MOLECULAR CHARACTERIZATION OF *STREPTOCOCCUS AGALACTIAE* ISOLATED FROM PREGNANT WOMEN IN THE EASTERN CAPE, SOUTH AFRICA AND WINDHOEK, NAMIBIA AND ANTIBACTERIAL ACTIVITIES OF SOME MEDICINAL PLANT EXTRACTS ON THE ISOLATES

MUNYARADZI MUKESI

DEPARTMENT OF BIOCHEMISTRY AND MICROBIOLOGY

FACULTY OF SCIENCE AND AGRICULTURE

UNIVERSITY OF FORT HARE

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DECLARATION

I, the undersigned, declare authorship of this thesis entitled "Molecular characterization of *Streptococcus agalactiae* isolated from pregnant women in the Eastern Cape, South Africa and Windhoek, Namibia and antibacterial activities of some medicinal plant extracts on the isolates" submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Microbiology in the Faculty of Science and Agriculture. The work contained herein is my original work, with exemptions to the citations and that the work has not been submitted to any other University for the award of any degree or examination purposes.

Name: Munyaradzi Mukesi

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I, **Munyaradzi Mukesi**, student number: 201415927 hereby declare that I am fully aware of the University of Fort Hare's policy on plagiarism and I have taken every precaution to comply with the regulations.

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CERTIFICATION

This thesis titled "Molecular characterization of *Streptococcus agalactiae* isolated from pregnant women in the Eastern Cape, South Africa and Windhoek, Namibia and antibacterial activities of some medicinal plant extracts on the isolates" meets the regulation governing the award of degree of Doctor of Philosophy of the University of Fort Hare and is approved for its contribution to scientific knowledge.

……..	……
Prof CL Obi (Supervisor)	Date
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Prof SR Moyo (Co-supervisor)	Date
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Prof AI Okoh (Co-supervisor)	Date
……	……
Dr BC Iweriebor (Co-supervisor)	Date

DEDICATION

This thesis is dedicated to Cathrine, Takudzwa, Thandaza and Thandeka Mukesi and all the brave pregnant women who took part in this study in both Namibia and South Africa.

Joshua 1:8

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LIST OF ACRONYMS

ACE:	Angiotensin Converting Enzyme
AEMREG:	Applied and Environmental Microbiology Research Group
ALPs:	Alpha like Proteins
BBB:	Blood Brain Barrier
CA:	Chromogenic Agar
CAMP:	Christie Atkins Munch Petersen
CDC:	Centre for Disease Control
CNA:	Colistine Nalidixic Acid Agar
CLSI:	Clinical Laboratory Standards Institute
CMV:	CytoMegalo Virus
CNS:	Central Nervous System
Cov:	Control of virulence
CPS:	Capsular Polysaccharide
CSF:	Cerebro Spinal Fluid
DAMPs:	Damage Associated Molecular Patterns
DMSO:	Dimethyl Sulfoxide
DNA:	Deoxyribo Nucleic Acid

dsDNA:	double stranded DNA
ELISA:	Enzyme Linked Immunosorbent Assay
EOD:	Early Onset Disease
ESBL:	Extended Spectrum Beta Lactamase
FACS:	Fluorescent Activated Cell Sorting
FDA:	Food and Drug Administration
GAS:	Group A Streptococcus
GBS:	Group B Streptococcus
GBSDA:	Group B Streptococcus Differential Agar
GC-MS:	Gas Chromatography- Mass Spectrometry
GIT:	Gastro Intestinal Tract
GMRDC:	Govan Mbeki Research Development Centre
HIV:	Human Immunodeficiency Virus
HSV:	Herpes Simplex Virus
IAP:	Intrapartum Antibiotic Prophylaxis
ICR:	Inducible Clindamycin Resistance
ICU:	Intensive Care Unit
IKS:	Indigenous Knowledge System

IV:	Intravenous
LMIC:	Low to Medium Income Country
LOD:	Late Onset Disease
LTAs:	Lipo Teichoic Acids
LVS:	Low Vaginal Swab
MALDI-TOF:	Matrix Assisted Laser Desorption Ionization- Time of Flight
MHC:	Major Histocompatibility Complex
MIC:	Minimum Inhibitory Concentration
MLST:	Multi Locus Sequence Typing
MRSA:	Methicillin Resistant *Staphylococcus aureus*
NCBI:	National Centre for Biotechnology Information
NNA:	Neomycin Nalidixic Agar
NUST:	Namibia University of Science and Technology
OTC:	Over the Counter
PAMPS:	Pathogen Associated Molecular Patterns
PCR:	Polymerase Chain Reaction
PMNs:	Poly Morphonuclear Neutrophils
PROM:	Premature Rapture of Membranes

PRR:	Pattern Recognition Receptors
PSA:	*Pseudomonas aeruginosa*
RNA:	Ribo Nucleic Acid
RS:	Rectal Swab
SAMRC	South Africa Medical Research Council
SCP:	Streptococcal C5A Peptidase
SDGs:	Sustainable Development Goals
SDS-PAGE:	Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis
STI:	Sexually Transmitted Infection
TBE:	Tris/Borate/EDTA
THP:	Traditional Health Practitioners
UFH:	University of Fort Hare
UK:	United Kingdom
UNSDGs:	United Nations Sustainable Development Goals
USA:	United States of America
WHO:	World Health Organisation
WTAs:	Wall Teichoic Acids

Manuscripts Submitted for Publication

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GENERAL ABSTRACT

Streptococcus agalactiae (*S. agalactiae*) also known as group B Streptococcus (GBS) is one of the leading causes of bacterial morbidity and mortality among neonates worldwide. It is the cause of invasive Early Onset Disease (EOD), which occurs in the first 7 days of life and characterised by sepsis, pneumonia and meningitis and Late Onset Disease (LOD) occurring between 7 and 89 days of life. Late onset disease is characterised by meningitis and long term neurological sequelae such as cerebral palsy, hearing impairment and cognitive challenges. *S. agalactiae* does not only infect neonates, it also infects the elderly, immunocompromised individuals and pregnant and non-pregnant women, causing invasive disease.

In the world, 10-40% of healthy women are rectally or vaginally colonised with GBS and they face the risk of passing it to their babies during the process of childbirth. During parturition, a GBS colonized pregnant woman transfers the bacterium to her new-born as the baby passes through the ruptured membrane, thus infecting the child. However, GBS has been reported to be transferred even without rupture of membranes. Once it infects the membranes, it is transferred into the amniotic fluid and subsequently infects the baby. It can be aspirated into the lungs causing pneumonia or it can infect the blood stream and disseminated round the body causing septicaemia, meningitis and other infections. Once in the neonate's body, the bacteria is able to evade the immune system as the host immune system is not yet fully developed. Bacterial evasion of the immune system is enhanced by its various virulence factors which are deployed to help it escape the immune system. These include the polysaccharide capsule, haemolysin and the release of complement inactivating factors such C5a peptidase.

The World Health Organisation (WHO) (2010) recommends universal screening of pregnant women to identify those colonised and who are at risk of passing the bacterium to their babies during birth. WHO also recommends identification of at risk women and providing Intrapartum

Antibiotic Prophylaxis (IAP) using penicillin. However, problems arise in penicillin allergic women and while alternatives for IAP include erythromycin and clindamycin, there is increasing resistance to these drugs thereby limiting therapeutic options. Antimicrobial susceptibility testing is also not always possible in most resource constrained countries due to poor infrastructure, limited access to health care and the logistical problems in implementing the WHO guidelines.

Alternative therapeutic options to GBS infection include developing new and potent antibiotics, development of a vaccine, use of medicinal plants and the use of bacteriophage therapy. While these look like better alternatives there is massive scientific work to be carried out to ensure proper characterisation and efficiency of such alternatives. This process should be followed by *in vitro* diagnostic testing, experiments with animal models and clinical trials. The problems encountered during vaccine development to curtail GBS infection are compounded by the multiplicity of *S. agalactiae* capsular types which vary in different geographic locations. Medicinal plants are a cheap and convenient option since they are widely used in communities but the phytochemical components of the plants have to be identified and subjected to *in vitro* testing to evaluate their therapeutic efficacy as antimicrobial agents. This study therefore sought to isolate GBS from pregnant women between 35 and 37 weeks gestation in Windhoek (Namibia) and the Eastern Cape (South Africa), to determine the prevalence of GBS colonisation in the vagina and rectum of the pregnant women, characterise the isolates by molecular techniques, determine the antimicrobial resistance profiles and genes of the isolates and explore the efficacies of medicinal plant extracts as possible candidates for therapeutic options.

Chapter 1 provides a vivid account of the introduction to the study, statement of the research problem, hypothesis and the aim and objectives.

Chapter 2 is a comprehensive review of literature to the study beginning with a brief introduction to streptococci and *S. agalactiae*. The chapter reviews the pathogenicity, prevalence, management and various diagnostic methods used for recovery and identification of *S. agalactiae*. It also gives an account of the various methods used in descriptive epidemiology of *S. agalactiae* and in the control of the organism among individuals.

Chapter 3 focussed on the analysis of antimicrobial susceptibility patterns of GBS isolates from South Africa and Namibia using the Vitek (2) by determining the minimum inhibitory concentrations (MICs) of benzyl penicillin, ampicillin, cefotaxime, ceftriaxone, levofloxacin, erythromycin, clindamycin, linezolid, vancomycin, tetracycline and cotrimoxazole against GBS. The results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines, 2015. All isolates showed 100% sensitivity to all the antibiotics except cefotaxime (99.4%), clindamycin (76.6%), erythromycin (89.6%) and tetracycline (96.1%).

All isolates were screened for *bla* genes by PCR using oligonucleotide primer sequences specific for the respective genes and they were all negative. While antibiotic resistance has been reported to be increasing among bacterial species, isolates from both Namibia and South Africa showed low resistance to antibiotics.

Chapter 3 provides a desription on the identification of *Olea europaea* in the Cala community of the Eastern Cape, South Africa and the preparation of ethanol leaf extracts and essential oils from the plants. Leaf extracts were obtained using standard ethanolic extraction methods while essential oils were extracted from the leaves in a modified hydro distiller. Working solutions of ethanol leaf extracts and essential oils were prepared in Dimethyl sulfoxide (DMSO) for determination of MICs against GBS. Gas Chromatography-Mass Spectrometry (GC-MS) analysis of essential oils was performed on the Agilent 5977A MSD and 7890B GC system to unravel the phytochemical composition.

Antimicrobial susceptibility testing was performed using agar well diffusion while MICs were determined using serial dilutions. Essential oils and leaf extracts were bactericidal against GBS and were composed of flavonones, biophenols, benzoic acid derivatives, steroids and secoiridoids. The results showed that *O. europaea* extracts may be useful as an alternative source of antimicrobials against *S. agalactiae*.

Chapter 4 focussed on collection of rectal and vaginal swabs from pregnant women and processing them in order to ascertain the prevalence and capsular type distribution of *S. agalactiae* amongst the pregnant women. Pregnant women between 35 and 37 weeks gestation, comprising 530 in Namibia and 100 in South Africa were recruited into the study on a voluntary basis. The participants targeted were those attending Windhoek Central Hospital maternity clinic in Namibia while in the Eastern Cape Province, South Africa, samples were collected at Mduntsane, Dimbazi and Middle Drift clinics. The sample collection procedures were explained to the participants and a low vaginal swab (LVS) and rectal swab (RS) were obtained from each patient by qualified nurses.

Samples were placed in Amies transport medium (Rochelle Chemicals, Johannesburg, South Africa), bar-coded for identification, placed in a cooler box containing ice packs and transported to the laboratory. In Namibia, samples were transported to the Microbiology Laboratory, Faculty of Health and Applied Sciences, at the Namibia University of Science and Technology (NUST) for culture and presumptive identification of GBS isolates while in South Africa, samples were processed at the Applied and Environmental Microbiology Research Group (AEMREG) Laboratory, Department of Microbiology and Biochemistry, University of Fort Hare (UFH). All samples were cultured within 2 h of collection.

S. agalactiae was grown on Columbia blood agar containing 5% sheep blood which was incubated at 37^{o}C for 18 h in 5% carbon dioxide. Identification of presumptive GBS isolates

was based on the following tests: β-haemolysis on Columbia blood agar containing 5% sheep blood, Gram positive cocci in appearance, negative catalase reaction, Lancefield grouping with type B antisera (Becton Dickinson, New Jersey, USA) and the Vitek (Biomerieux) version 2. All presumptive isolates were stored in glycerol stocks at -80ºC until further analysis and were confirmed by molecular techniques using primer sequences specific for the *scp*B gene. Capsular typing was performed on all isolates in a multiplex PCR using oligonucleotide primer sequences specific for the ten capsular types.

The prevalence of *S agalactiae* among pregnant women in Namibia was 13.6% while in South Africa the prevalence was 37%. Most of the women were vaginally and rectally colonised simultaneously. Capsular types II, III and V were prevalent in both countries. These capsular types have been reported as being associated with adverse maternal outcomes.

Chapter 5 gives an account multi locus sequence typing (MLST) of *S. agalactiae* isolated from pregnant women in South Africa and Namibia using housekeeping genes to establish the profile of sequence types in each population and the genetic relatedness of the isolates. DNA extracted from the isolates was amplified using PCR and sequenced using the Sanger method. Sequences were edited using Geneious version 10.2.2, analysed using Bioedit and a dendogram was constructed using Mega 7.

The average number of alleles per housekeeping gene ranged from 2 to 7 and the range of polymorphic nucleotide sites was low (0.4 to 1.6%). The most common sequence type in both South Africa and Namibia was ST 24 and the isolates from both countries were genetically closely related. The presence of different sequence types among the isolates from the same population showed that the strains colonising pregnant women were genetically diverse. Although the isolates from each of the countries were genetically diverse, they were closely

related when the 2 groups of isolates were compared to each other. However, most of the housekeeping genes were conservative as shown by a low *dn* /*ds* ratio.

Chapter 6 describes the molecular basis of virulence among *S. agalactiae* isolates to unravel relatedness in virulence factors among the isolates from both South Africa and Namibia and for epidemiological purposes. All the isolates recovered from pregnant women were screened for *cps*, *cylE*, *scp*B, *hla*, *hlb*, *etB* and *LukM* genes using PCR. All the isolates expressed the capsular gene (*cps*), and the C5a peptidase gene (*scp*B). The haemolysin gene (*cylE*) was amplified in 94.2% of the isolates. However, 94.2% of the isolates the *cps*, *scpB* and *cylE* genes were amplified while none of the isolates had *hla*, *hlb*, *etB* or *LukM* genes amplified. The virulence genes not amplified are usually expressed by *Staphylococcus aureus* but have been reported in other Gram positive bacteria. The expression of virulence genes by most of the isolates suggested that most of them had the potential of causing invasive GBS disease in the pregnant women or their babies.

Chapter 7 gives an overview of the study by offering general conclusions and recommendations on future perspectives.

CHAPTER ONE

GENERAL INTRODUCTION

1.0 INTRODUCTION

Prevalence rates of GBS among women from different countries vary according to characteristics of the population studied. It is also affected by differences in pre-analytical and analytical procedures employed in establishing the prevalence while the nature of the swab used to collect GBS specimens affects recovery of the organism. When sample processing is delayed, a potent transport medium should be employed to preserve the organism as failure to do so results in false negative results which lowers prevalence rates. Method of isolation and identification of the bacteria affects recovery rates as a result of the sensitivity and specificity of the test methods employed (Numanovic *et al*., 2017). Standard methods for isolating GBS use selective liquid media such as Todd Hewitt or Lim broth, culture and subculture on 5% blood (sheep or horse) agar. Some studies enhance the sensitivity of their testing methods by using chromogenic agar such as Granada agar while others use a combination of both standard methods and chromogenic agar (Verhoeven *et al*., 2014).

Gram stain, catalase test and Christie Atkins Munch Petersen (CAMP) test are used as standard tests for presumptive identification of GBS. Definitive identification of GBS requires antigen testing (serological), and/ or molecular confirmation by amplification of the *scp*B or 16S rRNA gene.

Many challenges exist in determining prevalence rates and these have an effect on the results reported in studies as well as serotyping of GBS and molecular characterization of the isolates. These challenges range from difficulties in obtaining specimens, poor laboratory facilities, capacity and practices in the diagnosis and identification of GBS which are common problems in low to middle income countries (Huang *et al*., 2016).

General prevalence rates of GBS around the world vary from America (19.7%), Europe (19.0%) and South East Asia (11.1%) to Africa (22.4%) (Mengistu *et al*., 2016). Variations are

due to geographic locations, the population of women sampled and testing methods. In a recent study in Tuzla's region of Bosnia and Herzegovina, the prevalence of GBS was 7% in pregnant women while the same study reported a prevalence (10%) which was higher in non-pregnant adult women (Numanovic *et al*., 2017). A study comparing prevalence rates in different continents found that it was highest in Africa (24%). Prevalence rates in Europe (16%), America (22%) and Oceania (22%) were also high but lowest prevalence rate was recorded in Asia (7%) (Huang *et al*., 2016). Another study done in Italy found a very high prevalence rate of 25.5% among pregnant women compared to the average of 16% in Europe and 24% in Africa. In the Italian study, more women who were colonized with GBS were more from public than private consulting rooms highlighting that variations in population sampled influences the prevalence rates reported (Matani *et al*., 2016).

The vagina has a delicately balanced microflora living in its ecosystem. Predominant members include lactobacilli, staphylococci, streptococci, peptostreptococcus, bacteroides, *Escherichia* and *Candida* spp. The balance in this ecosystem is created and sustained with the contribution of many factors which include but are not limited to: the woman's age, frequency of sexual relations, number of sexual partners, use of contraceptives, menstrual cycle, pregnancy and infections. Contributing factors also include sex habits and practices which are varied in different parts of the world (Numanovic *et al*., 2017). Intravaginal practices which entail cleaning of the vagina or inserting substances into the vaginal canal to dry or tighten the vagina are common practices in Africa. The use of vaginal creams to enhance sexual satisfaction is a common practice in Europe. This practice is associated with upsetting the balance of the normal flora and increased risk of carrying *Streptococcus agalactiae* (*S. agalactiae)* (Cools *et al*., 2016). *S. agalactiae* infection is not in itself a sexually transmitted infection (STI) even though sexual behaviour has been linked to increased risk of GBS colonization. Influence of sexual

behaviour on GBS transmission is however still a matter of debate (Seale *et al*., 2016; Cools *et al*., 2016).

Geographic differences in GBS colonization have long been acknowledged. Effect of race and geographical distribution of GBS are related to differences in nutrition and socioeconomic status in the different parts of the world. Other factors which vary according to race and geographical location include host immune status, sexual practices, and the STIs prevalent in different areas (Jahromi *et al*., 2008 and Kim *et al*., 2011).

In Africa, a recent study done by Seale *et al*., (2016) in Kenya reported a 12% prevalence of GBS among pregnant women while a similar study by Cools *et al*., (2016) in the same country reported the prevalence of GBS in pregnant women to be 14.3% which compares to the study by Seale *et al*., (2016). However, in the study by Cools *et al*., (2016) the prevalence was much higher in non-pregnant women (20.2%) while the pregnant women were only up to 14 weeks gestation instead of the standard 35-37 weeks gestational period for screening of GBS. These differences in gestational age could affect the prevalence as it was confirmed in the same study among South African women in which the prevalence of GBS was 23.2% in non-pregnant women and 10% in pregnant women (Cools *et al*., 2016).

Age has not been known to affect GBS colonization rates but in non-pregnant South African women, GBS colonization rates increased with age with younger people showing low colonization rates than older women. However, this age correlation was not the case in pregnant women (Cools *et al*., 2016). In a study in Ethiopia, the most colonized age groups were the 20-24 years (37.5%) and the 25-29 years (29.2%) age groups. Interestingly, in this Ethiopian study, colonization rates varied depending on sampling site such as the rectum (8.7%), vagina (4.8%) or both (5.6%) (Mengistu *et al*., 2016). In the study by Mengistu *et al*., (2016) other factors which were linked to GBS colonization were: educational status with women who reached only

secondary level education mostly colonized (37.5%), housewives (83.3%) as well as gravidity with multigravida women being heavily colonized (79.2%). The overall prevalence was 19% which was much higher than what had been reported in another Ethiopian study by Schmidt *et al*, (1989) 27 years earlier (9%). Differences in the reported prevalence rates were attributed to studies being conducted in different geographic regions of the same country and differences in study populations as the earlier study used post-partum women and the latter pregnant women.

Different studies have reported varying effects of Human Immunodeficiency Virus (HIV) infection on GBS colonization. HIV infection weakens the immune system and the microflora of the vagina in HIV positive women changes as the patients become prone to infections. However, prevalence has been found higher in women with a higher CD4 count than those with lower CD4 counts (Mengistu *et al*., 2016; Dauby *et al*., 2016). In a recent study in South Africa by Cools *et al* (2016), HIV positive women were found to have no GBS which was attributed to the effects of cotrimoxazole prophylaxis. However, having candidiasis was associated with GBS colonization.

In some African countries, GBS prevalence rates have increased due to the absence of screening as part of antenatal care and protocols for Intrapartum Antibiotic Prophylaxis (IAP) not being adopted as standard care. The prevalence of GBS in one Zimbabwean study by Moyo *et al*, (2000) was 31%. In this study by Moyo *et al*, (2000), sampling sites were associated with different colonization rates with more GBS isolated from the vagina (12.6%) than the rectum (6.3%). A similar study carried out in Zimbabwe in 2010 found the prevalence of GBS to be 60.3% (Mavenyengwa *et al*., 2010) yet both studies were carried out on pregnant women. In the study by Mavenyengwa *et al*., (2010), colonization varied with gestational age of the pregnancy from 20 weeks (47%), 26 weeks (24.2%) and at delivery (21%) while living in the rural areas was associated with GBS colonization.

Different epidemiological reports give varying accounts in different geographic locations as access to health care; the nature of diagnostic methods used and poor laboratory capacity affect the diagnosis and reporting of invasive GBS disease in neonates. Due to some of these factors, clinicians do empiric antibiotic treatment on suspected bacterial infections without microbiological diagnosis (Quan *et al*., 2016) and this affects the reported statistics of the problem. In Kenya, GBS has been associated with high incidence of still birth (0.91) and EOD (0.76) per 1000 births. Of note in this Kenyan study was that treated cases of Early Onset Disease (EOD) recorded high fatality (47%) in the first 24 hours (Seale *et al*., 2016).

Serotype distribution varies according to geographic locations with some serotypes being predominant is certain parts of the world than others. In a study involving New Zealand, South Korea and China, there were variations in serotype distribution although serotype III was consistently high in all countries. New Zealand reported III (29%), Ia (21%) and Ib (20%) as the main strains and South Korea had III (43.8%), V (20.3%) and Ia (12.1%) as the main strains while China had III (41.8%), Ia (21.4%) and V (14.9%) (Huang *et al*., 2016).

A study of serotype distribution in African countries reported different results with Kenya having Ia (27.3%), V (27.3%) and III (22.7%); South Africa: Ia (34.5%), V (31.0%) and IV (13.8%) while Rwanda had Ia (83.3%) and II (16.7%). In the study, a high prevalence of capsular types IV, VI and VIII were reported for the first time in sub Saharan Africa but there was no type Ib, which are found in the US (8.1%) and Europe (12.4%) (Cools *et al*., 2016). In a similar study on pregnant women in South Africa, serotypes III (29.7%), Ia (25.8%), II (15.6%), IV (8.6%) and Ib (8.6%) were the most predominant (Chukwu *et al*., 2016). A study of the burden of GBS disease in South African neonates found serotypes Ia, Ib and III contributing to 75.8% and 92.5% of EOD and Late Onset Disease (LOD) (Dangor *et al*., 2015)

with different populations within the same country presenting with predominantly different capsular types.

In Zimbabwe, the serotype distribution did not change over a 10 year period with serotypes III, V, Ia, Ib and II predominating in a study in 2000 (Moyo *et al*., 2000) and 2010 (Mavenyengwa *et al*., 2010). The studies were done in different parts of the country but had a similar capsular type pattern indicating that the same capsular types may be found in a country even when the population sampled changes.

Studies have reported that *S. agalactiae* isolates possess other virulence factors apart from the capsule which enable the isolates to cause invasive disease. These include *cps*, *scp*B and *cylE* gene and studies in China and Malaysia have reported that strains which possess these genes were more virulent than those which did not (Eskandarian *et al*., 2015 and Cheng *et al*., 2005). Studies have also used Multi Locus Sequence Typing (MLST) through amplification of housekeeping genes to characterise GBS, determine evolutionary changes among isolates and for epidemiological purposes (Jones *et al*., 2003).

Penicillin and ampicillin are the drugs of choice in prophylaxis or treatment of *S. agalactiae* disease while cefazolin, clindamycin, erythromycin and vancomycin are recommended as alternatives especially in penicillin allergic women. However, studies have reported increasing GBS resistance to these drugs (Huang *et al*., 2016; Frohlicher *et al*., 2014). A study by Matani *et al*., 2016 in Italy reported GBS isolates which were resistant to penicillin and ampicillin. For many years there have been reports of changes in penicillin Minimum Inhibitory Concentrations (MICs) to GBS but not outright resistance.

In the United States of America (USA), studies have reported high resistance of *S. agalactiae* to clindamycin (13-20%) and erythromycin (25-32%) (CDC, 2010). Such resistance has also been reported in Europe with a Swiss study in 2013 reporting resistance to clindamycin (28%)

and erythromycin (30%) (Capanna *et al*., 2013). A similar study in China by de Melo *et al*., 2016 reported even higher GBS resistance to clindamycin (54.4%) and erythromycin (64.9%). However, some studies in Africa have reported low resistance to antibiotics with studies in Ethiopia by Alemeseged *et al*., 2015 and Mengistu *et al*., 2016 reporting low or no GBS resistance to clindamycin and erythromycin. To the contrary, a recent study in South Africa by Bolukaoto *et al*., 2015 reported high resistance to clindamycin (17.2%) and erythromycin (25%).

Medicinal plants are used for the treatment of infections among a myriad of other uses. These include pregnancy associated bacterial infections such as GBS disease (Medicinal plants US, 2011). In India, *Acacia nilotica* (Mubarack *et al*., 2011) and *Moringa oleifera* (El Sohaimy *et al*., 2015) were reported to have antimicrobial action against GBS. Plant extracts of *Sambucus peruviana*, *Krameria triandra*, *Cestrum auriculatum* were reported to be inhibitory against GBS (Delfani *et al*., 2017). However, not all plant extracts are inhibitory against GBS as was reported in a study carried in Venda, South Africa (Sigidi *et al*., 2017). The study by Sigidi *et al*., 2017 reported that extracts of *Ziziphus mucronata* and *Pterocarpus angolensis* had no antimicrobial activity against clinical isolates of GBS. *Olea europaea* has been reported to have antimicrobial effects against various pathogenic microorganisms such as *Campylobacter jejuni*, *Helicobacter pylori*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* (Lee and Lee, 2010; Hashmi *et al*., 2015). However, there is scanty information on its antimicrobial effects on GBS.

Although GBS is one of the major causes of neonatal morbidity and mortality in the world, comparative or analogous studies in Southern Africa, particularly on its prevalence, capsular type distribution, antibiograms, antimicrobial activity of medicinal plants against isolates,

determination of genes coding for virulence and resistance, phylogenetic profiles have not been investigated.

1.1 STATEMENT OF THE RESEARCH PROBLEM

Twenty to thirty percent of healthy women are asymptomatically colonised with GBS in the world and are at risk of vertical transmission to their new-born babies (Chen *et al*., 2013). *S. galactiae* also infects pregnant women affecting the genital tract, placenta, amniotic sac and the blood stream. Ten to sixty percent of pregnant women colonised with GBS suffer miscarriage or still birth in developing countries as compared to 7-11% in developed countries (Dangor *et al*., 2015; Vincent *et al*., 2012). Data on the prevalence of GBS in resource limited countries are scarce and cannot be generalised because the rates vary according to geographical location. Several factors also affect data estimates such as sampling techniques, laboratory techniques for isolation, identification and confirmation of isolates and variations in implementation of CDC guidelines for screening of GBS.

S. agalactiae is one of the leading causes of neonatal morbidity and mortality in the world and has been since the 1970s (Dauby *et al*., 2016). Neonatal morbidity presents as EOD and LOD which occur in the first week of life and between 7 and 89 days respectively. Invasive GBS disease in neonates results in sepsis, bacteraemia, meningitis and pneumonia presenting in EOD. Those who survive EOD may suffer long term neurological sequelae such as hearing impairment, cerebral palsy, delayed mental development and cognitive challenges while mortality in EOD is estimated at 5% (Dauby *et al.*, 2016; Kobayashi *et al*., 2016). Mortality due to GBS EOS is thought to be higher in preterm infants (Dauby *et al*., 2016). Virulence factors are important during the development of invasive disease. However, the molecular basis

of these factors is poorly characterised and indepth understanding of their distribution assists in better clinical management of colonised or infected pregnant mothers and their neonates.

The World Health organisation (WHO) recommends IAP for pregnant women colonised with GBS or those at risk of vertical transmission to their babies. Penicillin is the drug of choice for IAP but there are challenges with penicillin allergic women and there have been reports of increasing MICs to penicillin in the last decade and recently outright resistance to penicillin was recorded in an Italian study (Matani *et al*., 2016). Alternative drugs to penicillin for IAP include erythromycin and clindamycin but there is growing resistance to these drugs in the world with resistance rates as high as 25% and 17.2% being recorded in a South African study recently (Bolukaoto *et al*., 2015).

Capsular types Ia, Ib, II and III are the most prevalent in the world but there are differences in capsular type distribution according to geographic location as seen in Japan, Brazil, Ghana and South Africa (Dangor *et al*., 2016; Dutra *et al*., 2014; Slotved *et al*., 2017; Madhi *et al*., 2016). This makes the development of a capsular polysaccharide based vaccine difficult as not one vaccine can be used in different geographic locations and even in some cases in the same geographic location. While the development of a potent vaccine is still in progress cases of invasive GBS diseases are increasing. Capsular types not normally considered as invasive are now being associated with GBS disease (Kobayashi *et al*., 2016). Capsular types have to be elucidated in each geographical area to help in the development or administration of potential vaccines. However, capsular types can vary even in the same geographical location.

The increasing resistance of GBS to antibiotics and the lack of a potent vaccine have heightened focus on medicinal plants as potential alternatives. Seventy to eighty percent of rural and urban communities in Africa use traditional medicine for primary health care purposes (Maroyi and Chiekhyoussef, 2015). Eighty percent of the South Africa population uses traditional medicine

and the country is home to 10% of the world's higher plant species (Street and Prinsloo, 2013). Medicinal plants have been used in the treatment of infections and although their chemical compositions, side effects and dosage are poorly understood, they are considered candidates for eventual drug design. Information on medicinal plants is from invitro diagnostic testing and herbalists rely on contemporary knowledge for their administration with no scientific evidence. The phytochemical compositions of some medicinal plants are not known making them unsafe to administer during pregnancy hence there is need for comprehensive scientific studying of medicinal plants before they can be recommended for use.

Phylogenetic analysis is used to establish congruency in gene expression among isolates over time. Such comparative studies have not been done on GBS in Sub Saharan Africa and evolutionary covariance among organisms in not known.

1.2 HYPOTHESES

- The prevalence of *S .agalactiae* capsular type distribution among pregnant women in the Eastern Cape, South Africa and Windhoek, Namibia is the same
- There is no resistance of *S. agalactiae* to antibiotics and antimicrobial activity of plant extracts.
- GBS isolates from Namibia and South Africa do not possess the same genes coding for resistance and virulence.
- GBS isolates from South Africa and Namibia have the same phylogenetic profile

1.3 AIM AND OBJECTIVES

Overall objective

The main aim of the study was to characterize *S. agalactiae* isolated from pregnant women between 35 and 37 weeks gestation in South Africa and Namibia by molecular techniques as well as determine their susceptibility to antibiotics and plants derived active compounds.

Specific objectives

The study seeks:

1. to isolate, identify and determine the prevalence of GBS in pregnant women between 35 and 37 weeks gestation in the Eastern Cape, South Africa and Windhoek, Namibia.
2. to assess the antimicrobial susceptibility profiles of GBS isolates to antibiotics and to screen for genes coding for resistance.
3. to detect the capsular type distribution among GBS isolated from South African and Namibian pregnant women.
4. to genetically screen for genes encoding for virulence factors in GBS isolates.
5. to determine the genetic relatedness of isolates from South Africa and Namibia using multilocus sequence typing.
6. to analyse the antimicrobial properties of *Olea europaea* subspecies Africana leaf extracts and essential oil on the GBS isolates.

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CHAPTER TWO

LITERATURE REVIEW

2.0 Introduction to Streptococci

2.1 Introduction

Streptococci are prokaryotic unicellular organisms which fall in the domain Bacteria. Various species of streptococci exist and these include *S. pyogenes, S. viridans, S. pneumoniae, S. agalactiae, S. mutans, S. bovis* and *S. dysgalactiae*. Streptococci are Gram positive cocci, non-motile, do not form spores and although they are facultative anaerobes with some being strict anaerobes they are all catalase negative. For most of the streptococci, enriched media such as blood agar is needed for growth (Black, 1917; Bascomb and Manafi, 1998).

2.2 Classification of Streptococci

Various methods are used to classify streptococci which include: colonial morphology and haemolysis, biochemical activity of the bacteria and specific bacterial antigenic determinants present on the cell wall (serologic specificity). Ability of streptococci to lyse red blood cells is another key factor used in their classification. When the bacteria lyse red blood cells completely, creating clear zones on blood agar it is classified as beta (β) haemolytic streptococci while incomplete haemolysis of red blood cells on blood agar that creates a green appearance around the bacterial colonies is classified as alpha (α) haemolysis. Gamma (γ) haemolytic streptococci do not show haemolysis on blood agar (Larsson *et al*., 2014; Rodriguez *et al*., 2015). Beta haemolytic streptococci are classified using carbohydrate cell surface antigens in a system designed by Rebecca Lancefield called Lancefield grouping. Figure 2.1 below shows the different streptococcal species.

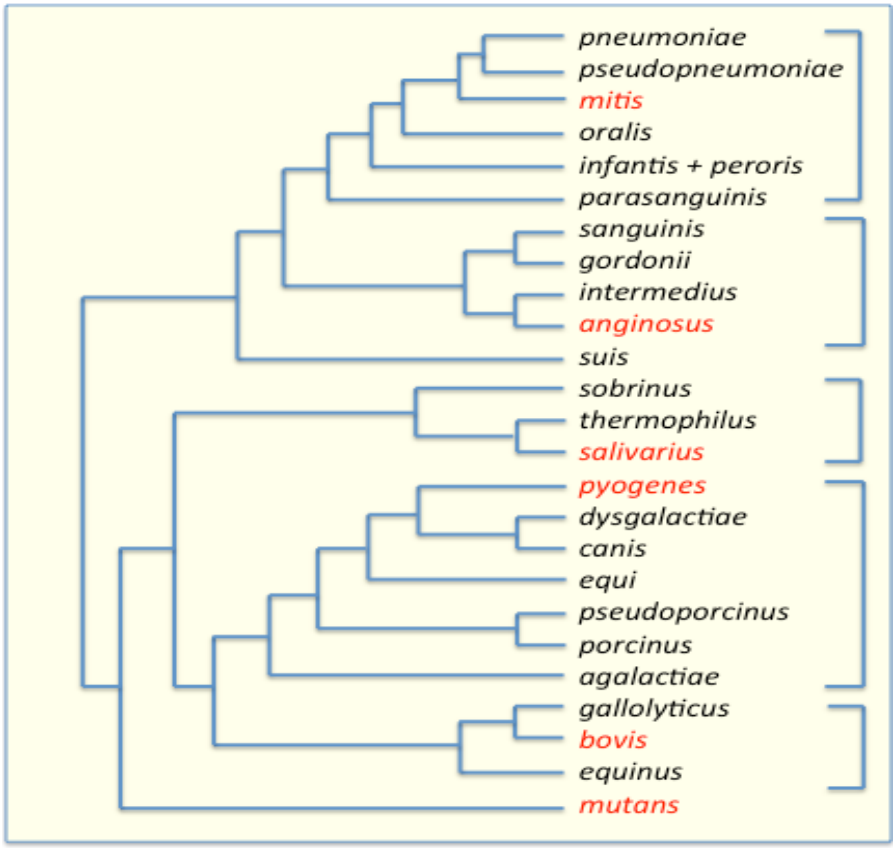

Figure 2.1: Phylogenetic analysis of *Streptococcus* species. Source: Nucleic Acids Res. 2014 Jan;42 (Database issue):D581-91. doi: 10.1093/nar/gkt1099

2.2.1 Lancefield grouping of streptococci

This method was initially designed to group coagulase negative and catalase negative β-haemolytic bacteria. However, there are two notable types which are not beta haemolytic (*Enterococcus* and *Streptococcus bovis*). The identification of haemolytic streptococci was originally based on culture characteristics and biochemical reactions. However, differentiating the different streptococci proved a problem and serologic testing was thought to be a possibility. A systematic way of classifying the bacteria would provide consistency in identification and reporting and was also be of epidemiological importance in providing the origin of the bacteria. Therefore, serological testing gave weight to designation of origin to streptococcal strains (Lancefield, 1933).

Classification of bacteria into distinct groups was based on carbohydrate antigen, the C substance. It was reported that group A streptococcus comprised of strains mainly from human beings and was linked to the development of puerperal sepsis while group B streptococcus was associated with strains from bovine and diary sources. Group B streptococcus was linked to the development of bovine mastitis and as saprophytes in women's throats and vagina (Lancefield & Hare, 1935).

2.3 Bacterial cell wall structure

Prokaryotic cells have five key components: nuclear material, ribosomes, cell membrane, cell wall and the surface layer while other essential components include the flagella, pili (fimbriae) and the envelope. The flagella and pili (fimbriae) are made of protein while the cell wall is composed of peptidoglycan (murein) complexed with teichoic acid in Gram positive bacteria and peptidoglycan surrounded by phospholipid, protein and lipopolysaccharide on the outer membrane. The plasma membrane is made of phospholipid and protein. The bacterial cell wall gives rigidity, shape, prevents osmotic lysis of the cell and protects the cell from the external environmental factors in Gram positive bacteria. In Gram negative bacteria the cell wall prevents osmotic lysis, confers rigidity, shape, acts as an outer membrane which is permeable and is associated with lipopolysaccharides and proteins which have multiple functions (Kumar *et al*., 2017; Sadovskaya *et al*., 2017).

Approximately 60% of the bacterial cell wall is made up of teichoic acids which are anionic glycopolymers with the anionic nature of these molecules allowing them to bind to metal cations. The binding of metal cations to bacterial cell walls alter some of the physiologic functions of the cell wall such as rigidity and porosity. In *Streptococcus* spp., about 40-60% of the cell wall polysaccharides are composed of rhamnose with some strains of *S. agalactiae* containing rhamnose in their repeating units (Mistou *et al*., 2016). Cell surface proteins are

important for adhesion and interaction with host cells during infection (Johnson *et al*., 2013) but the composition of cell surface proteins changes according to the environment as the bacteria adapt.

Figure 2.2 is a diagrammatic representation of a cell wall structure for Gram positive bacteria.

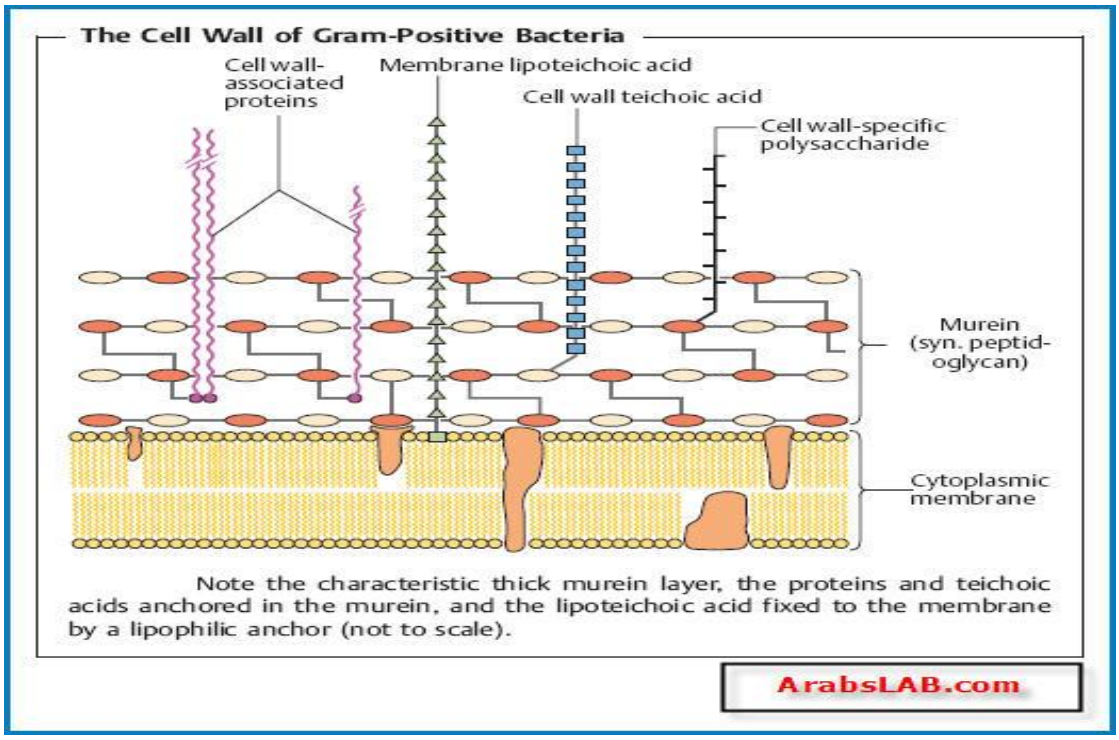

Figure 2.2: Gram positive cell wall structure.

Source: http://www.arabslab.com/vb/showthread.php

2.4 Vaginal flora

Vaginal microbiota is essential in maintaining normal microbial balance in the vagina which is part of the immune system in protecting against diseases caused by bacteria such as bacterial vaginosis and urinary tract infections. There is wide variation in the composition of the vaginal microbiome which may expose both the mother and neonate to greater risk of bacterial infection (Mendling, 2016).

Lactobacillus species is the predominant bacterial species in the vagina of healthy women but there is significant variation in vaginal flora in women which can be caused by changes in the balance of *Aerococcus, Anaeroglobus, Anaerotruncus, Atopobium, Coriobacteriaceae, Gardnerella* and other species. *Lactobacillus* species are important for the production of lactate which keeps the vagina at an acidic pH of around 4.5 (Roesch *et al*., 2017).

Various factors are responsible for change in the balance of the vaginal microbiota some of which are pregnancy related. Progression of pregnancy results in a relative change in the balance of Lactobacillus species such as *L.crispatus, L.jensenii, L.gasseri and L.vaginalis*. Administration of antibiotics alters normal flora favouring the growth of pathogenic bacteria in the birth canal of pregnant women within hours but studies have shown that IAP does not affect colonisation of GBS or *Staphylococcus aureus* although women in the third trimester become susceptible to *Escherichia coli* infection after IAP. However, women colonised with streptococcus receiving IAP will express low levels of *Lactobacillus* signifying a shift in the balance of bacterial composition due to the effect of antibiotics on normal flora (Roesch *et al*., 2017; Nasioudis *et al*., 2017).

2.5 *Streptococcus agalactiae* (Group B Streptococcus)

S. agalactiae also known as Group B Streptococcus following the discovery of the Lancefield group B antigen is a Gram positive organism causing β haemolysis on blood agar. Initially it was only known as a cause of bovine mastitis hence the name agalactiae, meaning no milk. It was only described as a cause of human disease in 1935 (Lancefield & Hare, 1935). It has emerged as the largest cause of bacterial morbidity and mortality in neonates and is also known to cause disease in immune-compromised individuals, diabetics and in malignancy (Lindahl *et al*., 2005). The primary virulent factor of GBS contributing to invasive disease and the basis of

its classification into different strains is its capsular polysaccharide. It is divided into ten serotypes, Ia, Ib and II-IX (Nishihara *et al*., 2016).

2.5.1 Pathogenicity of *S. agalactiae*

S. agalactiae is part of the normal flora of the vagina and rectum with 15 to 40% of women being asymptomatically colonised hence normal women can be colonised by GBS without symptoms. Due to the delicate relationship between GBS and the pregnant mother, it is precariously positioned to infect the neonate where both the innate and acquired immune systems are underdeveloped. During the neonatal period *S. agalactiae* is the main organism associated with the development of invasive disease caused by bacterial agents. In the last decade between 4 and 6% of neonates who were infected with GBS died. *S. agalactiae* causes meningitis in neonates and a notable percentage of those who survive the meningitis have long term problems which include mental retardation and loss of hearing (Lindahl *et al*., 2005).

One of the risk factors associated with GBS disease is preterm delivery while morbidity and mortality are high in neonates born prematurely. More than 95% of neonatal carriers of *S. agalactiae* are due to maternal colonization and subsequent transmission to the neonate during pregnancy, labour or parturition. Vertical transmission is known to occur in 80% of neonates whose mothers are vaginally or rectally colonised with GBS and invasive disease developing in 1-2% of those children. Risk factors to invasive GBS disease include: prolonged rapture of membranes during delivery, prematurity, route of delivery and extent of maternal GBS colonisation (Spellerberg, 2000). Serotypes IV, VI, VII and VIII have not been associated with invasive GBS disease in neonates.

S. agalactiae is the leading cause of sepsis, meningitis and long term neurological problems in babies younger than 90 days and is known to inhabit the upper respiratory tract and gastro and urinary tracts of pregnant women with vertical transmission to the neonate occurring during

the process of parturition. It invades the epithelial lining of tissue and the blood stream resulting in EOD which occurs in the first 6 days of life or LOD which occurs between 7 and 89 days of life. Group B streptococcus disease is known to be prevalent during pregnancy and after birth and is associated with adverse pregnancy outcomes such as still birth and preterm delivery. Efforts to reduce GBS disease in pregnant women such as administration of IAP have been successful but have not shown similar results in infants and data on the burden of GBS disease in resource limited settings is scarce or poorly characterised (Ji *et al*., 2017).

Group B streptococcus colonisation is a contributing factor to some of the cases of preterm delivery although its contribution is not as articulated as much as the contribution to EOD. A study in Australia to determine the aetiology of spontaneous abortions using autopsies and culture techniques found that GBS was the most common aetiological agent and sometimes the only organism associated with still birth in babies born to mothers with both ruptured and intact membranes. Pregnant and postpartum women have a higher risk of developing invasive GBS disease when compared to non-pregnant women with colonisation being associated with urinary tract infection, infection of the chorion and endometrium. While data in resource limited countries are scarce, in the USA GBS was found in the blood of pregnant women (43%) and those who had just given birth (32%), those with infection of the chorion (33%) and of the endometrium (25%). Other common problems associated with GBS colonisation of pregnant women include pneumonia, puerperal sepsis, poor obstetric history such as recurrent abortion, stunted foetal growth, premature delivery or rupture of membranes and infection of the chorion (Kobayashi *et al*., 2016; Perez-Moreno *et al*., 2017).

S. agalactiae has been implicated as a cause of disease in non-pregnant women with cases of invasive GBS disease doubling in recent years and in Thailand which is a Low to Medium Income Country (LMIC), GBS was identified as the leading cause of invasive disease. Other

infections associated with invasive GBS disease in non-pregnant women are infections of the bones, joints, endocardium, meninges and Intra Venous (IV) catheters. In South Africa, pneumonia was found to be commonly associated with GBS infection in 70% of cases causing a 35% mortality rate (Kobayashi *et al*., 2016).

2.5.1.1 Early Onset GBS Disease

Worldwide, the leading cause of neonatal deaths is bacterial infection. Reduction of preventable infection which leads to neonatal deaths is one of the Sustainable Development Goals (SDGs) of the WHO. In high income countries, GBS has been classified as the leading cause of neonatal sepsis and meningitis leading to high mortality and morbidity in neonates. However, the picture seems to be less appreciated in Africa (Dangor *et al*., 2015).

EOD occurs when GBS is acquired by the foetus or neonate from birth to six days after birth. In such cases, it is usually a result of vertical transmission from a mother who is vaginally or rectally colonized to the neonate. Between 30 and 70% of neonates born from women who are colonized acquire GBS, however, 1-3% of them subsequently develop GBS disease (Nishihara *et al*., 2016). The bacteria move from the vagina to the amniotic fluid after the onset of labour or rupture of membranes but infection has been known to ascend in the absence of membrane rupture. From the amniotic fluid GBS disseminates and colonises various body sites such as the foetal skin and mucous membranes and the bacteria can be aspirated into the lungs predisposing the infant to invasive GBS disease. Half of pregnant women colonised with GBS will give birth to babies colonised with 1 to 2 percent of the babies progressing to developing invasive GBS disease (Seale *et al*., 2016).

In a recent study in South Africa, 122 cases of invasive GBS disease were reported in 12 months with a total of 95.5% cases of EOD being observed in the first 24 hours (h) (Dangor *et al*., 2015). Case fatality of EOD in the South African study was 22.7% with 63.6% of the deaths

occurring in the first 2 days post-delivery or post admission. Of the infants suffering EOD, 18.9% were admitted to the Intensive Care Unit (ICU) (Dangor *et al*., 2015).

Investigators have reported that β haemolysin produced by GBS promotes entry of the organism into epithelial cells of the lung from which it spreads into the blood stream, meninges and other parts of the body. Infection of the foetus can also occur when GBS invades the amniotic fluid and this occurs during prolonged rapture of membranes when the expectant mother goes into labour. Neonatal infection can occur during birth or when the amniotic sac is infected and after acquiring infection, 90% of neonates rapidly develop into EOD in the first 24 hours. More often than not, the disease manifests at birth (Nishihara *et al*., 2016).

Several risk factors associated with EOD in rich countries which include: a positive maternal GBS culture, prolonged rupture of membranes, preterm delivery, GBS bacteriuria during pregnancy, maternal fever associated with chorioamnionitis, age of the pregnant mother and levels of anticapsular type antibodies have been identified. Reports from LMIC have found that the same risk factors play a role in GBS disease as those found in rich countries with HIV infection being an added risk factor due to the presence of low levels of GBS antibodies in HIV infected mothers (Kobayashi *et al*., 2016).

2.5.1.2 Late Onset GBS Disease

LOD occurs between 7 and 89 days after birth but pathogenesis of LOD is poorly understood with various modes of transmission and risk factors proposed. Horizontal transmission from the environment, communities and as well as nosocomial infections have been indicated as causes of LOD. Breast milk has been proposed as a potential cause of LOD although some studies have found no evidence of its contribution (Pintye *et al*., 2015). In one study, 6% of mothers with high GBS bacterial counts presented with mastitis but most of the breast milk samples with GBS were from mothers not presenting with mastitis and with low bacterial

counts. Hence, breast milk was reported as a cause of LOD in infants with or without the development of apparent mastitis (Berardi *et al*., 2012). LOD is also thought to be acquired during birth as studies have noted that about 50% of infants who suffer LOD have the same GBS strain as the mother (Pintye *et al*., 2015).

Some studies have defined LOD as occurring between 3 and 89 days from birth. For LOD to occur, GBS has to invade the blood stream after adhesion to and invasion of epithelial membranes. Transmission of GBS from mother to neonate during birth is a contributing factor to LOD but not as much as it does in EOD and in countries which have adopted and implemented IAP, cases of EOD have decreased but not LOD. The risk factors for LOD are poorly understood as compared to EOD but they are thought to include: nosocomial transmission, horizontal transmission from mother to infant after birth, transmission during breast feeding, preterm rupture of membranes and maternal GBS colonisation. Prevention strategies for LOD GBS disease have not yet been identified but recent studies are increasingly linking preterm delivery to LOD (Mengistu *et al*., 2016).

In a South African study, neurological problems were seen in about 22-50% of infants who survived GBS related meningitis. Just like in most African countries, GBS screening and IAP are not part of standard antenatal care due to resource constrains, poor capacity and infrastructure and overburdened health care systems due to HIV infection. Case fatality rate for LOD in the South African study by Dangor *et al*., (2015) was 12.5% while the incidence of LOD was five times in infants born to HIV positive women than those from HIV negative mothers (Dangor *et al*., 2015). The actual impact of LOD on permanent sequelae in one study could not be assessed due to a high number of deaths in the early stages of diseases (Berardi *et al*., 2012).

Developed countries have a much lower mortality rate of invasive GBS disease (7-11%) than in developing countries (10-60%) because they have made GBS screening and IAP standard antenatal care. In South Africa where GBS is not part of standard antenatal care the incidence of invasive GBS disease has remained at 2.38 per1000 live births in the last two decades (Dangor *et al*., 2015).

2.5.2 Prevalence of *S. agalactiae*

The prevalence of GBS in a population is established by collecting genitourinary specimens from individuals for the isolation and identification of the organism. Samples collected can be vaginal swabs only or rectovaginal swabs and several factors can influence the recovery of GBS from the specimens. These factors include the timing of sample collection, the sampling technique, specimen transport, culture media and technique used and identification methods. Studies have shown that collection of rectovaginal swabs results in higher recovery of GBS when compared to vaginal swabs only. Other samples which can be used for the recovery of GBS are cervical swabs, low vaginal swabs and urine. Use of selective media has also been found to result in higher recovery of GBS when compared to using non selective media (Wollheim *et al*., 2017).

Genital colonisation with GBS is estimated at 13% globally but rates are higher in studies which used adequate preanalytic and analytic conditions for the recovery of GBS. Prevalence rates vary across several geographic regions such as India and Pakistan (12%), Asia/Pacific (19%), sub Saharan Africa (19%), Middle East/ North Africa (22%) and the Americas, Africa (16.5-31.6%) and South America (1.4-36.7%) (Kobayashi *et al*., 2016; Wollheim *et al*., 2017). A study in Mozambique on the aetiology of meningitis in children under 5 years found that GBS was isolated in only 3% of cases and was the fourth most common organism after

Streptococcus pneumoniae (32.8%), *Haemophillus influenzae* (12.2%), and *Neisseria meningitides* (4.3%) (Nhantumbo *et al*., 2015).

Comprehensive research has to be carried in China to establish the prevalence of GBS across the country as the present data available is from sporadic studies in isolated cases. A study done in the Dongguan province of China found a prevalence rate of 8.2% which was low when compared to the global and Southeast Asia estimates but similar to prevalence rates reported in Japan (8.3%), South Korea (8.0%), Myanmar (7.1%) and Philippines (7.5%). Several factors can be attributed to the variation in prevalence rates which include variation in population demographics, time of sampling, source of samples, culture and identification methods (Ji *et al*., 2017). A Ghanaian study using both lower vaginal and rectal swabs for the recovery of GBS recorded high prevalence rates in two areas studied in the Greater Accra region of 25.5% and 28.0%. These prevalence rates were higher than those reported in other countries such as South Africa (Slotved *et al*., 2017).

2.5.3 GBS disease burden

2.5.3.1 Disease burden in the world

Young infants below 89 days of life are the most affected by GBS disease in Africa with the incidence doubling in sub Saharan Africa with serotypes Ia, Ib, II, III and V contributing 90% of invasive disease around the world. Southeast Asia has the lowest incidence of GBS disease recorded but the understanding of the extent of the burden is limited by scarcity of data in both Africa and Southeast Asia. Data on the contribution of GBS disease to adverse maternal outcomes are scarce due to the lack or no standardisation of case definitions though studies in Kenya and South Africa have shown the incidence of GBS associated still births is greater than that observed in EOD. Factors affecting data estimates include: sampling techniques especially during EOD, poor laboratory infrastructure for the recovery of GBS, variations in

implementation of IAP protocols and the lack of base line data for comparative purposes (Rivera *et al*., 2015).

The USA has seen a drop in cases of EOD by about 80% due to the effective implementation of IAP although this strategy has not seen a corresponding decrease in LOD as more than 2000 cases of LOD are still recorded annually. This strategy has been criticised for exposing an estimated 30% of new-born babies to antibiotics creating fears of a potential alteration in their normal flora which could predispose them to other infections. Numerous programs have been used in Europe to lower the incidence of GBS disease but it still remains the leading cause of bacterial meningitis in infants recording high mortality rates and long term sequelae. Countries such as England and Netherlands have reported an increase in the incidence of EOD and LOD with the prevalence of serotypes Ia, Ib, II, III and V (Kobayashi *et al*., 2016; Elikwu *et al*., 2016).

2.5.3.2 Disease burden in Africa

One of WHO Sustainable development Goals (SDGs) aims to reduce maternal and neonatal deaths by 2030 but currently bacterial infections account for 2.9 million neonatal deaths worldwide with mortality rates in Africa 4 to 5 higher than the Americas. GBS is a major cause of invasive disease in high income countries and the burden though poorly understood in resource limited countries is thought to be higher. While IAP has been lauded as a success story in the reduction of GBS disease in pregnant women and their neonates the same cannot be generalised to resource limited settings such as sub Saharan Africa. In these settings challenges for effective implementation of such a program range from lack of or poor health infrastructure, inadequate funding to problems of identifying women at risk and in need of IAP (Sinha *et al*., 2016).

There is an increased risk of LOD in children born from HIV positive mothers than those from HIV negative mothers although the risk of EOD is not different in the two groups. With a high prevalence of maternal HIV in sub Saharan African countries such as South Africa (29.5%) there is an increased risk of neurologic sequelae burden in neonates due to GBS (Dangor *et al*., 2015).

The incidence of GBS EOD and LOD in sub Saharan Africa was reported to be 0.92 to 2.09 and 0.56 to 1.0 per 1000 live births which was much higher when compared to North Africa and South Asia. Prevalence rates varied considerably in the region according to geographical location with the rates being thought to be much lower than expected as several factors affect their estimation. These factors include high cases of home deliveries, poor access to health care facilities, empirical treatment of cases before laboratory based confirmation and lack of diagnostic facilities for effective microbiologic testing of specimens such as blood cultures and cerebrospinal fluid (CSF) (Quan *et al*., 2016).

2.5.4 GBS disease management and prevention

The large intestine and lower genital tract are reservoirs of GBS but colonisation is not permanent and the bacterial load changes with time. Factors which lower the chances of vertical transmission of GBS from the pregnant mother to the neonate are administration of IAP, caesarean section before onset of labour and presence of high maternal immunoglobulin levels to specific capsular type antigens. *S. galactiae* is generally susceptible to β lactam antibiotics such as benzyl penicillin and ampicillin and these can be used for empirical treatment of GBS disease. However, after decades of reported MICs of β lactams to GBS a recent study in Italy reported outright resistance to β lactams by GBS limiting the therapeutic options (Matani *et al*., 2016). Other drugs which can be used are erythromycin and vancomycin (Nishihara *et al*., 2016).

WHO recommends intravenous IAP administration for women with GBS infection or colonisation to reduce the chances of vertical transmission to the new born baby and the empirical treatment of neonates at risk of contracting GBS with ampicillin and gentamicin. Neonates are at risk when membranes rupture for more than 18 hours where there is presence of maternal fever or foul smell and when pus is involved. IAP is indicated for pregnant women with preterm pre-labour rupture of membranes and not those with intact membranes or with rupture of membranes at or close to full gestational term. High income countries select pregnant women for IAP by profiling for risk factors as universal screening of GBS is not feasible in many settings and this is even more complex in low and medium income countries. WHO also recommends that pregnant women should have antenatal care examinations at least four times and studies have reported that sub Saharan Africa recorded 80% of women attending at least one visit with the regional range spreading from 41% in Ethiopia to 99% in Swaziland (Kobayashi *et al*., 2016).

Other methods used to reduce the risk of vertical transmission, morbidity and mortality of GBS disease are early recognition of neonatal sepsis, chlorhexidine washes and the use of a capsular polysaccharide based GBS vaccine (Nishihara *et al*., 2016).

2.5.5 Methods for isolation and identification of GBS

Methods for the identification of *S. agalactiae* from clinical specimens such as vaginal and rectal swabs, cerebrospinal fluid and blood cultures involve culture techniques, serological methods and molecular based techniques for rapid identification of the organism. Revised Centre for Disease Control (CDC) guidelines for the universal screening of pregnant women recommend the culture based method as the gold standard for GBS isolation in women between 35 and 37 weeks gestation. There are expanded methods for detecting the bacteria in clinical

specimens recommended by CDC such as Polymerase Chain Reaction (PCR) based assays (Wollheim *et al*., 2017).

Specimens collected for isolation of GBS are inoculated into selective enrichment broth media such as Todd-Hewitt or LIM broth supplemented with gentamicin (8µg/mL) and nalidixic acid (15µg/mL) and incubated at 33-37ºC for 18 to 24 hours after which the broth is streaked onto 5% sheep (or horse) blood agar. The blood agar is incubated at 33-37ºC for 18 to 24 hours after which it is inspected for β haemolysis. However, some GBS colonies present as non-haemolytic requiring further confirmatory testing such as the CAMP test after which positive samples are regarded as GBS isolates. Isolates can also be tested with Lancefield group B specific antibodies for confirmation as GBS isolates (Wollheim *et al*., 2017; Adler *et al*., 2008).

PCR based methods amplify the DNA of genes specific to GBS such as the *scp*B, *cfb* or any of the house keeping genes (*atr, phes, sdh, glcK, gln, adh* and *tkt*). The PCR products are electrophoresed on agarose gel and detected using a gel imaging system. In a study by Wollheim *et al*., (2017), PCR based methods detected GBS in 26% of cases while culture only and culture combined with the CAMP test detected GBS in 22.5% and 19.6% of the cases respectively. The PCR methods showed 100% sensitivity and 95.6% specificity with a Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of 86.8% and 100% respectively (Wollheim *et al*., 2017; de-Paris *et al*., 2011).

Another study comparing culture based methods and PCR found that Islam culture media detected GBS in 25.3% of cases while *cfb* and *scp*B gene based PCR detected 30.6% and 30% cases respectively showing 100% sensitivity and 92.85% and 93.75% specificity respectively in both PCR methods (Shabayek *et al*., 2010). A study comparing culture based methods only found that culturing in LIM broth followed by subculture on chromogenic GBS differential

agar was better than culturing in LIM broth followed by direct antigen detection (Adler *et al*., 2008).

2.5.6 Maternal factors influencing GBS transmission

The large intestine and lower vagina are major reservoirs of GBS (Nishihara *et al*., 2016). Colonisation of the rectum and vagina is not always persistent but it can be transient or intermittent. Heavy GBS colonization of the rectum or lower vagina during pregnancy is a risk factor for infection of the amniotic fluid and development of EOD and LOD. Bacteriuria occurs in 2-7% of pregnant women who are heavily colonised. Worldwide between 10 and 40% of women are vaginally or rectally colonized with GBS with a 19% colonization rate for women in sub Saharan Africa. High GBS prevalence has been reported in South Africa (30.9% and 23%) in 2015 and 2016 (Chukwu *et al*., 2016; Cools *et al*., 2016), 21% in Zimbabwe (Mavenyengwa *et al*., 2010), 23% in Tanzania (Joachim *et al*., 2009), 28.8% in Uganda and 20.2% in Kenya (Namugongo *et al*., 2016; Cools *et al*., 2016].

A study in Malawi showed a direct relationship between HIV infection and GBS colonization. Group B streptococcus was more prevalent in HIV positive women with a CD4 count more than 500cell/µl than lower CD4 counts. At low CD4 count, the acquired immune response is reduced and women are prone to bacterial vaginosis and changes in vaginal microflora which reduces GBS colonization. In a study done in HIV positive women, they had lower serum anti GBS antibodies which exposed their neonates to EOD and LOD (Nishihara *et al*., 2016).

Maternal vaginal and rectal colonization of GBS is linked to vertical transmission to the neonate. Intrapartum antibiotic prophylaxis is one method used to reduce chances of vertical transmission through reduction of GBS load, providing ample antibiotic concentrations, to confer protection to the neonate from bacterial infection and providing enough antibiotic, to the amniotic fluid to prevent GBS proliferation (Matani *et al*., 2016; Kim *et al*., 2011).

The most commonly used drugs are β -lactams with benzyl penicillin or ampicillin being the first choices for pregnant mothers with no known penicillin allergy. The CDC recommends a minimum of 4 hours between IAP and giving birth. Universal screening and a risk based approach are used in identifying suitable candidates for IAP. In the universal screening method, CDC recommends collection of both rectal and vaginal swabs for GBS identification at between 35 and 37 weeks gestation and for IAP to be effected to GBS carriers during labour. Other women considered will be pregnant women with GBS bacteriuria and a history of a child who suffered EOD (CDC, 2010). The USA adopted universal screening of pregnant women and has recorded a notable fall of GBS EOD from 1.7 cases per 1000 live births reported in the 90s, down to 0.26 cases per 1000 live births by 2014. Despite these tremendous results in reduction of EOD, LOD has remained unmoved at 0.3 cases per 1000 live births (Nishihara *et al*., 2016).

The risk based approach assesses factors related to the development of EOD in the new-born. These factors predispose the foetus or neonate to acquisition of GBS from the screened or unscreened mother. These factors include the following: preterm labour, prolonged rapture of membranes (PROM) of more than 18 hours and an intrapartum temperature of more than 38˚C (CDC, 2010; Kim *et al*., 2011). Countries which do not support or cannot afford universal bacteriological screening adopt strategies to implement IAP when risk is identified. An example is the UK Royal College of Obstetricians and Gynaecologists which has adopted the risk based approach over universal bacteriological screening (Nishihara *et al*., 2016).

2.5.6.1 GBS in breast milk

Cases of GBS in breast milk have been documented in very few studies. Comparisons of the findings from the different studies are difficult due to variations in the methods used. Studies have shown that the incidence of GBS in breast milk is higher in mothers of full term babies

(0.82%) as compared to mothers with preterm infants (0.4%) (Le Doare & Kampmann, 2014). Maternal breast milk has been found to contain between 10^3 and 10^9 colony forming units (CFU)/mL of GBS. The incidence of GBS has been found to be highest in donated breast milk, ranging from 3.5% to 10%. *S. agalactiae* can be reduced in donated breast milk by pasteurisation but the process depletes the breast milk of antibodies and cellular components. Strategies have been proposed to reduce transmission of GBS through breast milk when there is recurrent infection or when the mother has mastitis but these have not been widely adopted (Le Doare & Kampmann, 2014).

In the majority of cases where GBS was found in maternal breast milk, mastitis was not indicated. During delivery an infant can acquire GBS in the oropharynx and ascending infection from the neonate infects the mother's milk ducts during breast feeding. The bacteria multiply in the maternal breast milk and re-infects the neonate during breastfeeding. However, breast milk can offer protective immunity against GBS with immunoglobulin A (IgA) blocking the carbohydrate mediated cellular attachment of GBS to host cells. Capsular Polysaccharide (CPS) antibodies of type III GBS were detected in 63% of women in Sweden. Types Ia, II and III IgA was found in 10% of women in the USA (Le Doare & Kampmann, 2014).

2.5.7 *S. agalactiae* virulence

The interplay between the innate immune system and bacterial cells ensures that the body is in a position to resist infection. Bacterial infectivity comes in when this balance between the immune system and the ability of the bacteria to invade the body is altered. The ability of a pathogenic organism to cause disease is called virulence and it confers the microbe with its pathogenicity. This process is largely dependent on the microbe's ability to evade the components of the immune system, mainly phagocytosis, ability to invade tissue and destroy them. Once the bacteria breach epithelial barriers they encounter an environment devoid of iron

which is important for its metabolic process as the host's body sequesters the iron. The bacteria invade tissue in search of essential elements for survival and multiplication. However, the target of the microbes such as bacteria is not to harm the host or kill it but to multiply since there is no gain to the microbe by killing the host which provides the optimum conditions for its survival and multiplication (Beceiro *et al*., 2013).

Rapid multiplication and mutation by bacterial cells provide a positive selection advantage against hostile environmental and host factors aimed at killing the bacteria. However, the host is equipped to deal with such changes in the bacteria hence bacterial virulence cannot be dissociated from host interaction and this interplay defines the course of clinical disease. Several factors have been reported to contribute to the pathogenicity of the organism. These molecules are either released by GBS into the immediate surrounding or are present as cell surface molecules.

Development of the Blood Brain Barrier (BBB) continues from the late stages of pregnancy to the postnatal period but during this period there is increased permeability of the BBB to toxins, drugs and pathological damage which can later result in neurological damage. Vertical transmission of GBS occurs from a rectally or vaginally colonised pregnant woman's genital tract to the amniotic fluid during labour although GBS is also known to infect intact membranes. Bacteria are aspirated into the lungs where it causes pneumonia and moves into the blood stream causing septicaemia. The bacteria can be transferred through blood into the Central Nervous System (CNS) where it can cross the BBB transcellularly using laminin binding protein without leaving evidence in infected phagocytes. Various properties enable GBS to evade the immune system and invade tissue causing these problems. These include the polysaccharide capsule, expression of the *scp*B gene, β haemolysin, C proteins and pili among other factors (Barichello *et al*., 2013).

2.5.7.1 Bacterial capsule

The capsule is an outer envelope of a bacterial cell which is made of polysaccharides. It is found in both Gram positive and Gram negative bacteria and is known as the K antigen. The main causative agents for pneumonia and meningitis such as *S. pneumoniae, Haemophilus influenza, Neisseria meningitides, Klebsiella pneumoniae, E. coli* and *S. agalactiae* contain the capsule (Kennedy *et al*., 2015; Kay *et al*., 2016; Limoli *et al*., 2015). The capsule is made of polysaccharides consisting of repeating oligosaccharides of two to four repeating monosaccharide units to form approximately 0.2µm of a viscous layer with the final structure made of 98% water (Toniolo *et al*., 2015).

The function of the capsule is to prevent the cell from drying, protection from temperature effects, mechanical injury and to allow the bacterial cell to attach to other surfaces in biofilm formation. The capsule also acts as a source of nutrition when nutrients are low, enables cells to repel each other, prevents attachment of bacteriophages to bacterial cell surface and acts as an antiphagocytic component of bacterial cells (Grimwood *et al*., 2015; Hajishengallis *et al*., 2013).

In *S. agalactiae* the capsular polysaccharide is made up of five to seven repeating monosaccharides but the composition varies among the different strains of the organism resulting in different capsular types of the same bacteria. The *cps* operon encodes for enzymes which are needed for the transport and assembly of capsular polysaccharides and it consists of 18 genes separated into three sets which code for serotype, glycosyltransferase and polymerase. *Cps E-L* encodes for the capsule serotype and *cps A to D* are conserved in capsulated bacteria including *S. agalactiae* and *S. pneumoniae*. They encode for enzymes needed for sialic acid formation (Toniolo *et al*., 2015). In GBS twelve genes make up the capsule synthesis cluster

and these are *lysR*, *cpsR*, *cpsA-cpsJ* with different strains of GBS serotypes expressing conserved capsule synthesis genes.

The capsule is important for the attachment of bacterial cells to epithelial cells of the host and inhibiting phagocytosis by macrophages and neutrophils. The sialic acid prevents the deposition of the complement C3b to the bacteria and because C3b is a major opsonin the prevention of C3b attachment prevents opsonisation and ultimately phagocytosis. *S. agalactiae* produces enzymes which inactivate complement components. The *scpB* gene encodes for C5a peptidase, an enzyme which inactivates the complement component C5a which is an inflammatory component encouraging complement activation and it is also a chemotactic factor and anaphylatoxin (Rosini *et al*., 2015).

The capsule is the major virulence factor for GBS and is used for epidemiological purposes and its virulence is through molecular mimicry as the host immune system fails to recognise pathogen components bringing in a level of non-self-tolerance (Rajagopal, 2009).

2.5.7.2 Haemolysin

About 90% of human GBS strains express β haemolysin, a very important virulence factor which is also used in the microbiological identification of GBS. It plays a key role in GBS virulence by allowing the bacterial cells to penetrate human cell barriers such as epithelial and endothelial cells (Sagar *et al*., 2013). This allows GBS to colonise and infect the lungs and the blood brain barrier causing pneumonia and meningitis. The β- haemolysin activates the inflammatory response of infected individuals. However, GBS strains which have high β-haemolysin activity have been shown to have increased virulence. These strains have been isolated from cases of preterm labour. Non haemolytic GBS strains are rarely associated with neonatal GBS disease (Rosa-Fraile *et al*., 2014) and the isolation of non-haemolytic GBS

strains in samples like blood cultures is not common. This is due to reduced virulence as haemolysin is an important virulence factor (Sigge *et al*., 2008).

Haemolysin is considered one of the major virulence factors of GBS and is crucial in the invasion of tissues such as epithelium and endothelium by destabilising the cell membranes. This leads to impaired tissue or organ function such as cardiac and liver function and induces inflammatory responses which can lead to long term neurological sequelae. Haemolysin activity has been shown to be inhibited by phospholipids such as phosphatidylcholine and phosphatidylethanolamine. The haemolytic toxin is thought to be non-immunogenic but there are conflicting reports on the effect of the haemolysin on phagocytic cells with some reports stating that it makes GBS susceptible to phagocytic killing while others state it causes macrophage apoptosis. However, invasive GBS disease has been found to be caused by haemolytic strains with hyper haemolysis being associated with invasive disease in neonates (Rajagopal, 2009; Sagar *et al*., 2013; Whidbey *et al*., 2013).

The GBS β-haemolysin is encoded in the *cyl* gene cluster while non haemolytic GBS strains harbour mutations in the gene cluster and these mutations occur in 1-5% of human GBS isolates. The *cyl* operon is comprised of the following genes: *cyl*X, *cyl*D, *cyl*G, *cyl*C, *cyl*Z, *cyl*A, *cyl*B, *cyl*E, *cyl*F, *cyl*I, *cyl*J and *cyl*K. The operon is linked to haemolytic activity and pigment production by GBS strains. Mutation in *cyl*E gene has been demonstrated to result in the non-haemolytic phenotype. Therefore, cylE represents the GBS haemolysin phenotype (Rosa-Fraile *et al*., 2014).

2.5.7.3 C5a peptidase

Scp genes are found in group A streptococcus (*scp*A), group B streptococcus (*scp*B) and group G streptococcus (*scp*G). The *scp*B gene encodes for scpB protein which is a cell surface protein with the ability to cleave C5a and allows binding to fibronectin. C5a is a chemoattractant which

recruits neutrophils to the site of inflammation helping with fast elimination of bacteria while fibronectin is a high molecular weight protein which allows binding of cells to extracellular matrix. The C5a protein has an N-terminal end which has catalytic activity promoting binding of the protein to human tissue using integrins. Sialic acid impairs deposition of the complement C3 component on to the bacteria cell reducing the production of C5a which together with the action of scpB protein further diminishes C5a levels subsequently reducing neutrophil recruitment. *S. agalactiae* immunogenic bacterial adhesion molecule (BibA) binds C3bp which is important for phagocytosis through the classical complement pathway. Furthermore, GBS binds factor H blocking the deposition of C3b further inhibiting formation of C5a (Maisey *et al*., 2008). A study in Spain found *scp*B to be frequently significant among strains causing invasive neonatal disease (Lopez *et al*., 2017). In addition, in an Egyptian study 100% of isolates from pregnant women had the *scp*B gene (Sadaka *et al*., 2017).

C5a peptidase is a serine protease which is used by bacteria to inactivate the human C5a complement component. C5a is a neutrophil chemoattractant which is produced during the complement cascade. The protease contributes to *S. agalactiae* virulence by interfering with the recruitment of neutrophils to the inflammatory site. This results in a diminished response of the inflammatory process and subsequent failure to subdue infection in tissues invaded by bacteria allowing the bacterial cells to infect epithelial tissue. All human isolates of GBS contain the *scp*B gene but only a minority of bovine isolates of GBS contain the gene (Taylor *et al*., 2016).

The *scp*B inactivates C5a from humans and not from other several species and this means *scp*B protease is a virulent factor in human hosts causing development of invasive GBS disease in humans. Some strains of capsular type III GBS do not express C5a peptidase activity although they contain the *scp*B gene. Those strains which do not express C5a peptidase activity have

high capsular sialic acid content and their capsule effectively inhibits the activation of complement and C5a peptidase production and acts as the major virulent factor (Lindahl *et al*., 2005).

2.5.7.4 CAMP factor

The Christine Atkins Munch-Petersen (CAMP) factor is 226 amino acids long and is heat stable. It is non haemolytic and is used for identification of GBS isolates (Sagar *et al*., 2013). The usual phenotype of haemolysis on blood agar cannot be used to identify non haemolytic streptococci but the CAMP factor can be used as it is positive in non-haemolytic GBS strains (Sigge *et al*., 2008). The CAMP factor is a 25.3kDa protein which interacts with the cell membrane of red blood cells resulting in red cell lysis. It also binds the Fc portion of IgG and IgM antibodies but the role of the CAMP factor in pathogenesis is unclear. However, some researchers suggest that its release by bacteria impairs host immune responses (Gase *et al*., 1999).

2.5.7.5 Hyaluronidase

Hyaluronate lyases are enzymes encoded by the *hyl*B gene which break down hyaluronate. The enzyme breaks down the bond between N-acetyl-β-D-glucosamine and D-glucosamine. Hyaluronic acid is a major component of connective tissue, placenta and amniotic fluid. Hyaluronate lyases therefore allow GBS strains to break down barriers where hyaluronic acid is a component of the structure giving the bacteria invasive properties. *S. agalactiae* strains that are isolated from neonates by sepsis have demonstrated high hyaluronate lyase (hyaluronidase) activity. Most of the hyaluronate lyase activity has been observed in GBS serotype III (Baker *et al*., 1997).

2.5.7.6 The lmb protein

This is a surface protein of *S. agalactiae* which has been linked to adhesion and metal transport in the bacteria which is expressed by most GBS strains. It was discovered as a laminin binding protein of GBS involved in colonization and invasion of epithelial tissue although there are conflicting reports on the role of the protein. This makes it an interesting target in pathogenicity and potentially vaccine development against GBS. The *scp*B and *lmb* region in GBS is located on a transposon and this arrangement may allow for transfer of the genes horizontally. Most bovine GBS isolates lack both the *scp*B and *lmb* genes. This suggests that these two genes are inherited or lost together and may also be suggestive that both genes are important in the invasiveness of GBS (Lindahl *et al*., 2005).

2.5.7.7 Lipoteichoic acid

Teichoic acids are anionic glycoproteins which are major parts of Gram positive bacteria cell walls. They are a diverse family of glycopolymers which have phosphodiester linked polyol units. Lipoteichoic acids (LTAs) are teichoic acids anchored in the cell walls of bacteria covalently bound to the peptidoglycan of bacteria and constituting approximately 60% of bacterial cell wall. Wall teichoic acids (WTAs) are important for regulation of cell morphology, cell division, muramidases and for ion homeostasis and offering protection to the bacteria from host defences and antibiotics. WTAs give bacteria an anionic charge and hydrophobicity which affect the binding of extracellular molecules on the bacteria. The bacteria without WTAs cannot tolerate high temperature or high salt concentration and are susceptible to fatty acids from humans which have antibacterial properties (Brown *et al*., 2013).

LTAs are released from bacterial walls after lysozomal lysis or due to the action of leucocyte peptides or beta lactams. LTAs that are bound to host can interact with immunoglobulins hence can activate complement. They trigger inflammation through release of reactive oxygen and

nitrogen radicals and other molecules such as cationic proteinases, cationic peptides, growth factors and cytokines. They confer virulence to Gram positive bacteria and play a significant role in infections and long term problems observed in humans due to infections caused by Gram positive bacteria such as GBS (Ginsburg, 2002). They contain conserved portions and due to their conserved nature, they potent as targets for new antibiotics, vaccines and for diagnostic purposes (Weidenmaier & Peschel, 2008).

2.5.7.8 *hla* and *hlb*

Secretion of enzymes and toxins is a major virulence factor for bacteria including Gram positive species. Bacteria such as *S. aureus* and *S. agalactiae* produce haemolysins with the expression of haemolysin genes such as *hla, hlb, hlgc* and *hld* being linked to the development of invasive disease (Zhang *et al*., 2016). The haemolysins have a high affinity for mammalian red blood cells destabilising the cell membranes. In a study on *S. aureus*, all isolates expressed haemolysis and were amplified for *hla* gene with almost half of the isolates being amplified for the *hlb* gene (Ferreira *et al*., 2016).

2.5.7.9 *etA* and *etB*

Exfoliative toxin A and B (etA and etB) are virulence factors commonly found in *S. aureus* but have been thought to contribute to the virulence of other Gram positive bacteria such as streptococci. A study by Johnson *et al.,* (1991) screened for the exfoliative toxins A and B in strains of streptococcus species which produced pyrogenic exotoxins using PCR (Johnson *et al*., 1991; Kong *et al*., 2016). Studies have shown a close resemblance of streptococcal pyrogenic exotoxin A to staphylococcal strains (Sharma *et al*., 2000).

2.5.7.10 *lukM*

Leukocidin is a virulence factor commonly found in *S. aureus* and other Gram positive organisms which is cytotoxic to phagocytes causing pore formation and death of the phagocytic cells. They are potent cytotoxins which form pores through the aggregation of a class F component and a class S component. Several class F components (LukF-PV, -R, -D, '-PV, -1 and HlgB) and class S components (LukS-PV, -R, E, M, -1, HlgA and HlgC) exist (Jayasinghe and Bayley, 2005). *S. aureus* is comparable to *S. agalactiae* due to the expression of leukotoxicity which reduces host defences facilitating rapid colonisation or development of invasive disease in neonates, the elderly and immunocompromised individuals (Schlotter *et al*., 2012). *lukS*, *lukF* and *lukM* have been isolated from cows with bovine mastitis (Yamada *et al*., 2005). Leukocidins bind to C5a receptors on the surface of phagocytes' facilitating the formation of lytic pores on their surface (Spaan *et al*., 2015).

Some of the GBS virulence genes which are important in the development of invasive GBS disease are *sodA*, *covR*, *lmb* and *fbsA*. *SodA* encodes for superoxide dismutase which converts superoxide ions into oxygen and hydrogen peroxide and the enzyme requires manganese as a cofactor. The *covR* gene encodes for a CovR protein which is a DNA binding response regulator protein. *lmb* gene encodes for a laminin binding protein which is important in GBS colonisation and movement into the intravascular system and the *fbsA* gene encodes for a fibrinogen binding protein for adherence of bacteria to epithelia (Korir *et al*., 2017).

2.5.8 Host defences to GBS infection

When GBS enters sterile body sites, the innate immune system is set to clear the invading bacteria through the process of phagocytosis by polymorphonuclear leucocytes. Phagocytosis of GBS is enhanced by opsonisation through complement components or immunoglobulins.

Anti GBS antibodies are IgG serotype specific antibodies which can cross the placenta and confer passive immunity to the foetus (Henneke *et al*., 2005).

S. agalactiae employs various mechanisms to impair opsonisation and phagocytosis. The polysaccharide capsule inhibits activation of the alternative complement pathway by blocking attachment of the complement component C3 (Hyams *et al*., 2010). Antibodies binding to GBS are neutralized when their Fc portion is non-specifically bound by the CAMP factor and β C protein. In inflammation, the recruitment of phagocytic cells to the site of infection is crucial in containing disease (Spellerberg, 2000; Landwehr-Kenzel & Hennek, 2014). Chemotaxins such as C5a are important as chemical mediators in attracting the phagocytic cells. However, GBS produces C5a peptidase, an enzyme which catalyses the breakdown of C5a hampering its action as an effective chemo attractant. This helps GBS to evade the immune system and invade body tissue. High sialic acid of GBS strains is another factor considered for limiting complement activation (Morello *et al*., 2015).

The GBS cells which are phagocytosed are resistant to killing by oxygen radicals even if they lack the enzyme catalase. This means they can survive for 24 hours inside a phagosome and spread to body tissues. The ability of GBS to invade eukaryotic cells is the primary cause of invasive disease. *S. agalactiae* strains treated with trypsin showed capacity to invade cells in one experimental study on animal models giving an impression that cell surface proteins of the bacterium are crucial in host cellular invasion (Spellerberg, 2000).

Bacterial cells which evade phagocytosis and enter cerebral capillaries move into the cerebro spinal fluid (CSF). Entry of bacterial cells into the CSF initiates an inflammatory response in the subarachnoid space which is marked by protein elevation and an increase in leukocyte numbers (Khan, 2016). Antigens from the bacterial polysaccharide cell wall induce cytokine release which initiates leukocyte chemotaxis. This inflammatory response slows blood flow to

parts of the brain causing cerebral ischemia and is the ultimate factor in the development of permanent neurological damage even if the neonate survives meningitis (Spellerberg, 2000).

2.5.9 Laboratory diagnosis of GBS

2.5.9.1 Culture

Recovery of GBS is very crucial for identification, characterisation, treatment and management of GBS disease especially in immunocompromised individuals, pregnant women and in neonates. Center for Disease Control and Prevention (CDC) guidelines recommend isolation of GBS from both rectal and vaginal swabs. Sampling from both the lower vagina and rectum significantly increases the chances of GBS recovery as compared to sampling the cervix or the vagina. Use of enrichment media significantly increases GBS growth and in carrier women as much as 50% false negative results occur if enrichment broth is not used (CDC, 2010). However, with the use of selective broth the culture procedure could take as much as 48 hours which is a long period to wait for results so a variety of media is used for recovery of GBS including both agar and broths.

2.5.9.2 Granada medium

This media is a modification of Islam's medium and it contains serum and starch as additives. On media containing serum and starch, GBS anaerobically synthesizes an orange pigment granadaene which is an ornithine glycopolyene. The appearance of orange pigmented bacterial colonies is a positive identification of GBS. Culture and identification of GBS is done in a single step hence the method is fast. An example of such GBS differential agar is GBSDA produced by Becton Dickinson. Granada agar may not be suitable for the detection of GBS from primary specimens as the absence of the orange pigment does not mean there are no GBS colonies. *S. agalactiae* is effectively recovered by subculture from broth (te Witt *et al*., 2012)

while some studies have found the routine use, Granada medium in the recovery of GBS from clinical specimens to be accurate, easy and highly sensitive (Rolland *et al*., 2003).

2.5.9.3 Chromogenic Agar (CA)

CA agar does not require anaerobic incubation and *S. agalactiae* colonies grow and appear as pink to red and round colonies while other bacterial species are inhibited altogether. For other bacterial colonies which manage to grow they will have a different colour. This type of agar is produced by Biomerieux. However, the constituents of the media are not specified by the manufacturer (Aila *et al*., 2010).

2.5.9.4 Columbia Colistine Nalidixic Acid Agar (CNA)

Columbia Colistine Nalidixic Acid Agar (CNA) with 5% sheep blood is a selective medium for the isolation of Gram positive bacteria mostly staphylococci, streptococci, and enterococci. It is mostly used for recovery of bacteria in clinical specimens. The medium is enriched with 10 mg colistin and 15 mg nalidixic acid per litre in Columbia base. It inhibits growth of *Proteus, Klebsiella* and *Pseudomonas* spp. (BD, March 2013). When compared to other selective media like Neomycin Nalidixic acid Agar (NNA), CNA has low sensitivity and recovery of GBS (64.2% against 77.9% for NNA). Recovery of GBS on CNA was even found to be lower when compared to selective broth medium (Dunne, 1999).

2.5.9.5 Broth enrichment

Todd Hewitt and Lim broth are commonly used for enrichment of GBS in clinical specimens. On top of the necessary growth nutrients for bacteria, Todd Hewitt broth contains sodium carbonate (Na_2CO_3) and disodium phosphate (Na_2HPO_4) as buffers for protection of haemolysin produced by streptococci. If it is not protected, haemolysin will be destroyed by

acid which is formed during fermentation of dextrose (Atlas, 2010). Todd Hewitt broth is also recommended when producing streptococci for serotyping.

Lim's broth is made of Todd Hewitt base with additives to inhibit growth of Gram negative bacteria. Todd Hewitt is supplemented with either: gentamicin (8µg/mL) and nalidixic acid (15µg/mL) [TransVag broth] or colistin (10µg/mL) and nalidixic acid (15µg/mL)[Lim's broth] (CDC, 2010).

2.5.9.6 Gram stain

Gram staining is a simple, rapid and cheap method for presumptive classification and identification of organisms. Gram staining can be done on colonies growing on agar or on primary clinical specimens like vaginal and rectal swabs (Xu *et al*., 2017). Studies have reported that Gram staining can be used for provisional identification and confirmation of GBS in clinical samples while waiting for culture results to be produced between 24 to 48 hours. While Gram staining is neither specific nor sensitive, it has been used to make clinical decisions where GBS is suspected and treatment has to be initiated (Mahon *et al*., 2011).

2.5.9.7 Catalase test

The test is used for identification of catalase producing organisms. *S. agalactiae* is a catalase negative organism so the test differentiates the organism from *Staphylococcus* and *Enterococcus* species which are catalase positive (Black, 2008).

2.5.9.8 CAMP Test

The test is used for the presumptive identification of GBS and it rarely gives false positives results. The principle of the assay is based on the interaction of a β haemolysin produced by *S. aureus* with an extracellular factor produced by *S. agalactiae.* The interaction of the β

haemolysin and extracellular factor produces synergistic haemolysis which is easily observed on 5% sheep blood agar. This synergistic haemolysis is produced by both haemolytic and non-haemolytic *S. agalactiae* (Mahon *et al*., 2011).

2.5.9.9 Hippurate hydrolysis

Hippurate hydrolysis is a test used to separate *S. agalactiae* from other β haemolytic streptococci. However, there are other bacteria which are hippurate positive such as bacillus and enterobacteriaceae (Mahon *et al*., 2011).

2.5.9.10 Serology

Serological assays for detection of GBS are simple, cheap and reproducible with some being used for the determination of antibodies specific to GBS while others detect antigens associated with GBS infection or colonisation. In some assays, GBS CPS antigens are bound on solid phase and plasma from human subjects suspected of GBS infection containing immunoglobulins against CPS is added to the solid phase so that the antibodies can bind to the CPS antigens. The antigen-antibody reactions are detected by use of enzymes (Enzyme Immunoassays), fluorescence (Fluoro Immunoassays) or by chemiluminescent assays (Basham *et al*., 1996).

Lancefield typing is the generally accepted method for typing GBS. However, the number of non typable strains has been growing and the method is also long, time consuming and requires expertise. Latex agglutination tests are available for typing microorganisms including streptococci and GBS latex agglutination tests are available for rapid detection of GBS in primary clinical specimens like urine, cerebro spinal fluid (CSF), swabs and primary cultures. The assays are important in providing identification to non-haemolytic strains of *S. agalactiae* rapidly as compared to culture techniques and other serologic tests (Slotved *et al*., 2003). Latex

beads are coated with antibodies against GBS carbohydrate antigens and produce visible clumping in the presence of corresponding antigens.

Sandwich Enzyme Linked Immuno Sorbent Assays (ELISA) can be used to detect GBS. Anti GBS antibody bound to solid phase and bacterial cells are added to bind the antibody with an enzyme labelled secondary antibody being added to bind the bacterial cells. Antigen – antibody reactions are detected by adding appropriate substrate to the reaction mixture (Bu *et al*., 2015). Indirect ELISA is used to detect antibodies to GBS in human serum following exposure to the microorganism. Purified GBS antigens are fixed to a solid phase and serum or other body fluid containing antibodies to GBS is added while a secondary antibody with a reporter molecule is added to detect the reaction (Liu *et al*., 2013).

Sensitivity of serological assays is between 88 and100% while specificity is between 81 and 100%. Latex agglutination tests have been shown to detect GBS in fluids with as few as 10^5 colony forming units (CFU) per millilitre and sensitivity differences among assays means that latex agglutination cannot be used solely for screening purposes as some GBS cases can be missed (Ascher *et al*., 1991).

2.5.9.11 Vitek 2 System

The Vitek 2 is a card based system which was designed by Biomerieux (Missouri, USA) for the identification and antimicrobial susceptibility testing of organisms. The organisms tested range from Gram positive, Gram negative bacteria, *Bacillus* spp. to yeasts. It is an automated system which utilises growth characteristics of organisms to identify the organisms. The system uses colorimetric reagent cards which have wells with each well containing a substrate for evaluating the metabolic activity of the organism. Metabolic activities include acidification, enzymatic hydrolysis of molecules, alkalinisation and even growth. The cards are incubated

and interpreted automatically while the reactions are referenced to a database for interpretation (Machen *et al*., 2014).

For identification of Gram positive organisms the system uses an array of biochemical tests which measure utilization of carbon, enzymatic activities and resistance. Biochemical tests carried out include D-amygdalin, D-xylose, arginine dihydrolase, phosphatase, leucine arylamidase, urease, lactose and optochin resistance. Sensitivity of the Vitek 2 system in identification of Gram positive organisms is reported to be 96.5% (Pincus, 2006) while other studies have shown 98% sensitivity for positive identification of *Staphylococci*, *Streptococci* and *Enterococci* species and 97.7% for *S. agalactiae* (Ligozzi *et al*., 2002). American Type Culture Collection (ATCC) strains are used as positive controls in Vitek 2 analyses. However, there are various special considerations when using the automated Vitek 2 system and these include use of appropriate culture media for recovery of organisms prior to application in the Vitek, culture age, conditions of incubation and turbidity of the inoculum.

The Vitek 2 uses a card system for antimicrobial susceptibility testing with the cards containing a few microliters of antibiotics. Repeated measurement of turbidity is used to monitor growth of bacteria over a specified period after exposure to the antibiotic and antimicrobial testing of common Gram positive and Gram negative organisms is done over a 4 to 10 hour period (Reller *et al*., 2009).

2.5.9.12 Matrix Assisted Laser Desorption/Ionization- Time of Flight (MALDI-TOF)

Conventional microbiology methods for amplification and identification of bacteria from clinical isolates include stain based protocols, microbial culture on agar or in broth, biochemical and antigenic techniques for metabolic and phenotypic analysis. While these methods have been in use for long in the microbiology laboratory they suffer from delays due to slow or failure of bacteria to grow prompting a delay in results. MALDI-TOF mass

spectrometry is a method which was largely confined to clinical chemistry and haematology for characterization of proteins. The material to be analysed and the matrix co-crystallize and form a solid deposit when the mixture dries. The matrix supplies protons which help in the ionization of the clinical sample. This soft ionization of proteins enables large biomolecules like ribosomes and proteins to be analysed. The spectrum which is generated is compared to a database of references. It uses the principle of mass spectrometry in which the sample being analysed generates large amounts of ions which are sorted according to their mass to charge (m/z) ratio and expressed as a relative proportion (Clark *et al*., 2013; Rahi *et al*., 2016).

This method is finding widespread use in the microbiology laboratory due to its speed, accuracy and reproducibility. It was first introduced in 1987 but its use has been restricted to specialised areas due to its high cost of procurement. The samples for analysis are not limited to bacterial colonies as direct blood culture samples, urine, cerebrospinal fluid and other human material can be used (Calderaro *et al*., 2014).

MALDI-TOF mass spectrometry has demonstrated high power to identify β haemolytic streptococcus and it is noted to be better than the Vitek 2. It can detect antibiotic resistance in bacteria like methicillin resistant *S. aureus*, Vancomycin resistant *E. faecium* from susceptible strains and production of β lactamases. The system can identify GBS irrespective of the serotype while it has the ability to discriminate the highly virulent isolates and serotypes. It has been observed to be a promising method for direct detection of bacteria in primary clinical samples bypassing culture and staining protocols routinely used to amplify and identify bacteria (Singhal *et al*., 2015)

2.5.9.13 Molecular techniques

Identification of bacteria has largely been based in traditional methods employing phenotypic characteristics of the bacteria. However, these methods are not very accurate and are sometimes very subjective with more experience needed to distinguish specific aspects of reactions like haemolysis and other biochemical reactions. Genotypic methods for identification of bacteria are now becoming more common based on their specificity and in the past decade molecular methods have significantly improved although they are still more expensive than traditional microbiological methods for identification of bacteria (O'Connor *et al*., 2014).

2.5.9.13.1 16S rRNA gene amplification

The 16S rRNA gene is a part of DNA found in prokaryotic cells encoding for ribosomal RNA (rRNA) which is a constituent of ribosomes. It is required for maintenance of basic normal cellular function under all conditions (house-keeping gene) and it is the most common house-keeping gene which is present in almost all bacteria with a size of 1550 base pairs (Janda and Abbott, 2007). Due to conservativeness, the 16S rRNA gene is a target for many antimicrobial agents and mutations in the gene would render the antimicrobials ineffective (Clarridge, 2004).

The 16S rRNA gene is used to study gene sequences used for identifying bacterial phylogeny and taxonomy. It is used to provide genus and species identity to isolates which are poorly described, rarely isolated or whose phenotype does not match commonly used classification with traditional biochemical tests (Janda and Abbott, 2007). The reliability of the 16S rRNA gene sequencing method as a method for bacterial identification and classification is largely dependent on complete non-ambiguous nucleotide sequences being deposited into gene databases. These nucleotide sequences deposited in gene databases should be correctly labelled. About 90 0000 nucleotide sequences of the 16S rRNA gene were in the GenBank by 2004 (Clarridge, 2004).

Problems in identifying bacteria using the 16S rRNA gene sequencing method can arise due to the following: failure to recognise novel taxa, too few sequences deposited in nucleotide database, multiple gene labels assigned to single species resulting in classification errors and when species share similar nucleotide sequences (Janda and Abott, 2007; Vetrovsky & Baldrian, 2013).

Partial 16S rRNA sequences for identification of *S. agalactiae* commonly use a nucleotide sequence of 569 while accession number AB02574 has been reported to give 100% homology. GenBank accession numbers HM355957-70, HM452002, HM590574-75 and HM355971-83 carry GBS nucleotide sequences which can be used for identification of *S. agalactiae* isolates (Shome *et al*., 2012).

2.5.9.13.2 *Streptococcal C5a Peptidase B* Gene amplification

The Streptococcal C5a Peptidase B (*scp*B) gene encodes for the protease C5a peptidase which acts as a virulent factor for the β haemolytic streptococci GBS. The gene is 3450 bp producing a protein 1150 amino acids long and weighing 1.26KDa (Chmouryguina *et al*., 1996). All bacterial isolates of *S. pyogenes* and *S. agalactiae* (β haemolytic streptococcus) from humans produce a protease to inactivate C5a which acts as a chemoattractant for PMNs as part of the inflammatory response of the host. Cleaving of C5a inactivates recruitment of leukocytes to the site of infection allowing β haemolytic streptococci to be invasive. C5a peptidase enables adhesion of bacteria to fibronectin type III (Brown *et al*., 2005). Since the *scp* gene is found in all β haemolytic streptococci (*scp*A for *S. pyogenes* and *scp*B for *S. agalactiae*), it is used for the identification of the organism.

2.5.10 GBS surface anchored proteins

Surface anchored protein antigens are important in understanding the pathogenicity and epidemiology of GBS infection and these include alpha like proteins (Alps), C proteins and R proteins. Genes encoding for surface proteins are important for detecting invasive GBS disease while some of these surface proteins have been proposed as components of vaccines (Zhao *et al*., 2006).

2.5.10.1 Protein antigens

The C protein antigen is the first protein antigen identified in GBS and is made of an alpha and beta protein component but the two components are unrelated. Some GBS strains may exhibit either alpha or beta proteins while others express both, therefore, C antigens are known as either alpha c or beta c proteins. Antibodies to the c antigen have been known to confer immunity status to hosts against GBS in animal models. Therefore, the capsule is not the only immunogenic component of GBS as surface anchored proteins are also immunogenic (Zhao *et al*., 2006).

C protein is not expressed on GBS type III strain while Rib, another surface protein is expressed on type III and other strains eliciting protective immunity against GBS in hosts. Alpha like proteins (Alp) is a family of four members: α, Rib, R28 and Alp2 proteins and these proteins can be targets for vaccine development due to their immunogenicity. While the four Alp proteins are found in the majority of *S. agalactiae* strains some strains code for rare proteins like the Praque 25/60 strain which expresses Alp4 protein and some strains in Zimbabwe which were found to express the Z protein (Lindahl *et al*., 2005; Mavenyengwa *et al*., 2009).

Epitopes of surface anchored proteins can be components of a vaccine but alp proteins have repeating units which may cause the antigens not to elicit a strong immunologic response. In

one study, GBS strains containing few repeating units in the α chain were more immunogenic than strains containing more repeating units (Lindhal *et al*., 2005; Maeland *et al*., 2004).

2.5.10.2 β protein

The protein is unrelated to the α chain but forms part of the C protein together with the α antigen playing a role in protective immunity against GBS due to its interaction with components of the immune system. All strains of serotype Ib contain the β protein while a few strains of Ia, II and V contain the protein. The α protein is always found alone but all cells expressing the β protein express the α protein as well but the significance of this arrangement is unknown. High levels of β protein have been associated with virulence and the protein elicits production of protective antibodies making it a useful protein to consider in vaccine development as well. Pregnant women have been shown to express anti β IgG antibodies which can cross the placenta and give immunity to the infant against GBS disease. Other surface proteins expressed by GBS which are of interest as virulent factors or in future vaccine development include: the fibrinogen binding FbsA protein, the Sip protein, the Spb1 protein, R and X antigens ((Vasilyeva *et al*., 2015; Lindahl *et al*., 2005).

2.5.11 *S. agalactiae* capsular types

The *S. agalactiae* capsule is an important virulence and antiphagocytic factor and the typing of the capsule confers bacterial isolates with a serotype while the carbohydrate content of the capsule determines the serotype and pathogenicity. There are ten serotypes of *S. agalactiae* classified as Ia, Ib, II-IX (Sheppard *et al*., 2016) and direct agglutination is a preferred method for the serotyping of *S. agalactiae* isolates into the ten different serotypes. The method is an indirect passive latex agglutination test which employs antibodies directed against polysaccharides on the surface of the bacterial capsule. The method can result in non-typable isolates as some of them express low levels of capsular polysaccharides, rendering the method

not very effective. New methods using the polymerase chain reaction targeting the genes in the *cps* operon have been developed with the methods targeting the *cpsA* and *neuA* gene. Another method for serotyping of *S. agalactiae* isolates is flow cytometry analysis using monoclonal or polyclonal antibodies (Yao *et al*., 2013).

There is a close relationship among the polysaccharide repeating units of the capsule. Repeating units making types Ia and III; types Ia and Ib and types IV, V and VII are closely related. Although these repeating units are closely related, differences in nucleotide and protein content are quite significant (Cieslewicz *et al*., 2005). Glycosyltransferase assembles the capsule's repeating units on undecaprenylphosphate which a lipid molecule and the four monosaccharides forming the repeating units are Glc*p*, Gal*p*, Glc*p*NAc and Neu*p*NAc which is found on the terminus of the side chains. Polysaccharide units are made in the cytosol of the cell and are transferred to the cell wall by flippase and a polymerase joins the repeating units together while the enzymes involved in the production of the capsule are encoded by the *cps* genes (Berti *et al*., 2014).

There is loss of capsule in some of the bacterial isolates due to deletions, insertions, frame shifts and formation of premature stop codons in *cpsE*, *cpsG* and *cpsH* genes. The first step in the synthesis of the polysaccharide units involves the cpsE enzyme and non-encapsulated strains may have mutations in *cpsF*, *G*, *I*, *J* and *M* while in-frame deletion of *cpsA* also results in capsule loss (Hanson *et al*., 2012; Rosni and Magarit, 2015). Due to the loss of the capsule, agglutination tests have low sensitivity and specificity but sensitivity (96.1%) and specificity (95.9%) are high in real time PCR assays (Morozumi *et al*., 2014). However, a study by Belard *et al*., (2015) found congruency in serotyping using latex agglutination and PCR.

2.5.11.1 Capsular type distribution in the world

The most common serotypes in the world are Ia, Ib, II, III and V but there are geographical variations in the distribution of *S. agalactiae* serotypes. In European countries such as Portugal, Italy, Poland and Germany serotypes Ia, Ib, II, III and V are the predominant capsular types in adults (88%) and neonates (96%) while invasive neonatal disease is commonly caused by Ia, V and III. In China capsular type distribution is different with III, Ia, V, II and Ib being the predominant capsular types (Jiang *et al*., 2016). In USA, Europe and a few studies in South and South East of Brazil capsular types Ia, II, III and V have been reported as the predominant capsular types while capsular types VI and IX are rarely described. A Brazilian study encompassing all regions in the country noted that there variations in capsular type distribution according to geographic location although Ia, Ib, II and V were the most predominant capsular types. However, the study did not find capsular types VI and VIII and the results were different from those found in USA, Europe, Latin America and China (Dutra *et al*., 2014).

A study by Bjornsdottir *et al*., (2016) in Iceland on the changing epidemiology of group B streptococcus infection among adults from 1975 to 2014 reported capsular types Ia, Ib, II, III and V as being predominant while capsular types VII, VIII and IX were not detected for the entire study period. Serotype distribution varied according to age with serotypes Ia, III and II predominant in adults and serotype V being the leading cause of disease in the older population, a finding which was also described in Europe and North America. The study found an increase in the frequency of capsular type IV over time while Ib, III and IV & V were showing a decreasing and increasing trend respectively (Bjornsdottir *et al*., 2016).

Studies in Iran have found the same capsular types in that country but showing variations in distribution. A study by Emaneimi *et al*., (2015) reported that capsular type III (61%) was predominant followed by V, II, Ib and IV with type III being the most common cause of

invasive disease. The study described an increase in the frequency of isolation of capsular types V and IV, causing more invasive adult and neonatal disease with time and source of the isolation, ethnic profile of the sampled population, the geographic area and diagnostic techniques used for isolation, identification and capsular typing of the isolates determining the distribution of capsular types (Emaneini *et al*., 2015). Another Iranian study by Sadeh *et al*., (2016) on the distribution of *S. agalactiae* in pregnant and non-pregnant women reported capsular types III, II, Ia, V and Ib to be predominant showing a different distribution of capsular types from those described by Emaneini *et al*., (2015) (Sadeh *et al*., 2016).

In Japan most neonatal infections (53.2%) are caused by serotypes Ia, Ib and III with 70-80% being attributed to capsular type III and capsular types Ia, Ib and III accounting for 91.9% of severe infection in neonates such as meningitis and sepsis (Morozumi *et al*., 2014). A study in Ireland found capsular types Ia, III and V as the most prevalent and interestingly the study found evidence of capsular type switching among isolates (Meehan *et al*., 2014).

2.5.11.2 Capsular type distribution in Africa

Studies have shown that there is similar serotype distribution in Africa, Western Pacific, Europe, the Americas and Eastern Mediterranean regions with serotypes Ia, Ib, II, III and V being predominant. The distribution has barely changed in the last three decades (Le Doare and Heath, 2013). A study by Belard *et al*., (2015) in Gabon, situated in Central sub Saharan Africa, reported capsular types III (48.9%), Ia (22.9%), Ib (7.0%) were predominant with serotypes Ia, Ib, II, III and V accounting for more than 85% of the isolates while serotypes IV, VI, VII, VIII or IX were not found. However, a study by Slotved *et al*., (2017) in Ghana had different findings with serotypes VII and IX being predominant although there were differences in the capsular types according to region with one region showing higher frequency of capsular type

VII (42.9%) and IX (32.1%) than the other (38.5% and 26.9% respectively). The study did not find capsular types Ib and VI (Slotved *et al*., 2017).

Moyo *et al*., (2000), reported in a study in Zimbabwe a predominance of capsular types Ia, III and V while Mavenyengwa *et al*., (2010) in a similar study found capsular types Ia, Ib, II, III and V as predominant. In South Africa, studies have found similar capsular types with variations in distributions according to region and targeted population. In a study conducted in Soweto (Johannesburg) on pregnant women, capsular types Ia, III and V were predominant (84.4%) with type III being associated with persistent colonisation of pregnant women at 20-25, 26-30, 31-35 and more than 37 weeks gestation. In the study capsular type III contributed 49.2-57.7% of EOD when compared to type Ia (22.6 to 31%) (Kwatra *et al*., 2014). A similar study by Dangor *et al*., (2016) in Johannesburg which tracked the cases of invasive GBS disease noted a decline in the frequency of capsular type III from 70.3% in 2005 to 47.2% in 2014. The study found out that invasive disease caused by capsular type Ia increased from 16.2% in 2005 to 44.2% in 2012 but did not note any changes in the frequency of cases caused by capsular types V. Early onset disease was mostly caused by capsular types Ia, III and V in that order while late onset disease was commonly caused by capsular type III (73.8%) and Ia (19.6%). A study on pregnant women in the Limpopo province of South Africa found capsular types Ia, Ib, II, III, IV and V as predominant but did not find capsular types VI, VII, VIII and IX (Chukwu *et al*., 2015). The South African studies showed that capsular type distribution changes according to regions even in the same country and some serotypes which are not found in one region maybe a cause of invasive GBS disease in another.

2.5.11.3 Importance of capsular typing

Capsular typing is performed to elucidate the distribution of capsular types in a population while vaccines against *S. agalactiae* are targeted against the capsular polysaccharides which

are major virulence factors. Capsular polysaccharide based conjugate vaccines are crucial in the prevention of invasive GBS disease in neonates, elderly, immunocompromised individuals, pregnant and non-pregnant women and the characterisation of GBS capsular types is important for epidemiological purposes. Epidemiological distribution of *S. agalactiae* depends on a number of factors such as the geographical region, ethnicity of the population being studied and other characteristics of the population studied. Studies have shown variations in capsular type distribution of *S. agalactiae* when isolated from pregnant and non-pregnant women (Wang *et al*., 2015). One study reported that IgM was formed to capsular type V but there was weak class switching of the immunoglobulin class from mew (IgM) to gamma (IgG). The study deduced that capsular type V antigens were very close to self-antigens reducing the chances of stimulating a T cell immune response which triggers class switching so capsular typing can be used to monitor changes in the pathogenicity of *S. agalactiae*. There is an increase in the isolation of capsular type V in the world and capsular type IV is now being noticed as a common cause of neonatal invasive disease.

2.5.11.4 Methods for capsular typing

Capsular typing of *S. agalactiae* employs either antibodies to capsular polysaccharides or the amplification of genes which are specific to the organism. The method employed in capsular typing can influence the distribution of the capsular types among the isolates as some of them do not belong to any type.. The methods using serologic techniques, flow cytometry and molecular based techniques have been reported to have varying sensitivity and specificity.

2.5.11.4.1 Serologic methods

The most common serologic method is the latex agglutination test, which is a reverse passive agglutination test with known antibodies specific to different capsular types bound to latex particles. In the presence of specific capsular polysaccharides antigens the latex particles will

form visible agglutinates as the antibody paratopes on latex particles bind to epitopes on capsular polysaccharides. However, some of the immunologically predominant epitopes on the polysaccharides are structurally similar resulting in cross reactivity during serotyping being identified among capsular types IX, V and VIII. This has been attributed as one of the reasons it took long to discover capsular type IX until only recently with capsular polysaccharide V *cps* operon being thought to have originated from type IX *cps* by horizontal gene transfer (Berti *et al*., 2014). The Strep B latex kit (Statens Serum Institut, Copenhagen, Denmark) is one of the kits commonly used for serotyping of *S. agalactiae* isolates (Wang *et al*., 2015).

2.5.11.4.2 Flow cytometry

Flow cytometry uses Fluorescence Activated Cell sorting (FACS) based on anti-capsular polysaccharide antibodies which are mouse antibodies against capsular polysaccharides. The antibodies bind to capsular polysaccharides on bacteria and labelled goat anti mouse antibodies are used as reporter molecules to identify the reaction. The FACS calibur and BD FACS Canto are used for determining the capsular type (Berti *et al*., 2014).

2.5.11.4.3 Molecular methods

Molecular methods for capsular typing include singleplex PCR, multiplex PCR, real time PCR, Long range PCR and whole genome sequencing. Polysaccharide capsule genes have sections which are unique and can be amplified using oligonucleotide primers or probes. Oligonucleotide primers and probes can be synthesised to match selected regions of each of the ten capsular types and the nucleotide probes are labelled with a fluorescent tag so they can act as reporter molecules. Real time PCR is a technically challenging method for routine use but it provides an option to multiplex PCR but unlike multiplex PCR, real time PCR is not amenable to misinterpretation of results by the operator. Furthermore, multiplex PCR may not be able to detect new serotypes as that requires the specific oligonucleotide primers to be

included during the analysis. However, real time PCR largely depends on the conservativeness of the targeted region of the gene for each capsular type and is comparable to the latex agglutination assay (Breeding *et al*., 2016; Sheppard *et al*., 2016).

Multiplex PCR is fast and reliable in the assignment of serotypes and in singleplex and multiplex PCR, the reactions distinguish all the ten serotypes using distinctive bands which appear on agarose after electrophoresis of amplicons. The method is reproducible and easy to perform with the steps involving DNA extraction, addition of oligonucleotide primers, buffer, magnesium chloride, dATP, dCTP, dGTP and dTTP, Taq polymerase followed by thermocycling, gel electrophoresis while UV transillumination is used to view the amplified products (Imperi *et al*., 2010). PCR may not include all serotypes and there may be problems in distinguishing some closely related serotypes resulting in misidentification of some serotypes (Yao *et al*., 2013).

2.5.11.4 Anti-capsule antibodies

Anti-capsule antibodies have been shown to offer protection against GBS in high income countries while in low to medium income countries the association has not been established. Maternal capsular antibodies offer protection to the baby against invasive GBS disease as IgG type antibodies cross the placenta to the baby, offering passive immunity (Dangor *et al*., 2015). There is an increased risk of infant disease associated with a deficiency in maternal anti GBS antibodies (Fabbrini *et al*., 2016).

In a study in eight European Union countries involving colonised and non-colonised pregnant women, maternal serum IgG anti GBS antibodies were found to be significantly high in women who delivered healthy babies when compared to those whose babies were infected with GBS or were not colonised. In vitro tests showed that GBS anticapsular IgG mediated the killing of bacterial cells significantly reducing bacterial levels by between 78% and 81%. Recto vaginal

colonisation is significantly associated with increased levels of anti-capsular antibodies and in the European study; the control group had approximately four times more antibody levels than mothers whose babies had EOD. Studies have reported differences in antibody levels conferring different levels of passive immunity to babies which is dependent on the capsular type, variation in assays used and sampling techniques. Other studies have shown variation in antibody levels between African populations and those in the European Union and the United States of America (Fabbrini *et al*., 2016).

Anti-capsular immunoglobulins are predominantly IgG2 which facilitate opsonophagocytosis but are poorly transferred across the placenta when compared to IgG1. Antibody levels are lower in HIV infected women compared to uninfected women and lower antibody levels are also noted in HIV exposed uninfected infants when compared to unexposed HIV ones. HIV lowers transfer of antibodies across the placenta due to high levels of serum immunoglobulins present in these individuals and studies have shown that transfer of IgG1 to GBS serotype III is affected by maternal HIV infection. While it has been widely accepted that GBS anti-capsular antibodies are transferred across the placenta in large quantities, research has shown that high foetal anti capsular antibody levels are due to foetal IgG1 and not maternal IgG2 antibodies (Le Doare *et al*., 2016).

Formation of antibodies by the immune system to capsular polysaccharides is not efficient due to its poor immunogenicity (Yang *et al*., 2007). The sialic acid present on the capsule has immunosuppressant effects on dendritic cells which are the most potent phagocytic cells in tissue and are effective antigen presenting cells. Sialic acid inhibits the immune system through molecular mimicry coupled with inhibition of complement activation but dendritic cells recognise pathogen associated molecular patterns (PAMPS) through pattern recognition receptors (PRRS) on their surfaces. Interaction of PAMPS with PRRS modulates the adaptive

immune response through release of cytokines and chemokines by activated cells which triggers T independent B lymphocyte activation resulting in enhanced B cell proliferation, class switching and immunoglobulin secretion. Capsular polysaccharides do not induce production of essential cytokines such as IL-1β, IL-6 or TNF-α by monocytes or macrophages leading to low antibody levels (Snapper, 2012; Calzas *et al*., 2013).

Capsular polysaccharides are expected to be non-immunogenic due to their nature as they are carbohydrate antigens with repeating units and contain terminal sialic acid residues which are not immunogenic as they are present in human tissue while the NeuAc-α-(2->3)-Gal glycosidic linkage is found on red blood cell antigens which constitute blood group antigens. Sialic acid inhibits activation of the alternate pathway of complement fixation thus making it an important factor in the pathogenesis of GBS disease due to its suppressive effect on both the innate, cell mediated and humoral immune response of the body (Johnson, 2013).

2.6 Antimicrobial substances

The term antimicrobial refers to any substance which has activity against microorganisms such as fungi, protozoa, bacteria and viruses giving rise to antifungal, antiprotozoal, antibacterial and antivirals. The strict definition of an antibiotic is a substance produced by a microbe with activity against another microbe. Hence, synthetic and semi synthetic substances such as sulphonamides and penicillin, respectively and plants extracts such phytochemicals and animal products such as enzymes which inhibit bacterial growth are strictly not antibiotics (Davies and Davies, 2010).

2.6.1 History of antibiotic development

The modern antibiotic era was heralded by the transformative discovery of penicillin in 1928 with the antibiotic being widely used for treatment of infections in the 1940s and during the

Second World War. As early as the 1950s, the effectiveness of penicillin was beginning to face serious threat from antibacterial resistance as a result of indiscriminate usage. The demand for new therapeutic options in the face of failing penicillin efficacy led to the invention of new β lactams but shortly Methicillin Resistant *Staphylococcus aureus* (MRSA) was noted. Vancomycin use started in 1972 for the treatment of MRSA but microbial resistance to vancomycin surfaced by as early as 1979 (Ventola, 2015). The timeline for the development and rollout of other new drug classes is shown in Figure 2.3. However, due to a complex interplay of many factors there has not been a discovery of new drug classes since 1987. These factors include: poor research funding into new antibiotic targets, lengthy approval protocols, poor recovery of investment due to production of generic drugs, antibiotic resistance and a heightened interest in developing lifestyle related drugs and chronic medication which have high turnover rate.

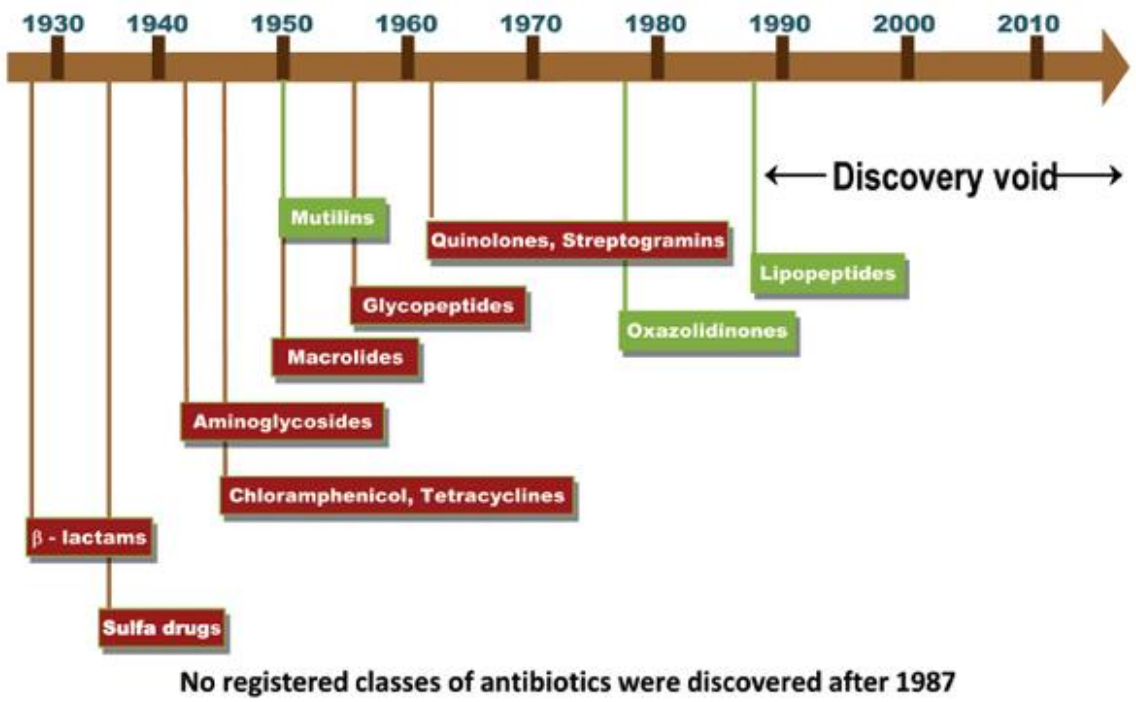

Figure 2.3: Timeline for the discovery of antibiotic classes

Reference: https://gomadforgonads.wordpress.com/2015/03/30/

2.6.2 Mechanisms of antibiotic action

Antibiotics affect target cells by various processes which involve the interaction of the drug with specific components of the target cell. This interaction results in altered synthesis of DNA, RNA, proteins or cell walls causing cell death or inhibiting cell growth resulting with antibiotics being referred to as bactericidal or bacteriostatic respectively. The effect of the drug on the target organism is an interplay of complex processes involving complex interactions of the drug and its target molecules occurring at basic molecular and cellular levels (Martinez and Baquero, 2014).

DNA gyrase inhibitors cause formation of breaks in double stranded DNA while rifamycins inhibit RNA synthesis from DNA. The antibiotics which inhibit DNA strands from re-joining through inhibition of DNA gyrase and topoisomerase causing bacteriostasis and cell death and include quinolones which are nalidixic acid derivatives. First generation quinolones are rarely used due to high toxicity while second and third generation quinolones include ciprofloxacin and levofloxacin respectively. Rifamycins inhibit RNA synthesis by binding to an RNA polymerase which is responsible for transcription and is encoded by the *rpoB* gene. These drugs are first line in the treatment of *Mycobacterium tuberculosis* (Hooper and Jacoby, 2015; Kim *et al*., 2017).

The bacterial cell wall has an envelope which is comprised of the cell membrane and peptidoglycan layers whose quantity varies in Gram positive and Gram negative bacteria. The peptidoglycan is made of cross linked β-(1-4)-*N*-acetyl hexosamine which gives mechanical strength to the bacterial cell containing osmotic pressure and environmental factors which may lyse the cell. Formation and maintenance of the peptidoglycan is done by transgylosylases and transpeptidases (Chapot-Chartier and Kulakauskas, 2014).

β lactams and glycopeptides alter particular steps which are important in cell wall synthesis thereby disrupting cell wall homeostasis causing disruption of cell integrity ending in cell lysis. β lactams such as penicillins, carbapenems and cephalosporins inhibit transpeptidase catalysed reactions resulting in failure of peptidoglycan to covalently crosslink. Most glycopeptides such as vancomycin bind to peptidoglycan subunits affecting synthesis of the cell wall through inhibition of transglycosylase and transpeptidase. Some antibiotics such as fosfomycin and bacitracin inhibit the synthesis and transport of peptidoglycan units respectively causing loss of cell wall synthetic ability while lipoproteins such as daptomycin induce cell membrane depolarization by inserting themselves into the cell membrane (Lin *et al*., 2015).

The process of protein synthesis involves transcription, translation and elongation and the translation phase is comprised of three steps: initiation, elongation and termination. The initiation phase is dependent on ribosomal subunits 50S and 30S and some antibiotic classes target the two ribosomal subunits and are classified as 50S inhibitors or 30S inhibitors. The 50S ribosomal inhibitors physically affect protein synthesis by inhibiting initiation of translation or translocation of tRNA in some cases. These drug classes include macrolides such as erythromycin, lincosamides such as clindamycin, amphenicols such as chloramphenicol and oxazolidinones such as linezolid. Tetracylines and aminocyclitol antibiotics inhibit the 30S ribosomal unit as well and the aminocyclitol class of antibiotics is comprised of streptomycin and aminoglycosides such as kanamycin and gentamycin which inhibit elongation by binding to the 16S rRNA of 30S (Kohanski *et al*., 2010).

2.6.3 Antimicrobial resistance

Antibiotic resistance occurs when bacteria does not respond to the effects of once effective antibiotics and in this case it is the bacteria which is not affected by the antibiotic and not the human being, resulting in the infections they cause being harder to treat. This leads to increased

morbidity, mortality and higher medical expenses. The solutions to antibiotic resistance are both multifactorial and multi-sectorial ranging from efforts by individuals, policy making, engaging health professionals and the agricultural sector. Individuals can help fight against antibiotic resistance by rational use of antibiotics, not demanding antibiotics when not necessary and preventing infection through proper and regular hand washing, practicing proper food hygiene, practicing safe sex and by partaking in vaccination programs. Policy makers should ensure national policies to fight antibiotic resistance are in place, perform surveillance, regulate appropriate use of antibiotics and increase health education in communities. Health professionals and the health care and agricultural sectors should ensure proper prescription of antibiotics as needed according to prescription guidelines, spearhead research in new antibiotics, vaccines and effective diagnostic methods, not to use antibiotics as growth promoters and to ensure safe disposal of effluent (WHO, 2017).

Antibiotic therapy is generally used to treat communicable diseases, endogenous infections and for prophylactic purposes. The global use of antibiotics now stands at over seventy billion doses per year with the most widely used antibiotic classes being penicillins, cephalosporins, macrolides, fluoroquinolones, trimethoprim and tetracyclines. There is now a change in focus on antibiotic use which is being shaped by increasing antibiotic resistance (Woolhouse *et al*., 2016). While the antibiotic resistance problem is largely attributed to misuse and overuse of antibiotics there is considerable contribution being made to this problem by the failure to develop new antibiotic options. Since the discovery of penicillin in 1928, there has been a steady increase in antibiotic resistant strains with MRSA being identified in the UK in 1962 and in the USA in 1968 as shown in Figure 2.4. Vancomycin, gentamycin, levofloxacin and other resistance followed in the coming years (Ventola, 2015).

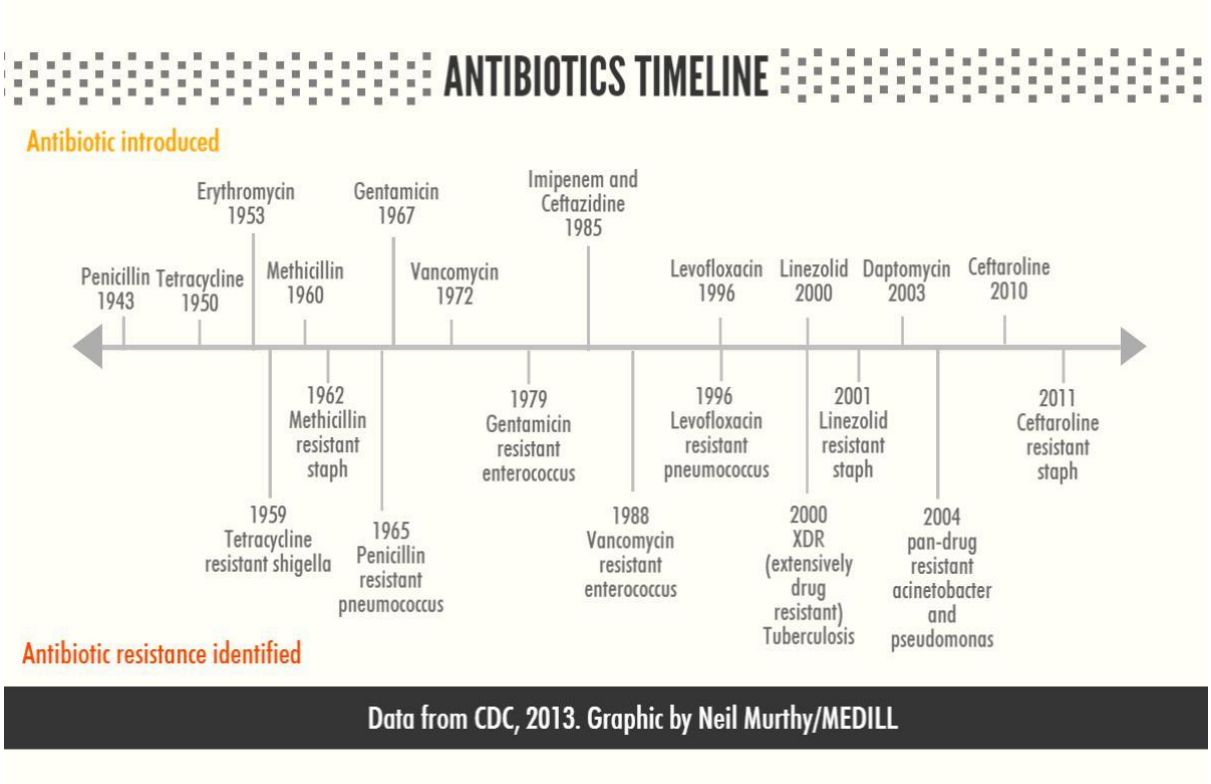

Figure 2.4: Timeline of antibiotic deployment and observed antibiotic resistance

Reference: http://chicago-mosaic.medill.northwestern.edu/antibiotic-resistance-superbugs/

2.6.4 Genetic basis of antibiotic resistance

Multi drug resistance by organisms was largely believed to be acquired from the hospital, but studies have shown that communities are another source of resistant strains of bacteria. Drug resistance is an evolutionary process which results in the development of mechanisms by bacteria to produce resistance to antibacterial agents. This could be through acquisition of genetic material or modification of genes resulting in resistance to antibiotics. It is crucial to understand the mechanisms and molecular basis of microbial resistance to antibiotics to allow for development of protocols and techniques to curb the spread of drug resistance and for the invention of new therapeutic options which can overcome current resistance mechanisms.

However, bacteria have effective mechanisms to overcome environmental factors and other selection pressures (Hughes and Andersson, 2017).

Mutational resistance occurs in a susceptible bacterial population which undergoes genetic mutation altering the effect of the antibiotic on the bacteria. The emergence of the resistant strain results in selection for the resistant strain and the elimination of the susceptible one rendering the antibiotic ineffective. Genetic mutations in bacteria result in change in a number of factors which contribute to resistance such as: modification of the drug target reducing affinity for the drug, altering uptake of the drug by the bacterial cell, activating mechanisms which result in drug efflux and change in metabolic pathways or their control mechanisms. Most of the antibiotic components currently used are made of components which are derived from the environment. While the bacteria was still in the environment these antibacterial components were in contact with the bacteria and it developed mechanisms to withstand the effects of the antimicrobial drugs. The mechanisms used to confer resistance include transduction and conjugation with genetic information conferring resistance being shared between chromosomes of different cells through plasmids and transposons (Schroeder *et al*., 2017).

Multiple processes are used by bacteria to ensure effective antibiotic resistance instead of using just one mechanism. Resistance to fluoroquinolones can occur due to coexistence of mutations of target sites, over expression of efflux pumps and protection of target sites. Gram negative bacteria produce β lactamases while Gram positive bacteria alter antibiotic target sites to foster resistance to β lactam antibiotics. However, production of β lactamases in Gram positive bacteria has been effective in some cases. Bacteria may produce enzymes which alter the antibiotic molecule rendering it ineffective and this is usually through genes acquired by both

Gram positive and Gram negative bacteria and the antibiotics commonly affected are those which inhibit protein synthesis (Munita and Arias, 2016).

2.6.5 B lactamases

Resistance by bacteria to β lactams is mainly through the production and release of enzymes which destroy antibiotics, known as β lactamases. The enzymes destroy the β lactam ring making the drug ineffective against the bacteria it is targeting but β lactams have been in existence since ancient times, even before penicillin was made available for clinical use. After the introduction of penicillin, infections which were resistant to it started to be of interest in the clinical setting with the mechanism of resistance being thought to be depended on penicillinase encoded for by a plasmid. New wide spectrum β lactams such as ampicillin were invented to overcome this resistance but new plasmids encoding new β lactamases emerged among Gram negative bacteria rendering ampicillin ineffective. The β lactamase was called TEM-1, after the patient from whom it was isolated (Munita and Arias, 2016).

Since then, any new generation of β lactams has been met with the rapid emergence of β lactamases destroying any novel compounds. B lactamase encoding genes are termed *bla* genes and more than 1000 β lactamases have been characterised. Extended Spectrum β Lactamases (ESBLS) hydrolyse penicillin, 3rd generation cephalosporins and monobactams while showing minimal activity against cephamycin and carbapenem. Class A β lactamases inhibit clavulanic acid and their many proteins have wide ranging catalytic properties hydrolysing penicillin (TEM-1 and SHV-1) and are ESBLS (e.g. CTX-M). Class C β lactamases produce enzymes called AmpC enzymes which hydrolyse 3rd generation cephalosporin without being inhibited by clavulanic acid and tazobactam. AmpC is encoded for by chromosomes but can also be transmitted via plasmids (Mohd Khari *et al*., 2016; Castellanos *et al*., 2017).

2.6.6 Antimicrobial susceptibility of GBS

Antibiotic use is widespread in combating bacterial infections and reducing invasive diseases like those caused by GBS. Antibiotic therapy against GBS is aimed at prophylaxis, treatment of GBS infection or reducing disease burden and GBS related deaths in neonates. CDC recommends penicillin and ampicillin for IAP in an effort to reduce vertical transmission of GBS from the mother to the neonate. Penicillin has a narrow spectrum and can cross the placenta from the mother to the baby and in place of penicillin, a broader spectrum *β lactam* ampicillin can be used while for women who are allergic to penicillin, erythromycin and clindamycin are the drugs of choice (Huang *et al*., 2016). The use of cefazolin is recommended in women with penicillin allergy and at low risk of anaphylaxis to it and if the GBS isolate is resistant to clindamycin, vancomycin is recommended (Frohlicher *et al*., 2014).

The emergence of pathogens which are resistant to multiple drugs is now posing a threat to use of antibiotics for therapeutic purposes. Multi drug resistant bacterial strains are on the increase and GBS is not spared either. The spread of these multidrug resistant strains limits therapeutic options, increases deaths due to invasive disease, prolongs hospital stay by patients and raises the cost of care and treatment of patients. This has resulted in the reintroduction of expensive drugs and those previously side-lined for their notable side effects like colistin (Marasini *et al*., 2015). There is need to closely monitor the antimicrobial susceptibility of clinical isolates as it is now becoming an issue of public health concern. It has become necessary to rationally select drugs which are sensitive to GBS for the control of GBS infection in both pregnant women and neonates (Huang *et al*., 2016). However, GBS resistance rates have geographical variations (Frohlicher *et al*., 2014).

Group B streptococcus resistance to clindamycin and erythromycin has been increasing in the last 20 years. In a recent study in Ethiopia, GBS resistance to erythromycin was 7 to 40% and

between 3 and 26.4% resistance to clindamycin. The study found very high resistance to tetracycline and cotrimoxazole which was attributed to the increasing and unregulated use of the antibiotics as members of the public could procure antibiotics without written prescriptions from physicians (Mengistu *et al*., 2016).

In one study, GBS resistance to clindamycin and erythromycin was found to be higher in Asian than non-Asian countries. This was also attributed to the widespread and indiscriminate use of the antibiotics in Asian countries. Resistance to tetracycline which is a broad spectrum antibiotic was also found to be higher among Asian isolates (78%) than non-Asian isolates (66%). High GBS resistance to tetracycline has also been reported in USA (96%), Germany (74.5%), France (88.1%), Kuwait (89.5%) and Tunisia (97.3%) (Huang *et al*., 2016).

Group B streptococcus had not shown outright resistance to penicillin and ampicillin until recently although isolates with increasing MICs had been reported in some studies since 2008 (Dutra *et al*., 2014). However, a study by Matani *et al*., (2016) in Italy a reported outright resistance of GBS to penicillin (12.07%) and ampicillin (12.90%). This is very worrisome as penicillin is used empirically for IAP in pregnant women and current guidelines do not recommend routine antimicrobial susceptibility testing during antenatal screening. In the same study, GBS recorded high resistance to clindamycin (43.75%), erythromycin (32.20%) and vancomycin (7.81%) (Matani *et al*., 2016).

The *erm*B gene has been associated with GBS resistance to clindamycin and macrolides in serotypes Ib and V while resistance to fluoroquinolones has been linked to mutations in GBS at codon 79 and codon 83 of par C (Wang *et al*., 2014). In a study in Taiwan, 40% of GBS isolates were resistant to erythromycin while 39% of the isolates were resistant to clindamycin and from 2001 to 2007 expression of the *erm*B gene increased from 72% to 90% (Janapatla *et al*., 2008). In Brazil, a similar study reported complete susceptibility to β lactams with very

low resistance to erythromycin (4.1%) and clindamycin (3%) while the *tet*M gene was detected in 99.3% of isolates which showed resistance to tetracycline (Dutra *et al*., 2014).

CDC recommends penicillin and ampicillin for IAP in pregnant women to reduce GBS disease in the pregnant women and EOD and LOD in their babies. The widespread rollout of penicillin prophylaxis has heightened fears of increased antibiotic resistance with reports of increasing Minimum Inhibitory Concentration (MIC) increasing in the last decade. Also worrying at present are reports of alterations in penicillin binding protein as reported in Japan (CDC, 2010; Matani *et al*., 2016). Clindamycin and erythromycin provide alternatives to penicillin especially in penicillin allergic women but there is increasing resistance to both antibiotics as reported in the USA of 25% to 32% and 13% to 20% for erythromycin and clindamycin, respectively. Other therapeutic options to GBS disease include cefazolin and vancomycin but CDC reported relatively high MICs in some isolates though it is generally recommended to report isolates susceptible to penicillin as susceptible to cefazolin (CDC, 2010).

High resistance to clindamycin (28%) and erythromycin (30%) was found in a Swiss study and of the isolates resistant to erythromycin, 92% were resistant to clindamycin although low inducible clindamycin was reported (Capanna *et al*., 2013). A similar study in China reported much higher resistance to both clindamycin (54.4%), erythromycin (64.9%) and levofloxacin (25.9%) although there was 100% susceptibility to penicillin. This was in contrast to a study in Brazil which reported low resistance to both clindamycin (2.2%) and erythromycin (8.1%) while resistance to tetracycline in the same study was 82.3%. Tetracycline is a broad spectrum antibiotic which was widely used in the 1970s and due to its low cost and availability as an over the counter (OTC) medication it was overused and misused even in the agricultural sector for treatment of animals (de Melo *et al*., 2016).

Studies in Africa have shown varying degrees of GBS resistance to antibiotics with one study in Ethiopia showing 100% sensitivity of GBS to penicillin, vancomycin, ampicillin, erythromycin and gentamicin (Alemeseged *et al*., 2015) and a similar study in Ethiopia showing low resistance to clindamycin (3.2%), erythromycin (6.5%) and Cotrimoxazole (29%) (Mengistu *et al*., 2016). A study in Gabon (Central Africa) found 100% sensitivity to penicillin, clindamycin and vancomycin with low resistance to erythromycin (13%) (Belard *et al*., 2015) while in Zimbabwe (Southern Africa) one study showed 100% sensitivity of GBS to penicillin and clindamycin and 14% resistance to erythromycin. In this study two isolates showed intermediate sensitivity to penicillin (Moyo *et al*., 2001).

A study in Malawi reported GBS isolates as susceptible to *β lactams* while 96% were resistant to tetracycline (Gray *et al*., 2007). In South Africa, GBS showed resistance to erythromycin (21.1%), clindamycin (17.2%) and tetracycline (94.5%) and the *erm*B gene was expressed in 55% of the isolates while the *mef*A gene was expressed in 3.4% of the isolates. Isolates which were resistant to both clindamycin and erythromycin had constitutive macrolide, lincosamide and streptogramin B (MLS_B) and inducible MLS_B (69% and 17.4% respectively) (Bolukaoto *et al*., 2015) which is considered as resistance in GBS (Frohlicher *et al*., 2014).

2.7 Methods for antimicrobial susceptibility testing

Antimicrobial Susceptibility Testing (AST) is important for evaluating the efficacy of antibiotics to microbes, for epidemiological purposes and in determining the usefulness of drugs as therapeutic agents. In vitro AST is performed using various methods such as the agar disc diffusion method, broth dilution method, E test and the Vitek technology.

2.7.1 Agar disc diffusion

Having been developed in 1940, the agar disc diffusion method still remains the method of choice for routine clinical AST. The method is not applicable to some organisms but standardised protocols for many other organisms are regularly updated and published for clinical application by the Clinical and Laboratory Standards Institute (CLSI). Agar plates are inoculated with a standardised preparation of the test organism (0.5 McFarland) to obtain a lawn growth and 6mm diameter filter papers impregnated with a defined concentration of the antibiotic are placed on the agar and left to diffuse and interact with the test organism (Mal *et al*., 2016).

Agar plates are incubated at optimum conditions favouring the growth of the bacteria and the plates are inspected for zones of inhibition after expiry of the prescribed incubation time. Zones of inhibition are measured and compared to published CLSI guidelines to determine if the organism is showing susceptibility, intermediate susceptibility or resistance to the antibiotic. The method is qualitative and cannot provide MICs but it is simple, cheap and easy to interpret and numerous antibiotics can be concurrently tested against one isolate (Balouiri *et al*., 2016).

2.7.2 E test

The E test also known as the antimicrobial gradient method is a method which combines the dilution method with the agar disc diffusion method. Test strips are commercially prepared by impregnating them with the antibiotic providing an ascending gradient of antibiotic concentration when the test strip is placed on the agar containing a lawn inoculum of the test organism. The MIC is determined by evaluating the point of intersection of the strip and bacterial growth while the results are interpreted using CLSI guidelines to determine the organism's susceptibility, intermediate susceptibility or resistance to the antibiotic. While the strips are easy to use they are expensive, making it unfavourable for routine clinical use or

when a large volume of isolates is to be tested. However, the method can be applied to determine the synergistic effect of antibiotics (Ogata *et al*., 2014; Khalili *et al*., 2012).

2.7.3 Vitek and Mindray card based technology

For AST on the Vitek, test organisms are grown on solid agar media and standardised 0.5 McFarland suspensions are made from the agar plates and inoculated onto the Vitek cards as per the manufacturer (Biomerieux)'s instructions for the identification and AST of bacteria. MICs generated by the Vitek are used to determine the susceptibility or resistance of an organism to a specific antibiotic in accordance with CLSI guidelines (Jo *et al*., 2016). However, AST using Vitek has challenges as false susceptibility has been reported especially when testing against ESBLs such as CTX-M-15 (Bobenchik *et al*., 2014).

The Mindray Technical Dedicated Reasonable (TDR) card based technonolgy uses suspensions made from agar plates. Standard bacterial suspensions are inoculated onto reagent cards which contain reagents for biochemical reactions necessary in the identification of organisms. The reagent cards also contain antibiotics for sensitivity testing (Sugiartha *et al*., 2017).

2.8 The GBS vaccine

The main mechanism for killing GBS by host defences is phagocytosis and subsequently intracellular killing. Attachment of specific antibody or complement components is a prerequisite for opsonisation and phagocytosis to occur. GBS prevents complement activation but sufficient amounts of CPS antibodies can effectively trigger GBS phagocytosis. New-born babies do not readily form antibodies and cannot mount an immune response to antigens. They have a small percentage of neutrophils with most leukocytes being lymphocytes soon after birth. Neonatal monocytes which transform into macrophages are immature at birth hence

defence against CPS before and after birth is dependent on passive immunity by direct transfer of maternal antibodies to the baby (Johri *et al*., 2006).

S. agalactiae has many virulent factors but the main virulent factor is the capsular polysaccharide which is used to delineate GBS strains into the different 10 serotypes. Studies have reported that anti GBS capsular maternal antibodies confer immunity to neonates against the development of invasive GBS disease. Neonates born to mothers with serum anti GBS capsular antibodies do not suffer from invasive GBS disease and they have circulating anti GBS capsular antibodies in their blood. The anti GBS capsular antibody is IgG type and can therefore cross the placenta from the mother to the baby. Maternal anti GBS capsular antibodies to GBS serotype IIIcorrelate well with protection of neonate from invasive disease (Kobayashi *et al*., 2016). Because of these findings, vaccination of women against GBS is a potentially effective method of conferring passive immunity to the neonate against invasive GBS. A GBS vaccine could also be very useful in the elderly and immunocompromised individuals like HIV positive people at risk of GBS disease (Chen *et al*., 2013).

2.8.1 Types of GBS vaccines

Early GBS vaccines were made of native GBS capsular polysaccharide antigens and when injected into humans they caused production of anti GBS capsular antibodies for the different capsular types. Serotype II stimulated the biggest immunological response in individuals while type Ia caused the least immunological response in subjects. The CPS initiated a T cell independent immunological response in study subjects (Kobayashi *et al*., 2016).

A T cell independent immunological response to GBS does not cause T helper cell activation. Antigen dependent B cell lymphopoiesis stimulated by GBS is limited in response with no production of both memory T helper cells and memory B cells. Subsequent boosters to amplify the immunological response do not cause a proportional increase in antibody levels. This means

that maternal antibody levels do not go up enough to confer passive immunity to the neonate during normal immune responses to GBS infection. Preterm babies get only 29-51% of maternal antibodies and to give sufficient passive immunity to preterm babies and protect them against LOD mothers have to develop higher antibody levels than mothers of full term babies (Chen *et al*., 2013; Kobayashi *et al*., 2016).

The second generation vaccines were glycoconjugates and these were made to address the problems encountered during the development and trials of polysaccharide vaccines. In these vaccines, a protein was conjugated to the capsular polysaccharide and B lymphocytes processed the glycoprotein and presented the antigen to T helper cells in association with major histocompatibility complex (MHC) class II. The activated T helper cells produced cytokines which in turn activated T helper cells and B lymphocytes to proliferate and differentiate into effector T helper cells (producing more cytokines) and B cells (plasma cells). The other cell populations produced from the differentiation of T helper and B cells are memory cells (Patras *et al*., 2015).

Production of memory T helper cells and memory B cells means that on subsequent booster, the immunological response is higher thereby producing much higher antibody titres. T cell dependent antibody production causes class switching from IgM to IgG type antibodies and there is also affinity maturation resulting in antibodies being more specific for the antigen. These vaccines were very potent with reported high maternal antibody levels which also correlate with high cord serum antibodies while antibody levels in the neonates remained high two months after birth. Women immunised with this vaccine have been reported to have a decreased vaginal and rectal colonization with GBS which is quite a remarkable feat (Chen *et al*., 2013).

In the third generation of vaccines, purified GBS surface proteins which were components of pilli where produced and injected into animal models. The technique of reverse vaccinology was used to produce vaccine components as the method sought to identify previously unknown GBS antigens. The major challenge was that out of four proteins that were produced, only one was universally expressed on GBS strains (Nuccitelli *et al*., 2015).

Some companies have already put GBS vaccines on trial in South Africa for pregnant women. Norvatis (Basel, Switzerland) developed a conjugated carbohydrate vaccine while Minervax (Boston, USA) produced a protein vaccine from N-terminal domains of the Rib and Alpha C surface proteins (Heath, 2016). Vaccines using GBS protein targets and CPS conjugated to carrier molecules have undergone testing in animal models and are undergoing trials in South Africa.

In clinical trials, NCT01193920 and NCT01150123 where the vaccine had Ia, Ib and III CPS antibody levels, were significantly higher on all CPS compared to the placebo group and remained so after a year. A four-fold increase in antibody levels against type III CPS was seen in 95% of women compared to their pre vaccination levels while cord serum antibody levels were 70% of maternal levels. The vaccination conferred passive immunity for both EOD and LOD to neonates as antibodies levels in infants persisted for 3 months after birth. Response to the vaccine was much lower in HIV positive mothers and their neonates when compared to HIV negative mothers. While the clinical trials have shown great potential, there is still need for development and trial of vaccines for all the other CPS associated with EOD and LOD (Kobayashi *et al*., 2016).

In South Africa, serotypes Ia, Ib and III are linked to 76% and 93% of EOD and LOD, respectively and a trial CRM197 trivalent vaccine is undergoing trials. Vaccination of pregnant women did not affect GBS colonisation and different serotypes stimulated the production of

different levels of antigen specific antibodies. Maternal-foetal antibody transfer was similar to that seen in other studies but a separate study showed that high maternal antibody levels affected colonisation as the vaccine activated a cell mediated immune response (Madhi *et al*., 2016).

2.9 Alternative therapy to invasive GBS disease

2.9.1 Medicinal plants

Indigenous knowledge is the basis for many community-based decisions and solutions to pressing problems especially in rural communities. To improve the conditions of rural communities, scientists and policy planners need to be able to incorporate indigenous knowledge into community programmes. One major aspect of indigenous knowledge systems is the use of medicinal plants for primary health care. Medicinal plants form part of a system of knowledge, practices, skills which is based on the understanding, beliefs and history which originates within a culture whether it works or not. This knowledge is used in primary health for management, treatment or prevention of disease or general maintenance of health through use of plants which are thought to contain pharmaceutical, nutraceutical or insecticide extracts. These extracts could be found in the plant's leaves, stem, flowers or its fruit and traditional medicine is also referred to as complementary or alternative medicine (WHO, 2000).

There has been a major resurgence in interest and use of medicinal plants. This could be attributed to the increasing cost of developing new drugs, fewer drugs being developed especially antimicrobials, increasing antimicrobial resistance and the increasing awareness of medicinal plants and the active chemical composition of such plants. In the USA, people prefer to use medicinal plants to minimise intervention by modern health care methods. There is grave concern among some people against the development of permanent sequelae after use of modern medicine like candida infections or drug resistant bacterial strains (Bent, 2008).

A total of 70-80% of rural and urban communities on the African continent use medicinal plants (Maroyi & Cheikhyoussef, 2015) and the majority of the South African population (approximately 80%) uses traditional medicine for their primary health care needs. South Africa is home to about 30 000 flowering plant species which constitutes about 10% of the world's higher plant species. Despite all this diversity, few plant species have been used to their full potential although essential oils and other extracts of plants have been used in diabetic, cancer, HIV patients and those suffering from infectious disease (Street and Prinsloo, 2013). A comparative ethnobotanical study was carried in Namibia and Zimbabwe and it was found that knowledge of medicinal plants was similar in application in the two countries (Maroyi & Cheikhyoussef, 2015).

In the USA medicinal plants are given to women who give birth at home when infections like GBS are suspected after risk assessment or when they have a positive culture during universal screening (Medicinal plants US, 2011). Home midwives are not allowed to administer intravenous drugs and it is difficult for women giving birth at home to access antibiotics. After conducting risk assessment for vertical transmission of infections such as GBS, midwives give herbs prenatally to reduce chances of bacterial transmission to the neonate. However, herbal treatment for *S. agalactiae* is effective in lowering bacterial load if started a fortnight before labour. Home deliveries in the USA were 1.26% in 2011 and 1.36% in 2012 and they ranged from 3-6% in different states (Mc Dorman *et al*., 2014; Medicinal plants US, 2011).

A study on babies born outside the health sector in South Africa reported that this occurred due to planned home delivery which is usually assisted by a skilled health professional or it was unplanned and was done with no assistance from a health professional. Birth before arrival at a Health facility was associated with hypothermia, hypoglycaemia, low birth weight, neonatal sepsis and even admission to the intensive care unit (ICU). Maternal factors associated with

birth before arrival at a health facility were young mothers, multi parity, poor educational level, inaccessible transport and health care facilities and poor uptake of antenatal care. In South Africa, poverty, living in rural settings, unwanted and teenage pregnancies contributed to a high prevalence of home deliveries accounting for 73.8% of deliveries and 70.5% of the deliveries had no birth attendant (Parag *et al*., 2014).

With so many deliveries occurring at home with or without the assistance of a qualified health professional, antenatal screening programs for GBS might not achieve the intended goal of reducing neonatal morbidity and mortality. The majority of the neonates will be at home in the critical moments for IAP prophylaxis or other intervention. As seen in the South African study, the majority of the neonates only got to a health care facility 48 hours after birth (Parag *et al*., 2014). These are the critical hours for GBS EOD associated with high morbidity and mortality and the use of medicinal plants portends as viable for both therapeutic and prophylactic purposes. Medicinal plants are indigenous to communities and can be self -administered or be administered by community members with the knowledge creating little or no risk to both the mother and neonate. Some communities have identified indigenous plants with antimicrobial activity and have used them to treat infections (Kipkore *et al*., 2014).

In Nepal, the following plants have been identified to be effective in eliminating various infections: *Artemisia vulgaris* is effective against diarrhoea and acts as an antiseptic; *Centella asiatica* is used to treat skin diseases and urinary tract infections; *Cinnamomum camphora* is used for treating bronchitis, broncho pneumonia and as an antiseptic; *Cuscuta reflexa* is used for fever, cuts and wounds while *Drymaria cordata* is used as an antifebrile. All these plants were tested against *S. agalactiae* and their extracts were found to have antimicrobial effects against GBS (Marasini *et al*., 2015).

In India, water and ethanol extracts of *Acacia nilotica* were found to be very potent against GBS from veterinary sources at very low MICs (Mubarack *et al*., 2011). *Moringa oleifera* was found to have a wide range of antimicrobial activity and was very effective against *Streptococcus* isolates. Extracts were however more effective when using water as a solvent than 70% ethanol or 70% methanol (El-Sohaimy *et al*., 2015).

No clinical trials have been done for the efficacy of medicinal plants against GBS infections while information available is from *in vitro* diagnostic testing. The medicinal plants could be effective against GBS but may not be safe for administration during pregnancy. Herbalists rely on traditional knowledge or contemporary knowledge of infection or disease states and herbal pharmacology to choose or administer medicinal plants. In many communities medicinal plants remain a novel approach in the prevention and treatment of conditions like GBS disease in pregnant mothers and neonates. Studies should be carried out to identify such plants and their efficacy in *in vitro* diagnostic testing before optimising them as potent pharmaceuticals (Marasini *et al*., 2015; Biasi-Garbin *et al*., 2015).

2.9.2 Indigenous Knowledge Systems

Synonyms for IKS include traditional science, folk knowledge, local knowledge or indigenous knowledge. The application of IKS touches every facet of human existence from diet, agriculture, health care, traditional medicine, midwifery, to ecological knowledge. The knowledge is important for the day to day activities in a community and for survival while the richness of indigenous knowledge is based on gathering empirical evidence from observations and from interacting with the ecosystem. It is passed through oral tradition, rituals and other traditional practices from generation to generation (Morton Ninomiya *et al*., 2017).

IKS can denote the community's interests, cultural beliefs and spirituality which is sometimes embedded in the community's interaction with ecological factors, ancestry, religion and

cosmology. The use of IKS in some communities is guarded by a traditional code of ethics, usually inherited which guards and oversees the traditional practices in a community and any deviation from the code is deemed offensive to the local community and may be punishable (Browne *et al*., 2016; Cheikhyoussef and Embashu, 2013).

Due to the many convoluted factors which guide IKS and govern its practices, it is challenging to properly identify and scientifically substantiate some of the claims of IKS. Due to its deep rooted connection to spiritual forms other than science, it is sometimes difficult to provide physical evidence of the effectiveness of IKS even in the face of overwhelming claims that it works in the communities. There are wide claims of the effectiveness of medicinal plants in therapy including antimicrobial activity but in the absence of evidence from randomised clinical trials which make use of control groups and placebos, there is no concrete evidence to this claim (Alebie *et al*., 2017; Maneenoon *et al*., 2015).

2.9.3 Therapeutic effects of medicinal plants

While about two-thirds of the world's population employ the use of traditional medicine as part of primary health care, only 15% of the world's 250 000 flowering plants have known phytochemical components of which only 6% have known biological activity (Budovsky *et al.*, 2015). In 2013, the market worth of medicinal plants and plant products was estimated at US$100 billion (Safowora *et al*., 2013). More than US$25 billion in the USA is used for treatment of wounds which affect over six million people although more wounds are treated with traditional medicines (more than 30%) as compared to modern drugs (1-3%) (Budovsky *et al*., 2015). The emphasis on medicinal plants was centred on treatment of diseases rather than preventive measures.

In Central and Southern Africa medicinal plants are used for the treatment of infections commonly caused by pathogenic microorganisms. Apart from treatment of infections, medicinal plants have a wide variety of uses including mental health, heart treatments as well as aphrodisiacs. However, widespread side effects have been reported after administration of medicinal plants and the effects range from metabolic to immunologic complications which are a result of neurotoxins, cytotoxins and other metabolic toxins found in medicinal plants (Kamsu-Foguem and Foguem, 2014).

Medicinal plants are important in prevention and treatment of disease. This involves both communicable and non-communicable diseases. Information on early use of medicinal plants in prevention of disease is found in Chinese and Indian literature. African knowledge is passed from one generation to another through oral tradition and knowledge on medicinal plants is yet to be fully documented. However, there is a lot of research currently ongoing to provide scientific evidence on the use of various medicinal plants in African communities for therapeutic purposes (Sofowora *et al*., 2013).

Medicinal plants are used for the prevention of cancer with *Pygeum africanum* and *Azadirachta indica* used to prevent prostate cancer and benign prostatic hypertrophy while *Glycine soya* (soya milk) binds to oestrogen receptors preventing choriocarcinomas. *Zingiber officinale* (ginger) is being studied as a potential phytochemical against malignant tumours. Garlic has been demonstrated in thirty five clinical trials to reduce cholesterol levels while *Cynara scolymus* reduces cellular oxidative stress. *Commiphora mukul* (engler) has been used to treat hypercholesterolemia and hyperlipidaemia (Sofowora *et al*., 2013).

In Kashmir and India medicinal plants are used for the treatment of dermatological problems, colds and flu, fever, arthritis and disorders of the gastrointestinal tract, urinary tract, respiratory tract, as analgesics and in pregnancy related diseases (Gairola *et al*., 2014). In Africa medicinal plants are used for the treatment of skin infections, sustaining pregnancy, as oxytotic components and some are thought to have occult powers performing functions beyond scientific scope or proof (Sofowora *et al*., 2013).

A study by Mugisha *et al*., (2014) in Uganda reported that some medicinal plants had medicinal and nutritious effects in people with HIV infection and AIDS. Medicinal plants had beneficial effects against anaemia, cough, skin infections, diarrhoea, respiratory infections and were crucial as part of nutritional support (Mugisha *et al*., 2014). In a review of medicinal plants used for treatment of diarrhoea in South Africa and Zimbabwe, *Sclerocarya birrea* was found to be mostly linked to treatment of rotavirus and bacteria causing diarrhoea such as *E. coli*, *Shigella*, *Campylobacter*, *Salmonella*, *Yersinia*, *Vibrio cholerae* and parasitic infections such as *Giardia* and *Entamoeba histolytica*. There is notable cross cultural acceptance of the use of medicinal plants for primary healthcare in different geographical locations (Maroyi, 2016).

In central Zimbabwe a study was carried to evaluate the medicinal plants prescribed to pregnant women and it was reported that elephant dung was commonly used on pregnant women due to its medicinal properties. Although the people who prescribed the medicine and the recipients did not have an understanding of the actions of the plants they had faith in its effectiveness. The plants had questionable effects in women with complicated obstetric history but this did not stop people taking the medicine (Panganai and Shumba, 2016).

In South Africa over 120 medicinal plants are used for treatment of oral infections (Akwalwaya *et al*., 2017), chest problems, asthma, high blood pressure, fertility problems and for purification of blood (Maema *et al*., 2016). Traditional medicine has been noted to be effective against pathogenic fungi such as *Cryptococcus neoformans*, *Candida albicans* with other plants possessing antioxidant activities (Mahlo *et al*., 2016). In the Eastern Cape of South Africa medicinal plants are used as herbal medicines, food and for ethno veterinary purposes (Maroyi, 2017).

2.9.3.1 Components of medicinal plants

Various studies in different geographic locations have found that medicinal plants contain a host of chemical components which include: alkaloids, tannins, saponins, steroids, terpenoids, flavonoids, phlobatannin and glycosides. When administered to an individual, these chemicals induce physiological changes in the body (Marusk *et al*., 2010; Ashiq *et al*., 2017; Dhandapani and Sabna, 2008; Neffati *et al*., 2017; Kozlowska *et al*., 2015).

Steroidal compounds have actions similar to sex hormones while alkaloids and flavonoids are important medicines. Minerals such as Mg, Ca, cr, Mn, Fe, Co, Cu and Zn are essential in human health. Iron is a component of haemoglobin, myoglobin, cytochromes and respiratory enzymes, copper is important in oxygen transport, erythropoiesis, nucleic acid and protein synthesis and collagen formation. Zinc is important in haemoglobin synthesis, collagen metabolism, as an enzyme cofactor, in reproduction, for wound healing, growth and immune system function. Cobalt is a constituent of vitamin B12 while chromium increases the effects of insulin. Manganese is an enzyme activator which promotes growth, reproduction, cholesterol metabolism, and bone and tissue formation. The minerals act as antioxidants and scavenge for free radicals while cellular oxidative stress is associated with the development of

cancer, cardiovascular and inflammatory disease (Daur, 2015; Bhowmik *et al*., 2008).The variation in biotic and abiotic factors to which the plants are exposed creates a chemically diverse group of metabolites in plants which are structurally and chemically similar. These complex compounds formed include alkaloids and flavonoids (Alebie *et al*., 2017).

Various medicinal plants have been reported to contain chemicals which are useful to human beings. *Aloe vera* contains phenolic compounds such as chromone, anthrone, anthraquinone, aloin and emodin which have antibacterial effects. These components also possess anti-inflammatory effects through the inhibition of the cyclooxygenase pathway. *Alstonia boonei* contains alkaloids such as echitamine, echitamidine, voacangine, tannins, steroids, saponin glycosides and triterpenoids (lupeol, ursolic acid). These chemical components are thought to possess potential inhibitory effects against *Mycobacterium ulcerans*. *Pupalia lappacea* has widespread uses and contains steroids such as stearic acid, setosterol-3-O-D-glycopyranoside (Tsouh Fokou *et al*., 2015).

Tannins are polyphenolic compounds which precipitate proteins and interfere with the function of macromolecules. Tannins have been demonstrated to interfere with angiotensin converting enzyme (ACE). Plants contain antioxidants such as phenolic compounds which mop up reactive oxygen radicals which have the potential of causing cardiovascular disease such as hypertension and congestive heart failure. ACE inhibitors contained in plants are compounds such as captopril whose synthetic form is a common antihypertensive drug (Sharifi *et al*., 2013).

Bisi-Johnson *et al*., (2017) demonstrated the effectiveness of several plants found in South Africa against multidrug resistant diarrhoeal agents (Bisi-Johnson *et al*., 2017). A study by Wintola and Afolayan (2015) in the Eastern Cape, South Africa on the root extracts of *Hydnora Africana* which is commonly used for treatment of dysentery found the plant to contain

antibacterial properties, phenols, flavonoids, tannin, proanthocyanidin, saponins, alkaloids and antioxidant activity. The same compounds were also found in *Bulbine abyssinica*, a plant commonly found in the Eastern Cape, South Africa (Kibiti and Afolayan, 2015).

2.9.4 Medicinal plants as antibiotics

There is increasing resistance to drugs by microorganisms being reported across the world including resistance by bacteria to antibiotics which is now a global health issue (Anyanwu & Okoye, 2017). In over 80 years, about 140 antibiotics have been developed but discovery of new antibiotics has drastically slowed down in the last few decades due to a variety of reasons. There is need to reinvent scientific methods for drug discovery as the old methods used to discover old antibiotics are not as efficient now (Spellberg, 2014).

Bacteria have also evolved and there is need to develop scientific techniques to overcome bacterial evolution. Pharmaceutical companies have redirected their efforts to the more lucrative option of developing drugs taken over a long period for conditions like hypertension, HIV infection and hypercholesterolemia. To the contrary antibiotics are short course therapies and they cure their disease (Spellberg, 2014).

Bacteria are now resistant to extended β lactams, vancomycin, and carbapenems and the misuse and abuse of antibiotics has led to renewed efforts on alternative antimicrobials. Medicinal plants are a viable option as they were once in widespread use before the advent of therapeutic drugs. A lot of scientific work is being devoted to understanding the active antimicrobial components of medicinal plants at present (Anyanwu & Okoye, 2017). Figure 2.5 below illustrates the fall in antibiotic development and approval compared to the rise in resistant bacteria.

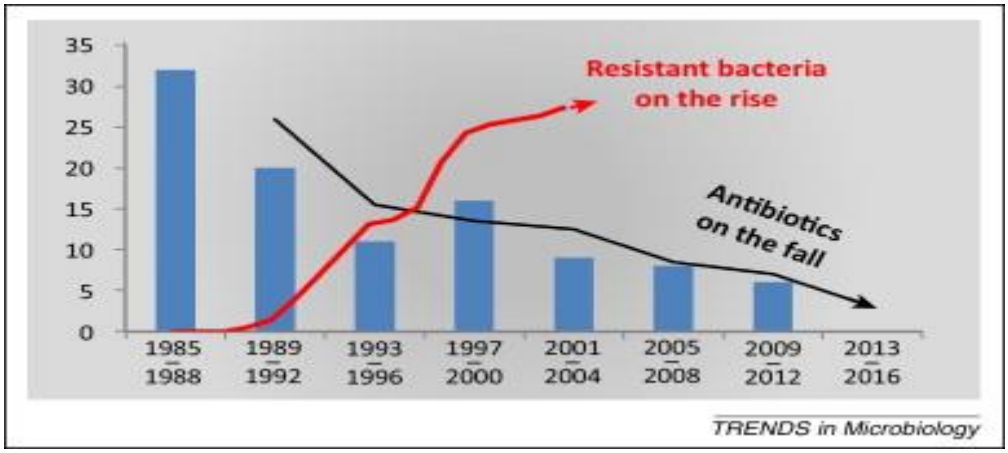

Figure 2.5: Antibiotic approval against rise in bacterial resistance

Reference: http://www.cell.com/cms

Medicinal plants have been used to cure disease caused by microbes and plants and plant extracts have been known to exhibit antimicrobial activity. In some countries medicinal plants are widely used by the general population while in others the use of medicinal plants is largely confined to the rural areas. In Pakistan methanol and ethanol extracts from thirty four medicinally valuable plants prepared in Dimethy sulphoxide (DMSO) were shown to contain E. coli inhibitory properties. These methanol and ethanol extracts contained polyphenols which are potent antimicrobial components. Plants such as *Withania somnilera, Justicia adhatoda, Althaea otticinalis, Azadirachta indica, Mentha longifolea* and *Delonix regia* were demonstrated to contain antimicrobial substances against E. coli. Plant extracts were also prepared with butanol, water, ethyl acetate, chloroform and n-hexane and they still showed antimicrobial activity (Adnan et al., 2014).

A study done in Vietnam on the in vitro activity of medicinal plants found that some plants exhibited antimicrobial activity against both Gram positive and Gram negative bacteria. Most of the plants had broad spectrum antibacterial activity. Medicinal plants such as *Cratoxylum formosum* and *Pedilanthus tithymaloides* had broad spectrum antibiotic activity. However, *P.*

aeruginosa showed resistance to some of the plants used in the study indicating that not all plants extracts have broad spectrum antibacterial activity. There was variation in the efficacy of the plant extracts according to the solvent used in the extraction process (Vu *et al*., 2016). In a similar study in Nepal MRSA, imipenem resistant *P. aeruginosa*, multi drug resistant *Salmonella typhi* and *Salmonella typhimurium* showed considerable resistance to plant extracts. *Cynodon dactylon* extracts showed activity against multi drug resistant bacterial isolates (Marasini *et al*., 2015).

In a study conducted in Cameroon, plant extracts were tested against Gram negative bacteria including organisms which were multidrug resistant. Most of the plant extracts contained phenols, tannin, anthraquinone and cardiac glycosides. All the plant extracts showed inhibitory capacity to a small number of the isolates (14.3%) but some of the plant extracts such as *Betula platyphylla* and *Erigeron floribundus* had broad spectrum antibacterial capabilities. PSA and *Enterobacter aerogenes* showed resistance to most of the plant extracts (Djeussi *et al*., 2016).

Several studies have been done in South Africa on the antibacterial effect of medicinal plants. A study by Obi *et al*., (2007) in the Limpopo province reported that plant extracts from *Bauhinia galpinni*, *Ficus sycomorus* and *Zornia milneana* had antibacterial activity against *Aeromonas* species. A study done in the Mpumalanga region on medicinal plants reported that most of the methanol extracts had low activity against *E.coli* while extracts from *Croton megalobotrys* were effective against *Enterecoccus faecalis* and *Croton steenkapianis* extracts had low activity against PSA (Selowa *et al*., 2010). A similar study by van Vuuren *et al*., (2015) in kwaZulu-Natal on the antimicrobial effects of plants used for treatment of diarrhoea in the region showed that only a few plants had antibacterial activity. Plants such as *Acacia burkei* and *Brachylaena transvaalensis* had considerable antibacterial activity while the most susceptible organisms were *Shigella flexineri* and *Bacillus cereus* (van Vuuren *et al*., 2015).

Studies on *Hydnora africana* in the Eastern Cape Province revealed that the plant had phenols, flavonoids, tannin, proanthocyanidin, saponins and alkaloids and the extracts had antibacterial activity against *E.coli*, *P. aeruginosa*, *Shigella sonnei*, *Vibrio*, *Bacillus cereus* and *Streptococcus pyogenes*. *Salmonella typhimurium* showed considerable resistance to some of the plant extracts (Wintola and Afolayan, 2015).

2.9.4.1 Problems associated with use of medicinal plants

The growing use of medicinal plants is creating new questions into their effectiveness and side effects. However, with few medicinal plants undergoing through clinical trials to establish their efficacy and safety it is difficult to monitor problems associated with their usage. With most plants remaining untested there are no effective ways of ensuring their safe and rational use (Randriamiharisoa *et al*., 2015; Tinitana *et al*., 2016).

There is a lot of dissatisfaction with current conventional medicines due to their high cost, numerous side effects, efficacy and low discovery of certain classes of drugs such as antibiotics. This has led to the misplaced belief that medicinal plants are superior and effective therapies, increasing the risk of self-medication. There is increased spiritual belief in the utilisation of medicinal plants and people are inclined to accept therapies based on spiritual beliefs and instinct. This is still in light of the inadequate knowledge on the mode of action of medicinal plants in local communities (Isah *et al*., 2012).

Medicinal products administered to people should be licenced on a criteria of safety, efficacy and quality but a lot of these medicinal products are classified under dietary components or supplements. This means they do not go through rigorous regulatory requirements necessary for antibiotics and other therapeutic substances. The monitoring of adverse effects gets complicated as they are part of a normal diet while there is a misconception that medicinal plants do not have adverse effects. This misconception increases self-medication and tampering

with dose and unregulated dosage predisposes people to the toxic effects of medicinal plants. Many countries do not have the technology to screen medicinal plants to provide safe and processed products which ensures quality production standards and products while poor regulatory mechanisms make toxicity monitoring less effective. Toxicity of medicinal plants can occur at cellular level, with medicinal plants affecting DNA or other processes hence there is need for effective toxicology screening (Shetti *et al*., 2011).

Regulation of medicinal practices is met with varying challenges in different countries. In some communities the understanding of traditional medicine practices is poor and has no scientific backing. Regulation is usually left to local communities and traditional healers who cannot ensure an effective assessment of the safety, efficacy and quality control of the medicinal plants. These processes are easier to perform on conventional medicine than medicinal plants since medicinal plants are administered as whole plant components like roots, leaves, bark or fruit and sometimes as a concoction of different parts of the same plant or different plants. Each component of a plant may contain several active phytochemical components and it gets more complex when different parts of a plant or plants are administered concomitantly (Shaw *et al*., 2012).

The phytochemical composition of a plant is not only dependent on the plant's genetic factors as several other factors influence the phytochemical composition and quality of plant extracts used for therapy. These factors include how the plant was grown and climatic conditions like temperature, rainfall pattern and humidity. Selection and collection method, identification, transport, storage, sanitation and preparation of the plant are other factors which affect the phytochemical composition of medicinal plants. The wide variation in phytochemical composition of plants even those of the same species makes standardisation of doses even more difficult (Ekor, 2013).

Knowledge on medicinal plants is based on history and experience by trial and error and there is very little or no reference made to contraindications. Due to the availability of conventional medicine, there is a risk of concomitant administration with medicinal plants resulting in reactions and toxicities not normally encountered in monotherapy. Generally, patients do not report that they are on medicinal plants when visiting healthcare professionals (Ekor, 2013).

2.9.5 Global distribution of medicinal plants

The use of medicinal plants dates back to ancient times for all cultures and with 80% of population in the world including young people relying on traditional medicine, the use of medicinal plants plays a greater role in therapy. It is not only developing countries which rely on medicinal plants as a quarter of prescriptions in the USA is made of plant extracts. The general belief has always been that medicinal plants are just available. However, some medicinal plants are under threat of extinction with very little knowledge and effort put into conservation by traditional societies, whose very existence is also under threat. Variations in habitat, climatic changes, extremes of climate and the interaction with other environmental factors are putting pressure on the survival of medicinal plants (Chen *et al*., 2016; Si-Yuan *et al*., 2013; Umair *et al*., 2017).

There are more than one thousand three hundred medicinal plants in Europe. About 79% of the USA's top prescription drugs are made from natural resources as compared to only about 25% of prescription drugs in developing countries which are made from natural resources. Destruction of forests for human habitat and excessive harvesting has left a lot of plants facing extinction in countries like China, India, Kenya, Nepal, Tanzania and Uganda and reports have noted uneven distribution of medicinal plants across the world with China, India, Colombia, South Africa and USA (Hong *et al*., 2015; Corlett, 2016).

There are about 50136 seed producing plant species in sub Saharan Africa which accounts for a quarter of the world's total angiosperm population. Over 5400 of these plants are used as medicinal plants with over 16300 medicinal uses although a small number of plant species have been commercialised in Southern Africa (14) when compared to Europe (336). The most notable commercialised plants in Southern Africa are *Aloe vera*, *Aspalathus linearis* (rooibos tea), *Hoodia gordonii* (Hoodia), *Hypoxis hemerocallidea* (African potato) and *Tulbaghia alliacea* (wild garlic). Also widely researched is *Harpagophytum procumbens* (the devil's claw) which is common in Southern African countries such as Angola, Zimbabwe, South Africa, Botswana and Namibia. It is used for treatment of arthritis, indigestion and anorexia. *Aloe vera* has topical uses and is used as a health drink and rooibos is used as an anti-ageing and anticarcinogenic agent. The African potato is used as a traditional tonic and for treatment of benign prostatic hyperplasia while wild garlic is used for relief of colds and fever (van Wyk, 2011).

There is uneven distribution of plant species in sub Saharan Africa and Southern Africa but the South western tip is species rich while there is poor distribution in the West African interior. Cape Town (South Africa) is home to about 25% of the continent's plant species and documentation of medicinal plants in South Africa started as far back as 1847 with publications on the status of medicinal plant use in the Eastern Cape dating as far as 1888. Comprehensive documentation of medicinal plants in South Africa only happened in 2002. While Africa has a vast collection of medicinal plants there is very little scientific evidence supported by in vivo studies to substantiate the claims of therapeutic benefits of these plants (van Wyk, 2011).

2.9.6 Common infections in pregnancy

Infections common in pregnancy include influenza virus, hepatitis E virus and malaria and in the USA pregnant women are at a higher risk of contracting influenza virus than non-pregnant

women resulting in hospitalisation, admission to intensive care units and even death. There seems to be increased susceptibility to infection during pregnancy with organisms such as bacteria, viruses and parasites (Sappenfield *et al*., 2013). Infections maybe asymptomatic or may manifest in many different forms and may infect the mother, placenta, foetus or neonate and can lead to pregnancy loss, stillbirth, preterm delivery, postpartum neonatal infections or long term sequelae (Silasi *et al*., 2015).There is no strong evidence to suggest systemic immune suppression during pregnancy however, humoral changes especially in the later period of pregnancy seem to affect the immune system's responses to infection. Physiological changes which occur during pregnancy such as alterations in blood flow and urinary flow rate seem to favour survival and proliferation of microorganisms (Hodgins *et al*., 2016).

Major hormonal changes occur during pregnancy and these changes coupled with a myriad of other factors interact with the immune system altering the pregnant woman's response to infection. Serum levels of estradiol and progesterone alter immune cell function with low estradiol levels increasing the response of T helper 1 cells which are important for response to intracellular pathogens. High estradiol levels favour the proliferation of T helper 2 cells which are important in the immune system's response to helminths and allergens while progesterone is important in regulating Th1 and Th2 cellular responses (Lissauer *et al*., 2015).

Progesterone and estradiol are important in maintenance of innate immune function during the second and third trimester by altering the function of cellular components of the innate immune system such as phagocytes, neutrophils and dendritic cells. Levels of cytokines such as interleukins and interferons are reduced, altering inflammatory responses. There is a decrease in the adaptive immune response of the body in late pregnancy reducing the numbers of functional T lymphocytes and natural killer cells delaying clearance of bacteria, fungi, viruses

and parasites causing an increased susceptibility to infection. Therefore, the generalised view that pregnancy causes immunosuppression is oversimplified and not true (Kourtis *et al*., 2014).

Malaria is a common parasitic infection in pregnancy (De Beaudrap *et al*., 2016; Moore *et al*., 2017) while herpes simplex virus (HSV) (Perti *et al*., 2014; Aebi-Popp *et al*., 2016; Johnston *et al*., 2016), cytomegalovirus (CMV) (Saldan *et al*., 2016; Saldan *et al*., 2017), rubella (Lambert *et al*., 2015; Dimech *et al*., 2016), hepatitis (Zhao *et al*., 2016; Ogholikhan & Schwarz, 2016), herpes and influenza (Giakoumelou *et al*., 2016) are common viruses. Common bacterial infections during pregnancy include: *E.coli* (Saez-Lopez *et al*., 2016; Pathak *et al*., 2013; Ramos *et al*., 2012), *Klebsiella* spp (Khatri *et al*., 2015; Matuszkiewicz-Rowinska *et al*., 2012; N G *et al*., 2014), *Staphylococcus* spp (Freitas *et al*., 2017; Nuriel-Ohayon *et al*., 2016; Unger *et al*., 2015), *S. pyogenes* (Golinska *et al*., 2016; Hamilton *et al*., 2013), *S. agalactiae* (Cools *et al*., 2016), *E. faecalis* (Labi *et al*., 2015; Mueller *et al*., 2016) and Listeria (Lamont *et al*., 2011; Li *et al*., 2016).

Pregnant women are normally colonised by microbes as part of the vaginal normal flora including candida, *S. agalactiae,* coagulase negative staphylococcus, lactobacillus and bacteroides species. Overgrowth of normal flora or change in the pregnant woman's immune status can change the balance in the microbiota leading to infection which can be transmitted to the neonate during delivery (Honda *et al*., 2014; Kraal *et al*., 2014).

2.9.7 Use of medicinal plants in pregnancy

Medicinal plants play a crucial role in women's health and there is deep rooted belief in the efficacy of traditional medicine with 80% of the women using complementary medicine. It is a readily available health care option in resource limited African settings used to remedy female fertility, for birth control, abortion and during pregnancy. During pregnancy medicinal plants

are used to control emesis, during labour and for the control of fungal and bacterial infections and they are also important as nutritional supplements (Shewamene *et al*., 2017).

In a study by Ali-Shtayeh *et al*., (2015) in Pakistan, there was widespread use of medicinal plants during pregnancy, parturition, postpartum period and in infant care. The most common reasons for use of medicinal plants during pregnancy were to control anaemia, constipation and urinary tract infections (Ali-Shtayeh *et al*., 2015). In a similar study by Al-Ramahi *et al*., (2013) in Pakistani, 40% of women used herbs during pregnancy and most of the women (82.5%) thought medicinal plants were better than conventional medicines. Almost all the women (99.2%) did not report side effects after use of medicinal plants (Al-Ramahi *et al*., 2013).

In South East Asia medicinal plants are used in women's health care for fertility, abortion, birth control, control of menstruation, during delivery and in treatment of infection (de Boer and Cotingting, 2014). A study in Brazil by Yazbek *et al*, (2016) reported widespread use of medicinal plants in control of menstruation, inducing abortion and treating infections during pregnancy including sexually transmitted infections. However, the study noted that scientific work was needed to reveal the phytochemical components of the medicinal plants while the pharmacological and toxicological effects of the medicinal plants were also unknown (Yazbek *et al*., 2016).

Studies in Africa have revealed the widespread use of medicinal plants in African communities during pregnancy with studies in Mali (Nergard *et al*., 2015), Cameroon (Yemele *et al*., 2015) and Cote d'Ivoire (Malan and Neuba, 2011) reporting that most pregnant women studied used medicinal plants. The reasons for use of medicinal plants during pregnancy included treatment of malaria symptoms, reducing oedema and treatment of urinary tract infections. The women believed the medicinal plants did not have adverse effects on them or their pregnancies.

There was low level use of medicinal plants in pregnancy in a study conducted in Ethiopia with only 12% of the women using medicinal plants. The use of medicinal plants was significantly associated with the educational level of the women and a small percentage of the women (12.5%) who used medicinal plants during pregnancy disclosed the information to health care professionals. A fifth of these women used medicinal plants concurrently with conventional medicine while common reasons for use of medicinal plants included treatment of malaria, pain alleviation and treatment of infections (Mothupi, 2014).

A study by Moteetee and Seleteng (2016) in Lesotho reported that medicinal plants were used to relieve pregnancy related complications although the phytochemical components, pharmacological and toxicological effects of the plants were poorly understood (Moteetee and Seleteng, 2016). In South Africa over 80% of the population uses traditional medicine just as in other African countries. Pregnant women do not disclose the use of traditional medicine to health care workers for fear of affecting the care they will receive from health professionals. However, traditional medicine is used for various conditions in different trimesters including prevention of miscarriage, controlling high blood pressure, treatment of infections including sexually transmitted infections and inducing labour. Just as in other African countries, pregnant women are not fully aware of the adverse effects of traditional medicine or the danger of concomitant use of traditional medicine and conventional medicine. Different plants are used in different trimesters with the common ones being *Zingiber officinale* (1st trimester), *Agapanthus africanus* (2nd and 3rd trimester) and *Rubusidaeus* (3rd trimester) (Maputle *et al*., 2015).

2.9.7.1 Challenges of using medicinal plants in pregnancy

Medicinal plants do not have specific elicit reactions while in contrast synthetic drugs contain a purified component which has a specific function. When using synthetic drugs the risk of

adverse effects to beneficial effects is weighed and although medicinal plants are considered safe for use, pregnancy is a contraindication. Though there are no reports of teratogenicity of medicinal plants, the risk is better avoided (Nasri & Shirzad, 2013).

The most common toxicities of medicinal plants are seen in different organs of the body including kidneys (nephrotoxicity), liver (hepatotoxicity), heart (cardiotoxicity) and central nervous system (neurotoxicity). When conventional drugs are taken concomitantly with medicinal plants it exacerbates the toxic effect of each class of medicine while other possible causes of toxic effects include contamination with microorganisms, aflatoxins and heavy metal poisoning. The idea that medicinal plants do not have adverse effects or are non-toxic might after all just be a myth (Nudrat & Naira, 2016; Efferth & Kaina, 2011).

2.9.8 Medicinal plants for treatment of *S. agalactiae*

The use of medicinal plants for the treatment of infection is common and technological advancement has enabled the extraction and characterisation of the components of medicinal plants. However, despite the advancement in medicinal plants treatment of infections has not changed. Approximately 35% of healthy women and 2% of new-born babies are colonised with *S. agalactiae* but ethanol extracts of *Sambucus peruviana*, *Krameria triandra*, *Cestrum auriculatum*, *Annedera diffusa* and *Papaver bracteatum* have been proved by in vitro testing to be effective against *S. agalactiae* (Delfan *et al*., 2017).

Eugenol which is an extract of many medicinal plants' essential oils has antibacterial effects against *S. agalactiae* and other microorganisms. A study by Biasi-Garbin *et al*., (2015) reported that a combination of Eugenol and silver nanoparticles was more potent than Eugenol without silver nanoparticles (Biasi-Garbin *et al*., 2015). *Cortex phellodendri* and *Rhizoma coptidis* extracts are used to make berberine which is an alkaloid and has broad spectrum antimicrobial activity with little or no effect on host cells. The chemical disrupts cellular constituents like the

cell wall, leaving intracellular components to leak out. *Coptis* which is a genus of about fifteen flowering plants has an effective in vitro antibacterial effect against *S. agalactiae* (Peng *et al*., 2015).

In a study done in Turkey on the in vitro antibacterial activity of *Xylaria hypoxylon* (*X.hypoxylon*), it was reported that the plant had inhibitory effects against several bacteria including *S. agalactiae*. In a similar study in Nepal on the activity of various medicinal plant extracts against pathogenic bacteria, plants such as *Acorus calamus, Adhatoda vasica*, *Centella asiatica, Curcuma longa, Cynodon dactylon, Drymaria cordata, Ginkgo biloba* and *Rauwolfia serpentina* were effective against *S. agalactiae* (Canli *et al*., 2016; Marasini *et al*., 2015).

A study by Sigidi *et al*., (2016) assessed the antimicrobial activities of two indigenous Venda (South Africa) medicinal plants against selected bacteria of clinical importance. The study reported that the plant extracts of *Ziziphus mucronata* (Buffalo thorn) and *Pterocarpus angolensis* ((Bleedwood tree or Mutondo) had no antibacterial effect against bacteria of clinical importance such as *S. agalactiae.* However, in the community these plants were known to be effective in treating wound and eye infections cleansing blood of various ailments *(Pterocarpus angolensis)* and boils and skin infections *(Ziziphus mucronata)* (Sigidi *et al*., 2016). This shows that despite the claims made in traditional communities of the effectiveness of medicinal plants in treating infection not all such plants will show positive results in vitro testing.

2.9.9 *Olea europaea* (*O. europaea*) Subsp. africana

The Oleaceae is a family of angiosperms made of thirty genera and approximately 600 species with Oleae as one of the numerous tribes. The name Olea has Spanish and Greek origin but the genera has many names. While the Oleae are known to grow well in the tropical and temperate areas of the Far East they also grow in the tropics and subtropics of Africa. *O. europaea* is the most common species of the Olea genus and is a common food component. The use of Olea

dates back to ancient times and has had spiritual and religious significance as it is cited multiple times in the Holy Bible (Old and New Testaments) and the Holy Quran. In Africa it grows well in Northern Africa but it is also found in Southern Africa as the plant is drought resistant (Ryan and Robards, 1998). In South Africa *Olea europaea* L sub species africana is widely distributed in the Cape (Northern, Eastern and Western), KwaZulu-Natal, Limpopo and Mpumalanga provinces. It is known by different names among the different communities: Olienhout (Afrikaans), mutlhwari (Venda), motlhwane (Tswana) and umnquma (Zulu, Xhosa and Swati) (SANBI, 2017).

O. europaea is a food component but has a multitude of medicinal uses most of which are still under scientific investigation for validation. These include: lowering of serum levels of glucose, lipids and urate; treatment of infections such as in intestinal, respiratory and urinary tracts; treatment of metabolic conditions such as diabetes and hypertension. Different components of the tree (bark, fruit, leaves, wood, seeds and essential oils) have specific applications although they are used in combination in some cases. The oil from seeds is used as a laxative and for anti-inflammatory purposes while the leaves and fruit are used for treating infections but tree components are used differently in different communities and geographical locations. In Japan Olea leaves and essential oil are used for treatment of stomach and intestinal diseases while in Italy the essential oil of the fruit is used for treatment of calculi. The Greeks, Moroccans and Algerians use leaf extracts as antihypertensive medication while in East Africa the bark is used as an anti-helminth. The phytochemical components of the plant have been characterised and it has been reported to contain pharmacologically active substances such as secoiridoids, iridoids and phenols. Apart from medicinal properties, *O. europaea* has other social uses in the communities such as making of furniture, ornaments, fence posts and ink (Vogel *et al*., 2015; Hashmi *et al*., 2015). Figure 2.6 shows the branch, leaves and fruits of *O. europaea* sub species africana.

Figure 2.6: *O. europaea* sub species africana branches, leaves and fruits.

Reference: https://www.google.com.na/search?q=olea+europaea+subsp.+africana

2.9.9.1 Botanical description of *O. europaea* sub species Africana

It is a dense green plant which can be classified as a tree or shrub as it grows up to 10 metres and can spread to between 9 and 12 metres. The bark is grey with evergreen leaves while the numerous white flowers are small and bisexual. The fruit is small with a hardened kernel surrounded by a fleshy outer layer and is popular with both humans and animals such as monkeys, baboons and birds. The tree flowers in spring and early summer (October to February) and the fruits follow between March and July (Long *et al*., 2010).

2.9.9.2 Phytochemical components of *O. europaea*

All the different parts of the *O. europaea* plant contain similar phytochemicals but the plant contains primary and secondary metabolites in different parts. The most common phytochemicals are phenolic compounds, flavonoids, secoiridoids and its glycosides and derivatives of benzoate. Although the different parts of the plant contain similar

phytochemicals, the distribution of the constituents varies among the different parts depending on growth patterns of the plant (natural or cultivated), climatic conditions and geographic location (Vogel *et al*., 2015).

The leaves and fruit contain oleuropein (a secoiridoid glycoside) as the main component but the fruits and seeds have many other phytochemicals such as flavonoids and phenolics. Methanol extracts of leaves have been reported to contain flavonoids and secoiridoids while the essential oil is rich in phenolic compounds. The bark is rich in secoiridioid glycosides and iridoids among other phytochemicals. The phytochemicals have pharmacological actions including antidiabetic, anticancer, antimicrobial, antioxidant, antienzymatic, antihypertensive, anti-inflammatory and antinociceptive activities (Vogel *et al*., 2015; Hashmi *et al*., 2015).

2.9.9.3 Antimicrobial activities of *O. europaea*

O. europaea has been used in communities as a medicinal plant for the treatment of various microbial infections. The plant extracts such as phenols and α,β-unsaturated aldehydes have broad spectrum antimicrobial activity. Ethanolic leaf extracts have been demonstrated to have inhibitory effects against *C. jejuni, H. pylori, S. aureus, P. aeruginosa, E. coli, K. pneumoniae, C. albicans* and *C. neoformans* (Lee and Lee, 2010; Hashmi *et al*., 2015). In a recent study *O. europaea* leaf extracts reduced the pathogenicity of *L. monocytogenes* by removing the flagella and the extracts had inhibitory effects against *S. aureus* and *S. enteritidis* while purified extracts of the plant were more potent than crude extracts (Liu *et al*., 2017). In one study *O. europaea* leaf extracts were reported to possess inhibitory effects against *E. coli* and *S. aureus*. The extracts had synergistic interactions with ampicillin showing that while the leaf extracts are potent individually they work better when administered concomitantly with other drugs like ampicillin (Lim *et al*., 2016; Rempe *et al*., 2017).

Phenolic compounds in *O. europaea* leaf extracts act by disrupting bacterial cell membranes of both Gram positive and Gram negative bacteria. However, it is not clear whether the membrane disruption effect is due to interaction of phenolic compounds with membrane components or by interaction with intracellular processes. Other antibacterial processes of Olea leaf extracts are through non membrane mechanisms such as DNA intercalation, protein kinase inhibition and DNA gyrase inhibition. Different phenolic compounds have varying effects on different bacterial species. Chlorogenic acid has cell membrane disruption effect against Gram positive and Gram negative bacteria such as *S. pneumoniae, S. aureus, Bacillus subtilis, E. coli, S. dysenteriae* and *S. typhimurium*. Quercetin has cell membrane disrupting effects, DNA intercalation and inhibition of protein kinases which affects *E. coli* and *H. pylori* while Apigenin has dehydratse and protein kinase inhibition effects against *H. pylori* (Rempe *et al*., 2017).

Many plant components commonly considered as food have no notable toxicity to human beings. However, in the absence of clinical trials to provide conclusive scientific evidence it is difficult to rule out adverse effects when the plant is administered for medicinal purposes in unregulated concentrations or formulations.

2.10 Bacteriophages

Bacteriophages (phages) are viruses which infect bacteria and replicate within the bacteria, ultimately lysing the bacteria to release progeny virions. An estimated 10^{31} phages with more than 10^{6} distinct species exist (Pastagia *et al*., 2013). Double stranded DNA (dsDNA) bacteriophages replicate inside the host and release two types of proteins, endolysins and holins which are necessary for host cell to lyse. The production cycle for progeny phages takes about 30 minutes (Pastagia *et al*., 2013). These phages which lyse bacterial cells as they exit the host cell are termed lytic phages. Other phages incorporate their DNA into the host DNA and when

the host replicates the phage DNA is also replicated. They do not lyse host cells and are termed lysogenic. However, they can be activated to be lytic through external stimuli. Below is a diagrammatic representation of the bacteriophage life cycle (Figure 2.7).

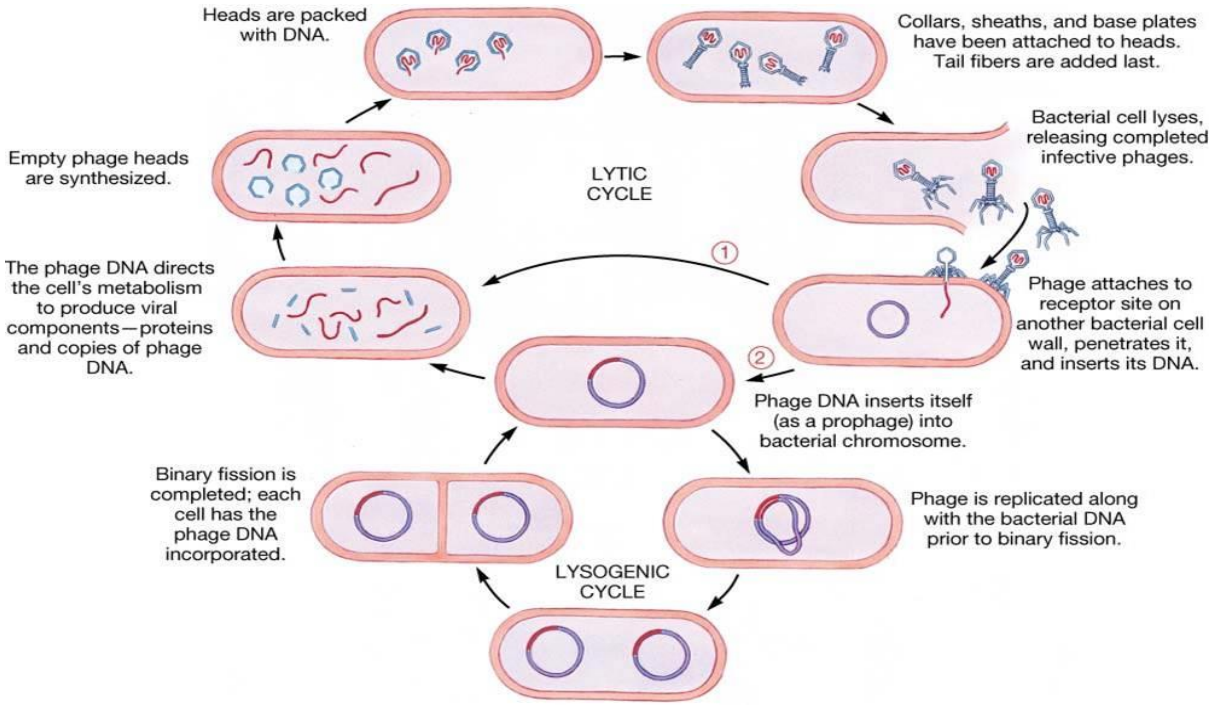

Figure 2.7: Bacteriophage life cycle.

Reference: https://online.science.psu.edu/micrb106_wd/node/6231

Bacteriophages have been used since the 1900s for treatment of bacteria which causes infectious diseases (Loc-Carrillo & Abedon, 2011). The rapid proliferation of multidrug resistant bacteria and slow pace of drug development has necessitated the option of alternative treatment options especially to multidrug resistant invasive bacteria (Kutateladze & Adamia 2010).

Phages were first used in 1917 by Felix d'Herelle for treatment of bacterial dysentery while research in phage and its use was wide during the 1930s but stopped after the Second World War due to discovery of broad spectrum antibiotics. Phage action and its characteristics were

poorly understood while there were no protocols for phage therapy and hosts were poorly identified although it was known then that phage had a narrow range of hosts (Haq *et al*., 2012).

Bacteria do not develop resistance to phage as it gains entry into the bacteria through a conservative bacterial cell wall but some bacteria have shown resistance to phages by restricting phage attachment to the bacterial cell wall (Pastagia *et al*., 2013). While drugs generally have a broad spectrum of activity against bacteria unlike phages, the bacteria eventually develop resistance due to selection pressure. Ten years after the discovery of penicillin, reports of bacterial resistance to the drug were documented while it took almost 30 years for vancomycin resistance to be noticed.

In one study, GBS lysin gene was expressed in *E. coli* and used produce a lysin (PlyGBS) which was purified and tested against GBS isolates. PlyGBS killed all tested GBS serotypes it was tested against in vitro and also GBS in the vagina and oropharynx of mouse when it was tested in animal models. It was postulated that the lysin potent as alternative therapy for elimination of GBS in pregnant women (Cheng *et al*., 2005).

2.11 Chlorhexidine wash

Chlorhexidine gluconate is a cationic bisbigaunide which is effective against both Gram positive and Gram negative bacteria. Its broad spectrum antimicrobial activity allows it to be commonly used as a skin disinfectant. However, it is non-sporicidal hence it is not effective against spore forming bacteria (Nerandzic & Donskey, 2015).

In studies done on dressings impregnated with chlorhexidine gluconate, it was found to be effective in reducing catheterisation related septicaemia (Wibaux *et al*., 2015). However, in another study, it was found not to be able to reduce bacterial count of potential pathogens of the mouth when used as a decontaminant (Scannapieco *et al*., 2009). When four percent

chlorhexidine gluconate was compared to normal saline as a predisinfection skin scrub solution during surgery, chlorhexidine was found to be similar to normal saline in efficacy (Hsieh *et al*., 2014).

Chlorhexidine gluconate has been proposed as a powerful membrane disinfectant which can be used to reduce GBS colonisation of the vagina and bacterial load during birth. This action reduces the chances of vertical transmission of GBS from the mother to the baby. In four clinical trials which were conducted on the effectiveness of chlorhexidine, there was no reduction of EOD or pneumonia associated with its use (Nishihara *et al*., 2016).

2.12 Genetic mutations in GBS

A mutation refers to a change in the nucleotide sequence of a genome which is permanent and can be transmitted to offsprings. The nucleotide sequence of a genome is made up of a backbone of four nitrogenous bases composed of two pyrimidines (thymine and cytosine) and two purine (guanine and adenine). The pyrimidines are heterocyclic organic compounds made of a single ring while purines are heterocyclic aromatic compounds made of a double ring. The purine ring is composed of a pyrimidine ring fused with an imidazole ring with nucleotide bases pairing as: thymine (T)-adenine (A), adenine-thymine, guanine (G)-cytosine (C) and cytosine-guanine in DNA. A unique sequence of three of the four nucleotides in DNA is called a codon and encodes for a specific amino acid among the common 20 amino acids. The number and sequence of amino acids joined together makes a protein and gives the protein its function (Stoval *et al*., 2014; Acuna-Hidalgo *et al*., 2016).

The section of a gene which carries the nucleotide sequence which results in a product is the coding region which carries the genetic code for the gene product, usually a protein. The gene also contains a regulatory region which is responsible for regulating the transcription of a gene (Lane & Fan, 2015).

2.12.1 Causes of mutations

Errors may occur when DNA is replicating when wrong nucleotide pairing occurs during DNA synthesis resulting in substitution of a base. A tautomer may also mispair during DNA replication causing a mutation while translesion synthesis is a process which copies damaged sites of a DNA template during synthesis allowing completion of DNA replication. DNA lesions can stop the enzymes (polymerases) which enable DNA to replicate so translesion is an important cellular process that responses to DNA which is not repaired during replication (Zhou *et al*., 2010). While translesion synthesis is a very important process, it is highly mutagenic due to the high potential of miscoding damaged nucleotides. It is a significant contributing factor to mutations arising from environmental processes. This gives rise to spontaneous mutagenesis (Kochenova *et al*., 2015) because cells are constantly exposed to endogenous and exogenous factors which cause DNA lesions to develop. Despite this, the genome is supposed to be protected to ensure that damaged DNA is not transferred to daughter cells failure of which causes changes in chromosomes.

Several processes ensure that damaged DNA is not transferred to daughter cells during the cell cycle which include DNA break detection, checkpoint arrest and DNA damage repair. Failure of these processes results in the transfer of damaged DNA, mutagenesis and chromosomal rearrangements (Genois *et al*., 2014). When a chemical change occurs to DNA it does not constitute a mutation but results in damaged DNA or a DNA lesion. The damaged DNA is repaired and during the S phase of the cell cycle the DNA is replicated in daughter cells. However, the nucleotide sequence may not be correct due to the repair process to the damaged DNA and this change in the nucleotide sequence is what constitutes a mutation. These mutations are termed induced mutations and arise due to exposure to chemicals and radiation (Brash, 2015).

2.12.2 Types of mutations

Mutations can be classified according to the effect of the mutation on the DNA structure. Substitution mutations exchange a single nucleotide for another which can be a purine for a purine, a pyrimidine for a pyrimidine, a purine for a pyrimidine or a pyrimidine for a purine. When the mutation forms a codon which codes for the same amino acid it is called a silent mutation but when the mutation results in the coding of a different amino acid it is called a missense mutation while nonsense mutations code for a stop codon. Insertions occur due to the addition of one or more nucleotides into the DNA while the removal of one or more nucleotides from the DNA is called a deletion (Housden *et al*., 2017; Jung *et al*., 2015).

2.12.2.1 Transitions

A transition is a result of a point mutation which results in substitution of a pyrimidine for a pyrimidine (T for C or C for T) or a purine for a purine (A for T or T for A). Transitions are the commonest form of point mutations making up more than two-thirds of single nucleotide polymorphisms. The main causes of transitions include oxidative deamination and tautomerization (Luo *et al*., 2016).

2.12.2.2 Transversions

A transversion is the substitution of a purine for a pyrimidine or a pyrimidine for a purine. The substitution of a double ring purine for a single ring pyrimidine or the reverse is more difficult than a transition hence transitions are more common than transversions. Transversions are more likely to result in amino acid change encoded by a codon than transitions resulting in change in the amino acid sequence of a protein, protein structure, function, organism function and may cause death of the organism if the protein is a functional protein (Alexander *et al*., 2013).

2.12.2.3 Nonsynonymous mutations

Genetic change in organisms does not usually result in notable change in the organism hence is considered as neutral. Changes in or around a gene alters its expression and may affect its phenotype hence understanding genetic mutations helps in understanding protein structure, protein expression and interaction. A single base change in the coding region of a gene which results in the change of an amino acid or protein is considered a nonsynonymous mutation. The change in protein structure or function maybe have serious consequences to the organism and most of the mutations that occur in organisms are harmful and are eliminated through purifying selection. Those mutations which are not harmful can propagate in a population and establish themselves contributing to genetic variability in a population (Ng & Henikoff, 2006).

Predicting the changes in an organism which occur as a result of genetic mutations is crucial in understanding the impact of nonsynonymous mutations (Fariselli *et al*., 2015). Organisms that are complex are better suited to compensate for deleterious mutations than less complex organisms. However, mutation in less complex organisms is less harmful when compared to complex organisms as the mutation is less likely to result in the change of an important process. Beneficial nonsynonymous mutations are more common in less complex organisms than complex organisms while a decreased number of nonsynonymous mutations is a good predictor of positive selection (Huber *et al*., 2017).

Single nucleotide polymorphisms can occur in both the coding and non-coding region of a gene and these polymorphisms are associated with complex Mendelian diseases and appreciating the contribution of a mutation to change in phenotype is crucial in linking genetic variation to survival of an organism or disease as studies have linked changes in protein interaction to genetic polymorphisms (Zhao *et al*., 2014).

2.12.2.4 Synonymous mutations

Synonymous mutations are mutations which do not result in change in the amino acid coded by a codon and alternative codons can code for the same amino acid which means the genetic code is redundant. While it is widely acknowledged that synonymous mutations do not cause change in the amino acid phenotypic changes can still occur and synonymous mutations may affect or impair the initiation of a translational process and also the speed and accuracy of translation (Knoppel *et al*., 2016; Waters *et al*., 2016).

Synonymous mutations are referred to as silent mutations and they are usually ignored in many studies because of the belief that they do not show phenotypic change. However, other studies have challenged the neutrality of synonymous mutations. Distinguishing synonymous mutations with neutral evolution from selection pressure is a very difficult task when phenotypic change is minimal. Almost half of synonymous mutations undergo purifying selection with some of the genes being selected against but some synonymous mutations have been linked to disease (Gotea *et al*., 2015). The frequency of synonymous mutations varies between individuals according to genes and studies have reported that synonymous mutations have an effect on the stability of nucleic acids, protein structure, concentration and function of proteins which occurs without alteration of the amino acids (Hunt *et al*., 2014; Bali and Bebok, 2015).

2.12.3 Effects of mutations

DNA codes for proteins on which a cell depends for normal function and a mutation can result in the coding of a protein which is non-functional or inadequate, thereby effectively rendering the organism unable to properly function or survive. However, not all mutations are harmful to the organism as some mutations may render the mutant organism more resistant to certain factors better than the primary organism (wild-type) (Wang *et al*., 2016; Swings *et al*., 2017).

Mutations result in genetic variation which is a prerequisite to evolution and the evolutionary process is important in the survival of an organism (Hershberg, 2015). The principle of natural selection which facilitates evolution depends on genetic variation with good genes being found among the genes which are inherited by an individual. Each gene inherited has selection value and the benefits can either be beneficial or deleterious (Liberles *et al*., 2012). A random mutation gives rise to a new allele which results in a phenotype irrespective of the allelic combinations in a gene and if the new allele is beneficial to the survival of the organism, it is propagated by natural selection from the parent organism to the offspring giving it the desired phenotype (Burmeister and Smith, 2016).

There are variations in the rate of mutations across the genome with no uniform distribution of the rate of genetic mutations in different genes. Mutation hotspots are common in genes which interact with each other and with the environment (Lind *et al*., 2015) and these mutation hotspots are common in genes under selection pressure. A high *dn*/*ds* ratio is indicative of mutations which result in phenotypic change, putting the organism under selection pressure (Livnat *et al*., 2013).

2.12.4 Multi Locus Sequence Typing

Multi Locus Sequence Typing (MLST) is a molecular technique for characterization of bacterial isolates using a number of house-keeping genes, usually seven which involves the amplification of DNA using primers targeting the specific genes and is followed by Sanger sequencing of the amplicons. For each gene, the different sequences identified within one species are marked as an allele and the alleles of each of the different loci define an allelic profile or a sequence type (Ludovic *et al*., 2004) and these allelic profiles are due to mutations in the DNA of bacterial isolates.

Mutations are transversions when a purine is substituted for a pyrimidine (or the reverse) or transitions when a purine is substituted for a purine and pyrimidine for a pyrimidine. Mutations are also classified as synonymous when the nucleotide mutation does not result in the alteration of an amino acid sequence or nonsynonymous when the nucleotide mutation results in the alteration of an amino acid sequence (Guo *et al*., 2017). Sequencing of the 450-500 base pair (bp) fragments of the seven house-keeping genes of a pathogen have been used to unravel its population structure. This method has been applied to organisms like *Neisseria meningitidis*, *S. pneumoniae* and *S. agalactiae* (Jones *et al*., 2003) and can separate and precisely distinguish GBS isolates (Carvalho-Castro *et al*., 2017).

In the MLST for GBS, seven house-keeping genes are used and these are: alcohol dehydrogenase, *adhP;* phenylalanyl tRNA synthetase, *pheS;* amino acid transporter, *atr;* glutamine synthetase, *glnA;* serine dehydratase, *sdhA;* glucose kinase, *glcK; and* transketolase, *tkt*. These house-keeping genes code for proteins which are important for sustaining basic normal cellular metabolic processes and are present in an organism under normal or pathological conditions (Glaser *et al*., 2002; Skaare *et al*., 2014).

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CHAPTER THREE

CHEMICAL COMPOSITION AND ACTIVITY OF ETHANOLIC LEAF EXTRACTS AND ESSENTIAL OILS OF *OLEA EUROPAEA* AGAINST *S. AGALACTIAE* ISOLATED FROM PREGNANT WOMEN ATTENDING SPECIFIC CLINICS IN NAMIBIA AND SOUTH AFRICA: PROFILE OF RESISTANT GENES.

Abstract

Background: The emergence of multidrug resistant bacteria is now a major health concern in the world. Many factors contribute to the problem of antibiotic resistance including genes that confer resistance to drugs. Medicinal plants are important in the prevention and treatment of both communicable and non-communicable diseases, pregnancy related conditions and nutrition related disorders. They are used for treatment of infections caused by pathogenic microorganisms. *Olea europaea* is known to contain a wide range of phytochemical components with medicinal properties and it has been reported to have inhibitory effects against *Campylobacter jejuni, Helicobacter pylori, Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli* and *Listeria monocytogenes*. This study therefore, sought to explore the antimicrobial effects of selected antibiotics and *O. europaea* leaf extracts and essential oils against *S. agalactiae* isolates. **Methodology**: Benzyl penicillin, ampicillin, cefotaxime, ceftriaxone, levofloxacin, erythromycin, clindamycin, linezolid, vancomycin, tetracycline and cotrimoxazole and *O. europaea* leaf extracts and essential oils were tested against *S. agalactiae* isolates from both South Africa (37 isolates) and Namibia (115 isolates). Minimum inhibitory concentrations (MICs) of antibiotics were obtained using the Vitek and results were interpreted using Clinical and Laboratory Standards Institute (CLSI) guidelines (2015). All the isolates were screened for *β lactamase* (*bla*) genes by PCR using primers specific for the respective genes. *O. europaea* was identified in the Cala community of the Eastern Cape, South Africa. Ethanol leaf extracts and essential oils were extracted and tested against *S. agalactiae* isolates. MICs were determined using agar well diffusion and dilution methods. **Results**: The isolates showed 100% sensitivity to benzyl penicillin, ampicillin, ceftriaxone, levofloxacin, linezolid, vancomycin and Olea leaf extracts and essential oils. Only one isolate (0.6%) was resistant to cefotaxime. Thirty six isolates (23.4%) and sixteen isolates (10.4%) were resistant to clindamycin and erythromycin, respectively. *Bla* genes were not

amplified in any of the isolates. **Conclusion:** *S. agalactiae* isolates were sensitive to *β lactams*, Olea leaf extracts and its essential oils.

Key words: *Streptococcus agalactiae,* Antimicrobial, *β Lactams*, Resistance

3.0 INTRODUCTION

Antibiotic resistance has been in existence since the beginning of the antibiotic era but the problem was not given much attention until recently. Recent developments such as the increase in the frequency of emergence of drug resistant and invasive bacteria, a reduction in the development and approval of new drugs and projections of an imminent post antibiotic era have created interest in antibiotic resistance. The estimated excess health care costs on resistant infections was reportedly $20 billion in USA and 1.6 billion euros in Europe while the cost of unnecessarily prescribed antibiotics was estimated at $1.1 billion (Fair and Tor, 2014). Over prescription of antibiotics, poor public management of antibiotic doses and the unlegislated use of antibiotics in the agricultural sector have increased selection pressure on bacteria. This has resulted in the development and propagation of resistance factors in bacteria (Fair and Tor, 2014).

Current practices of Intrapartum Antibiotic Prophylaxis (IAP) against *S. agalactiae* involve the administration of β-lactam antibiotics, penicillin and ampicillin. Penicillin has a narrow antibiotic spectrum hence it is therefore less likely to select for resistant organisms. The alternatives to penicillin include: cefazolin, clindamycin, erythromycin and vancomycin but these alternative antibiotics have not been assessed in clinical trials and data in their concentration levels in amniotic fluid, foetal blood and tissue are limited (CDC, 2010).

There is an increasing prevalence of multidrug resistant bacterial pathogens which are a major threat to existing antibacterial therapeutic options. Of note are extended spectrum β lactamase producing *E. coli* (ESBL-EC), extended spectrum β lactamase producing *Klebsiella pneumoniae* (ESBL-KP), carbapenem resistant enterobacteriacae, *Pseudomonas aeruginosa*, Methicillin Resistant *S. aureus* (MRSA) and vancomycin resistant enterococcus (VRE) (Marasini *et al*., 2015). *S. agalactiae* is generally sensitive to β-lactams antibiotics but isolates with reduced minimum inhibitory concentrations (MICs) have been described from as far back as 1995 (Biasi-Garbin *et al*., 2015), while MIC changes of cefazolin to GBS have been described from 1999 (CDC, 2010). In a recent study in Italy, outright resistance to GBS was noted for the first time. Out of 65 GBS isolates recovered in the study, 12.07% were resistant to benzyl penicillin (Matani *et al*., 2016).

Antibiotic resistance can arise due to the presence of genes that confer resistance to drugs in an organism. The most clinically significant genes coding for resistance are those genes which encode for enzymes responsible for hydrolysis of *β-lactams* (*bla* genes). These genes confer high level bacterial resistance to *β-lactam* antibiotics like cephalosporins (including 4th generation cephalosporins), penicillins and monobactams (except cefamycins). *Bla* genes include *bla*$_{SHV}$, *bla*$_{CTX-M}$, *bla*$_{TEM}$, and *amp*C while *bla*$_{TEM}$ group consists of more than 220 alleles (Adesoji and Ogunjobi, 2016) and the presence of these genes causes the production of SHV β lactamases, TEM β lactamases and CTX-M β lactamases, respectively. The *bla* genes can be borne on plasmids or transposons which are transferable and this facilitates horizontal spread of antibiotic resistance (Ojdana *et al*., 2014).

While erythromycin and clindamycin were indicated as alternative therapy to GBS IAP, there has been increasing resistance of GBS to these drugs and in the United States of America (USA), clindamycin and erythromycin resistance have been recorded at 13-20% and 25-35%,

respectively (CDC, 2010). Across the world, resistance has been estimated at 14.5-70% and 8.2-70% for erythromycin and clindamycin, respectively (Biasi-Garbin *et al*., 2015). The CDC guidelines removed erythromycin as a second line prophylactic antibiotic in women with penicillin allergy due to the high resistance of GBS to it while clindamycin is still acceptable but only after susceptibility testing (Back *et al*., 2011).

Medicinal plants have been used since ancient times mostly instinctively with very little information on the pathology of the disease or the pharmacological action of the plant while decisions were based on experience (Petrovska, 2012). Medicinal plants were widely used before the discovery of synthetic drugs but increasing antimicrobial resistance coupled with the slow pace of new antibiotic invention or approval has brought back attention to such plants (Sofowora *et al*., 2013).

O. europaea is known to contain a wide range of phytochemical components with medicinal properties from as far back as 1854. A number of phytochemical components have been isolated from different parts of *O. europaea.* The bark, fruits, seeds, leaves, olive oil and wood all contain phenolic compounds, flavonoids and secoridoids. Due to the presence of many compounds in different parts of the plant, *O. europaea* has an array of uses ranging from control of plasma concentrations of glucose, cholesterol and urate. In different countries, the plant has different uses although some uses are common across different cultures (Hashmi *et al*., 2015).

Leaf extracts and essential oils from *O. europaea* have been reported to have antimicrobial activities against many microorganisms including bacteria, viruses, fungi and parasites. While research has shown that the plant does not have broad spectrum antimicrobial activity it has been shown to be potent against some Gram positive and Gram negative bacteria such as *S. aureus* and *E. coli,* respectively (Darsanaki *et al*., 2012). Antimicrobial activity has also been shown against fungi such as *C. albicans* and *C. neoformans*. Other studies have shown

antimicrobial activity of the plant against *H. pylori*, *C. jejuni*, *B. subtilis* and *P. aeruginosa* (Hashmi *et al*., 2015). With the increasing resistance of GBS to many antibiotics and with the recently documented outright resistance to penicillin by Matani *et al*., (2016) in Italy, it is now imperative to seek alternative therapeutic options to GBS treatment.

This study therefore documents the antibiotic susceptibility profiles of GBS isolates as well as the genes coding for resistance to antibiotics in order to provide data for clinical epidemiological purposes and for effective management of patients. It also seeks to determine the antimicrobial susceptibility of GBS isolates to leaf extracts and essential oil from *O. europaea* collected from the Cala community in the Eastern Cape, South Africa.

3.1 MATERIALS AND METHODS

3.1.1 Ethical considerations

Ethical approval for the research was granted by Govan Mbeki Research and Development Centre (GMRDC), University of Fort Hare (UFH). Ethical clearance was granted by the Ministry of Health and Social Services (Namibia) and the Department of Health, Eastern Cape (South Africa). Written informed consent was obtained from each participant prior to sample collection which was carried out by qualified midwives.

3.1.2 Sample collection, culture and identification of GBS

A rectal and vaginal swabs were collected from each participant who were between 35 and 37 weeks gestation by a registered nurse. In South Africa (Eastern Cape), 100 participants were included in the study while in Namibia (Windhoek), 530 participants took part in the study. All swabs collected in South Africa were processed at the AEMREG Laboratory, University of

Fort Hare, Alice while samples in Namibia were processed at the Namibia University of Science and Technology, Microbiology Laboratory.

Samples were inoculated onto Columbia blood agar and Todd Hewitt broth (Rochelle Chemicals and Laboratory Equipment, Johannesburg, South Africa) and incubated at 37 °C for 24 h. Inocula in Todd Hewitt broth were subcultured onto blood agar and incubated at 37°C for 24 h and the cultures were checked for colonies exhibiting β-haemolysis on the blood agar. The colonies were tested for the catalase reaction and catalase negative colonies were further screened using the Lancefield grouping antisera (Becton Dickinson, New Jersey, USA). Colonies which tested positive for the latex agglutination assay were presumptively identified as GBS isolates and were confirmed using the Vitek 2 and by PCR. All presumptive isolates were stored in glycerol stocks at -80 ^{o}C until further analyses.

3.1.3 Molecular confirmation of isolates

Glycerol stocks were resuscitated in Muller Hinton broth for 24 h at 37^{o}C and streaked on Columbia blood agar containing 5% horse blood. *S. agalactiae* isolates were confirmed by molecular techniques using a pair of primers specific for the *scp*B gene. A single colony of GBS was picked from Columbia blood agar containing 5% horse blood and suspended into 160µL of nucleic acid free water in a 2mL Eppendorf conical tube. The suspended colony in a microcentrifuge tube was placed on a heating block and allowed to boil at 100˚C for 15 minutes. The mixture was centrifuged at 10,000 revolutions per minute (rpm) for 5 minutes and the supernatant containing GBS DNA was decanted in a clean 1.5mL microcentrifuge and stored at -80˚C. Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25units/ml One Taq DNA polymerase, was mixed with 6µL each of nucleic acid free water,

1µL of 10 pMol each of reverse and forward primers and 5 µL of DNA in a total reaction volume of 25 µL. Primer sequences are given in Table 3.1 below.

Table 3.1: Primers for molecular confirmation of GBS

Name	**scpBF**	**Length**
Sequence	ACAACGGAAGGCGCTACTGTTC	22 bases
Name	**scpBR**	**Length**
Sequence	ACCTGGTGTTTGACCTGAACTA	22 bases

(Adopted from Elbaradie *et al*., 2009).

The cycling conditions were as follows: an initial denaturation at 94˚C for 4 min followed by 35 cycles of: denaturation at 93˚C for 1 min, annealing at 57˚C for 1 min and extension at 72˚C for 1 min. A final elongation step of 72˚C for 7 min was followed by a hold at 4˚C. Amplicons were loaded on 1 % agarose gel stained with 10µL ethidium bromide and electrophoresed for 45 mins at 110V in 0.5X Tris/Borate/EDTA (TBE) buffer and viewed under transilluminator and photographed.

3.1.4 Antimicrobial sensitivity testing using Vitek

Presumptive GBS isolates were confirmed using the Vitek 2 before antimicrobial sensitivity testing was performed. Antimicrobial sensitivity testing was performed using Vitek Sensitivity Card (AST-ST01) on the following antibiotics: Benzyl penicillin, Ampicillin, Cefotaxime, Ceftriaxone, Levofloxacin, Erythromycin, Clindamycin, Linezolid, Vancomycin, Tetracycline, Cotrimoxazole and for Inducible Clindamycin Resistance (ICR). MICs were determined for each antibiotic on all the isolates and the results were interpreted according to the Clinical Laboratory Standards Institute (CLSI, 2015) guidelines. The results were classified as sensitive (S), intermediate (I) or resistant (R). All isolates were evaluated for inducible clindamycin resistance.

3.1.5 Screening for resistant determinants

All bacterial isolates (154) were screened for various genes coding for resistance to β lactams. The genes were: *bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$, *bla*$_{AmpC}$ and *bla*$_{CMY}$. Primer sequences of *bla* genes are shown in Table 3.2.

Table 3.2: Primer sequences for *bla* genes used in the study

Gene	**Annealing temperature**	**Primers**	**Reference**
bla$_{SHV}$	58◦C	SHVF GCGAAAGCCAGCTGTCGGGC SHVR GATTGGCGGCGCTGTTATCGC	Adesoji & Ogunjobi, 2016
bla$_{TEM}$	58◦C	TEMF AAAGATGCTGAAGATCA TEMR TTTGGTATGGCTTCATTC	Speldooren *et al*., 1998
bla$_{CTX-M}$	56◦C	CTXF GTGCAGTACCAGTAAAGTTATGG CTXR CGCAATATCATTGGTGGTGCC	Adesoji *et al*., 2015
bla$_{CTX-M-1}$	54◦C	CTX-M-1F GCTGTTGTTAGGAAGTGTGC CTX-M-1R CATTGCCCGAGGTGAAG	Kameyama *et al*., 2013
bla$_{CTX-M-9}$	56◦C	CTX-M-9F CAGATAATACGCAGGTG CTX-M-9R CGGCGTGGTGGTGTCTCT	Adams-Sapper *et al*., 2013
bla$_{AmpC}$	56◦C	AmpCF GAAATCCTCAAGCGACTTGC AmpCR GCGCCGAACAAACCGATACTG	Kurpiel & Hanson, 2011
bla$_{CMY}$	56◦C	CMYF CATGCTACGCATTCACTGC CMYR GAACTGTCGCTTGATGATGC	Kurpiel & Hanson, 2011

Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25units/mL One

Taq DNA polymerase, was mixed with 6µL of nucleic acid free water, I uL each of 10 pMol of reverse and forward primers for the respective capsular types, 5uL of DNA template to make a final reaction volume of 25uL.

The cycling conditions were as follows: 94˚C for 4 minutes as an initial denaturation followed by 35 cycles of denaturation at 93˚C for 1 min adapt in all, annealing at the respective annealing temperature as shown in Table 4.2 above for 1 minute and extension at 72˚C for 1 minute with a final elongation step of 72˚C for 7 minutes followed by a hold at 4˚C. Amplification was verified in a 1% agarose gel electrophoresis at 120 volts for 45 minutes in a 0.5X TBE buffer and thereafter viewed in a transilluminator and photographed. ESBL *E. coli* was used a positive control.

3.1.6 Identification of *O. europaea* and preparation of extracts

Olea europaea plant was identified in the Cala community, geographical coordinates 31.52.30ºS, 27.69.80ºE, Eastern Cape Province, South Africa during the winter of 2016. The plant was identified by Cala Traditional Health Practitioners using the vernacular name Umnquma and was authenticated in Selmar Schnland herbarium by a plant taxonomist. The Voucher (No ADE 2016/2) was deposited in the Giffen Herbarium, University of Fort Hare.

Leaves were harvested from the plants and washed in water to remove dust. The leaves were rinsed in distilled water to cleanse them of impurities and were dried in air for 3 days. The leaves were pulverized as previously described by Darsanaki *et al*., 2012. Fifty grams of leaf powder was mixed with one litre of ethanol and placed on a shaker to solve and thereafter, the solution was filtered and the solvent dried to obtain a crude ethanol extract of the *Olea europaea*. For the extraction of essential oils, 200g of dried pulverized leaves was mixed with 200 mL of water and the mixture placed in a modified hydro distiller or a Clevenger's type apparatus and allowed to hydro distillate for three hours for complete extraction of the essential

oils from the leaves. The procedure was repeated thrice to obtain adequate essential oil for experimental assays and the extracted essential oil was dried using anhydrous sodium sulphate and extracts stored at -20ºC in actinic bottles from which working solutions were prepared with Dimethyl Sulfoxide (DMSO) for use in experiments.

3.1.7 GC-MS analysis

The GC-MS analysis of essential oil was performed on the Agilent 5977A MSD and 7890B GC system, Chemetrix; Agilent Technologies, DE (Germany) with a Zibron-5MS column. GC grade helium at 2mL/min flow rate and spitless 1mL injection was used and temperatures were set at 280ºC (Injector), 280ºC (Source) and 70ºC (oven) while the ramp settings were set as follows and held for 3min; 15ºC/min to 120ºC, followed by 10ºC/min to 180ºC and finally 20ºC/min to 270ºC (Igwaran *et al*., 2017).

3.1.8 Preparation of plant extracts for susceptibility testing

Plant extract and essential oils were diluted with 10% DMSO using serial dilution methods to make 300mg/mL of essential oil and 0.5mg/mL of leaf extract. These solutions were used as stock solutions for all antimicrobial testing.

One hundred microliters of 0.5 McFarland standard of each isolate was prepared using sterile normal saline. Twenty microliters of each 0.5 McFarland preparation was inoculated onto Mueller Hinton agar and spread for a lawn growth of the bacteria on the agar. Standard bores of eight millimetres in diameter were made in the agar plates using a sterile cork-borer. Ten percent DMSO was used as a negative control while 5µg of ciprofloxacin served as a positive control. One hundred microliters each of the test extracts, positive control and negative control were put in labelled wells and allowed to stand for 1 h and culture plates incubated at 37 °C for 24 h (CDC, 2010). The test extracts were compared to the negative and positive control to

determine the antimicrobial activity of the extracts on the isolates. Ten isolates each from both South Africa and Namibia were used in the experiment. The isolates were selected using systematic sampling.

3.1.9 MIC determination

Four hundred microliters of broth was put in each of four sterile tubes and serial dilutions were made using 100µL of extract creating five-fold dilutions of each extract. Broth was used as a negative control while 5µg of ciprofloxacin was used as a positive control. One hundred microliters of broth was placed in six labelled tubes for each experiment. One hundred microliters each of broth (negative control), 5µg ciprofloxacin (positive control) and each of the dilutions for both essential oil and plant extract were put in a respective labelled tube and 20 µL of 0.5 McFarland of the test isolate was inoculated in each tube and incubated at 37°C for 24 h (CDC, 2010). Thereafter, the test tubes were checked for turbidity and compared to the negative and positive control to establish the antimicrobial activity of the different concentrations of the extracts. Tubes without apparent growth were regarded as concentrations where the extract had growth inhibitory activities.

The bactericidal or bacteriostatic effect on the bacteria was determined by inoculating 20µL from each of the tubes above onto Mueller Hinton agar and the culture plates were incubated at 37°C for 24 h. Absence of growth in the broth and agar plate was use to confirm that the extract had bactericidal effect while absence of growth in broth and bacterial growth on solid agar plates, indicates bacteriostatic effect of the extract.

3.2 RESULTS

Results of antimicrobial sensitivity testing of antibiotics against GBS, genes coding for resistance and susceptibility to plant extracts are presented below. The sample size included

117 isolates from Namibia and 37 isolates from South Africa. Results for antibiotic susceptibility testing are presented in Table 3.3.

Table 3.3: Antibiotic sensitivity patterns of GBS isolates

Antibiotic	**MIC (µg/mL)**	**Frequency (%)**		
		Susceptible	**Intermediate**	**Resistant**
Benzyl penicillin (P)	≤ 0.12	154 (100.0)	0 (0.0)	0 (0.0)
Ampicillin (Amp)	≤ 0.25	154 (100.0)	0 (0.0)	0 (0.0)
Cefotaxime (CTX)	≤ 0.5	153 (99.4)	0 (0.0)	1 (0.6)
Ceftriaxone (CRO)	≤ 0.5	154 (100.0)	0 (0.0)	0 (0.0)
Levofloxacin (Lev)	≤ 2	154 (100.0)	0 (0.0)	0 (0.0)
Erythromycin (E)	≤ 0.25	138 (89.6)	0 (0.0)	16 (10.4)
Clindamycin (CD)	≤ 0.25	118 (76.6)	0 (0.0)	36 (23.4)
Linezolid (Li)	≤ 2	154 (100.0)	0 (0.0)	0 (0.0)
Vancomycin (Va)	≤ 1	154 (100.0)	0 (0.0)	0 (0.0)
Tetracycline (T)	≤ 2	6 (3.9)	0 (0.0)	148 (96.1)
Cotrimoxazole (SXT)	≤ 2	137 (89.0)	0 (0.0)	17 (11.0)

MICs determined interpreted according to CLSI guidelines, 2015

S. agalactiae did not show resistance to β lactams but there was resistance to clindamycin, erythromycin, tetracycline and Cotrimoxazole.

Table 3.4: Distribution of genes coding for resistance among the isolates

Gene	Resistance phenotype	Frequency (%)
bla_{SHV}	P, AMP, CTX, CRO, SXT	0(0)
bla_{TEM}	P, AMP, CTX, CRO, SXT	0(0)
bla_{CTX-M}	P, AMP, CTX, CRO, SXT	0(0)
$bla_{CTX-M-1}$	P, AMP, CTX, CRO, SXT	0(0)
$bla_{CTX-M-9}$	P, AMP, CTX, CRO, SXT	0(0)
bla_{AmpC}	P, AMP, CTX, CRO, SXT	0(0)
bla_{CMY}	P, AMP, CTX, CRO, SXT	0(0)

There were no *bla* genes amplified among all the 154 isolates from both Namibia and South Africa screened for 7 *bla* genes.

Table 3.5 shows the chemical composition of the essential oils of *O. europaea*.

Table 3.5: Chemical composition of essential oils of *Olea europaea*

S/N	Chemical constituents	Chemical formula	RT	Area %
1	2-Butenal, 2-ethenyl-	C_6H_8O	3.255	1.56
2	2-Hexenal, (E)-	$C_6H_{10}O$	3.292	2.95
3	1-Hexanol	$C_6H_{14}O$	3.365	0.75
4	Heptanal	$C_7H_{14}O$	3.638	1.00
5	(1S)-2,6,6-trimethylbicyclo[3.1.1] hept-2-ene	$C_{10}H_{16}$	3.975	1.89
6	Pyridine, 3-ethenyl-	C_7H_7N	4.204	2.21
7	2,4-Heptadienal, (E,E)-	C_2H_5CH	4.412	1.58
8	Octanal	$C_8H_{16}O$	4.450	0.77
9	alpha-Phellandrene	$C_{10}H_{16}$	4.530	1.72

10	4-Carene	$C_{10}H_{10}$	4.729	0.79
11	2-Octenal, (E)-	$C_8H_{14}O$	4.908	0.50
12	1-Octanol	$C_8H_{18}O$	4.972	1.87
13	Pyridine, 5-ethenyl-2-methyl-	C_8H_9N	5.198	0.88
14	Nonanal	$C_9H_{18}O$	5.271	10.57
15	1,6-heptadiene,2,5,5-trimethyl	$C_{10}H_{18}$	5.674	0.73
16	Acetaldehyde, (3,3dimethylcyclohexylidene),(E)	$C_{10}H_{18}O$	5.720	1.07
17	1-Methylpentyl cyclopropane	C_9H_{18}	5.772	0.80
18	Decanal	$C_{10}H_{20}O$	6.059	2.23
19	3-Isopropylidene-5-methyl-hex-4-en-2-one	$C_{10}H_{16}O$	6.278	0.99
20	Octane, 1-iodo-	$C_8H_{17}I$	6.347	0.57
21	2,6-Octadien-1-ol, 3,7-dimethyl-(Z)-	$C_{10}H_{18}O$	6.418	0.39
22	2-Decenal, (E)-	$C_{10}H_{18}O$	6.485	2.78
23	2,4-Decadienal	$C_{10}H_{16}O$	6.724	0.76
24	4-tert-Butylcatechol, dimethyl ether	$C_{12}H_{18}O_2$	6.809	4.63
25	9-Oxabicyclo [4.2.1] non-7-en-3-one	$C_8H_{12}O$	6.892	1.80
26	1-Oxaspiro [4.5] dec-6-ene, 2,6,10,10-tetramethyl-	$C_{13}H_{22}O$	6.995	1.10
27	2-Undecenal	$C_{11}H_{20}O$	7.206	0.78
28	cis-beta-Farnesene	$C_{15}H_{24}$	7.422	0.60
29	1-(3,6,6-trimethyl-1,6,7,7a-tetrahydrocyclopenta [c] pyran-1-yl) ethanone	$C_{13}H_{18}$	7.510	0.72
30	2-Buten-1-one,1-(2,6,6-trimethyl-1-cyclohexen-1-yl)-	$C_{13}H_{20}O$	7.631	0.50
31	Caryophyllene	$C_{15}H_{24}$	7.751	2.04
32	5,9-Undecadien-2-one, 6,10-dimethyl-	$C_{13}H_{22}O$	7.798	2.91
33	1H-Inden-1-one, 2,4,5,6,7,7a-hexahydro-4,4,7a-trimethyl-	$C_{12}H_{18}O$	7.931	0.46

34	1,4,7,-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	$C_{15}H_{24}$	7.974	0.44
35	trans-beta-Ionone	$C_{13}H_{20}O$	8.093	2.39
36	21.xi-methyl-17-isocholest-16-en-3beta-ol	$C_{27}H_{46}O$	8.150	0.47
37	Naphthalene, 1,2,3,4,4a,5,6,8a-octahydro-4a,8-dimethyl-2- (1-methylethenyl)-,[2R-(2 alpha, 4a alpha, 8a beta)]	$C_{15}H_{24}$	8.232	0.69
38	Naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-	$C_{15}H_{34}$	8.323	2.91
39	1,6,10-Dodecatrien-3-ol,3,711,trimethyl-, [S-(Z)]-	$C_{15}H_{26}O$	8.508	1.82
40	Benzoic acid, nonadecyl ester	$C_{26}H_{44}O_2$	8.631	0.79
41	1H_Cycloprop[e] azulen-7-ol, decahydro-1,1,7-trimethyl-4-methylene-,[1ar-(1aalpha, 4a alpha, 7 beta, 7a beta, 7b alpha)]-	$C_{15}H_{24}O$	8.750	0.80
42	Caryophyllene	$C_{15}H_{24O}$	8.809	1.49
43	4,2,8-Ethanylylidene-2H-1-benzopyran, octahydro-4-methyl-	$C_{14}H_{22}O$	8.923	0.55
44	1,4-Methano-1H-indene, octahydro-1,7a-dimethyl-4-(1-methylethenyl)-[1S-(1 alpha, 3a beta, 4 alpha, 7a beta)]-	$C_{15}H_{24}$	8.961	0.52
45	Bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene	$C_{15}H_{24}$	9.089	5.65
46	Heptadecane	$C_{17}H_{36}$	9.250	0.39
47	7-epi-cis-sesquisabinene hydrate	$C_{15}H_{26}O$	9.281	0.94
48	2,6,10-Dodecatrien-1-ol, 3,7,11,trimethyl	$C_{15}H_{26}O$	9.424	0.46
49	2-Pentadecanone, 6,10,14-trimethyl	$C_{18}H_{36}O$	10.058	1.66
50	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	$C_{16}H_{22}O_4$	10.235	0.72
51	Eicosane	$C_{20}H_{42}$	10.312	0.47
52	2,6,10,14,18-Pentamethyl-2,6,10,14,18-eicosapentaene	$C_{25}H_{42}$	10.467	0.97
53	Dibutyl phthalate	$C_{16}H_{22}O_4$	10.713	0.43

54	Eicosane	$C_{20}H_{42}$	10.810	0.42
55	Isopropyl palmitate	$C_{19}H_{38}O_2$	10.930	0.29
56	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl-	$C_{14}H_{44}$	11.235	0.09
57	Octadecane	$C_{18}H_{38}$	11.286	1.03
58	2-Myristynoyl-glycinamide	$C_{16}H_{31}BrN_2O_2$	11.336	0.77
59	Phytol	$C_{20}H_{40}O$	11.388	6.40
60	1H-Indole-2-carboxylic acid , 6-(4-ethoxyphenyl)-3-methyl-4-oxo-4,5,6,7-tetrahydro-, isopropyl ester	$C_{21}H_{25}$	11.741	0.55
61	Nonadecane	$C_{19}H_{40}$	12.174	1.09
62	Cyclotrisiloxane, hexamethyl	$C_6H_{18}O_3Si_3$	12.593	0.34
63	Heptasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13-tetradecamethyl	$C_{14}H_{44}O_6Si_7$	12.994	0.98
64	Octasiloxane, 1,1,3,3,5,5,7,7,9,9,11,11,13,13,15,15-hexadecamethyl	$C_{16}H_{48}O_7Si_8$	13.779	1.81
65	Tetrasiloxane, decamethyl-	$C_{10}H_{30}O_3Si_4$	14.201	0.37
66	Silicic acid, diethyl bis (trimethylsilyl) ester	$C_{10}H_{28}O_4Si_3$	14.201	0.20
67	Heneicosane	$C_{21}H_{44}$	14.659	3.37
68	Tris (tert-butyldimethylsilyloxy) arsane	$C_{18}H_{45}AsO_3Si_3$	15.741	1.94

Key: RT = Retention Time Area% = Percent area which equates to concentration

The GC-MS analysis of the plant extract showed that the plant contained flavonoids, flavonones, biophenols, benzoic acid derivatives, sterols and secoiridoids.

Table 3.6: Screening for antimicrobial activities of plant extracts against GBS isolates

Extract (Concentration)	**Zone of inhibition on MH (Average diameter)**	
Essential oils (300 mg/ml)	Present	25mm
Leaf extract (0.5 mg/ml)	Present	28mm
5µg Ciprofloxacin (Positive control)	Present	35mm
10% DMSO (Negative control)	Not present	0mm

Essential oils and leaf extracts exhibited antimicrobial activity against GBS isolates and leaf extracts showed a bigger zone of inhibition compared to essential oils.

Table 3.7: MIC for essential oils against GBS isolates

	Concentration (mg/mL)				
	0.05	0.4	3.7	33.3	MIC
Isolates	A B C	A B C	A B C	A B C	Essential oils
GBS in broth	+ + -	+ + -	+ + -	- + -	33.3
GBS on MH	+ + -	+ + -	+ + -	- + -	33.3

Key: A = Essential oil, B = 10% DMSO, C = Ciprofloxacin, + = Growth, - = no growth

Essential oils showed antibacterial activity to GBS at an MIC of 33.3 mg/mL and had bactericidal effect on the isolates at that concentration.

Table 3.8: MIC for leaf extracts against GBS isolates

	Concentration (mg/mL)				
	0.0008	0.004	0.02	0.1	MIC
Isolates	A B C	A B C	A B C	A B C	Leaf extracts
GBS in broth	+ + -	+ + -	- + -	- + -	0.02
GBS on MH	+ + -	+ + -	- + -	- + -	0.02

Key: A = Leaf extracts, B = 10% DMSO, C = Ciprofloxacin, + = Growth, - = no growth

Leaf extracts showed antibacterial activity to GBS at an MIC of 0.02 mg/mL and had bactericidal effect on the isolates at that concentration.

3.3 DISCUSSION

In this study 117 isolates from Namibia and 37 isolates from South Africa were screened for their susceptibilities to antibiotics. All the isolates showed absolute sensitivity to benzyl penicillin, ampicillin, ceftriaxone, levofloxacin, linezolid and vancomycin, with only one isolate (0.6%) showing resistance to cefotaxime as shown in Table 3.3. In reports from other studies done in other African countries, such as in Malawi (Gray *et al*., 2007), Ethiopia (Gebremeskel *et al*., 2015), Zimbabwe (Moyo *et al*., 2001), South Africa (Bolukaoto *et al*., 2015) and Nigeria (Ezeonu and Agbo, 2014), GBS isolates did not show any resistance to the *β lactams*. In a study in Brazil, GBS isolates were not resistant to *β lactams* (Dutra *et al*., 2011). However, resistance to *β lactam* group of antibiotics has been evolving as noted in reduced MICs for more than a decade and recently an Italian study recorded outright resistance by some GBS isolates to penicillin (Matani *et al*., 2016).

Thirty six isolates (23.4%) and sixteen isolates (10.4%) were resistant to clindamycin and erythromycin respectively and the results of this study are comparable to a report from recent study in Pretoria, South Africa where GBS isolates had 17.2% and 21.16% resistance to clindamycin and erythromycin, respectively (Bolukaoto *et al*., 2015). The results also fall within world statistics of clindamycin resistance (8.2-70%) and erythromycin resistance (14.5-70%) (Biasi-Garbin *et al*., 2015). However, a study in Brazil recorded low GBS resistance to clindamycin (3.0%) and erythromycin (4.1%) (Dutra *et al*., 2011) and 8.1% and 2.2% for erythromycin and clindamycin, respectively. Resistance to erythromycin can be a result of the efflux pump or methylation of 23S rRNA blocking the 50S ribosomal subunit with clindamycin being another 50S ribosomal inhibitor. Approximately 8 to 10% of pregnant women are allergic to penicillin and with the emerging resistance of GBS to penicillin, high resistance to erythromycin and clindamycin are a major cause for concern (CDC, 2010). These high levels of GBS resistance to recommended antibiotics may pose problems to empirical treatment without AST especially in resource limited settings where health care and laboratory facilities are limited (Capanna *et al*., 2015).

There was high resistance of GBS to tetracycline (96.1%) in this current study and this appears consistent with other studies which have reported high GBS resistance to tetracycline (86.7%) in South Africa (Bolukaoto *et al*., 2015) and 97% in Brazil (Dutra *et al*., 2011) and this could be due to the indiscriminate use of tetracycline and its availability as a non-prescription drug. Tetracycline is commonly used at farms as a growth promoter and for treatment purposes. Resistance to the drug could spread through the environment due to agricultural runoff and contaminated effluent while antibiotic residue in farm produce like meat is another serious concern which perpetuates drug resistance as low levels of drugs in farm produce exert persistent selection pressure on bacteria. This gives rise to the emergence of resistant strains (Al-Bahry *et al*., 2013).

Resistance to cotrimoxazole was low in this study (11.0%) contrary to its reported high resistance level among GBS isolates from other studies in other African countries such as Ethiopia (29.0%) (Mengistu *et al*., 2016). In Tanzania, just like many other sub Saharan African countries burdened by HIV infection, cotrimoxazole is used for prophylactic purposes in all AIDS patients including pregnant women before 36 weeks gestation. Resistance to cotrimoxazole in Tanzania ranged from 56.0% in *Escherichia coli* to 87.5% in *Pseudomonas aeruginosa* with no difference in prevalence among patients on cotrimoxazole prophylaxis. The high resistance to cotrimoxazole could be attributed to the excessive use of the drug for prophylactic purposes in HIV positive patients. In a South African study, 85.7% of *E. coli* isolates from HIV infected children were resistant to cotrimoxazole (Madhi *et al*., 2000) while another South African study showed a significant increase in resistance to cotrimoxazole by organisms from 1999 to 2002 (Crewe-Brown *et al*., 2004). The World Health Organization noted that although cotrimoxazole was a broad spectrum antibiotic, increased use of the drug for prophylactic purposes would spur development of drug resistance which could ultimately render the drug ineffective when applied to common community pathogens (Coutsoudis *et al*., 2010).

There was no comparative data for Namibia on bacterial resistance to cotrimoxazole from past studies. However, the drug is still used in Namibia for prophylactic purposes as recommended by WHO and in treatment of other bacterial infections. This would point to potential high antimicrobial resistance to cotrimoxazole, which is not the case in this study. The low resistance of GBS reported might be an isolated case until a full picture of drug resistance is established. On the other hand it might be an indicator of good prescription practices by clinicians and good compliance to antibiotic use by patients.

The findings of this study showed very low resistance to *β-lactam* antibiotics by GBS as only one isolate (0.6%) showed resistance to cefotaxime. There was no phenotypic resistance to other *β lactams* (benzyl penicillin, ampicillin and ceftriaxone) as shown in Table 3.4. All the organisms were however screened for *bla* genes irrespective of the phenotypic resistance and there were no *bla* genes amplified among the isolates. Even the one isolate which had phenotypic resistance to a *β lactam* antibiotic did not exhibit any of the *bla* genes screened as showed in Table 3.4. While *bla* genes are generally screened in Gram negative bacteria some Gram positive bacteria have been shown to express β lactamases and with the increasing resistance of GBS to β lactams it is prudent to screen for these genes which may be acquired from the environment through horizontal gene transfer via plasmids or transposons (Munita and Arias, 2016).

Resistance to erythromycin by bacterial strains has been attributed to the *ermB*, *ermA* and *mefA* genes and the *mefA* gene is known as an efflux gene (Wang *et al*., 2015). Investigators have reported that resistance to clindamycin alone is due to the *lnu(B)* gene. In a study by Srinivasan *et al*., 2011, *S. agalactiae* isolates resistant to macrolides had the following genes amplified: *ermB* (70%) and *lnuB* (94%) and none of the isolates expressed *ermA* (0%) or *mefA* (0%). The study also reported that mutations in resistant coding genes resulted in bacterial susceptibility to antibiotics targeted by the gene. The *mreA* gene has been reported as a resistant determinant to macrolides and clindamycin through drug efflux but the gene has been amplified in erythromycin susceptible strains (Clarebout *et al*., 2001).

In tetracycline non-susceptible bacterial strains, *tet* genes have been reported as resistant determinants and these include *tet(M), tet(O)* and *tet(L)* (Da Cunha *et al*., 2014). In a study by Fischer *et al*., 2013 on *S. agalactiae*, all tetracycline non-susceptible strains had the *tet(M)* gene amplified while they were negative for the *tet(O)* gene. These results were identical to those

reported in a study by Compain *et al*., 2014. The study also reported the presence of *tet(L)* and *tet(K)* as resistant determinants.

This current study found that the essential oils were made up of 68 compounds and the main components were: nonanal (10.57%), phytol (6.40%), bicyclo[4.4.0]dec-1-ene, 2-isopropyl-5-methyl-9-methylene (5.65%), 4-tert-Butylcatechol, dimethyl ether (4.63%), 2-hexanol (2.95%), 5,9-Undecadien-2-one, 6,10-dimethyl- (2.91%) and naphthalene, 1,2,4a,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)- (2.91%) as shown in Table 3.5. The findings of this study are different from those of a similar study done by Boukhebti *et al*., (2015) in Algeria in which the essential oils were composed of 38 components. In the study palmitic acid (14.71%), z-nerolidol (9.45%), octacosane (6.32%), ceryophyllene oxide (4.77%), tetracosane (4.06%) and 4-hydroxy-4-methy-2-pentane (4.04%) were the main components of the essential oils (Boukhebti *et al*., 2015). Phytochemical composition of medicinal plants changes with change in geographical location.

A study by Upadhyay (2014) in India reported *O. europaea* plants to contain 24 compounds with the main components being 2-propanone (8.80%), 2,4imidazolidinedione (14.92%) and z-(13,14,-Epoxy) tetra dec-11-en-1-ol acetate (7.77%). Different studies in Tunisia reported differences in phytochemical composition of Olea extracts. Plant extracts have variations in total phenols, colour, composition of polysaturated fatty acids and water content (Chebab *et al*., 2013; Brahmi *et al*., 2015; Dabbou *et al*., 2011). The variation in chemical composition is attributed to differences in climatic conditions under which the plant grew or cultivation conditions such as irrigation and the ripening stage of the plant (Gomez-Rico *et al*., 2006; Chebab *et al*., 2013). Water content affects activity of enzymes responsible for synthesis of phytochemicals like phenols and increased water supply to plants increases the content of phenolic components, alcohols and esters such as (E)-2-hexanol (Chebab *et al*., 2013).

Extracts from *O. europaea* had antimicrobial activity against GBS isolates from both South Africa and Namibia as shown in Table 3.6. Studies have shown that *O. europaea* has antimicrobial activity against other bacteria although the extracts do not have broad spectrum antimicrobial activity (Hashmi *et al*., 2015). While *Olea* extracts were reported to have antimicrobial properties in several countries including Spain, Israel, Palestine, plants in different geographical locations exhibit varying phytochemical constitutions therefore resulting in different properties and ethno botanical uses of the same plant (Hashmi *et al*., 2015; Wadood *et al*., 2013).

Olea essential oils showed bactericidal activity against GBS at 33.3 mg/mL as shown in Table 3.7 and other investigators showed that essential oils had antimicrobial activity against a wide range of bacteria (Darsanaki *et al*., 2012; Hashmi *et al*., 2015).

Olea plant extracts have been reported to have antimicrobial effects on both Gram positive and Gram negative organisms such as *C. jejuni, S. aureus, P. aeruginosa, K. pneumoniae, C. albicans, C. neoformans, L. monocytogenes, S. enteretidis, E. coli, S. pneumoniae* (Upadhyay, 2014; Hashmi *et al*., 2015; Lee and Lee, 2010; Liu *et al*., 2017; Rempe *et al*., 2017). The antimicrobial effects of these plants are due to the action of phenolic compounds on the bacterial cells. The effects can be membrane based such as disruption of membrane structure causing lysis or non-membrane based effects such as inhibition of intracellular enzymes or other metabolic processes in the bacterial cells (Rempe *et al*., 2017). Plant extracts contain numerous compounds which work antagonistically or synergistically but have a defined combined effect on physiologic functions. The essential oil used in this study had 68 components and they had a combined inhibitory effect on bacteria.

Extracts from the leaves exhibited bactericidal activity at very low concentration (0.02 mg/mL) compared to essential oil as presented in Table 3.8. The antimicrobial activity could be

attributed to phytochemical components present in the *Olea* plant extract such as flavonoids, iridoids, secoiridoids, biophenols and benzoic acid derivatives (Biasi-Garbin *et al*., 2015; Marasini *et al*., 2015; Hashmi *et al*., 2015). While different parts of a plant contain the same phytochemical components, the composition of the components varies with respect to the parts of the plant. The difference in phytochemical composition in parts of the same plant can result in different physiologic functions or inhibitory capabilities of the extracts which could explain why the inhibitory MICs of essential oils were different from the MIC of ethanolic leaf extracts.

3.4 CONCLUSION

S. agalactiae isolates from both South Africa and Namibia did not show marked resistance to commonly used antibiotics. All isolates were susceptible to penicillin and there was no genotypic resistance shown based on the *bla* genes screened. Olea leaf extracts and essential oil had inhibitory effects against *S. agalactiae* isolated from pregnant women in both South Africa and Namibia while Olea ethanol leaf extracts were more potent against bacterial isolates than essential oils. Olea leaf extracts and essential oil potent as alternative antimicrobials against GBS.

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CHAPTER FOUR

PREVALENCE AND CAPSULAR TYPE DISTRIBUTION OF *S. AGALACTIAE* ISOLATED FROM PREGNANT WOMEN ATTENDING SPECIFIC CLINICS IN NAMIBIA AND SOUTH AFRICA

Abstract

Background: *Streptococcus agalactiae* or Group B Streptococcus (GBS) is one of the leading cause of neonatal morbidity and mortality resulting in septicaemia, bacteraemia and meningitis. Long term problems of GBS disease in children range from loss of hearing to mental retardation. While Intrapartum Antibiotic Prophylaxis (IAP) has reduced the incidence of *S. agalactiae* infection, it still remains the leading cause of disease in neonates. *S. agalactiae* has ten capsular types whose distribution varies across the world. **Objective:** This study sought to determine the prevalence of GBS in Namibia and South Africa amongst pregnant women between 35 and 37 weeks gestation and elucidate the capsular types. **Methodology:** Lower vaginal and rectal swabs were collected from pregnant women between 35 and 37 weeks gestation. Five hundred and thirty pregnant women were recruited into the study in Windhoek, Namibia while one hundred pregnant women were recruited in the Eastern Cape, South Africa. The swabs were cultured on 5% sheep blood agar for isolation of GBS. Presumptive isolates were confirmed using both the Vitek (2) and molecular techniques targeting the *scp*B gene. Capsular typing was performed in a multiplex PCR with capsular specific primer pairs. **Results:** The prevalence of GBS in Namibia was 13.6% and 37% in South Africa respectively. In both countries most women were colonised both rectally and vaginally by GBS. Capsular types II, III and V were the most prevalent in both countries. **Conclusion:** The predominant capsular types in this study are the ones commonly associated with adverse maternal outcomes.

Key words: Prevalence, capsular type, *Streptococcus agalactiae*, pregnant, women

4.0 INTRODUCTION

S. agalactiae primarily colonizes both the gastrointestinal and genital tracts but can also be found in the oropharynx. In the 1970s, GBS emerged as the leading cause of neonatal sepsis and meningitis with case fatality rates of up to 50% and since then, it has remained the leading cause of neonatal sepsis and meningitis in the United States (Lo *et al*., 2017). The pathogenesis of these infections is based on GBS colonisation of the mother vaginally or rectally followed by transmission of the organism during labour or delivery (Whidbey *et al*., 2013). Colonization rates vary between countries but as many as 20-40% of healthy women are asymptomatically colonized and are at risk of transmitting it to their new-born babies (Chen *et al*., 2013). It can cause invasive disease in neonates, pregnant women and immune-compromised patients. In pregnant women the disease presents as infection of the genital tract, placenta, amniotic sac or simply bacteraemia. While infection does not cause death in pregnant women, in 10-60% of the cases it results in either miscarriage or stillbirth in developing countries when compared to with 7-11% in developed countries (Dangor *et al*., 2015; Dagnew *et al*., 2012; Edmond *et al*., 2012).

Prevalence rates for GBS vary in the world according to geographic regions with a colonisation rate of 19% reported for pregnant women in sub Saharan Africa (Kobayashi *et al*., 2016). Several factors influence the reported prevalence rates in studies and these include timing of sample collection, sampling technique used, specimen transport, media and culture technique used and identification methods (Wollheim *et al*., 2017). In sub Saharan Africa GBS prevalence vary as reported in South Africa (30.9% and 23%) in 2015 and 2016 (Chukwu *et al*., Cools *et al*., 2016), Zimbabwe (21%) (Mavenyengwa *et al*., 2010), Tanzania (23%) in 2009 (Joachim *et al*., 2009), Uganda (28.8%) and Kenya (20.2%) in 2016 (Namugongo *et al*., 2016; Cools *et al*., 2016).

Vertical transmission of GBS from colonized mothers to their new-borns can result in early onset GBS infection which occurs in the first 7 days of life and is a leading cause of invasive bacterial infection in neonates. Mortality in early onset disease is estimated at 5% and is characterized by bacteraemia, pneumonia and meningitis (Nathoo *et al*., 1991; Dauby *et al*., 2016). Mortality is higher in preterm infants with case fatality rates of up to 20% and as much as 30% in those born before 33 weeks of gestation (Chen *et al*., 2013). Late onset disease refers to infections from one week to 90 days of age and this is attributed primarily to transmission after birth from the mother or other external sources to the neonate. Late onset disease presents primarily with meningitis and associated sequelae such as deafness and developmental disabilities (Shabayek *et al*., 2014).

Surface associated polysaccharide capsules are common in both Gram positive and Gram negative bacteria. Microorganisms develop capsules for protection against environmental factors and survival against the host's defence mechanisms. In human beings, it allows the microorganisms to escape phagocytosis, complement mediated killing and acquired immune responses by masking bacterial antigenic determinants, mimicking host antigens and interfering with complement mediated killing. However, the capsule can also act as a target for specific antibodies, conferring the host with immunity to GBS infection (Le Doare and Kampman, 2014).

Capsular serotyping has been one of the mainstays in the descriptive epidemiology of GBS as currently ten capsular serotypes 1a, 1b, 11-IX, have been described based on the antigenicity of their capsular polysaccharides. These capsules represent one of the major virulence factors of GBS and of these, serotype III has been noted to be responsible for the majority of GBS infections including meningitis in neonates (Karunakaran *et al*., 2009). Other studies have reported GBS serotypes Ia, Ib, II, III and V as being responsible for most early onset disease

(Joubriel *et al*., 2015; Lin *et al*., 1998). Sialic acid residues incorporated in the capsular polysaccharide primary structure are associated with development of disease.

Capsular polysaccharides are composed of repeating units of four to seven monosaccharides with a backbone and side chains (Berti *et al*., 2014). Of the ten serotypes, eight are closely related genetically and structurally with serotype VIII being distantly related. This suggests that despite the evolutionary pressures toward antigenic variation exerted by hosts' acquired immune responses, GBS capsular polysaccharides still remain highly conserved.

Capsular polysaccharide distribution varies even within geographical areas. In South Africa in 2011, serotype III was found to be common among mothers (37.3%) and new-borns (36.2%). Serotypes Ia, Ib and III were more prevalent among pregnant women (74.1%) and new-borns (69.6%) with serotypes III and Ia causing 53.9% of invasive disease in infants aged 7 to 90 days as compared to Ib, II, IV and V (<6%) (Madzivhandila *et al*., 2011). A study carried out on pregnant women in Zimbabwe in 2000 reported serotypes III and V as the most common (Moyo *et al*., 2000) and in a recent study on pregnant women in Zimbabwe, Mavenyengwa *et al* detected serotypes Ia, Ib, II, III and V (Mavenyengwa *et al*., 2010).

Studies in Malawi (Gray *et al*., 2007), Finland (Kalliola *et al*., 1999), Sweden (Gudjonsdottir *et al*., 2015), England (Lamagni *et al*., 2013), United States (Teatero *et al*., 2016), Zimbabwe (Mavenyengwa *et al*., 2010), Gambia (Le Doare *et al*., 2016), Egypt (Shabayek *et al*., 2009) and in Brazil (Barbosa *et al*., 2016) have shown variations in serotype distribution around the world. This study therefore sought to determine the prevalence of GBS and capsular type distribution of isolates colonising pregnant women in Namibia and South Africa.

4.1 MATERIALS AND METHODS

4.1.1 Ethical considerations

Ethical approval for the research was granted by Govan Mbeki Research and Development Centre (GMRDC), University of Fort Hare (UFH). Ethical clearance was granted by the Ministry of Health and Social Services (Namibia) and the Department Health, Eastern Cape (South Africa). Informed consent was obtained from each participant prior to sample collection which was carried out by qualified midwives.

4.1.2 Description of study area

The study was carried out in Windhoek, Namibia and the Eastern Cape Province of South Africa. The geographical coordinates of Windhoek are: 22.5609ºS, 17.0658ºE while those of the Eastern Cape Province are: 32.2968ºS, 26.4194ºE. Figure 4.1 below shows the geographical map of Windhoek while Figure 4.2 represents the geographical map of the Eastern Cape Province.

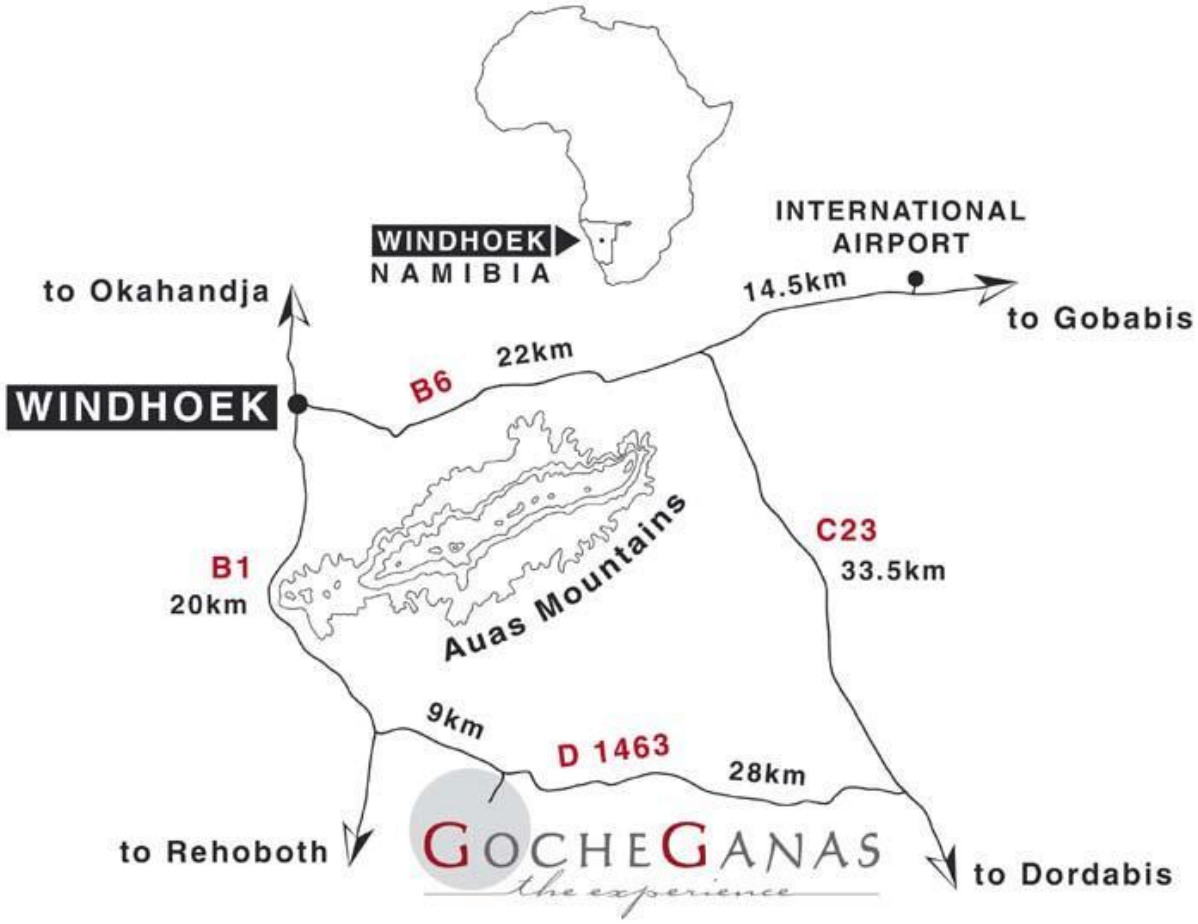

Figure 4.1: Geographical map of Windhoek, Namibia

Reference: http://www.map-of-namibia.com/namibiatourmaps/tourmap-07.html

Figure 4.2: Geographical map of the Eastern Cape, South Africa

Reference: http://www.citiestips.com/city/EasternCapeSouthAfrica

4.1.3 Sample collection, culture and identification of GBS

Pregnant women between 36 and 37 weeks gestation were recruited into the study on a voluntary basis. Participants targeted were those attending Windhoek Central Hospital maternity clinic, Windhoek (Namibia) while in the Eastern Cape, samples were collected from Mduntsane, Dimbazi and Middle Drift clinics. The sample collection procedure was explained to the participants and a low vaginal swab (LVS) and rectal swab (RS) were collected from each patient. Samples were placed in Amies transport medium (Rochelle chemicals, Johannesburg, South Africa), bar-coded for identification, placed in a cooler box containing ice packs and transported to base laboratories. In Namibia, samples were transported to the Microbiology laboratory, Faculty of Health and Applied Sciences, Namibia University of

Science and Technology (NUST) for culture and presumptive identification of GBS while samples collected in South Africa were processed at the Applied and Environmental Microbiology Research Group (AEMREG) laboratory, Department of Microbiology and Biochemistry, University of Fort Hare (UFH). Efforts were made to culture samples were within 2 h of collection and molecular confirmation and characterization was done at AEMREG laboratory.

Samples were inoculated onto Columbia blood agar containing 5% horse blood and incubated at 37ºC for 18 h under 5% carbon dioxide atmosphere. Presumptive isolates were identified as GBS based on: β-haemolysis on Columbia blood agar containing 5% horse blood, Gram positive cocci with Gram staining technique, negative catalase reaction, Lancefield grouping with type B antisera (Becton Dickinson, New Jersey, USA) and using the Vitek (Biomerieux) version 2. All presumptive isolates were stored in 30% glycerol stocks at -80ºC until further analysed.

4.1.4 Molecular confirmation of GBS strains

Presumptive isolates in glycerol stocks were resuscitated in Todd Hewitt broth for 24 h at 37 ºC and streaked onto Columbia blood agar containing 5% horse blood. *S. agalactiae* isolates were confirmed by molecular techniques (PCR) using a pair of primers specific for the *scp*B gene. A single colony of GBS was picked from Columbia blood agar containing 5% horse blood and emulsified with 2mL of nucleic acid free water in a 2mL microcentrifuge tube. The tube was boiled at 100˚C for 15 minutes on a Heating Block and thereafter centrifuged at 10,000 revolutions per minute (rpm) for 5 minutes and supernatant containing GBS DNA was separated from the pellet and stored at -80˚C. Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630,

0.05% Tween^{R} 20 and 25 units/mL One Taq DNA polymerase, 6uL of water of PCR grade, 1uL of 10 pMol of both forward and reverse primers were mixed with 5µL of DNA template to make up a total reaction volume of 25uL. *Scp*B primer sequences are presented in Table 4.1.

Table 4.1: Oligonucleotide primers for molecular confirmation of GBS

Name	***scp*BF**
Sequence	ACAACGGAAGGCGCTACTGTTC
Name	***scp*BR**
Sequence	ACCTGGTGTTTGACCTGAACTA

(Adopted from Elbaradie *et al*., 2009).

The cycling conditions were as follows: an initial denaturation of 94˚C for 4 minutes followed by 35 cycles of denaturation at 93˚C for 1 min, annealing at 57˚C for 1 min and extension at 72˚C for 1 min and a final elongation step of 72˚C for 7 min followed by a hold at 4˚C as described by Desjardins *et al*, (2004). The following ATCC reference strains (and respective capsular type) were used as positive controls during capsular typing: BAA-1138 (Ia), BAA-1174 (Ib), BAA-2675 (II), BAA-2674 (III), BAA-2673 (IV), BAA-2672 (V), BAA-2671 (VI), BAA-2670 (VII), BAA-2669 (VIII) and BAA-2668 (IX).

Two microliters of amplicons were loaded on 1 % agarose gel stained with 10µL ethidium bromide and electrophoresed for 45 minutes at 110˚C in a 0.5X Tris/Borate/EDTA (TBE) buffer. Amplification was verified in a gel documentation system and photographed.

4.1.5 Determination of capsular types of the isolates using multiplex PCR

Table 4.2: Oligonucleotide primers for capsular typing

Primer name	Sequence	Band size (bp)
Ia-F	GGTCAGACTGGATTAATGGTATGC	521
Ia-R	GTAGAAATAGCCTATATACGTTGAATGC	
Ib-F	TAAACGAGAATGGAATATCACAAACC	770
Ib-R	GAATTAACTTCAATCCCTAAACAATATCG	
II-F	GCTTCAGTAAGTATTGTAAGACGATAG	397
II-R	TTCTCTAGGAAATCAAATAATTCTATAGGG	
III-F	TCCGTACTACAACAGACTCATCC	1 826
III-R	AGTAACCGTCCATACATTCTATAAGC	
IV-F	GGTGGTAATCCTAAGAGTGAACTGT	578
IV-R	CCTCCCCAATTTCGTCCATAATGGT	
V-F	GAGGCCAATCAGTTGCACGTAA	701
V-R	AACCTTCTCCTTCACACTAATCCT	
VI-F	GGACTTGAGATGGCAGAAGGTGAA	487
VI-R	CTGTCGGACTATCCTGATGAATCTC	
VII-F	CCTGGAGAGAACAATGTCCAGAT	371
VII-R	GCTGGTCGTGATTTCTACACA	
VIII-F	AGGTCAACCACTATATAGCGA	282
VIII-R	TCTTCAAATTCCGCTGACTT	

(Adopted from Poyart *et al*., 2007).

Capsular types were grouped according to annealing temperature as follows: Types 1a, 1b, II &III at 58˚C; IV, V, VI & VII at 59˚C and VIII at 56˚C. Twelve microliters of One Taq^{R} Master

Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^R CA-630, 0.05% Tween^R 20 and 25units/mL One Taq DNA polymerase, was mixed with 6µL of nuclease free water, I uL each of 10 pMol of reverse and forward primers for the respective capsular types, 5uL of DNA template to make a final reaction volume of 25uL..

The cycling conditions were as follows: 94˚C for 4 minutes as an initial denaturation followed by 35 cycles of denaturation at 93˚C for 1 min, annealing at the respective annealing temperature for 1 minute and extension at 72˚C for 1 minute with a final elongation step of 72˚C for 7 minutes followed by a hold at 4˚C. Amplification was verified in a 1% agarose gel stained with ethidium bromide and electrophoresed at 120 volts for 45 minutes in a 0.5X TBE buffer and thereafter viewed in a transilluminator and photographed.

4.2 RESULTS

Out of the 530 pregnant women recruited in Namibia, 72(13.6%) colonised with GBS vaginally, rectally or both while in South Africa, out of 100 women screened, 37 (37%) were colonized with GBS. All the isolates were confirmed as GBS by molecular techniques. The frequency of distribution of GBS in Namibia and South Africa is shown in Table 4.3 while that of the capsular type in both countries are shown in Tables 4.4 respectively.

Table 4.3: Frequency of GBS isolates from Namibia and South Africa

Colonization site	**Frequency (%)**	
	Namibia	**South Africa**
Vagina only	18 (25.0)	5 (13.5)
Rectum only	11 (15.3)	2 (5.4)
Dual colonization	43 (59.7)	30 (81.1)
Total	72 (100)	37 (100)

Most of the pregnant women in Namibia and South Africa were both rectally and vaginally colonized with GBS while few were rectally colonized. *S. agalactiae* was recovered from 72 pregnant women in Namibia and 37 pregnant women in South Africa.

Table 4.4: Frequency of capsular types among GBS isolates from Namibia and South Africa

Capsular type	**Frequency (%)**	
	Namibia	**South Africa**
II	69 (60.0)	35 (52.2)
III	29 (25.2)	12 (17.9)
V	12 (10.4)	11 (16.4)
Ia	3 (2.6)	6 (9.0)
IV	2 (1.7)	1 (1.5)
1b	0 (0)	2 (3.0)
Total	115 (100)	67 (100)

Capsular types II, III and V were predominant in Namibia and South Africa while capsular type 1b was reported only among South African isolates and not Namibia isolates.

4.3 DISCUSSION

The prevalence of GBS colonization varies around the world according to different geographical locations as shown in studies on the prevalence of GBS conducted on both non-pregnant and pregnant women (Alp *et al*., 2016; Namugongo *et al*., 2016). The prevalence of GBS among pregnant women between 35 and 37 weeks gestation in Windhoek was 13.6% which is low compared to findings of other Southern African countries such as South Africa (30.9% & 23%) in 2015 and 2016 and Zimbabwe (21%-47%) in 2010 (Mavenyengwa *et al*., 2010; Chukwu *et al*., 2015; Cools *et al*., 2016).

In South Africa, this current study showed a GBS prevalence of 37% in the Eastern Cape which is much higher than the prevalence in Windhoek (Namibia) and other South African studies done by Chukwu *et al*., (2015) and Cools *et al*., (2016). Sampling of different populations could contribute to differences in prevalence rates of GBS colonization as a similar study in Zimbabwe in 2006, reported a prevalence of 60% in a rural population and 46% in an urban population (Mavenyengwa *et al*., 2006). The Windhoek population was predominantly urban while in the Eastern Cape, Amatole Municipality which is largely rural is ranked among the poorest municipalities in South Africa (Baiyegunhi *et al*., 2014). In a study conducted by de Steenwinkel *et al*., (2008) in Mozambique, the reported prevalence of GBS was 1.8% in one of the poorest communities in Maputo which is much lower than the prevalence from this current study. The low prevalence of GBS was speculated to be linked to cultural norms and personal hygienic habits including male circumcision.

The prevalence of GBS in both Namibia and South Africa as shown in this study were not very different from those reported for other African countries such as Tanzania (23%), Uganda (28.8%) and Kenya (20.2%) (Joachim *et al*., 2009; Namugongo *et al*., 2016; Cools *et al*., 2016. The differences in the prevalence rates could be attributed to difference in study designs, prevalence at different gestational ages and different colonization rates in the different geographical regions (Cools *et al*., 2016).

Prevalence rates around the world vary between 10 and 40% (Dangor *et al*., 2015) as the prevalence rates of GBS in Europe among pregnant women vary between 6.5 and 36% with more than a third of the studies reporting a prevalence of 20% or greater. Eastern Europe had a prevalence of 19.7-29.3%, Western Europe 11-21%, Scandinavia 24.3-36% and Southern Europe 6.5-32% (Barcaite *et al*., 2008) and these are similar to those recorded in African studies, including results of this study.

As shown in Table 4.3, 18 (25.0%) of study participants from Namibia were vaginally colonised and 11 (15.5%) were rectal colonised while 43 (59.7%) were both vaginally and rectally colonised. In the South African study participants, 5 (13.5%) were vaginally colonised while 30 (81.1%) were both vaginally and rectally colonised with GBS. In both populations, most of the women were dually colonized. In a similar study in Tanzania, colonisation rates were 12.3% in the vagina and 5% in the rectum (Joachim *et al*., 2009). *S. agalactiae* colonises both the vagina and the rectum with colonization of either site resulting in vertical transmission to the baby during labour and or delivery while dual colonization of both the rectum and vagina increases the risk of vertical transmission to the new-born so CDC recommends rectovaginal sampling for detection of GBS.

S. agalactiae capsular types vary in different geographical locations. Capsular types Ia, II, III and V are common around the world. Capsular type II was prevalent in this study in the selected

areas of both Namibia (59.4%) and South Africa (52.2%) as shown in Table 4.4 Capsular types II, III and V constituted 94.1% of the Namibian isolates and 86.5% of the South African isolates in this study. These capsular types have been reported to be commonly associated with adverse pregnancy outcomes as well as neonatal morbidity and mortality (Lamagni *et al*., 2013) and they are predominantly the capsular types which have been isolated in other Southern Africa countries such as Zimbabwe and South Africa. However, a report from a study in South Africa in 2015 found a high prevalence rate of capsular types III (29.7%), Ia (25.8%) and V (10.9%) (Chukwu *et al*., 2015). In a similar study in South Africa (Johannesburg), Cools *et al*., (2016), reported a high prevalence rate of the same capsular types Ia (36.8%), V (26.3%) and III (14.0%).) while in Zimbabwe, Mavenyengwa *et al*., (2010) reported similar findings with the following prevalence rates: Ia (15.7%), Ib (11.6%), II (8.3%), III (38.8%) and V (24.0%). Similar studies in Europe have equally reported capsular types II, III and Ia as common (Barcaite *et al*., 2008).

Even though the results of this study are similar to those of other African studies and are comparable to European countries, most of the women were dually colonised rectally and vaginally predominantly by capsular types commonly associated with adverse maternal outcomes and this poses a risk to both the mother and baby. However, the investigator was not able to make any follow up on the outcomes of pregnant mothers and their babies.

4.4 CONCLUSION

It is concluded that most of the pregnant women were both vaginally and rectally colonised with GBS which may increase the risk of vertical transmission to the babies during delivery. The GBS capsular types predominant in pregnant women were the same capsular types prevalent in other African studies and commonly reported as being associated with adverse

pregnancy outcomes. Failure to make a follow up on both mothers and their babies may result in participants developing GBS disease, especially babies if they acquire it by vertical transmission during birth.

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CHAPTER FIVE

MULTI LOCUS SEQUENCE TYPING OF *S. AGALACTIAE* ISOLATED FROM PREGNANT WOMEN ATTENDING SELECTED CLINICS IN NAMIBIA AND SOUTH AFRICA

Abstract

Background: Multilocus sequence typing (MLST) is the reference method for the descriptive epidemiology of *Streptococcus* species. However, capsular serotyping is widely used for molecular epidemiological classification of GBS isolates. Understanding the epidemiology of GBS helps to unravel the genetic relatedness of isolates in the region which is key in the prevention and management of GBS disease. The study aimed to assess the evolutionary changes in GBS isolated among pregnant women in South Africa and Namibia and determine the genetic relatedness of those isolates using MLST. **Methods:** Thirty bacterial strains of *S. agalactiae* were isolated from pregnant women between 35 and 37 weeks gestation in the Eastern Cape, South Africa and Windhoek, Namibia. DNA was extracted from the bacterial isolates, Housekeeping genes were amplified using PCR and sequenced using the Sanger method. The sequences were edited using Geneious version 10.2.2 and analysed using Bioedit and sequences were compared to homologous *S. agalactiae* MLST: http://sagalactiae.mlst.net to determine transitions, transversions, nonsynonymous and synonymous mutations and to elucidate the MLST. Only isolates with complete sequences were considered for results analysis. **Results:** The number of alleles per gene ranged from two to seven and the range of polymorphic nucleotide sites was 0.4 to 1.6% for the six housekeeping genes used. The G+C% content was 35.9 to 43.4% for the six housekeeping genes and transitions were two thirds or more when compared to transversions. Five genes had a *dn/ds* ratio of less than one which indicated stabilizing selection while one gene had a ratio greater than one which suggested Darwinian selection. The isolates from South Africa and Namibia were genetically closely related. **Conclusion:** The housekeeping genes showed varying degrees of mutations while most of the genes were still conservative showing stabilizing selection with only one gene showing Darwinian selection. This agreed with studies done on other organisms like *S. pyogenes, N. gonorrhae* and *C. jejuni* in which some housekeeping genes remained conservative while

others showed evolutionary changes. Although the isolates had varying degrees of mutations, they were genetically closely related because there was no distinct clustering of isolates on the dendogram based on Sequence Type (ST).

Key words: *S. agalactiae*; mutations; MLST; analysis

5.0 INTRODUCTION

Multi Locus Sequence Typing (MLST) is a molecular technique for characterization of bacterial isolates using a number of house-keeping genes, usually seven. It involves the amplification of DNA using primers targeting the specific genes which is followed by Sanger sequencing of the amplicons. In *S. agalactiae* the housekeeping genes encode for alcohol dehydrogenase (*adhP*), phenylalanyl transfer RNA synhetase (*pheS*), amino acid transporter protein (*atr*), glutamine synthetase (*glnA*), 1-serine dehydratase (*sdhA*), glucose kinase (*glcK*) and transketolase (*tkt*) (Carvalho-Castro *et al*., 2017).

Several studies have elucidated the molecular epidemiological variations in *S. agalactiae* but MLST was reported as the most suitable method for characterizing and distinguishing isolates from various sources. For each gene, the different sequences identified within one species are marked as an allele and the alleles of each of the different loci define an allelic profile or a sequence type (Ludovic *et al*., 2004). The allelic profiles are due to mutations in the DNA of bacterial isolates. Comparing variations in the genetic composition of organisms allows for distinction of isolates from different sources such as fish, humans, bovine and dolphins (Pang *et al*., 2017). A study by Pang *et al*., (2017) in China reported that *S. agalactiae* isolates causing mastitis in dairy farms were similar to those reported in Brazil, France, UK and USA, while specific STs were associated invasive disease.

While MLST is good at tracking the evolutionary history among isolates, it is not efficient at differentiating strains in different hosts according to source. It is also good at establishing the distribution and association of isolates from different disease outbreaks caused by streptococci. Whole-genome MLST (wgMLST) is being proposed as a possible method for distinction of closely related isolates and is rapidly being applied in epidemiological studies. The use of only seven loci in MLST is thought to result in loss of valuable genetic information resulting from lateral gene transfer or bacteriophage genes inserted in host genome (Barony *et al*., 2017).

Comparative genomics has improved understanding of invasive GBS disease, not only in human beings but also in bovine, canine, feline and aquatic mammals. This study therefore sought to determine the sequence types among GBS isolates from pregnant women in South Africa and Namibia.

5.1 MATERIALS AND METHODS

5.1.1 Ethical considerations

Refer to the designated preceding chapter.

5.1.2 Strain collection

A rectal and vaginal swab were collected from each participant between 35 and 37 weeks gestation by a registered nurse. In South Africa (Eastern Cape), 100 participants were included in the study while in Namibia (Windhoek), 530 participants took part in the study. All swabs collected in South Africa were processed at the AEMREG Laboratory, University of Fort Hare Alice, while samples in Namibia were processed at the Namibia University of Science and Technology, Microbiology Laboratory.

5.1.3 Identification of GBS

Samples were inoculated onto Columbia blood agar and Todd Hewitt broth (Rochelle Chemicals and Laboratory Equipment, Johannesburg, South Africa) and incubated at 37 °C for 24 h. Inoculums in Todd Hewitt broth were sub cultured onto blood agar and incubated at 37°C for 24 h. The cultures were checked for colonies exhibiting β haemolysis on the blood agar. The colonies were tested for the catalase reaction and catalase negative colonies were tested using the Lancefield grouping antisera (Becton Dickinson, New Jersey, USA). Colonies which tested positive for the latex agglutination assay were presumptively identified as GBS isolates and were confirmed using the Vitek 2 and by molecular techniques. All presumptive isolates were stored in glycerol stocks at -80 ^{o}C until further analyses.

5.1.4 DNA extraction

Glycerol stocks were resuscitated in Muller Hinton broth for 24 h at 37^{o}C and streaked on Columbia blood agar containing 5% horse blood. *S. agalactiae* isolates were confirmed by molecular techniques using a pair of primers specific for the *scp*B gene. A single colony of GBS was picked from Columbia blood agar containing 5% horse blood and emulsified into 160µL of nucleic acid free water in a 2mL Eppendorf conical tube. The emulsified colony was incubated at 100˚C for 15 minutes on a heating block. The mixture was centrifuged at 10,000 revolutions per minute (rpm) for 5 minutes and the supernatant containing GBS DNA was separated from the pellet and stored at -80˚C. Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25units/ml One Taq DNA polymerase, was mixed with 6µL each of nucleic acid free water, 1 µL of 10 pMol of reverse and forward primers and 5µL of DNA in a total reaction volume of 25 µL. The oligonucleotide primers used are shown in Table 5.1.

Table 5.1: Oligonucleotide primers for *S. agalactiae scp*B gene amplification

Name	***scp*BF**	**Length**
Sequence	ACAACGGAAGGCGCTACTGTTC	22 bases
Name	***scp*BR**	**Length**
Sequence	ACCTGGTGTTTGACCTGAACTA	22 bases

(Adopted from: Elbaradie *et al*., 2009)

The cycling conditions were as follows: an initial denaturation at 94˚C for 4 min followed by 35 cycles of: denaturation at 93˚C for 1 min, annealing at 57˚C for 1 min and extension at 72˚C for 1 min. A final elongation step of 72˚C for 7 min was followed by a hold at 4˚C as described by Desjardins *et al*., (2004). Amplicons were loaded on 1 % agarose gel stained with 10µL ethidium bromide and electrophoresed for 45 mins at 110V in 0.5X Tris/Borate/EDTA (TBE) buffer and viewed under transilluminator and photographed.

5.1.5 Amplification and nucleotide sequence determination

Bacterial isolates (n=30) had housing keeping genes (*adh*P, *atr*, *gln*A, *sdh*A, *glc*K and *tkt*) amplified using PCR. A representative sample of isolates was selected using systematic sampling. Amplification was as follows:

Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25units/mL One Taq DNA polymerase, was mixed with 6µL of nucleic acid free water, 1 µL each of 10 pMol of reverse and forward primers, and 5 µL of DNA making a total of 25 µL reaction volume. Primer sequences of the house keeping genes are shown in Table 5.2.

Table 5.2. Oligonucleotide primers for GBS MLST

Locus	Use	Name and sequence of primer		Amplicon size (bp)
		Forward (5′ to 3′)	Reverse (5′ to 3′)	
adhP	Amplification	GTTGGTCATGGTGAAGCACT	ACTGTACCTCCAGCACGAAC	672
	Sequencing	GGTGTGTGCCATACTGATTT	ACAGCAGTCACAACCACTCC	498
pheS	Amplification	GATTAAGGAGTAGTGGCACG	TTGAGATCGCCCATTGAAAT	723
	Sequencing	ATATCAACTCAAGAAAAGCT	TGATGGAATTGATGGCTATG	501
atr	Amplification	CGATTCTCTCAGCTTTGTTA	AAGAAATCTCTTGTGCGGAT	627
	Sequencing	ATGGTTGAGCCAATTATTTC	CCTTGCTCAACAATAATGCC	501
glnA	Amplification	CCGGCTACAGATGAACAATT	CTGATAATTGCCATTCCACG	589
	Sequencing	AATAAAGCAATGTTTGATGG	GCATTGTTCCCTTCATTATC	498
sdhA	Amplification	AGAGCAAGCTAATAGCCAAC	ATATCAGCAGCAACAAGTGC	646
	Sequencing	AACATAGCAGAGCTCATGAT	GGGACTTCAACTAAACCTGC	519
glcK	Amplification	CTCGGAGGAACGACCATTAA	CTTGTAACAGTATCACCGTT	607
	Sequencing	GGTATCTTGACGCTTGAGGG	ATCGCTGCTTTAATGGCAGA	459
tkt	Amplification	CCAGGCTTTGATTTAGTTGA	AATAGCTTGTTGGCTTGAAA	859
	Sequencing	ACACTTCATGGTGATGGTTG	TGACCTAGGTCATGAGCTTT	480

(Adopted from Jones *et al*., 2003)

5.1.6 Computational analyses

Amplicons were sequenced using the Sanger method and sequences were edited using Geneious version 10.2.2 (Biomatters Limited) while the generated nucleotide sequences were analysed using Bioedit version 7.6.2.1. The nucleotide sequences were subjected to homology search by means of the BLAST 2.0 program in the National Centre for Biotechnology

Information (NCBI). Sequences were compared with homologous *S. agalactiae* sequences in the GenBank.

5.1.7 Allele and sequence type assessment

All isolates were evaluated for changes in base sequences and two types of changes were assessed, change of a purine for a purine or pyrimidine for a pyrimidine (transition) and substitution of a purine for a pyrimidine or vice versa (transversion) and any change in the nucleotide sequence irrespective of its effect on the amino acid sequence was considered an allele. The alleles were compared to those deposited in the *S. agalactiae* MLST at http://sagalactiae.mlst.net to determine the allelic profiles of the isolates. The allelic profile of each isolate was used to determine its sequence type by comparison to isolates deposited in the gene bank. Molecular phylogenetic analysis was performed using the Maximum Likelihood method while evolutionary analyses were conducted in MEGA7.

5.2 RESULTS

Six housekeeping genes of thirty *S. agalactiae* isolates were amplified using sequence specific oligonucleotide primers. Twenty isolates from Windhoek (Namibia) and ten from the Eastern Cape (South Africa) were sequenced using the Sanger method. Nucleotide sequences were edited using Geneious version 10.2.2 and analysed using the BLAST tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). Table 5.3 presents the properties of loci used for *S. agalactiae* MLST and Table 5.4 shows the distribution of mutations among the housekeeping genes.

Table 5.3: Properties of loci used for *S. agalactiae* MLST

Locus	**Putative function of gene**	**Size of sequence fragment**	**No of alleles identified**	**No (%) of polymorphic nucleotide sites**	**% G+C**	***dn/ds***
adhP	Alcohol dehydrogenase	498	3	2 (0.4)	43.4	0.5
sdhA	Serine dehydratase	519	2	7 (1.3)	41.6	0.3
tkt	Transketolase	480	3	5 (1.0)	39.2	1.5
glnA	Glutamine synthetase	498	4	6 (1.2)	35.9	0.5
glcK	Glucose kinase	459	4	5 (1.1)	42.5	0.3
atr	Amino acid transporter	501	3	8 (1.6)	37.3	0.3

Most of the housekeeping genes had a *dn/ds* ratio of <1.0 except *tkt* and the number of alleles identified per gene was low. However, most the genes had more than 5 polymorphic nucleotide sites.

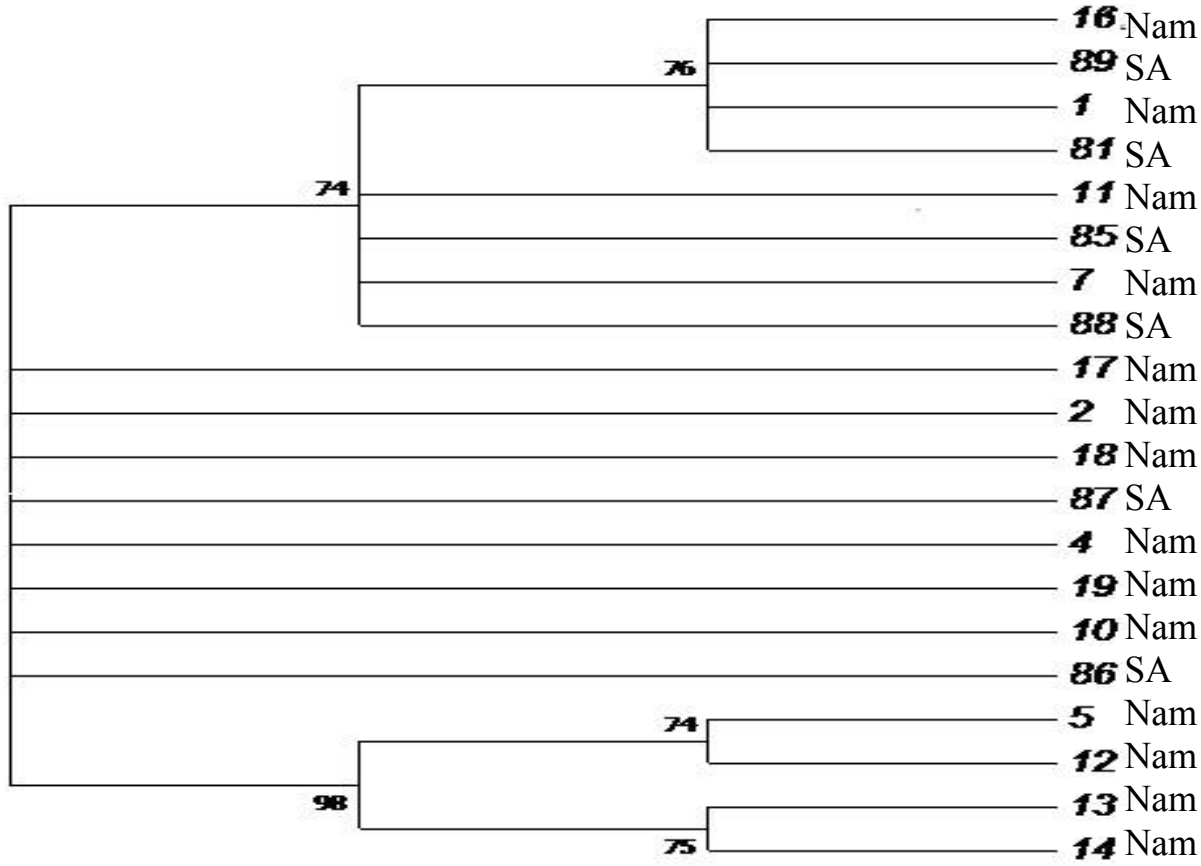
Table 5.4: Distribution of mutations among the housekeeping genes

Locus	**Allelic profile**	**Transitions** **n (%)**	**Transversions** **n (%)**
adhP	1,2,5	1 (50.0)	1 (50.0)
sdhA	1,2	7 (87.5)	1 (12.5)
tkt	1,2,3	4 (80.0)	1 (20.0)
glnA	2,3,5,10	4 (66.6)	2 (33.3)
glcK	1,2,3	4 (80.0)	1 (20.0)
atr	1,3,4,6	6 (75.0)	2 (25.0)

All the genes exhibited more transitions than transversions with transitions being at least two-thirds more than transversions in all genes except *adhP*. Table 5.5 is a frequency distribution table for STs among GBS isolates.

Table 5.5: Distribution of sequence types among GBS isolates

Sequence type	**Allelic profile**	**No of strains**	**Country of origin**
19	1,3,2,2,2,2	1	Namibia
24	5,4,3,2,3,3	4	SA, Namibia
48	2,1,2,2,1,1	2	Namibia
109	2,1,10,1,1,1	1	Namibia
144	5,1,3,2,1,2	1	Namibia
171	2,3,2,1,1,1	1	Namibia
185	2,1,2,2,1,3	2	SA
206	5,1,3,2,1,1	1	Namibia
331	1,3,5,2,2,3	1	Namibia
457	2,6,3,2,1,3	2	SA, Namibia
464	5,4,3,2,3,1	1	SA
670	2,4,2,1,1,1	1	Namibia
1010	5,4,2,1,3,3	1	Namibia
1174	5,1,2,2,1,3	1	Namibia



Key: SA denotes isolates from South Africa; Nam denotes isolates from Namibia

Figure 5.1. Dendogram illustrating the phylogenetic relationship of *S. agalactiae* isolated from pregnant women in SA and Namibia.

The evolutionary history was established by using the Maximum Likelihood method based on the Tamura-Nei model. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 60% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated

using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. Codon positions included were $1^{st}+2^{nd}+3^{rd}$+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 480 positions in the final dataset and evolutionary analyses were conducted in MEGA7.

5.3 DISCUSSION

The MLST of *Streptococcus agalactiae* isolates in this study was based on six (*adhP, sdhA, tkt, glnA, glcK and atr*) housekeeping genes as described by Jones *et al*., 2003. The isolates were from South Africa (10 isolates) and Namibia (20 isolates). The DNA was sequenced using the Sanger method and the size of the sequenced fragments ranged from 459bp (*glcK*) to 519bp (*sdhA*) as shown in Table 5.2.

The highest number of alleles identified on a locus were on *glnA* (4) with *sdhA* (2) registering the least number of alleles per locus with an average of 3.2 alleles per locus (Table 5.3). This was much lower than the alleles registered per locus in a similar study by Jones *et al* (2003) in which *glcK* had the least (4) and *adhP* had the highest (11). The frequency of alleles on different loci in this study was lower than that reported in *Neisseria gonorrhoeae* (*N. gonorrhoeae*) in which the alleles varied from 2 to 18 with an average of 8.3 alleles per locus (Viscidi *et al*., 2003). Studies on *Streptococcus pyogenes* (*S. pyogenes*) showed more variation in the number of alleles per locus for the housekeeping genes with 21 to 35 with an average of 28.1 in one study (Enright *et al*., 2001) and 36 to 66 in another similar study (McGregor *et al*., 2004).

Polymorphic nucleotide sites in the current study ranged from 0.4 to 1.6% among the genes with the most polymorphic locus being *atr* and *adhP* being the least variable site. In a study by Jones *et al* (2003) *glnA* (1.2%) and *sdhA* (2.5%) were the least and most variable sites

respectively but a similar study on *S. pyogenes* showed a higher variation in genetic polymorphism ranging from 1.4 to 6.1% (Enright *et al*., 2001).

The proportion of nonsynonymous mutations to synonymous mutations (*dn/ds*) was less than one for all the genes except *tkt* (1.5) as shown in Table 5.3. A proportion *dn/ds* proportion of less than one shows that there is selection against amino acid change and a proportion greater than one could be an indicator of positive Darwinian selection. It is assumed that synonymous mutations are selectively neutral and do not lead to changes in the amino acid sequence or protein coded for by a gene (Wielgoss *et al*., 2011). In a similar study by Suebaum *et al*., (2001) on *C. jejuni*, the proportion of nonsynonymous to synonymous mutations was less than one in all isolates indicating that housekeeping genes were quite conserved (Suerbaum *et al*., 2001). In a study on *N. gonorrhae* by Viscidi *et al*., (2003) out of 16 housekeeping genes two of the genes showed possible positive selection with *dn/ds* greater than one. Ratios of less than one are indicative of stabilizing selection in the genes and gene products are likely not to change while ratios greater than one are likely to give rise to new genetic variants. While the ratio of nonsynonymous mutations to synonymous mutations is a useful tool in detecting evolutionary patterns in protein coding genes. It is limited in detecting mutations which adaptively alter phenotype, evolutionarily alter relevant phenotype or are due to hitchhiking (Bloom, 2017).

The guanine and cytosine content of DNA (G+C %) is an indicator of how stable the DNA of a gene or whole genome is. A high G+C % indicates a gene is more stable than a gene with a low content. Mutational G+C % variation in bacteria is often assumed to be approximately between 20% and 80% (Hershberg and Petrov, 2010) and in this study G+C % content was lowest in *glnA* (35.9%) and highest in *adhP* (43.4%) as shown in Table 6.3. This was consistent with a similar research done by Jones *et al* (2003) in which *glnA* (35.7%) and *adhP* (43.1%) had the lowest and highest G+C% content respectively. Studies have shown that selection

favours bacteria with a high G+C% although there is wide intra-genomic heterogeneity (Brocchieri, 2014; Lassale *et al*., 2015)

Transitions are substitutions of a purine for a purine or a pyrimidine for a pyrimidine while transversions are substitutions of a purine for a pyrimidine or vice versa and transversions are more likely to result in an amino acid substitution impacting on the structure and function of a protein formed from a gene (Guo *et al*., 2017). Although there are two possible transversions there is one one possible transition. Transitions are two thirds more likely to occur as compared to transversions. The proportion of transitions to tranversions was 3:1 in all genes except *adhP* (1:1) in this study (Table 5.4). The results are consistent with other studies (Liu, 2012; Seplyarskiy *et al*., 2012; Duchene *et al*., 2015). Transitions are less likely to result in an amino acid substitution and many persist as silent substitutions. Substitution of a single ring structure with a single ring structure or a double ring structure with a double ring is easier than substituting a single ring structure for a double ring or vice versa (Luo *et al*., 2016).

The ratio of transitions to transversions for *adhP* in this study could be an indicator of the change in the protein encoded by the gene. This has implications on the survival of the organism as *adhP* is a housekeeping gene which encodes for a protein that acts as a metabolic enzyme (alcohol dehydrogenase). On the *sdhA* gene, transitions on position 188 and 337 resulted in amino acid change from valine to alanine and asparagine to aspartic acid, respectively. This causes a change in protein structure and function potentially threatening the survival of the organism (Guo *et al*., 2017; Duchene *et al*., 2015).

The most common sequence types in the data set were ST24 (two each from SA and Namibia), 48 (Namibia), 185 (SA) and 457 (SA and Namibia) as shown in Table 5.5. Sequence types 24 and 457 were found in isolates from both countries. Isolates from Namibia and SA were distributed across 12 sequence types and 4 sequence types, respectively. The presence of

different sequence types among isolates recovered from pregnant women in the same population showed that GBS strains colonising the pregnant women were genetically diverse. The presence of different alleles in the sequence types of the same organism could be indicators of horizontal gene transfer as seen in other organisms such *S. pneumoniae* and *S. pyogenes* (Jones *et al*., 2003; Enright *et al*., 2001).

The phylogenetic tree (Figure 5.1) shows a few branches indicating that nucleotide sequences from the Eastern Cape, SA were genetically closely related to those from Windhoek, Namibia and there was little evolutionary diversity. The *S. agalactiae* isolates from the two countries are not distinguishable using the six genes and it is a relatively uniform group with low variability showing close evolutionary relationships. Isolates from the same species or same group clustered together and isolates which do not cluster showed variation in genetic composition indicating that they belonged to the same evolutionary clade (Liu *et al*., 2013; Gao *et al*., 2014).

There was no immediate distinct clustering observed on the dendogram. However, samples 5, 12, 13 and 14 from Namibia were seen to form a subgroup of their own and interestingly, these samples are all rectal swabs. There was no distinction of isolates on the dendogram according to ST as there was no distinct clustering. A study by Evans *et al*., (2008) reported no clustering of *S. agalactiae* isolates based on ST. However, in a study by Jones *et al*., (2003) *S. agalactiae* isolates were distinguishable of the dendogram according to ST.

5.4 CONCLUSION

S. agalactiae isolates from South Africa and Namibia were found to be closely related and they did not show wide variations in the house keeping genes as reported elsewhere in the world.

The house keeping genes largely remained conservative with very few changes in the amino acid sequences.

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CHAPTER SIX

DISTRIBUTION OF SELECTED VIRULENCE GENES AMONG *S. AGALACTIAE* ISOLATES FROM PREGNANT WOMEN ATTENDING SPECIFIC CLINICS IN NAMIBIA AND SOUTH AFRICA

Abstract

Background: In spite of the array of virulence factors associated with GBS isolates across the globe, studies to unravel such factors from isolates obtained from pregnant women in selected sites in South Africa and Namibia have received cursory attention. The spectrum of such virulence factors will shed more light on the pathogenicity of GBS isolated from the region. Studies have associated virulence factors to GBS prevalence rates and development of invasive disease in different geographic locations. This study was therefore undertaken in two countries in Southern Africa in order to discern any relatedness in virulence factors among GBS isolates and for epidemiological profiling. **Method:** One hundred and fifty four GBS isolates from Namibia and South Africa recovered from pregnant women between 35 and 37 weeks gestation were screened for genes encoding virulence using PCR. Primer sequences specific for each gene were used to screen for the following genes: *cps*, *cylE*, *scp*B, *hla*, *hlb*, *etB* and *lukM*. **Results:** All the isolates were capsulated and had the *scp*B gene which encodes for C5a peptidase while the haemolysin gene (*cylE*) was expressed by 94.2% of isolates. In this study 94.2% of the isolates expressed the capsule, *scp*B and haemolysin concurrently. None of the isolates (0%) expressed the *hla*, *hlb*, *etB* or *lukM* gene. **Conclusion:** Most of the isolates (94.2%) had three virulence encoding genes (*scp*B, *cps* and *cyl*E) amplified. The presence of these genes in the bacterial isolates suggests that the isolates had the potential of causing invasive GBS disease in the pregnant women, their babies or both.

Key words: Virulence, *Streptococcus agalactiae*, Women, Invasive

6.0 INTRODUCTION

Bacteria possess properties which allow them to survive and evade the host's immune system. The bacterial properties facilitate tissue invasion, evasion of the immune system and propagation of the bacteria in the host (Zheng *et al*., 2012). Organisms such as group A Streptococcus have haemolytic toxins, streptolysin O (SLO) and streptolysin S (SLS) which are also found in *Staphylococcus aureus*, *Clostridium botulinum* and *Listeria monocytogenes*. The haemolysin SLS is encoded for by the *sag* gene. *S. agalactiae* possesses virulence factors such as *hylB, lmb, scpB, cylE, bac* and *bca* genes (Souza *et al*., 2013; Rosa-Fraile *et al*., 2014.

In a study carried out by Otaguiri *et al*., 2013 in Brazil all isolates had the *cylE* and *hylB* gene and the *cylE* is reported to be responsible for the haemolysis seen around GBS isolates on blood agar. However, 3% of the isolates were non-haemolytic and studies have shown that 1-5% of human isolates are non-haemolytic (Rosa-Fraile *et al*., 2014). A similar study by Dutra *et al*., 2014 in Brazil reported that out of 434 GBS isolates, all of them had the *lmb* and *scpB* gene amplified. The isolates also had *bca* (54.4%) and *bac* (13.1%) amplified although another study by Souza *et al*., (2013) in Brazil reported low prevalence of both these genes.

A study by Rosa-Fraile *et al*., 2014, reported that cylE was also expressed by other organisms such as *Propionibacterium jensenii* and *Bacillus cereus* and mutations in the *cylE* gene resulted in non-haemolytic phenotypes. Some investigators have found a high prevalence of virulence genes in the same isolates such as *scpB* (93.1%), *lmb* (90.8%), *cylE* (92.0%), *hylB* (18.4%), *bca* (60.9%) and *bac* (24.1%) (Jiang *et al*., 2016; Sadaka *et al*., 2017; Rajagopal, 2009). The presence of these virulence factors makes the GBS isolates invasive causing invasive GBS disease such as meningitis and septicaemia in neonates, preterm babies and pregnant women (Jiang *et al*., 2016; Sadaka *et al*., 2017).

The invasiveness of GBS is related to its ability to evade the immune system through various means. It has been reported that in developing countries mortality rate from invasive GBS disease is 10 to 60% while in developed countries it is 7 to 11% (Quan *et al*., 2016). Children with invasive GBS will present with Early Onset Disease (EOD) which is characterised by sepsis, meningitis and pneumonia. They may also present with Late Onset Disease (LOD) characterised by neurological sequelae (Quan *et al*., 2016; Madhi and Dangor, 2017.

The highest incidence of GBS disease is in Africa especially sub Saharan Africa where case fatality rates are 2 to 3 times greater in Africa (20-38%) than the rest of the other countries such as Hong Kong (10%), and USA (4-6%) (Kobayashi *et al*., 2016). The range of GBS virulence factors is poorly understood and there is need for proper characterisation of GBS contribution to still births in low income countries although it is believed to be high in Africa. There has been a high incidence of invasive GBS disease in South Africa (1.1 per 1000 live births) for the last two decades which is twice the overall incidence in Africa (Madhi and Dangor, 2017). Low incidences of GBS disease have been reported in some provinces of South Africa (<0.5 per 1000 live births) and these were attributed to poor diagnostic methods, poor health care practices, babies being born at home, poor health care facilities and empiric treatment of GBS disease (Kobayashi *et al*., 2016; Madhi and Dangor, 2017). The study was therefore undertaken to discern any relatedness in virulence factors among GBS isolates and for epidemiological profiling.

6.1 MATERIALS AND METHODS

6.1.1 Ethical considerations

Refer to the section in preceding chapter.

6.1.2 Sample collection, culture and identification of GBS

Refer to the preceding chapter

6.1.3 Molecular confirmation of GBS strains

Presumptive isolates in glycerol stocks were resuscitated in Todd Hewitt broth for 24 h at 37 ºC and streaked on Columbia blood agar containing 5% horse blood. *S. agalactiae* isolates were confirmed by molecular techniques using a pair of primers specific for the *scp*B gene. A single colony of GBS was picked from Columbia blood agar containing 5% horse blood and emulsified with 2mL of nucleic acid free water in a 2mL microcentrifuge tube. The tube was boiled at 100˚C for 15 minutes on a Heating Block and the mixture was centrifuged at 10,000 revolutions per minute (rpm) for 5 minutes and supernatant containing GBS DNA was separated from the sediment and stored at -80˚C. Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom) containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25 units/mL One Taq DNA polymerase, 6uL of water of PCR grade, 1uL of 10 pMol of both forward and reverse primers were mixed with 5µL of DNA template to make up a total reaction volume of 25uL. *Scp*B primer sequences are presented in Table 6.1.

Table 6.1: Oligonucleotide primers for molecular confirmation of GBS

Name	***scp*BF**
Sequence	ACAACGGAAGGCGCTACTGTTC
Name	***scp*BR**
Sequence	ACCTGGTGTTTGACCTGAACTA

(Adopted from Elbaradie *et al*., 2009)

The cycling conditions were as follows: an initial denaturation of 94˚C for 4 minutes followed by 35 cycles of denaturation at 93˚C for 1 min, annealing at 57˚C for 1 min and extension at 72˚C for 1 min and a final elongation step of 72˚C for 7 min followed by a hold at 4˚C as described by Desjardins *et al.*, 2004. Amplification was verified in a 1% agarose gel electrophoresis at 120 volts for 45 minutes in a 0.5X TBE buffer and thereafter viewed in a transilluminator and photographed.

6.1.4 Screening of virulence genes among isolates using multiplex PCR

The isolates were amplified for seven virulence genes which encode for various virulence factors: *cps* (capsular polysaccharide), *scp*B (C5a peptidase), *cyl*E (haemolysin), *hla* (haemolysin A), *hlb* (haemolysin B), *etb* (exfoliative toxin B) and *lukM* (leucocidin). Primers for *cps* are those used in capsular typing.

Table 6.2: Oligonucleotide primers for virulence genes

Gene	Annealing temperature (°C)	Primers	Amplicon size	Reference
scpB	57	scpBF: ACAACGGAAGGCGCTACTGTTC scpBR: ACCTGGTGTTTGACCTGAACTA	255	Elbaradie *et al*., 2009
cylE	55	cylEF:TGACATTTACAAGTGACGAAG cylER: TTGCCAGGAGGAGAATAGGA	248	Jain *et al*., 2012
hla	56	hlaF: CTGATTACTATCCAAGAAATTCGATTG hlaR: CTTTCCAGCCTACTTTTTTATCAGT	209	Jarraud *et al*., 2002
hlb	57	hlbF:GTGCACTTACTGACAATAGTGC hlbR: GTTGATGAGTAGCTACCTTCAGT	309	Gharsa *et al*., 2015
etb	47	etbF:CAGATAAAGAGCTTTATACACACATTAC etbR: AGTGAACTTATCTTTCTATTGAAAAACACTC	612	Jarraud *et al*., 2002
lukM	57	lukMF: TGGATGTTACCTATGCAACCTAC lukMR: GTTCGTTTCCATATAATGAATCACTAC	780	Jarraud *et al*., 2002

Twelve microliters of One Taq^{R} Master Mix with standard buffer (New England Biolabs, United Kingdom), containing: 20mM Tris-HCl, 1.8mM $MgCl_2$, 22mM NH_4Cl, 22mM KCl, 0.2mM dNTPs, 5% glycerol, 0.06% IGEPAL^{R} CA-630, 0.05% Tween^{R} 20 and 25units/mL One Taq DNA polymerase, was mixed with 6µL of nucleic acid free water, I uL each of 10 pMol of reverse and forward primers for the respective capsular types, 5uL of DNA template, to make a final reaction volume of 25uL. *Staphylococcus aureus* was used a positive control during PCR.

The cycling conditions were as follows: 94˚C for 4 minutes as an initial denaturation followed by 35 cycles of denaturation at 93˚C for 1 min adapt in all, annealing at the respective annealing

temperature for 1 minute and extension at 72˚C for 1 minute with a final elongation step of 72˚C for 7 minutes followed by a hold at 4˚C. Amplification was verified in a 1% agarose gel electrophoresis at 120 volts for 45 minutes in a 0.5X TBE buffer and thereafter viewed in a transilluminator and photographed.

6.2 RESULTS

All the GBS isolates from Namibia and South Africa recovered from pregnant women between 35 and 37 weeks gestation were screened for genes encoding virulence factors. The sample size was 117 isolates from Namibia and 37 isolates from South Africa. The genes screened in the isolates were: *cps*, *cylE*, *scp*B, *hla*, *hlb*, *etB* and *lukM*. Table 6.3 below shows the frequency distribution of isolates for genes coding for virulence factors among isolates

Table 6.3: Frequency distribution of isolates for genes coding for virulence

Gene	**Phenotype**	**Frequency (%)**
*scp*B	C5a peptidase	154 (100)
cps	Polysaccharide capsule	154 (100)
cylE	Haemolysin	145 (94.2)
hla	Alpha haemolysin	0 (0)
hlb	Beta haemolysin	0 (0)
etB	Exfloiative toxin B	0 (0)
lukM	Leukocidin	0 (0)

The *scpB* and *cps* genes were amplified in all isolates while the most of the isolates had the hamolysin gene (*cylE*).

6.3 DISCUSSION

Virulence factors are important in the pathogenesis of a disease resulting in the development of invasive disease. GBS virulence factors are associated with the development of disease in pregnant women, neonates, non-pregnant women and those immunocompromised. Invasive GBS diseases include chorioamnionitis, septicaemia, EOD, LOD, meningitis and long term neurological sequelae such as hearing impairment, cerebral palsy and cognitive challenges (Tevdorashvili *et al*., 2015).

In this study, all the isolates had a capsule as shown in Table 6.3. The capsule is the major virulence factor for GBS which enables the bacteria to evade phagocytosis and is associated with the development of EOD and LOD in neonates. Out of the ten capsular polysaccharide types, capsular types III, Ia, Ib, II, V and IV have been linked with the development of invasive GBS disease (Dangor *et al*., 2015). However, other capsules still facilitate bacterial cells in evading the immune system and invade host tissue. Studies have shown that encapsulated GBS is not invasive and is susceptible to phagocytic killing. This study did not observe any encapsulated bacterial strains and the capsule has been identified as a good candidate for vaccine development (Sheppard *et al*., 2016).

All the isolates in this study had the *scp*B gene which encodes for C5a peptidase, a protein which cleaves the complement component C5a reducing complement activation and clearance of bacteria from the host. This may lead to increased colonisation, bacterial load and risk of developing invasive GBS disease in pregnant women, neonates and immunocompromised individuals. Other studies have found that the invasiveness of GBS is due to its ability to traverse tissue and survive in epithelial cells and macrophages and bacteria with a mutated *scp*B gene is significantly less invasive compared to that with no mutation (Cheng *et al*., 2002).

While C5a peptidase has been demonstrated in studies to assist in bacterial invasiveness, studies have also demonstrated that pyogenic Streptococci can inactivate C3 which is a central molecule in the complement cascade system. Cleaving of C3 slows down neutrophil recruitment and reduces: chemotaxis, opsonisation, phagocytosis and bacterial elimination. This coupled with C5a peptidase activity spurs GBS virulence resulting in severe invasive GBS disease. However, the expression of *scp*B and other virulence genes varies in different GBS strains with upregulation and downregulation of some genes based on strain and period of infectivity (Lynskey *et al*., 2017; Korir *et al*., 2017).

The haemolysin gene was amplified in 94.2% of isolates in this study. The expression of haemolysin by GBS in a host reduces GBS clearance by neutrophils leading to increased colonisation of epithelial linings in organs such at the vagina. This increases the risk of vertical transmission from the mother to the baby during birth. Haemolysin disrupts epithelia and endothelia allowing the bacteria to penetrate tissue causing invasive GBS disease. This leads to sepsis, meningitis, pneumonia in neonates and chorioamnionitis and pneumonia in pregnant women. Haemolysin is a major virulence factor in GBS and approximately 5% of GBS among carriers are non haemolytic but non haemolytic strains have been isolated from cases of invasive GBS disease presenting with meningitis, pneumonia and sepsis (Rodriquez-Granger *et al*., 2015; Six *et al*., 2016).

In this study 94.2% of the isolates expressed the capsule, *scp*B and haemolysin concurrently which makes the bacteria very virulent and capable of evading the immune system causing invasive GBS disease. The findings of this study are in agreement with a study done in Malaysia in which almost all isolates had *scp*B and haemolysin (Eskandarian *et al*., 2015).

lukM, hla, *hlb* and *etB* are virulence factors commonly expressed in *S. aureus* leading to invasive disease. The virulence factors have been found to be expressed by other Gram positive

bacteria. However, this study found that the *S. agalactiae* isolates did not express any of those virulence factors as shown in Table 6.3 (Jayasinghe and Bayley, 2005; Schlotter *et al*., 2012; Spaan *et al*., 2015).

6.4 CONCLUSION

All isolates from both Namibia and South Africa were amplified for the *scp*B and *cps* genes, while 94.2% of the isolates expressed the *cyl*E gene. Most of the samples (94.2%) had all the three genes (*scp*B, *cps* and *cyl*E) amplified. The presence of these genes in the bacterial isolates showed that the isolates had the potential of causing invasive GBS disease in the pregnant women, their babies or both. None of the isolates (0%) had the *hla*, *hlb*, *etB* or *lukM* gene amplified.

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CHAPTER SEVEN

GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 General conclusions

Based on the results of this study it is concluded that:

- In Windhoek the prevalence of GBS was low while in the South African population the prevalence was high (13.6% and 37% respectively). The Namibian population was predominantly urban while the South African one was rural and this difference can cause a disparity in prevalence rates.

- The GBS isolates from both Namibia and South Africa expressed major virulence genes such as the *cps*, *cylE* and *scp*B encoding the capsule, haemolysin and C5a peptidase, respectively. This is an indication that the isolates had the potential to cause invasive GBS disease in both the pregnant woman and the baby resulting in EOD and LOD in the neonates.

- The capsular types identified in this study are the same as those identified in other South African and African studies. Although the capsular types are the same there are differences in distribution of the various capsular types between isolates from Namibia and South Africa. The predominant capsular types are those commonly associated with invasive GBS disease.

- There was no marked antimicrobial resistance to antibiotics used for empiric treatment except for notable resistance to erythromycin, clindamycin and cotrimoxazole which were reported in other studies. However, medicinal plants seem to be offering alternatives to antibiotic treatment as plant extracts showed antimicrobial activity

against GBS isolates. Both ethanolic leaf extracts and essential oil of *O. europaea* were bactericidal against GBS.

- The *S. agalactiae* isolates from South Africa and Namibia showed stabilising selection with the mutations in the genes not causing amino acid or protein changes.

7.2 Recommendations

The following recommendations are made on the backdrop of the findings of the study:

- The high prevalence of GBS in the rural South African population maybe an indication of the need for continuous surveillance and screening of pregnant women between 35 and 37 weeks gestation as proposed by CDC with a view to offer IAP.

- This study did not look at the colonization or infection of neonates and further research will be crucial in determining the transmission to neonates and the subsequent acquisition of EOD and LOD. Future studies could also focus on the morbidity and mortality of invasive GBS disease in pregnant women as the bacteria was shown to possess very potent virulence factors in this study.

- With reports of continuing reduction of penicillin MICs and outright resistance by GBS in some parts of the world there is need for continuing surveillance of resistance patterns and instituting AST based treatment with antibiotics.

- Future studies focussing on identifying and characterising the active phytochemical components of plant extracts which can target GBS are necessary.