

Epidemiology and multilocus sequence typing of group B streptococcus colonising pregnant women and their neonates at Dr George Mukhari Academic Hospital, Pretoria.

By

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Declarations

I declare that the dissertation "Epidemiology and multilocus sequence typing of group B streptococcus colonising pregnant women and their neonates 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.



12/ February/2014

SIGNATURE

DATE

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May her soul rest in peace

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Abstract

Background: Group B streptococcus (GBS) is regarded as one of the most important causes of maternal and neonatal morbidity and mortality in many parts of the world. GBS recto-vaginal colonization is important in the health of a mother and her neonate, especially in developing countries. Maternal vaginal colonization with GBS at the time of delivery can cause vertical transmission to the neonate. Multilocus sequence typing (MLST) is a technique used to characterize microbial isolates by means of sequencing internal fragments of housekeeping genes and has the advantage of reproducibility and has been shown to correlate with the other typing techniques and thus has emerged as the standard for delineating the clonal population of GBS. The study aimed to investigate the epidemiology of GBS colonization among pregnant women and their neonates, and to characterize the isolates by multilocus sequence typing technique at Dr George Mukhari Academic Hospital, Pretoria.

Methodology: A total of 413 pregnant women who visited the antenatal clinic were recruited and screened. Participants were interviewed using a questionnaire to gather demographic and other relevant information such as history of current pregnancy, previous miscarriages and still births. Samples from maternal rectum and vagina as well as neonate ear and umbilical cord were taken for culture using colistin and nalidixic acid (CNA) blood agar and incubated for 24-48 hours. If negative after 48 hours, Todd-Hewitt broth was subcultured after 18-48 hours onto sheep blood agar. Multilocus sequence typing (MLST) was used to characterize seven group B streptococcus isolates collected at Dr George Mukhari academic hospital. Fragments of seven housekeeping genes were amplified by polymerase chain reaction (PCR) for each strain and sequenced. CLC bio software (Inqaba biotech, South Africa; Pretoria) was used to analyse sequenced loci and UPGMA dendrogram was constructed.

Results: The colonization rate for GBS in pregnant women and their neonates was 30.9% and 0%, respectively. A higher proportion of GBS were isolated from the rectum (37.9%) as compared to the vagina (20.6%). Most socio-economic, demographic and obstetric factors analysed were not significantly associated with

GBS colonization. On 128 positive samples, the results of Todd-Hewitt enrichment broth and direct plating method using CNA were compared. A total of 45.3% of colonised were positive on direct selective agar (CNA); an additional 54.7% samples were recovered from Todd-Hewitt broth. Three genes (*adhP*, *glnA* and *tkt*) were sequenced successfully for six samples (1, 2, 4, 6, 12 and 65). The UPGMA tree with 1000 bootstrap showing the relationship between six samples was drawn.

Conclusion: This study revealed that pregnant women of all ages are at risk of group B streptococcus colonization. Group B streptococcus was common among pregnant women at Dr George Mukhari Academic Hospital. No socio-economic risk factor was associated with group B streptococcus colonization. Results confirm that the combination of Todd-Hewitt broth and CNA agar plate is a time saving and sensitive method. The allelic profile, characteristics such as G+C (guanine+cytosine) content and dN/dS ratio were not analysed because of the smaller sample size used in this study, which shows that the MLST method was unsuccessful in this study. The UPGMA tree based on differences in consensus of the isolates showed that all group B streptococcus isolates are clustered and descend from a single node.

Keywords: *Epidemiology, Group B streptococcus, Multilocus sequence typing (MLST), colonization, pregnant women, neonates, Dr George Mukhari Academic Hospital.*

,

List of abbreviations and symbols

<i>adhP</i> :	alcohol dehydrogenase
AIDS:	Acquired Immunodeficiency Syndrome
ANC:	Antenatal clinic
Bp:	base pairs
β :	beta
<i>atr</i> :	amino acid transporter
CC:	clonal complex
CNA:	Colistin and Nalidixic Acid
CO ₂ :	Carbon dioxide
CPS:	Capsular polysaccharide
DGMH:	Doctor George Mukhari Academic Hospital
DNA:	Deoxyribonucleic acid
ELISA:	Enzyme-linked immunosorbent assay
EOD:	Early-onset disease
H ₂ O ₂ :	hydrogen peroxide
HIV:	Human immunodeficiency virus
g/ml:	gram per millilitre
GBS:	Group B streptococcus
<i>glcK</i> :	glucose kinase
<i>glnA</i> :	glutamine synthetase
LOD:	Late-onset disease
μ l:	microliters
MLST:	Multilocus sequence typing
NICD:	National Institute for Communicable Diseases
PCR:	Polymerase chain reaction
PFGE:	Pulse-field gel electrophoresis
<i>pheS</i> :	phenylalanyl tRNA synthetase
%:	percentage
<i>sdhA</i> :	serine dehydratase
ST :	sequence type
<i>tkt</i> :	transketolase

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CHAPTER 1

1. INTRODUCTION

Streptococcus agalactiae, known as group B streptococcus (GBS), is a Gram-positive opportunistic pathogenic bacterium of the genus *Streptococcus*. It is a commensal organism in humans but can cause life threatening infection in susceptible hosts such as neonates, pregnant women and non-pregnant adults with chronic illnesses. Group B streptococcus exists as normal flora in genitourinary and gastrointestinal tracts in humans (Shen *et al.*, 2001). The bacterium can be transferred from the vagina to the newborn babies during parturition, and this is frequent in developing countries (Javanmanesh and Eshraghi, 2013).

Group B streptococcus causes invasive disease such as early-onset sepsis and pneumonia (Johri *et al.*, 2006; Busetti *et al.*, 2007) and late-onset infections such as meningitis in newborn babies making it one of the leading neonatal causes of morbidity and mortality (Yang *et al.*, 2012). In pregnant women, it causes chorioamnionitis, urinary tract infection and wound infection in immunocompromised non-pregnant adults or those with underlying conditions (McKenna and Lams, 1998; Gonza'lez-Outeirin~o *et al.*; 2004).

The major reservoirs for group B streptococcus are reported to be the vagina and the rectum, and the colonization of these regions is a risk factor for subsequent infection in pregnant women and newborns (Sharmila *et al.*, 2011; Yang *et al.*, 2012). Vertical transmission from the colonized mother to the newborn is the major risk factor for early-onset infection, including ascending intra-uterine and intrapartum infection, especially during vaginal deliveries (Busetti *et al.*, 2007; Yang *et al.*, 2012).

Maternal factors predisposing to invasive neonatal infection include the following: high genital inoculums with group B streptococcus at delivery and prolonged rupture of membranes. Group B streptococcus infection may cause premature delivery, stillbirth, chorioamnionitis, group B streptococcus bacteriuria (Suara *et al.*, 1998; Law., *et al.*, 2005). Ethnicity, maternal age and parity, marital status, education and smoking are factors that have been reported to influence the prevalence of colonization, with studies that was done in the United States, have shown that

occupation, black race, Hispanic ethnicity, age, high parity and high body mass index might be associated with group B streptococcus colonization in pregnancy (Stapleton *et al.*, 2005).

Group B streptococcus possess genotypic and phenotypic markers which are important in epidemiological settings (Doran and Nizet, 2004; Mavenyengwa *et al.*, 2009). Group B streptococcus is divided into nine serotypes (Ia, Ib, and II-VIII) according to distinct capsular polysaccharides, and serotype IX was identified in 2007, which appears to be mostly related to serotype Ib, although its *cps* gene cluster is related to those of serotype V, Ia and IV (Slotved *et al.*, 2007). The specific capsular polysaccharides are composed of different arrangements of glucose, galactose, N-acetylglucosamine and sialic acid (Doran and Nizet, 2004; Cieslewicz *et al.*, 2005). Almost all serotypes of group B streptococcus have been associated with invasive disease in neonates, group B streptococcus capsular type III is the most frequent isolate and cause majority of late onset disease and meningitis (Polin *et al.*, 1982).

Common serotypes reported in South Africa are Ia, Ib, and III. Serotype III was reported as being the most pathogenic strain (Madzivhandila *et al.*, 2011). A recent systematic review and meta-analysis of group B streptococcus found that in low and high-income countries, the main serotypes are Ia, Ib, II, III and V, thus a vaccine including these serotypes could prevent invasive group B streptococcus (Edmond *et al.*, 2012).

To gain insight into epidemiology and the phylogeny of the organism such as group B streptococcus typing method is performed. Molecular typing methods have several advantages over serotyping method, among the generation of more ambiguous results and they bypass the problem of immunological cross-reactivity. More advanced molecular methods such pulse-field gel electrophoresis and multilocus sequence typing (MLST) have the ability to differentiate strain collections into many types (Radtke, 2012)

Traditionally, descriptive epidemiology of group B streptococcus has relied on capsular serotyping or genetic typing; this includes that determined by pulse -field gel electrophoresis, and multilocus sequence typing (Jones *et al.*, 2003; Tien *et al.*, 2011). In order to compare experiments, it is required that DNA sequencing and analysis are performed using portable standard, one such standard is MLST; a nucleotide sequence based approach for the unambiguous characterisation of

isolates of bacteria and other organisms and is particularly suitable for epidemiological studies because it provides data that can be compared easily between laboratories over the internet (Jones *et al.*, 2003). The aim of MLST is to provide a portable, accurate, and highly discriminating typing system that can be used for most bacteria and some other organisms. It is envisaged that this approach will be particularly helpful for the typing of bacterial pathogens. Therefore; the study aimed to investigate the epidemiology of group B streptococcus colonization among pregnant women and to characterize group B streptococcus isolates by multilocus sequence typing at Dr George Mukhari Academic Hospital, Pretoria.

1.2. Problem statement

Bacterial infections are frequently the direct or principal underlying cause of human neonatal deaths. The group B streptococcus is regarded as the dominant group of gram-positive bacteria that are responsible for severe or life threatening infections (Lachnauer *et al.*, 2002). The epidemiology of group B streptococcus has not been well studied in South Africa. Recent studies on maternal factors associated with high attack rates for early-onset group B streptococcus disease have reported black race, history of previous miscarriages, young adults of 20 years of age and preterm delivery as risk factors (Farley, 2001; Batalis *et al.*, 2007). Traditional and molecular typing schemes for the characterization of pathogenic microorganisms are poorly portable because their index variation that is difficult to compare among laboratories and some less frequently used molecular typing methods involves indirect characterization of gene alleles through electrophoresis of gene products.

1.3. Objectives of the study

1.3.1. Main objective

To investigate the epidemiology of group B streptococcus colonization among pregnant women and to characterize group B streptococcus isolates by multilocus sequence typing at Dr George Mukhari Academic Hospital, Pretoria.

1.3.2. Specific objectives

- ✓ To estimate the group B streptococcus colonization among pregnant women and their neonates.
- ✓ To establish sensitivity of different media in group B streptococcus detection.
- ✓ To characterize group B streptococcus serotype isolates using multilocus sequence typing.

1.4. Motivation of the study

This study was to provide baseline information on the prevalence of group B streptococcus. Data on the rate of group B streptococcus colonization; these will be useful in the development of guidelines for screening of group B streptococcus in order to be able to manage the patients. MLST is one of the most reliable tools for

group B streptococcus typing, with sufficient discrimination for use in epidemiology and unambiguous characterization of isolates. Sequence data can be compared readily between laboratories, such that a typing method based on the sequences of gene fragments from a number of different housekeeping loci is fully portable. Data stored in a single expanding central multilocus sequence database can be interrogated electronically through the internet to produce the powerful resource for global epidemiology (Jones *et al.*, 2003).

CHAPTER 2

2. LITERATURE REVIEW

2.1. Group B streptococcus

Streptococcus agalactiae is characterized by the presence of group B Lancefield antigens and gram positive facultative anaerobic diplococcus of genus streptococcus with an ultrastructure similar to other gram positive cocci, which can grow on a variety of microbiologic media (Baker *et al.*, 1977; Edwards and Baker, 2001). When cultured on sheep blood agar, they appear as glistening grey-white colonies with a narrow zone of beta haemolysis larger than *Streptococcus pyogenes*, usually found on gram stain to be arranged in chains, frequently pigmented after anaerobic incubation on Columbia agar and grow in the presence of bile on MacConkey agar (Mims *et al.*, 2004).

Culture of group B streptococcus is routinely performed on agar supplemented with sheep's blood, and colonies are detected by the breakdown of red blood cells, producing a characteristic zone of hemolysis, termed beta hemolysis (Schrug *et al.*, 2002). The presence of group B streptococcus may be confirmed by performing Gram staining, CAMP (Christie, Atkins, and Munch-Peterson) testing, or typing via an agglutinin reaction on selected beta haemolytic colonies (Verani *et al.*, 2010). This is often labour intensive, and in cases in which the patient's sample contains many different species, beta hemolysis may be missed (Schwartz *et al.*, 2012; Adler *et al.*, 2008). There is a risk that samples may be overgrown with competing organisms, such as *Proteus* or *Enterococcus*, and false negatives are not uncommon. When hemolysis is identified, follow-up testing is often required to prevent a false positive from being reported (Bou *et al.*, 2005). While culture continues to be regarded as the gold standard, it is labour intensive and is not without shortcomings (Faro *et al.*, 2013).

Group B streptococcus, is a very invasive encapsulated organism capable of producing severe disease in immunocompromised hosts. This pathogen can be recovered from sterile sites on a selective blood agar, however there is a standard method to isolate Group B streptococcus from genital or rectal sites, where a selective broth medium (Todd-Hewitt or Lim broth) that contains antibiotics such as

nalidixic acid, colistin or gentamicin to inhibit other bacteria (Baker *et al.*, 1977). This bacterium has group B cell wall antigen, which is composed of rhamnose, galactose and N-acetylglucosamine. Group B streptococcus is able to hydrolyze sodium hippurate, is resistant to bacitracin, and is sensitive to bile; therefore, these bacteria will lyse in the presence of bile (Shen *et al.*, 2001).

Group B streptococcus isolation from blood, cerebrospinal fluid (CSF), another usually sterile site, or a site of focal suppuration is only means by which the diagnosis of invasive infection can be documented. Detection of group B streptococcus antigen in serum or CFS using methods such as latex agglutination may be established as a presumptive diagnosis. Antigen tests have poor sensitivity and variable specificity, with the results that are infrequently useful in diagnosis establishment, antigen testing of urine is not recommended (Forbes *et al.*, 2007).

2.2. Group B Streptococcus colonization

Group B streptococcus is part of the normal flora of the gut and genital tract in humans. About 16-40% of healthy adults are asymptotically colonized with group B streptococcus in human genitals (Brimil *et al.*, 2006). When compared to group A streptococcus and *Streptococcus pneumonia*, however, little is known about the pathogenesis, natural history and transmission dynamics of group B streptococcus (Manning, 2003). Group B streptococcus can colonize vaginal and/ or rectal sites of women without showing any symptoms, from which can be transferred to the newborn babies during parturition, since group B streptococcus exist as normal flora in genitourinary and gastrointestinal tracts (Shen *et al.*, 2001). Accordingly, colonization is rare in neonates delivered by caesarean section before the onset of labour (Law *et al.*, 2005).

After the establishment of neonate colonization, the systematic infection may occur through the umbilical cord, respiratory tract or skin abrasions. The ascent of organisms to the foetus from the mother's genital tract through ruptured or intact membranes, with aspiration or ingestion of infected amniotic fluid by the foetus is a second important source of infection occurring before birth (Law *et al.*, 2005). The study indicated that, African women were at a higher risk (29%) as compared to Europeans and Asian women, of which were at lower risk (13%) for group B streptococcus carriage (Valkenburg-van den Berg *et al.*, 2006). A study done in

Malawi showed lower colonization rate, with the overall colonization rate of group B streptococcus to be 16.5 % (Dzowela *et al.*, 2005) as compared with the three separate studies in Zimbabwe where group B streptococcus colonization rate was shown to be 20 %, 31 %, and 32% (Mason *et al.*, 1989; Stoll and Schuchat., 1998). A study from Maputo, Mozambique indicated low prevalence of group B streptococcus colonization among pregnant women between 35 and 37 weeks of pregnancy (1.8%). The method used was a rectovaginal swab which was taken from women between 35 and 37 weeks of pregnancy who visited the clinic for antenatal consultation (De Steenwinkel *et al.*, 2008).

The colonization rates have been found to vary by geographic areas, term of pregnancy and age group. In another study conducted in Zimbabwe by Mavenyengwa *et al.*, (2006); mother cumulative colonization rate after sampling three times was found to be significantly higher in the rural areas (60%) as compared to the urban areas (46%). Group B streptococcus colonization persistence showed to be more in rural (48%) than in urban women (12%). Baby colonization was also shown to be more in the rural (23%) than in urban area (5%). The prevalence of group B streptococcus colonization during pregnancy is variable; in one study, among women who had positive GBS cultures between 26 and 28 weeks of gestation, only 65% remained colonized at term, while 8% of those with negative prenatal cultures were positive for GBS at term (Shet and Ferrieri, 2004).

The study in Hong-Kong showed the prevalence of group B Streptococcus colonisation in antenatal population was found to be 10.4%. The majority of carriers were identified by low vaginal swabs (78%), while high vaginal swabs and rectal swabs only identified 31% and 30% of the carriers, respectively. Pregnant women, who work outside home, yielded a higher carrier rate than housewives (21% vs10%). There was no increase in preterm delivery rate in group B Streptococcus carriers indicated in this study (Tsui *et al.*, 2009).

The rate of group B streptococcus colonization in the vagina and/or rectum among pregnant women varies with ethnic group, geographic area and age (Shet and Ferrieri, 2004). Group B streptococcus colonization may be intermittent during pregnancy. A positive test for group B streptococcus in pregnant women might

therefore be a poor predictor for colonization at delivery. However, few studies have reported results of repeated tests during pregnancy. Such a study has recently been conducted in Denmark. In the study conducted in Denmark, group B streptococcus colonization in a cohort of 77 women was relatively stable during pregnancy and up to a period of a year after delivery. In African women, little is known of group B streptococcus colonization during pregnancy as well as the predictive value of colonization at different stages for colonization of group B streptococcus at delivery (Mavenyengwa *et al.*, 2010).

2.3. Group B Streptococcus prevalence

Until recently, Group B streptococcus was infrequently reported in the developing world. Yet the prevalence of maternal carriage of group B streptococcus in developing countries, including the populations in tropical Africa, is similar to the identified populations in the United States (Stoll and Schuchat., 1998; Dawudo *et al.*, 1983). Studies from Kenya (Berkley *et al.*, 2005; English *et al.*, 2003 ; Laving *et al.*, 2003), South Africa (Bomela *et al.*, 2001; Madhi *et al.*, 2003), Zimbabwe (Nathoo *et al.*,1990) and Malawi (Milledge *et al.*,2005) suggested that group B streptococcus is emerging as an important cause of neonatal sepsis in Africa. Studies done in South Africa have also showed that the burden of invasive GBS disease in South Africa is greater than in developed countries (Madhi *et al.*, 2003). The prevalence among black pregnant women in South Africa and United States has shown to be higher than in women of other racial groups. Most documented data on group B streptococcus has come from Europe and North America and to date only Zimbabwe and Malawi in Africa have active research programme on group B streptococcus colonization and burden diseases (Moyo, *et al.*, 2002; Dzowela *et al.*, 2005).

The prevalence may differ due to culture technique differences, the location differences and number of sites cultured and the population studied. Women of Caribbean origin, and black women, previously were reported to be at greater risk of colonization than those of Mexican origin and white women, which make a suggestion of a role for ethnic or genetic factors (Valkenburg-van den Berg, *et al.*, 2006). Prevalence of group B streptococcus is 16.5% in Malawi (Dzowela *et al.*, 2005), 19.0% in Ivory coast, 20.0% in Nigeria, 22% in Gambia (Stoll and Schuchat, 1998) and 32% in Zimbabwe (Moyo *et al*, 2002).

The incidence among black American infants increased during the years 2003-2005 (70%), whilst incidence rates among white Americans infants decreased. In the year 2005, among black infants in the United States, the rate of early onset group B streptococcus disease infection was 0.84 per 1000 live births compared to 0.24 per 1000 deliveries in white infants (Pulver *et al.*, 2009). Geographic variations in early onset group B streptococcus disease infection rates have also been noted, ranging from 0.53 cases per 1000 live births in Tennessee to 0.14 in Oregon (CDC, 2002). Generally in the United States prior to the introduction of intrapartum prophylaxis the overall incidence of neonatal group B streptococcus infections were approximately 2 per 1000 live births (Baker and Edwards, 2001).

In South Africa, during the period 1997 to 1999, the burden of neonatal group B streptococcus was 2, 06 cases/1000 lives births and 1case/1000 live births for early and the late onset disease respectively (Madhi *et al.*, 2003), and in Netherlands, the incidence of early onset group B streptococcus disease in the period from 1997-1998 was estimated at 1.9 per 1000 live births, with a fatality case rate of 5% (Valkenburg-van den Berg., *et al.*, 2006). In the study conducted to determine the prevalence of group B streptococcus and to identify group B streptococcus colonisation risk factors in a multicultural population of pregnant women in Netherlands, it was found that carrier rate of group B streptococcus in late pregnancy was 21% (Valkenburg-van den Berg *et al.*, 2006).

Previous studies suggested that maternal rectal colonization may be the important acquisition source of group B streptococcus in newborns. The rectal swabs exclusion to detect group B streptococcus colonization may compromise the sensitivity in group B streptococcus colonization detection (Stoll and Schuchat, 1998). Previous research studies reported that the sensitivity of detecting group B streptococcus colonization in pregnant women by undertaking both vaginal swabs and rectal is higher (18, 5 -51%) when compared to taking only vaginal swabs (Dzowela *et al.*, 2005) since both the vaginal and rectal flora contain numerous microorganisms. The use of selective enrichment broth is recommended to maximise the isolation of group B streptococcus and to avoid the overgrowth of the other microorganism (Ferrieri *et al.*, 1977).

When agar plate is used instead of enrichment broth, many women who are group B streptococcus carriers give false-negative culture results. This was proved by (Ferrieri *et al.*, 1977) and Kubota (2002) who did not use selective culture medium and only found the prevalence rates of 5.6% and 11.4%, whereas with studies that used selective medium reported rates of 19.8% and 18.6% (Madzivhandila *et al.*, 2011; Valkenburg-van den Berg, *et al.*, 2006).

2.4. Group B streptococcus disease

Group B streptococcus, was originally known for causing bovine mastitis and was not demonstrated to be a human pathogen until the year 1938, after description of three patients with fatal puerperal sepsis (Fry, 1938). The bacteria remained unknown to most clinicians until the years around 1970s, when the observations of dramatic increase of group B streptococcus septicaemia and meningitis in neonates occurred in different parts of the world (Law *et al.*, 2005).

2.5. Disease in newborn babies

Group B streptococcus is known as the important leading neonatal pathogen worldwide that causes morbidity and mortality in neonates (Baker, 2000; Beitune *et al.*, 2005). Despite the decrease in the last decades, early onset group B streptococcus disease remains a serious neonatal condition, which may cause a serious neurological damage (Valkenburg-van den Berg *et al.*, 2006). These bacteria cause invasive infections such as, meningitis, pneumonia and sepsis in newborn babies (Gonza'lez-Outeirin'ó *et al.*; 2004; McKenna and Iams, 1998).

Group B streptococcus can pass through the cervix without causing serious cervicitis, and cross-intact amniotic fluid causing amnionitis thereby infecting the foetus in the uterus (Dzowela *et al.*, 2005). Most of neonatal group B streptococcus infections occur during gestation and at delivery due to the failure to produce and transmit cross-placental sufficient serum antibody against group B streptococcus by the mother (Suara *et al.*, 1998), Group B streptococcus pathogen is the most prevalent infection in the first week of life, presenting in two forms: early-onset and late-onset. Early onset infection will flare-up in the first week and mainly (90%) in the first 12 hours (Javanmanesh and Eshraghi, 2013). Late-onset disease (LOD) occurs after the first week until 3 months and accounts for most meningitis cases and deaths (Dzowela *et al.*, 2005).

2.5.1. Early onset disease

The group B streptococcus that causes early-onset infection is usually due to transmission from a mother to the baby due to amniotic fluid infection or from the birth canal of a colonized mother. Of all infants born of these women who are colonized with group B streptococcus, 1 to 2% will develop early onset invasive disease (Matlova *et al.*, 2004). The incidence of early-onset disease is about 10 times higher in premature than in term neonates (Boyer *et al.*, 1983). The newborn will present with symptoms such as breathing problems, blood pressure and heart rate instability, gastro-intestinal and urinary system disturbances, sepsis, pneumonia and meningitis (Javanmanesh and Eshraghi, 2013).

2.5.2. Late-onset disease

Late-onset group B streptococcus, results from contamination through direct contact with other colonized individuals in the community. The bacterium causes pneumonia, bacterial sepsis, and meningitis in most of newborn babies (Beal and Dancer, 2006; Schrag *et al.*, 2000; Javanmanesh and Eshraghi, 2013). Despite early antimicrobial treatment in neonatal intensive care, up to 10% neonatal invasive group B streptococcus infections are lethal and about 25-35% of surviving infants with meningitis experience permanent neurological sequelae (Baker, 2000).

2.6. Group B streptococcus disease in pregnant women

Group B streptococcus is known as the important leading neonatal pathogen worldwide that causes maternal infections (Baker, 2000; Beitune *et al.*, 2005). The mortality rate from invasive group B streptococcus infection in pregnant women is extremely low. Group B streptococcus colonised pregnant women, have high risk factors associated with mother to child transmission including: prolonged rapture of membranes, maternal pyrexia during labour and preterm delivery (Schrag *et al.*, 2000). These bacteria can cause chorioamnionitis, urinary tract infection and wound infection in pregnant women (González-Outeirón *et al.*, 2004; McKenna and Iams, 1998).

Group B streptococcus is able to infiltrate the amniotic cavity even if the membranes are intact or not, and infect the foetus through lung epithelium (Beal and Dancer, 2006; Schrag *et al.*, 2000). Culture collection between 35 and 37 weeks' gestation is recommended to improve the sensitivity and specificity of the identification of women who are colonised at the time of delivery (Valkenburg-van

den Berg *et al.*, 2006). In the study performed by Boyer *et al.*, (1983), it was observed that women at parturition with negative vaginal but positive rectal cultures had a group B streptococcus vertical transmission rate of 17% to their new born babies.

2.7. Group B streptococcus disease in adults

Black race is associated with an increased risk of group B streptococcus infection in most adults and this reflects socio-economic factors (Schrag *et al.*, 2000). The incidence of disease among non-pregnant adults, particularly among adult with underlying conditions such as diabetes mellitus, HIV-AIDS and malignant diseases, has increased over the past decade. In non-pregnant adults with underlying medical conditions, group B streptococcus can cause urosepsis, pneumonia, as well as a skin and soft tissue infections (Farley, 2001).

2.8. Group B streptococcus antigens

Group B streptococcus; a Gram-positive coccus surrounded by a thick cell wall that acts as an exoskeleton to guarantee resistance to mechanical stresses and maintenance of cell shape. Understanding the organization and the functioning of the cell wall is very important as this cellular compartment is essential to bacterial physiology and the target of many antibiotics. Originally, Rebecca Lancefield defined two cell wall carbohydrate antigens in group B streptococcus: the group B-specific antigen common to all strains and the capsular antigen which currently defines ten different serotypes (Ia, Ib, II to IX) (Lancefield, 1934; Slotved *et al.*, 2007). The complex multiantennary structure of group B carbohydrate based on arrangement of four different oligonucleotides (rhamnose, galactose, N-acetylglucosamine and gluticoll) was solved in a series of seminal studies at the end of 1980s (Michon *et al.*, 1987; Michon *et al.*, 1988). The capsular polysaccharide and the group B carbohydrate were shown to be covalently bound to the peptidoglycan at separate sites (N-acetylglucosamine and N-acetylmuramic acid respectively). The type V capsular polysaccharide has a unique repeating unit structure consisting of a trisaccharide backbone with two distinct side chains. Antibodies against the serotype V capsule do not cross react with other group B streptococcus capsular types and appear to recognize an epitope which is not as heavily dependent on the presence of terminal side chain of salic acid residue as that of types Ia, II, and III GBS polysaccharides (Baker *et al.*, 2003).

2.9. Group B streptococcus capsular types

Group B streptococcus is one of many serologically distinct species within the genus streptococcus, however; Group B streptococcus is identified based on the presence of a polysaccharide type-specific antigen that is common to all Group B streptococcus strains determined by serological testing (Harrison *et al.*, 1998). This common group B cell wall antigen are composed of rhamnose, galactose, and N-acetylglucosamine (Bhushan *et al.*, 1998).

Therefore, group B streptococcus capsular polysaccharide is composed of pentasaccharide repeating units of N-acetylglucosamine, N-acetylneuraminic acid, glucose and galactose. Maintaining the conformation of the antigenic epitope of group B streptococcus the N-acetylneuraminic acid play the critical role. High specific binding of antibody to the group B streptococcus type III capsular polysaccharide is dependent on negatively charged N-acetylneuraminic acid's presence positioned as the terminal saccharine of the disaccharide side chain (Paoletti and Johnson, 1995).

The polysaccharide capsule, which is the basis for serotyping, is the major known virulence factor among group B streptococcus, almost all group B streptococcus isolated from invasive cases are encapsulated (Manning *et al.*, 2003). The differentiation of group B streptococcus into types based on capsular polysaccharides antigens has provided a valuable tool for defining the epidemiology of human infection. There are nine serotypes identified based on their capsular polysaccharide structure labelled Ia, Ib, II, III, IV, VI, VII, VIII and IX that were isolated from human and these are used as epidemiologic markers (Slotved *et al.*, 2007).

2.10. Group B streptococcus capsular types distribution

Some capsular types are geographically specific, for example, capsular type III that is found almost exclusively in Japan. Capsular polysaccharides dominate the surface of some bacteria and are the major target of the immune system's attack, which primarily involves antibodies, complement, and phagocytic cells (Guttormsen *et al.*, 2002). Group B streptococcus capsule primary function is thought to be protection of the organism by downregulating the complement system and prevention from phagocytosis by the host immune system (Burnham and Tyrell, 2003). Capsular

polysaccharide has been shown to elicit type specific antibodies against group B streptococcus, which protects against invasive infections (Lancefield *et al.*, 1975).

Strains of four classical serotype; Ia, Ib, II and III are of particular importance because they have been reported to account for many of group B streptococcus infections in infants in most cases of neonatal meningitis (Harrison *et al.*, 1998). Although all group B streptococcus serotypes may cause infections, type Ia, II and III form the majority of Group B streptococcus disease (Harrison *et al.*, 1998). Reports from diverse geographic regions documented type V as a frequent cause of colonization and invasive disease, first in neonates then later in adults (Blumberg *et al.*, 1996; Davies *et al.*, 2001). The emergence of type V is not due to a single clone, but most type V isolates do have one pulse field gel electrophoresis pattern that has been present in the united states since 1975 (Elliot *et al.*,1998).

Type Ia has increased in prevalence and corresponding decline has occurred in type II strains causing perinatal disease (Zaleznik *et al.*, 2000). Group B streptococcus type III polysaccharide capsule as suggested by immunological and epidemiological data is a major factor contributing to the virulence of the organism, and is more invasive than other serotypes, and this may be due to the response of specific maternal antibody against the bacterium strain (Capsular type III) (Davies *et al.*, 2001). Group B streptococcus capsular type III cause significant percentage of early onset disease in newborns and cause majority of late onset disease in newborns which manifest as meningitis and causes most of the mortality rate (Takahashi *et al.*, 1999).

Serotype III has been studied in the greatest detail, primarily because of its association with invasive neonatal disease. Serotypes III are more resistant to opsonophagocytic killing by interfering with host complement factor C3b and are more virulent. The sialic acid component of the capsule inhibits the alternative pathway of component by preventing deposition of active C3 on the surface of group B streptococcus. Eventually if C3 does deposit, conversion of C3b to I C3b on the bacterial surface is promoted by a capsule, resulting in the organism being resistant to uptake and killing by neutrophils (Burnham and Tyrell, 2003). Types VI, VII, VIII and IX rarely cause human disease in United States or United Kingdom, but in Japan

types VI and VIII are the predominant strains causing disease in neonates and healthy Japanese women (Lanchenauer *et al.*, 1999).

Identification of group B streptococcus is based on the group B specific cell wall antigens common to all strains. Methods of which each uses hyperimmune group specific antisera have been developed to detect the group B streptococcus antigen: enzyme-linked immunosorbent assay (ELISA), counter current immunofluorescence, staphylococcal agglutination and latex agglutination. Latex agglutination method is widely used due to commercial availability and simplicity. The suggested adequate method for the differentiation of group B streptococcus from other β -haemolytic serogroups of streptococci is the combination of the CAMP test with bacitracin sensitivity and the esculin reaction (Facklam *et al.*, 1979).

2.11. Expressed virulence factors

Many group B streptococcus researchers have performed an evaluation on the expression of specific virulence factors *in vitro* (Beta-haemolysin, CAMP factor, oligopeptidase, hyaluronate lyase and carbohydrate toxin CM101) or in animal models, which has contributed greatly to understanding of disease pathogenesis (Manning, 2003). Group B streptococcus pathobiology, indicates that the bacterium must encounter a number of diverse cell types such as macrophages, epithelial cells and endothelial cells during the process of invasive disease. So for this bacterium to overcome these defensive barriers and survive in the host, group B streptococcus must possess a variety of virulence factors (Burnham and Tyrrell, 2003).

Some virulence factors are only expressed in the host and not in the laboratory, thus the expression levels measured may not accurately reflect *in vivo* levels. This variable expression often decreases the reproducibility of the results. Like many pathogenic bacteria, group B streptococcus encodes a myriad of virulence factors that are critical for its ability to cause disease. There are GBS virulence factors, such as the pore-forming toxins and the sialic acid-rich capsular polysaccharide (CPS) (Manning, 2003), capsule, lipoteichoic acid, camp factor, beta haemolysin, hyaluronate lyase, oligopeptidase activity, C- protein, superoxide dismutase, C5a peptidase, penicillin binding protein, induction of apoptosis in macrophage and two component regulatory system (Burnham and Tyrell., 2003).

2.12. Group B streptococcus infection prevention efforts

Based on theory, early-onset and late-onset group B streptococcal disease could be prevented only if susceptible hosts were not exposed to the microorganism or if exposure occurred in the setting of protective immunity. Several approaches to prevention have been advocated; conceptually, these are directed at eliminating exposure or enhancing host resistance by chemoprophylaxis or immunoprophylaxis. Both strategies have limitations with respect to implementation, but could be targeted for the prevention of maternal and neonatal infections and are theoretically achievable (Baker, 1990).

Different strategies for identification of high risk mothers and infants and provision of intrapartum prophylaxis may reduce the rate of neonatal sepsis, though they are unlikely to eliminate the problem (Shet and Ferrieri, 2004). Limitations to the use of intrapartum antibiotics include the emergence of antibiotic-resistant group B streptococcus and ineffectiveness in preventing late-onset disease. Intrapartum vaginal and newborn chlorhexidine washes have not prevented sepsis in independent trials in South Africa and Pakistan, leaving resource-poor countries with no feasible alternative to vaccination (Cutland *et al.*, 2009; Saleem *et al.*, 2010), to elicit protective levels of antibodies in mothers (Lin *et al.*, 2001). Active maternal immunization may prevent peripartum maternal and neonatal disease by transplacental transfer of protective IgG antibodies. Transport across the placenta is limited before 34 weeks of gestation, so preterm babies are less well protected (Phares *et al.*, 2008; Madhi *et al.*, 2003).

From the nine recognised serotypes of GBS: a 4-valent vaccine (types Ia, Ib, III, V) would cover an estimated 85% of cases on the basis of distributions of invasive disease serotypes in industrialised countries and South Africa (Phares *et al.*, 2008; Madhi *et al.*, 2003). Vaccines that produce antibodies against the group B streptococcus capsule have been made available, and would hopefully prevent both early and late-onset neonatal infection. Glycoconjugate vaccines with type specific polysaccharide covalently linked to tetanus toxoid have shown to be protective in experimental animals and glycoconjugate vaccine composed of the type III polysaccharide and C protein could in theory protect against infection (Beal and Dancer, 2006).

The evaluation of investigational group B streptococcus vaccines, however, will require large sample sizes, because of reduced incidence of disease that has resulted from the use of intrapartum antibiotics, and would take a long time to evaluate efficacy if the vaccine were administered to women before pregnancy. Evaluating the safety of administering the vaccine to pregnant women would be difficult, due to the incidence of birth defects and again their occurrence with vaccination would be difficult to evaluate (Lin *et al.*, 2001). Robins *et al.*, 1995 proposed that licensure be granted for group B streptococcus vaccines that induce protective levels of group B streptococcus type-specific antibody without efficacy trials in order to avoid the problems. To simplify the licensure of group B streptococcus vaccines, Lin *et al.*, (2001) conducted a seroepidemiological study to estimate the protective antibody level, as measured by a standardized ELISA.

Group B streptococcal conjugate vaccine formulations for group B streptococcus have undergone Phase 1 and Phase 2 trials, including one small trial of maternal vaccination in the USA that showed safety and sustained functional antibody responses in mothers and infants two months after delivery (Baker *et al.*, 2003). Phase 2 trials with serotype III conjugate showed moderate protection against acquisition of serotype III group B streptococcus in the vagina and rectum (Hillier *et al.*, 2009).

2.13. Epidemiological typing

Serologic or molecular typing of capsular Polysaccharides and surface proteins distinguish between isolates of a microbial population, but may be insufficient in detecting virulent clones in a local outbreak and are also inadequate for evolutionary studies. Molecular typing methods may identify clones or closely related strains recovered from an outbreak, describe the population in an area and show the relatedness of isolates from different geographic areas to common ancestors. Several methods of molecular typing have been developed; the two most widely used methods in epidemiological studies of GBS at present are pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

2.14. Multilocus sequence typing

To study the epidemiology of group B streptococcus, molecular methods such as multilocus sequence typing are used (Delannoy *et al.*, 2013). Data from MLST can be used to investigate evolutionary relationships among bacteria. While PFGE is a gel-based analysis and dependent on visual interpretation, MLST is a more unambiguous genotyping method and sequence data are transferable between laboratories. MLST consisted of the amplification by PCR and sequencing of seven housekeeping genes (Figure 2.1) (*alcohol dehydrogenase (adhP)*, phenylalanyl tRNA synthetase (*pheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose kinase (*glcK*), and transketolase (*tkt*)), these method provides the sequences of 450-500 base pairs from fragments of the above listed seven “housekeeping genes encoding central metabolic enzymes in the organism (Jones *et al.*, 2003) and has been used successfully to type strains and investigate the population structure of a number of human bacterial pathogens, including *Neisseria meningitidis* and *Streptococcus pneumoniae*. (Jones *et al.*, 2003; Delannoy *et al.*, 2013). For each housekeeping gene, the nucleotide sequences may vary. The variants of the genes are assigned as alleles, and the combination of alleles at each of the seven loci represent an allelic profile which unambiguously defines the sequence type (ST) of each isolate (Maiden, 2006; Maiden *et al.*, 1998).

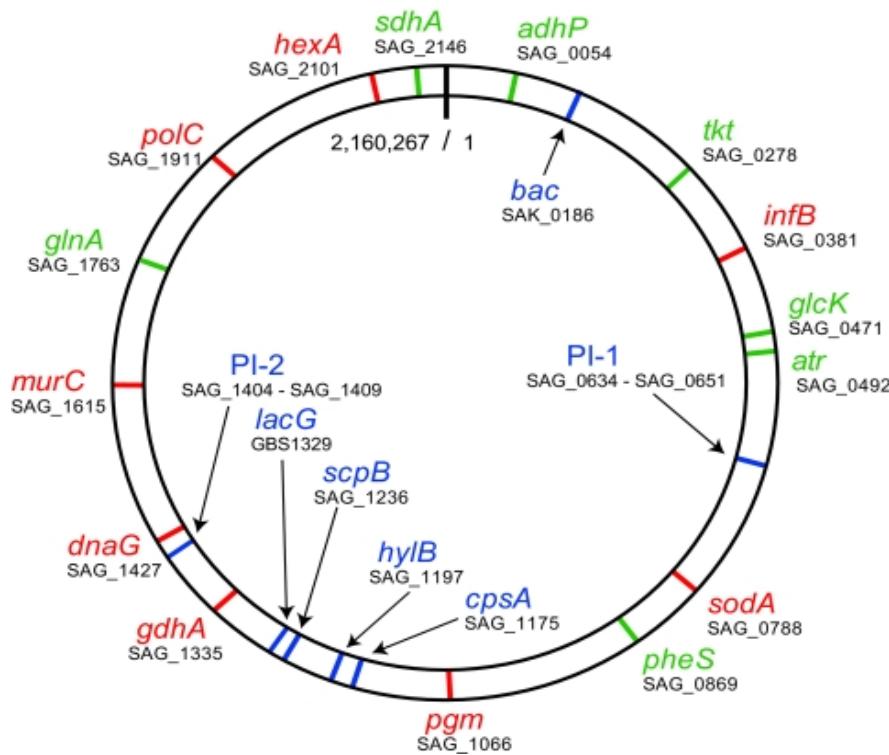


Figure 2.1. Schematic map of the group B streptococcus genome. Genes used in conventional MLST are shown in green adapted from (Sorensen *et al.*, 2010).

In MLST, the number of nucleotide differences between alleles is ignored, and sequences are given different allele numbers whether they differ at a single nucleotide site or at many sites (Maiden *et al.*, 1998). The rationale for this is that a single genetic event resulting in a new allele can occur by a point mutation (altering only a single nucleotide site), or by a recombinational replacement that will often change multiple sites. Weighting according to the number of nucleotide differences between alleles would imply that the latter allele was more distantly related to the original allele than the former, which would be true if all nucleotide changes occurred by mutation, but not if the changes occurred by a recombinational replacement (Maiden, 2006; Maiden *et al.*, 1998).

The clonal structure of the group B streptococcus population has been demonstrated by a variety of techniques, including multilocus enzyme electrophoresis, restriction endonuclease digest patterns of chromosomal DNA, pulsed-field gel electrophoresis

and gene analysis, and, most recently, by multilocus sequence typing (Cooper and Feil, 2004).

These studies have demonstrated that serotype III group B streptococcus associated with human disease derive largely from two distinct phylogenetic lineages. Although these two lineages can be identified by any of these techniques, MLST has the advantage of reproducibility and has been shown to correlate with the other techniques and thus has emerged as the standard for delineating the clonal population of group B streptococcus (Delannoy *et al.*, 2013). MLST is particularly suitable for epidemiological studies because it provides data that can easily be compared between laboratories over the Internet (Jones *et al.*, 2003), and is an important tool for molecular epidemiology because the MLST databases for individual pathogen species currently cover far more isolates than have been characterized based on whole genome sequencing. Similarly, isolates that have been characterized by three-set genotyping still outnumber isolates that have been characterized by whole genome sequencing, thus providing a less detailed but broader frame of reference than offered by whole genome sequences (Delannoy *et al.*, 2013).

CHAPTER 3

3. MATERIALS AND METHODOLOGY

3.1. Study Area

The study was conducted at Dr. George Mukhari Academic Hospital in Ga-Rankuwa, Gauteng province; Pretoria, which services the city of Tshwane municipality. The hospital serves as the teaching hospital for University of Limpopo (Medunsa campus). Ga-Rankuwa has mixed population comprising of various African tribal groups. The hospital's antenatal clinic and paediatric outpatient department serve the immediate surrounding areas and distant areas such as Limpopo and North West provinces.

3.2. Study design and population

This was a descriptive, prospective study, where pregnant patients presenting to the antenatal care (ANC) clinic at Dr. George Mukhari Academic Hospital from 4-40 weeks were recruited to form a cohort group. After signing an informed consent (Appendix A), samples were collected to investigate for *Streptococcus agalactiae*. Those with positive results were managed according to the departmental policy and their hospital file of record was tagged such that the Registrar on call can identify them as study participants. The colonised parturient women were followed up, to repeat the samples when they present in labour. Screening samples were also taken from the babies immediately after delivery. A research nurse was hired to assist with recruitment and follow-up of the cohort group.

3.3. Sample size

The prevalence of *Streptococcus agalactiae* in South Africa is estimated at 10%, as such a prevalence of 10% was used to calculate sample size. Based on estimation of 30 pregnant women seen at the ANC per day, the estimated population was 20 000 women over a two year period. At an expected frequency of 10%, confidence level of 95% and a margin of error of 5% sample size were calculated to be 138 using EpilInfo v6. Due to the challenges of retention which were expected with a cohort, a sample size was increased to 200 to accommodate for lost to follow-up and home deliveries. An additional 100 pregnant women whom were not seen at ANC and only presenting at labour were also be included in the study to make sample

size to a total of 300. To obtain valid and statistically relevant results, a clutch size of 300 samples was finally considered. Sampling took place over a period of a year. Due to lack of cooperation in the clinic and loss of participants, sample size was increased to a total of 413.

3.4. Sampling

Every pregnant woman (4-40 weeks gestation) seen at the antenatal clinic (ANC) was recruited until sample size of 413 was reached. This formed the cohort that was followed at delivery and post-delivery including their babies.

3.4.1. Inclusion criteria

- Consented pregnant women, who attended antenatal clinic at Dr George Mukhari Academic Hospital from 4 to 40 weeks gestations to form a cohort aged 18 years and above was included in the study.

3.4.2. Exclusion criteria

- Pregnant women who did not sign the consent form.
- Pregnant women whom had been on antibiotics for the past 2 weeks.
- Very sickly pregnant women

3.5. Methods

3.5.1. Data collection by questionnaire (Appendix B)

A questionnaire was designed and used to collect information regarding socio-economic data, obstetric history and the current pregnancy from each of the study participants at recruitment. Data from the hospital and clinic records included information routinely collected at the time of delivery from the mother and the neonates. Information of socio-economic characteristics (level of education, employment and marital status), demographic data and obstetric history (parity, miscarriages, stillbirths and any serious illness or vaginal discharge requiring treatment during pregnancy) were collected by a questionnaire at recruitment.

3.5.2. Specimen collections and processing

Collection of specimens was carried out for a period of a year, after permission to carry out the study was received from all relevant authorities, namely; Higher Degrees Committee ethics and Medunsa Research and Ethics Committees, Dr. George Mukhari Academic Hospital Management and Unisa College of Agriculture and Environmental Sciences. Convenient sampling method was used. An overview

of type of data and specimens that were collected during pregnancy and at delivery is shown in Table 3.1. During the recruitment (prenatal visit), low vaginal and rectal swabs was collected by a registrar/research nurse using a cotton wool swab and placed in Amies transport medium (Rochelle Chemicals & Lab equipment, SA). They were accurately labelled and placed in a cooler box filled with ice blocks for group B streptococcus investigation. The specimens collected was transported from antenatal clinic and processed at Dr. George Mukhari Academic Hospital medical microbiology laboratory. At birth the infants were swabbed on the umbilicus and ear area for group B streptococcus cultures.

Table 3.1: Types of data and specimen collection from women and babies

	Pregnant women	Babies
	Recruitment	Delivery
Questionnaire	✓	
GBS culture	✓	✓

All vaginal-rectal swab specimens were cultured on a CNA agar plate and if negative, the swabs were inoculated into the Todd-Hewitt broth supplemented with 8 g/ml colistin and 15g/ml gentamicin as to inhibit other normal flora (Manning *et al*; 2006). The plates and tubes were incubated overnight at 37°C in the presence of carbon dioxide (CO₂). After 24 hours of incubation, primary plates were examined for β-hemolytic or non-hemolytic streptococcal colonies typical of group B streptococcus. A single colony from each isolate was picked and sub-cultured onto blood agar. Plates showing no growth were further incubated for another 24 hours. Todd Hewitt broth was sub-cultured onto 5% sheep blood agar plates after overnight incubation. Two CAN agar plates were used to culture the swabs, one plate was divided into two to culture high vaginal and lower vaginal swabs and one plate was used to culture rectal swabs.

3.5.3. GBS identification and confirmation

Isolates were grown on a 5% sheep blood agar and identified as group B streptococcus by the following criteria: β-hemolysis on a 5% sheep blood agar, Gram staining showing gram positive cocci in pairs or short chains, negative reaction with

catalase reagent and Lancefield grouping with type B antisera (streptex; Biomereux and Remel Ltd, Europe).

3.5.4 Catalase test

A drop of hydrogen peroxide (H_2O_2) (30%) was placed on a clean slide, using a sterile inoculation loop; suspected colonies due to the presence of haemolytic activity were picked from the plate and be placed on a clean slide with H_2O_2 to determine the presence of Catalase enzyme in the isolate, The absence of gas bubbles indicated that the colony might be the GBS, since GBS is catalase positive.

3.5.5 Gram staining

A gram stain was done to catalase negative suspected colonies from a culture medium (CNA). Colonies that were Gram positive cocci were picked and subcultured onto 5% sheep blood agar (National Institute for Communicable Diseases (NICD)). After 18-24 hour incubation, colonies were picked for serological confirmation.

3.5.6 Latex agglutination test for identification of group B streptococcus

Seroagglutination was carried out by a slide agglutination technique using commercially prepared Lancefield group B Antisera. Agglutination patterns were linked to group B streptococcus species following manufacturer's (Biomeurex) instructions. All isolates that are confirmed to be GBS were stored in microbanks (which it comprises of a unique cryovials System incorporating treated beads and a special cryopreservative solution) with different numbers and placed in a -70°C fridge until further analysis.

3.5.7 Multilocus sequence typing (MLST)

Multilocus sequence typing was performed as described by Jones *et al.*, (2003). Initially DNA from each isolate was extracted with a genomic DNA isolation kit (ZR fungal/bacterial Zymo Research Corp, USA) following manufacturer's instructions. Briefly, 100 mg (wet weight) GBS bacterial cells that have been resuspended in 200 μ l of distilled water and 750 μ l lysis solutions was added to a ZR Bashing Bead lysis tube, and were secured in a bead beater fitted with a 2 ml tube holder assembly (Disruptor Genie) and were processed at a maximum speed for 5 minutes. The ZR Bashing Bead lysis tube were centrifuged at 10 000 Xg for a minute, then 400 μ l supernatant were transferred to a zymo-spin™ IV spin filter in a collection tube and centrifuge at 7000 rpm for a minute. To the filtrate in the collection tube, 1200 μ l

fungal/bacterial DNA Binding Buffer, 800 µl of the mixture was then transferred to a zymo-spin™ IIC column in a collection tube and centrifuged at 10 000 x g for a minute, the step was repeated. DNA Pre-wash buffer (200 µl) was added to the zymo spin™ IIC column in a new collection tube and centrifuged at 10 000 x g for a minute, then 500 µl fungal/bacterial DNA wash buffer to the zymo spin™ IIC column and centrifuge at 10 000 x g for a minute. The zymo spin™ IIC column was transferred to a clean 1.5 ml microcentrifuge tube and 100 µl DNA elution buffer was then added directly to the column matrix and centrifuged at 10 000 x g for 30 seconds to elute the DNA. The supernatant PCR template was stored at -20°C for the subsequent PCR amplification.

3.5.8 Locus selection and PCR

The following seven housekeeping genes; *alcohol dehydrogenase (adhP)*, phenylalanyl tRNA synthetase (*pheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose kinase (*glcK*), and transketolase (*tkt*) (Table 3.2) used for group B streptococcus characterization were amplified from DNA extracts by Polymerase Chain Reaction (PCR). PCR products were amplified with oligonucleotide primer pairs designed from the Integrated DNA technologies (Canada). Each 50 µl amplification reaction mixture comprised of 25 µl PCR master mix (Thermo scientific), 1 µl of each PCR primer (Integrated DNA technologies, Canada) and 5µl of group B streptococcus chromosomal DNA. The PCR reaction was performed in an automated thermal cycler (Applied Biosystems, Foster City, CA, USA). The reaction conditions were denaturation at 94°C for 1min, primer annealing at 55°C for 45 seconds and extension at 72°C for 1min for 30 cycles.

3.5.9 Detection of amplified products

Amplified products were detected by ethidium bromide (Bio-Rad) stained agarose gel electrophoresis. This was done as follows: A 1.5 % gel was prepared by adding 100ml of 0.5 X Tris-Borate-EDTA (TBE) buffer solutions to 1.5 g of agarose. The solution was dissolved in a microwave oven and then cooled. Five micro litre of the intercalating agent ethidium bromide was added to visualize amplified products when exposed to fluorescent light. The solution was poured into a gel tray, a comb was inserted and the gel left to set. Ten µl of amplified product and 5µl of loading dye were added into the wells of the gel. A 50 bp molecular weight marker

(HyperladderTM, Bioline) was used in combination with amplified product from group B streptococcus isolates. The gel was run at 75 V for 1hour in a 0.5 x TBE buffer solution. A photographic copy of the amplified products on the gel was taken with a Gel DocTM EZ Imager (Bio-Rad).

Extracted DNA was PCR amplified with primers listed in Table 3.2 and partial segments (450-500) of the PCR products were sequenced (Inqaba Biotec, Pretoria, South Africa). Genetic comparison of the strains was performed by constructing a dendrogram using CLC-bio (Inqaba Biotec, Pretoria, South Africa).

Table 3.2: Oligonucleotide primers for group B streptococcus MLST (Jones *et al.*, 2003)

Locus use	Name of the sequence of primer		Ampli- con size (bp)	
	Forward (5'-3')	Reverse (5'-3')		
<i>adhP</i>	Amplification	GTTGGTCATGGTGAAGCACT	ACTGTACCTCCAGCACGAAC	672
	Sequencing	GGTGTGTGCCATACTGATT	ACAGCAGTCACAACCAACTCC	498
<i>pheS</i>	Amplification	GATTAAGGAGTAGTGGCACG	TTGAGATCGCCCATTGAAAT	723
	Sequencing	ATATCAACTCAAGAAAAGCT	TGATGGAATTGATGGCTATG	501
<i>atr</i>	Amplification	CGATTCTCTCAGCTTGTAA	AAGAAATCTCTTGCGGGAT	627
	Sequencing	ATGGTTGAGCCAATTATTTC	CCTTGCTCAACAATAATGCC	501
<i>glnA</i>	Amplification	CCGGCTACAGATGAACAATT	CTGATAATTGCCATTCCACG	589
	Sequencing	AATAAAGCAATGTTGATGG	CATTGTTCCCTTCATTATC	498
<i>sdhA</i>	Amplification	AGAGCAAGCTAATAGCCAAC	ATATCAGCAGCAACAAGTGC	646
	Sequencing	AACATAGCAGAGCTCATGAT	GGGACTTCAACTAACCTGC	519
<i>glcK</i>	Amplification	CTCGGAGGAACGACCATTAA	CTTGTAAACAGTATCACC GTT	607
	Sequencing	GGTATCTTGACGCTTGAGGG	ATCGCTGCTTAATGGCAGA	459
<i>tkt</i>	Amplification	CCAGGCTTGATTAGTTGA	AATAGCTTGGCTTGAAA	859
	Sequencing	ACACTTCATGGTGTGGTTG	TGACCTAGGT CATGAGCTT	480

3.5.10 Phylogenetic Analysis

The consensus of all six isolates was drawn in CLC bio software (Inqaba Biotec, Pretoria, South Africa). This consensus was aligned in CLC bio software to get global consensus sequence. The consensus was used to draw a UPGMA dendrogram and study variations in different isolates of the different serotypes of group B streptococcus.

To draw a phylogenetic tree of the six isolates of group B streptococcus, only three genes (*adhP*, *glnA* and *tkt*) were used. All sequences were first aligned in the CLC bio software and aligned file was then subjected to UPGMA method to draw a phylogenetic tree. Neighbour-joining and maximum parsimony methods were used for the construction of phylogenetic tree. The above methods were used in combination with bootstrap method.

3.5.11 Reliability, validity of the study and Statistics

All tests were done according to standard operating procedures to ensure reliability and validity of the results. Data were collected and analysed using Windows Office Excel and SPSS software (SPSS Inc.).

3.5.12 Ethical considerations

The study was conducted after an approval from the Higher Degrees Committee ethics and Medunsa Research and Ethics Committees, Dr. George Mukhari Academic Hospital Management (MREC/P/02/2011: IR) and Unisa College of Agriculture and Environmental Sciences (Appendix D). Pregnant Women were informed about the study and those who agreed to participate, signed an informed consent statement (see Appendix A) indicating their approval to participate. The information and consent statement was available in both Tswana (local) and English languages. The information obtained from the pregnant women was treated with confidentiality. All women were informed that they were free to withdraw from the study at any time without this having any effect on their further treatment from the clinic or hospital.

CHAPTER 4

4. RESULTS

4.1. Socio-demographic of the participants

The socio-demographic characteristics of participating pregnant women are shown in Table 4.1. Four hundred and thirteen (413) pregnant women from different areas were recruited into the study over a period of 9 months from February 2012 to October 2012.

All the pregnant women enrolled in the study were of the same racial group (blacks) within the age range of 18-45 years with the median age of 30 and the mean age of 30. The pregnant women were categorised into different age groups in years as follows: <20, 20-24, 25-29, 30-34 35-39 and ≥40. The majority of the participants were between the ages of 25-29 years (27.3%).

The study participants were from different geographic areas (urban semi-urban and rural) surrounding Dr George Mukhari Academic Hospital. The majority of the participants were either single or divorced (53%), followed by married (24.3%) then cohabiting (22.1%) (Table 4.1).

The participants' educational levels were documented as follows: below matric, matric and tertiary level. Most had an educational level below matric (56.8%), followed by those who had educational level matric (29.2%) and then tertiary level (16.6%). Out of 413 participating pregnant women 107 were below matric, 229 were in matric whereas 67 were in tertiary level and there were missing data of 10 participants.

Table 4.1: Socio-demographic characteristics of the pregnant women investigated for group B streptococcus colonization at Dr George Mukhari Hospital, Ga-Rankuwa.

Socio demographic characteristics	Category	Frequency	Percentage
Age in years			
	<20	12	2.98%
	20-24	75	18.6%
	25-29	110	27.3%
	30-34	95	23.6%
	35-39	81	20.1%
	≥40	30	7.4%
Geographical areas			
	Urban	359	89.1%
	Semi-urban	36	8.9%
	Rural	6	1.5%
Marital status			
	Married	98	24.3%
	Cohabiting	89	22.1%
	Single/widowed/divorced	214	53%
Educational level			
	Below matric	229	56.8%
	Matric	118	29.2%
	Tertiary	67	16.6%
Employment			
	Unemployed	225	55.8%
	Employed	148	36.7%

4.1.1 Overall colonization

Pregnant women were classified as being group B streptococcus colonized, if the bacterium was isolated from the vagina, the rectum or both and confirmed by antigen detection. The participants were regarded as non-colonised if there was no group B streptococcus isolated from all the sites mentioned above. Group B streptococcus

colonization was detected in 128/413 (30.9%) of pregnant women with gestational age from 4-37 weeks.

4.1.2 Comparison between media used to culture

The importance of selective enrichment broth and selective plating is shown in Table 4.2 below, the comparison between the selective direct plate (CNA agar plate) and selective broth enrichment (Todd-Hewitt) was performed on 413 samples of which 128 of the samples tested positive. Seventy (54.7%) of Group B streptococcus positive samples were recovered from using Todd-Hewitt broth, whereas 45.3% were recovered from CNA agar plate. From 58 colonised pregnant women, 12 had strains cultured only from vaginal swabs, while 22 had strains isolated only from the rectal swabs and another 24 had strains isolated simultaneously from both the vaginal and rectal swabs when cultured on a CNA agar plate.

Table 4.2: Comparison between Todd-Hewitt broth and CNA media

GBS colonised pregnant women	Type of medium		Site of recovery from CNA media			
	CNA	Todd-Hewitt broth	RS	LVS	HVS	All sites (RS,LVS,H VS)
128	CNA	Todd-Hewitt broth	RS	LVS	HVS	All sites (RS,LVS,H VS)
	58	70 (37.9.2%)	22 (15.5%)	9 (5.2%)	3 (41.4%)	24

4.1.3 Variables associated/not-associated with GBS colonization

The age range (18-45) distribution of the group B streptococcus colonised pregnant women were compared. Among the group B streptococcus colonised pregnant women, group B streptococcus colonization rate was higher in the 25-29 age group and the colonization rate was lower in <20 age group (Figure 4.1).

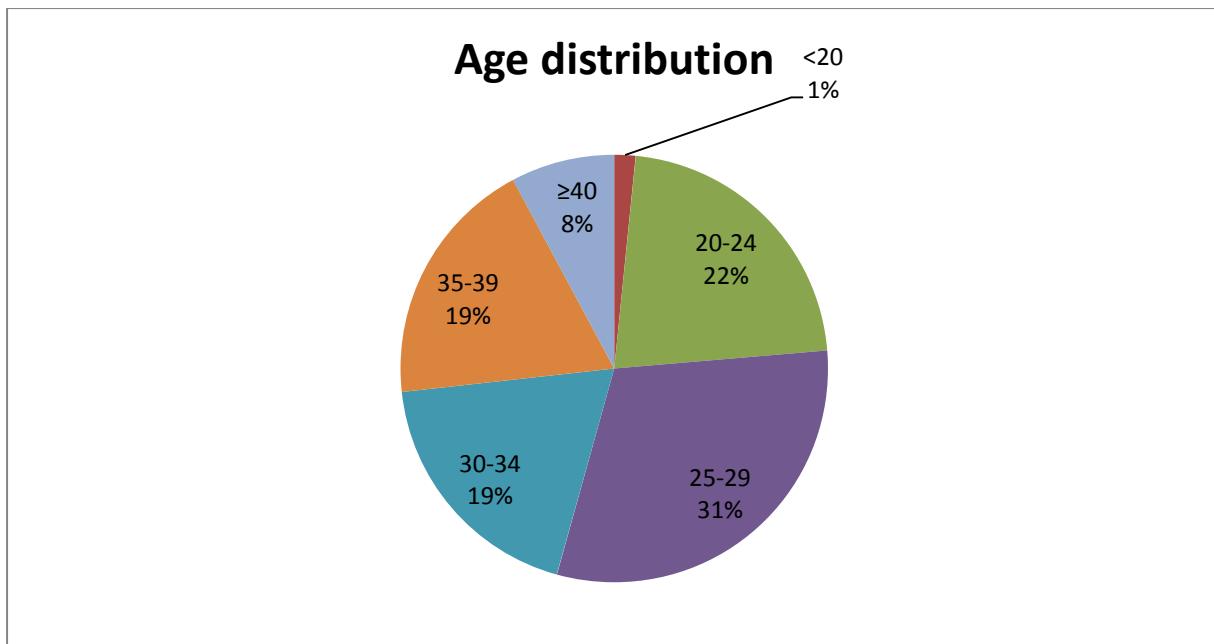


Figure 4.1: Distribution of 128 group B streptococcus isolated among different age interval of the study participants.

4.2 Adverse pregnancy outcomes

Adverse pregnancy outcomes were categorised into miscarriages, HIV positive and still birth while pregnant women who were currently colonised with group B streptococcus and had miscarriages previously were 32/85 and those who had stillbirth were 12/23 (Table 4.3).

Table 4.3: Variables associated with group B Streptococcus colonization in pregnant women, George Mukhari academic hospital; Ga-Rankuwa.

	N (number)	%GBS colonization
HIV status		
Positive	52	40
Negative	72	56
Parity		
0	38	37
1-2	68	30
≥3	19	28
Still birth		
0	113	33
1-2	12	55
≥3	0	0
Total GBS colonisation	12	52
Miscarriage		
0	93	29
1-2	29	37
≥3	3	43
Total GBS colonisation	32	38

4.1.4 Neonates born to colonised pregnant women

Out of 128 colonised pregnant women, only 39 of their newborn babies were involved in the study. This was because most pregnant women gave birth without giving notice to the registry or a nurse involved in the study for the swabs to be taken. The neonates were supposed to be classified as being colonized if group B

streptococcus was isolated from any of the two specimens (ear or cord). However, there were no positive results obtained.

4.3. Multilocus sequence typing

4.3.1. PCR and Sequencing

Due to time and resource limitations, a representative proportion of each capsular type was randomly selected for further analysis using multilocus sequence typing. In this study, a total of seven group B streptococcus isolates were used, each representing serotype group (Ia, Ib, II, III, IV, and V) recovered at Dr George Mukhari Academic hospital. Genomic DNA was extracted from seven isolates and seven housekeeping gene fragments were amplified (PCR), were negative control was used to ensure that there was no contamination and sequenced using the corresponding primers that are listed in Table 3.2 (in a previous chapter).

To check if the PCR had worked and that there was no contamination in the negative controls, the samples were run on an agarose gel to separate DNA molecules according to their size and ensure purity. Most of the genes could be amplified successfully except the following gene and samples; *glcK* 113, *adhP* 1 and 113, *glnA* 4 and 113, *sdhA* 6, *pheS* 6, and *atr* 1 and 2. Clear specific bands of expected sizes were observed and it was easy to differentiate these from non-specific bands (Appendix C). Table 4.5 gives the summary of agarose gel electrophoresis (Appendix C) results from PCR products amplified with corresponding oligonucleotide primers of seven housekeeping genes. The negative sign (-) shows that there was no band observed when visualizing amplified products exposed to fluorescent light, whereas positive (+) sign shows that the band was available, which proves that the PCR has worked.

Table 4.4: Summary of agarose gel electrophoresis (Appendix C) results from PCR products amplified with corresponding oligonucleotide primers.

Isolates		<i>adhP</i>	<i>pheS</i>	<i>atr</i>	<i>glnA</i>	<i>sdhA</i>	<i>glcK</i>	<i>tkt</i>
ID								
1	-	+	-	-	+	+	+	+
2	+	+	-	-	+	+	+	+
4	+	+	+	-	-	+	+	+
6	+	-	+	-	+	-	+	+
12	+	+	+	-	+	+	+	+
65	+	+	+	-	+	+	+	+
113	-	+	+	-	-	+	-	+

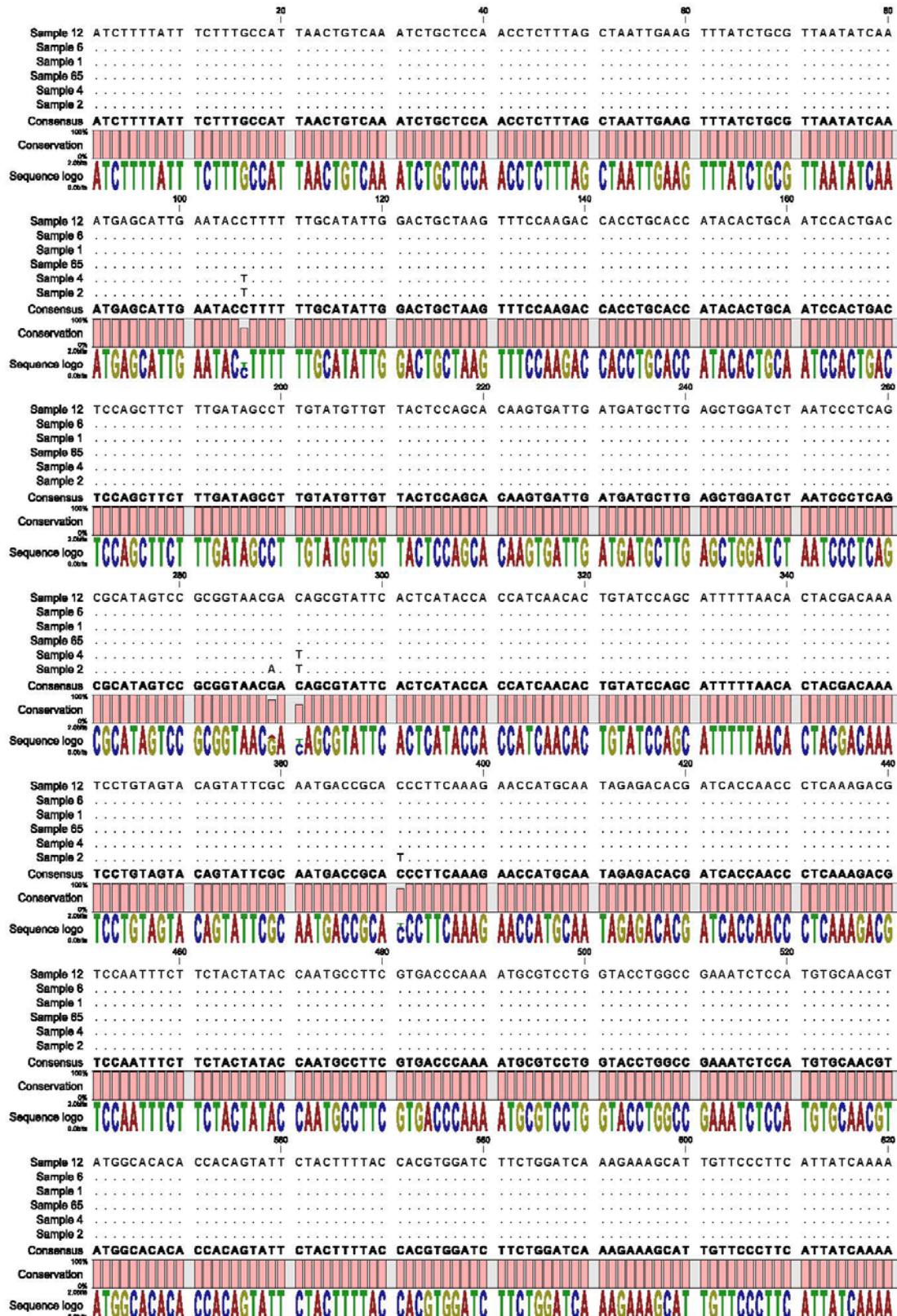
Isolate 1 represent GBS serotype III, isolate 2 GBS IV, isolate 4 GBS serotype III, isolate 6 represent GBS serotype Ia, isolate 12 represent GBS V, isolate 65 represent GBS Ib and isolate 113 represent serotype II.

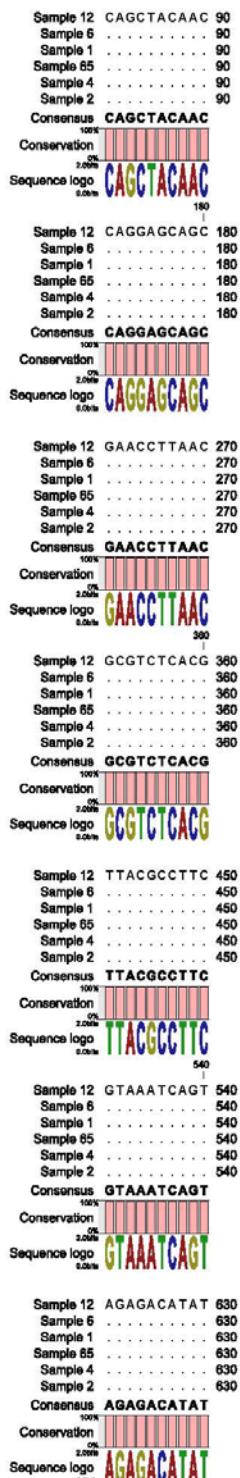
ID=Identity, *adhP*= alcohol dehydrogenase, *pheS* = phenylalanyl tRNA synthetase, *atr* =amino acid transporter, *glnA*= glutamine synthetase, *sdhA* = serine dehydratase, *glcK* = glucose kinase, and *tkt* = transketolase, (-) = negative, (+) = positive.

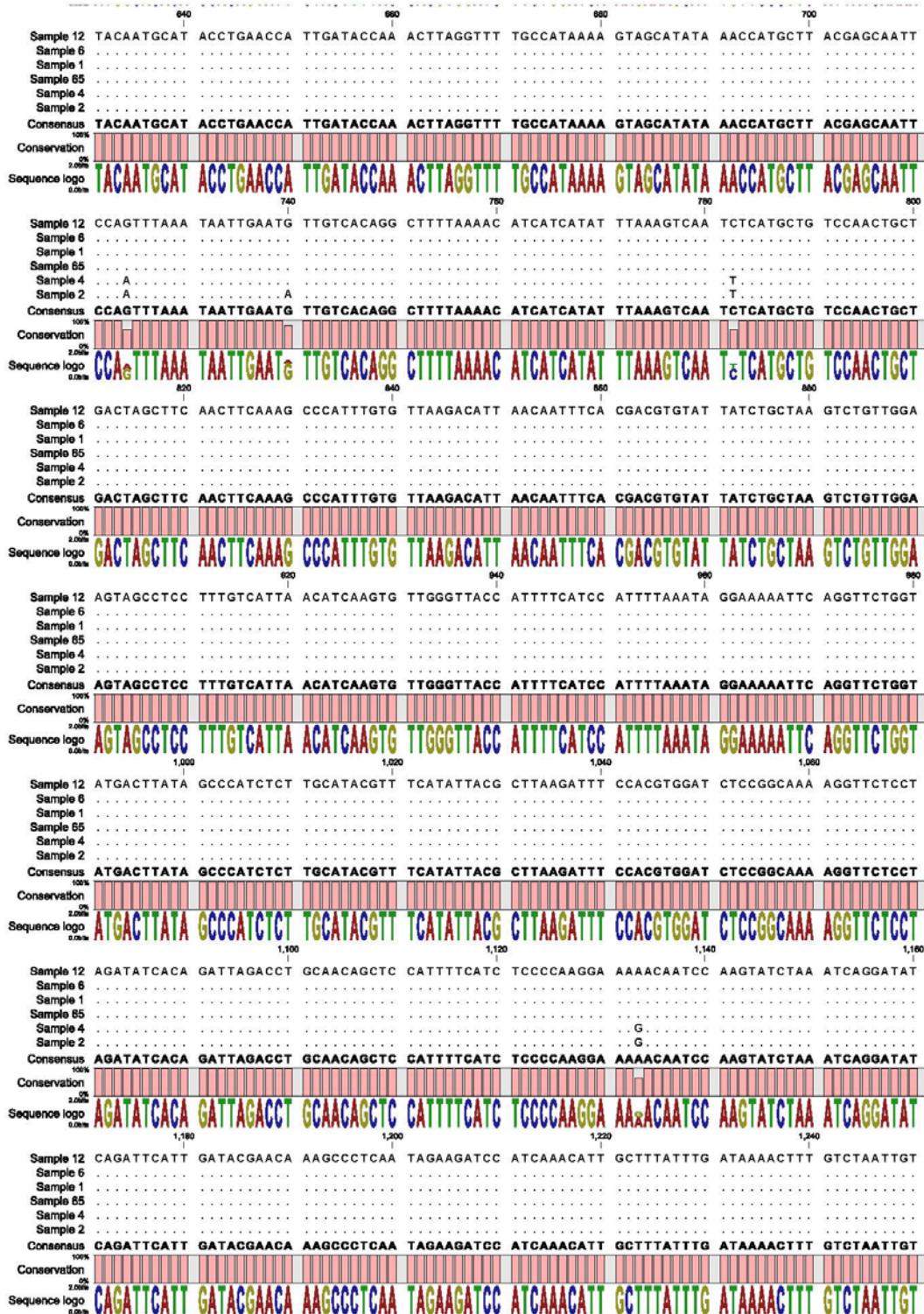
To proceed with the sequencing, PCR products undergone quality control test, where they were purified to remove all the primers, nucleotides, enzymes, salts and other impurities from the DNA samples that might interfere with the sequencing process. In this study, 49 samples (which comprises of seven isolates and seven genes from each isolates) undergone quality control test, where only 10 samples out of 49 samples failed the quality control test, therefore only 39 samples were sequenced. The following samples; *atr* 12, *sdhA* 113 and *pheS* 4 were unable to be edited because they contained mixed templates that was confirmed by a second sequencing reaction and therefore, they were not used for analysis.

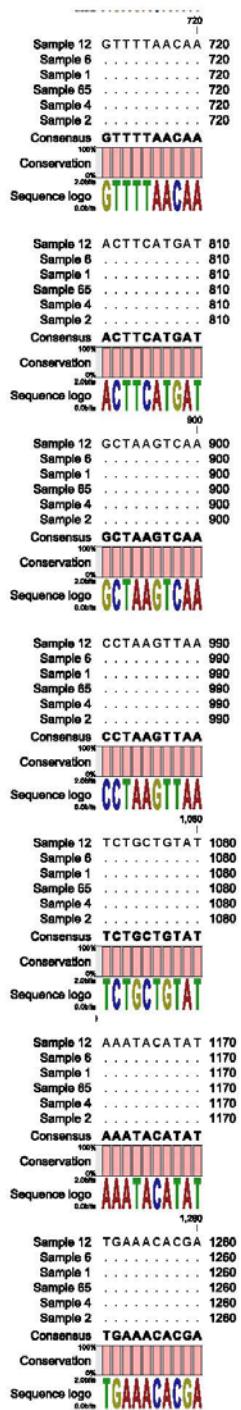
4.4. Consensus Sequences of the housekeeping genes

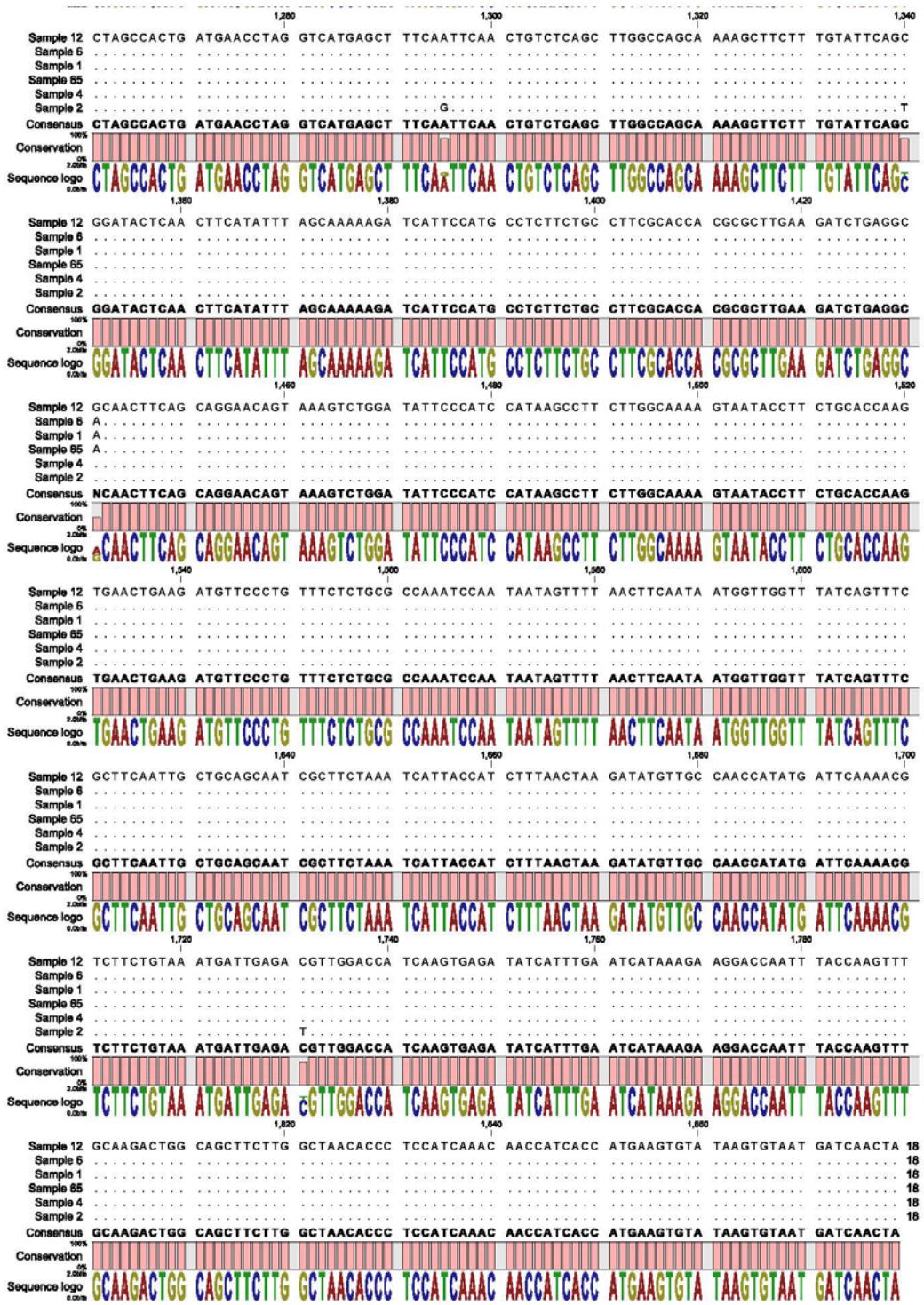
For the seven isolates of the group B streptococcus, the sequences obtained at each of the seven loci were compared with each other. Figure 4.2 shows the alignment of the consensus sequence based on three genes (*adhP*, *glnA* and *tkt*) sequenced successfully for the six isolates of the group B streptococcus, the global consensus sequence is shown at the base and identical bases are marked with dots. Conserved residues are shown with their corresponding symbols. The alignment of all the consensus sequences helped to study the highly conserved residues in the isolates of group B streptococcus. Figure 4.3 shows the UPGMA dendrogram (CLC bio software); that represent the relatedness of group B streptococcus isolates. A default value of 1000 was used in bootstrap analysis and the values are present at each branch. The different sample numbers at the ends of the branches are external nodes. The lengths of the branches between the nodes illustrate the differences in nucleotide sequences between the isolates. Thus, the tree illustrates phylogenetic relationships between the sequence types. The relationships are presented as a rooted tree with branch lengths being proportional to the estimated genetic distance between the strains of group B streptococcus. The scale bars represent the number of nucleotide differences. Since not all the genes were covered for all the samples; the dendrogram was constructed using the most complete set of genes of the following isolates; 1, 6, 65, 2, 4 and 12, except for 113 isolate. The allelic profiles for the isolates were not recorded.











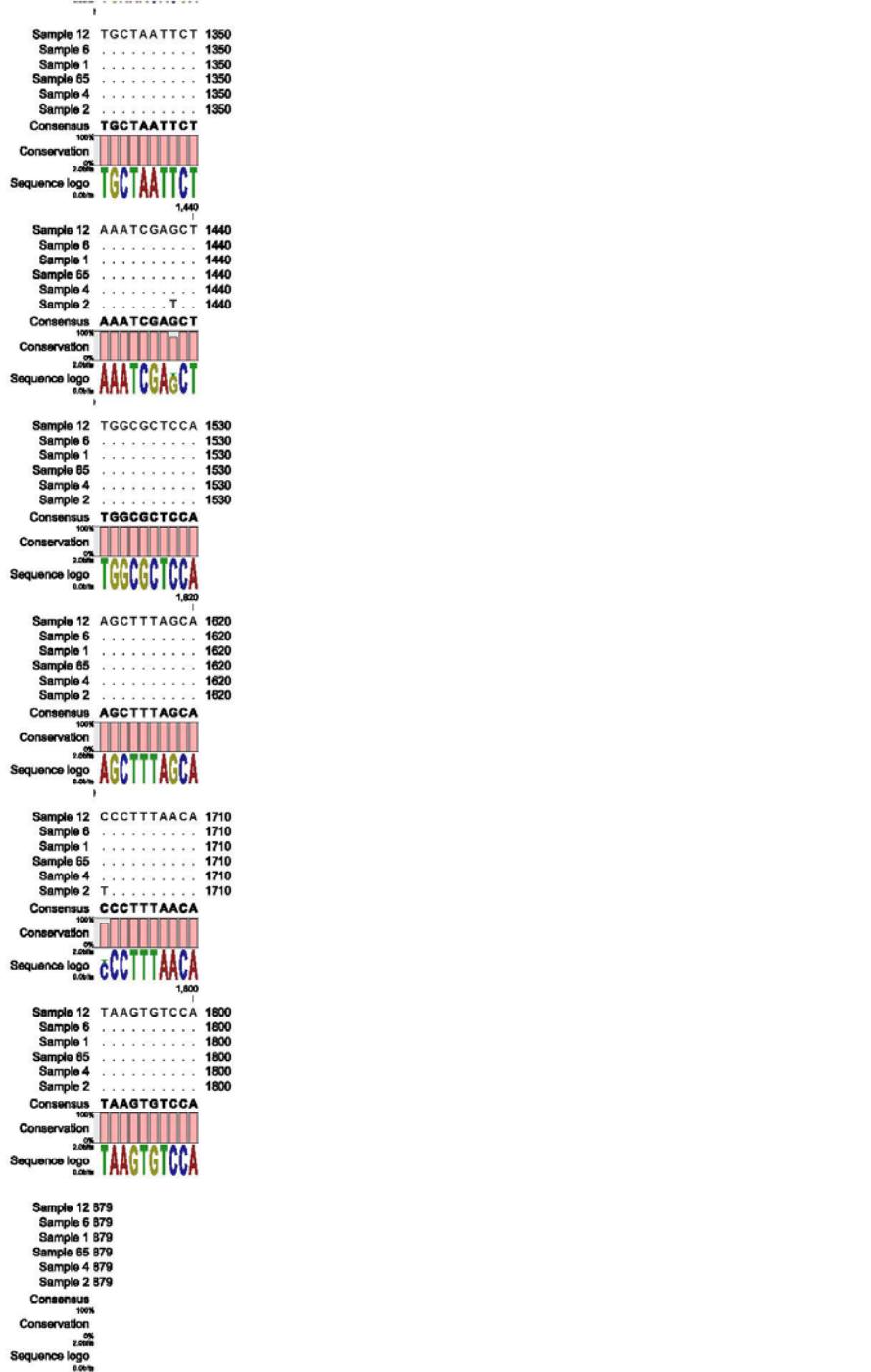


Figure 4.2: Multiple sequence alignment of consensus sequences of the isolate one to six (1, 6, 65, 2, 4 and 12) of the group B streptococcus. A reduced form alignment showing only the differences for the data set was used in the UPGMA tree.

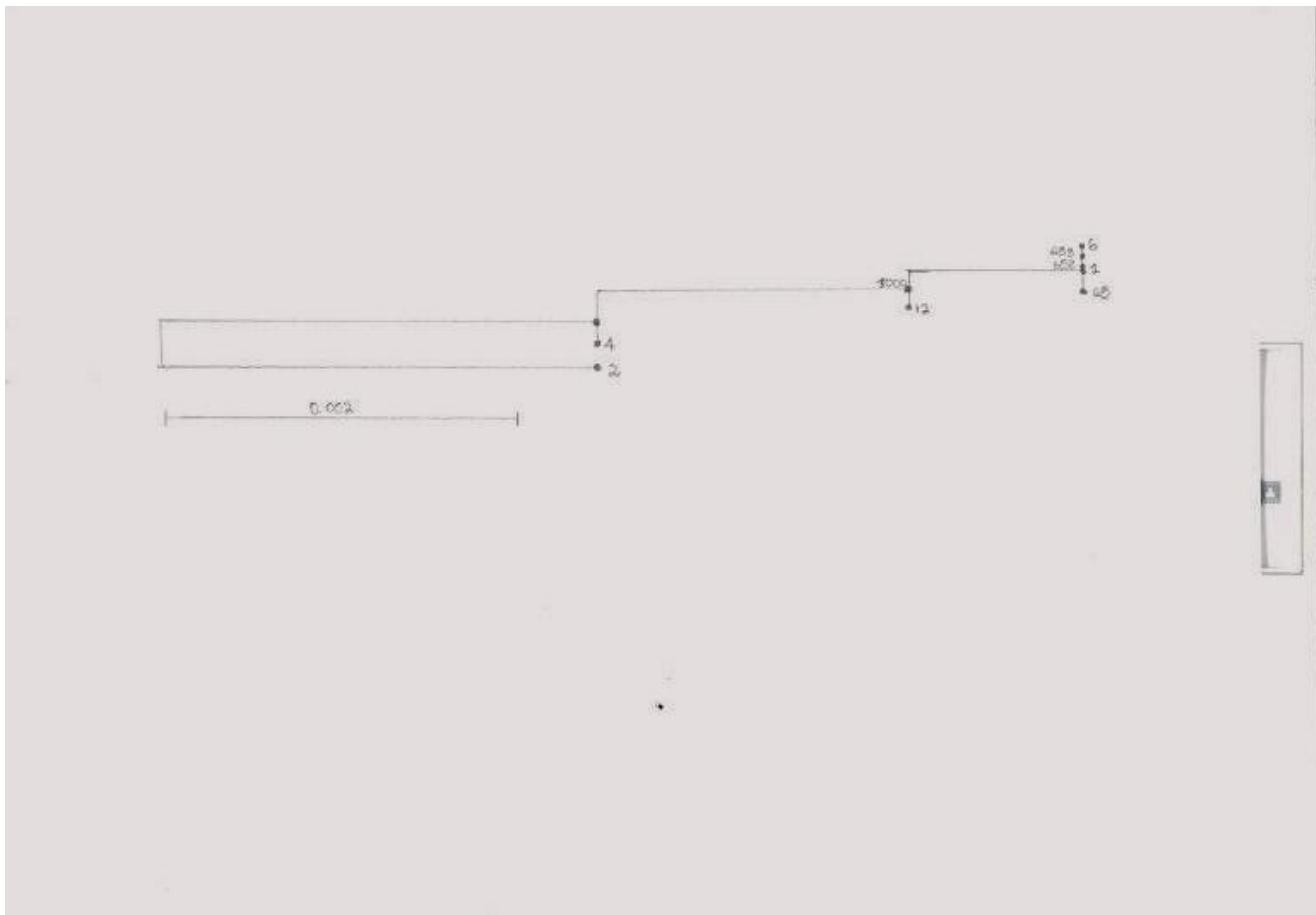


Figure 4.3: Phylogenetic tree of group B streptococcus consensus alignment tree isolates sequences constructed using CLC Bio. The dendrogram was a consensus of 1000 bootstrap trees generated with the neighbour-joining algorithm with the use of sequence data for three genes (*adhP*, *glnA* and *tkt*).

CHAPTER 5

5. DISCUSSION

5.1. Epidemiology of group B streptococcus

This was a descriptive, prospective study with a population of 413 pregnant women seeking antenatal care at Dr George Mukhari Academic Hospital. However, selection bias might have occurred. Some women attending antenatal care may not have been recruited into the study due to lack of staff and time at the study sites. However, these pregnant women were likely not to differ from the study participants. The numbers of pregnant women not recruited due to this reason, as well as the number of women who declined recruitment to the study were not recorded. Data on participants' socio-demographic were not available for ten (2.4%) of the pregnant women originally recruited to the study. Group B streptococcus culture was performed on every specimen. The missing data during this part of the study are therefore, unlikely to represent a bias with respect to group B streptococcus status.

5.1.1. Colonization rate

The key findings of the study were that, the estimated maternal colonization rate of group B streptococcus colonisation was 30.9%. The finding of this study is high when compared to colonization rate from some other African countries like Mozambique which reported colonization rate of 1.8% (De Steenwinkel *et al.*, 2008). The difference in colonization rate reported from Mozambique and the present study may be due to source of blood used for culture for isolation of group B streptococcus and geographic difference can also contribute to the difference. However, higher rates have been reported from two previous studies (35% and 54%) in Europe (Bergh *et al.*, 2004; Bergseng *et al.*, 2008) and the other study (47%) from Zimbabwe (Mavenyengwa *et al.*, 2009). The reasons for this significant colonization rate in this study were unclear, though; the contributory factors might include the specimen collection and culture medium used. The use of selective enrichment broth is recommended to maximise the isolation of group B streptococcus and to avoid the overgrowth of the other microorganism, because vaginal and in particular rectal flora contains numerous microorganisms (Ferrieri *et al.*, 1977). The cohort's gestation age in this study was from 4-40 weeks which differs with what Bergeron *et al.*, (2000) used in their study, where they cultured specimens at 35-37 weeks gestation.

When direct agar plating was used instead of selective enrichment broth, as many as 50% of women who are group B streptococcus carriers have false-negative culture results (Laboratory practices for prenatal Group B streptococcal screening and reporting, 1999). The isolation rate of group B streptococcus strains was improved by selective enrichment broth (Todd-Hewitt broth) in this study, where the swabs that showed no colonization when cultured on a CNA agar plate, tested positive when inoculated in a Todd-Hewitt broth. These false-negative culture results were proved by Ferrieri *et al.*, (1977) and Kubota (2002) who did not use selective culture medium and only found the prevalence rates of 5.6% and 11.4%, whereas with studies that used selective medium, reported rates of 19.8% and 18.6% (Madzivhandila *et al.*, 2011; Valkenburg-van den Berg, *et al.*, 2006).

In some scientific reports, the different positive culture rates in the variety of culture media was shown, and in the different areas of specimen collection (Yang *et al.*, 2012), the rectal swabs exclusion to detect group B streptococcus colonization may compromise the sensitivity in group B streptococcus colonization detection (Stoll and Schuchat., 1998). Transmission rates range from 29% to 85% in most studies, low rates as 12% have been reported from countries where low colonization rates has been found irrespective of the adequate microbiological methods used (Edwards and Baker., 2000; McKenna and Iams., 1998). The culture methods used in this study was an internationally renowned method using lower vaginal and rectal swabs cultured both in enrichment broth and on a solid agar plate media.

In various studies there are no significant differences in colonization rates noted on the basis of age or parity, but increasing age and parity (Anthony *et al.*, 1978; Yow *et al.*, 1980) have also sometimes been associated with lower rates of carriage. However, Regan *et al* (1991) described group B streptococcus carriage as being more common among older women and women of lower parity. In this study, no association between colonization and age or parity was found. This correspond to study done by Valkenburg-van den Berg, *et al.*, (2006), where they found no association between colonization, parity and age.

5.1.2. Group B streptococcus and Ethnic groups

Characteristics of the population studied can also explain differences in group B streptococcus colonization rates. The group B streptococcus colonization rate

among black pregnant women both in United States and South Africa has been shown to be higher (Dzowela *et al.*, 2005). Comparison between different ethnic groups was limited in this study, since all pregnant women enrolled were of the same ethnic group.

5.1.3. Risk factors for GBS colonization

There are many predisposing factors which have previously been reported to be associated with an increased risk of group B streptococcus colonization and as a result the neonate has an increased risk of group B streptococcus infection. The factors that were investigated in this study include reproductive history and illness, delivery methods and HIV status. The study aimed at correlating the pregnant women's current group B streptococcus status with their past pregnancy history, because group B streptococcus was previously reported as being associated with pre-term delivery, miscarriages, still birth and parity.

Although the importance of infection as a cause of preterm delivery is gaining recognition, little is known about the role of group B streptococcus infection in miscarriages. McDonald *et al.*, (2000) stated that group B streptococcus was a key pathogen in unsuspected intrauterine infections underlying spontaneous midgestation abortions. The study of Daugaard (1988) demonstrated an association between the occurrence of group B streptococci in the urine and cervix and late spontaneous abortions, but El Kersh *et al.* (2002) found no correlation with a history of repeated spontaneous miscarriages. There was no relation between pregnant women who had a history of miscarriages, stillbirth and group B streptococcus carriage, though there was a report, of Group B streptococcus colonised pregnant woman originally from Zimbabwe who was reported to give birth to a macerated baby (foetus), and due to the condition, swabs were not taken for analysis, so there is no prove of group B streptococcus being the cause of the maceration. There was another report of group B streptococcus pregnant woman who was admitted in a hospital because she experienced no foetus movement for a period of a week, and she was found to have uterine foetus death. Due to the situation, no swabs were taken, there's no prove of group B streptococcus being the cause of the foetus death.

The majority of group B streptococcus colonised pregnant women were unable to report back when they come back to give birth, this resulted in having a smaller cohort of the neonates than it was expected. Only 39 (30.5%) neonates from 128 colonized pregnant women participated in the study; represented the whole population of the neonates. It was documented that, most neonates were born by caesarean and this limited the comparison between colonized neonates born by caesarean and those born by normal birth.

5.2. Multilocus sequence typing (MLST)

Typing of bacterial strains and isolates remains an important aspect in epidemiology and microbiological research. The process of MLST analysis is very specific and therefore it is unlikely that strains have the same sequence type due to chance alone (Jones *et al.*, 2003). The samples analyzed in this study were limited in number due to time and resource limitations but a representative of each capsular types (which was done in the other part of the study) was randomly selected for further analysis using multilocus sequence typing to provide a snapshot of a larger cohort and information about the strains of group B streptococcus circulating in the population of interest at Dr. George Mukhari Academic hospital. Some of the amplification products (samples) were not edited because they contained mixed templates. No analysis on the sample was done. Thus, only samples with no mixed templates were edited and used for analysis.

The group B streptococcus polysaccharide capsule is determined by genes in the capsular polysaccharide locus, which encodes enzymes for polysaccharides synthesis (Cieslewicz *et al.*, 2005 and Yim *et al.*, 1997). No isolate of serotype VI, VII and VIII was found among the strains isolated from Dr George Mukhari Academic Hospital but only Ia-V. Isolates representing Ia, Ib, II, III, IV and V was used for MLST to represent the population. The most common strain was serotype III, which is the reason why two strains, representing III, were used for multilocus sequence typing (MLST) analysis.

Multiple alignment construction corresponds to developing a hypothesis of how a number of sequences have evolved through the processes of character substitution, insertion and deletion. The input to multiple alignment algorithms is a number of homologous sequences; that is sequences that share a common ancestor and most

often also share molecular function. The generated alignment in figure 4.2 where each row corresponds to an input sequence and each column corresponds to a position in the alignment. An individual column in the table represents residues that have all diverged from a common ancestral residue. Gaps in the table (represented by a '-') represent positions where residues have been inserted or deleted and thus do not have ancestral counterparts in all sequences.

The allelic profile, characteristics such as G+C (guanine+cytosine) content and the proportions of nucleotide alterations that led to a change in the amino acid sequence (non-synonymous substitution, dN) and the proportions of nucleotide alterations that did not lead to a change in the amino acid sequence (synonymous substitution, dS) ratio were not analysed or calculated because of the smaller sample size used in this study as it is compared with the study done by Jones *et al.*, (2003) where they used MLST to characterize a collection of 152 of globally and ecologically diverse human strains of group B streptococcus that included representatives of capsular types Ia, Ib, II, III, V, VI and VIII. The combination of their alleles of their results at the house keeping loci provided an allelic profile or sequence type for each isolate. Similar results might have been obtained if the same sample size was used. By saying that, the MLST method was unsuccessful in this study. With the phylogenetic analysis, the UPGMA tree based on differences in consensus of the isolates showed that all group B streptococcus isolates are clustered and descend from a single node.

5.3. Limitations

There was a high number of absenteeism of colonised pregnant women to give birth, which resulted in a smaller cohort of the neonates to the study. Most neonates who were involved in the study were born by caesarean section. Larger cohorts would have enabled the comparison between neonates born from colonised pregnant women by caesarean and normal vaginal birth. While this research serves as a good pilot study for further MLST analysis, major limitations exist. The most crucial of these limitations of the study was the small sample size, especially with MLST analysis. For characterization of GBS isolates a representative selection of seven of 128 GBS Isolates in total were chosen. The isolates were randomly chosen, but were selected to represent all serotypes isolated. There was a low quality of PCR products and therefore low quality sequencing results, only a small percentage of the original cultured data was available for MLST analysis. The resources that were

supposed to be used to achieve objectives were quite limited. This ultimately posed a major problem for achieving the set objectives.

5.4. Conclusions

The population used for this study was unique due to the age, socioeconomic background, and ethnic group in the patients visiting the study clinic at Dr George Mukhari academic hospital. This study revealed that pregnant women of all ages are at risk of group B streptococcus, and screening process must be implemented and every pregnant woman must be screened for group B streptococcus. A number of studies have documented that the accuracy of prenatal screening cultures in identifying intrapartum colonization status can be enhanced by careful attention to the timing of cultures, the anatomic sites swabbed and the laboratory procedures used for culture and detection of the organisms (Schrag *et al*, 2002). This study has provided the valuable knowledge regarding group B streptococcus colonisation at Dr George Mukhari Academic Hospital. This had also provided a foundation for further studies on group B streptococcus prevalence in pregnant women and their babies. The results confirmed that the combination of Todd-Hewitt broth and CNA agar plate is a time saving and sensitive method. A larger sample size would have enabled trends in data to be shown with more statistical power and certainty. Only three loci for six samples were successfully sequenced out of seven housekeeping genes.

5.5. Recommendations

1. More research studies need to be done in diverse and rural communities in South Africa to determine group B streptococcus prevalence.
2. Future studies should also investigate the persistence of group B streptococcus
3. The molecular studies on group B streptococcus using MLST and other advanced and sensitive techniques should be carried out.

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APPENDIX A

Consent Form

This is a Research Project of University of South Africa in collaboration with the University of Limpopo Medunsa campus and the Polytechnic of Namibia.

Title of research project: Epidemiology and multilocus sequence typing of group B streptococcus colonising pregnant women and their neonates at Dr George Mukhari Hospital, Pretoria.

Participant (mother)'s informed consent

You (mother) and your newborn baby are being invited to take part in a research study that investigate group B streptococcus. Group B streptococcus is a bacterium that is found naturally in birth canal and digestive system in pregnant women and it can cause infection such as in blood (septicaemia), lungs (pneumonia) and can damage brain linings (meningitis), body surface (sepsis). Group B streptococcus can lead to miscarriage, stillborn, or even death after being born.

Procedures

You (mother) will be asked to provide a sample of blood (5 ml) and vaginal-rectal swab specimens. The results of the study of your samples will be used for research purposes only and will remain confidential.

Privacy:

Your research record is confidential. Your identity and all personal and confidential information about you will not be divulged to any participating scientist. A coded number will be given to the specimen, and used by the investigators for reference only. Participating scientists will not have access to your identity.

Risks and benefits:

The risks associated with this study are slight discomfort when blood sample is collected. It is important to know you will not benefit from the study, as this is not a

treatment study. We cannot and do not guarantee or promise that you will receive any benefits from this study and you will not get paid to participate in this study.

Time involvement:

Your participation will take approximately 10 minutes

If I sign, can I withdraw from the research later?

If you decide to participate, you are free to withdraw from participating in this study at any time. After any revocation, your study data will no longer be used.

B. I hereby consent voluntarily to participate in the above mentioned study:

I (Mother 's name)hereby voluntarily consent and allow me and my newborn baby.....to take part in the above mentioned project.

I declare that:

- I have read or had read to me this information and consent form and I understand what is required of me.
- I have had a chance to ask question and all my questions were adequately answered.
- I understand that taking part in this study is voluntary and we have not been pressurized to take part.
- I may choose to leave the study at any time and will not penalized or prejudice in anyway.
- My decision whether or not to participate in this study will not affect my medical care.
- My privacy will be maintained in all published and written data resulting from the study.

Signed at.....on.....2012
(Place) (Date)

Signature signature of the witness.....

Declaration by a Senior Researcher

I (Name).....declare that:

- I have explained the information in this document to the participant of this study
- I have encouraged her to ask questions and take time to answer them.
- I have endeavored to make her understand all aspects of the research.

Signed at.....on.....2012
(Place) (Date)

.....
Signature of the Senior Researcher

Signature of the witness..... Date.....

APPENDIX B

GBS STUDY QUESTIONNAIRE

PART I

Patient Name: _____

Date Recruited |_____|_____|_____|_____|
(DD/ MM/ YY)

Study Number |_____|_____|_____|

Hospital/Clinic Number |_____|_____|_____|_____|

Home Address _____

Contact Tel No _____

Date of birth |_____|_____|_____|_____|
(DD/ MM/ YY)

Age (years) |_____|

Socio-economic characteristics

Marital status Married/ Single/ Divorced/ Widowed/Cohabiting
|_____|_____|_____|_____|____|

Level of Education Reached 1. Below Matric 2. Matric 3. Tertiary level
|____| |____| |____|

Employed Yes No
|_____|____|

Salary per month (Rand) |_____|_____|_____|_____|____|

Occupation Managerial Semi-Skilled skilled Unskilled
|____| ||____| |____|

Husband/Partner employed |_____|____|
Yes No

Husband/Partner Level of Education Reached |____| |____| |____|
1. Below Matric 2. Matric 3. Tertiary level

Husband/Partner Salary per month (Rand) |_____|_____|_____|____|

Accommodation Own Rent Lodge Under guardianship of relative
|____| |____| |____| |____|

Dwelling most during the year Urban Rural Semi-urban

 |__| |__| |__|

Obstetric History

Yes No

Antibiotic treatment in the last 14 days |__| |__|

Parity |__|__|

No. of previous miscarriages |__|__|

No. of previous stillbirths |__|__|

No. of previous normal

Vaginal deliveries |__|__|

Present pregnancy

Last menstrual period (LMP) |__|__| |__|__| |__|__|__|
(DD/ MM/ YY)

Sure Unsure Unknown

Date |__| |__| |__|

Booked Unbooked

Booking status |__| |__|

Expected date of delivery |__|__| |__|__| |__|__|__|
(DD/ MM/ YY)

History of Flu-like illness Yes No

in early pregnancy |__|__|

If yes, for how long (in weeks) |__|__|

	Yes No	
Trauma in pregnancy	<input type="checkbox"/> <input type="checkbox"/>	
Specify	(If yes) _____ _____ _____	
Any vaginal discharge requiring		
	Yes No	
Treatment during current pregnancy	<input type="checkbox"/> <input type="checkbox"/>	
Height (cm)	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
Weight (Kg) at first ANC visit	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
Subsequent Weight	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/> Date.....	
Mid-arm circumference (cm)	<input type="checkbox"/> <input type="checkbox"/> <input type="checkbox"/>	
Blood group	_____	
	Yes No	
VDRL test	<input type="checkbox"/> <input type="checkbox"/>	
	Yes No	
TPHA test	<input type="checkbox"/> <input type="checkbox"/>	
HIV status	Pos Neg If Pos: CD count	
	<input type="checkbox"/> <input type="checkbox"/>	
On HAART	PMTCT	None.
<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>
Comorbid illness:		

APPENDIX C

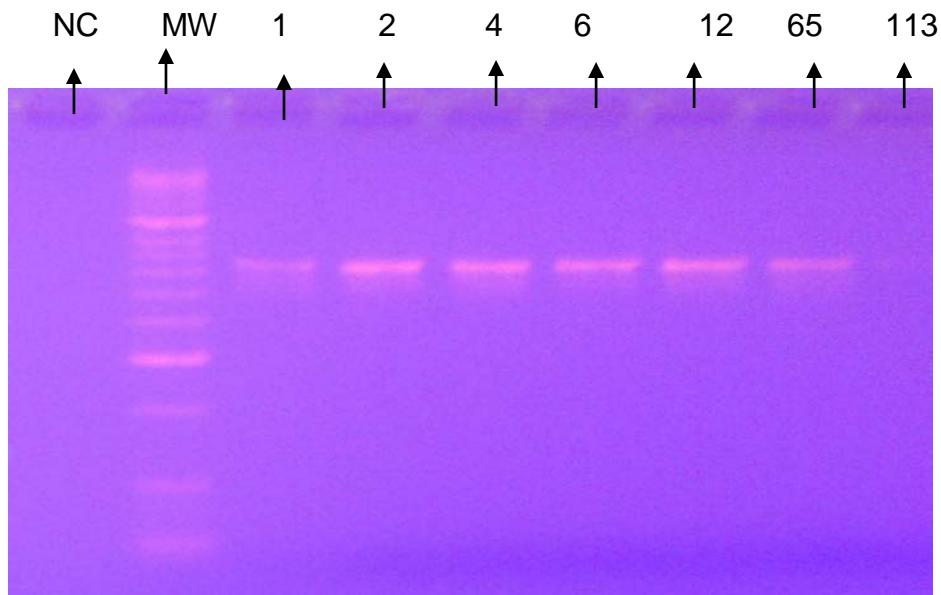


Fig C1: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *glcK* (glucose kinase). Showing the 607bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

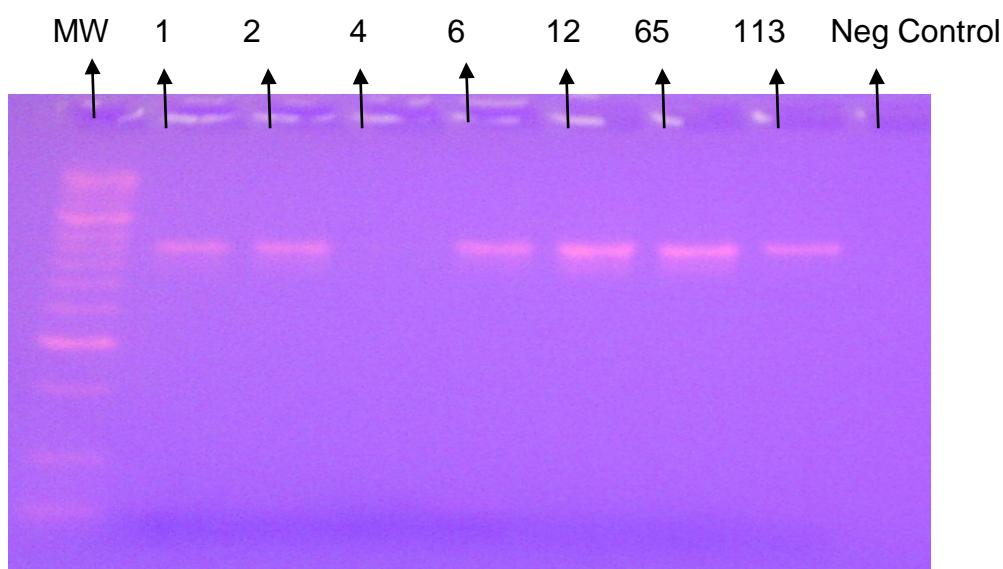


Fig C2: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *glnA* (glutamine synthetase). Showing the 589bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

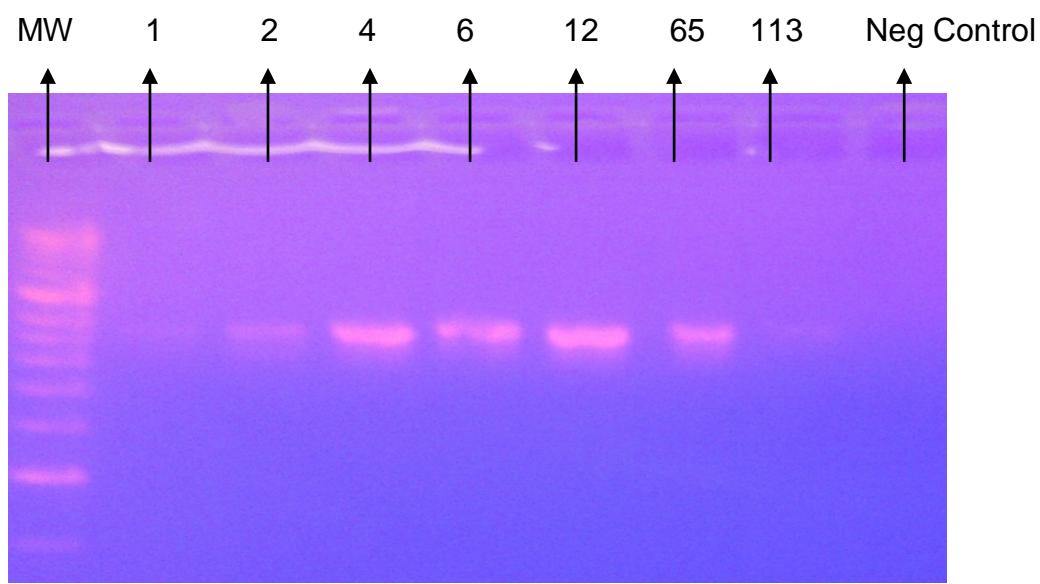


Fig C3: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *adhP* (alcohol dehydrogenase). Showing the 672bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

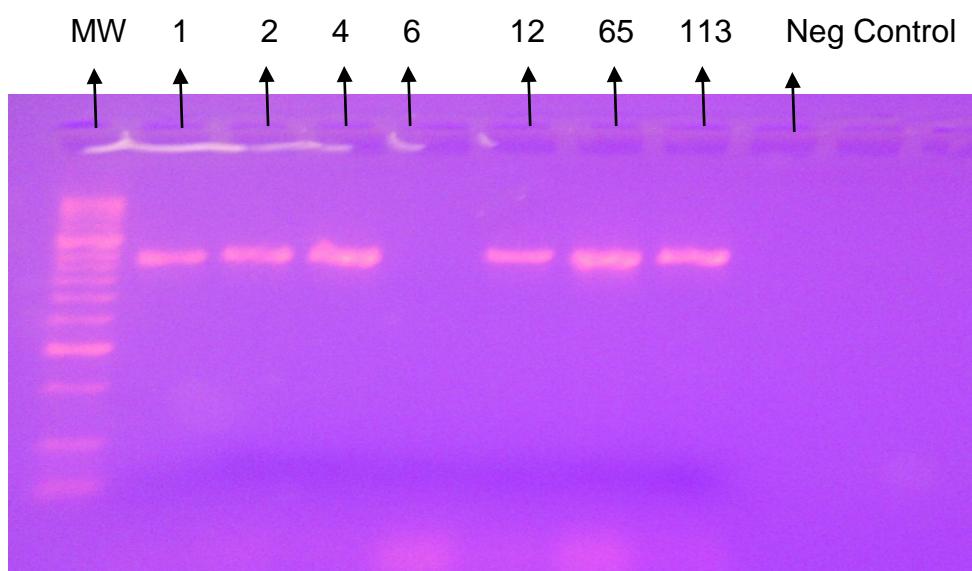


Fig C4: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *sdhA* (serine dehydratase). Showing the 646bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

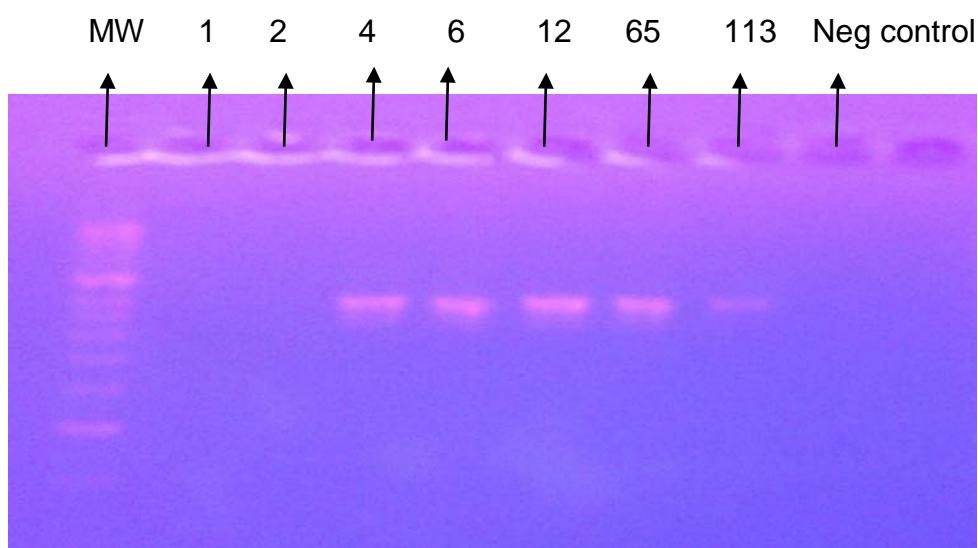


Fig C5: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *atr* (amino acid

transporter). Showing the 627bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

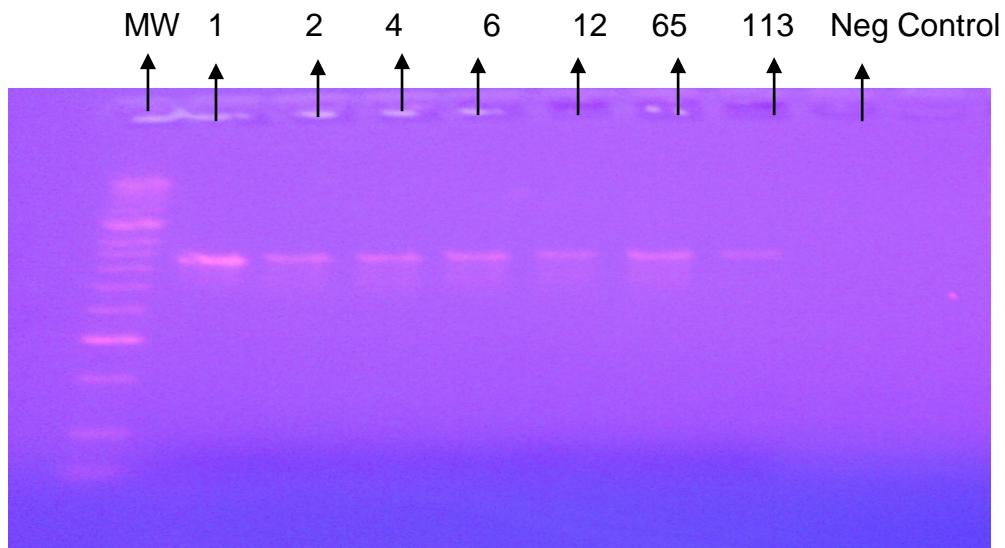


Fig C6: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *tkt* (transketolase). Showing the 859bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

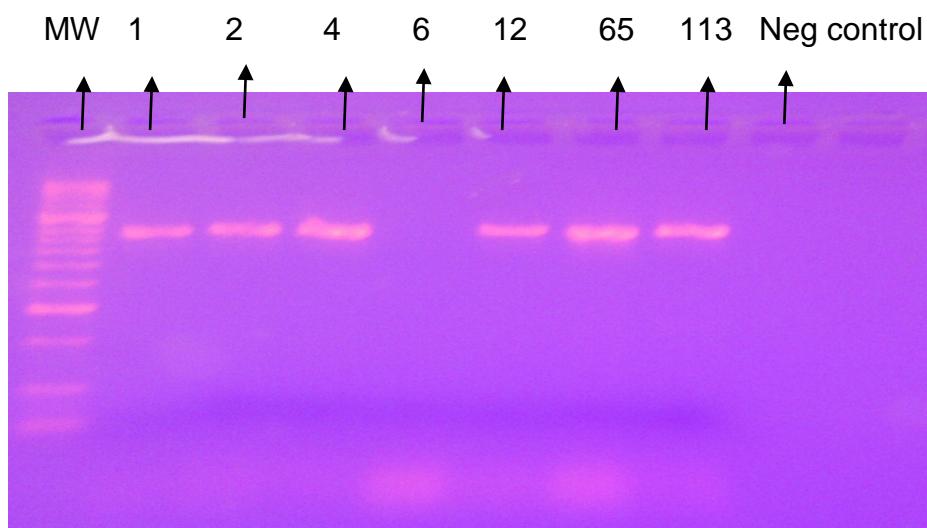


Fig C7: Genomic DNA from group B streptococcus strains (different serotypes; Ia, Ib, II, III, IV and V) subjected to PCR with oligonucleotide primer pairs of *pheS* (phenylalanyl tRNA synthetase). Showing the 723bp PCR product (lanes 1-7). Lane MW, molecular weight marker (50bp ladder).

APPENDIX D

UNIVERSITY OF LIMPOPO Medunsa Campus



MEDUNSA RESEARCH & ETHICS COMMITTEE

CLEARANCE CERTIFICATE

P O Medunsa

Medunsa

0204

SOUTH AFRICA

MEETING: 01/2011

Tel: 012 - 521 4000

PROJECT NUMBER: MREC/P/02/2011: IR

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)

Dr P Shembe (DGMH)

Hospital Superintendent: Microbiological Pathology

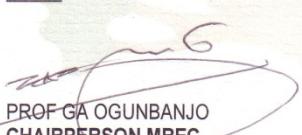
Department: Pathology

School: 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.

African Excellence - Global Leadership