

**INCIDENCE AND MECHANISM OF ANTIBIOTIC RESISTANCE  
OF *STREPTOCOCCUS AGALACTIAE* ISOLATES  
FROM PREGNANT WOMEN AND THEIR BABIES AT  
DR GEORGE MUKHARI ACADEMIC HOSPITAL, PRETORIA**

by

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**UNIVERSITY OF SOUTH AFRICA**

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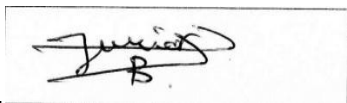
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## DECLARATION

Student number: 45898375

I, declare that INCIDENCE AND MECHANISM OF ANTIBIOTIC RESISTANCE OF *STREPTOCOCCUS AGALACTIAE* ISOLATES FROM PREGNANT WOMEN AND THEIR BABIES AT DR GEORGE MUKHARI ACADEMIC HOSPITAL, PRETORIA is my own work and that all the sources that I have used or quoted have been indicated and acknowledged by means of complete references.



.....  
SIGNATURE  
(Mr YJ Bolukaoto)

...08 October 2014...

DATE

## **DEDICATION**

In memory of those who contributed enormously in my life and in this study but passed on very soon without benefiting the fruit of their hard work:

My parents **Alexis Bolukaoto Bolika and Elisabeth Batokande Ngandi**,  
whom their sweat and blood flow in my veins;

My co-supervisor **Dr. Snow Teffo Malibe**, who was among the initiators of  
this research project;

You will always be remembered and may your souls rest in perfect peace.

*“There are many projects in a man’s heart;  
But it is the destiny of the Lord that shall stand”  
Prov. 19: 21*

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Most of all, I would like to thank and bless God, the almighty, for the breath, strength and his mercy on me, Let his name be praised.

## ABSTRACT

**BACKGROUND AND OBJECTIVES:** *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) is the leading cause of neonatal infections and deaths in human. It can also cause infections in pregnant women and non-pregnant adults. Penicillin and ampicillin are antibiotics of choice for the treatment of GBS infections. Erythromycin and clindamycin are used as alternative therapy in penicillin allergic patients, however resistance to these agents has been increasingly observed. This present study was undertaken to determine the colonization rate of GBS, susceptibility profile and the mechanism of antibiotic resistance in pregnant women and their babies at Dr. George Mukhari Academic Hospital in Pretoria.

**METHODS:** Rectal and vaginal swabs were collected from pregnant women; ear and umbilical swabs from newborns over an 11 month period. Samples were cultured on selective media (CNA agar and Todd-Hewitt broth) and GBS positively identified using morphological and biochemical tests including Gram staining, hemolytic activity, catalase test, bile esculin, CAMP test and Latex agglutination test. The susceptibility testing was done using the Kirby-Bauer and E-test methods. The D-test method was used to determine the inducible clindamycin resistance. Multiplex PCR with were used to detect different genes coding for resistance.

**RESULTS:** Out of the 413 patients evaluated, 128 (30.9%) were positive with GBS. All isolates were sensitive to penicillin and ampicillin. Erythromycin and clindamycin resistance was 21.1% and 17.2% respectively; of which 69% harbouring constitutive  $MLB_B$ , 17.4% inducible  $MLS_B$ . The alteration of ribosomal target encoded by *ermB* genes was the commonest mechanism of resistance observed in 55% of isolates, 38% of isolates had both *ermB* and *linB* genes and efflux pump mediated by *mefA* genes was detected in one of isolates.

**Conclusion:** This study reaffirms the appropriateness of penicillin as the antibiotic of choice for treating GBS infection. However it raises the challenges of resistance to the macrolides and lincosamides. More GBS treatment options for penicillin allergic patients need to be researched.

### Keys terms

Group B *streptococcus* (GBS); antibiotic susceptibility; antibiotic resistance; gene of resistance; mechanism of resistance; Multiplex polymerase chain reaction; pregnant women; newborn babies; Pretoria, South Africa.

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## LIST OF ABBREVIATIONS

<b>ABCs</b>	Active Bacterial Core surveillance
<b>AIDS</b>	Acquired Immuno Deficiency Syndrome
<b>ANC</b>	Antenatal clinic
<b>ATCC</b>	American type culture collection
<b>BP</b>	base pairs
<b>BNF</b>	British National Formulary
<b>CAES</b>	College of Agriculture and Environmental Science
<b>CAMP</b>	Christie Atkins and Munch-Petersen
<b>CDC</b>	Centers for Disease Control and prevention
<b>CLSI</b>	Clinical Laboratory Standards Institute
<b>CNA</b>	colistin nalidixic acid
<b>COD</b>	childhood onset disease
<b>CO<sub>2</sub></b>	carbon dioxide
<b>CPSs</b>	capsular polysaccharides
<b>CSF</b>	cerebrospinal fluid
<b>CVR</b>	Cervico-vaginal-rectal
<b>DGMAH</b>	Doctor George Mukhari Academic Hospital
<b>dH<sub>2</sub>O</b>	distilled water
<b>DMP</b>	Division Medical Products
<b>DNA</b>	Deoxyribose nucleic acid
<b>dNTPs</b>	Deoxynucleotide triphosphates
<b>ds</b>	double stranded
<b>EOD</b>	early-onset disease
<b><i>erm</i></b>	erythromycin ribosomal methylation
<b>ES</b>	ear swab

<b><i>et al.</i></b>	and others
<b>g</b>	gram
<b>GBS</b>	Group B <i>streptococcus</i>
<b>GMOs</b>	Genetically modified organisms
<b>h</b>	hour
<b>H<sub>2</sub>O<sub>2</sub></b>	Hydrogen peroxide
<b>HIV</b>	Human immunodeficiency virus
<b>HOD</b>	Head of Department
<b>HVS</b>	High vaginal swab
<b>IgG</b>	Immuno globulin G
<b>i/v</b>	intravenously
<b>Kb</b>	kilobase
<b>l</b>	liter
<b>LOD</b>	late-onset disease
<b>LVS</b>	Lower vaginal swab
<b>Medunsa</b>	Medical University of Southern Africa
<b>MIC</b>	Minimal inhibitory concentration
<b>mg</b>	milligram
<b>MgCl<sub>2</sub></b>	Magnesium chloride
<b>ml</b>	milliliter
<b>MLS<sub>B</sub></b>	Macrolide-lincosamide-streptogramin type B
<b>cMLS<sub>B</sub></b>	constitutive macrolide-lincosamide-streptogramin type B
<b>iMLS<sub>B</sub></b>	inducible macrolide-lincosamide-streptogramin type B
<b>mm</b>	millimeter
<b>MREC</b>	Medical Research Ethics Committees
<b>NAAT</b>	Nucleic acid amplification test
<b>NHLS</b>	National Health Laboratory Services

<b>PBPs</b>	Penicillin-binding proteins
<b>PCR</b>	Polymerase chain reaction
<b>PCR<sub>P</sub></b>	Polymerase chain reaction products
<b>PFGE</b>	Pulsed-field gel electrophoresis
<b>pH</b>	Potential of Hydrogen (Hydrogen ion concentration)
<b>QRDR<sub>s</sub></b>	quinolone resistance-determining regions
<b>RS</b>	Rectal swabs
<b>SA</b>	South Africa
<b>SOP</b>	Standard operation procedures
<b>UNISA</b>	University of South Africa
<b>US</b>	umbilical swab
<b>UTI</b>	urinary tract infection
<b>VRE</b>	vancomycin-resistant enterococci
<b>μg</b>	microgram
<b>μg/ml</b>	microgram per milliliter
<b>μl</b>	microliter
<b>°C</b>	degree Celsius
<b>&gt;</b>	greater than
<b>&lt;</b>	less than
<b>≥</b>	greater or equal to
<b>≤</b>	less than equal to

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# CHAPTER I

## INTRODUCTION

### 1.0 Brief Introduction

*Streptococcus agalactiae* (Group B *Streptococcus*; GBS) was recognized primarily as an important cause of bovine mastitis. However, since the 1970s, GBS has become the leading cause of neonatal infections in humans and an important cause of illness in pregnant women, the elderly, and adults with underlying diseases such as diabetes (Lammler *et al.*, 1995; Murayama *et al.*, 2009). GBS forms part of normal flora of the gastrointestinal and genital tract and is found in 10 – 40% of pregnant women (Mandell *et al.*, 2010; Manning *et al.*, 2003).

Colonization rate differs among ethnic groups, geographic area, and ages; however, rates are almost similar for pregnant and non - pregnant women (Dzowela *et al.*, 2005; Mavenyengwa *et al.*, 2010, CDC 2010). Different GBS colonization rates have been reported in African; Zimbabwe (32%), Gambia (22%), Nigeria (20%), Ivory Coast (19%), and 16.5% in Malawi (Dzowela *et al.*, 2005). In Tanzania, maternal colonization of GBS was confirmed in 23% of pregnant women (Joachim *et al.*, 2009). In a South African study, GBS colonization rate of 21.5% was reported (Madzivhandila *et al.*, 2011). GBS prevalence has been shown to be higher in African countries and studies show that black race, poor socio-economic status of women are usually implicated among the risk factors for GBS colonization (Schrag *et al.*, 2002; Dzowela *et al.*, 2005; Edmond *et al.*, 2012).

The incidence of GBS disease ranges from 0.5 to > 2 per 1000 live births in different geographic areas (Fisher *et al.*, 2001; Lyytikäinen *et al.*, 2003; Yu *et al.*, 2011; Edmond *et al.*, 2012). Several reports on the incidence of GBS infection have been documented; 2.7 per 1000 live-births in Aberdeen - Scotland (Skinner *et al.*, 1978); 0.72 per 1000 live-births in UK and Republic of Ireland (Heath *et al.*, 2004).

In USA, due to the CDC's Active Bacterial Core surveillance (ABCs) in the emerging infection program network, reliable epidemiologic data on invasive infection due to

GBS were obtained for a population of 17 million people during 1999 to 2003. GBS infection resulted in about 20,400 cases and 2,200 deaths with the incidence of 1.7 infants per 1000 live births. The highest attack rates were observed for patients less than 1 year and + 65 years old (Murray *et al.*, 2007; CDC, 2009). However in recent years, with the application of high prevention efforts, this incidence of GBS dramatically declined from 1.7 cases per 1,000 live births to 0.34–0.37 cases per 1,000 live births. GBS caused approximately 1,200 cases of early-onset invasive disease per year; and approximately 70% of cases were among babies born at term or  $\geq 37$  weeks' gestation (CDC, 2010).

In Malawi, the incidence of GBS infections of 0.90 per 1000 live-births was reported (Gray *et al.*, 2007). In South Africa, the first report of GBS infections in neonates was reported in Kwazulu Natal, and the incidence was reported to be 2.65 per 1000 live births (Haffejee *et al.*, 1991).

### **1.1 Morphology and Growth conditions**

The species *Streptococcus agalactiae* is a Gram positive bacterium, coccoid in shape, catalase negative, encapsulated, facultative anaerobe, with  $\beta$ -hemolysis on blood containing agar and gives a positive CAMP test (Willey *et al.*, 2011). GBS grow on most bacteriologic media and occur predominantly in pairs and in chains formation (Figure 1). Encapsulation may be evident in direct smears of purulent exudates. On 5% sheep blood agar, GBS produces smooth large gray to whitish-gray colonies of > 0.5mm, and surrounded by hazy, a weak zone of  $\beta$ -hemolysis of red blood cells in the culture medium. However, the use of enriched selective media (CNA agar and Todd-Hewitt broth) for 18-24 hours remains critical for the sensitivity of GBS culture results. Sensitivity increases by approximately 50% with use of enrichment media (CDC, 2010; Farley *et al.*, 2001; Willey *et al.*, 2011).





**Figure 1.** *Streptococcus agalactiae* ATCC<sup>(R)</sup> 13813 (Cat. no A300; adapted from Catalog.hardydiagnostic.com)

## 1.2 Transmission

Mother to child transmission, vertical transmission, commonly occurs via the ascending route from the maternal genital tract into the amniotic fluid, which is then aspirated by infant *in utero* or at delivery (Baker *et al.*, 1974; Moyo *et al.*, 2002). Predisposing factors include birth complication, stillbirth, prolonged rupture of membrane (or difficult labor) and lack of antibodies to the invading GBS strain in the mother and hence the neonate (Bottone, 2004; Greenwood *et al.*, 2007).

Neonatal GBS infection is classified as early-onset disease (EOD), when occurring within 12h – 6 days after birth (70% - 80% of cases); and late-onset disease (LOD), when it occurs more than 7 - 90 days after birth and the childhood onset disease (COD, age > 90 days). Late-onset disease is transmitted from mother, day care or health care personnel to infants (Madhi *et al.*, 2003; Martins *et al.*, 2011). The aspect of infections beyond early Infancy is often used for patients with GBS diseases and who are older than 3 months and younger than 18 years old. Many of these infections are rare and occur among very low birth weight infants (Mandell *et al.*, 2010).

In adults, GBS is a part of normal flora of the gut and genital tract and found in both male and female. Due to the diverse obstetric complications and manipulations, the bacteria can become active and be transmitted. In elderly people the bacteria can be transmitted from one person to another during a long stay in hospital and nursing facility residents (Narayanan *et al.*, in 2006)

### 1.3 Frequency of Antibiotic Resistance in GBS

Antibiotic resistance is the ability of bacteria or other microbes to resist the effects of an antibiotic. It occurs when bacteria change in a way that reduces or eliminates the effectiveness of drugs, chemicals, or other agents designed to cure or prevent infections. The bacteria survive and continue to multiply causing more harm (Beith *et al.*, 2008).

Antibiotic resistance has actually become a serious public health problem worldwide. However, a large part of the problem is due to the massive use or misuse of antibiotics in the biosphere which has had serious consequences for the antibiotic guidelines for the treatment of infections (Greenwood *et al.*, 2007; Beith *et al.*, 2008). Evolution of bacteria towards resistance has been considerably accelerated by selective pressure exerted by over prescription of drugs in clinical settings; self-medication; inappropriate antibiotic treatment; the failure of taking the entire prescribed course of antibiotics; and the use of antibiotics to treat viral infections. Those are among other factors that promote emergence of antibiotic resistance (Walsh *et al.*, 2003; Beith *et al.*, 2008).

Several studies, carried out at various centers around the world, have assessed the antimicrobial susceptibility profile and determined the mechanism of resistance in GBS. These studies, in accordance with CDC 2010 guidelines, found that penicillin is the antibiotic of choice for the treatment of GBS infections, followed by ampicillin and the 1<sup>st</sup> generation cephalosporins (Law *et al.*, 2005; CDC, 2010); and have reported that there is an increase worldwide in the resistance to two most commonly used antibiotics for penicillin allergic patients with high risk of anaphylaxis namely macrolides (erythromycin) and linconsamide (clindamycin), living no alternative drugs for penicillin-allergic individual (Pearlman *et al.*, 1998; Arpin *et al.*, 1999; Ghearrhadi *et al.*, 2007; Culebras *et al.*, 2002; Malbruny *et al.*, 2004; Betrui *et al.*, 2004; Wehbeh *et al.*, 2005; Dipersio *et al.*, 2006).

The erythromycin resistance mechanism in GBS is mostly due to ribosomal modification encoded by *erm* genes (Arpin *et al.*, 1999; Zeng *et al.*, 2006, Gyax *et al.*, 2007) and Clindamycin resistance in GBS is due to ribosomal translocation encoded by *linB* genes (Heelan *et al.*, 2004).

It has been shown by various studies that erythromycin and clindamycin resistance has been the most frequently observed among GBS. This resistance has been observed in both developed and developing countries (Dahesh *et al.*, 2008). A recent study in Italy, by Lambiase *et al.*, 2012, reported a higher resistance to both erythromycin and clindamycin; ranging from 16.5% to 69.9% in the 4 years study period (from 2005 to 2008). They concluded that this increase of *in vitro* resistance of GBS to macrolides and clindamycin was clearly evident and suggested that the discordance with reports from different countries emphasizes the crucial role of microbiological methods in setting possible therapeutic strategies.

In Japan, macrolide-resistant strains were detected in both children and adults and the difference was statistically significant different, with the majority resistance being due to *erm(B)* genes. One of the collected strains had mutations of the *pbp2x* gene, responsible for reduced penicillin susceptibility in GBS (Murayama *et al.*, 2009).

Simoes *et al.*, 2004, in USA, determined the *in vitro* resistance of GBS to 12 antibiotics currently used for GBS chemoprophylaxis in pregnant women. In which 25% of isolates were resistant to erythromycin and 19% resistant to clindamycin. In France, the level of erythromycin and clindamycin resistance from GBS isolates was a concern and led to the recommendation that alternative prophylactic therapy for pregnant women who are allergic to penicillin should be guided by susceptibility testing (De Mouy *et al.*, 2001).

To establish the susceptibility of Zimbabwean GBS strains isolated from hospitalized patients, a study was performed in four regions of this country. One hundred percent of isolates showed resistance to tetracycline, 14% to erythromycin, 8% to clindamycin and 2% of isolates showed intermediate susceptibility to penicillin (Moyo *et al.*, 2001). In Malawi, serotype Ia and III were reported to be responsible for 77% of disease. All isolates were susceptible to penicillin, but 21% were resistant to erythromycin (Gray *et al.*, 2007). In Tanzania, the study performed in Dar es Salaam has shown a resistance rate to clindamycin and erythromycin of 17.6% and 13% respectively (Joachim *et al.*, 2009).

Several other studies have been carried out to assess the resistance patterns. However these results cannot be used to compare with other areas due to difference in study design and also difference in geographic region (Berkowitz *et al.*, 1990; Chohan *et al.*, 2005).

The mechanisms of resistance of GBS to these antibiotics are well known. Numerous molecular methods such as Multiplex PCR, Quantitative real-time PCR and Individual gene specific PCR have come to the fore in detection of genes of resistance in GBS (Gygax *et al.*, 2006; Brochet *et al.*, 2007; Kimura *et al.*, 2008; Gosiewski *et al.*, 2012).

#### **1.4 Rational and Motivation**

This study aimed to investigate the incidence of antibiotic resistance of GBS isolates at Dr. George Mukhari Academic Hospital (DGMAH) in Pretoria. GBS antibiotic susceptibility surveys demonstrate increasing prevalence of resistance to alternative drugs (erythromycin and clindamycin) used for penicillin allergic patients (Back *et al.*, 2012). Because of various mechanisms employed by GBS to counteract the effects of these therapeutic agents, and if steps are not taken to prevent it, antibiotic resistance in GBS will reach an era where all antibiotics might be rendered ineffective (Fitoussi *et al.*, 2001), creating a situation similar to the pre-antibiotic era. Hence, routine surveillance, susceptibility profiles of GBS to these antibiotics as well as understanding of resistance mechanisms are warranted.

In South Africa, this organism has received minimal attention; and thus local data that seek to investigate the incidence of antibiotic resistance in GBS infections are scanty in our setting. Therefore the purpose of this study was to determine the colonization rate of GBS disease, antibiogram, antibiotic resistance and molecular basis of resistance of GBS isolates from pregnant women and their newborns at Dr. George Mukhari Academic Hospital, in Pretoria.

## **1.5 Aims and Objectives of the study**

### **1.5.1 Aim**

The aim of this study was to determine the anti-biogram and mechanisms of antimicrobial resistance in GBS isolates from pregnant women and their newborns at Dr. George Mukhari Academic Hospital (DGMAH), in Ga-rankuwa, Pretoria.

### **1.5.2 Objectives**

- To determine the colonization rates of GBS isolates from pregnant women and their babies at DGMAH
- To assess the susceptibility profile of GBS isolates to penicillin, erythromycin, clindamycin, ampicillin, high level gentamicin, vancomycin, chloramphenicol, tetracycline and ciprofloxacin.
- To determine genetic basis of resistance in GBS isolates colonizing pregnant women and their babies at DGMAH.
- To determine the mechanisms of antibiotic resistance (erythromycin and clindamycin) in GBS isolates from pregnant women and their babies at DGMAH.

## **1.6 Significance of the study**

This study will provide baseline information on prevalence, antibiotic susceptibility and resistance profiles of GBS in pregnant women and their newborns at Dr. George Mukhari Academic Hospital, Pretoria.

Data on antibiogram and molecular profiles will be useful as a source of information to guide good management of patients, treatment regiment design, routine surveillance of antibiotic resistance patterns, and also will help to raise awareness and inform policy on prevention against GBS infections in newborns born to colonized mothers; and will provide templates for practical epidemiological application.

## CHAPTER II

### LITERATURE REVIEW

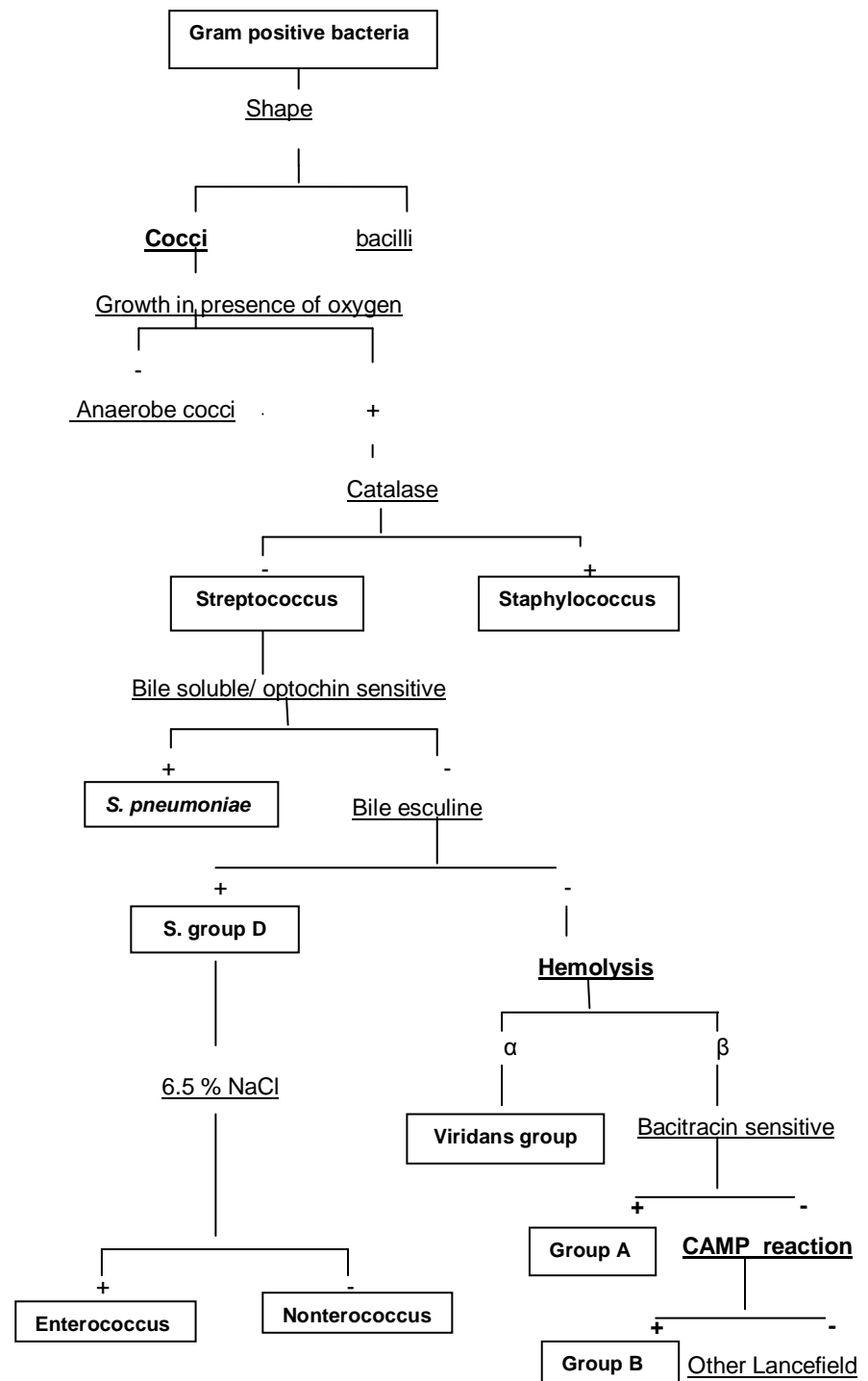
#### 2.1 Classification of Streptococci

The genus *Streptococcus*, of the family *Streptococcaceae*, is comprised of diverse group of facultatively anaerobic to strictly anaerobic species; and divided into three genera, *Streptococcus*, *Enterococcus* and *Lactococcus* (Willey *et al.*, 2011). The genus *Streptococcus* includes different species such as *S. agalactiae*, *S. anginosus*, *S. bovis*, *S. canis*, *S. constellatus*, *S. cristatus*, *S. dysagalactiae*, *S. equi*, *S. equines*, *S. gordonii*, *S. milleri*, *S. mitis*, *S. mutans*, *S. oralis*, *S. parasanguis*, *S. pneumonia*, *S. porcinus*, *S. pyogenes*, *S. ratti*, *S. salivarius*, *S. sanguinis*, *S. sobrinus*, *S. suis*, *S. Uberis*, *S. vestibularis* (Willey *et al.*, 2011).

They have been classified on the basis of the colony size, the interaction with erythrocytes in agar media as  $\alpha$ -hemolytic (greening due to hemoglobin conversion),  $\beta$ -hemolytic (clear zone of hemolysis around colonies) and  $\gamma$ -hemolytic (no zone); and the presence of Lancefield antigens. The taxonomy of Streptococci has experienced a number of changes during the last 20 years due to the application of molecular biology techniques such as 16S rRNA gene sequencing; and DNA – DNA re-association experiments; and still source and object of different studies. However, the traditional streptococcal classification system is well established and still of value to the clinical microbiologist (Murray *et al.*, 2007).

Differentiating GBS strains by phenotypic molecular techniques, such as serotyping enabled the earliest molecular epidemiologic studies to be conducted. Recently, genotypic techniques, such as pulsed-field gel electrophoresis (PFGE) and nucleotide sequence analyses have been utilized to better characterize the GBS strains in circulation, and to gain a better understanding of disease pathogenesis, and possible transmission modes (Murray *et al.*, 2007; Willey *et al.*, 2011).

Figure 2 below shows the general scheme for the classification and identification of streptococci.



Source: Willey *et al.*, 2011

## **2.2 Isolation and Screening of GBS**

GBS can be isolated from blood, cerebrospinal fluid ( CSF), urine, vagina, rectal and/ or site of local suppuration. The reliable detection of colonization with GBS in pregnant women before delivery is an essential requirement for effective prophylaxis (Narayanan *et al.*, 2006).

Screening for GBS consists of obtaining and identifying by culturing vaginal and anal specimens at 35 to 37 weeks' gestation. The mother's vaginal and ano-rectal swabs are collected and placed into an appropriate transport medium such as Amie's or Stuart; streaked on a single 5% defibrinated sheep blood agar plates containing antibiotics such as Columbia colistin and nalidixic acid (CNA), and inoculated into selective broth media, such as Lim broth or Todd-Hewitt broth, with the same antibiotics and similar concentration as CNA agar, and incubated for 24hours in a CO<sub>2</sub> enriched environment. The broth culture be sub-cultured onto blood agar and plates read after 24- 48 hours (Bergeron *et al.*, 2000; Manning *et al.*, 2003; CDC, 2010 ).

Colonization with GBS in pregnant women can be identified rapidly, accurately and reliably by molecular assays such as PCR for prenatal, nucleic acid amplification test (NAAT) etc. The performance of these tests allows for rapid intrapartum detection of GBS in 45 - 100 minutes before delivery (Bergeron *et al.*, 2000; Manning *et al.*, 2003; Bergseng *et al.*, 2007; CDC, 2010)

## **2.3 Typing of GBS**

Group B Streptococci are identified because of their content of rhamnose-containing "group specific" polysaccharide. The capsule of the organism is composed primarily of a sialic acid containing type-specific polysaccharide, which is the basis for further classification (Chan *et al.*, 2000). The pathogenesis of GBS can be grouped into the adherence to epithelial surfaces, penetration of host cellular barrier, avoidance of immunologic clearance mechanisms and inflammatory activation (Musa *et al.*, 2012).



The Lancefield classification scheme serologically groups  $\beta$ -hemolytic streptococci on the basis of their carbohydrate cell wall antigens; this differs from typing of group A streptococci for which protein antigens are utilized (Lammler *et al.*, 1995; De Azavedo *et al.*, 1999). To date, based on the polysaccharide antigen, ten capsular types of GBS have been described (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX); and in several reports, capsular types III is the most common and up to 90% of isolates from infants with meningitis or from infants with late-onset disease caused by serotype III (Dele *et al.*, 2001; Mavenyengwa *et al.*, 2008; Florindo *et al.*, 2011). In addition to serological methods for typing, recently developed multiplex PCR and DNA sequencing-based techniques allow the detection of capsular serotypes (Manning *et al.*, 2003).

#### **2.4 Antibodies against GBS**

Antibodies to type-specific capsular polysaccharides (CPSs) of GBS in the serum of experimental animals and human neonates correlate with protection from GBS disease. The absence of antibody to GBS in infants is a risk factor for infections. Up to 90% of pregnant women may be deficient in type III CPS-specific antibody, which suggests a lack of priming by this antigen (Larsson *et al.*, 2006). The precise concentration of CPS-specific antibody needed for protection of neonates may differ by serotype, bacterial inoculum, and possibly other factors; and the relative risk for colonization or invasive infection is related to the degree (inoculum size) of maternal colonization (Dele *et al.*, 2001; Larsson *et al.*, 2006).

The GBS cell surface proteins  $\alpha$  and Rib elicit protective immunity in animal models and have been suggested as potential antigens in a vaccine against human GBS neonatal infection. These proteins have been extensively characterized and belong to a family of streptococcal proteins with extremely repetitive sequence (Larsson *et al.*, 2006). Because antibodies to GBS provide protection against disease in animal models, there is an ongoing interest in vaccination as an approach for reducing the incidence of GBS colonization in healthy women (Lammler *et al.*, 1995; Woods *et al.*, 2010).

## 2.5 Clinical manifestation

### 2.5.1 GBS infections in newborns

Group B *Streptococcus* is an important agent of serious neonatal infections (Figure 3). The most important risk factor for the development of invasive neonatal disease is the colonization of the maternal urogenital or gastrointestinal tract with GBS (Larsoon *et al.*, 2006). In fact, about 1% of children born to mothers infected with GBS are prone to developing severe neonatal diseases such as neonatal sepsis, pneumonia, and meningitis (Rosetti *et al.*, 1997; Mandell *et al.*, 2010).

The presenting signs of early-onset-disease in GBS infections include poor feeding, lethargy, raised temperature, grunting respiration, hypotension, etc. In Late-onset-Disease, some signs of EOD generally occur but in association with fever ( $t^{\circ} \geq 38^{\circ}\text{C}$ ). Bone and joints infections are other clinical forms of LOD. Newborn infections such as conjunctivitis, cellulitis, otitis media, and endocarditis can also be due to GBS (Mandell *et al.*, 2010). Studies done elsewhere show that 25 - 50% of survivors of GBS meningitis, whether early- or late - onset, have permanent neurologic sequelae such as deafness, loss of hearing, mental retardation, etc. (Schrag *et al.*, 2002; Mandell *et al.*, 2010).



**Figure 3:** Neonate infected with GBS. Observe the Greenish liver visible through the abdomen (Larsoon *et al.*, 2006).

### **2.5.2 GBS infections in adults**

Group B *Streptococcus* is an important cause of illness in pregnant and non-pregnant adults (female and male). Infection with GBS is commonly asymptomatic, this means GBS colonization does not result in symptoms and is not harmful (Faye-Kette *et al.*, 1991; Narayanan *et al.*, 2006; CDC, 2010; Gray *et al.*, 2011). However, colonization with GBS in pregnant women has been associated with adverse outcomes of the pregnancy, and hence the need for review of policy on screening and intrapartum eradication of GBS in the recto-vaginal tract. Invasive GBS infection causes substantial morbidity and mortality among the elderly people (+65 years old) and adults with underlying conditions, such as diabetes mellitus, liver disease, renal failure, neurologic impairment, cardiovascular disease, pulmonary disease, urologic disease, malignancy, cirrhosis, and immuno-compromised patients with alcoholism, including HIV-AIDS patients (Farley *et al.*, 2001; Slaven *et al.*, 2007; Mandell *et al.*, 2010; Gray *et al.*, 2011).

Most adult GBS infections occur in association with one of several expressions of infections; including primary bacteremia, meningitis, infection in female genital tract, and a variety of clinical manifestation, most common of these are endometritis and wound infection both associated with cesarean section; and pneumonia, and also skin and soft tissue infections, which includes foot ulcers, abscesses and cellulitis associated with foreign bodies (Manning *et al.*, 2003; Gibbs *et al.*, 2004; Murayama *et al.*, 2009, Mandell *et al.*, 2010).

### **2.5.3 Spectrum of systemic infections caused by GBS**

#### **2.5.3.1 Bacteremia**

Bacteremia is the presence of bacteria in the bloodstream. This may occur through a wound or injection, or through surgical procedure. Bacteremia may cause no symptoms and resolve without treatment, or it may produce fever and other infections. If the immune system is damaged, septicemia may develop (Narayanan *et al.*, 2006)

### **2.5.3.2 Sepsis**

Sepsis is a severe illness caused by overwhelming infection of the bloodstream by toxin producing bacteria. Neonatal GBS sepsis is characterized by clinical features such as fever, tachypnea and tachycardia. Common sites include the kidneys, liver, skin, and lung. Infection is confirmed by a positive blood culture (Humphreys *et al.*, 2000).

### **2.5.3.3 Meningitis**

Meningitis is the inflammation of the meninges (membranes covering the brain or spinal cord), usually due to infections. Neonatal GBS meningitis occurs by route such as nosocomial infection. It often leads to permanent neurologic sequelae such as cerebral or cranial nerve palsy, epilepsy, mental retardation or hydrocephalus (Mandell *et al.*, 2010). In adults, GBS meningitis is an important but uncommon manifestation of invasive GBS disease, and may account for up to 4% of all cases of bacterial meningitis. It is almost always associated with anatomical abnormalities contiguous with, or of, the central nervous system, usually as a result of neurosurgery. A small but significant proportion of survivors are left with neurologic sequelae such as permanent hearing loss (Farley *et al.*, 2001).

### **2.5.3.4 Pneumonia**

Pneumonia is an infection in one or both lungs. GBS pneumonia is rare and has few unique features. It generally occurs in older adults with diabetes and neurological impairment resulting from conditions such as cerebrovascular disease or dementia. In many cases aspiration is either documented or suspected. Pleural effusions are uncommon, and lung tissue necrosis is rare (Farley *et al.*, 2001; Narayanan *et al.*, 2006).

### **2.5.3.5 Skin and Soft tissue infections**

These are the most frequently reported clinical syndromes associated with invasive GBS. These infections most often present as cellulitis, decubitus ulcers, and infected foot ulcers. Many of these patients are diabetic (Bayer *et al.*, 1976; Narayanan *et al.*, 2006). GBS have occasionally been associated with wound and burn infections in

non-pregnant adults. Cases of necrotizing fasciitis and toxic shock–like syndrome associated with GBS have been reported (Tyrrell *et al.*, 1996).

#### **2.5.3.6 Bone and joint infections**

GBS osteomyelitis most often occurs by contiguous spread or direct inoculation. The bones of the foot are frequently involved; this involvement is linked with vascular insufficiency and overlying ulcers and spreads from adjacent skin and soft-tissue infection. Vertebral osteomyelitis, usually in the lumbosacral area, is another common form of GBS osteoarticular infection (Farley *et al.*, 2001; Narayanan *et al.*, 2006).

#### **2.5.3.7 Urinal tract infections**

Urinal tract infections (UTI) are more common in older individuals (mean age, 71 years). Many patients with GBS urosepsis are nursing facility residents. In fact, between 5% and 23% of non-pregnant adults with invasive GBS disease are presented with a urinary tract infection. Most patients have significant predisposing conditions, such as diabetes mellitus, prostate disease, an indwelling urinary catheter, and anatomic abnormalities of the urinary tract. The presence of a neurogenic bladder has been associated with significantly increased risk for invasive GBS disease (Farley *et al.*, 2001).

Other conditions to be considered associated with GBS infections include otitis media, endocarditis, neurologic deficit, cellulitis, chorioamnionitis, diabetic foot, line infection, post-partum infections, septic arthritis, etc. (Farley *et al.*, 2001; Narayanan *et al.*, 2006).

## **2.6 Treatment approach in GBS infections**

There are several regimens for the treatment of infections with GBS. Antibiotics currently used for management of GBS include penicillin G, ampicillin, vancomycin, cefuroxime, cefotaxime, ceftriaxone, erythromycin, clindamycin, gentamicin, chloramphenicol, ciprofloxacin, and amoxicillin. However susceptibility testing must be done before any treatment is administered (Mandell *et al.*, 2010). Duration of treatment depends on the clinical syndrome, risk of complications, response to therapy and the age of the patients (Simoes *et al.*, 2004; Woods *et al.*, 2010).

### **2.6.1 Treatment of GBS infection in newborns**

Therapy depends on the infection focus. As presentation in neonates is non-specific, all neonates are treated almost the same once GBS infection is confirmed. “Blind” antibiotic treatment is always recommended as soon as CSF and blood samples have been taken for Gram stain and culture (Mims *et al.*, 2004).

The first-line treatment is benzylpenicillin (Penicillin-G) or ampicillin, plus gentamicin. For children with penicillin allergy, a 2<sup>nd</sup> or 3<sup>rd</sup> generation cephalosporin (e.g. cefuroxime, cefotaxime or ceftriaxone) may be appropriate depending on the type of allergy, or vancomycin with or without gentamicin (British National Formulary (BNF), 1993).

Vancomycin is not suitable as monotherapy if meningitis is present. In the case of septic arthritis, joint must be aspirated and a formal washout in operating room is strongly recommended. Treatment course is 3 to 4 weeks. An infectious-disease specialist should be consulted if polymicrobial infection is suspected (Narayanan *et al.*, 2006; Woods *et al.*, 2010).

### **2.6.2 Treatment of GBS infection in pregnant women**

The most common infections are UTI, chorioamnionitis, sepsis, postpartum endometritis, and postpartum wound infection. The choice of antibiotic in pregnant women is influenced by the potential effects on the fetus. Likewise, the choice of antibiotic in postpartum infections is influenced by penetration into the breast milk (BNF, 1993).

Penicillin or ampicillin work synergistically with gentamicin as the 1<sup>st</sup> line treatment of chorioamnionitis and endometritis. For patients with penicillin allergy, a 2<sup>nd</sup> or 3<sup>rd</sup> generation cephalosporin may be appropriate depending on the type of allergy, also clindamycin, erythromycin or vancomycin plus gentamicin. Treatment course is 10 days. Infection is often polymicrobial (Narayanan *et al.*, 2006; Woods *et al.*, 2010).

### **2.6.3 Treatment of GBS infection in non-pregnant adults**

Benzylpenicillin or ampicillin is the first-line treatment for sepsis, cellulitis, septic arthritis and meningitis. A second or third generation cephalosporin or a macrolide, or clindamycin may be appropriate for patients with penicillin allergy, depending on the type of allergy. Gentamicin may be considered as adjunctive therapy in selected cases, but advice from an infectious-disease specialist must be sought, as the presentation is rare and management complicated. The duration of treatment course is a minimum of 10 days for sepsis and cellulitis; and 14 to 21 days for meningitis; and 3 to 4 weeks for septic arthritis (BNF, 1993; Narayanan *et al.*, 2006; Woods *et al.*, 2010).

In case of conjunctivitis, sinusitis, otitis media, endocarditis, soft-tissue infection, osteomyelitis and intra-abdominal infections, seek advices from an infectious-disease specialist since these infections are often polymicrobial. As GBS infection occurs more commonly in older patients, hepatic and renal impairment must be taken into account when selecting dose of some drugs (Mandell *et al.*, 2010; Woods *et al.*, 2010).

In case of urinary tract infections, the first-line treatment is amoxicillin or ampicillin. Penicillin or ampicillin with gentamicin is used for complicated infections such as pyelonephritis. Patients with penicillin allergy should be given trimethoprim, or nitrofurantoin, or vancomycin with or without gentamicin. The duration of treatment course is 3 to 14 days (Narayanan *et al.*, 2006).

For pneumonia, 1<sup>st</sup> line treatment is benzylpenicillin or ampicillin. Patients with penicillin allergy, a 2<sup>nd</sup> or 3<sup>rd</sup> generation cephalosporin may be appropriate depending on the type of allergy. Vancomycin, linezolid, a macrolide, or a quinolone may also be used but antibiotic susceptibility testing must be done before any

treatment. Rifampicin or gentamicin may be considered as adjunctive therapy in selected cases (Narayanan *et al.*, 2006; Woods *et al.*, 2010).

## 2.7 Antimicrobial agents in GBS

Table 1 below lists some antibiotics used for GBS therapy infections, their antimicrobial spectra, origin and mode of action.

Antibiotic	Organism producer	Active against	Mode of action	Effect
Penicillin	<i>Penicillium spp</i>	Gram + bacteria	Inhibit cell wall synthesis	Bacteriocidal
Erythromycin	<i>Streptomyces erythreus</i>	Common Gram +	Interfere with protein synthesis	Bacteriocidal
Clindamycin	<i>Streptomyces lincolnensis</i>	Gram <sup>+</sup>	Interfere with protein synthesis	Bacteriostatic
Ciprofloxacin	<i>Streptomyces spp</i>	Gram+	Block DNA replication	Bacteriocidal
Gentamicin	<i>Micromonospora spp</i>	Variety of Gram +	Induce abnormal protein synth.	Bacteriocidal
Tetracycline	<i>Streptomyces spp</i>	Many Gram +	Interfere with protein synthesis	Bacteriostatic

**Source: Antibiotics used for Chemotherapy of GBS infections (Pelczar *et al.*, 1993)**

### 2.7.1 Penicillin

Penicillin is a beta-lactam antibiotic. Penicillin-G (Benzylpenicillin) is a powerful antibacterial agent widely used to inhibit the polymerization and attachment of new cell wall peptidoglycan synthesis by binding to the penicillin-binding proteins (PBPs). Penicillins diffuse well into body tissues and fluids; but penetration into the cerebrospinal fluid (CSF) is poor except when the meninges are inflamed (BNF, 1993; Laurence *et al.*, 1998).

With its bacteriocidal effect, it is the first-line agent (drugs of choice) for the treatment of GBS infections (Laurence *et al.*, 1998; CDC, 2010). An important feature is that so far there have been only very few cases or not at all substantiated reports of penicillin resistance (Mims *et al.*, 2004; Murray *et al.*, 2007).

During the course of treatment, serious allergy to penicillins may occur in the form of an immediate (type1) hypersensitivity reaction, anaphylactic shock, rash, convulsion, diarrhea, platelet dysfunction, sodium overload. Penicillin-G can also produce neurotoxicity if given in high doses, particularly in patient with renal impairment. This toxicity is manifest as fits, unconsciousness and hallucinations (BNF, 1993; Laurence *et al.*, 1998; Mims *et al.*, 2004).



### **2.7.2 Ampicillin**

Ampicillin is a beta-lactam drug (broad spectrum penicillin) active against Gram positive and some Gram-negative organisms. Ampicillin is well excreted in the bile and urine. It is principally indicated for the treatment of exacerbations of chronic bronchitis, UTI, otitis media, sinusitis, invasive salmonella, and gonorrhoea (Laurence *et al.*, 1998; Mims *et al.*, 2004).

In GBS infections, no ampicillin resistance has been reported. Ampicillin can be given intravenously as an intrapartum antimicrobial prophylaxis in term women with culture evidence of recent vaginal or rectal GBS infection; starting 4hrs before delivery (CDC, 2010).

### **2.7.3 Vancomycin**

Vancomycin is a very large molecule from the glycopeptide antibiotics family. It has bactericidal activity and is used mainly for the treatment of infections caused by Gram-positive cocci and Gram-positive rods that are resistant to beta-lactam drugs. It is also used as alternative for patients who are allergic to penicillin (Laurence *et al.*, 1998; Mims *et al.*, 2004).

Because it is a very large molecule, it has difficulty moving through the outer membrane to the peptidoglycan of Gram-negative cells; which makes them “naturally” resistant. Some organisms may acquire resistance to vancomycin; this is the case of vancomycin-resistant enterococci (VRE) (Laurence *et al.*, 1998).

A variety of resistance phenotypes have been described which can be differentiated by transferability (e. g. plasmid association), inducibility and extent of resistance. The genes associated with vancomycin resistances are *vanA*, *vanB*, and *vanD* which encode a ligase producing pentapeptides terminating in D-alanine-D-lactate (Mims *et al.*, 2004).

In GBS infections, no vancomycin resistance has been reported.

#### 2.7.4 Erythromycin

Erythromycin is the most important macrolide that exerts its action by binding to 50S subunits of bacterial ribosome; and inhibits (or blocks) protein synthesis of most Gram positive bacteria, because they accumulate the drug more efficiently than Gram negative bacteria (Laurence *et al.*, 1998; Mims *et al.*, 2004; Woods *et al.*, 2010).

It is usually administered by the oral route, but can also be given intravenously (i/v). It is well distributed in the body and penetrates mammalian cells to reach intracellular organisms. The drug is concentrated in the liver and excreted in the bile. A small proportion of the dose is recoverable in the urine (Laurence *et al.*, 1998; BNF, 1993).

In GBS infections, erythromycin provides useful alternative therapy for penicillin-allergic patients, but increase in the incidence of resistance has been observed. The erythromycin resistance mechanism is due to ribosomal modification conferred by a family of methyl-transferase enzyme (methylase) encoded by *erm* (erythromycin ribosomal methylation) genes. Basically *erm* genes methylate 23S rRNA and induce ribosomal alteration which result in loss of binding and promote the so-called cross-resistance to macrolides-lincosamides-streptogramin resistance “MLS resistance” or MLS<sub>B</sub> (Back *et al.*, 2012). The erythromycin resistance is also due to efflux pump mediated by the plasmid-encoded *mef(A)* gene that cause resistance to 14- and 15-membered macrolides (Weisblum *et al.*, 1995).

The resistance can either be inducible (iMLS<sub>B</sub>), where the methylase is in the presence of the inducer; or constitutive (cMLS<sub>B</sub>), where the methylase is produced constitutively. Erythromycin is a better inducer of resistance than lincosamides (Quiroga *et al.*, 2008; Khan *et al.*, 2011). Strains resistant to erythromycin will also be resistant to lincomycin and clindamycin. Several erythromycin resistance determinants and genetic carrying elements in GBS strains have been reported such as *ermB*, *ermA/TR*, *mef(A)*, *mef(E)*, *orf*, *intTn* (Arpin *et al.*, 1999; De Azavedo *et al.*, 1999, De Mouy *et al.*, 2001; Zeng *et al.*, 2006).

### 2.7.5 Clindamycin

Clindamycin is a bacterial protein synthesis inhibitor and represents the most important drug in the class of lincosamides. It binds to the 50S ribosomal subunit. Its antibacterial spectrum is similar to erythromycin (they both have bacteriostatic effect) and to penicillin (Laurence *et al.*, 1998; Mims *et al.*, 2004). Clindamycin is usually given orally, but can be administered intramuscularly (i/m) or intravenously (i/v). It penetrates well into bone, but not into CSF, even when the meninges are inflamed (BNF, 1993; Laurence *et al.*, 1998).

In GBS infections, clindamycin provide useful alternative therapy for penicillin-allergic patients, but an increase in the resistance to this alternative drug has also been observed. Clindamycin resistance in GBS is due to ribosomal translocation encoded by *linB* genes. In this case, the resistance mechanism is the methylation of the 23S binding site. If this occurs then the bacteria are resistant to both macrolides and lincosamides (Heelan *et al.*, 2004). As clindamycin is a less potent inducer of 23S rRNA methylase as mentioned in MLS, erythromycin-resistant strains may appear susceptible to clindamycin *in vitro*. However, resistance will be manifest *in vivo* (Mims *et al.*, 2004).

### 2.7.6 Gentamicin

Gentamicin is the most important antibiotic of the aminoglycosides family widely used for the treatment of serious infections. With the bacteriocidal effect, it is active against many Gram-negative organisms and some Gram-positive (Laurence *et al.*, 1998; Tazi *et al.*, 2012).

Generally, gentamycin acts by binding to specific proteins in the 30S ribosome subunit, where it interferes with the binding of formylmethionyl-transfer RNA thereby preventing the formation of initiation complexes from which protein synthesis proceeds (Fluegge *et al.*, 2004; Mims *et al.*, 2004). It is not active against streptococci when used alone, but it acts in synergy with the beta-lactam agents (Laurence *et al.*, 1998; Fluegge *et al.*, 2004). Higher doses are occasionally indicated for serious infections, especially in the neonate or compromised host. A lower dose in association with Benzylpenicillin is sufficient for endocarditis due to oral and gut streptococci (Liddy *et al.*, 2002).

Production of aminoglycoside-modifying enzymes is the principal cause of resistance to aminoglycosides. The genes for these enzymes are often plasmid-mediated, located on transposons, and transferable from one bacterial species to another (Tazi *et al.*, 2012). Resistance to aminoglycoside antibiotics may occur by alteration of the 30S ribosomal target protein, but also through alterations in cell wall permeability or in the energy dependent transport across the cytoplasmic membrane (Mims *et al.*, 2004).

### **2.7.7 Tetracycline**

Tetracyclines are a family of large cyclic structures that have several sites for possible chemical substitutions. They are bacteriostatic and inhibit protein synthesis by binding to the small ribosomal subunit (30S ribosome) in a manner that prevents aminoacyl-tRNA from entering the acceptor sites on the bacterial ribosome; their selective action is due to higher uptake by bacterial than human cell (Laurence *et al.*, 1998; Mims *et al.*, 2004).

Generally, tetracycline resistance is due to a decrease in the levels of drug accumulation; and in most cases it is the plasmid encoded and inducible. The widespread use of this drug in human and also to their use as growth promoters in animal feed is one of the common causes of resistance (Mims *et al.*, 2004).

In GBS, tetracycline resistance is common; and genes are often found on the same mobile unit as erythromycin resistance; carried on transposon and usually due to ribosomal protection encoded by genes *tet(M)* and *tet(O)* or less commonly to an efflux pump encoded by *tet(K)* (Betrucci *et al.*, 2003).

### **2.7.8 Ciprofloxacin**

Fluoroquinolones have bactericidal effect. They bind and inhibit the activity of enzyme DNA gyrase and topoisomerase IV and prevent them from decatenating. Because of their safety and tolerability, they are commonly used as alternatives to beta-lactam antibiotics for treating a variety of infections (Mims *et al.*, 2004).

Ciprofloxacin is the 2<sup>nd</sup> generation of quinolones that is active against Gram-negative bacteria, also has moderate activity against Gram-positive bacteria. Uses for ciprofloxacin include infections of respiratory, urinary tracts, and of gastro-intestinal

system and septicemia caused by sensitive organisms (Laurence *et al.*, 1998; Mims *et al.*, 2004).

Mutations at key sites in DNA gyrase or topoisomerase IV can decrease their binding affinity to quinolones, decreasing the drug's effectiveness and create resistance (Wu *et al.*, 2008). In GBS infections, the resistance is caused by mutation position in the quinolone resistance-determining regions (QRDR<sub>S</sub>) of the genes coding for type II topoisomerase enzymes, i.e. DNA gyrase (*gyrA/gyrB*) and topoisomerase IV (*parC/parE*) (Wehbeh *et al.*, 2005; Wu *et al.*, 2008).

### **2.7.9 Chloramphenicol**

Chloramphenicol is a relatively simple molecule containing a nitrobenzene nucleus, and prevents peptide bond synthesis, with a bacteriostatic result. It may be bactericidal against *H. influenza*, *N. meningitis* and *S. pneumonia* (Laurence *et al.*, 1998).

It acts by binding on the 50S ribosome subunit, where it blocks the action of peptidyl-transferase, thereby preventing peptide bond synthesis. It has been used in the treatment of bacterial meningitis since the drug achieves satisfactory concentration in the CSF. It is active against both Gram-positive bacteria and Gram-negative bacteria, but it is a potent and potentially toxic. Its toxicity renders it unsuitable for systemic use except in some circumstances (Laurence *et al.*, 1998; Mims *et al.*, 2004). The most common mechanism of chloramphenicol resistance involves the inactivation of the drug by a plasmid mediated enzymatic, mechanism which is easily transferable to bacterial population, especially Gram-negative bacteria (Mims *et al.*, 2004).

### **2.8 Multiplex Polymerase Chain Reaction in determining GBS resistance**

Multiplex Polymerase Chain Reaction (PCR) is a variant of PCR which enable simultaneous amplification of many targets of interest in one reaction by using more than one pair of primers. It consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences (Chamberlain *et al.*, 1988).

Multiplex PCR is becoming a rapid and convenient screening assay in both clinical and research laboratories. This method has been applied in many areas of DNA testing, including analyses of deletions, mutations, and polymorphisms, or quantitative assays and reverse transcription PCR (Henegariu *et al.*, 1997). For a successful multiplex PCR assay, strategic planning and multiple attempts to optimize reaction conditions are required. The relative concentration of the primers, concentration of the PCR buffer, balance between magnesium chloride and deoxynucleotide concentrations, cycling temperatures, and amount of template DNA and Taq DNA polymerase are important (Markoulatos *et al.*, 2002).

In GBS infections, multiplex PCR has been used to detect antibiotic resistant genes. In a study performed in four countries Australia, New Zealand, South Korea and China, multiplex PCR methods detected 7 genes encoding antibiotics resistance against GBS, *erm(A/TR)*, *erm(B)*, *mef(A)*, *tet(M)*, *tet(O)*, *aphA-3*, and *aad-6*; and antibiotic related gene (*int-Tn*) and *mreA*, encoding flavokinase, were also present in all GBS isolates. This study has shown the higher rate of phenotypic resistance to erythromycin, clindamycin and tetracycline which varied with geographic area (Zeng *et al.*, 2006).

Gygax *et al.*, in 2007 used a multiplex PCR assay to detect erythromycin and clindamycin resistance genes, *ermB*, *ermTR*, and *mefA/E*, in GBS clinical isolates and in DNA extracted from the corresponding cervico-vaginal-rectal (CVR) swabs. The results were compared to the standard erythromycin / clindamycin double disk diffusion assay of 46 isolates. The PCR could accurately detect resistance genes and predict the resistance phenotype from purified GBS isolates.

Recently in Poland, in 2012, standardization of multiplex PCR with the use of seven primer pairs was performed on 81 bacterial strains representing different GBS isolates and other Gram-positive cocci. As a result, seven genes important for screening of GBS infection were detected: *cfb* gene encoding the CAMP factor presented in every GBS; the *cps* operon genes such as *cps1aH*, *cps1a/2/3IJ*, and *cps5O* specific for capsular polysaccharide types Ia, III, and V, respectively were also detected; the macrolide resistance genes *ermB* and *mefA/E* were detected; and

finally the *gbs2018* S10 region specific for ST17 hyper-virulent clone were also detected (Gosiewski *et al.*, 2012).

## **2.9 Prevention of GBS infections**

Pregnant women with the following conditions are at higher risk of having a baby with GBS disease: previous baby with GBS disease, urinary tract infection due to GBS, fever during labor, prolonged or difficult labor, rupture of membranes before 37 weeks of pregnancy, and rupture of membrane 18 hours before delivery (Murray *et al.*, 2007).

The Centre for Disease Control and Prevention (CDC) recommends the use of either risk assessment or screening for GBS colonization in pregnant women to identify candidates for intrapartum prophylaxis. Risk assessment is performed at the onset of labor and conditions sus-mentioned and considered indicative of the need for prophylaxis. The current screening approach to prevention of GBS infections in pregnancy has become standard. This approach requires intra-partum antimicrobial prophylaxis in term women with culture evidence of recent vaginal or rectal GBS infection. The affected pregnant women can be given intravenous antibiotics during labor; starting 4h before delivery. These recommendations are based on the fact that administration of antimicrobial agents during labor to women at risk of transmitting GBS to their newborns could prevent invasive disease in the first week of life (Bergseng *et al.*, 2007; Murray *et al.*, 2007; CDC, 2010).

## **2.10 Vaccines against GBS Disease**

GBS vaccines have been recognized as a powerful tool for reducing maternal colonization and preventing transmission to neonates, but no licensed vaccine is yet available (CDC, 2010). Sufficient amounts of GBS capsular polysaccharide type-specific serum IgG in mothers have been shown to protect against invasive disease in their infants. Phase I and II clinical trials among healthy non-pregnant adults of monovalent polysaccharide-protein conjugate vaccines of GBS have shown these vaccines to be well tolerated and immunogenic (Baker *et al.*, 1988; CDC, 2010).

Vaccine development was once promising, but shifting serotypes of GBS responsible for clinical disease have limited this approach. Other factors that have made this

approach less attractive include problems related to access to vaccination by women of childbearing age and the emotion and possible litigation associated with vaccination during pregnancy (Bergseng *et al.*, 2007; Woods *et al.*, 2010).



## **CHAPTER III**

### **MATERIALS AND METHODS**

#### **3.1 Study area**

This study was conducted from February 2012 to December 2012 at Dr. George Mukhari Academic Hospital, formerly Ga-Rankuwa Hospital, in Pretoria (Tshwane); South Africa. This is an academic Hospital associated with the University of Limpopo, MEDUNSA Campus (Medical University of Southern Africa), located about 37 km north of Pretoria in Gauteng Province. It lies at an altitude of about 1.350m (4.500 ft) above the sea level; in a Longitude of 25°37'14" S and a Latitude of 28°1'1" E.

#### **3.2 Ethical Consideration**

The study was approved by the Medical Research and Ethics Committee of South Africa (MREC/P/02/2011: IR) and Directorate for Health and Social Affairs (MEDUNSA) and the College of Agriculture and Environmental health Sciences, University of South Africa (CAES – UNISA).

#### **3.3 Experimental design**

This was a qualitative and prospective study. The study population constituted pregnant women and newborn babies. Samples were obtained from patients attending the antenatal clinic (ANC) from 16 weeks of gestation to form a cohort group.

Specimens were collected from the mothers (high vaginal swab, lower vaginal swab and rectal swab), and from babies (ear swab, umbilical swab and cord blood) and transported to the laboratory for culture within 4 hours of collection. Processing of samples followed the standard operating procedures (SOPs) developed for the study.

### **3.4 Sampling**

#### **3.4.1 Sample size**

Based on an estimation of 30 pregnant women seen at the antenatal clinic (ANC) per day, the estimated population was 20 000 women over a 2 year period. At an expected frequency of 10%, confidence level of 95% and a margin of error of 5% sample size was estimated at about 300. Due to the challenges of loss to follow up which are expected with a cohort, the sample size was increased to 500 to accommodate for anticipated lost follow-up and home deliveries. However only 413 samples were collected from pregnant women and 39 samples were collected from babies. MS Excel and Epi info was used to capture the data and analysis was partially by Epi info and PSSP.

#### **3.4.2 Sample Collection**

Random convenience sampling method was used. All pregnant women seen at the ANC were informed about the nature and implication of the study and those who agreed (volunteered) to participate were requested to sign a consent form (Appendix A) before they were recruited. Recruitment continued until sample size of 413 was reached. This then formed the cohort that was followed-up to delivery and post-delivery including their babies.

A questionnaire (Appendix B) was also used to gather demographic, obstetric and current pregnancy history data from all pregnant women recruited. At delivery the baby's details were also recorded (Appendix C).

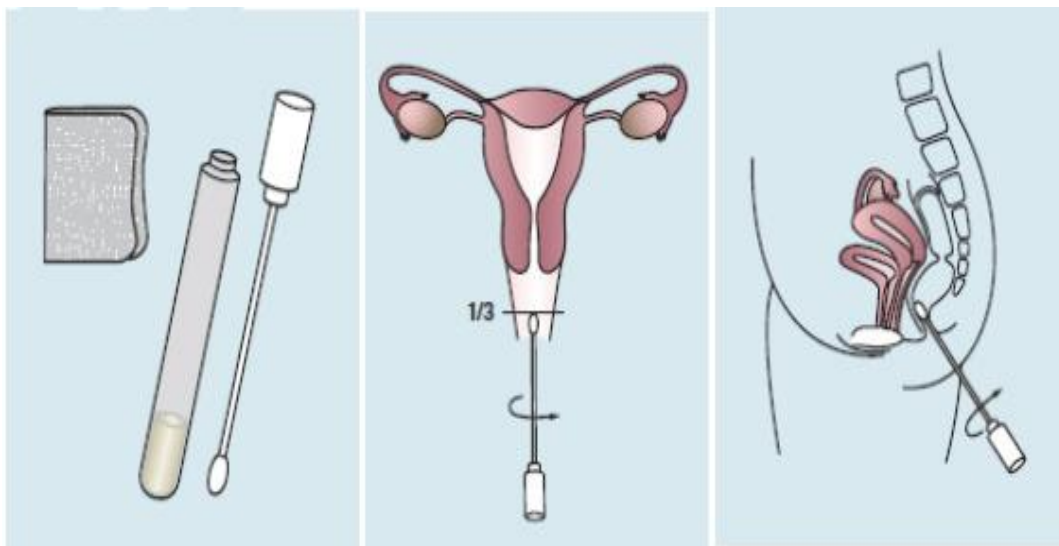
Gynecologist, obstetrician, pediatrician and the research nurses collected the specimens. The procedure for the collection was explained to the patients before the specimens were taken (Figure4). Samples were taken aseptically. High vaginal swabs (HVS), low vaginal swabs (LVS) and rectal swabs (RS) were collected from pregnant women (figure 4) attending ANC at DGMH, at the gestational period of 16 – 38 weeks, who signed informed consent, and received no antibiotics for at least two weeks prior to sample collection. At delivery repeat samples were collected from pregnant women and umbilical swabs (US) and ear swabs (ES) were collected from their newborn babies too.

**Inclusion criteria:**

Pregnant woman attending ANC at DGMAH from 16 weeks gestation, aged of 18 years and above, and babies born from identified positive women

**Exclusion criteria:**

Pregnant women who were not willing or unable to give consent; those who were on antibiotic treatment 2 weeks prior to recruitment



**Figure 4:** Site for collection of recto-vaginal specimen for GBS isolation

(Adapted from BD diagnostics [www.geneohm.com](http://www.geneohm.com); Musa *et al.* 2012).

**3.4.3 Culture of specimens**

Swabs were placed into a Amie's transport medium, (Rochelle Chemicals & Lab equipment, SA), properly labeled and put into a cooler box containing ice packs, and transported to the laboratory of Microbiological Pathology department, University of Limpopo, Medunsa campus within 2 - 4 hours of collection. Specimens collected from mothers and babies were cultured on selective medium, 5% blood of Columbia colistin and nalidixic acid (CNA) agar (DMP – NHLS, SA); and all incubated for 24 - 48hours in a 5% CO<sub>2</sub> atmosphere at 37°C. Growth was quantified as abundant or +++ (more than 21 colonies per plates), moderate or ++ (11 - 20 colonies) and sparse or + (1 - 10 colonies). The negative swabs from CNA were then inoculated in enriched selective GBS broth, Todd-Hewitt broth (DMP-NHLS, SA), with the same antibiotics concentration as in CNA (15 mg/l nalidixic acid and 8 mg/l gentamicin).

Growth was quantified as heavy, moderate or scanty considering the growth from the main inoculum and the streaks.

Todd Hewitt broth was incubated overnight at 37°C sub-cultured onto blood agar and plates read after 24 – 48 hours of incubation in a CO<sub>2</sub> enriched environment at 37°C. Isolates with colony morphological description similar to GBS were further identified for confirmation.

#### **3.4.4 Confirmatory tests for identification of GBS**

Isolates were confirmed as GBS by using the following methods: morphology of bacteria, hemolytic activity, Catalase test, microscopy (Gram's stain), bile esculin, and CAMP reaction following by latex agglutination test (Streptex – Slidex® Strepto Plus – BioMérieux – France, Randburg – SA) for antigen detection.

For validation of the tests, *S. agalactiae* ATCC 12403 was used as positive control as previously described in the literature (Kimura *et al.*, 2008);

##### **3.4.4.1 Hemolytic activity**

Any blood agar plate showing a complete destruction of red blood cells leaving a clear zone ( $\beta$ -hemolysis) were scored as a positive reaction indicating GBS growth. Some isolates were non-hemolytic but positive for GBS.

##### **3.4.4.2 Gram stain**

The objective was to differentiate bacteria in two groups depending on their abilities to retain a particular stain in the cell wall. After the process of staining of the fixed smear containing organism with a primary stain (crystal violet) for 1 minute; then the application of Gram's iodine (Mordant) for 1 minute; then the washing of the stained slide for 30 seconds with a decolorizing agent (95% ethanol); and finally the application of counterstain (Safranin) for 3 minutes. Gram-positive bacteria stained purple and Gram-negative pink. It should be noted that GBS is a gram positive coccus.

#### **3.4.4.3 Catalase test**

The objective of this test was to determine the presence of catalase enzyme in isolate using hydrogen peroxide ( $H_2O_2$ ) tests. Few drop of  $H_2O_2$  (3%) were placed on a clean slide, using sterile applicator sticks, isolates were mixed with  $H_2O_2$ . The vigorous gas bubbles production indicated positive reaction. GBS is catalase negative.

#### **3.4.4.4 Bile Esculin test**

The objective of this test was to distinguish GBS from group D streptococci and Enterococci; bacteria able to hydrolyse esculin in the presence of bile. Bile esculin medium contains esculin and peptone for nutrition and bile to inhibit Gram-positive bacteria other than Group D streptococci and enterococci. Ferric citrate is added as a colour indicator. Organisms that split the esculin molecules and use the liberated glucose to supply energy needs release esculetin into the medium. The free esculetin reacts with ferric citrate in the medium to form a phenolic iron complex, which turns the agar slants dark brown to black. An agar slant that is more than half darkened after no more than 48 hours' incubation is bile-esculin positive. If less than half the slant has darkened, the result is negative. It should be noted that GBS is bile negative. *Enterococcus faecalis* ATCC 29212 was used as positive control.

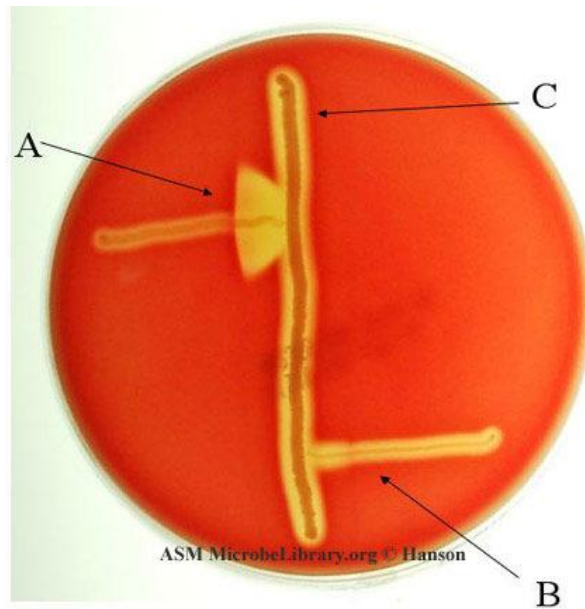
#### **3.4.4.5 Latex agglutination test**

Lancefield group B antigen detection was performed according to the manufacturer's instruction. Latex agglutination test is a rapid test detecting protein antigen. Colonies were identified as GBS using a commercial latex agglutination test or streptex (Slide® Strepto Plus - BioMérieux® – France; Randburg – SA).

#### **3.4.4.6 CAMP reaction (Christie Atkins and Munch-Petersen)**

The objective of this test was to separate GBS from other  $\beta$ -hemolytic species. GBS produce a peptide, the CAMP substance, which acts in concert with the  $\beta$ -haemolysis produced by some strains of *Staphylococcus aureus*, resulting in an increased haemolytic effect (Figure 5). Sheep blood agar plate was taken. Using a sterile loop, a single line of *S. aureus* was streaked on agar, and another line of the isolate was streaked as a "T" without allowing the horizontal lines to touch the vertical line. And plate was incubated at 37°C in an inverted position. Upon

successful completion of CAMP reaction, we noted a formation of an arrowhead pattern of hemolysis at the intersection of the two isolates. *Staphylococcus aureus* ATCC 29213 was used for the test.



**Figure 5:** CAMP test for the identification of *S. agalactiae* (Adapted from ASM MicroLibrary.org)

- (A) *Streptococcus agalactiae* (group B) shows a positive CAMP reaction.
- (B) *Streptococcus pyogenes* (group A) shows a negative reaction
- (C) *Staphylococcus aureus*

### 3.4.5 Storage of isolates

All isolates that were confirmed to be GBS were stored at  $-80^{\circ}\text{C}$  in cryovials (microbank) containing cryo-preservative fluid for future use. A data sheet and work sheet were developed (Appendix D and E).

## **3.5 Antibiotic Susceptibility Testing**

### **3.5.1 Phenotypic testing**

To determine the phenotypic resistance, the susceptibility testing was done qualitatively using Disk Diffusion (Kirby-Bauer) method and Double-disk Diffusion (D-test) as previously described (Quiroga *et al.*, 2007; Khan *et al.*, 2011); and quantitatively using the Epsilometer tests (E-Tests) as previously described (Manning *et al.*, 2001). Agar based susceptibility testing methods (Disk Diffusion and E-Tests) are widely used due to their simplicity, reproducibility, and lack of requirement for specialized equipment comparatively to the automated system or Vitek (Tang *et al.*, 2004).

#### **3.5.1.1 The Disc Diffusion Test (Kirby-Bauer method)**

This is a standardized qualitative testing method used to measure the effectiveness of a variety of antibiotics on specific bacteria. A minimum of two pure small colonies were selected from an overnight positive GBS plate, suspended into normal saline and adjusted until the turbidity was equivalent to the 0.5McFarland standard (Rochelle Chemicals). The sterile swab was dipped into the suspension and used to inoculate onto Mueller-Hinton agar (MHA) supplemented with 5% sheep blood. By using the dispenser or sterile forceps, the antibiotic impregnated paper disks were placed onto agar; and incubated at 37°C for 20-24h in 5% CO<sub>2</sub> environment. The plates were read the following day; the zones of growth inhibition were measured to the nearest whole millimeter using a sliding caliper or ruler. Each isolate was classified as susceptible, intermediate or resistant to each antibiotic tested.

The susceptibility to ampicillin (AMP) (10µg), vancomycin (VA) (30µg), ciprofloxacin (CIP) (5µg), high level gentamicin (CN) (120µg), chloramphenicol (C) (30µg), and tetracycline (TE) (30µg) was tested (Oxoid, Davies-Diagnostics, South Africa).

Results were interpreted according to Clinical Laboratory Standard Institute (CLSI) guidelines 2012, breakpoints criteria M100-S22, as indicated in Table II.

### **3.5.1.2 The Double Disk Diffusion Test (D-test)**

This qualitative method was used for phenotypic characterization and detection of inducible clindamycin resistance.

Erythromycin (15µg) and clindamycin (2µg) disks (Oxoid, Davie's – Diagnostics, SA) were placed 12 mm apart, edge to edge (CLSI, 2012) on Mueller-Hinton agar supplemented with 5% sheep blood agar (DMP – NHLS, SA) which were inoculated with a 0.5 McFarland suspension of the organism. The plates were incubated for 20 – 24 h at 37°C in 5% CO<sub>2</sub> atmosphere.

Blunting was defined as growth within the clindamycin zone of inhibition proximal to the erythromycin disk, indicating MLS<sub>B</sub>-inducible methylation. Resistance to both erythromycin and clindamycin indicated MLS<sub>B</sub>-constitutive methylation. Resistance to erythromycin but susceptibility to clindamycin without blunting indicated an efflux mechanism (M phenotype). And finally resistance to clindamycin but susceptible to erythromycin was referred as L phenotype as previously described (Desjardins *et al.*, 2004; Quiroga *et al.*, 2007; Khan *et al.*, 2011).

### **3.5.1.3 The Epsilometer test (E-Test)**

This quantitative method was done following the manufacturer's procedure. Briefly, a commercially available paper strip (AB Biodisk, Solna – Sweden, Davie's Diagnostics – South Africa), which is soaked with antibiotic gradients (antibiotic concentration decreased from one point of the paper strip to the other) was placed onto Mueller-Hinton agar supplemented with 5% sheep blood (DMP – NHLS, SA) following bacterial inoculation (0.5 McFarland of bacterial suspension). The plates were incubated at 37°C for 24 h, in a 5% CO<sub>2</sub> enriched environment at 37°C. MICs were read as the lowest concentration of antibiotic that inhibit the bacterial growth.

Susceptibility to penicillin, erythromycin and clindamycin were tested. And results were interpreted according to CLSI guidelines 2012.



**Table II: Interpretation of size of zone of inhibition, Breakpoints criteria (CLSI, 2012: M100-S22)**

Antibiotic	Disc Concentration	Diameters breakpoints (mm)		
		Susceptible (S)	Intermediate (I)	Resistant (R)
<b>E – test</b>				
- Penicillin	10units	≤ 0.12	-	-
- Erythromycin	15µg	≤ 0.25	0.5	≥1
- Clindamycin	2µg	≤ 0.25	0.5	≥1
<b>Disc Diffusion</b>				
- Penicillin	10U	≥ 24	-	-
- Ampicillin	10µg	≥ 24	-	-
- Vancomycin	30µg	≥ 17	-	-
- Erythromycin	15µg	≥ 21	16-20	≤15
- Clindamycin	2µg	≥ 19	16-18	≤15
- Chloramphenicol	30µg	≥ 21	18-20	≤17
- Tetracycline	30µg	≥ 23	19-22	≤18
- Gentamicin	120µg	≥ 15	13-14	≤12
- Ciprofloxacin	10µg	≥ 21	16-20	≤15

**Source:** OXOID, 2012; CLSI, 2012. Performance standard for antimicrobial susceptibility testing, M100-S22, Table 2H-1, Wayne, Pa: CLSI recommends disk diffusion (M-2) or broth micro-dilution testing (M-7) for susceptibility testing of GBS.

### Quality Control

The Clinical and Laboratory Standards Institute (CLSI) antimicrobial susceptibility testing standards were used to derive a correlation between the zone of inhibition and the Minimum Inhibitory Concentration (MIC) of the test organism; and results were interpreted according to CLSI guidelines for streptococcus spp, β-hemolytic group 2012 breakpoint for disk diffusion.

A penicillin-susceptible *S. agalactiae* ATTC 12403 and *S. pneumoniae* ATCC 49619 were used as positive quality control strains for measurement of the exact MICs as previously described (Kimura *et al.*, 2008; CLSI, 2012).

### **3.5.2 Genotypic Identification**

During the study, molecular techniques were used for the identification of genotypic resistance among all the erythromycin and clindamycin resistant isolates for the detection of resistant genes.

#### **3.5.2.1 DNA extraction**

DNA extraction was done using the Zymo Research–DNA Mini-Kit (Zymo-Research–USA; Inqaba Biotec – SA) and following the manufacturer instructions (Appendix G).

Briefly, 200 µl of sterile water was added into 1.5ml micro-centrifuge tube. Bacterial cells (from overnight growth) were suspended into the above water and 100 mg (wet weight/colonies) of the suspension was added into ZR Bashing-Bead Lysis tubes (provided by supplier). 750 µl of lysis solution was added to the tube (ZR Bashing-Bead Lysis tubes) and secured in a bead beater (Disruptor Genie) and processed at maximum speed for 5 minutes.

The ZR Bashing-Bead Lysis Tubes were centrifuged at 10,000 x g (10,000 RPM) for 1 minute. Up to 400µl of supernatant was transferred to a Zymo-Spin IV Spin Filter (orange top) in a collection tube and centrifuged at 7,000 x g (7,000 RPM) for 1 minute. 1200 µl of Bacterial DNA Binding buffer was added to the filtrate in the collection tube from step above. 800 µl of the mixture from step above was transferred to a Zymo-Spin IIC column in a collection tube and centrifuged at 10,000 x g (10,000 RPM) for 1 minute; the flow from the collection tube was discarded (this step was repeated twice). 200 µl DNA Pre-Wash Buffer was added to the Zymo-spin IIC column in a new collection tube and centrifuged at 10,000 RPM (10,000 x g) for 1 minute. 500 µl Bacterial DNA Wash Buffer was added to the Zymo-spin IIC column and centrifuged at 10,000 RPM (10,000 x g) for 1 minute. The Zymo-Spin IIC column was transferred to a clean 1.5 ml microcentrifuge tube. Finally 100 µl DNA Elution Buffer was added directly to the column matrix and centrifuged at 10.000 RPM (10,000 x g) for 30 seconds to elute the DNA.

A total of 5 µl of the Ultra-pure DNA was used in the experiments

### 3.5.2.2 Primer sets used for Multiplex PCR

To ensure easy detection of fragments, multiplex PCR was designed. The primers were separated according to their fragment sizes, to make reading of the band sizes much easier. Three genes for macrolide resistance, *ermB*, *ermTR*, *mefA* and one gene for clindamycin resistance *linB* were identified with sets of specific primers as previously described (Sutcliffe *et al.*, 1999; De Azavero *et al.*, 2001; Desjardins *et al.*, 2004; Gygax *et al.*, 2007).

Table III below shows specific primers (synthesis at Inqaba Biotec – SA) used for the detection of resistance genes in GBS, their gene targets and their product size.

**Table III:** Specific primers used for the detection of resistance genes in GBS

Gene targets	Primers name	Primers sequence (5'- 3')	Products size: bp	References
<i>ermB</i>	<i>ermB1</i>	5'_-GAA AAG GTA CTC AAC CAA ATA-3'_ (F)	640	Sutcliffe <i>et al.</i> , 1999; De Azavado <i>et al.</i> , 2001
	<i>ermB2</i>	5'_-AGT AAC GGT ACT TAA ATT GTT TAC-3'_ (R)		
<i>ermTR</i>	<i>ermTR1</i>	5'_-GAA GTT TAG CTT TCC TAA-3'_ (F)	400	Desjardins <i>et al.</i> , 2004 Gygax <i>et al.</i> , 2007
	<i>ermTR2</i>	5'_-GCT TCA GCA CCT GTC TTA ATT GAT-3'_ (R)		
<i>mefA</i>	<i>mefA1</i>	5'_-AGT ATC ATT AAT CAC TAG TGC-3'_ (F)	348	Sutcliffe <i>et al.</i> , 1999; Gygax <i>et al.</i> , 2006
	<i>mefA2</i>	5'_-TTC TTC TGG TAC TAA AAG TGG-3'_ (R)		
<i>linB</i>	<i>linB1</i>	5'_-CCT ACC TAT TGT TTG TGG AA-3'_ (F)	944	Gygax <i>et al.</i> , 2006; Gygax <i>et al.</i> , 2007
	<i>linB2</i>	5'_-ATA ACG TTA CTC TCC TAT TC-3'_ (R)		

### 3.5.2.3 Amplification of erythromycin and clindamycin genes

An SOP was developed and different genes of resistance to GBS were detected by using Multiplex PCR and conditions as previously described (Gygax *et al.*, 2007).

A 50µl PCR reaction contained 2.5 mM Tris-HCl pH 8.6, 2.5mM KCl, 2.5 mM MgCl<sub>2</sub>, 5 mM dNTP, 0.5U Taq DNA polymerase (Thermo Scientific – Phusion Flash High-Fidelity PCR Master Mix), PCR water, and 1 µM primers pairs (Inqaba - Biotec - SA). A total of 5µl template DNA was used in the PCR.

The cycling conditions on a My Cycler™ thermal cycler (BioRad) consisted of a single cycle of 95°C for 3 minute followed by 35 cycles of denaturation at 95°C for 1 minute, annealing at 57°C for 1 minute and extension at 72°C for 1 minute. A final

extension step of 72°C for 5 minutes was followed by a hold at 4°C (Desjardins *et al.*, 2004).

#### **3.5.2.4 Detection of amplified products**

Electrophoresis on 1% agarose gels in 40 mM Tris acetate–2 mM EDTA buffer was used to distinguish PCR products (PCRPs) as previously described (De Azavedo *et al.*, 2001). The different genes of resistance were analyzed based on presence or absence of bands in the agarose gel

A culture of GBS ATTC 49447 was used as negative control.

#### **3.6 Results analysis**

All data were captured using Microsoft Excel 2010. The different variants were analyzed based on the parameter considered; and finally IBM-SPSS Software Version 21 was partially used to analyze data.

## CHAPTER IV

### RESULTS

Over a period of 11- months; February to December 2012, a total of 413 adult black pregnant women at Dr. George Mukhari Academic Hospital were recruited; and at delivery 39 samples were collected from the babies born to colonized mothers. This study population comprised only of the pregnant women who agreed to participate in the study and who met the selection criteria as listed in point 2.3.2.

#### 4.1 GBS culture

Of the 413 pregnant women recruited, 128 (30.9%) were colonized with GBS and of 39 babies recruited none of them (0%) was colonized with GBS (see the Table IV below)

**Table IV: GBS colonization by site and by use of GBS selective media.**

Number of samples collected	Mothers					Babies	
	413					39	
Number of positive GBS isolated	CNA				TH	CNA	
	RS	LVS	HVS	All sites	70	ER	US
	22	9	3	24		-	-
	58					-	-
	128 (30.9%)					0	
GBS negative	285 (69.1%)					39 (100%)	

**Key:** CNA : Colistin nalidixic acid agar

TH : Todd-Hewitt broth

RS : Rectal swab

LVS : Lower vaginal swab

HVS : Higher vaginal swab

ES : Ear swab

US : Umbilical swab

The table IV shows that 128/413 (30.9%) pregnant women were GBS positive and 285 (69%) were GBS negative. The difference between the GBS positive and the negative GBS was not statistically significant.

From the 128 positive GBS isolated; 70 (54.6%) were recovered from Todd-Hewitt broth and 58 (45.3%) from CNA agar. The difference was no significant between positive GBS recorded from Todd-Hewitt broth and CNA agar. From CNA agar, rectal site was found to be the commonest site of the colonization; with an overall of 22/58, following by LVS (9/58) and HVS (3/58) and finally 24/58 isolates were found in all sites at the same time. Thirty-nine samples were collected from the babies born to colonized mothers and none of them were GBS positives. Most of these babies were born by cesarean section.

## 4.2 Study Participants

### 4.2.1 Demographic Data

The questionnaire was used to collect and record demographic data. The socio-demographic characteristics of 413 pregnant women screened for GBS colonization against the positive women (n=128) at Dr. George Mukhari Hospital, Ga-Rankuwa in Pretoria (Feb 2012 - Dec 2012) is presented in tables Va, Vb, Vc and Vd below.

**Table Va: Age distribution of women recruited (n=413) in comparison to women colonized with GBS (n=128)**

Age in years	18 – 19	20 – 24	25 – 29	30 – 34	35 – 39	40 – 45
Women recruited (%)	12 (2.9)	75 (18.1)	110 (26.6)	95 (23.0)	81 (19.6)	30 (7.2)
GBS Positive (%)	2 (1.5)	28 (21.8)	39 (30.4)	24 (18.7)	24 (18.7)	10 (7.8)

Table Va shows the difference in age distribution between participants recruited and GBS positive women was not significant. The age range of participants was between 18–44 years with the majority of the participants recruited 110/413 (26.6%) being between the ages of 25 – 29 years old and 30.4% of them being colonized with GBS. Of 95 (23%) women recruited were between 30 – 34 years old and 24 (18.7%) of them were GBS positive. Women of age range 18 – 19 years had the lowest rate of

GBS colonization (1.5%). During the recruitment time and processing, ten data were lost and one of them was GBS positive.

**Table Vb: Comparison of marital Status of women recruited (n=413) against women colonized with GBS (n=128)**

Marital status	Single	Married	Divorced	Widowed	Co-habiting
Women recruited (%)	209 (50.6)	98 (23.7)	03 (0.7)	02 (0.4)	89 (21.5)
GBS Positive (%)	68 (53.1)	27 (21)	1 (0.7)	0 (0)	31 (24.2)

Considering the marital status of the participants and GBS colonized pregnant women, the difference was not significant. The table Vb shows that almost half of the participants were single (50.6%); followed by 23.7% of participants who were married; 21.5% of participants were co-habiting; 3 (0.7%) and 2 (0.4%) participants were divorced and widowed, respectively.

**NB.** The term “**Cohabiting**” refers to the arrangements where two people who are not married live together in an intimate relationship, particularly an emotionally and/ or sexually intimate one, on a long-term or permanent basis. This is a common pattern among people for a number of reasons such as financial, housing, or seeing no need to marry (and some people don’t believe in marriage), etc.

**Table Vc: Educational level of women recruited (n=413) in relation to women colonized with GBS (n=128)**

Educational level	Below-matric	Matric	Tertiary
Women recruited (%)	107 (25.9)	229 (55.4)	67 (16.2)
GBS Positive (%)	36 (28.1)	76 (59.3)	15 (11.7)

Considering the educational level, the table Vc shows that approximately 55.4% (229/413) of participants recruited reached the matric level and 59.3% (76/128) of them tested positive for GBS; while 107/413 (25.9%) were below matric and 36/128 (28.1%) were GBS positive; and 67/413(16.2%) with tertiary level education of which 15 (11.7%) were GBS positive.

**Table Vd: Employment status of women recruited (n=413) in comparison to GBS colonization (n=128)**

Occupational status	Employed	Unemployed	Skilled	Semi-skilled	Unskilled
Women recruited (%)	148 (35.8)	255 (61.7)	41 (9.9)	67 (16.2)	18 (4.3)
GBS Positive (%)	51 (39.8)	76 (59.3)	10 (7.8)	22 (17.1)	3 (2.3)

Out of 413 participants recruited, 225 (61.7%) were unemployed and 59.3% of them were colonized with GBS and 35.8% of participants employed where 39.8% of them tested positive for GBS. Forty-one participants (9.9%) were skilled, 67 (16.2%) were semi-skilled, 18 (4.3%) were unskilled.

#### **4.2.2 Data on obstetric and history of current pregnancy**

The obstetric and history of current pregnancy were recorded. Of the 128 colonized pregnant women, 22 (25%) reported previous history of miscarriage; 12 (9.3%) history of stillbirth and 62 (48.4%) had given normal vaginal delivery in which 25 (12.1%) had given birth by caesarean section (C/S) and 4 women (1.9%) had terminated their pregnancy due to several reasons.

Of the 128 colonized women, 56 (43.7%) complained of flu like illness during early pregnancy; 6 (4.6%) had trauma in pregnancy; and 22 (17.1%) had vaginal discharge requiring treatment.

From 128 GBS positive women, a total of 52 (40.6%) were HIV positive and 72 (56.2%) were HIV negative.



### 4.3 Antimicrobial susceptibility

Purification of isolates was done before susceptibility testing. The susceptibility pattern was performed on 128 positive GBS isolates against 9 antimicrobial agents and results are presented in Table VIa, VIb, VIc.

**Table VIa:** Susceptibility profile of GBS isolates using disc diffusion method (n=128)

Antibiotics	No of susceptible (%)	No of intermediate (%)	No of resistant (%)
Ampicillin	128 (100)	-	-
Vancomycin	128 (100)	-	-
Gentamicin-High level	128 (100)	-	-
Ciprofloxacin	104 (71.0)	17 (13.2)	7 (5.4)
Chloramphenicol	96 (65.6)	11 (8.5)	21 (16.4)
Tetracycline	07 (5.4)	10 (7.8)	111 (86.7)

Table VIa shows that all strains were 100% susceptible to ampicillin, vancomycin and to high level gentamicin; however 86.7% of the isolates were resistant to tetracycline, 16.4% to chloramphenicol and 5.4% to ciprofloxacin. The intermediate values were observed against ciprofloxacin (13.2%), chloramphenicol (8.5%) and tetracycline (7.8%). And when assimilated all the intermediate values to resistant; this gave the overall result of 94.5% of isolates resistant to tetracycline, 24.9% resistant to chloramphenicol and 18.6% resistant to ciprofloxacin. Figure 7 shows the result of susceptibility testing by Kirby Bauer.



**Figure 6:** Susceptibility testing results by disc diffusion method. Isolate no 83 sensitive to ampicillin, vancomycin, ciprofloxacin, high-level gentamicin; but resistant to chloramphenicol and tetracycline.

**Table VIb:** E – test MIC results for GBS isolates (n=128)

Penicillin		Erythromycin		Clindamycin	
MIC µg/ml	No of isolates (%)	MIC µg/ml	No of isolates (%)	MIC µg/ml	No of isolates (%)
< 0.12	128 (100)	< 0.25	101	< 1	106
0.19 – 1	-	0.50 – 1	12	0.50 – 1	12
> 1	-	> 1	15	> 1	10

All the isolates tested were 100% sensitive to penicillin with the MICs ranging from  $\leq 0.047 \mu\text{g/mL}$  to  $\geq 0.12 \mu\text{g/mL}$ . 101/128 (78.9%) and 106/128 (82.8%) isolates were sensitive to erythromycin and clindamycin respectively. The MICs for erythromycin and clindamycin both ranged from  $\leq 0.016 \mu\text{g/mL}$  to  $\geq 0.25 \mu\text{g/mL}$ .

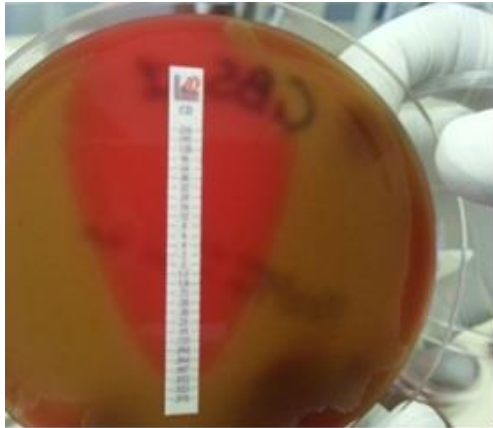
Resistant strains to erythromycin and clindamycin were observed in 21.1% and 17.2% of the isolates, respectively. All erythromycin and clindamycin resistant isolates were screened for resistance genes.

**Table VIc:** D-test (Double disk diffusion) results for GBS isolates (n=128)

Antibiotics	No of isolates	Percentage (%)
<b>Erythromycin vs. clindamycin</b>		
<b>iMLS<sub>B</sub> positive</b>	5	3.9
<b>cMLS<sub>B</sub> positive</b>	20	15.6
<b>M phenotype positive</b>	2	4.6
<b>L phenotype positive</b>	2	0.7

- iMLS<sub>B</sub> (MLS<sub>B</sub>-inducible methylation): Blunting defined as growth within the clindamycin zone of inhibition proximal to the erythromycin disk. See figure 9
- cMLS<sub>B</sub> (MLS<sub>B</sub>-constitutive): Resistance to both erythromycin and clindamycin.
- M phenotype: Resistance to erythromycin but susceptibility to clindamycin without blunting; indicated an efflux mechanism.
- L phenotype: Resistance to clindamycin but susceptible to erythromycin

Figures 7 and 8 below illustrate the E-test result of one isolate, susceptible to clindamycin and the D-test result of one isolate susceptible to clindamycin and resistant to erythromycin, showing the D-zone.

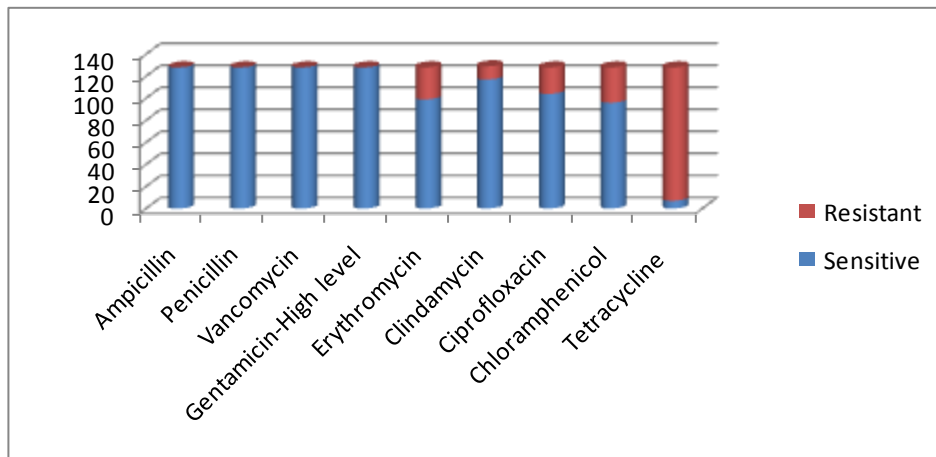


**Figure 7:** susceptibility testing result by E-test isolate isolate no 1 sensitive to clindamycin zone



**Figure 8:** Susceptibility testing result by D-test Isolate no 83. Observe the blunting showing D-zone

The overall result of the susceptibility testing of nine antibiotics tested and the number of positive pregnant women is presented in the graph below.



**Figure 9:** Overall results of nine antibiotics tested and the number of positive pregnant women. With 100 % of isolates sensitive to ampicillin, penicillin, vancomycin and high-level gentamicin; 21.1% of isolates were resistant to erythromycin, 17.2% resistant to clindamycin, 94.5% to tetracycline, 24.9% to chloramphenicol and 18.6% resistant to ciprofloxacin.

#### 4.4 Molecular Identification

Table VIIa and VIIb show the MICs of GBS isolates resistant to erythromycin and clindamycin and their phenotype values (D-shape) and resistant genes detected by multiplex PCR. The PCR products of isolates with resistant genes were distinguished by agarose gel electrophoresis and are presented in Figure 10 and 11 below.

**Table VIIa: MICs of erythromycin and clindamycin for resistant isolates and the screened genes for resistance (n=29)**

No	MIC ( $\mu\text{g/ml}$ )		D-shape	MLS	Genes
	Erythromycin	Clindamycin			
1	R-1	I-0.50	Negative	cMLS <sub>B</sub>	<i>ermB</i>
2	R-3	I-0.75	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
3	I-0.75	I-0.75	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
4	I-0.50	S-0.047	Negative	M phenotype	<i>mefA</i>
5	I-0.75	R-1	Negative	cMLS <sub>B</sub>	<i>ermB</i>
6	R-4	I-0.50	Negative	cMLS <sub>B</sub>	<i>ermB</i>
7	R-1	I-0.38	Negative	cMLS <sub>B</sub>	<i>ermB</i>
8	I-0.75	R-1	Negative	cMLS <sub>B</sub>	<i>ermB</i>
9	I-0.75	R-4	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
10	R-4	R-7	Negative	cMLS <sub>B</sub>	<i>ermB</i>
11	R-2	S-0.047	Positive	iMLS <sub>B</sub>	<i>ermTR</i>
12	R-1.5	S-0.016	Positive	iMLS <sub>B</sub>	<i>ermB</i>
13	I-0.75	I-0.50	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
14	R-8	I-0.38	Negative	cMLS <sub>B</sub>	<i>ermB</i>
15	S-0.25	R-2	Negative	L phenotype	<i>linB + ermB</i>
16	R-8	R-1	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
17	I-0.75	R-1	Negative	cMLS <sub>B</sub>	<i>ermB</i>
18	R-3	I-0.38	Negative	cMLS <sub>B</sub>	<i>ermB</i>
19	R-1	I-0.38	Negative	cMLS <sub>B</sub>	<i>ermB</i>
20	R-1.5	S-0.25	Positive	iMLS <sub>B</sub>	<i>ermB</i>
21	S-0.25	R-1	Negative	L phenotype	<i>linB + ermB</i>
22	R-3	S-0.047	Positive	iMLS <sub>B</sub>	<i>ermB</i>
23	I-0.50	I-0.75	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
24	R-2	R-8	Negative	cMLS <sub>B</sub>	<i>ermB</i>
25	I-0.75	I-0.50	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
26	I-0.75	S-0.094	Negative	M phenotype	<i>ermB</i>
27	R-4	S-0.023	Positive	iMLS <sub>B</sub>	<i>ermB</i>
28	I-0.50	R-1	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>
29	I-0.75	I-0.50	Negative	cMLS <sub>B</sub>	<i>linB + ermB</i>

**R=** Resistant

- **iMLS<sub>B</sub>** = MLS<sub>B</sub>-inducible methylation

**I=** Intermediate

- **cMLS<sub>B</sub>**= MLS<sub>B</sub>-constitutive

**S=** Susceptible

**Table VIIb: Erythromycin-clindamycin resistance phenotypes and genotypes among GBS isolates (n=29)**

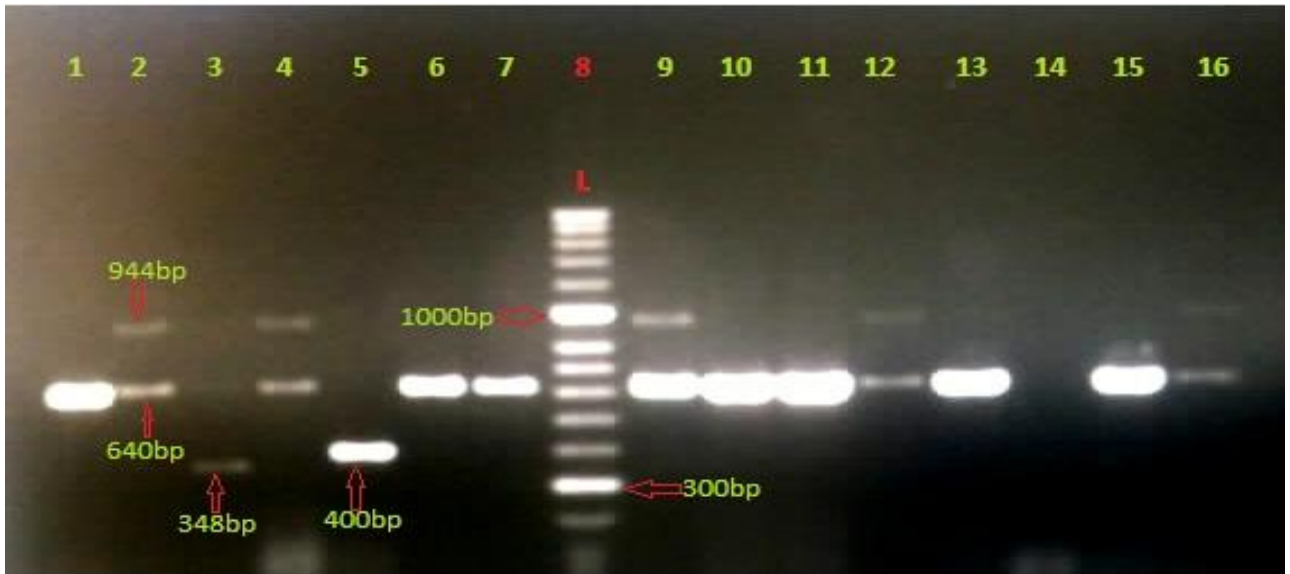
Phenotype		Genotype					
	No of isolates	Genes detected	No of isolates	Overall genes detected	No of isolates		
iMLS <sub>B</sub>	5	<i>ermB</i>	4	<i>ermB</i>	15		
		<i>ermTR</i>	1				
cMLS <sub>B</sub>	20	<i>ermB</i>	11		<i>ermB + linB</i>	11	
		<i>ermB + linB</i>	9				
		<i>ermTR</i>	0			<i>ermTR</i>	1
		<i>linB</i>	0				
M phenotype	2	<i>mefA</i>	1		<i>mefA</i>	1	
		<i>ermB</i>	1				
L phenotype	2	<i>ermB + linB</i>	2		<i>linB</i>	0	
		<i>linB</i>	0				

**iMLS<sub>B</sub>** : E(R), Cd(S) + D shape (*ermB* or *ermTR*)

**cMLS<sub>B</sub>** : E(R), Cd(R) (*ermB*, *ermTR*, *linB*)

**M phenotype** : E(R), Cd(S) – D shape (efflux – *mefA*)

**L phenotype** : E(S), Cd(R) (*linB*)



**Fig 10: Agarose gel electrophoresis of Multiplex PCR 1**

- Lane 8 :** DNA Molecular Weight Marker Hyper Ladder™ 50bp (Bioline)
- Lane 14:** Negative control (GBS ATTC 49743, susceptible to both erythromycin and clindamycin)
- Lane 3 :** Presence of *mefA* gene, **348 bp** (isolate no 60)
- Lane 5 :** Presence of *ermTR* gene, **400bp** (isolate no 83)
- Lane 1, 6, 7, 10, 11, 13, 15:** Presence of *ermB* genes, **640bp** (isolate no 15, 63, 65, 125, 148, 182, 183)
- Lane 2, 4, 9, 12, 16:** Presence of *ermB* genes and *linB* genes, **944bp** (isolate no 32, 57, 191, 159 & 184)



**Fig 11: Agarose gel electrophoresis of Multiplex PCR 2**

**Lane 8** : DNA Molecular Weight Marker HyperLadder™ 50bp (Bioline)

**Lane 7** : Negative control (*S. agalactiae* ATTC 49743, susceptible to erythromycin and clindamycin)

**Lane 1, 3, 5, 6, 9, 10, 12, 13, 14, 15, 17**: Presence of ***ermB*** genes, **640bp** (isolate no 100, 116, 117, 121, 70, 74, 68, 128, 86 & 129)

**Lane 2, 4, 11, 15, 16**: Presence of ***ermB*** genes, **640bp** and ***linB*** genes, **944bp** (isolate no 110, 131, 99, 71 & 104)

## CHAPTER V

### DISCUSSION

The primary objective of the present study was to isolate GBS from pregnant women and their babies at Dr. George Mukhari Academic Hospital (DGMAH). Secondly, to assess the antimicrobial susceptibility profile of those isolates to different antibiotics. Thirdly, to determine the incidence of antibiotic resistance in GBS isolates, and finally to investigate the mechanisms of antibiotic resistance in GBS isolates at DGMAH.

From February 2012 to December 2012, a total of 413 pregnant women were recruited and 39 samples were collected from the babies born to colonized mothers. A multiple profile questionnaire was completed for each participant, to establish the participant's demographic, obstetric and history of current pregnancy. Three swabs (RS, HVS, and LVS) were collected from each participant at recruitment and at delivery from the mothers; and ear and umbilical cord swabs from the babies at delivery; and all cultured onto selective media (CNA agar and Todd-Hewitt broth). GBS strains isolated were subjected to different tests of identification, including gram staining, catalase test, hemolytic activity, CAMP test, bile esculin and Latex agglutination test.

Of the 413 specimens subjected to culture and identification, 128 (31%) were positive with GBS (Table IV). Of all the 39 samples collected from the babies born to colonized mothers, none of them were positive for GBS. In view of low numbers of specimens from babies, it is difficult to reach conclusion regarding prevalence of GBS in babies born to colonized mothers.

The overall prevalence of GBS colonization among pregnant women of our findings is higher compared to the study conducted about 2 years ago in Johannesburg, South Africa, in which 21.5% of GBS was identified from the vaginal swabs only (Madzivhandila *et al.*, 2011). This difference in colonization rates might be due to the characteristics of study participants and laboratory diagnosis method including site of



sample collection. In fact, culturing specimen from both the ano-rectum and vaginal introitus increases the likelihood of GBS isolation by 5% - 27% over vaginal culture alone (Badri *et al.*, 1977; Dillon *et al.*, 1982; CDC, 1996; Madzivhandila *et al.*, 2011).

Similar situation was also reported in GBS studies conducted elsewhere in the world. In Zimbabwe, Moyo *et al.*, 2002 found the carrier rates of 31.6% and the rate of 35.7% was reported by Maveyengwa *et al.*, in 2010. Similar situation was also reported in Malawi, which 16.5% was found in Blantyre, Queen Elisabeth Hospital in 2005 by Dzowela *et al.*, and in 2011, in the same hospital, Gray *et al.*, reported the rate of 21%.

In Italy, Rosetti *et al.*, in 1997 found the colonization rate of 12.3%; and Lambiase *et al.*, when performing a series of investigations in the same country reported yearly increase in the colonization rates of vaginal-rectal cultures of 6.4% in 2005, 14.3% in 2006, 37.5% in 2007 and 41.7% in 2008. Studies conducted in USA indicated a colonization rate of 25% (Berkowitz *et al.*, 1990) and in 2007 Bergseng *et al.*, found a colonization rate of 34.7%.

Other different studies have reported different rates of GBS colonization, 29.5% in Québec – Canada (Bergeron *et al.*, 2000), 23% in Tanzania (Joachim *et al.*, 2009), 12% in Finland (Lyytikäinen *et al.*, 2003) and 7.6% in Argentina (Quiroga *et al.*, 2007). This affirms that differences in colonization rates might be due to differences in genetics, in sampling and culturing techniques and generally to the geographic location or characteristics of the population investigated (Schrag *et al.*, 2002; Edmond *et al.*, 2012).

Considering the socio-demographic characteristics, similar findings to ours have been reported in studies conducted elsewhere (Manning *et al.*, 2001; Schrag *et al.*, 2002; Dzowela *et al.*, 2005; Maveyengwa *et al.*, 2010 and Gray *et al.*, 2011). The findings of our study show that most GBS positive pregnant women were from between the ages of 25 – 34 years old. This is similar to the mean age of 27.3 found by Dzowela *et al.*, 2005 and 25.7 found by Maveyengwa *et al.*, 2010; but it is higher comparing to the study conducted by Manning *et al.*, in 2001 which reported the mean age of 22 years. This appears to be consistent with the findings by Narayanan

*et al.*, 2006, that young and middle-aged women are at risk because of obstetric and gynecologic manipulations.

There was significant difference between employment and GBS colonization. Our findings show that 59.3% of GBS positive women were unemployed and 39.8% were employed with 7.8% being skilled, 17.1% semi-skilled and 2.4% unskilled. The current study showed that the majority of GBS positive pregnant women were of the lower socio-economic groups. This is in agreement with what was reported by Schrag *et al.*, in 2002, Chohan *et al.*, in 2006 and Edmond *et al.*, in 2012 that poor socio-economic status is a risk factor for GBS colonization.

There was no significant difference between colonization and marital status of pregnant women. Our findings show that 53.1% of the GBS positive pregnant women were single, 21% were married and 24.2% were co-habiting. The educational level of GBS positive women ranged from below matric to tertiary level. At least 11.7% of the GBS positive pregnant women reached the tertiary level; 59.3% matric level and 28.1% were below matric. With these percentages, it was difficult to find a scientific relationship between educational level, occupational status and GBS.

In the current study of the 128 colonized women, 9.3% had history of stillbirths; this is higher when compared to 3.5% of stillbirths reported by Gray *et al.*, in 2011; and almost 25% of pregnant women reported history of previous miscarriage. In our study, 12.1% of GBS colonized women had given birth before by caesarean section (C/S) and this is consistent with the 20% reported by both Lyytikainen *et al.*, 2003 and Gray *et al.*, 2011. Of the 128 colonized women, 22 (17.1%) had vaginal discharge, 56 (43.7%) complained of flu like illness in early pregnancy and 6 (4.6%) had trauma in pregnancy. Overall, almost 40% of GBS positive women had poor obstetric history. This is almost similar to what was reported by Dzewela *et al.*, (2005) which reported that 36.1% of pregnant women admitted previously had bad pregnancy outcomes.

The HIV status of all participants in this study was recorded and there was no significant difference detected between GBS carriage (31%) and HIV positive women (40.6%). Of the 128 colonized women, 52 (40.6%) were HIV positive. This was

higher when compared to 20.1% and 21.7% reported by Mavenyengwa *et al.* in 2010 and Gray *et al.*, in 2011, respectively. Our findings significantly differ from previous studies from elsewhere; Mavenyengwa *et al.*, 2010 and Gray *et al.*, 2011 on these conflicting findings concerning the relationship between persons infected with HIV being at increased risk of invasive GBS infections. This has raised conflicting views among scientists; some of whom have found that persons with HIV were at an increased risk of invasive GBS infections, but others have not detected any association between GBS disease and HIV infection (Mavenyengwa *et al.*, 2010; Gray *et al.*, 2011).

During the course of our study, no maternal death was recorded but two neonatal deaths, three neonatal sepsis and one miscarriage were recorded, compared to the Malawian study in which 20 maternal deaths and 29 neonatal deaths were recorded (Gray *et al.*, 2011). However, extreme cases were reported in our study, two babies were born with congenital abnormalities and (one with hydrocephalus). One GBS pregnant mother was admitted in the hospital because there was no fetus movement for a week, and was found to have uterine fetus death; finally another GBS colonized woman, originally from Zimbabwe was reported to give birth to a macerated baby (fetus), and unfortunately swabs were not taken.

In the present study, the susceptibility testing was performed on 128 GBS isolated from pregnant women against 9 antimicrobial agents. All strains were 100% susceptible to penicillin, ampicillin, vancomycin and high level gentamicin. Our findings are in agreement with the study conducted in Germany, in which Fluegge *et al.*, (2004) found 100% of isolates sensitive to high-level Gentamicin, to penicillin, and to vancomycin. Similar findings were also observed in the USA by Manning *et al.*, (2003) which showed 100% isolates sensitive to penicillin, to ampicillin and to vancomycin.

This is also in agreement with an Ethiopian study where they found similar findings with 100% isolates sensitivity to ampicillin, to penicillin, to vancomycin and to gentamicin Musa *et al.* (2012). And in Argentina, Quiroga *et al.*, 2008 found that 100% of isolates were sensitive to ampicillin, to penicillin and to vancomycin.

Our findings are similar to the Zimbabwean study by Moyo *et al.*, (2001) in which almost 100% of isolates were sensitive to penicillin (with only 2% were intermediate susceptible to penicillin), and 14% of isolates were resistant to erythromycin, 8% resistant to clindamycin and 100% of isolates were resistant to tetracycline.

Our findings did not demonstrate isolates with an intermediate sensitivity or reduced MICs to penicillin using both disk diffusion and E-test methods compared to the findings by Simoes *et al.*, (2004) in USA in which 19% of GBS isolates had an intermediate susceptibility to penicillin. Rouse *et al.*, (1998) reported 10% of isolates having an intermediate susceptibility to penicillin; Liu *et al* (1997) in Taiwan reported 15% of GBS isolated with intermediate susceptibility to penicillin. Kimura *et al.*, 2008 and Murayama *et al.*, 2009 in Japan, reported the increasing MICs to penicillin in 14 noninvasive isolates among adults and also alterations in a penicillin-binding protein (pbp 2x) were found in all of the isolates.

In this study, 94.5% of the isolates were resistant to tetracycline, 24.9% resistant to chloramphenicol, 21.1% resistant to erythromycin, 18.6% resistant to ciprofloxacin and 17.2% resistant to clindamycin.

The rate of erythromycin and clindamycin resistance was almost similar to the Canadian study by Desjardins *et al.* 2004, in which they found erythromycin and clindamycin resistant rates of 17% and 8% respectively; and this is also similar to the Tanzanian study conducted by Joachim *et al.*, 2009 which reported GBS resistance rate of 17.6% and 13% erythromycin and clindamycin, respectively. The rate of erythromycin resistance (21.1%) found in our study is in agreement with the Malawian study by Gray *et al.*, 2007 where they found erythromycin resistance rates of 21%.

Our finding on erythromycin and clindamycin resistance was higher when compared to the Canadian study conducted by De Azavedo *et al* 2001, which reported erythromycin and clindamycin resistance rates of 8% and 4.5%. This current finding is lower when compared to the 38% and 21% of erythromycin and clindamycin resistant, respectively, reported by Gyax *et al* (2006). It is again far lower than the study conducted by Back *et al.* (2012) in the USA which reported 50.7% of erythromycin resistance and 38.4% clindamycin resistance; and far lower than the

study conducted by DiPersio *et al.* (2006) which reported erythromycin and clindamycin resistance of 54% and 33%, respectively.

The 94.5% tetracycline resistance rates found in our study is similar to the 96% reported by Gray *et al.*, (2007), and also 85.2% reported by Betrui *et al.*, (2003), 86.8% by Azavedo *et al.*, (2001), and 100% reported by Moyo *et al.* in 2001.

Resistance to tetracycline might be explained by wide and indiscriminate use of these antibiotics worldwide.

The molecular identification revealed that Multiplex PCR can be used as an effective screening method to detect different sequences important in GBS and to detect the major erythromycin and clindamycin resistance genes and also predict the phenotypic result of double disk diffusion test in the isolated GBS strains (Gygax *et al.*, 2007; Gosiewski *et al.*, 2012).

In our study, the phenotypic testing by double disk diffusion revealed that 29 isolates were resistant to both erythromycin and clindamycin in which 20 (69%) isolates had harbored cMLS<sub>B</sub>, 5 (17.2%) had harbored iMLS<sub>B</sub>, the M phenotypes was present in 2 (6.8%) isolates and the L phenotypes in 2 (6.8%). Our finding in phenotypic resistance by double disk diffusion was in agreement with the Canadian study by Desjardins *et al.* 2004 in which 47.2% had cMLS<sub>B</sub> resistance phenotype, 40% had an iMLS<sub>B</sub> resistance phenotype, and 12.7% of the isolates displayed M phenotypes. In Ireland, Khan *et al.*, 2011 observed similar findings with 40% of isolates that harbored iMLS<sub>B</sub>, 36% had cMLS<sub>B</sub>, 24% M phenotype and 0% L phenotype

The genotypic analysis by multiplex PCR (Table VIIb) shows that erythromycin and clindamycin resistance in GBS were mainly associated with *ermB* genes with 55% of isolates, *ermTR* genes harbored 3.4% and *mefA* genes 3.4% of the isolates and both *ermB* and *linB* genes together was harbored by 38% of the isolates. None of the strains carried both *ermB* and *ermTR* nor both *mefA* and *erm* nor *linB* alone.

Our findings were consistent with a French study conducted by Fitoussi *et al.* 2001 in which *ermB* was found in 47% of isolates, *ermTR* genes in 45% of isolates and *mefA* gene in 6% of the isolates. None of the strains carried both *ermB* and *ermTR* or both *mefA* and *erm* genes too just like in our study.

There were two isolates which were phenotypically sensitive to erythromycin however had *erm* genes detectable on molecular testing. This may be due to *erm* gene not being expressed, but will require further studies to confirm the interpretation. And two isolates were resistant to clindamycin but no resistance mechanism was found. This situation could be explained by the fact that isolates may harbor mutations in genes coding for 23S rRNA. Similar situation was reported in Ireland where no resistance mechanisms were found in nine isolates (Khan *et al.*; in 2011).

In South Africa, data to compare genetic mechanisms underlying erythromycin resistance in GBS was not obtainable.

## CHAPTER VI

### CONCLUSIONS

Our report shows that the colonization rate of GBS infections in pregnant women at DGMAH is 30.9% and may require review of antepartum screening policy.

This study confirms the appropriateness of penicillin as still being the antibiotic of choice for treating GBS infections. However, the main concern was the increase in the resistance to the macrolides and clindamycin, used as alternative drugs for penicillin allergic patients. More GBS treatment options for penicillin allergic patients need to be explored. The methylation of targets encoded by *ermB* and ribosomal translocation encoded by *linB* genes were the commonest mechanisms of resistance observed and efflux pump mediated by *mefA* genes was also found among the isolates.

## **LIMITATIONS**

- Some of the women delivered out of our facility and it was difficult to reach them for follow up; and as a consequence there was a low number of babies in the study
- During the molecular component of the study we could not find a positive control (ATCC strain) resistant to erythromycin and clindamycin to use.

## **RECOMMENDATIONS**

- Although penicillin is still the antibiotic of choice, more GBS treatment options for penicillin allergic patients need to be explored
- Constant surveillance is advisable and local statistics remain crucial for empirical antibiotic therapy
- There is need for a comprehensive GBS incidence study to acquire strains for an in depth study of invasive isolates in South Africa.
- More research studies need to be done in various areas and populations of RSA to determine GBS colonization



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## **APPENDIX**

### **APPENDIX A: CONSENT FORM**

**Study title: Incidence and mechanism of antibiotic resistance of *Streptococcus agalactiae* isolates from pregnant women and their babies at Dr. George Mukhari Hospital**

You are asked to participate in a research study conducted by Department of Life and Consumer Science at UNISA and Department of Microbiological Pathology at Medunsa. It will form part of my Master degree. Your participation in this study is entirely voluntary. Read the information provided below. You are allowed to ask questions about anything you do not understand, before deciding whether or not to participate.

#### **Purpose of the study**

The purpose of this study is to determine the incidence and to investigate the mechanisms of antimicrobial resistance in GBS isolated from pregnant women and their newborns at Dr. George Mukhari Hospital, Ga-Rankuwa, Pretoria - South Africa.

#### **Procedures**

If you volunteer to participate in this study, you will be asked to provide us with your vaginal and rectal swabs as well as the umbilical/ear swabs from your neonates. The swabs will be analyzed by researchers in the laboratory for any antibiotic resistance you may have. You will also be required to complete the questionnaire which will require information about you and your baby.

#### **Potential risks and discomforts**

The only minor discomfort may be experienced during collection of lower vaginal as well as rectal swabs from you and umbilical/ear swabs from your baby, although this will be done by experienced Gynecologist and Pediatrician and research nurse.

#### **Participation and withdrawal**

You can choose whether or not to be in this study. If you volunteer to be in this study, you may withdraw at any time without consequences of any kind or loss of benefits to which you are otherwise entitled. There is no penalty if you withdraw from the study. In the event of physical and/or mental injury resulting from participation in this research project, UNISA does not provide any medical, hospitalization or other insurance for participants in this research study, nor will UNISA provide any medical treatment or compensation for any injury sustained as a result of participation in this research study, except as required by law.

**Potential benefits to subjects and/or to society**

Participation in this research will help you to know if you/your neonate have developed any resistance toward antibiotics used for Group B streptococcus infection. This will enable the doctor to provide you with alternative treatment in case you are infected.

Being part of this study will also help us to have knowledge of antibiotic that are resistance to GBS infection at GMH Ga – Rankuwa. This research will also enable the government to work towards providing alternative treatment and preventions strategies.

**Confidentiality**

Any personal information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission or as required by law. Confidentiality will be maintained by using code name instead of your real name on samples.

**Declaration**

I have read the information about the proposed study and was provided with the opportunity to ask questions and given adequate time to rethink the issue. The aim and objectives of the study are sufficiently clear to me. I have not been pressurized to participate in any way.

I understand that participation in this study is completely voluntary and that I may withdraw from it at any time and without supplying reasons. This will have no influence on the regular treatment that holds for my condition neither will it influence the care that I receive from my regular doctor.

I am fully aware that the results of these this study is for research purpose only. I agree to this, provided my privacy is guaranteed.

I hereby give consent to participate in this study

.....

Name of participant

.....

Signature of participant

.....

Place.

.....

Date

.....

Signature of Witness



## APPENDIX B: QUESTIONNAIRE – PART I

### Personal information of Patient (Mother)

Full names.....Surname.....  
Languages.....Date of Birth.....  
**Race** : Black.....Coloured.....Indian.....White.....  
**Marital status** : Single .....Married.....Divorced.....Widowed....Cohabiting...  
**Residential address** : Urban.....Semi-urban.....Rural.....  
**Contact no**.....

**Education History:** Primary school..... Secondary school.... Diploma.... Degree.....

### Medical Information

#### Chronic illness

Are you suffering from any chronic illness? Yes.....No.....

If yes, which chronic illness.....

#### Pregnancy information

How old is your pregnancy?

Less than 3 months.....3-6 months.....7-9 months..... More than 9 months

Is this your first pregnancy? Yes.....No.....

If No how many pregnancies did you previously have? .....

#### Information about delivery

Have you had any miscarriage before? Yes.....No.....

Have you had any still birth before? Yes.....No.....

From the first month of your pregnancy till now, have you had any urinary tract infection?

Yes.....No.....

Did you have a vaginal discharge requiring treatment during the current pregnancy?

Yes.....No.....

#### Details of mother at delivery.....

Place of Delivery Hospital  Clinic  Home

Type of delivery NVD  C/S-

If other please specify \_\_\_\_\_

Time between rupture of membrane and delivery \_\_\_\_\_

Temperature \_\_\_\_\_

Antibiotic treatment \_\_\_\_\_

## APPENDIX C: QUESTIONNAIRE – PART II

### Details of the baby at delivery

Alive Yes  No

Stillbirth Fresh  Macerated

Congenital abnormality Yes  No

Specify (If yes) \_\_\_\_\_

Gestational age (weeks) at delivery |||

Swabs collected

(*Umbilicus, ear*) Yes  No

Cord blood collected Yes  No

Fetal: Apgar: 1 min ..... 5 min .....

Mass: Kg: .....

Resuscitation required: Yes  No

### ii) Details of the mother at delivery

Place of Delivery Hosp.  Clinic  Home

Type of delivery NVD  C/S  Forceps

Collect vaginal swab  rectal swab  venous blood

Time between rupture of membrane and delivery \_\_\_\_\_

Temperature \_\_\_\_\_

Antibiotic treatment \_\_\_\_\_

## APPENDIX D: DATA COLLECTION SHEET

Study no..... Specimen type..... Date.....

### 1. CULTURE

Colony Count	Quantity growth +, ++, +++		
	RS	LVS	HVS
CNA			
Todd Hewitt Broth			

Initials.....Date.....

### 2. IDENTIFICATION

GBS	RESULTS		
	RS	LVS	HVS
Hemolytic Activity			
Catalase Test			
Gram Stain			
Bile Esculin			
Agglutination (STREPTEX)			
CAMP test			

Initials.....Date.....

### 3. ANTIBIOTIC SUSCEPTIBILITY

GBS		Peni	Erythro	Clinda	Ampi	Vanco	Genta	chlora	Tetra	Cipro
Results	Disc D.									
	E-Test									
	D-Test									

Initials..... Date.....

### APPENDIX E: GBS WORK SHEET

DATE OF COLLECTION	STUDY No	TYPE OF SAMPLES				TIME RECEIVED in Lab	CULTURE RESULT						IDENTIFICATION					STORY/N			
		R S	L V S	H V S	BLD		RS		HVS		LVS		HEMO LYTIC ACTIV ITY	CATA LASE TEST	MICRO SCOPY	STREP TEX	CAMP Test				
					M		B	C	T	C	T	C							T		
								N A	H B	N A	H B	N A							H B		

**RS:** Rectal Swab; **LVS:** Low Vaginal Swab; **HVS:** High Vaginal Swab; **BLD:** Blood; **CNA:** Colistin Nalidixic Acid agar; **THB:** Todd Hewitt Broth; **M:** Mothers; **B:** Babies;

## **APPENDIX F: ANTIBIOTIC SUSCEPTIBILITY TESTING – PROTOCOL**

### **Materials & Media required**

The SOP was developed. Mueller-Hinton agar supplemented with 5% sheep blood, Blood agar or CNA, normal saline, McFarland standards, sterile loops and swabs, sterile forceps, CO2 incubator, antibiotics (discs & E-test), antibiotic disc dispensers, quality control strains, ruler or caliper for measuring zone size, CLSI guidelines 2012

### **Methods**

1. Label the blood agar plates with study identification numbers.
2. Open aseptically the cryovial (microbank) with GBS isolates, and using a sterile needle or forceps, remove one bead and directly streak onto blood agar (or CNA)
3. Incubate at 37°C in 5% CO<sub>2</sub> environment for 20-24h,
4. Perform gram stain, catalase, streptex or CAMP test for purification of the organism.
5. Select a pure colony from overnight positive GBS plates and suspend into normal saline
6. Adjust the culture until the turbidity is equivalent to the 0.5 McFarland standard
7. Inoculate the culture (using a sterile swab) onto Mueller-Hinton agar (+5% sheep) blood plates
8. Place antibiotics onto agar using dispenser or sterile forceps. 6 discs was placed into 1 plate (100mm apart); Place erythromycin and clindamycin discs 12mm distant to enable demonstration of MLS resistance phenotype
9. Place MIC strips (E-tests) in 1 plate.
10. Incubate at 37°C for 20-24h in 5% CO<sub>2</sub> environment
11. Read the plate the following day; use caliper or ruler to measure the zone of inhibition in mm, and interpret the results by using the CLSI guidelines 2012.

## **APPENDIX G: DNA EXTRACTION PROTOCOL**

### **(ZYMO RESEARCH – USA– DNA Mini-KIT – Inqaba Biotec, SA)**

**Product Contents:** ZR BashingBead Lysis Tubes (50), Lysis Solution (40ml), Bacterial DNA Binding buffer (100ml), Bacterial DNA Pre-wash Buffer (15ml), Bacterial DNA wash buffer (150ml), Elution buffer (10ml), Zymo-Spin IV spin filters/ Orange Tops (50), Zymo-Spin IIC Columns (50), Collection Tubes (150).

For optimal performance, add beta-mercaptoethanol/ optional (supplier) to the Bacterial DNA Binding buffer to a final dilution of 0.5% (v/v) i. e, 500µl per 100 ml.

### **STEPS**

- Add 200µl of sterile water or isotonic buffer (e.g. PBS) into 1.5ml microcentrifuge tube
  - Suspend bacterial cell (from overnight growth ) into the above water
  - Add 50-100 mg (wet weight/colony) of the suspension into ZR BashingBead Lysis tubes
  - Add 750 µl of Lysis Solution to the tube (ZR BashingBead Lysis tubes)
  - Secure in a bead beater fitted with a 2ml tube holder assembly (e.g. Disruptor Genie)
  - Process at maximum speed for 5 min
  - Centrifuge the ZR BashingBead Lysis Tubes in microcentrifuge at 10,000 RPM for 1'
  - Transfer up to 400 µl supernatant to a Zymo-Spin IV Spin Filter (orange top) in Collection Tube. Centrifuge at 7,000 x g (7,000 RPM) for 1min
  - Add 1200 µl of Bacterial DNA Binding buffer to the filtrate in the collection tube from step above
  - Transfer 800 µl of the mixture from step above to a Zymo-Spin IIC column in a collection tube. Centrifuge at 10,000 x g (10,000 RPM) for 1min
  - Discard the flow through from the collection tube and repeat the above step
  - Add 200 µl DNA Pre-Wash Buffer to the Zymo-spin IIC column in a new collection tube
  - Centrifuge at 10,000 x g (10,000 RPM) for 1min
  - Add 500 µl Bacterial DNA Wash Buffer to the Zymo-spin IIC column. Centrifuge at 10,000 x g (10,000 RPM) for 1min
  - Transfer the Zymo-Spin IIC column to a clean 1.5 ml microcentrifuge tube
  - Add 100 µl DNA Elution Buffer directly to the column matrix.
  - Centrifuge at 10,000 x g (10,000 RPM) for 30 seconds to elute the DNA
- The Ultra-pure DNA is now ready for use in your experiments

# UNIVERSITY OF LIMPOPO

Medunsa Campus



## MEDUNSA RESEARCH & ETHICS COMMITTEE

### CLEARANCE CERTIFICATE

P O Medunsa  
Medunsa  
0204  
SOUTH AFRICA

**MEETING:** 01/2011  
**PROJECT NUMBER:** MREC/P/02/2011: IR

Tel: 012 - 521 4000  
Fax: 012 - 560 0086

**PROJECT :**

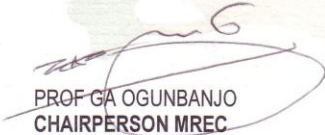
**Title:** *Streptococcus agalactiae*: in pregnancy women and their babies at the Dr George Mukhari Hospital, Pretoria

**Researcher:** Prof M Nchabeleng  
**Co-workers:** Dr MRB Maloba (Microbiology)  
Dr MPB Mawela (Paediatrics)  
Dr AN Muse (Obstetrics & Gynaecology)  
Prof S Moyo (UNISA)  
**Hospital Superintendent:** Dr P Shembe (DGMH)  
**Department:** Microbiological Pathology  
**School:** Pathology  
**Type of Research:** Independent Research

**DECISION OF THE COMMITTEE:**

MREC approved the project.

**DATE:** 10 February 2011

  
PROF GA OGUNBANJO  
CHAIRPERSON MREC



**Note:**

- i) Should any departure be contemplated from the research procedure as approved, the researcher(s) must re-submit the protocol to the committee.
- ii) The budget for the research will be considered separately from the protocol. PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES.

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