 reported that 10.19% of isolates showed phenotypic gelatinase activity, but 51.59% were
positive for gelE26. In the aforementioned study conducted in Iran by Saffari et al., 2017, 71.8% of positive isolates tested positive for gelE which did not compare well to this study with 16.7% prevalence in E. faecalis and 9.1% in the other Enterococci species22. This could be attributed to the fact that the study in Iran tested clinical isolates which are therefore already proven to be virulent as opposed to isolates that are colonising the vaginal and gastrointestinal tract. Unlike this current study where no esp genes were found, the study in Iran done by Saffari et al., 2017 had a prevalence of 76.7% of the esp gene 22. In a study done in India by Biswas et al, 2016 the most common virulence gene was gelE with 44.4% in the vancomycin resistant isolates and 16.4% in vancomycin sensitive isolates34. In a study done in Brazil by Soares et al., 2014 73.3% of the isolates were positive for gelE, 70% were positive for esp and 58.3% were positive for agg. This does not compare well to this study with only 16.7% positive for agg in the E. faecalis isolates and 16.7% and 9.1% of E. faecalis and other Enterococci positive for gelE respectively. A study done in Brazil by Comerlato et al., 2013 showed a gelE prevalence of 60% in clinical isolates, different from the 11.1% in this study 14. In a study in Brazil by Soares et al., 2014, 58.3% of the clinical isolates harboured the agg gene 25. And only 16.7% of E. faecalis had agg in the current study as shown in table 6. In a study done in India, 30.43% of E. faecalis and 27.77% of E. faecium tested positive for the gelE gene, compared to 16.7% of E. faecalis isolates and no E. faecium isolates testing positive in this study 8. In the same study 17.39% of E. faecalis and 11.1% of E. faecium isolates tested positive for the esp gene 8. The esp factor was not found in this study. A study in Brazil showed that 70% of the clinical isolates had the esp gene 25. In a study done in Bulgaria by Strateva et al., 2016 the prevalence of esp was 44.3%, agg at 38.4% and gelE at 64.3% in clinical Enterococcus isolates42. The virulence factors in that research were the same that were done in this research however the overall prevalence of agg and gelE were 16.7% each in E. faecalis isolates, with 9.1% of other Enterococci testing positive for gelE and no esp genes.

CONCLUSION

In this study samples from a total of 193 patients attending the Antenatal clinic at Windhoek Central hospital were tested, with 10.3% prevalence of Enterococci. In rectal swabs 30.8% of the isolates were E. faecalis, 7.7% were E. faecium and 61.5% were other Enterococci. In vaginal swabs, 33.3% were E. faecalis and 66.6% were other Enterococcus. Half the isolates found in urine were E. faecalis and the rest was other Enterococci. In E. faecalis, 16.7% were positive for vanA, 50.0% were positive for vanB, blaZ were positive for 33.3% and blaR1 were positive for 33.3% isolates. In the E. faecalis isolates 16.7% were positive for agg and 16.7% were positive for gelE. E. faecium did not test positive for any virulence or antibiotic resistance genes. The other Enterococcus species were not confirmed with molecular methods, with 9.1% being positive for vanA, 9.1% positive for vanB, 9.1%
positive for tetM, 60.0% positive for blaZ and 9.1% positive for blaR1. The other Enterococci were also positive for gelE with a prevalence of 9.1%. The majority of the isolates collected in this study were not the most common species found in human disease namely E. faecalis and E. faecium. This then becomes important to speciate, in order to better treat patients when faced with species with intrinsic resistance. Furthermore, virulence genes were only found in 3 isolates. Although there are resistance genes present in these isolates, empirical antibiotics would still be effective. This is good considering the global trend of resistance.

RECOMMENDATIONS
A study looking at the virulence factors and antibiotic sensitivities of Enterococcus in pregnant women should be carried out using a larger sample size and collected from different areas in Namibia.

More studies should be conducted around Enterococcus in general, for example in clinical isolates due to the lack of statistics available.

In future studies on Enterococcus the other species of Enterococcus should be identified through molecular techniques as well. The other Enterococcal species have intrinsic resistance harbouring genes such as vanC, making speciation important for treatment.

With a prevalence of 10.3% of Enterococcus in pregnant women, screening pregnant patients can be of importance considering the high rates of maternal and neonatal deaths as reported in Namibian newspapers.

Future studies can include the screening of neonates for Enterococcus to determine prevalence.

LIMITATIONS OF THE STUDY
This study was limited due to the small sample size and the collection of samples from only one Antenatal clinic.

All the species of Enterococcus weren’t identified and confirmed through molecular methods.

Two isolates in this study could not be revived after freezing and as such no further testing could be done on these isolates.

In future studies the phenotypic virulence of isolates can be tested as well as exploring more virulence and resistance genes.

The quality and concentration of DNA extracted could not be determined.

REFERENCES


