Serotype, pilus island distribution and molecular epidemiology of *Streptococcus agalactiae* isolates from colonization and invasive disease

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Declaration

I declare that this is my own unaided work. It is being submitted for the degree of Doctor of Philosophy in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa. The work contained in this thesis has not been submitted before for any degree or examination in this University or any other University.

Mashudu Madzivhandila 

30th Septermber 2013

Dedications

In memory of my Father

Chief Mavhungu. Andries Madzivhandila

1934-2010

Publications and presentation

Publications

1. **Madzivhandila, M**., Adrian, P. V., Cutland, C. L., Kuwanda, L., Schrag, S. J. & Madhi, S. A. (2011). Serotype distribution and invasive potential of group B streptococcus isolates causing disease in infants and colonizing maternal-newborn dyads. *Plos One,* 6, e17861.

2. **Madzivhandila, M**., Adrian, P. V., Cutland, C. L., Kuwanda, L. Madhi, S. A. and the PoPS trial team. (2012). Distribution of pilus islands of group B streptococcus associated with maternal colonization and invasive disease in South Africa. *J. Med. Microbiol*. 62, 249-253.

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Presentations

1. **Madzivhandila, M**., Adrian, P. V., Cutland, C. L., Kuwanda, L. & Madhi, S. A. Distribution of group B streptococcus pilus islands associated with maternal colonization and invasive disease in South Africa. *The Australian Society for Microbiology, Annual Scientific Meeting*, Brisbane, 1-4 July 2012

2. **Madzivhandila, M**., Adrian, P. V., Cutland, C. L., Kuwanda, L. & Madhi, S. A. Distribution of group B streptococcus pilus islands associated with maternal colonization and invasive disease in South Africa. *International African Vaccinology Conference*, Cape Town, 7-11 November 2012

Abstract

Background: Group B streptococcus (GBS) is a leading cause of invasive bacterial disease in neonates. The possibility of maternal immunization with GBS-vaccines is being explored. Vaccine candidates include serotype-specific polysaccharide-protein conjugates and GBS surface proteins, including pilus island proteins. In this project, we aimed to undertake capsular serotype identification, pilus island identification and genotypic characterization of GBS isolates associated with colonization in mother-newborn dyads and invasive disease in infants.

Methods: Colonizing GBS isolates were identified by vaginal swabbing of mothers (n=541) during active labor and from skin of their newborns post-delivery (n=395). Invasive GBS isolates from infants (n=284) were identified through laboratory-based surveillance. GBS serotyping was done by latex agglutination. Serologically non-typeable isolates were typed by a serotype-specific PCR method. The pilus islands from 541 colonizing isolates and 284 invasive isolates were characterized by real-time PCR targeting the ancillary protein 1 and 2. We undertook sequence typing based on the three most heterogeneous genes (*adhP*, *atr* and *glnA*) of multilocus sequence typing (MLST) on GBS isolates identified in young-infants with invasive disease (n=283) and those associated with maternal (n=525) and newborn colonization at birth (n=369). A total of 121 colonizing and 131 invasive disease GBS isolates that were representative of 55 and 35 clusters respectively were analyzed by the remaining four MLST genes. The gbs2018 locus was characterized by DNA sequencing.

Results: There were no significant differences in serotype distribution between vaginal and newborn colonizing isolates (P=0.77). Serotype distribution of invasive GBS isolates were significantly different to that of colonizing isolates (P<0.0001). Serotype Ia and III were the most dominant serotypes associated with maternal colonization (30.1% and 37.3%, respectively), invasive disease in neonates less than 7 days (57.7% and 84.3%, respectively) and infants between 7 to 90 days of age (22.6% and 13.9%, respectively). Relative to serotype III, other serotypes had lesser invasive potential: Ia (0.49; 95%CI 0.31-0.77), II (0.30; 95%CI 0.13-0.67) and V (0.38; 95%CI 0.17-0.83). All isolates carried at least one pilus island, and they were identified alone or in combinations. A combination of PI-1 and PI-2a was more common among colonizing (28.7%) compared with invasive isolates (17.6%). Conversely, a combination of PI-1 and PI-2b was more frequently associated with invasive disease (60.2%) compared to colonization (37.2%). PI-2a was identified in 92.6% of colonizing and 90.9% of invasive serotype-Ia isolates, whereas serotype III was associated with co-expression of a PI-1 and PI-2b among 84.6% of colonizing and 96.5% of invasive isolates. MLST identified 30 sequence-types in colonizing isolates grouped into six clonal complexes (CC), CC1 (19.8%), CC-10 (23.1%), CC17 (19.8%), CC-19 (15.8%), CC-22 (15.8%) and CC-23 (19.0%). Invasive disease isolates were classified into 22 sequence-types grouped within five clonal complexes. Clonal complex-17 and CC-23 were the most frequent clones associated with invasive disease within 7 days of birth (42.4% and 57.1%, respectively) and those from infants 7-90 days of age (23.7% and 34.3%, respectively. Three allelic variants of gbs2018 (A, B and C) which code for the cell wall anchored proteins were identified, and allele C was identified among all ST-17 lineage isolates.

Conclusion: In South Africa, a GBS vaccine including serotypes Ia, Ib and III has the potential of preventing 74.1%, 85.4% and 98.2% of GBS associated with maternal vaginal-colonization, invasive disease in neonates less than 7 days and invasive disease in infants between 7-90 days of age, respectively. Furthermore based on the homogeneity of pilus island distribution, a pilus based vaccine targeting a component of PI-1 and PI-2a would have a similar coverage in South Africa compared to Europe and North America. Our study identified molecular diversity among colonizing and invasive disease GBS isolates which were, however, similar to that observed in industrialized countries. Furthermore, this study confirmed that the allele C can be used in future as a rapid and cost effective way to detect ST-17 lineage GBS isolates.

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Abbreviations

adhP- Alcohol dehydrogenase

AP- Ancillary protein

atr- Amino acid transporter

BP- Backbone protein

CAMP- Christie Atkinson Munch-Petersen

CC- Clonal complex

CDC- Centers for Disease Control and Prevention

CPS- Capsular polysaccharides

C. diphtheriae- Corynebacterium diphtheriae

CSF- Cerebrospinal fluid

EOD- Early onset disease

ELISA- Enzyme linked immunosorbent assay

GAS- Group A streptococcus

GBS- Group B streptococcus

HIV- Human immunodeficiency virus

HvgA- Hypervirulent group B streptococcus adhesin

IAP- Intrapartum antibiotic prophylaxis

glnA- Glutamine synthetase

glcK- Glucose kinase

LOD- Late onset disease

MLST- Multilocus sequence typing

NT- Nontypeable

PheS- Phenylalanyl tRNA synthetase

PFGE- Pulse filled gel electrophoresis

PoPs- Prevention of prenatal sepsis

PI- Pilus island

RDP- Restriction digest pattern

sdhA- Serine dehydratase

ST- Sequence type

S. pneumoniae- Streptococcus pneumoniae

Srt- Sortase enzyme

tkt- Transkelotase

Chapter 1 Introduction

Streptococcus agalactiae (also known as Group B Streptococcus; GBS) is a bacterial commensal of the lower gastrointestinal and genitourinary tract (Motlova *et al.* 2004; Quinlan *et al.* 2000). Maternal ano-vaginal colonization is implicated as the major risk for early-onset disease (EOD); i.e. within first 7 days of life (Elbaradie *et al.* 2009; Faro *et al.* 2010). Maternal screening to detect GBS is recommended during pregnancy, and GBS positive or women who previously gave birth to an infant with invasive GBS receive intrapartum antibiotic prophylaxis (IAP) (CDC 2005; Isaacs 1998; Schrag *et al.* 2000). Despite the success of IAP in reducing GBS EOD in industrialized countries, the high costs and resource intensiveness of this strategy limits the feasibility of it being implemented in industrializing countries. Furthermore, IAP has not reduced the incidence of GBS occurring beyond the first week of life (Jordan *et al.* 2008).

Vaccination of pregnant women against GBS may offer a more suitable strategy for the prevention of invasive GBS disease in industrializing countries. Group B streptococcus vaccine candidates include polysaccharide-protein conjugate, in which polysaccharide epitopes of specific serotypes are conjugated to an immunogenic carrier protein. GBS serotype specific polysaccharide-protein conjugates containing serotypes Ia, Ib and III, have been evaluated for safety and immunogenicity (Baker *et al.* 1999; Baker *et al.* 2003). A trivalent vaccine, including serotypes Ia, Ib and III, conjugated to mutated *Corynebacterium diphtheria* (*C. diphtheria*) toxin (CRM_{197}) is currently under clinical evaluation (Clinical Trials Gov: NCT01193920, NCT01150123, NCT01446289,

NCT01412801). In addition, selected surface exposed proteins are also being explored as potential vaccine candidates, including the pilus islands proteins (Maione *et al.* 2005; Margarit *et al.* 2009).

Knowledge on the clinical and molecular epidemiology of GBS serotypes, pilus-island and genotypes, including the invasive potential of different serotypes and strains, would contribute toward deciding on vaccine formulations aimed at optimizing coverage against invasive GBS disease. There is, however, limited data on serotypes and genetic diversity associated with GBS ano-genital maternal colonization or invasive disease in industrializing countries, and no data on pilus-island distribution.

1.1 Background and study rationale

Group B streptococcus expresses a type specific polysaccharide capsule on the bacterial surface. Ten capsular types (Ia, Ib, II-IX) have been characterized based on the capsular phenotype (Buttery & Moxon 2002; Slotved *et al.* 2007). At least 95% of all neonatal invasive GBS disease is caused by the following serotypes, namely: Ia, Ib, II, III and V (Berg *et al.* 2000; Fluegge *et al.* 2005; Gray *et al.* 2007; Harrison *et al.* 1998; Madhi *et al.* 2003; Martins *et al.* 2007; Palacios *et al.* 2005; Zaleznik *et al.* 2000).

More recently, a pilus-like structure that protrudes on the surface of most GBS strains has been identified (Lauer *et al.* 2005; Rosini *et al.* 2006). Three genotypically distinct pilus-islands have been described, and each houses three proteins namely, the backbone (BP) and two ancillary proteins (AP1 and AP2) (Lauer *et al.* 2005; Rosini *et al.*

2006). The roles of external structures or surface molecules on GBS biology, pathogenesis and potential as vaccine candidates are being explored (Baker *et al.* 2003; Larsson *et al.* 1999; Margarit *et al.* 2009).

The rationale for maternal GBS vaccination in preventing neonatal sepsis is fostered by the observation of the inverse association between maternal specific anti-capsular antibody and development of invasive disease in the neonate (Baker *et al.* 2003; Lin *et al.* 2001; Lin *et al.* 2004). There is however concerns regarding the safety of vaccinating pregnant women, to avoid coincidental association with spontaneous abortion which is common in the first trimester, maternal immunization during 2^{nd} or early 3^{rd} trimester have been proposed as a promising option to prevent neonatal GBS disease. The success of maternal immunization in preventing young infant morbidity and mortality is best characterized by the success of tetanus vaccination program and inactivated influenza vaccination during pregnancy and demonstrated no adverse fetal effects (WHO position paper 2006; Zaman *et al.* 2008)

Although there are extensive data on serotype distribution of maternal-colonizing and invasive isolates from industrialized countries (Brimil *et al.* 2006; Croak *et al.* 2003; Hickman *et al.* 1999; Kunze *et al.* 2011; McKenna *et al.* 2003; Savoia *et al.* 2008; Tsolia *et al.* 2003), there are limited data comparing serotype distribution from colonized mothers and their newborns from industrializing countries (Al-Sweih *et al.* 2005; Suara *et al.* 1994). In addition there is paucity of data on serotypes causing invasive disease in infants from industrializing countries.

Furthermore, vaccine-candidates based on a combination of selected surface proteins, which may have the potential of providing protection against all GBS serotypes are currently under development (Cheng *et al.* 2002; Larsson *et al.* 1999; Maione *et al.* 2005). These include targeting recombinant GBS pilus island proteins (Margarit *et al.* 2009; Nuccitelli *et al.* 2011), whose potential in protecting against invasive disease has been established in animal models (Margarit *et al.* 2009; Nuccitelli *et al.* 2011). A successful pilus island based vaccine may also potentially prevent GBS colonization, since pili play a major role in bacterial adhesion to the uro-genital mucosa (Dramsi *et al.* 2006; Telford *et al.* 2006). Currently, there is no published data on the prevalence of pilus islands in isolates from vaginally colonized mothers and infants with invasive disease outside of Europe and the USA.

To supplement vaccine development research, there has been interest in the characterization of the genetic lineages of GBS associated with neonatal infections and colonization (Bohnsack *et al.* 2008; Jones *et al.* 2003; Martins *et al.* 2007). Investigation of the genetic diversity of GBS isolates is therefore important in understanding the genetic relationship between serotypes causing invasive disease and those that are simultaneously circulating in the same community. Although the polysaccharide capsule is the most important virulence factor in the pathogenicity of GBS, variation in the invasive potential of different serotypes in causing disease may further be influenced by its genotypic characteristics (Bohnsack *et al.* 2008; Fluegge *et al.* 2011; Marchaim *et al.* 2006). There is limited data on the genotypic characteristics and clonal distribution of GBS associated with colonization and none published to my knowledge in relation to invasive disease in Africa.

Furthermore, it has been previously documented that genes encoding GBS surface proteins constitute genetic markers that can be used as accurate identification of potentially invasive isolates (Imperi *et al.* 2011; Martin *et al.* 2002). Recently, a sequence type-17 (ST-17) specific surface protein called hypervirulent group B streptococcus adhesin (HvgA)/gbs2018C was identified (Lamy *et al.* 2006; Tazi *et al.* 2010). Given the burden of ST-17 associated GBS neonatal disease, HvgA may in future be an important target for the development of novel diagnostic, vaccine and antimicrobial strategies.

1.2 Aims and objectives

In this study we aimed to undertake capsular serotype identification, pilus island and genotypic characterization of GBS isolates associated with colonization in mother-newborn dyads and invasive disease in infants. Objectives of the study included to:

i. Characterize the serotype distribution of GBS associated with maternal-newborn dyads colonization and invasive disease in infants; and explore the invasive potential of different serotypes.
ii. Characterize the pilus island distribution of maternal colonizing and invasive GBS isolates from infants.
iii. Determine the clonal relatedness between maternal-newborn dyads colonizing and invasive disease isolates.
iv. Characterize the allelic forms of gbs2018 gene which encodes the surface protein HvgA.

Chapter 2 Literature review

2.1 Genus streptococcus

The genus streptococcus is a group of heterogeneous gram-positive pathogens, many of which form part of the normal microbiota of humans and animals and some of which have disease causing potential in humans (Facklam 2002). Classification and differentiation of the streptococcus genus have been based on several characteristics, including colony morphology, biochemical reactions, hemolytic activity and group-specific carbohydrate antigen. The most significant species responsible for human infections include group A streptococcus (GAS), GBS, and *Streptococcus pneumoniae* (*S. pneumoniae*). The pathogenic and epidemiological aspects of GAS, GBS, and *S. pneumoniae* have been more extensively studied than other species, partly because of their greater disease-causing potential in humans.

2.2 Characteristics of group B streptococcus

2.2.1 Identification and growth conditions

Group B streptococcus was first isolated from ungulates as a cause of bovine mastitis in the early 1930s (Lancefield 1934). The recognition of GBS as an etiological agent of severe human infections among neonates was first described in the 1960s (Eickhoff *et al.* 1964). The classification of GBS is based upon specific polysaccharide cell wall antigen reacting to hyperimmune serum under the Lancefield grouping (Bromberger *et al.* 1980; Ingram *et al.* 1982; Lancefield 1934).

Metabolically, GBS is a facultative anaerobe and when grown on blood agar, exhibit a β-hemolytic appearance producing a clear zone around colonies that results from the cytopathic effect of GBS released hemolysin completely lysing surrounding erythrocytes (Nizet 2002; Sigge *et al.* 2008). Selectivity of culture media for GBS growth can be enhanced in blood agar supplemented with gentamicin (8µg/ml) and nalidixic acid (15µg/ml). This selective enrichment supports the growth of GBS while inhibiting the growth of other ano-vaginal bacterial commensals. Group B streptococcus colonies appear grey or white, flat and slightly mucoidal when grown on blood agar plates (Liddy & Holliman 2002).

Verification of the suspected GBS colonies can be carried out using a group B antigen latex test or other specific method such as genetic probes and fluorescent antibodies (Boyer *et al.* 1981; Elliot *et al.* 2004; Kong *et al.* 2005). The Christie Atkinson Munch-Petersen (CAMP) test is used for presumptive identification of GBS. CAMP test involves the hemolytic activity of staphylococcal B-lysin on red blood cells which is enhanced by the CAMP factor produced only by GBS strains (Fuchs *et al.* 1978). Group B streptococcus in blood agar may be mistaken for *Enterococcus faecalis* (*E. faecalis*). To discriminate between the two, a bile esculin test is commonly used for presumptive identification of the *E. faecalis*. Bile esculin medium contains selective (bile salts) and differential component (esculin). Enterococci hydrolyze esculin to glucose and esculetin causing the medium to blacken, while GBS cannot hydrolyze esculin (Chuard & Reller 1998; Facklam & Moody 1970).

2.3 Epidemiology and transmission

2.3.1 Maternal ano-vaginal colonization

Heterogeneity in study design, differences in culturing techniques, culture media and sites of sampling limit the extent to which direct comparisons can be made between studies. The prevalence of GBS vaginal colonization among pregnant women varies regionally and between different ethnic groups, ranging from 1.3% to 36.0% (Goyal *et al.* 2004; Gupta & Briski 2004). The prevalence of maternal GBS colonization also varies based on individual characteristics such as age, parity, socio-economic status, race, presence of sexually transmitted disease and sexual behavior (Foxman *et al.* 2007; Hickman *et al.* 1999; Regan *et al.* 1991; Sharmila *et al.* 2011).

Recto-vaginal swabbing has been recommended to improve identification of GBS (Schrag *et al.* 2002). Previous studies have reported that the sensitivity of detecting GBS colonization in pregnant women by undertaking both rectal and vaginal swabs is 18.5%-51.0% higher compared to taking vaginal swabs alone (Dillon, Jr. *et al.* 1982; Kovavisarach *et al.* 2007; Quinlan *et al.* 2000). There is, however, no consensus in this regard as some studies from the USA have showed no difference in GBS detection rates from vaginal-perianal specimens compared with vaginal-rectal specimens 34% vs. 33.5% (Jamie *et al.* 2004) 15% vs. 15.7% (Hickman *et al.* 1999), and 26.5% vs. 28.7% (Orafu *et al.* 2002).

In Europe and the Americas, recto-vaginal GBS colonization among pregnant women varied from 6.6% to 36.0%, with the lowest prevalence being reported in Greece (6.6%) (Maniatis *et al.* 1996), France (8.0%) (van *et al.* 2009a) and in Mexico (13.3) (Gilson *et al.* 2000). In contrast, the prevalence of maternal colonization was 33.5% in

the state of Florida and 36.0% in Denmark (Gupta & Briski 2004; Hansen *et al.* 2004). Absence of standardization of methodology for sampling and processing of samples may, however, contribute in-part to the variability in the prevalence of GBS colonization between studies. Examples of this include that the low prevalence of GBS colonization in France may have been due to attempting to isolate GBS exclusively in the genital tract, whilst in Greece non selective culturing media was used (Chocolate and blood agar instead of colistin nalidixic acid) which could have decreased isolation rates. Maternal colonization prevalence in Australia and New Zealand varies between 12.9% to 22.0%, with the major difference between studies from Australia and New Zealand being the exclusion of rectal swabs to detect GBS colonization in the study from Australia which reported lower (12.9%) prevalence of colonization (Garland *et al.* 2000; Grimwood *et al.* 2002).

Studies that assessed the prevalence of GBS maternal colonization in sub-Saharan Africa including an intervention study from South Africa (21.6%), three cross sectional studies from Malawi (16.5%), Gambia (22.1%) and Tanzania (23.0%) reported a similar prevalence of colonization, despite differences in gestational age at sampling, the site/s of sampling, transport medium and culturing media used (Cutland *et al.* 2009; Dzowela *et al.* 2005; Joachim *et al.* 2009; Suara *et al.* 1994). A longitudinal study from Zimbabwe, reported a cumulative rate of 60.3% for GBS colonization from 20 weeks gestational age through delivery (Mavenyengwa *et al.* 2006; Mavenyengwa *et al.* 2010). A low prevalence of GBS has, however, been observed in Mozambique ranging from 0.9% to 1.8% in two studies conducted a decade apart (de Steenwinkel *et al.* 2008; Osman *et al.* 1995). Both these studies, however, did not use selective media for GBS

isolation and hence culture of GBS may have been compromised. Direct agar plating without the use of selective media reportedly reduces the yield of GBS recovery by 50% (CDC 1996).

The prevalence of GBS colonization among pregnant women in Asia and the Middle East varies considerably, ranging from 1.3% in India (Goyal *et al.* 2004) to 31.6% in Saudi Arabia (Zamzami *et al.* 2011). The low prevalence in India is consistent with other South/South-East Asian countries including Pakistan (8.5%), Philippines (7.5%) and Myanmar (7.1%), irrespective of whether single or multiple sites were sampled (Chaudhry *et al.* 2010; Nahaei *et al.* 2007; Whitney *et al.* 2003). The reason for the lower prevalence of GBS colonization in Asian countries and geographical variation in general remains unknown.

When the prevalence of GBS colonization was stratified with respect to the human immunodeficiency virus (HIV) status, there were no significant differences (P=0.28) observed in a study from Brazil in GBS colonization between HIV-infected (19.2.%) and uninfected (14.1%) pregnant women (El *et al.* 2006). In South Africa, maternal HIV infection was not associated with increased risk of maternal vaginal GBS colonization, GBS colonization was detected in 17.0% of HIV infected women compared with 23.0% of HIV uninfected women (Cutland *et al.* 2012). Similarly GBS colonization was respectively associated with 19.4% and 21.7% in HIV infected and HIV uninfected in Malawi (Gray *et al.* 2011). In contrast, a study done in Belgium reported a significantly (P<0.001) higher colonization prevalence amongst HIV-infected (29.0%) compared to HIV uninfected pregnant women (19.0%) (Epalza *et al.* 2010).

2.3.2 Transmission of group B streptococcus to neonates

Group B streptococcus is transmitted vertically from colonized mothers to their newborns peri-partum and involves colonization of the skin or mucous membranes in 10.0% to 50.0% of newborns born to mothers with ano-vaginal colonization (Beal & Dancer 2006). The vertical transmission rates vary substantially in different geographical regions, and rates as low as 2.1% in the Netherlands (Valkenburg-van den Berg AW *et al.* 2006) and as high as 61.0% in Sweden (Hakansson *et al.* 2008) have been reported. Neonatal colonization is defined by a GBS positive culture swab taken from a neonate's umbilicus, throat, and ear after birth. The number of sites sampled may consequently influence the detected rate of colonization in newborns. Swabbing of the neonate's umbilicus, throat and ear yielded higher isolation rates, 47.2% compared to 23.2% when only one site was sampled (Sensini *et al.* 1997).

Neonates are at a greater risk of acquiring GBS when the mother is heavily colonized with GBS during labor (Sensini *et al.* 1997). In an Italian study, the rates of vertical transmission were substantially higher among women with high density of colonization (50.0%) compared to those with a lower density of colonization (30.4%) (Sensini *et al.* 1997). Density of colonization was based on the semi-quantitative four zone streaking method, and the colony forming unit defines the GBS growth. Despite higher prevalence of maternal GBS colonization, studies from Netherlands (21%) and Brazil (27.6%) reported low vertical acquisition rates i.e. 2.1% and 3.1%, respectively (Valkenburg-van den Berg AW *et al.* 2006). Conversely, in countries with low maternal colonization, including India (2.52%), Iran (9.1%) and Italy (11.3%), vertical

transmission rates were 60.0%, 60.0%, and 41.5%, respectively (Joachim *et al.* 2009; Kuruvilla *et al.* 1999; Sensini *et al.* 1997). An additional factor determining the acquisition of GBS in newborns is the prolonged rupture of amniotic sac membranes (PROM). i.e. vertical transmission was 73.3% in membrane rupture of more than 12 hours before delivery compared to 38.4% in less than 12 hours before delivery (Hickman *et al.* 1999).

Neonatal colonization has also been reported in neonates born from non-colonized mothers, indeed 2.0-5.6% of neonates born to non-colonized mothers are colonized by GBS during birth (Cutland *et al.* 2009; Eren *et al.* 2005; Hickman *et al.* 1999). These findings may suggest sources of acquisition other than from the mother, or reflect limitations on culturing techniques or the sensitivity of identifying maternal colonization as well as exclusion of rectal swabbing.

2.3.3 Group B streptococcus disease in infants

Neonates with invasive GBS disease may present with septicemia, meningitis and pneumonia, with septicemia being the most common (Luck *et al.* 2003; Puopolo *et al.* 2005). Invasive neonatal GBS diseases have been reported in both early-onset (EOD) and late-onset (LOD) of the disease. Early onset disease is classically defined as occurring within seven days of birth, and LOD as occurring between 7 to 90 days after birth. Group B streptococcus was responsible for 86.1% of bacterial meningitis within the first two months of age in the USA between 1998 and 2007 (Thigpen *et al.* 2011).

2.3.4 Early onset disease

Early onset disease is thought to occur as a consequence of invasion by fetal aspiration of GBS infected amniotic fluid or following colonization during birth (Berkowitz & Papiernik 1993; Lyytikainen *et al.* 2003). Recently a systemic review that estimated the current global burden of EOD in low, middle and high income countries reported an overall incidence of 0.43 per 1000 live births. The incidence per 1000 live births was found to be high in Africa (0.53), followed by the Americas (0.50) and Europe (0.45). The lowest incidence rate of 0.11 per 1000 live births was reported in South-East Asia (Edmond *et al.* 2012). In countries where IAP is widely implemented, a decrease in GBS EOD has been evident, including from 3.2 in 1987-1989 to 2.0 in 1990-1992 and even further to 0.37 between 1997-2003 in the USA (Chen *et al.* 2005; Puopolo *et al.* 2005).

Increased use of the IAP in most European countries during the 1990s has also successfully led to the significant decrease in the incidence of EOD. There is, however, a high residual incidence (per 1000 live births) of EOD disease even in some industrialized countries where IAP has been implemented, including Finland (0.6-0.7), Israel (0.80), Spain (0.89) and Czech Republic (0.7-1.0). The high incidence of GBS in these countries implementing IAP suggests that there is a need for an alternative intervention.

In Africa and some Asian countries where IAP, based on screening for GBS colonization during pregnancy, has not been implemented, the incidence of GBS remains high. In South Africa the incidence of EOD did not changed over a decade. An initial

study from Soweto, South Africa between 1997 and 1999 reported an incidence of 2.06 per 1000 live births for EOD (Madhi *et al.* 2005), which was more recently confirmed (i.e. 2.0 per 1000 live births) in a cohort study in the same setting undertaken between 2005-2007 (Cutland *et al.* 2009). A high incidence of EOD (0.92) has also been reported in Malawi (Gray *et al.* 2007).

In some Asian countries, including Taiwan and Japan where neither universal GBS screening during pregnancy nor risk-based prophylaxis is practiced, comparable incidences of EOD (0.7-1.83) (Matsubara *et al.* 2007; Yu *et al.* 2011) to those of the sub-Saharan countries have been reported. There has been a suggestion that there might be underestimation of true rates of GBS incidence in some Asian and other African countries including India (0.17) and Nigeria (0.24) (Kuruvilla *et al.* 1999; Ojukwu *et al.* 2006). This may be attributed to inadequate laboratory facilities in rural regions, in addition to which cases occurring among home-births may be missed should there be limited access to health care (Shet & Ferrieri 2004).

2.3.5 Late onset disease

Development of LOD is less well understood, however, it is suggested that it is closely related to a delayed manifestation of a vertically transmitted infection or nosocomially and community acquired infection (Jordan *et al.* 2008). It has also been shown that breast feeding may be a source of infection resulting in either late onset or recurrent neonatal disease (Kotiw *et al.* 2003). In a review assessing the incidence of LOD GBS, the mean rate was 0.24 per 1000 live births with the highest rates in Africa

(0.71) followed by the Americas (0.31) (Edmond *et al.* 2012).

In industrialized countries such as the USA and Europe, there also remains a substantial burden of LOD, 0.47 and 0.36 per 1000 live births, respectively (CDC 2005). In industrializing regions including sub-Saharan Africa, studies from South Africa and Malawi have reported late onset incidence to be 1.0 and 0.89 per 1000 live births, respectively (Gray *et al.* 2007; Madhi *et al.* 2005). In 2005 GBS bacteremia was the leading bacterial cause of early death in young infants from Kenya compared to any other gram-positive pathogen (Berkley *et al.* 2005). The study from Kenya however, did not stratify the incidence of GBS according to the onset of infection, and reported the incidence of 0.66 per 1000 live births in infants less than 28 days of age (Berkley *et al.* 2005).

More studies are needed especially in the Asian countries, to accurately estimate the burden of LOD due to GBS. Currently the incidences of LOD in Asia remain poorly documented compared with other geographical regions. The explanatory variables for a lack of data from Asia may be linked to high case fatality before specimen collection and incomplete periods for data collection (Edmond *et al.* 2012).

In 1996, the Centers for Disease Control and Prevention (CDC) recommended two strategies for prevention of perinatal GBS disease, the screening based strategy and the risk based strategy (CDC 1996). The guidelines for GBS prevention were revised, and the screening based strategy was adopted as the only strategy after it was found to be >50% more effective than the risk factor based strategy (Schrag *et al.* 2002)..

2.4 Pathogenesis and virulence factors

Several virulence factors of GBS have been identified, this includes capsular polysaccharide, GBS pili, HvgA and β-hemolysin (Lamy *et al.* 2006; Rosini *et al.* 2006; Spellerberg 2000). The role of capsular polysaccharide has been extensively studied for many years and has been documented as the primary factor that enable GBS to survive in the host by preventing activation of complement pathways involved in opsonophagocytosis (Edwards *et al.* 1982; Edwards *et al.* 1993; Vallejo *et al.* 1996). GBS pili and HvgA are important in the adhesion and attachment of GBS to the host cells (Krishnan *et al.* 2007; Tazi *et al.* 2010), the roles of GBS pili and HvgA are described in sections 2.7 and 2.8, respectively. β-hemolysin is implicated in the pulmonary and endothelial cell injuries, which contribute to severe pneumonia in infants because of its cytopathic effects on the pulmonary epithelial cells (Nizet *et al.* 1996).

2.5 Capsular polysaccharides

Type specific capsular polysaccharide is the most common feature of the streptococcus genus. The antigenic variations in the repeating units of oligosaccharides result in characterization of at least ten different capsular polysaccharide compositions (Cieslewicz *et al.* 2005). Majority of serotypes (Ia, Ib, II, III, IV,V, and VII) consists of the monosaccharide glucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid (Jennings *et al.* 1983a; Jennings *et al.* 1983b; Kogan *et al.* 1995; Wessels *et al.* 1989; Wessels *et al.* 1991), whilst type VI and VIII contain rhamnose in place of N-acetylglucosamine (Kogan *et al.* 1996; von *et al.* 1993).

Although the repeating unit structure in GBS serotypes are similar in their constituent monosaccharide composition and certain structural motifs, differences exist in the synthesis of distinct capsular polysaccharide precursor oligosaccharides repeating units (Chaffin *et al.* 2005). The majority of type specific capsular polysaccharides have five sugar residues, with the exception of IV and II which contain six and seven sugar residues respectively (Wessels *et al.* 1989). Sialic acid, which is present as a terminal side chain residue in the repeating structure of each GBS polysaccharide, enhances GBS virulence abilities by interfering with the host innate immune recognition and specifically opsonic components of the complement system (Weiman *et al.* 2010).

Type specific capsular classification has traditionally been performed by capillary precipitation, commonly known as the Lancefield method (Lancefield 1934). There are several other serologic methods that are used for serotype determination, including latex agglutination (Slotved *et al.* 2003), and inhibition enzyme linked immunosorbent assay (iELISA) (Arakere *et al.* 1999). The Lancefield method and latex assay involves subjective interpretation as end points, while the iELISA assay relies on a different principle to the Lancefield method and latex assay, and involves detection of GBS serotypes based on the specific interaction between the type specific capsular polysaccharide and GBS directed antibodies from a pooled serum. The Lancefield method involves the detection of a precipitate when antiserum diffuses through acid extracted type specific capsular polysaccharide in a capillary tube (Severin 1972). The Lancefield method has been superseded by the latex agglutination which is a rapid and sensitive slide agglutination test and involves reacting type specific antibodies coated on latex particles to capsular polysaccharide (Afshar *et al.* 2011; Slotved *et al.* 2003).

More recently a new proposed serotype designated as serotype-IX has been described (Slotved *et al.* 2007), implying that some non-typeable strains could belong to as yet uncharacterized serotypes not screened for by currently available antisera. Molecular typing based techniques for the identification of the genes coding for type specific capsular polysaccharide have been developed to overcome limitations in serotype classification. Molecular techniques were seen as highly desirable because of the high discriminatory power, reproducibility, and specificity (Imperi *et al.* 2010; Murayama *et al.* 2009; Poyart *et al.* 2007), however the mosaic nature of the capsular polysaccharide loci make it difficult to use molecular typing as a gold standard.

2.6 Group B streptococcus serotype epidemiology

2.6.1 Group B streptococcus serotypes associated with colonization in pregnant women

The global systematic review of serotypes associated with recto-vaginal maternal colonization is summarized in Table 2.1. Pubmed database was used as a search engine, and using search terms “Streptococcus agalactiae, group B streptococcus, serotype and maternal colonization”. The selection criteria included studies conducted from 1990-2012, however studies not written in the English language or those for which the full text article could not be accessed were excluded from the table.

Distribution of GBS serotypes and the proportion of individual serotypes may vary regionally even within a country (Hong *et al.* 2010; Seo *et al.* 2010). Two studies

from the state of Ohio in America conducted a year apart showed a difference in the frequency of serotypes associated with colonization of pregnant women (see Table 2.1). In a study by Croak *et al*, 2003 serotype V was isolated most frequently (31.7%), followed by serotype Ia (17.2%) and III (16.6%). Whilst McKenna *et al*, 2003 reported serotype III (31.9%) as dominant followed by Ia (23.2%) and low prevalence of serotype V (7.2%). The reason for this variability is not understood, however, the discordance may be due to the limitation in detecting concurrent multiple colonizing serotypes. It has been previously documented that 21.6% women are colonized with more than one serotype (Ferrieri *et al.* 2004).

Despite the differences in the proportionality of individual serotypes, the majority of studies done in the USA have consistently reported the dominance of colonization by serotype III (24.2%-27.1%) and Ia (24.2%-28.5%) (Blumberg *et al.* 1996; Hickman *et al.* 1999; Ippolito *et al.* 2010; McKenna *et al.* 2003). These findings suggest that the most common GBS serotypes responsible for colonization have remained relatively stable over the years. Serotypes IV and VI which are the least reported serotypes were identified in two studies from the USA (Ippolito *et al.* 2010; McKenna *et al.* 2003), whilst no colonizing isolates belonged to serotypes VII or VIII.

The epidemiology of the most common GBS serotypes from pregnant women in European countries does not differ significantly between countries. The majority of studies from Europe have reported serotype III as the most common colonizing serotype, ranging between 21.7% to 34.5%; Table 2.1. Netherlands and Greece reported the predominance of serotype Ia (26.1%) and serotype II (26.9%) respectively, which

marginally exceeded serotype III at 21.7% and 22.4% in the respective countries (van *et al.* 2009b). The difference between the dominant serotype associated with colonization in the Netherlands and Greece, indicates that the propensity of GBS to colonize is not entirely dependent on serotype but may include other factors.

No significant differences in the GBS serotype distribution associated with colonization have been observed over time within geographical regions in Europe (Blumberg *et al.* 1996; Brimil *et al.* 2006; Dore *et al.* 2003; Kieran *et al.* 1998; Kunze *et al.* 2011; Savoia *et al.* 2008; Sensini *et al.* 1997). In agreement with serotype data from the USA, serotype Ib, IV and VI were the least reported serotypes associated with colonization in pregnant women in Europe. Serotype VIII which is rare in most countries was only reported in one isolate from Greece (Tsolia *et al.* 2003).

Studies done in Africa showed geographical differences in the serotype distribution; Table 2.1. Two studies from West Africa reported serotype V as the most common serotype, accounting for 44.0% in Senegal and 37.0% in the Gambia (Brochet *et al.* 2009; Suara *et al.* 1994). The low prevalence of serotype III (7.4%) in the Gambia was attributable to the significantly higher antibody levels to serotype III (geometric mean level of 3.28±0.67 µg/mL) in women colonized with serotype V (Suara *et al.* 1998). This suggests that serotype-specific anti-capsular antibody may have a role in protecting against serotype-specific colonization.

There are some unexplained geographical differences in the serotype epidemiology of GBS maternal colonization between countries of the Middle-East and

South/East Asia compared to the USA, Europe and Africa. For example, in the United Arab Emirates (UAE), serotype IV was the dominant colonizing serotype (26%), followed by serotypes Ia (24.6%) and III (17.5%) (Amin *et al.* 2002); Table 2.1. In contrast serotype VI and VIII accounted for over 25.1% of GBS serotypes isolated from Japanese pregnant women (Lachenauer *et al.* 1999; Matsubara *et al.* 2002; Terakubo *et al.* 2003). Serotype IV which accounted for 26.3% in the Arab Emirates, is indeed also common among colonizing isolates from Brazil (13.1% of colonizing isolates) (Palmeiro *et al.* 2011). In France the emergence of serotype IV has been linked to the capsular switch from serotype III to serotype IV, probably due to the selective immune pressure (Bellais *et al.* 2012). The reason why the capsular switching of serotype III is confined to serotype IV and does not include other serotypes remains to be explored.

Serotype distribution from Thailand reflected temporal variation over a decade, an earlier study by Whitney *et al*, 2004 conducted in 1999-2001 reported serotype V (50.0%) as the most prevalent whilst between 2009-2010 serotype II (24.0%) was the most frequent serotype (Turner *et al.* 2012). Despite the use of different serotyping techniques, non-typeable isolates were frequently isolated in studies from the middle-east, 15.8%, 17.7%, and 28.5% in the UAE, Kuwait and Lebanon respectively (Al-Sweih *et al.* 2005; Amin *et al.* 2002; Seoud *et al.* 2010). The high rates of non-typeable isolates in the Middle East countries may be a result of low CPS synthesis or production such that it could not be detected by the typing sera. This poor correlation in serotype distribution between countries, particularly in Asia will pose a challenge for the success of a serotype specific trivalent vaccine which is being evaluated to reduce maternal colonization.

Table 2.1: Distribution of group B streptococcus serotypes in colonizing isolates from pregnant women in different countries

Countries (Reference)	Year of study	Typing method	Ia	Ib	II	III	IV	V	VI	VII, VIII	NT
United States											
Georgia (Blumberg *et al.* 1996)	1992-1993	CP	41.0	12.0	3.0	19.0		25.0			
Texas (Hickman *et al.* 1999)	1994-1995	OID	24.2	9.2	26.1	24.2		11.8	0.7		4.0
Ohio (Croak *et al.* 2003)	1998-2000	CP	17.2	7.6	11.7	16.6	3.4	31.7	0.7		11.0
Ohio (McKenna *et al.* 2003)	2001-2002	CP	23.2	2.9	8.7	31.9	1.4	7.2			24.6
Madigan (Ippolito *et al.* 2010)	2009	PCR	28.5	12.1	12.1	27.1	1.9	16.9	1.0		0.5
Europe											
Ireland (Kieran *et al.* 1998)	Not stated	TS	23.3	7.0	16.3	30.2	0.8	13.2			9.3
Ireland (Dore *et al.* 2003)	1997-1999	LA	17.2	9.2	14.9	34.5	2.3	16.1			5.7
Ireland (Whitney *et al.* 2004)	1999-2001	CP	35.0	5.0	5.0	30.0		20.0			5.0
Portugal (Martins *et al.* 2007)	2002-2004	LA	15.6	5.2	17.1	21.9	2.2	21.9		1.9	14.1
Germany (Brimil *et al.* 2006)	2001-2003	OID	17.3	14.6	21.3	28.0	2.7	16.0			
Germany (Kunze *et al.* 2011)	2004	LA	16.0	19.0	12.0	28.0	6.0	15.0			4.0
Italy (Sensini *et al.* 1997)	1993-1995	OID	16.2	27.0	21.6	21.6	2.7	5.4		5.4	
Italy (Savoia *et al.* 2008)	2005-2006	LA	20.5	6.8	5.5	32.9	8.2	26.1			
Sweden (Hakansson *et al.* 2008)	2005	LA, PCR	11.0	13.0	16.0	24.0	15.0	19.0	0.5	1	0.8
Greece (Tsolia *et al.* 2003)	2000-2001	LA	19.4	11.9	26.9	22.4	3.0	9.0	3.0	4.5	
Netherlands (van *et al.* 2009b)	1995-2004	LA	26.1	6.5	13.0	21.7	7.6	15.2	7.6		
France (van *et al.* 2009a)	20012003	PCR	25.6	7.7	7.7	41.0		17.9			
UK (Jones *et al.* 2006a)	2001-2002	LA	25.8	15.7	9.4	26.4	0.4	18.9	0.4	0.8	2.0
Spain (Perez-Ruiz *et al.* 2004)	Not stated	TS	29.0	12.9	12.9	38.7		6.5			
Africa											
Zimbabwe (Whitney *et al.* 2004)	1999-2001	CP	9.5	14.3	4.8	23.8		42.9			4.8
Zimbabwe (Moyo *et al.* 2002)	2000	AbTyp	14.5	5.1	4.3	45.3	5.1	23.9			2.0
Gambia (Suara *et al.* 1998)	1992-1993	AbTyp	18.5	3.7	25.9	7.4	3.7	37.0			3.7
Senegal (Brochet *et al.* 2009)	2005-2006	PCR	17.3	5.3	12.0	21.3		44.0			
Central African Republic (Brochet *et al.* 2009)	2005-2006	PCR	27.3	8.0	22.7	17.0		25.0			

Countries (Reference)	Year of study	Typing method	Ia	Ib	II	III	IV	V	VI	VII, VIII	NT
Middle East											
UAE (Amin *et al.* 2002)	1998-1999	ID	24.5		3.5	17.5	26.3	12.3			15.8
Kuwait (Al-Sweih *et al.* 2005)	2004	TS	8.9	2.4	8.1	26.6	0.8	21.8	13.7		17.7
Lebanon (Seoud *et al.* 2010)	2004	PCR	14.6	6.6	10.9	16.1	0.7	22.6			28.5
Turkey (Eren *et al.* 2005)	2000-2001	TS	25.9	3.7	29.6	18.5	1.9				20.4
Israel (Bisharat *et al.* 2005)	2000	LA	11.5	8.6	22.1	25.0		17.3			15.4
Asia											
Japan (Lachenauer *et al.* 1999)	1992-1994	iELISA	6.8	8.2		11.0		4.1	24.6	35.6	9.6
Japan (Terakubo *et al.* 2003)	1992-2001	TS	8.6	6.4	1.6	10.2		8.6	26.6	31.6	6.4
Japan (Matsubara *et al.* 2002)	1999-2000	ELISA	8.3	12.5	8.3	10.4		6.3	18.8	27.1	8.3
Korea (Uh *et al.* 1997)	1995-1996	TS	24.1	48.3		20.7					6.9
Korea (Seo *et al.* 2010)	2004-2007	DB	17.0	9.3	5.1	29.8		27.7	6.9	3.7	
Korea (Hong *et al.* 2010)	2006-2007	TS	13.0	6.8	5.6	35.6	2.3	24.3			12.4
Taiwán (van *et al.* 2009b)	1995-2004	LA	22.4	8.6	3.4	32.8		25.9	3.4		
Malaysia (Dhanoa *et al.* 2010)	2003	LA	11.5	1.5	5.5	12.0	10.0	19.0	17.0	6.5	17.0
Myanmar (Whitney *et al.* 2004)	1999-2001	CP	14.3			35.7		35.7			14.3
Thailand (Whitney *et al.* 2004)	1999-2001	CP	20.9		12.5	12.5		50.0			4.2
Thailand (Turner *et al.* 2012)	2009-2010	LA	16.0	2.0	24.0	13.0	6.0	13.0	16.0	6.0	4.0
Oceania											
New Zealand (Grimwood *et al.* 2002)	1998-1999	OID, PCR	21.2	19.2	5.8	28.8		19.2	1.9	1.9	1.9
South/Central America											
Brazil (Simoes *et al.* 2007)	2003-2004	ID	18.5	23.9	19.6	5.4	6.5	8.7			17.4
Mexico (Gonzalez Pedraza *et al.* 2002)	1999-2001	LA	61.4		25.7	12.9					

[OID]- Ouchterlony immunodiffusion, [CP]- Capillary precipitation, [PCR]- Polymerase Chain Reaction, [LA]- Latex assay, [AbTyP]- Antibody typing, [TS]- Typing Sera, [iELISA]- Inhibition Enzyme Linked Immunosorbent Assay, [ID]- Immunodiffusion with rabbit antisera

2.6.2 Group B streptococcus serotype associated with invasive disease in infants

Serotype distribution in invasive disease is summarized in Table 2.2 and Table 2.3, this includes studies written in the English language conducted between 1990 and 2012. Invasive GBS disease isolates in these studies mainly involved isolates identified from normally sterile sites such as blood, cerebrospinal fluid (CSF) and pleural fluid of infants younger than three months.

Although GBS acquisition by newborns from maternal recto-vaginal colonization is an established risk factor for GBS sepsis within the first 7 days of life, there are differences in the relative contribution of individual serotypes that cause invasive disease compared to their relative prevalence in colonization. These differences appear to be related to an increased invasive disease potential of certain GBS serotypes, rather than due to increased risk of transmission of these serotypes from mothers to newborns (Bisharat *et al.* 2004; Martins *et al.* 2007).

Despite geographical differences in the proportion of serotype distribution, serotype III remains the most frequently identified invasive serotype globally (48.9%) and together with serotype Ia (22.9%) accounted for over 70.0% of GBS invasive disease (Edmond *et al.* 2012). A systematic review on serotype distribution in infants under three months reported that five serotypes (Ia, Ib, II, III, and V) accounted for more than 85.0% of serotypes in all regions, including 98.0% in Africa, 96.0% in the Americas, 93.0% in Europe, 89.0% in the Western Pacific and 88.0% in the Eastern Mediterranean (Edmond

et al. 2012).

When serotype distribution was stratified on the basis of the onset of invasive disease, 37.0% of EOD was due to serotype III compared to 53.0% of LOD. Conversely, 40.0% of EOD was caused by serotype Ia compared with 30.0% of LOD (Edmond *et al.* 2012). This review, however, reflected that the majority of studies on serotypes causing invasive disease in infants originate from industrialized countries, whereas there was limited data from Africa and the Eastern Mediterranean and none from South East Asia; Figure 2.1.

In Africa there were only three published studies on serotype distribution of invasive GBS isolates in infants presenting with either EOD or LOD (AitMhand *et al.* 2000; Gray *et al.* 2007; Madhi *et al.* 2003). Based on these published studies, serotype Ia and III were consistently found to be the dominant serotypes, collectively associated with 80% and 77% of EOD in South Africa and Malawi, respectively. Similarly both serotypes accounted for 100% and 92% of LOD in South Africa and Malawi, respectively (Gray *et al.* 2007; Madhi *et al.* 2003). Similar findings were observed in Morocco, where serotype Ia and III accounted for 72.6% in EOD and 65.2 % in LOD (AitMhand *et al.* 2000). In Africa, serotype III was more likely to be isolated from LOD (72.0-75.7%) than in EOD (49.2-51.8%), and conversely serotype Ia was more common in EOD (26.0-31.0%) compared to LOD (20.0%-24.3%). This breakdown was consistent with invasive neonatal serotype epidemiology from the USA and European countries; Table 2.2 and Table 2.3.

Serotype V which has become more common among invasive disease GBS isolates was associated with 11.8% of EOD in Morocco (AitMhand *et al.* 2000). Similarly, studies from Europe and the USA have reported EOD due to serotype V in 23.0% and 15.0% respectively (Blumberg *et al.* 1996; Harrison *et al.* 1998; Lin *et al.* 1998; Persson *et al.* 2004; Weisner *et al.* 2004). The preponderance of serotype V in the above mentioned countries cannot be generalized as its relative contribution in other countries remains modest (Ekelund & Konradsen 2004; Gray *et al.* 2007; Hoshina *et al.* 2002; Madhi *et al.* 2003; Poyart *et al.* 2008). This is corroborated by two studies from sub-Saharan Africa where lower proportion of EOD was attributed to serotype V in Malawi (3.7%) and South Africa (5.6%) (Gray *et al.* 2007; Madhi *et al.* 2003).

Despite the slight difference in their prevalence between EOD and LOD, on average serotype Ib and II seems to have remained stable in Africa over a period of years. Serotype Ib and II have less often been associated with invasive GBS disease among infants. In Africa, serotype Ib was responsible for approximately 7.0% of EOD in South Africa and Malawi, and 3.9% in Morocco (AitMhand *et al.* 2000; Berg *et al.* 2000; Gray *et al.* 2007; Madhi *et al.* 2003). In South Africa (7.0%) and Morocco (3.9%) the prevalence of serotype II was similar to that of serotype Ib, whereas the Malawi study reported a relatively higher contribution of serotype II (11.1%) in EOD compared to the other African countries. Although serotype Ib was not significantly responsible for most of the GBS invasive disease cases in Africa, reports from Europe and the USA substantiate the inclusion of serotype Ib in the trivalent vaccine currently under clinical trial since in Europe and the USA serotype Ib was responsible for as much as 15.9% and 10.1% of EOD respectively (Oladottir *et al.* 2011; Zaleznik *et al.* 2000).

The distribution of the least common GBS serotypes (IV, VI, VII and VIII) differs between countries, and their identification is largely associated with EOD; Table 2.2. In sub-Saharan Africa none of the above mentioned serotypes were identified in either EOD or LOD whilst 7.8% of EOD isolates were associated with serotype IV in Morocco**;** Table 2.2 and Table 2.3. In Europe, serotype IV and VI were associated with invasive disease in some of the countries, including 10.0% of LOD being serotype IV in Sweden (Persson *et al.* 2004). Notably, strong evidence suggest that serotypes VI, VII and VIII are only prevalent in Asia and combined they were responsible for 15.5% of neonatal invasive disease in Japan, similar to the prevalence (6.9%-35.6%) of maternal colonization by serotypes VI, VII and VIII in Japan and Korea (Lachenauer *et al.* 1999; Seo *et al.* 2010).

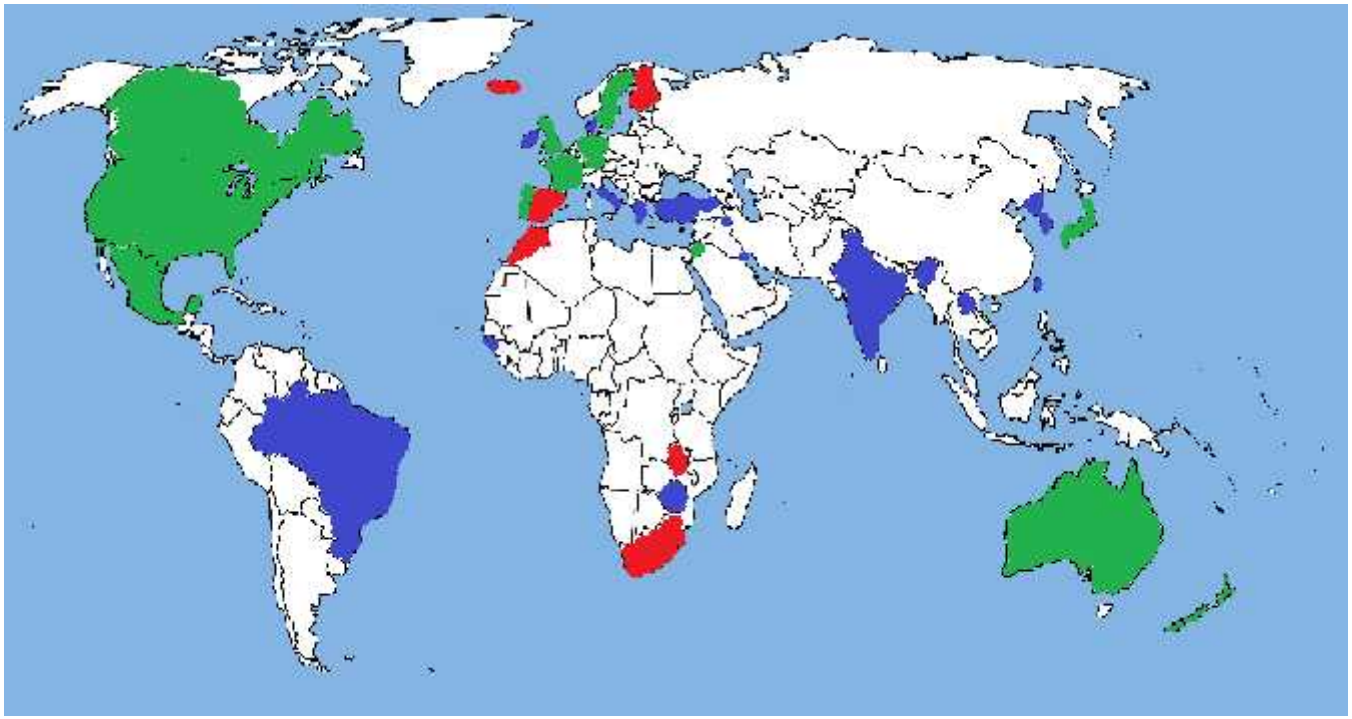
When serotype distribution was stratified on the basis of clinical syndrome and site of isolations of invasive GBS disease, between 34.0% and 76.9% of LOD GBS isolates recovered from the CSF were serotype III (Berg *et al.* 2000; Gray *et al.* 2007; Madhi *et al.* 2003; Weisner *et al.* 2004). Based on the published data regarding GBS serotype distribution within and between countries, the majority of serotypes causing invasive disease will be covered by a trivalent (Ia, Ib and III) GBS conjugate vaccine in most regions of the world; Figure 2.1. This may, however, be less so in countries such as Japan and Korea where serotypes VI, VII and VIII are important causes of neonatal disease. The potential coverage of the tri-valent GBS conjugate vaccine is estimated at 68.5%, 86.0% and 85.0% for EOD in the USA, South Africa and Malawi, respectively and higher coverage rates for LOD.

Table 2.2: Serotype distribution of early-onset invasive disease in neonates

Countries (Reference)	no of isolates	Year	Typing method	Ia %	Ib %	II %	III %	IV %	V %	VI, VII, VIII %	NT %
Africa											
Morocco (AitMhand *et al.* 2000)	51	1992-1993	LA	31.4	3.9	3.9	41.2	7.8	11.8		
South Africa (Madhi *et al.* 2003)	71	1997-1999	ELISA	31.0	7.0	7.0	49.2		5.6		
Malawi (Gray *et al.* 2007)	29	2004-2005	LA	26.0	7.4	11.1	51.8		3.7		
Europe											
France (Lamy *et al.* 2006)	21	1990-2005	LA	23.8	4.8		71.4				
Spain (Martins *et al.* 2011)	123	1994-2009	LA	26.0	6.5	8.1	43.9	1.6	9.8		4.1
Sweden (Berg *et al.* 2000)	64	1995-1996	TS	20.3	3.1	6.3	54.6	3.1	10.9		1.6
Iceland (Oladottir *et al.* 2011)	53	1995-2006	LA	22.7	15.9	11.4	34.1	2.3	13.6		
Netherlands (Trijbels-Smeulders *et al.* 2006)	142	1997-1999	TS	21.1	3.5	10.6	47.9		9.2		7.0
Sweden (Persson *et al.* 2004)	40	1998-2001	TS	12.5	2.5	2.5	60.0		22.5		
UK (Weisner *et al.* 2004)	231	2000-2001	TS	32.0	6.0	5.0	37.0		13.0	2.5	4.0
Portugal (Martins *et al.* 2007)	42	2000-2002	TS	31.0	2.4	19.0	28.6	2.4	16.7		
Germany (Fluegge *et al.* 2005)	168	2001-2003	TS	17.2	5.4	7.7	57.7	1.2	8.9		1.8
France (Poyart *et al.* 2008)	39	2006-2007	PCR	28.2	5.1	2.6	61.5		2.5		
USA											
Georgia (Blumberg *et al.* 1996)	64	1992-1993	CP	36.0	9.0	8.0	30.0		14.0		3.0
Houston (Zaleznik *et al.* 2000)	129	1993-1996	OID	37.2	10.1	11.6	26.4		14.0	0.7	
Maryland (Harrison *et al.* 1998)	149	1992-1993	CP	36.2	1.3	10.7	38.3		13.4		
USA (Lin *et al.* 1998)	67	1995-1997	TS	40.0	9.0	6.0	27.0		15.0		3.0
North America											
Canada (Davies *et al.* 2001)	75	1993-1999	OID	26.7	5.3	8.0	37.3		17.3		5.3
Asia											
Japan (Hoshina *et al.* 2002)	224	1993-1997	ND	17.0	11.2	6.7	38.4	4.0	2.7	13.8	6.3
Israel (Bisharat *et al.* 2005)	50	2000	LA	20.0	4.0	8.0	38.0		18.0	2.0	10.0
Oceania											
New Zealand (Zhao *et al.* 2008)	105	1994-2005	PCR	29.0	12.3	10.4	35.1	1.9	8.4	1.9	

Table 2.3: Serotype distribution of late-onset invasive disease in infants

Countries	no of isolates	Year	Typing method	Ia %	Ib %	II %	III %	IV %	V %	VI, VII,VIII %	NT %
Africa											
Morocco (AitMhand *et al.* 2000)	8	1992-1993	LA	37.5			25.0				37.5
South Africa (Madhi *et al.* 2003)	37	1997-1999	ELISA	24.3			75.7				
Malawi (Gray *et al.* 2007)	28	2004-2005	LA	20.0	4.0	4.0	72.0				
Europe											
France (Lamy *et al.* 2006)	31	1990-2005	LA				100				
Spain (Martins *et al.* 2011)	89	1994-2009	LA	17.0	6.0	1.0	72.0	1.0	2.0		1.0
Sweden (Berg *et al.* 2000)	14	1995-1996	TS	7.0			93.0				
Iceland (Oladottir *et al.* 2011)	34	1995-2006	LA	19.2	7.7		61.5	3.8	7.7		
Netherlands (Trijbels-Smeulders *et al.* 2006)	56	1997-1999	TS	12.5	1.8	1.8	73.2		1.8	1.8	7.1
Sweden (Persson *et al.* 2004)	10	1998-2001	TS		10.0		60.0	10.0	20.0		0
UK (Weisner *et al.* 2004)	122	2000-2001	TS	17.0	7.0	2.0	67.0	5.0	0		2.0
Portugal (Martins *et al.* 2007)	22	2000-2002	TS	22.7	4.5	0	63.6	4.5	0		4.5
Germany (Fluegge *et al.* 2005)	124	2001-2003	TS	11.3	4.0	1.6	76.6	0	6.4		0
France(Poyart *et al.* 2008)	70	2006-2007	PCR	7.1	7.1	0	82.9	0	2.9		0
USA											
Georgia (Blumberg *et al.* 1996)	17	1992-1993	CP	23.0	6.0	0	71.0	0	0		0
Maryland(Harrison *et al.* 1998)	47	1992-1993	CP	23.4	4.3	2.1	61.7	0	6.4		2.1
North America											
Canada (Davies *et al.* 2001)	43	1995-1999	OID	2.3	4.7	0	81.4	0	9.3		2.3
Asia											
Japan (Hoshina *et al.* 2002)	58	1993-1997	ND	6.9	12.1	0	77.6	0	1.7	1.7	
Oceania											
New Zealand (Zhao *et al.* 2008)	154	1994-2005	PCR	24.0	13.5	1.0	48.1	1.0	12.5		



Indicates region where there was no serotype data from colonization and invasive disease

Indicates region which has serotype distribution data from colonization and invasive disease

Indicates region which only has serotype distribution data from invasive disease

Indicates region which only has serotype distribution data from colonization

Maternal colonization ([a]) and EOD invasive disease ([b])

Africa (n=7)
Ia=13.4%[a]/29.5%[b]
Ib=6.6/7.3
III=29.6/47.4

Asia (n=17)
Ia=15.5/18.5
Ib=8.2/7.6
III=21.0/38.2

Oceania (n=2)
Ia=21.2/29.0
Ib=19.2/12.5
III=28.8/35.1

Europe (n=23)
Ia=20.7/27.7
Ib=11.4/7.9
III=27.9/56.5

USA (n=8)
Ia=26.7/37.4
Ib=8.9/7.4
III=23.7/30.4

N/America (n=3)
Ia=13.4/29.5
Ib=6.6/6.1
III=29.6/47.4

S/America (n=3)
Ia=39.4/17.4
Ib=12.0/23.9
III=38.6/4.3

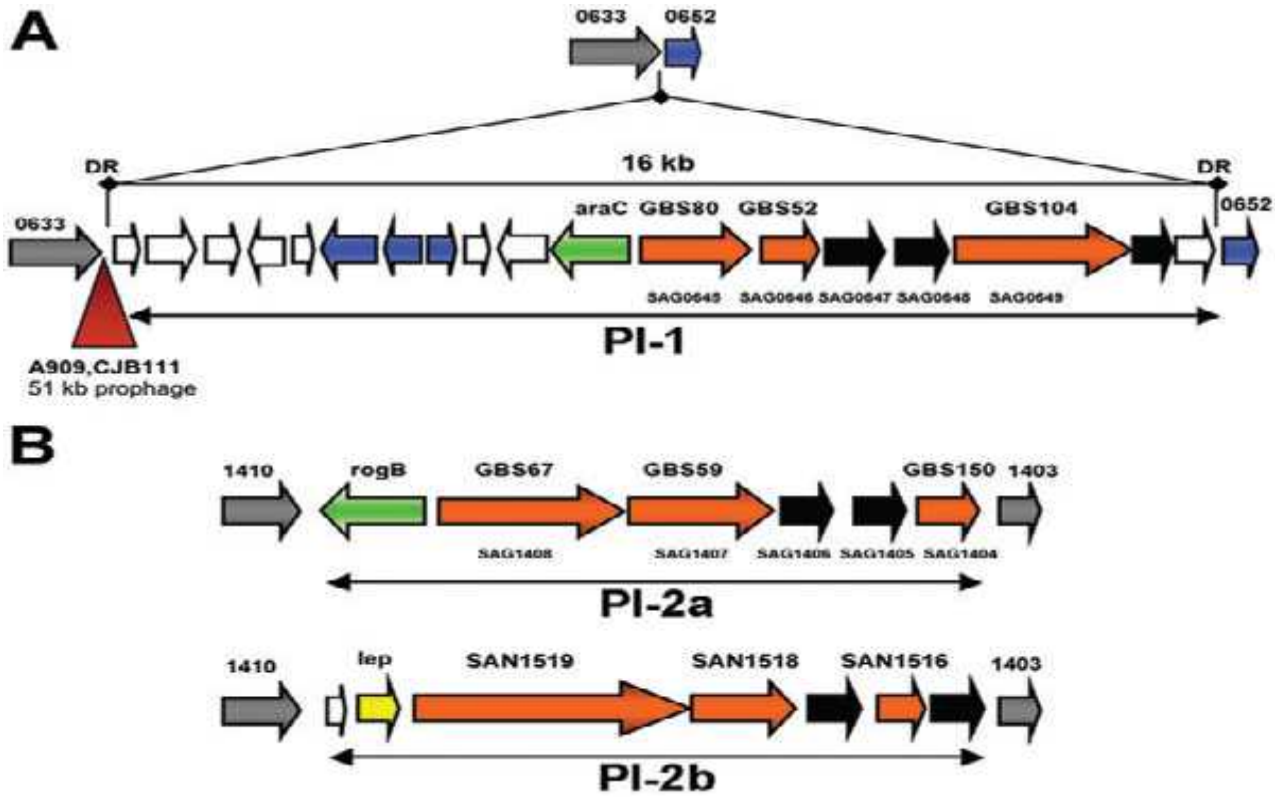
Figure 2.1: Regional distribution of serotypes included in the group B streptococcus trivalent vaccine

2.7 Pili

Pili are long proteinaceous filaments that were historically associated with gram-negative bacteria such as *Escherichiae coli* and *Neisseria* species (Sauer *et al.* 2000; Silverman 1997; Taha *et al.* 1998; Virji *et al.* 1993). They have been recently described in gram positive organisms including *C. diphtheria* (Ton-That & Schneewind 2003), GAS (Mora *et al.* 2005), and *S. pneumoniae* (Barocchi *et al.* 2006). Pili are important in establishing successful colonization and invasion of the respiratory, urinary and intestinal tract. Pili in gram-positive bacteria are formed by polymerized proteins, and these proteins have an N-linked conserved signal peptide LPXTG motif on the C-terminal (Gaspar & Ton-That 2006).

2.7.1 Pili in Group B streptococcus

Three pilus islands (PI-1, PI-2a, and PI-2b) have been identified in GBS isolates (Rosini *et al.* 2006). Each pilus island houses genes that encode three LPXTG motif carrying proteins, namely backbone protein (BP), two ancillary proteins (AP1 and AP2) and two sortase enzymes (Margarit *et al.* 2009; Nobbs *et al.* 2008); Figure 2.2. Pilus island-1 and PI-2a were the first to be identified. Subsequent analysis of eight sequenced GBS genomes revealed an additional pilus island (PI-2b), an allele of PI-2a, which varies with respect to gene organization and sequences; Figure 2.2.



In A (PI-1), GBS80 represents the BP, while the other two pili structures, GBS104 and GBS52 are AP1 and AP2 respectively. In B (PI-2a), GBS59 represents BP whereas GBS67 and GBS150 are AP1 and AP2 respectively. In B (PI-2b), SAN1518 represents BP and SAN1519 and SAN1516 are AP1 and AP2 respectively.

Figure 2.2: Schematic representation of group B streptococcus pilus island regions

Genes coding for LPXTG-containing proteins are represented with orange arrows, whereas transcriptional regulators are in green and conserved flanking genes are in grey. Genes coding for the sortase enzymes are in black, while a signal peptidase is present in PI-2b (yellow arrow). In PI-1, transposable elements are also present (blue arrows) as well as interrupted or frame-shifted genes (white arrows) (Rosini *et al.* 2006).

2.7.2 Pilus island-1

Analysis of sequences from a large panel of GBS strains showed that genes coding for the respective proteins were relatively conserved across different strains. There were, however, minor sequence variability amongst investigated strains including amino acid substitutions and point mutations resulting in frame-shifts and premature termination in BP and AP-1 (Margarit *et al.* 2009). The BP and AP-1 of PI-1 were found to be highly exposed on the surface of the bacteria, whilst AP-2 was unexposed, this was evident when mouse antisera raised against AP-2 failed to stain the bacterial surface (Rosini *et al.* 2006). Immunization of mice with BP and AP-1 were found to confer protection against virulent GBS strains, and as expected AP-2 immunization did not confer any protection. The protection offered by BP and AP-1 antibodies can be attributed to the relative abundance of these two antigens on the bacterial surface (Maione *et al.* 2005; Margarit *et al.* 2009).

2.7.3 Pilus island-2a

The BP and AP-1 were implicated in phagocyte resistance and systemic virulence in the mouse model. Additionally, AP-1 contributes to GBS adherence whilst BP is implicated in the invasion of the human brain endothelial cells (Maisey *et al.* 2007). The BP is also associated with GBS translocation of differentiated epithelial cells (Pezzicoli *et al.* 2008). Genes encoding for the BP of PI-2a are sub-classified into six major variants with sequence identities ranging from 48% to 98% (Margarit *et al.* 2009). DNA sequence analysis of the genes coding for structural components of PI-2a showed that AP-1 and

AP-2 were well conserved, with the exception of BP, which is grouped into six main different immunologically variants (Margarit *et al.* 2009). Mice immunization with the BP of PI-2a showed variant specific protection, which questioned the prospects of this target as a vaccine epitope. In an attempt to resolve this limitation, a structural vaccinology approach to design a synthetic protein with multivalent activity was developed (Nuccitelli *et al.* 2011).

2.7.4 Pilus island-2b

Pilus island-2b occupies the same position of the GBS genome PI-2a and thus never occurs in the same isolate. Unlike PI-2a, the DNA sequence analyses of the three structural proteins of PI-2b have been shown to be highly conserved among GBS bearing PI-2b isolates. The level of protection against GBS strains carrying PI-2b correlated with the surface antigen exposure, i.e. both BP-2b and AP1-2b were found to induce island specific protective antibodies (Margarit *et al.* 2009). This observation suggests that BP and AP-1 from all three pilus islands elicit protective antibodies in vivo against GBS strains expressing corresponding antigens.

2.7.5 Group B streptococcus pilus islands distribution

Considering that the identification of pili in GBS is a recent discovery (Lauer *et al.* 2005; Rosini *et al.* 2006), there is limited data regarding GBS pilus island distribution in colonizing and invasive disease isolates. Knowledge of the prevalence of pilus islands in GBS isolates is limited to two studies which reported that all GBS strains carried at

least one of the three pilus islands and all isolates had either PI-2a or its variant PI-2b (Margarit *et al.* 2009; Martins *et al.* 2010).

A study by Margarit *et al,* 2009 provided more insight on the type of pilus islands associated with colonization and invasive disease based on the identification of the three proteins specific for each island. It was reported that dissimilar to the serotypes, in most circumstances the majority of GBS isolates expresses or carries more than one pilus-island. The most common pilus island carried alone was PI-2a, accounting for 33.3% and 26.4% of colonizing and invasive disease isolates respectively, whereas PI-2b alone was identified in 1.4% of invasive disease isolates. Pilus island-1 was identified together with either PI-2a or PI-2b in colonizing (55.6% and 11.1%, respectively) and invasive disease (45.4% and 26.8%, respectively) isolates. No significant differences were reported in pilus island distribution for isolates causing EOD and LOD (Margarit *et al.* 2009).

In a study from Portugal, the distribution of pilus islands was not stratified by the source of isolation nor the onset of disease (Martins *et al.* 2010). Sortase enzyme coding genes specific for each island was used for the identification of three pilus islands. Pilus island-2a was identified in all isolates alone (40.7%) or in combination with PI-1 (59.3%) and no isolates carried PI-2b. Despite the use of different genes for the identification of the pilus islands, the two studies consistently showed all isolates carried at least one of the PI-2 alleles. The ubiquitousness of PI-2 in GBS isolates can be explained by its importance in biofilm formation, a process important for the persistence of GBS colonization (Rinaudo *et al.* 2010).

It has become apparent that over the years GBS has evolved mechanisms by which it expresses other novel virulence factors such as the pili. Depending on the pilus island carried by an isolate, a correlation between a pilus island and capsular serotype was established. The relationship between pilus island and capsular serotype has been reported by both of the above studies (Margarit *et al.* 2009; Martins *et al.* 2010). Association between capsular serotypes and pilus islands showed that majority of serotypes represent a two pilus combination. For example, serotype Ib (85.7%), and V (95.7%) were significantly associated with a combination of PI-1 and PI-2a, whilst serotype III (68.9%) was significantly associated with a combination of PI-1 and PI-2b. The majority of serotype Ia (91.0%) carried PI-2a alone (Margarit *et al.* 2009).

Consistent with the previous study by (Margarit *et al*, 2009), a study by (Martins *et al*, 2007) reported 90.0% of serotype Ia isolates to have carried PI-2a alone, whilst all serotype Ib and 90% of serotype V isolates carried a combination of PI-1 and PI-2a. Although there were minor inconsistencies between the two studies the similarities were sufficient to confirm that the presence of a particular pilus island was largely dependent on the capsular serotype. The relationship between capsular serotype and pilus-island is of importance regarding the proposed vaccine exclusively constituted by pilus components of the three pilus islands. On the basis of the effectiveness of functional pilus specific antibodies in mouse models, a pilus based vaccine composed of AP-2 of each pilus island can theoretically confer protection against all isolates of GBS.

2.8 Hypervirulent group B streptococcus adhesion

Hypervirulent GBS adhesin (HvgA) is a surface protein, and harbors an LPXTG motif that enables covalent attachment to the cell wall in a sortase dependent manner. Genes encoding HvgA are located within a genomic locus (gbs2018 allele C), there are three allelic forms or clusters of gbs2018 (A, B and C) (Lamy *et al.* 2006). The flanking genes from each allele display >90% sequence identity whereas their internal parts vary considerably; Figure 2.3. Isolates that belong to a particular sequence type lineage are associated with a certain allelic cluster, for example, HvgA is a ST-17 specific surface protein, thus because of an association between HvgA and ST-17, cluster C comprises isolates belonging to ST-17. Cluster A comprises the isolates belonging to ST-19 and ST-23, whilst cluster B includes multiple sequence types (ST-1, ST-2, ST-6, ST-8, ST-9, ST-10 and ST-41) (Lamy *et al.* 2006).

The pathogenic importance of HvgA has been extensively studied because of its greater disease causing potential. The hyper adherence role of HvgA was first demonstrated by the low adherence of HvgA non-expressing isolates to the intestinal and blood brain barrier. Moreover it was further shown that HvgA mediates gut colonization and translocation across intestinal barrier (Tazi *et al.* 2010).

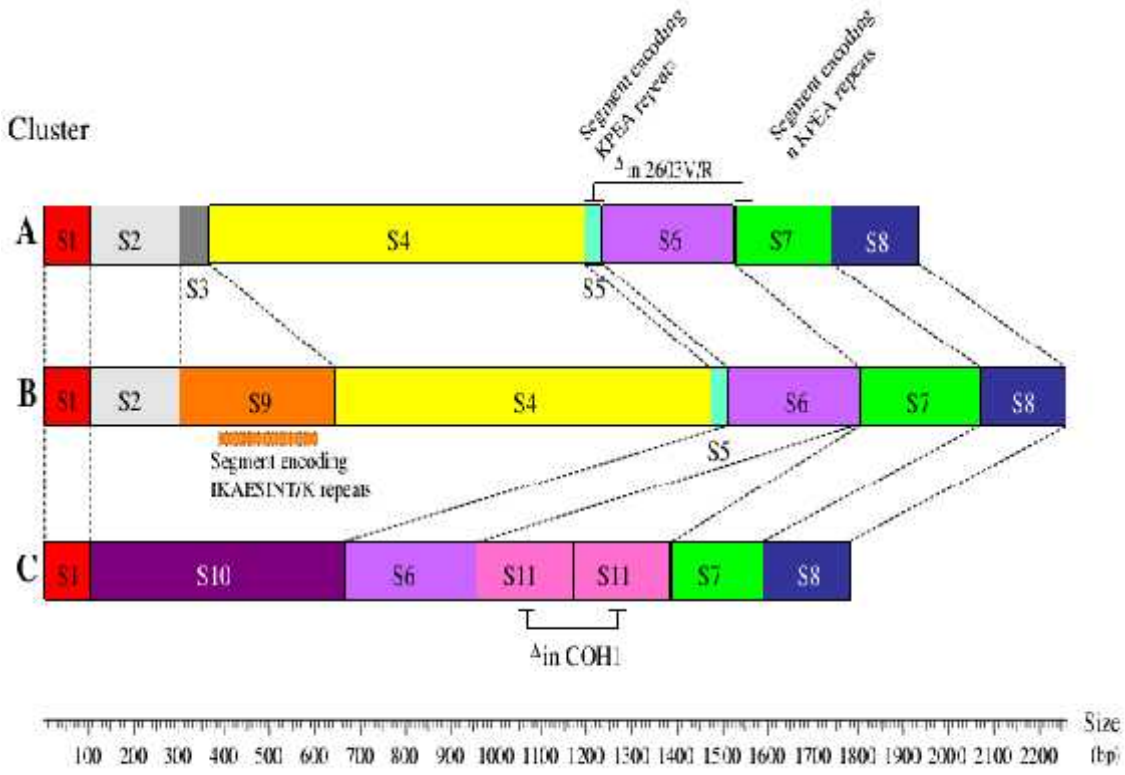


Figure 2.3: Schematic representation of genes encoding gbs2018 mosaic variants

Segments with identical nucleotide sequences are depicted by the same color. The 5' segment (S1) encodes the signal peptide and the 3' segment (S8) encodes the sorting signal made of the LPXTG motif (Lamy *et al.* 2006).

2.9 Group B streptococcus molecular epidemiology

A combination of phenotypic classification and molecular typing has been successfully used in epidemiological investigations of GBS to study clonal lineages associated with colonization or invasive disease (Savoia *et al.* 2008; Toresani *et al.* 2001). A diverse range of molecular techniques have been applied to determine the population structure of GBS, including pulse field gel electrophoresis (PFGE) (Gordillo *et al.* 1993) restriction digest pattern (RDP) (Nagano *et al.* 1991) and multilocus sequence typing (MLST) (Jones *et al.* 2003).

Multilocus sequence typing has several advantages over restriction digest based molecular typing techniques in that it uses standardized protocols, and provides specific data on single nucleotide changes rather than crude estimates of approximate fragment length. Furthermore, the MLST data from different laboratories can be stored and compared on an open access online database (http://pubmlst.org/agalactiae/), and hence it has become the preferred method to compare the genetic relatedness of GBS isolates (Cooper & Feil 2004).

Multilocus sequence typing has been successfully used to characterize the clonal diversity of a number of human bacterial pathogens including *Neisseria meningitides* and *S. pneumoniae* (Brueggemann *et al.* 2003; Maiden *et al.* 1998). Multilocus sequence typing is based on the DNA sequencing of seven housekeeping GBS genes, which include alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*PheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*), glucose kinase (*glcK*) and transkelotase (*tkt*) (Jones *et al.* 2003). The sequence polymorphisms at each

locus are classified and each allele is given a number. The combination of alleles found in each isolate defines the sequence type, and sequence types having allelic profiles that share at least five or six identical alleles are grouped within a particular clonal complex (CC) (Spratt *et al.* 2004).

There is, however, limited information from industrializing countries on the genetic relatedness between colonizing and invasive GBS isolates; and the relationship these isolates have with those from industrialized countries. The majority of studies which identified the clonal lineages among isolates responsible for either colonization or invasive disease were performed on isolates from Europe and the USA (Bergseng *et al.* 2009; Bohnsack *et al.* 2008; Davies *et al.* 2004; Fluegge *et al.* 2011; Jones *et al.* 2006b; Luan *et al.* 2005; Manning *et al.* 2008; Salloum *et al.* 2011; Tien *et al.* 2011).

2.9.1 Multilocus sequence typing population of maternal and neonatal colonizing group B streptococcus

Based on the MLST data, a number of different sequence types (STs) have been described in maternal and neonatal colonizing GBS isolates. Despite variability in the proportionality of clonal lineages, molecular characterization of GBS colonizing isolates has shown a limited number of major sequence types as associated with colonization regardless of geographical and epidemiological distinction (Bohnsack *et al.* 2008; Marchaim *et al.* 2006). There are four major MLST defined genetic lineages (ST-1, ST-17, ST-19 and ST-23), with ST-1 and ST-19 being responsible for over 80% of sequence types in colonizing isolates (Jones *et al.* 2003).

It is challenging to compare the GBS population on the basis of described sequence types, as single allele variability may reflect a different lineage. Hence, related sequence types are grouped within particular clonal complex. A number of common sequence types make up a clonal complex, for example CC-1 is composed of ST-1, ST-139 and ST-2; CC-17 includes ST-17, ST-31, ST-32 and ST-109; CC-19 includes ST-19, ST-27 and ST-107; and CC-23 encompasses ST-23, ST-24 and ST-114.

Four clonal complexes (CC-1, CC-17, CC-19 and CC-23) account for the majority of colonizing isolates; Table 2.4. Collectively, they are associated with 63.7% in Europe, 74.4% in the USA, 65.8% in Africa, 69.0% in Canada and 72.2% in the Middle East (Bohnsack *et al.* 2008; Brochet *et al.* 2009; Davies *et al.* 2004; Fluegge *et al.* 2011; Jones *et al.* 2003; Manning *et al.* 2009; Marchaim *et al.* 2006; Sadowy *et al.* 2010). These findings indicate that GBS is less diverse, more particularly when one considers the clonal diversity of other bacterial pathogens such as *S. pneumoniae* and GAS (Brueggemann & Spratt 2003; Enright *et al.* 2001).

Temporal variations with respect to the most common clonal complex associated with maternal colonization may exist within geographic regions. In Canada, (Davies *et al.,* 2004) reported CC-1 and CC-23 as the common clonal complexes accounted for 53.0% of isolates, whereas, in a later study (Manning *et al*, 2009) reported a low prevalence of both CC-1 (3.6%) and CC-23 (1.8%).

Furthermore a study from Canada that determined whether distinct clones were associated with different stages of colonization, compared genotypic diversity of GBS from colonized women before and after delivery (Manning *et al.* 2008). It was reported

that there is an association between particular clones and their temporal association to pregnancy. For example, CC-10 was significantly associated with GBS clearance, whilst CC-17 and CC-19 were associated with persistent colonization after pregnancy (Manning *et al.* 2008). This finding explains the preponderance of both CC-17 and CC-19 in GBS isolates, suggesting that specific sequence types are less likely to be eradicated after acquisition and therefore more likely to be transmitted to the newborn during birth.

A study from Africa that characterized isolates collected from Senegal and Central African Republic showed genotypic overlap between the two regions. In Senegal, CC-1 (32.0%) was significantly higher compared to the Central African Republic (10.2%). In contrast, CC-19 (43.2%) was significantly more common in the Central African Republic compared to Senegal (12.0%; $P<0.0001$). Despite some differences in the population of colonizing isolates circulating in Senegal and Central African Republic, there was some similarity between the two countries, such as the detection of a rarely described CC-26, in 20.0% and 11.4% of isolates from Senegal and Central African Republic, respectively.

It has generally been reported that the prevalence of CC-17, which is mostly associated with invasive disease, remains modest in GBS isolates from colonized pregnant women; Table 2.5. In contrast, a study by Davies *et al*, 2004 found no significance difference in the relative frequency of CC-17 when comparing invasive and colonizing isolates ($P=0.80$). The higher proportion of CC-17 in colonizing isolates from a Canadian study could be attributed to only serotype III isolates having been characterized. The association between serotype-III and CC-17 has been widely documented by others (Lamy *et al.* 2006; Luan *et al.* 2005; Tien *et al.* 2011).

Table 2.4: Global group B streptococcus clonal complexes distribution in maternal and neonatal colonizing isolates

Location and reference	Year of study	Source	no of isolates	Clonal Complex (%)	Comment
USA (Bohnsack *et al.* 2008)	1995-1999	Colonized neonates	770	CC1 (16.0) CC12 (9.1) CC17 (11.9) CC19 (17.9) CC23 (40.5) Others/singletons (4.5)	
Canada (Davies *et al.* 2004)	1995-1999	Pregnant women	55	CC1 (3.6) CC17 (30.9) CC19 (63.6) CC23 (1.8)	Included only capsular serotype III isolates
Poland (Sadowy *et al.* 2010)	1996-2005	Maternal colonized	14	CC1 (35.7) CC17 (7.1) CC 19 (28.6) CC23 (28.6)	
Canada (Manning *et al.* 2009)	1998-2000	Pregnant women	232	CC1 (28.0) CC12 (18.0) CC17 (6.0) CC19 (17.0) CC23 (25.0) Others/singletons (6.0)	
UK (Jones *et al.* 2006b)	2000-2003	Pregnant women	190	CC1 (20.5) CC10 (17.4) CC17 (12.1) CC19 (20.0) CC23 (23.2) Others/singletons (7.1)	
Germany (Fluegge *et al.* 2005)	2001-2003	Colonized Neonates	46	CC1 (4.3) CC17 (30.4) CC19 (50.0)	MLST performed only in serotype III isolates

Location and reference	Year of study	Source	No of isolates	Clonal Complex (%)	Comment
Germany (Fluegge *et al.* 2005)	2001-2003	Colonized Neonates	46	CC23 (6.5) Others/singletons (8.8)	MLST performed only in serotype III isolates
Senegal (Brochet *et al.* 2009)	2005-2006	Pregnant women	75	CC1 (32.0) CC17 (17.3) CC19 (12.0) CC23 (16.0) CC20 (20.0) Others/singletons (2.7)	
Central African **Republic** (Brochet *et al.* 2009)	2005-2006	Pregnant women	88	CC1 (10.2) CC10 (9.1) CC17 (6.8) CC19 (43.2) CC23 (17.0) CC26 (11.4) Others/singletons (2.3)	
Israel (Marchaim *et al.* 2006)	2000	Maternal colonized	72	CC1 (58.3) CC17 (9.7) CC22 (15.3) CC23 (13.9) Other/singletons (2.8)	

2.9.2 Multilocus sequence typing population of invasive disease group B streptococcus

There are not many studies that have determined the prevalence of clonal complexes in EOD and LOD independently (Bohnsack *et al.* 2008; Davies *et al.* 2004; Lamy *et al.* 2006; Salloum *et al.* 2011; Tien *et al.* 2011). Available data, however, suggests that CC-17 and CC-23 are responsible for most of EOD and LOD, suggesting that invasive disease is caused by a limited set of clonal lineages which are similar to those associated with maternal colonization (Jones *et al.* 2006b).

Clonal complex-17 has consistently been found to be associated with invasive disease; Table 2.5, indicating intrinsic genetic properties of CC-17 isolates to cause invasive disease regardless of timing of onset of disease or geographic location (Bisharat *et al.* 2005; Jones *et al.* 2003). In EOD isolates, CC-17 accounted for between 17.0% in Canada (Manning *et al.* 2009) to 67.1% in France (Salloum *et al.* 2011). A higher proportion of LOD is associated with CC-17 compared to EOD, with prevalence ranging from 38.1% in Norway (Bergseng *et al.* 2008) to 81.6% in Italy (Imperi *et al.* 2011). The limitation for this analysis is that not all studies had both EOD and LOD isolates.

In both EOD and LOD, CC-17 is dominated by ST-17, which is of bovine origin (Bisharat *et al.* 2004). Sequence type-17 has been identified as having enhanced invasiveness in neonates (Jones *et al.* 2006b). The high virulence abilities of ST-17 clone has been attributed to the genomic locus (gbs2018C) that encodes a cell wall anchored protein HvgA (Lamy *et al.* 2006). Gbs2018C has been exclusively found in ST-17

isolates and the gene encoding for gbs2018C is being used as a rapid detection for this highly virulent GBS clone (Lamy *et al.* 2006). Genes coding for HvgA was present in (40/156; 25.6%) of GBS isolates, and all HvgA positive isolates were confirmed as ST-17 by MLST (Lamy *et al.* 2006).

Clonal complex-19 is more common among EOD than in LOD isolates; Table 2.5. The prevalence of CC-19 in EOD corroborates that maternal colonization is the major risk factor for EOD development. The overall prevalence of CC-19 in EOD ranged between 5.8% in France to 57.1% in Canada (Davies *et al.* 2004; Salloum *et al.* 2011), whilst in LOD it is prevalent between 5.3% in Italy and 20.0% in Canada isolates (Imperi *et al.* 2011; Manning *et al.* 2009). Clonal complex-19 is dominated by a single genetic lineage (ST-19), accounting for over 72.0% of all of CC-19 sequence types with the remainder being other sequence types (Bohnsack *et al.* 2008; Jones *et al.* 2003). Clonal complex-23 also lacks diversity in sequence types representation and >87% of this clonal complex is comprised of ST-23.

Table 2.5: Global group B streptococcus clonal complexes distribution associated with early and late onset infant invasive disease

Location	Year of study	Source	No of isolates	Clonal Complex		Reference
				EOD (%)	LOD (%)	
***Sweden** (Luan *et al.* 2005)	1988-1997	Infants	102	CC1 (11.8) CC17 (33.3) CC19 (28.4) CC23 (14.7) Other/singletons (11.8)		
USA (Bohnsack *et al.* 2008)	1995-1999	Neonates	129	CC 1 (18.6) CC12 (11.6) CC17 (17.8) CC19 (10.1) CC23 (36.4) Others/singletons (5.4)		
[+]**Canada** (Davies *et al.* 2004)	1995-1999	Infants	28	CC17 (35.7) CC19 (57.1) CC23 (7.1)		MLST performed only in serotype III isolates
Canada (Manning *et al.* 2009)	1995-2002	Infants	181	CC1 (19.0) CC12 (8.0) CC17 (17.0) CC19 (27.0) CC23 (24.0) Other/singletons (5.0)	CC1 (8.0) CC12 (11.0) CC17 (57.0) CC19 (20.0) CC23 (4.0)	
UK (Jones *et al.* 2006b)	2000-2003	Neonates	190	CC 1 (25.0) CC10 (10.9) CC17 (26.6) CC19 (17.2) CC23 (15.6) Others/singletons (4.7)		

Location	Year of study	Source	No of isolates	Clonal complex		Reference
				EOD (%)	LOD (%)	
***Taiwan** (Tien *et al.* 2011)	2001-2004	Infants	17	CC 1 (5.9) CC12 (11.8) CC17 (41.2) CC19 (17.6) CC23 (23.5)		
Germany (Fluegge *et al.* 2011)	2001-2003	Infants	185	CC17 (66.7) CC19 (22.9) CC23 (6.3) Other singletons (3.1)	CC17 (79.8) CC19 (13.5) CC23 (2.2) Other singletons (4.5)	MLST performed only in serotype III isolates
***France** (Salloum *et al.* 2011)	2003	Infants	67	CC1 (4.5) CC17 (67.1) CC19 (5.8) CC23 (13.4) Others/singletons (9.0)		
Italy (Imperi *et al.* 2011)	2005-2008	Infants	75	CC1 (13.5) CC17 (48.6) CC19 (10.8) CC23 (13.5) Others/singletons (13.5)	CC17 (81.6) CC19 (5.3) CC23 (2.6) Others/singletons (10.5)	
Norway (Bergseng *et al.* 2009)	2006-2007	Infants	96	CC1 (31.5) CC17 (25.9) CC19 (20.4) CC23 (3.7) Others/singletons (18.5)	CC1 (21.4) CC17 (38.1) CC19 (14.3) CC23 (14.3) Others/singletons (11.9)	

[CC] Clonal complex, [*] included infants younger than 3 months, [+] included infants younger 7 days

2.9.3 Distribution of clonal complex among colonizing and invasive capsular serotype

Certain clonal complexes, including CC-17, CC-19 and CC-23, are confined within particular capsular serotypes (Bergseng *et al.* 2008; Bohnsack *et al.* 2008; Manning *et al.* 2009; Martins *et al.* 2007). The correlation has been observed in isolates responsible for both colonization and invasive disease. Over 90.0% of isolates belonging to CC-17 and CC-19 are associated with serotype III and at least 90.0% of CC-23 isolates are associated with serotype Ia (Bohnsack *et al.* 2008; Imperi *et al.* 2011; Jones *et al.* 2006b; Luan *et al.* 2005). Until recently, CC-17 was the only clonal complex exclusively associated with serotype III, however, a recent report has identified serotype IV to be also associated to CC-17 (Bellais *et al.* 2012). Comparative analysis of serotype IV isolates belonging to CC-17 showed that they share more genes to strains representing serotype III, suggesting the capsular switch may have occurred due to selective pressure imposed by host immune factors.

There is, however, some degree of variability between the proportion of colonizing and invasive disease isolates of serotypes III which are grouped either to CC-17 or CC-19. The majority of serotype III invasive isolates (56.4%-73.1%) are associated with CC-17 whereas 18.8%-21.8% of serotype III colonizing isolates are grouped within CC-19 (Brochet *et al.* 2009; Jones *et al.* 2003; Salloum *et al.* 2011). In contrast, 95% and 66.7% of serotype II isolates from Central African Republic and Senegal respectively belonged to CC-19. Isolates representing serotype Ia irrespective of whether they are colonizing (68.7%-96.4%) or invasive isolates (66.6%-84.0%) are predominantly

grouped under a single clonal complex, i.e. CC-23 (Brochet *et al.* 2009; Imperi *et al.* 2011; Jones *et al.* 2003; Salloum *et al.* 2011).

There are differences in association between certain clonal complexes and capsular serotypes, for example, CC-1 and CC-10 are composed of isolates of varying serotypes and the reason for this is unknown. Clonal complex-1 comprises of isolates representing mostly serotypes Ib, II and V, the association of serotypes Ib, II and V in the same clonal complex is interesting considering the difference in their invasive potential and prevalence. Clonal complex-26 which is rarely reported was found in Senegal and in the Central African Republic, and in both countries it was associated with serotype V. This observation, however, implies a distinct geographical lineage corresponding to serotype-V, as in Europe and USA serotype-V was associated with CC-1 (Bohnsack *et al.* 2008; Jones *et al.* 2006b).

Chapter 3 Materials and Methods

3.1 Ethical consideration

Signed informed consent for collection of the isolates was obtained from study participants of the Prevention of prenatal sepsis (PoPS) trial (HREC number M030207) and the sepsis surveillance trial (HREC number M031007) which were approved by the Human Research Ethics Committee (HREC) on Human Subjects at the University of the Witwatersrand. The PoPs trial was registered in ClinicalTrials.gov (Trial number NCT00136370). Analysis of the GBS isolates for this study was approved by the HREC (MM080366).

3.2 Study population

The study was conducted at a secondary-tertiary care hospital (Chris Hani-Baragwanath Hospital; CHBH) which provides health care to an urban population of 1.5 million indigenous Africans living in Soweto, South Africa. There are approximately 28 000 births annually in Soweto, 75% are born at CHBH and the remainder at one of seven surrounding primary health care clinics. The majority of Soweto children requiring hospitalization are admitted to CHBH.

Group B streptococcus isolates from mother-newborn dyads were identified from participants involved in the PoPS trial undertaken at CHBH between April 2004 to October 2007 as reported (Cutland *et al.* 2009). Briefly, mothers were randomized during active labor to receive either intra-vaginal chlorhexidine washes (0.5%) or external genitalia wipes with water (placebo) to evaluate the efficacy of 0.5% chlorhexidine wipes of the birth canal during labor and of the infant at birth in reducing: i). Vertical

transmission of leading pathogenic bacteria from mother to child during labor and delivery; and ii). Incidence of early-onset neonatal sepsis and maternal peripartum infections. Vaginal swabbing with a sterile Dacron swab was undertaken prior to initiation of the study-intervention, and swabs were obtained from the newborn's ears, nose and umbilicus shortly after birth. The swabs were transported in Amies transport medium without charcoal (Medical Wire, MW170) and processed with standard microbiology techniques as described in 3.2.1-3.2.3.

The PoPS study concluded that intra-vaginal chlorhexidine wipes were not associated with any reduction in vertical acquisition of GBS in the newborns (Cutland *et al.* 2009). Evaluation of isolates in the current study was, nevertheless, limited to mother-newborn dyads who had been randomized to the placebo arm of the PoPS trial.

In addition, prospective surveillance of infant sepsis at CHBH was undertaken from January 2004 to December 2008. GBS isolates from blood and CSF from infants with invasive disease, identified as part of routine medical care, were retrieved from the laboratory.

3.3 Bacterial isolates, identification and growth conditions

Maternal lower vaginal swabs and neonatal skin swabs were inoculated onto three different media: 5% horse blood agar, CNA agar 5% horse blood with 10µg/ml colistin and 15µg/ml nalidixic acid and Todd-Hewitt broth (2ml) supplemented with 8µg/ml gentamicin and 15µg/ml nalidixic acid followed by inoculation onto 5% horse blood agar. The plates and the broth were incubated overnight at 37^0C in 5% CO_2 incubator (Sanyo Electric Biomedical, Japan). The identity of GBS was confirmed by

microbiological assays described in 3.3.1-3.3.3. Group B streptococcus positive isolates were stored at -70^{o}C in a broth containing skim milk, tryptone, glucose and glycerol (STGG) at the Respiratory and Meningeal Pathogens Research Unit (RMPRU), Johannesburg, South Africa, where serotyping, pilus island identification and MLST characterization was subsequently undertaken. Reference strains used in this study are listed in Table 3.1

Table 3.1: Group B Streptococcus reference strains used in this study

Strain	Serotype	Pilus island	GBS2018
SS-615[a]	Ia		
SS-618[a]	Ib		
SS-619[a]	II		
SS-620[a]	III		
SS-1243[a]	IV		
SS-1168[a]	V		
SS-1354[a]	VI		
SS-1355[a]	VII		
SS-1356[a]	VIII		
27412[b]	IX		
2603V/R[c]	V	PI-1 + PI-2a	Allele A
COH1	III/Ia	PI-1 + PI-2b	Allele C
A909[c]	Ia	PI-1 + PI-2b	
S.aureus 25923[c]			
Enterococcus faecalis 29212[c]			

[a]CDC, [b]Statens Serum Institut (SSI) [c]American type culture collection (ATTC)

3.3.1 CAMP test

The hemolytic activity of staphylococcal β-lysin on red blood cells is enhanced by an extracellular factor produced by GBS called the CAMP factor as shown in Figure 3.1, wherever the zone diffusion of the two enzymes overlap in sheep blood agar, a β-hemolytic reaction occurs. Briefly a single streak of *Staphylococcus aureus* (*S. aureus*)

was made on the middle of a sheep blood agar plate, positive (GBS), negative control (*E. faecalis*) and test isolates were also streaked out on either side, 5mm away and perpendicular to the *S. aureus* streak as shown in Figure 3.1. The plate was incubated at 37^{o}C with 5% CO_2 for 18-24 hours. Group B streptococcus positive isolates produced a zone of complete lyses in the shape of an arrowhead at the junction of the streak line. In contrast the *E. faecalis* showed no enhanced hemolytic reaction near junction of the two organisms.

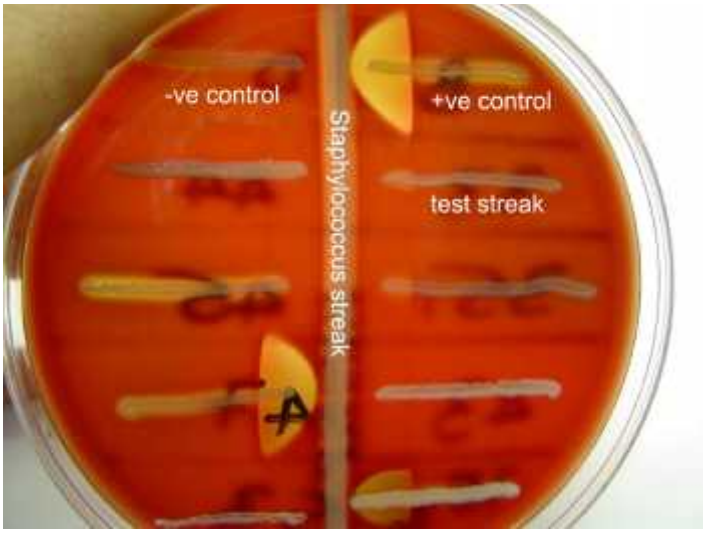


Figure 3.1: CAMP test indicated by the formation of an arrowhead of β-hemolysis at the junction of group B streptococcus and staphylococcal β-lysins

3.3.2 Bile esculin test

The bile esculin test is used to differentiate *E. faecalis* from bile intolerant species (i.e. GAS, *S. aureus* and *S. pneumoniae*) and furthermore those that are bile tolerant but cannot hydrolyze esculin including GBS (Chuard & Reller 1998). Briefly the bile esculin

agar plate was divided into quadrants, a GBS and *E. faecalis* controls colony were streaked along with the test isolates onto different quadrants of the bile esculin agar plate. The plate was incubated at 37^{o}C overnight with 5% CO_2 for 18-24 hours. A black ferric precipitate indicates *E. faecalis* and growth with the absence of a black precipitate indicates GBS, Figure 3.2**.**

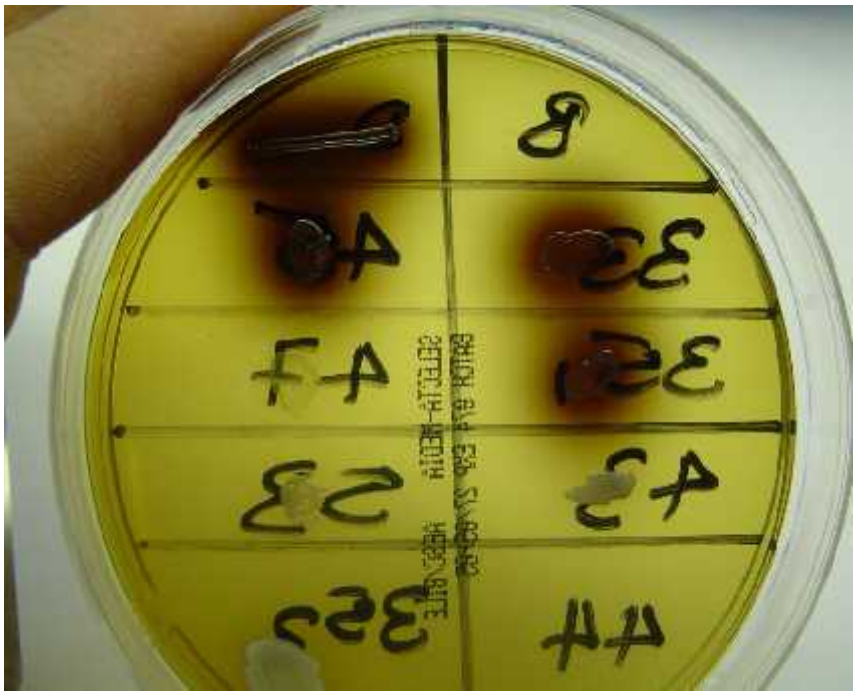


Figure 3.2: Bile esculin test, blackened quadrant shows positivity for *Enterococcus faecalis* and clear with growth quadrant shows negativity for group B streptococcus

3.3.3 Streptex agglutination test

The Lancefield grouping is used for the classification of streptococcal species, and the grouping is based on the species specific polysaccharide cell wall antigen that each streptococcus produces. Briefly 2-3 colonies were suspended into 400µl of

extraction enzyme, and incubated for 10 minutes in a water bath at 37^{o}C shaking every 5 minutes to release the polysaccharide from the cell wall. The extract was mixed with strep B latex particles coated with group B streptococcal antigens (Omega Diagnostics, Scotland, UK), and a positive reaction was recorded as any sign of agglutination within 1 minute.

3.3 Group B streptococcus capsule serotyping

Capsular serotyping was performed with the latex agglutination method as described by (Slotved *et al.* 2003) with particles of latex coated with rabbit antisera specific for GBS serotypes Ia, Ib and II to IX (SSI, Sweden). Briefly, a single colony of GBS was inoculated into 5ml of Todd-Hewitt broth and grown overnight at 37°C with 5% CO_2. Ten microliters of overnight culture was placed onto a glass microscope slide, an equal amount of the antiserum was added and mixed with the bacterial culture.

A positive reaction was recorded as any sign of agglutination within 10-15 seconds for that particular serotype. Isolates that were reactive to sera against serotype III were further tested with sera against serotype VI to test for cross reactivity. Isolates that tested negative with all antisera were re-subcultured and confirmed as GBS by CAMP test, bile esculin test and Streptex agglutination. Isolates that tested positive for CAMP and Streptex agglutination, inability to hydrolyze bile esculin and negative with all type specific antisera were designated as serologically non-typeable. Isolates designated as non-typeable by latex agglutination were further characterized by a molecular capsular typing assay as described in 3.4.

3.4 Molecular capsular typing

3.4.1 Deoxyribonucleic acid extraction

DNA was extracted with the QIAamp DNA mini kit (Qiagen GmbH, USA). One GBS colony sampled from a CNA agar plate was re-suspended in 500µl of 1X phosphate buffer saline (1XPBS). The tube was centrifuged at 5000g for 1 minute to pellet the bacteria. One hundred and eighty microliters of 20mg/ml lysozyme was added to the retained pellet, and incubated at 37^{o}C for 1 hour to lyse the bacterial cell wall. Thereafter 20µl of 1mg/ml proteinase K and 200µl of buffer AL were added and the lysate was incubated for 30 minutes at 56^{o}C to digest and denature indigestible protein. The supernatant was passed through a filter to remove proteinase K residues using 200µl of absolute ethanol as an elution solution. The filtrate was pipetted into the QIAamp mini spin column and centrifuged at 6000g for 1 minute. The filtrate was discarded and the nucleic acids were washed with 500µl AW1 and AW2 buffers at 6000g for 1 minute and 3 minutes respectively, 200µl of buffer AE was added to elute and recover the DNA at 6000g for 5 minutes. The DNA was stored at -20^{o}C until use.

3.4.2 Polymerase chain reaction (PCR) setup

Capsular typing was performed with a singleplex endpoint PCR method as described by (Poyart *et al.* 2007) for serotypes Ia, Ib, II, III, IV and V with primer sequences shown in Table 3.2. The genomic DNA was added to tubes containing a PCR master mixture for individual capsular types; Table 3.3. Each master mix was tested with an additional positive control consisting of DNA from a reference strain, and a no DNA negative control. DNA amplification was carried out with a mastercycler gradient thermocycler (Eppendorf, Germany) under the following cyclic conditions, initial

denaturation at 94^{o}C for 2 minutes, followed by 40 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55.1^{o}C for 30 seconds, extension at 72^{o}C for 2 minutes and final extension at 72^{o}C for 10 minutes.

Table 3.2: Specific primer sequences for the detection of group B streptococcus capsular types Ia-V

Serotype	Sequence (5'-3')	Amplicon size(bp)
Ia	Forward 5'-GGTCAGACTGGATTAATGGTATGC-3' Reverse 5'-GTAGAAATAGCCTATATACGTTGAATGC-3'	1826
Ib	Forward 5'-TAAACGAGAATGGAATATCACAAACC-3' Reverse 5'-GAATTAACTTCAATCCCTAAACAATATCG-3'	770
II	Forward 5'-GCTTCAGTAAGTATTGTAAGACGATAG-3' Reverse 5'-TTCTCTAGGAAATCAAATAATTCTATAGGG-3'	397
III	Forward 5'-TCCGTACTACAACAGACTCATCC-3' Reverse 5'-AGTAACCGTCCATACATTCTATAAGC-3'	1826
IV	Forward 5'-GGTGGTAATCCTAAGAGTGAACTGT-3' Reverse 5'-CCTCCCCAATTTCGTCCATAATGGT-3'	578
V	Forward 5'-GAGGCCAATCAGTTGCACGTAA-3' Reverse 5'-AACCTTCTCCTTCACACTAATCCT-3'	701

Table 3.3: Polymerase chain reaction setup for detection of group B streptococcus serotypes Ia, Ib, II and V

Reagents	Stock concentration	1 reaction (µl)	Final Concentration
10X PCR Buffer	2X	2.5	1X
DNTPs	2mM	2.5	0.2 mM
$Mgcl_2$	50mM	0.75	1.5 mM
Primer mix	10 µM	1	0.4 µM each
Platinum Taq	1.25 units	0.1	
Nuclease free water		13.6	
DNA template		5	
		25	

Table 3.4: Polymerase chain reaction setup for detection of group B streptococcus serotypes III and IV

Reagents	Stock concentration	1 reaction (µl)	Final Concentration
EconoTaq Master Mix	2X	12.5	1X
Primer mix	10µM	1	0.4 µM each
Nuclease free water		8.5	
DNA template		2	
		25	

3.4.3 Agarose gel electrophoresis and interpretation of the results

One percent agarose gels were prepared by boiling 1g of agarose (Molecular Biology Grade, Melford) in 100ml of Tris-acetic-EDTA (TAE) buffer (pH 8.0). The gel was allowed to cool down to about 60^{o}C before adding DNA intercalator 1mg/ml of ethidium bromide and cast into a gel mould. The gel was allowed to set for at least 45 minutes after which the gel was submerged into the gel tank containing TAE buffer. Three microliters of the PCR product was mixed with 1µl of the tracking dye and loaded

into the wells of the gel. The gel was run at 100V with an Elpho power supply (Life sciences, Austria) for 60 minutes. The PCR product was visualized by illuminating the gel with ultraviolet (UV) light on a trans-illuminator (UVP, USA). The relative size of the PCR products were determined by comparing them to a positive control band and the 1kb DNA ladder (Gene Ruler, Fermentas, Life Sciences: # SM0311).

3.5 Pilus island identification characterization

To identify the three pilus island of GBS by PCR, we designed sets of primers specific for each island targeting the AP-1 and AP-2. The primers were designed based on the sequences of GBS strains as described in Figure 3.3

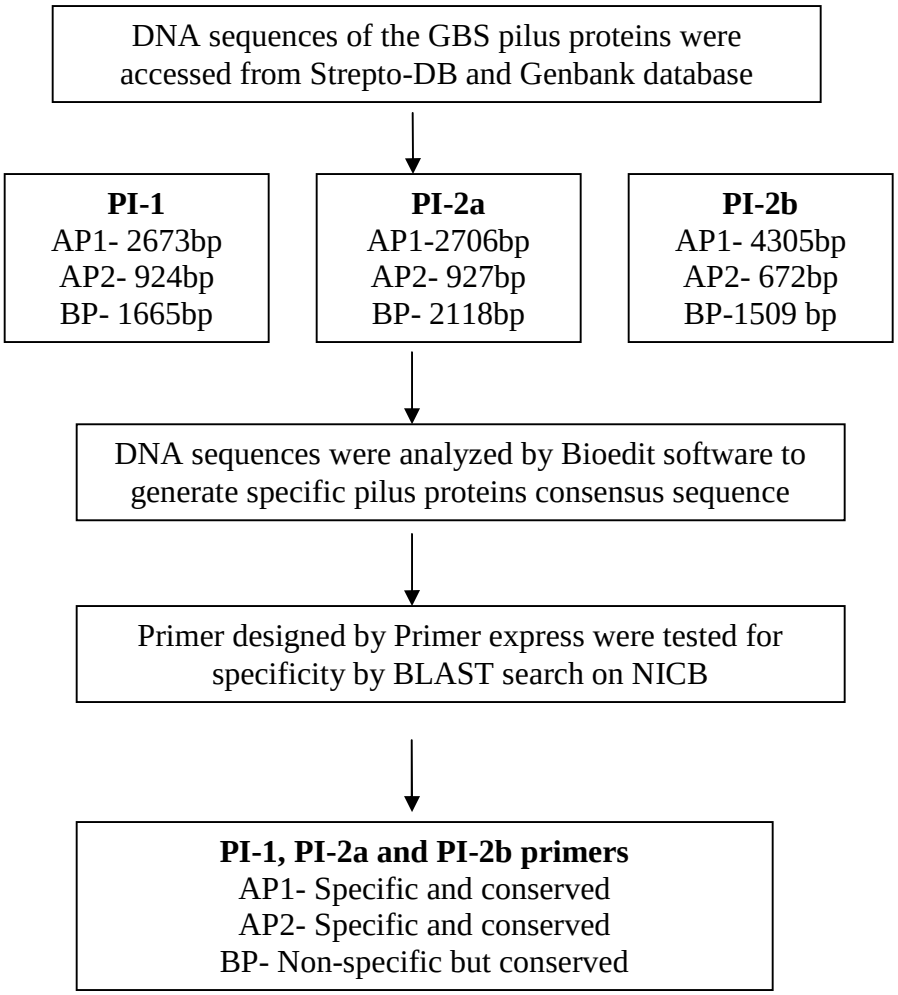


Figure 3.3: Design of group B streptococcus pilus islands primers

3.6.1 Primer and probe design

Ancillary protein 1 was selected as a real-time PCR target, to identify and discriminate between the three pilus islands. The ancillary protein was selected because it was highly variable between different pilus islands and conserved within a particular island. Oligonucleotide primers and Taq-Man flouregenic minor groove binding (MGB) probes targeting the ancillary protein genes were designed with the Primer Express 3.0 software (Applied Biosystems, USA); Table 3.5: The selected primers and probes targeting the ancillary proteins were validated with the NCBI primer blast search to ensure that they were specific for each island. Isolates which tested negative for the AP-1 gene from the three pilus islands were re-amplified with a second set of primers representing conserved regions of the ancillary protein 2; Table 3.5:

3.6.2 Sample preparation

Bacterial isolates were subcultured on CNA agar and incubated overnight at 37^{o}C with 5% CO_2. One GBS colony was suspended in 300µl of nuclease free water and the suspension was heated at 95^{o}C for 10 min. The tubes were centrifuged at 9000g for 1 minute to pellet the cell debris. The resulting supernatant was stored at -70 ^{o}C.

Table 3.5: Group B streptococcus strains and accession numbers used to design the primers for pilus island identification

Pilus island	Locus (gene target) and accession number	
	AP1	AP2
PI-1	2603V/R (SAG0649) CJB111 (SAM_0665) NEM316 (gbs0632) A909 (SAK_0780) COH1 (SAN_0702)	2603V/R (SAG0646) CJB111 (SAM_0662) A909 (SAK_0777) COH1 (SAN_0699) EU929429-EU929433
PI-2a	2603V/R (SAG1408) 18RS21 (SAJ_1417) NEM316 (gbs1478) H36B (SAI_1512)	2603V/R (SAG1404) 18RS21 (SAJ_1413) NEM316 (gbs1474)
PI-2b	COH1 (SAN_1519) EU929390-EU929393	EU929701-EU929710

Table 3.6: Oligonucleotide sequences of the primers and probes for detecting ancillary protein 1 and ancillary protein 2

Pilus protein	Sequence (5'-3')	Amplicons bp
PI-1AP1	Forward 5'-GGTGAAACCCAAGATACCAATCA-3' Reverse 5'-CTTTGCCTAATGGTGTAGCATTGT-3' Probe 5'-6FAM CACTTGGAAAAGTAATTG MGB-3'	82
PI-2aAP1	Forward 5'-ACTGCGGTCCCAAGAGCTT-3' Reverse 5'-GATGGCCCAAATTTTCAAAGG-3' Probe 5'-VIC AGCTTTCTTGGCTTTATT MGB-3'	65
PI-2bAP1	Forward 5'-TCTATGCAGGTTGGCAAAAGGTA-3' Reverse 5'-TCGGCCTCCGTTTGGA-3' Probe 5'-NED CATATCGTGTCAATATTG MGB-3'	59
PI-1AP2	Forward 5'-CCGGGCTCATCCATGGGGACT-3' Reverse 5'-ACAGCAGTCTCTGCCTGTCCGA-3'	82
PI-2aAP2	Forward 5'-ATGGTTCGTACTTTGGTCGTGCTT-3' Reverse 5'-TCGGCCTGTTTCAACTTTTCGCT-3'	137
PI-2bAP2	Forward 5'-ACCGGCTGAAGGTATTGTTGCGA-3' Reverse 5'-GCCACCATACTTGTCCAGTAAACGG-3'	251

3.6.3 Real-Time PCR for identification of genes coding for Ancillary protein-1

The detection of PI-2b was performed as a singleplex reaction whilst duplex reaction was used for detection of PI-1 and PI-2a. PCR reactions were run on an AB-7500 instrument (Applied Biosystems, USA). The reaction mixture (25µl) of PI-2b consisted of 250nM of a probe, 900nM of each primer, 12.5µl of 10X *Taq*Man universal PCR Master Mix (Applied Biosystems, 4304437), 3.38µl of free nucleases water, and 4µl of DNA template. The reaction mixture (25µl) for PI-1 and PI-2a consisted of 250nM of probe of each target, 300nM of each forward primer, 110nM of each reverse primer, 12.5µl of 10X *Taq*Man universal PCR Master Mix, 4µl of DNA template, and the final volume was adjusted to 25µl with nuclease free water. Positive control consisting of DNA from a reference strain and a no DNA negative control were added to respectively check the presence of any PCR inhibitors and give an indication of false positive PCR. DNA amplification was carried out under the following cyclic conditions, AmpErase activation at 30^{o}C for 2 minutes and *Taq* DNA polymerase activation at 95^{o}C for 10 minutes followed by 40 cycles of denaturation for 15 seconds at 94^{o}C and extension at 60^{o}C for 1 minute.

3.6.3 Polymerase chain reaction for identification of genes coding for AP2

The PCR was run as a single reaction for each pilus island on a Mastercyler gradient. The reaction mixture consisted 0.2µM of each primer, 12.5µl of 10X Econo*Taq* Master mix (Inqaba Biotec, LU 630035-1), 1µl of DNA template and the final volume was adjusted to 25µl with nuclease free water. DNA amplification was carried out under the following cyclic conditions, initial denaturation at 94^{o}C for 2 minutes, followed by 35

cycles of denaturation at 94^{o}C for 30 seconds annealing at 59.1^{o}C for 1 minute, extension at 72^{o}C for 2 minutes and final extension at 72^{o}C for 10 minute. Positive control consisting of DNA from a reference strain and a no DNA negative control were added to respectively check the presence of any PCR inhibitors and give an indication of false positive PCR. The PCR products were visualized by 2% agarose gel electrophoresis as described in 3.4.3.

3.7 Multilocus sequence typing

Multilocus sequence typing of GBS is based on the sequencing of the seven housekeeping genes (*adhP*, *atr* and *glnA*, *PheS*, *sdhA*, *glcK* and *tkt)* and despite its good reproducibility and high discriminatory power MLST remains an expensive typing tool. Due to cost constraints, maternal and newborn colonizing and infant invasive disease isolates were pre-screened using the three most heterogeneous MLST housekeeping genes (*adhP*, *atr* and *glnA*) and clustered according to the detected alleles; Figure 3.4. Colonizing and invasive disease isolates were randomly selected from the pre-screening clusters, and were further genotyped with four additional housekeeping genes (*PheS*, *sdhA*, *glcK* and *tkt*) in order to categorize the isolates in MLST derived sequence types, as described previously (Jones *et al.* 2003). Where more than one serotype occurred within a cluster, then isolates with the different serotypes were selected. This approach may, however, lead to missing a few clonal lineages which may reduce the discriminatory power if all MLST genes were analyzed. Notwithstanding these challenges, sequencing of the three most heterogeneous core loci will provide a reliable insight in the population structure and remain applicable to the local epidemiology.

The gene encoding the GBS surface antigen gbs2018 has been shown to exist in three distinct allelic forms (A-C). We sequenced this allele from a selection of invasive disease and colonizing isolates with different sequence types to determine their association with the different allelic forms of gbs2018.

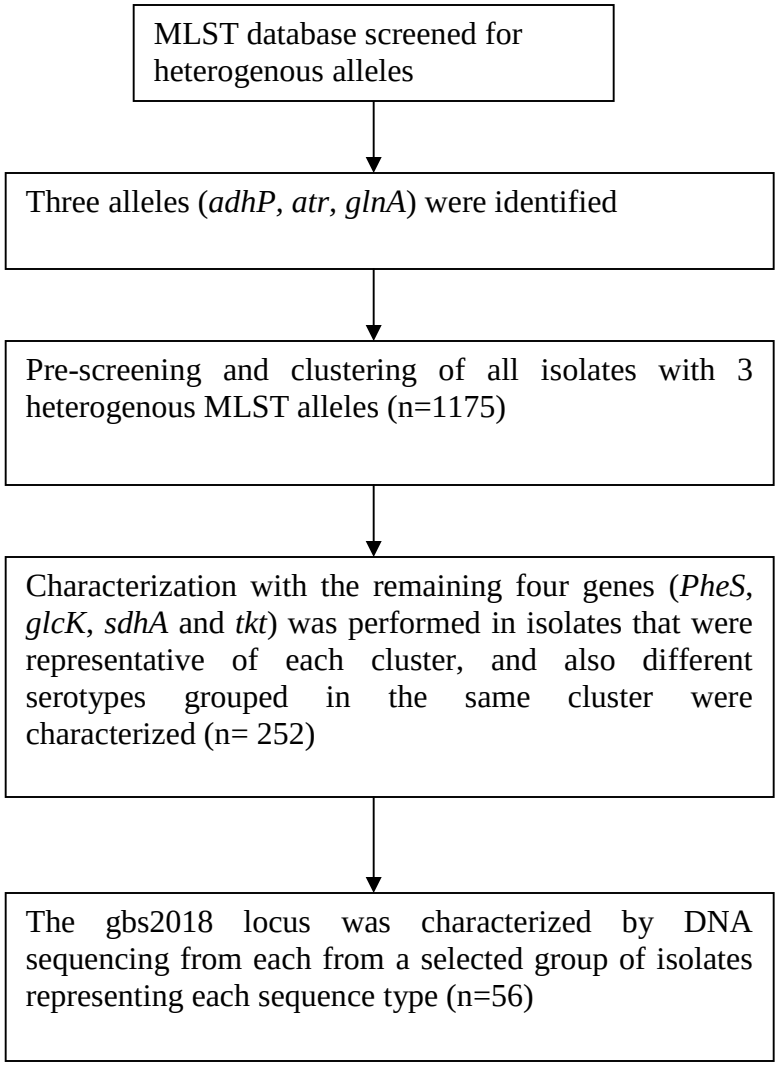


Figure 3.4: Rationale and workflow for selection of group B streptococcus isolates characterization by multilocus sequence typing and gbs2018 sequence typing

3.7.1 Deoxyribonucleic acid extraction

Total nucleic acid extraction was performed with the NucliSens easyMAG instrument (bioMeriux, France). The Nuclisens easyMAG platform is intended for the automated isolation (purification and concentration) of total nucleic acid (RNA/DNA) from biological specimens. The Nuclisens easyMAG only isolate DNA from liquid

specimen and the first step involves lyses of any cellular matter to release the nucleic acids, i.e. bacteria, viral particle or fungi. The isolation process is initiated by the binding of magnetic silica to the nucleic acids under high salt condition, increased temperature allow the nucleic acid to be released from the magnetic silica and then recovered in an elution buffer.

Briefly, GBS isolates were grown overnight on CNA agar plate at 37^{o}C with 5% CO_2. One colony was suspended in 300µl of 1XPBS, and 200µl of suspension was added to the sample cartridge. The samples were incubated for 10 minutes with 500µl of NucliSens easyMAG lyses buffer to lyse the bacteria and inactivate any nucleases. Thereafter, 50µl of the NucliSens easyMAG magnetic silica was added. The magnetic silica is then washed several times, a step that involved three buffers, buffer 1 (guanidine Thiocyanate), buffer 2 (organic buffer and biocide solution), and buffer 3 (inorganic buffer and biocide solution). Washed nucleic acids were eluted from the magnetic silica and concentrated in an elution buffer under an elevated temperature and stored at -70^{o}C until required.

3.7.2 Multilocus sequence type and characterization of gbs2018 locus

Polymerase chain reaction was used to amplify fragments of GBS housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) and gbs2018 allelic variants with primers described in Table 3.7. The reaction mixture consisted of 0.40µM of each primer, 10X Econo*Taq* Master mix (Inqaba Biotec, LU 630035-1), 1µl of DNA template and the final volume was adjusted to 25µl with free nuclease water. The reactions conditions for DNA amplification was carried out under the following conditions, DNA denaturation at

94^{o}C for 1 minute, followed by 35 cycles of denaturation at 94^{o}C for 30 seconds, annealing at 55^{o}C for 30 seconds, extension at 72^{o}C for 90 seconds and final extension at 72^{o}C for 10 minutes. DNA was analyzed by 1% of agarose gel electrophoresis to determine the quality and quantity of the PCR product for sequencing as described in 3.4.3.

DNA sequencing of the PCR products was performed by Macrogen Inc (Seoul, Korea), with standard Sanger sequencing protocols using the ABI prism 3730x1 DNA analyzer (Applied Biosystems). Sequence run files were received as WINRAR ZIP archives files, and were read and analyzed with the Bioedit Sequence Alignment Editor to determine the integrity of the sequence data. Samples with no signal were repeated, and sequencing of the antisense strand was performed if there was an abrupt signal loss, or to resolve any discrepancies in the read.

Table 3.7: Oligonucleotide sequences of the primers for group B streptococcus multilocus sequence typing

Locus	Sequence (5' to 3')	Amplicon size (bp)
adhP **amplification and Sequencing**	Forward 5'-GTTGGTCATGGTGAAGCACT-3' Reverse 5'-ACTGTACCTCCAGCACGAAC-3' Forward 5'-GGTGTGTGCCATACTGATTT-3' Reverse 5'-ACAGCAGTCACAACCACTCC-3'	672 498
pheS **amplification and Sequencing**	Forward 5'-GATTAAGGAGTAGTGGCACG-3' Reverse 5'-TTGAGATCGCCCATTGAAAT-3' Forward 5'-ATATCAACTCAAGAAAAGCT-3' Reverse 5'-TGATGGAATTGATGGCTATG-3'	723 501
atr **amplification and Sequencing**	Forward 5'-CGATTCTCTCAGCTTTGTTA-3' Reverse 3'-AAGAAATCTCTTGTGCGGAT-3' Forward 5'-ATGGTTGAGCCAATTATTTC-3' Reverse 5'-CCTTGCTCAACAATAATGCC-3'	627 501
glnA **amplification and Sequencing**	Forward 5'-CCGGCTACAGATGAACAATT-3' Reverse 5'-CTGATAATTGCCATTCCACG-3' Forward 5'-AATAAAGCAATGTTTGATGG-3' Reverse 5'-GCATTGTTCCCTTCATTATC-3'	589 498
sdhA **amplification and Sequencing**	Forward 5'-AGAGCAAGCTAATAGCCAAC-3' Reverse 5'-ATATCAGCAGCAACAAGTGC-3' Forward 5'-AACATAGCAGAGCTCATGAT-3' Reverse 5'-GGGACTTCAACTAAACCTGC-3'	646 519
glcK **amplification and Sequencing**	Forward 5'-CTCGGAGGAACGACCATTAA -3' Reverse 5'-CTTGTAACAGTATCACCGTT-3' Forward 5'-GGTATCTTGACGCTTGAGGG-3' Reverse 5'-ATCGCTGCTTTAATGGCAGA-3'	607 459
tkt **amplification and Sequencing**	Forward 5'-CCAGGCTTTGATTTAGTTGA-3' Reverse 5'-AATAGCTTGTTGGCTTGAAA-3' Forward 5'-ACACTTCATGGTGATGGTTG-3' Reverse 5'-TGACCTAGGTCATGAGCTTT-3'	859 480
gbs2018 amplification and sequencing	Forward 5'-GAAAAACAGCTTATGGTTTGG-3' Reverse 5'- TTGCTAAGAGTGGACTTGCG -3'	1569-1893

3.7.3 Assignment of sequence types and clonal complexes grouping

Group B streptococcus MLST database was used to identify allelic profiles and sequence types of complete sequences. An allele number was assigned to each fragment based on its sequence. Sequence type assignment was based on the allelic profile of the seven housekeeping genes (Jones *et al.* 2003). Isolates that had a new MLST profile were submitted for sequence assignment under identity number 20120328_002 and 20121204_004. The "based upon sequence type (eBurst)" online program was used to group closely related sequence type within a particular clonal complex (CC). Clonal complexes were defined as isolates that shared at least five identical alleles. To determine whether genes encoding specific allelic form of gbs2018 were associated with certain genetic marker, randomly selected invasive disease and colonizing isolates representing different sequence types as determined by MLST were analyzed. The gbs2018 locus was characterized by DNA sequencing, and emphasis was on ST-17 lineage because of the suggestion that identification of gbs2018 cluster C can be used as a rapid and cost effective for detecting ST-17 lineage GBS isolates.

3.8 Statistical analysis

3.8.1 Serotype distribution

Data were analyzed with Graphpad Prism version 4.01 and STATA version 8.0. Differences in serotype distribution between maternal and neonatal colonizing isolates, and isolates causing invasive disease were determined by a two tailed Fisher's exact test. Logistic regression was used to determine the association of frequency of serotypes in relation to the timing of onset of infant sepsis and disease syndrome. As maternal colonization is a major risk factor for early onset (<7days of age) GBS disease, analyses

of the invasive potential of GBS isolates was restricted to episodes within 7 days of birth. Invasive potential of individual serotypes was estimated by calculating the odds ratio (OR) using serotype III as a fixed reference serotype, since it has been consistently shown to be the most prevalent serotype in both colonizing and invasive disease isolates. This method has an advantage over calculating OR by reference to all other serotypes because the resulting estimate is a robust measure of invasive potential.

The relative serotype-specific invasive potential was calculated based on the formula of odds ratio (OR) = (ad)/ (bc) described by Brueggemann *et al* (Brueggemann *et al.* 2004). Where "a" is the number of early onset isolates of a specific serotype; "b" is the number of early onset isolates of serotype III; "c" is the number of that specific serotype from maternal colonizing isolates; and "d" is the number of serotype III from maternal colonizing isolates. Associated 95% confidence intervals (95% CI) were estimated.

3.8.2 Pilus islands distribution

Distribution of pilus islands in maternal colonizing and invasive disease isolates was determined by a two tailed Fisher's exact test. A multiple regression model was used to test for the association between pilus island and serotype and the time of onset of illness. X^2 test was used to compare pilus island distribution in infants aged <7 days, between 7 and 90 days and older than 90 days. A P value of < 0.05 was considered significant.

3.8.3 Multilocus sequence typing

Cluster analysis was carried out with phylodendron version 0.8d (http://pubmlst.org/analysis/) using the unweighted pair group method with arithmetic mean (UPGMA) to construct the dendrogram from the matrix of pairwise allelic differences between the sequence types. A Phylogenetic tree of different chimeric forms of gbs2018 was constructed using the molecular evolutionary genetics analysis, version 5.1 (MEGA, 5.1). Clonal relationship between invasive disease and colonizing isolates was determined by X^2 test. A P value of < 0.05 was considered significant.

Chapter 4 Results

4.1 Serotype distribution

4.1.1 Serotype distribution of maternal and newborn colonizing isolates

This study included 551 (21.5%) GBS isolates from 2561 women and 402 (15.8%) GBS isolates from 2542 newborns born to these women who were swabbed. Group B streptococcus was identified in 289 (52.5%) newborns of the 551 mothers who were identified to be vaginally colonized by GBS during labor. In addition, GBS was also identified in a further 113 (5.6%) newborns born to 2,010 mothers in whom GBS was not detected on vaginal swabbing.

Serotyping was done on 541 (98.2%) of 551 available isolates obtained from colonized mothers and on 396 (98.5%) of 402 available isolates from colonized newborns. A total of 106 (19.6%) maternal and 76 (19.2%) newborn colonizing GBS isolates were serologically non-typeable; Table 4.1. Molecular capsular typing was successful in identifying a capsular serotype gene in 173 (95.1%) of the 182 serologically non-typeable colonizing isolates. The frequency of serotype distribution between serologically typeable isolates and serologically non-typeable differed as follows: Ia (32.4% vs. 14.8%, respectively; $P<0.0001$), Ib (2.4% vs. 23.1%, respectively; $P<0.0001$), II (13.3% vs. 4.4%, respectively; $P=0.0004$), III (40.3% vs. 22.5%, respectively; $P<0.0001$); IV (1.3% vs. 15.4%, respectively; $P<0.0001$) and V (10.3% vs. 14.8%, respectively; $P=0.09$).

Overall, serotype III was the most common colonizing serotype in mothers (37.3%) and newborns (36.2%); Table 4.1. Collectively, serotypes Ia, Ib and III accounted for 74.1% (401/541) of maternal and 69.6% (275/395) of newborn colonizing isolates. There were no differences in the relative frequencies of serotypes between colonized mothers and colonized newborns (P=0.77). Isolates were available for serotyping from 280 (96.9%) of 289 mother-newborn pairs who were both colonized with GBS, among whom there was 90.7% (254/280) concordance in serotype between the mother and newborn isolates. There were no differences in the serotype distribution between isolates from HIV-infected women (n=119) compared to HIV-uninfected women (n=418; P=0.55).

Table 4.1: Serotype distribution among maternal and newborn colonizing group B streptococcus isolates

Serotype	**Maternal colonizing isolates**			**Neonatal colonizing isolates**			**P value Comparing total of proportion of maternal vs. newborn serotypes**
	Latex no=541	PCR[3] no=106	Total no=541	Latex no=395	PCR no=76	Total no=395	
Ia	146 (27.0)[1]	17 (16.0)	163 (30.1)	98 (24.8)	10 (13.2)	108 (27.3)	P= 0.38
Ib	12 (2.2)	24 (22.6)	36 (6.7)	6 (1.5)	18 (23.7)	24 (6.1)	P= 0.79
II	55 (10.2)	6 (5.7)	61 (11.3)	45 (11.4)	2 (2.6)	47 (11.9)	P= 0.84
III	177 (32.7)	25 (23.6)	202 (37.3)	127 (32.2)	16 (21.1)	143 (36.2)	P= 0.73
IV	5 (0.9)	15 (14.2)	20 (3.7)	5 (1.3)	13 (17.1)	18 (4.6)	P= 0.51
V	40 (7.4)	15 (14.2)	55 (10.2)	38 (9.6)	12 (15.8)	50 (12.7)	P= 0.61
NT	106 (19.6)	4 (3.8)		76 (19.2)	5 (6.6)		P= 0.22

[1]Figure in rounded parenthesis is a percentage, molecular serotype identification by PCR was done by primers targeting serotypes 1a to V.

4.1.2 Serotype distribution of invasive disease isolates

A total of 284 GBS isolates were obtained from 282 infants with invasive disease. These included 222 from blood (78.2%), 62 from CSF (21.8%) and two cases in which GBS was obtained from blood and CSF. The age distribution of infants with invasive disease isolates included 137 (48.2%) under 7 days of age (EOD), 108 (38.0%) between 7 to 90 days of age (LOD) and 39 (13.7%) from children older than 90 days of age.

The dominant serotypes causing invasive disease in EOD and LOD were serotype III (57.7% vs. 84.3%, respectively; P<0.0001) and serotype Ia (22.6% vs. 13.9%, respectively; P=0.01); Table 4.2: Collectively, these serotypes accounted for 80.3% of EOD and 98.2% of LOD. Serotypes III and Ia together accounted for 53.9% of invasive disease isolates occurring in children older than 90 days of age. Also, of the three episodes were both colonizing and invasive disease isolates from newborns were available for serotyping, the invasive disease was due to the same serotype that colonized the newborns during birth.

Individually, serotypes Ib, II, IV, and V accounted for less than 6.0% of invasive disease isolates in infants less than 90 days of age. No invasive disease isolate was reactive to sera against serotypes VI, VII, VIII and IX. After adjusting for the type of specimen from where the isolate was recovered (i.e. blood only vs. CSF) and age group, serotype III remained the dominant cause of invasive disease in LOD (Adjusted odds ratio: 3.60; 95%CI 1.91-6.78; P<0.0001), but was not more frequently associated with meningitis compared to other serotypes (P=0.36). Serologically non-typeable isolates were less common from invasive GBS isolates (20/284; 7.0%). Genotypic serotyping by

PCR was successful in attributing a serotype to all serologically non-typeable invasive disease isolates.

Table 4.2: Serotype distribution among early onset, late onset diseases and in children older than 90 days.

Serotype	Early Onset Disease (<7 days age) no=137	Late Onset Disease (7-90 days age) no=108	>90 days age no=39	P value EOD vs. LOD
Ia	31 [1][1] (22.6) [2]	15 [1] (13.9)	9 [0] (23.1)	P= 0.99
Ib	7 [2] (5.1)	0 [0] (0)	6 [2] (15.4)	P= 0.009
II	7 [2] (5.1)	0 [0] (0)	6 [2] (15.4)	P= 0.009
III	79 [3] (57.7)	91 [3] (84.3)	12 [1] (30.8)	P< 0.0001
IV	5 [1] (3.6)	0 [0] (0)	0 [0] (0)	P= 0.07
V	8 [0] (5.8)	2 [1] (1.9)	6 [2] (15.4)	P= 0.19

[1] Figure in squared parenthesis indicates number of isolates which were serologically non-reactive but typed by PCR and included in the total number. [2]Figure in rounded parenthesis is a percentage.

4.1.3 Estimates of the relative invasive potential of serotypes

There were significant differences in the serotype distribution between invasive disease isolates from EOD and maternal colonizing isolates. A higher proportion of invasive isolates in EOD were serotype III (79/137; 57.7%) compared to maternal colonizing isolates (202/537; 37.6%; P<0.0001). Conversely comparing invasive disease isolates in EOD to maternal colonizing isolates, serotype II [7/137 (5.1%) vs. 61/537 (11.3%); respectively, P=0.0008] and serotype V [8/137 (5.8%) vs. 55/537 (10.2%), respectively; P=0.014] were less common as invasive disease isolates; Table 4.3.

Table 4.3: Estimation of invasive potential of group B streptococcus serotypes

Serotype	Maternal colonizing isolates no= 537	Early Onset Disease (<7 days age) no=137	Total no. of isolates (%)	OR (95% CI)
Ia	163 (30.3)	31 (22.6)	194 (28.8)	(0.49; 0.31-0.77)
Ib	36 (6.7)	7 (5.1)	43 (6.4)	(0.50; 0.22- 1.18)
II	61 (11.4)	7 (5.1)	68 (10.1)	(0.30 0.13- 0.67)
III	202 (37.6)	79 (57.7)	281 (41.6)	(1.00)
IV	20 (3.7)	5 (3.6)	25 (3.7)	(0.65; 0.23- 1.79)
V	55 (10.2)	8 (5.8)	63 (9.3)	(0.38; 0.17- 0.83)

In our data two isolates of the same serotype (i.e. III) were obtained from the same infant, and only one isolate was included in the analysis.

4.2 Pilus island distribution

The real time PCR assay was able to detect the AP1 gene for PI-2a and PI-2b in 48.5% and 35.2% of isolates respectively. Additional screening with PCR assays for the AP2 gene of PI-2a and PI-2b negative isolates revealed that all isolates harbored either PI-2a or PI-2b in 54.6% and 45.3% of isolates respectively. PI-1 was found in 69.9% of all isolates, and was associated with PI-2a in 45.5% of the time. In contrast, the majority (99.5%) of PI-2b positive isolates were associated with PI-1.

4.2.1 Pilus islands distribution in maternal colonizing isolates

Pilus islands in maternal colonizing isolates were identified alone or in combinations at the following overall hierarchical frequency PI-1+PI-2b (n=201; 37.2%), PI-2a (n=183; 33.8%), PI-1+PI-2a (n=155; 28.7%), and PI-2b (n=2; 0.4%**),** Figure 4.1. More than one type of pilus island was identified in 356 (65.9%) of maternal colonizing isolates.

4.2.2 Pilus islands distribution in invasive isolates

Similarly, pilus islands in invasive disease isolates were identified alone or in combinations at the following overall frequency PI-1+PI-2b (n=171; 60.2%), PI-2a (n=63; 22.2%), PI-1+PI-2a (n=50; 17.6%). More than one type of pilus island was identified in 221 (77.8%) of 284 invasive disease isolates; Figure 4.1.

4.2.3 Comparison between colonizing and invasive disease isolates

There were significant differences when the pilus island distributions were stratified by colonization or invasive disease. A combination of PI-1 and PI-2b was more common among invasive disease isolates (171/284; 60.2%) than colonizing isolates (201/541; 37.2%; P<0.0001), and isolates with both PI-1 and PI-2a were more common in colonizing (155/541; 28.7%) than invasive disease isolates (50/284; 17.6%; P=0.0005); Figure 4.1.

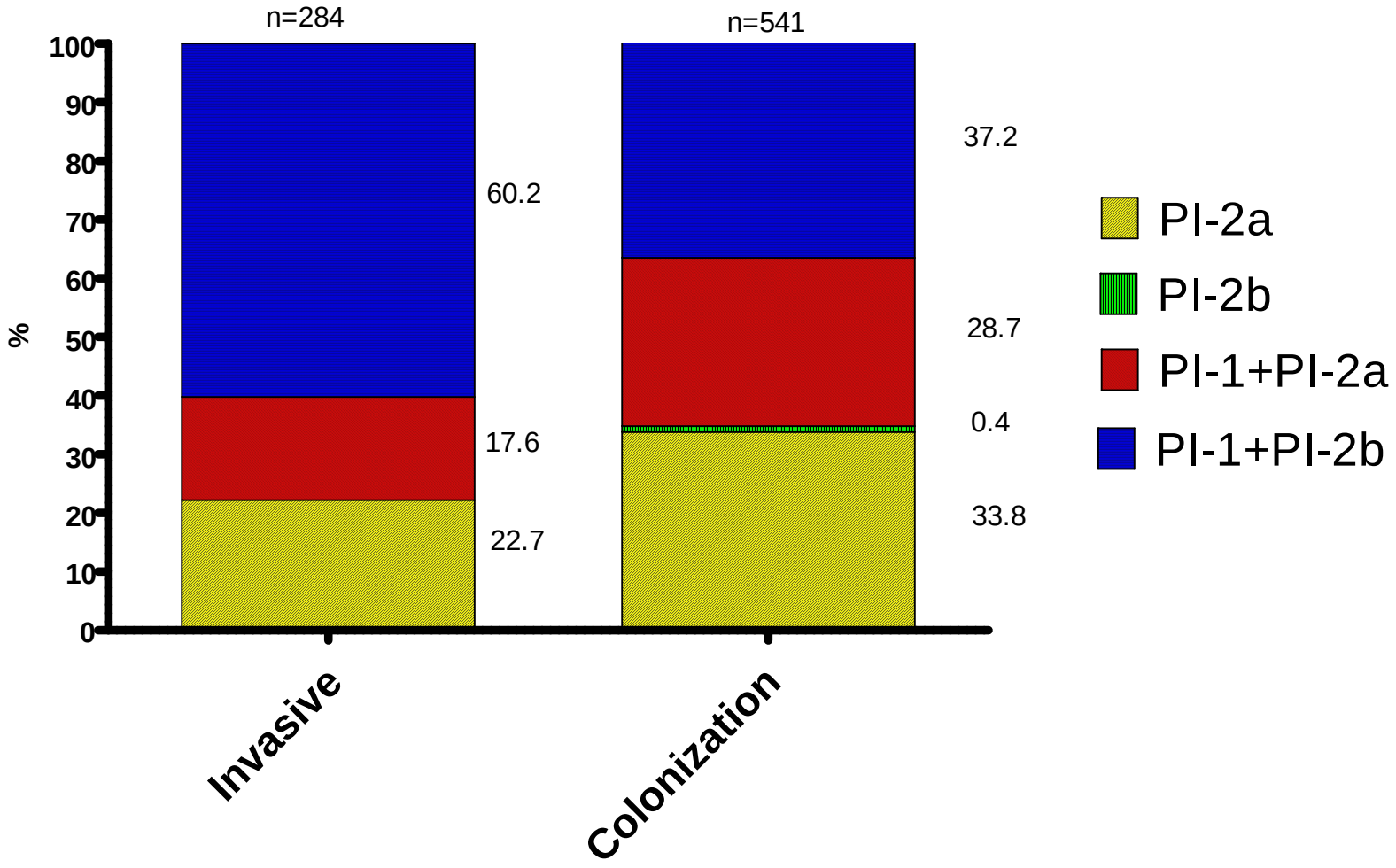


Figure 4.1: Group B streptococcal pilus islands distribution among invasive disease isolates and maternal colonizing isolates

4.2.4 Pilus island distribution according to age of onset

When the pilus island distribution in invasive disease isolates was stratified according to age of disease onset, there were significant differences between EOD and LOD. A combination of PI-1 and PI-2b was more frequently associated with LOD (54.7% v/s 80.6%; P<0.0001), whilst PI-2a alone or in combination with PI-1 were more likely to associated with EOD (31.4% v/s 17.6%; P=0.02, respectively) and (13.9 v/s 1.9%; P=0.0008, respectively), **Error! Reference source not found.** The distribution of pilus islands alone or in combination from infants older than 90 days were PI-2a (33.3%), PI-1+PI-2a (38.5%) and PI-1+PI-2b (28.2%).

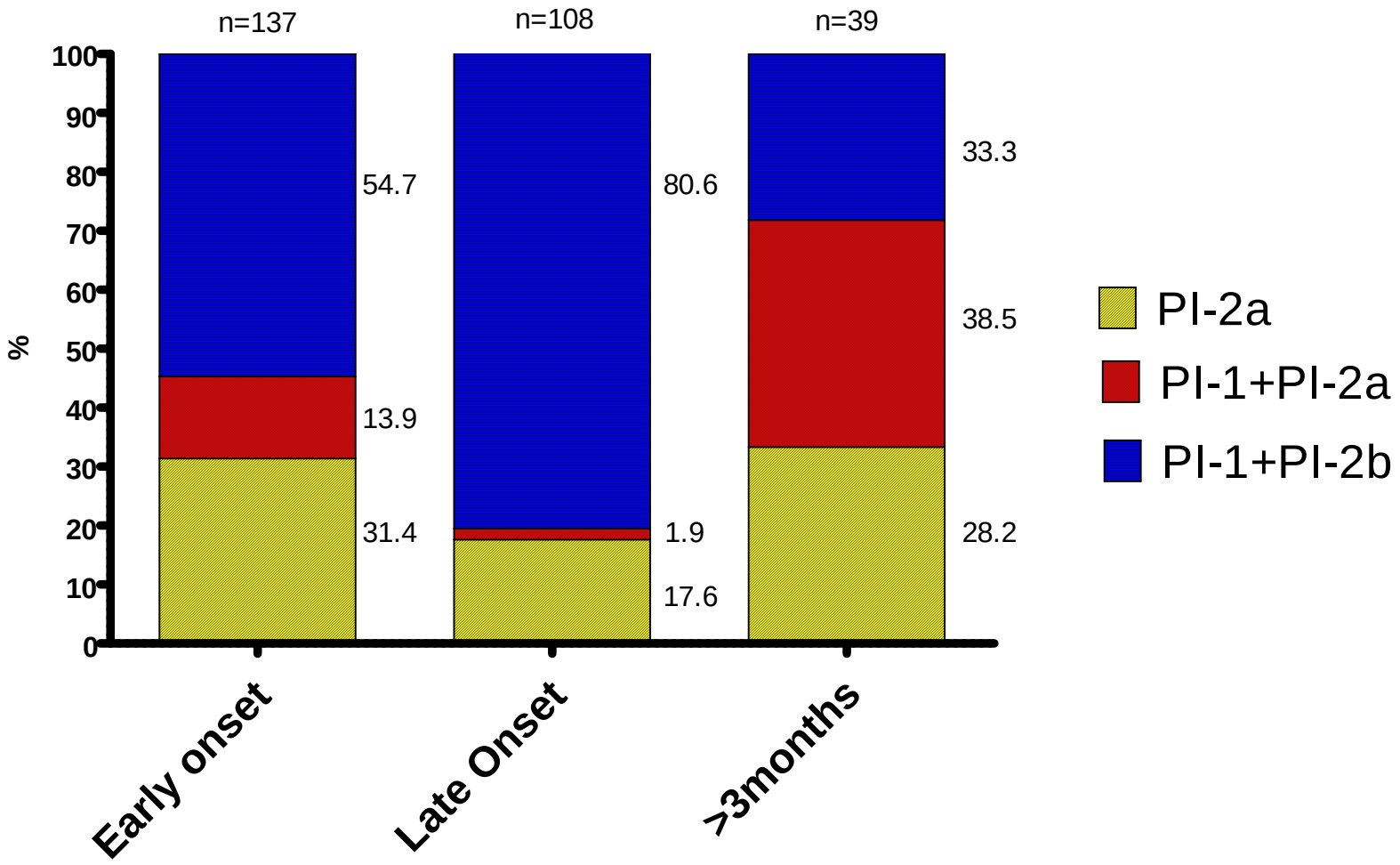


Figure 4.2: Group B streptococcal pilus islands distribution among invasive isolates from early onset, late onset diseases and in children older than 90 days

4.2.5 Pilus island and capsular polysaccharide serotype

There was a significant association between the capsular serotype and pilus island combinations irrespective of whether isolates were from colonization or invasive disease. The majority of colonizing (84.2%, P<0.0001) and invasive disease isolates (90.7% P<0.0001) attributed to serotype III carried a combination of PI-1 and PI-2b. Pilus island-2a alone was identified in 92.6% and 90.9% of colonizing and invasive disease serotype Ia isolates, respectively. Serotype Ib, II and V were associated with a combination of PI-1 and PI-2a, altogether accounting for 71.6% of colonizing and 56.0% of invasive disease isolates. Three of four non-typeable colonizing isolates were associated with a combination of PI-1 and PI-2a; Figure 4.3:

When the association between the capsular serotype and pilus island was analyzed with respect to the onset of disease, serotype Ia was associated with 82.9% and 87.5% of EOD and LOD respectively in isolates carrying PI-2a. In contrast serotype III was associated with 88.0% in EOD and 98.9% in LOD isolates carrying a combination of PI-1 and PI-2b. When pilus island distribution was adjusted with respect to serotype and the disease onset, serotype Ia was the dominant predictor in the distribution of PI-2a alone in EOD (Adjusted odds ratio: 0.38; 95%CI 0.3-0.5; P<0.0001), whilst serotype III was the dominant predictor in the distribution of PI-1 and PI-2b combination in LOD (Adjusted odds ratio: 2.55; 95%CI 2.00-3.26; P< 0.0001).

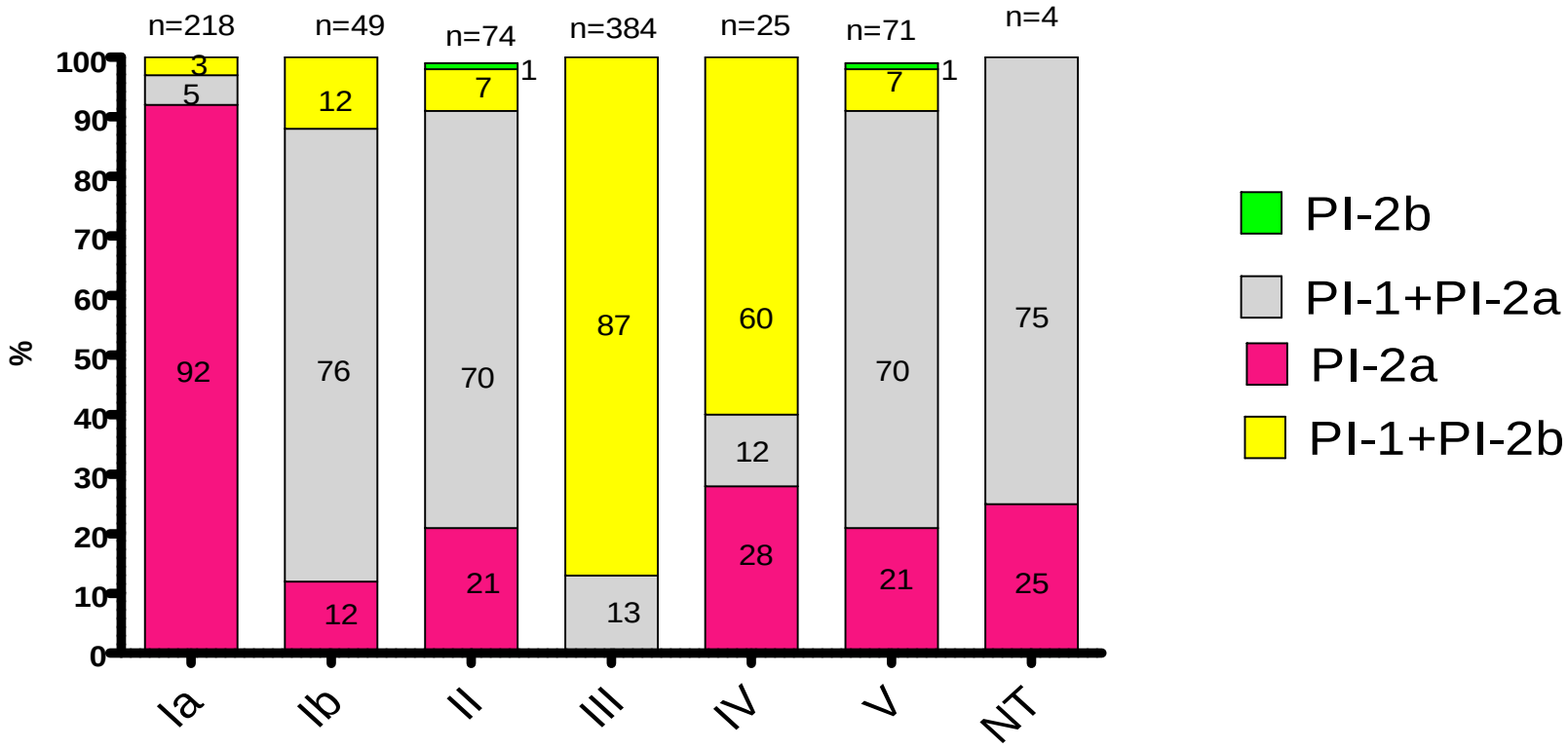


Figure 4.3: Group B streptococcal pilus islands distribution among invasive disease and maternal colonizing isolates grouped by capsular polysaccharide serotype

4.2 Genetic diversity of colonizing and invasive disease isolates

A total of 1175 GBS isolates were pre-screened by partial MLST for *atr, adhP and glnA* genes, including 523 maternal colonizing-isolates, 369 newborn colonizing isolates and 283 infant invasive disease isolates.

4.2.1 Genetic diversity among maternal and neonatal colonizing isolates

Twenty six different allele combinations were identified in maternal and newborn colonizing isolates, and the combinations were stratified into six clusters; Table 4.4. Paired maternal-newborn dyads isolates were available for genetic characterization from 257 (92.1%) of 289 colonized newborns which revealed 91.1% (234/257) concordance. There were no differences in the relative frequencies and clusters distribution among isolates colonizing mothers and newborns. Over two-thirds of maternal and newborn colonizing isolates involved three clusters, including clusters A (32.0% vs. 33.9%), B (30.1% vs. 29.3%) and D (14.1% vs. 11.1%); Table 4.4. A strong association was observed between serotype and specific clonal clusters among maternal and newborn colonizing isolates. This included serotype Ia with cluster B (97.5% and 98.1%) in maternal and neonatal colonizing isolates respectively, serotype III with cluster A (82.7% v/s 86.0%) of maternal and neonatal colonizing isolates respectively. Serotype V was associated with cluster E in 87.0% of maternal and 87.8% of newborn colonizing isolates. Serotype Ib and IV were respectively associated with cluster D in 86.1% and 90.0% of maternal and in 100% and 87.5% of newborn colonizing isolates, while serotype II was associated with cluster D or E in 96.0% of maternal and 90.7% of newborn

colonizing isolates.

4.2.2 Genetic diversity among invasive disease isolates

Fifteen different allelic combinations were identified among invasive disease isolates associated with EOD (n=136), LOD (n=108) and from episodes in those >90 days of age (n=39). There were significant differences when the cluster distributions were stratified by maternal colonization or invasive disease, the frequency of cluster distribution between invasive disease compared to maternal colonizing isolates were as follows A (59.4% v/s 32.3%, respectively; P<0.0001), B (21.6% v/s 30.0%, respectively; P=0.010), C (1.1% v/s 1.5%, respectively; P=0.76), D (3.2% v/s 14.3%, respectively; P<0.0001), E (7.1% v/s 10.5%, respectively; P=0.13) and F (7.8% v/s 11.3%; respectively; P=0.14). When adjusted for differences in serotypes there were no significant differences between invasive disease and maternal colonizing isolates belonging to serotype Ia, for example 94.5% of invasive disease and 96.3% of maternal colonizing isolates belonging to serotype Ia were associated with cluster B (P=0.70). Whilst 90.1% of invasive-disease and 82.7% of maternal colonizing isolates belonging to serotype III were associated with cluster A (P=0.04), and 87.0% of invasive disease and 81.3% of maternal colonizing isolates belonging serotype V were associated with cluster E (P=0.27).

There were significant differences between cluster distribution in relation to the timing of invasive disease onset, with cluster A being associated with 80.6% of LOD compared to 52.2% in EOD (P<0.0001). There was, however, no significant differences between EOD and LOD when adjusted for serotype Ia distribution, 90.3% of serotype Ia in EOD was associated

with cluster B compared with 93.3% in LOD (P=1.00). In contrast, 86.1% of serotype III of EOD was associated with cluster A compared with 96.6% of serotype III in LOD (P=0.03). Individually cluster A and B accounted for 25.6% of invasive disease isolates from infants older than 90 days, while the remaining isolates were stratified into cluster E and F (17.9% each), cluster C (10.3%) and cluster D (2.6%).

Table 4.4: Group B streptococcus housekeeping genes (*adhP*, *atr* & *glnA*) associated with maternal (M), neonatal (N) colonization and invasive disease (Inv) isolates from infants grouped by capsular polysaccharide serotype

				Serotype																			
Genes				Ia			Ib			II			III			IV			V			NT	
adhP	Atr	glnA	Group (cluster)	M	N	Inv	M	N	Inv	M	N	Inv	M	N	Inv	M	N	Inv	M	N	Inv	M	N
2	1	10	**A (A)**										40	26	57			1					
2	1	2	**B (A)**						1				126	97	107	1		2					
4	4	1	**C (D)**	1			27	16	3	5	1	1				1							
5	6	3	**D (B)**	139	96	49			1		1	1			2					2	2		
9	4	1	**E (D)**		1		1	5	1	20	11	3			1	17	7						
1	3	3	**F (F)**							1		1											
1	4	2	**G (C)**						1										5	2	1		
1	3	5	**H (F)**		1	1	2			22	25	6	1	1								1	
1	2	1	**I (E)**			1			1	1	2		8	4	2		1	1	39	35	12		
1	3	1	**J (F)**				2		2	1	2	1								1		3	5
5	4	3	**K (B)**	4	2	3									1								
5	1	3	**L (B)**	14	8				2														
1	6	1	**M (E)**	5		1			1														
2	4	2	**N (C)**				1																
4	6	1	**O (D)**				1																
4	3	1	**P (D)**				2																
1	3	4	**Q (F)**										1										
1	3	2	**R (F)**							1			22	15	11				1				
13	1	3	**S (A)**							1													
2	6	2	**T (C)**										1										
1	5	4	**U (F)**										1										
5	6	2	**V (C)**										1		1								
2	1	3	**W (A)**										1										
36	2	1	**X (E)**													1							
86	2	1	**Y (E)**								1								1		1		
62	2	1	**Z (E)**																	1			
				163	**108**	**55**	**36**	**21**	**13**	**52**	**43**	**13**	**202**	**143**	**182**	**20**	**8**	**4**	**46**	**41**	**16**	**4**	**5**

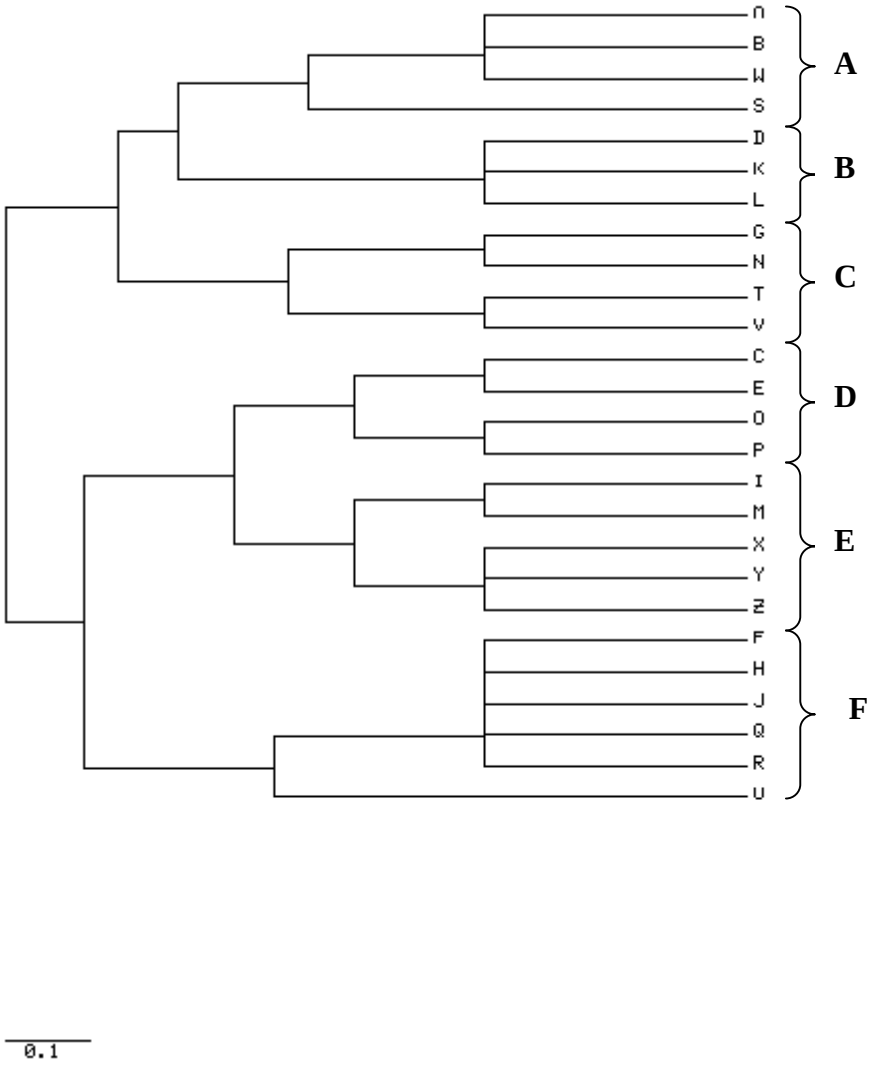


Figure 4.4: Phylogenetic relationship of group B streptococcus and clusters, as shown in Table 4.4

4.2.3 Multilocus sequence type analysis

4.2.3.1 Sequence types and clonal complexes among colonizing isolates

Of the 121 colonizing isolates with complete MLST profile, thirty different sequence types were identified including six novel sequence types; Table 4.5. The most frequently occurring colonizing isolates sequence types were ST-1 (14.9%) and ST-10 (9.9%), while 8.3% of isolates stratified into ST-17, ST-23, ST-109 and ST-144 each; Table 4.5. The eBURST analyses grouped related sequence types into six clonal complexes, in the following order of frequency CC-10 (23.1%), CC-1 (19.8%), CC-17 (19.8%), CC-23 (19.0), CC-19 (15.7%) and CC-22 (2.3%). Notably, three serotype II isolates were identified in the rarely described CC-22, which consisted of ST-22 and ST-37. Colonizing isolates from seven mother-newborn pairs who had discordant phenotypic serotypes were also identified to be genetically heterogeneous and grouped in different clonal complexes.

4.2.3.2 Sequence type and clonal complex among invasive disease isolates

Multilocus sequence typing classified 131 invasive disease isolates into 22 sequence types, including three novel sequence types; Table 4.5. The most common sequence types included ST-23 (22.1%), ST-17 (20.6%) and ST-109 (16.8%). ST-17 and ST-23 were significantly associated with invasive disease isolates compared to maternal colonizing isolates ($P<0.0001$), in contrast ST-1 and ST-10 were more likely to be associated with maternal colonization compared to invasive disease isolates ($P=0.06$). The eBURST analyses

revealed six major clonal complexes associated with invasive disease isolates; Figure 4.5. There were significant differences in the frequency of clonal complexes between invasive disease and maternal colonizing isolates as CC-1 (15.3% v/s 15.6%, respectively; P=0.41), CC-10 (7.6% v/s 25.3%, respectively; P<0.0007), CC-17 (42.7% v/s 22.9%, respectively; P<0.0001), CC-19 (6.9% v/s 15.7%, respectively; P=0.03), CC-23 (27.5% v/s 19.3%, respectively; P=0.14). There were no significant differences when the clonal complex distribution was stratified with respect to age of disease, CC-17 was associated with 42.4% of EOD compared to 57.1% of LOD (P=0.17), whilst CC-23 was associated with 22.0% of EOD and 34.3% of LOD (P=0.19).

Table 4.5: Multilocus sequence typing characterization of group B streptococcus from colonizing and invasive disease grouped into clonal complex

CC and ST	Allelic profile	No of isolates	Invasive disease	Colonization
CC-1 (n=44)				
1	1 1 2 1 1 2 2^{+}	34	16	18
2	1 1 3 1 1 2 2	3	3	
139	1 1 4 1 1 2 2	1		1
153	36 1 2 1 1 2 2	1		1
167	1 1 6 1 1 2 2	2	1	1
462	86 1 2 1 1 2 2	2		2
619*	62 1 2 1 1 2 2	1		1
CC-10 (n=38)				
8	4 1 4 1 3 3 2	13	4	9
10	9 1 4 1 3 3 2	16	4	12
327	1 1 4 2 20 3 2	6	2	4
332	4 1 3 1 3 3 2	1		1
579*	9 1 4 1 2 3 2	1		1
583*	4 14 1 1 3 1	1		1
CC-17 (n=80)				
17	2 1 1 2 1 1 1	37	27	10
109	2 1 1 10 1 1 1	32	22	10
126	2 1 1 2 1 1 10	1	1	
180	2 4 1 2 1 1 1	1	1	
174	2 1 1 3 1 1 1	1		1
291	2 25 1 2 1 1 1	3	2	1
576*	2 1 6 10 1 1 1	1	1	
577*	2 4 1 10 1 1 1	1	1	
578*	2 1 6 2 1 1 1	1		1

CC and ST	Allelic profile	No of isolates	Invasive disease	Colonization
582*	5 1 6 2 1 1 1	2	1	1
CC-19 (n=28)				
19	1 1 3 2 2 2 2	10	3	7
27	1 1 3 4 2 2 2	1		1
28	1 1 3 5 2 2 2	13	5	8
175	1 1 3 3 2 2 2	2	1	1
182	1 1 3 2 18 2 2	1		1
580*	9 1 3 5 39 2 2	1		1
CC-22 (n=3)				
22	13 3 1 3 1 1 1	2		2
37	13 3 9 3 1 1 1	1		1
CC-23 (n=59)				
23	5 4 6 3 2 1 3	39	29	10
55	5 1 6 3 2 1 3	1	1	
144	5 4 1 3 2 1 3	12	2	10
163	5 4 4 3 2 1 3	4	3	1
249	5 4 6 1 2 1 3	1		1
427	5 1 6 3 2 1 2	1	1	
581*	5 4 6 3 9 1 1	1		1

*Asterisk indicates novel sequence types + Indicates allelic profile for each MLST gene in the following order: *adhP, PheS, atr, glnA, sdhA, glnA* and *tkt*

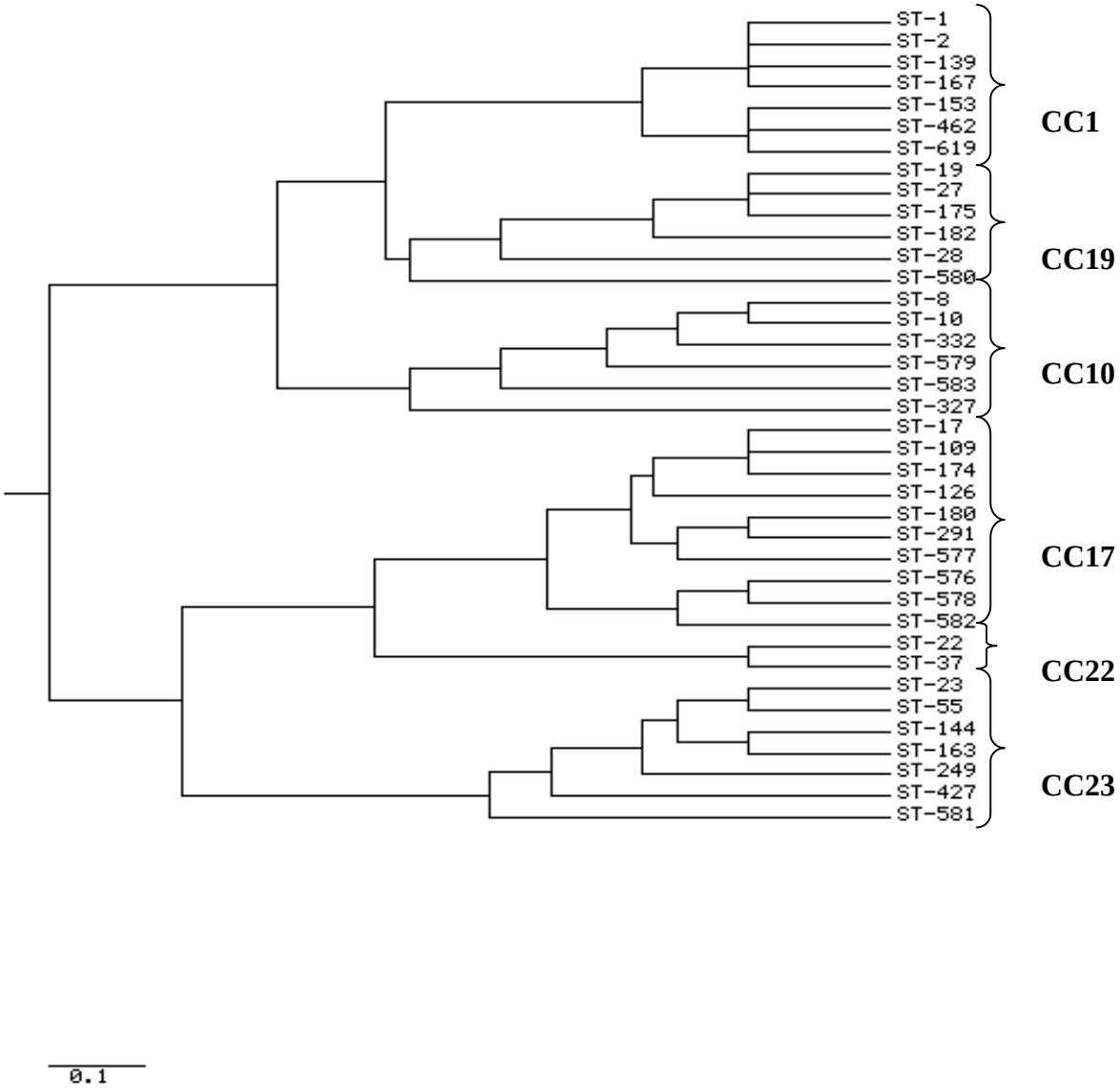


Figure 4.5: Phylogenetic relationship among sequence types from group B streptococcus isolates associated maternal and neonatal colonizing and invasive disease isolates from infants

4.3.2 Sequence types, clonal complexes and capsular serotype

There were strong associations between capsular serotype and sequence type, and were consistent between colonizing and invasive disease isolates; Table 4.6. For example, the majority of invasive disease isolates (92.9%) within CC-17 were associated with serotype III, and of these 87.5% were either ST-17 (48.2%) or ST-109 (39.3%). In maternal colonizing isolates, 95.8% of the isolates within CC-17 were associated with serotype III, of which 78.9% were either ST-17 (36.8%) or ST-109 (42.1%). Invasive disease CC-23 isolates were mainly serotype Ia (83.3%), of which 66.7% were ST-23, while maternal colonizing isolates within CC-23 were associated with 91.3% of serotype Ia, of which 75.0% were either ST-23 (31.3%) or ST-144 (43.6%). The majority of invasive disease CC-1 isolates (65.0%) were serotype V, and all of serotype V isolates belonged to ST-1, while 61.5% (P=0.37) of maternal colonizing isolates within CC-1 were serotype V, and of which 53.8% stratified into ST-1. There were no significant differences between EOD and LOD when the clonal complex distribution was analyzed by capsular serotypes. The majority of CC-17 EOD (84.0%) and LOD (100%) were associated with serotypes III (P=0.12), and in CC-23 78.6% of EOD and 91.7% of LOD were associated with serotype Ia (P=0.60).

Table 4.6: Clonal complexes grouped according to capsular serotype of group B streptococcus from colonization and invasive disease

Serotype	Clonal Complex											
	CC-1		CC-10		CC-17		CC-19		CC-22		CC-23	
	Col	ID	Col	ID	Col	ID	Col	ID	Col	ID	Col	ID
Ia	1	1	1	0	0	0	0	0	0	0	21	30
Ib	1	3	11	5	0	1	1	0	0	0	0	3
II	1	2	7	4	0	0	10	6	3	0	1	1
III	0	0	0	0	23	53	8	3	0	0	1	0
IV	2	1	5	0	1	2	0	0	0	0	0	0
V	12	13	3	1	0	0	0	0	0	0	0	2
NT	7	0	1	0	0	0	0	0	0	0	0	0
Total	24	20	28	10	24	56	19	9	3	0	23	36

[Col]- Colonizing isolates; [ID]- Invasive disease isolates; [NT]- Nontypable

4.3.3 Relationship between sequence types, clonal complexes and pilus islands

The genetic population structure of invasive disease and colonizing isolates were related to the pilus island alleles of the isolates. There were, however, no differences between invasive disease and maternal colonizing isolates when clonal complexes were analyzed in relation to the pilus island distribution; Table 4.7. The majority of invasive disease isolates (89.3%) within CC-17 carried a combination of PI-1 and PI-2b, of which 76.8% were ST-17 (37.5%) and ST-109 (39.2%). Whereas 94.7% of maternal colonizing isolates within CC-17 were associated with a combination of PI-1 and PI-2b, of which 78.9.3% were ST-17 (36.8%) and ST-109 (42.1%). The majority of invasive disease (88.9%) isolates within CC-23 were

associated with PI-2a alone, of which 80.6% were ST-23, while 81.3% in maternal colonizing-isolates were associated with PI-2a alone (P=0.66). There was no significant difference when clonal complex distribution was analyzed in relation to the timing of disease onset. The majority of EOD isolates (88.0%) and LOD (90.0%) within CC-17 carried a combination of PI-1 and PI-2b (P=1.00), whilst 92.9% of EOD and 100% of LOD CC-23 isolates carried PI-2a alone (P=1.00).

Table 4.7: Clonal complexes grouped according to pilus island of group B streptococcus from colonization and invasive disease

Serotype	Clonal Complex											
	CC-1		CC-10		CC-17		CC-19		CC-22		CC-23	
	Col	ID	Col	ID	Col	ID	Col	ID	Col	ID	Col	ID
PI-2a	4	7	2	2	0	0	0	0	1	0	13	32
PI-1+PI-2a	9	13	14	7	1	6	13	7	0	0	3	1
PI-1+PI-2b	0	0	5	1	18	50	0	2	0	0	0	3
Total	13	20	21	10	19	56	13	9	1	0	16	36

In the colonization group, pilus island data was only available from maternal colonizing isolates; [Col]- Colonizing isolates; [ID]- Invasive disease isolates.

4.3.4 Relationship between gbs2018 clusters and sequence types

Analysis for gbs2018 allelic variants included 56 GBS isolates from colonizing and invasive disease clonal complexes representing different sequence types [ST-1 (n=5), ST-2 (n=1) ST-8 (n=2), ST-10 (n=2) ST-17 (n=20), ST-23 (n=4) ST-28 (n=3), ST-109 (n=14), ST-167 (n=1), ST-291 (n=2) and ST-327 (n=1) ST-582 (n=1)].

A phylogenetic tree of the DNA sequences of gbs2018 was constructed (Figure 4.6) and showed that this allele clustered in three distinct allelic forms (A, B and C), with minor variation being found between isolates from each cluster. There was a strong association between the allelic forms of gbs2018 and specific sequence types that the isolate carried, and in all cases individual sequence types were confined within a specific allele clusters. Cluster C was the only cluster that exclusively contained isolates representing the ST-17 lineages, in contrast cluster A (ST-19, ST-23, ST-28 and ST-327) and B (ST-1, ST-2, ST-8, ST-10 and ST-167) contained multiple sequence types.

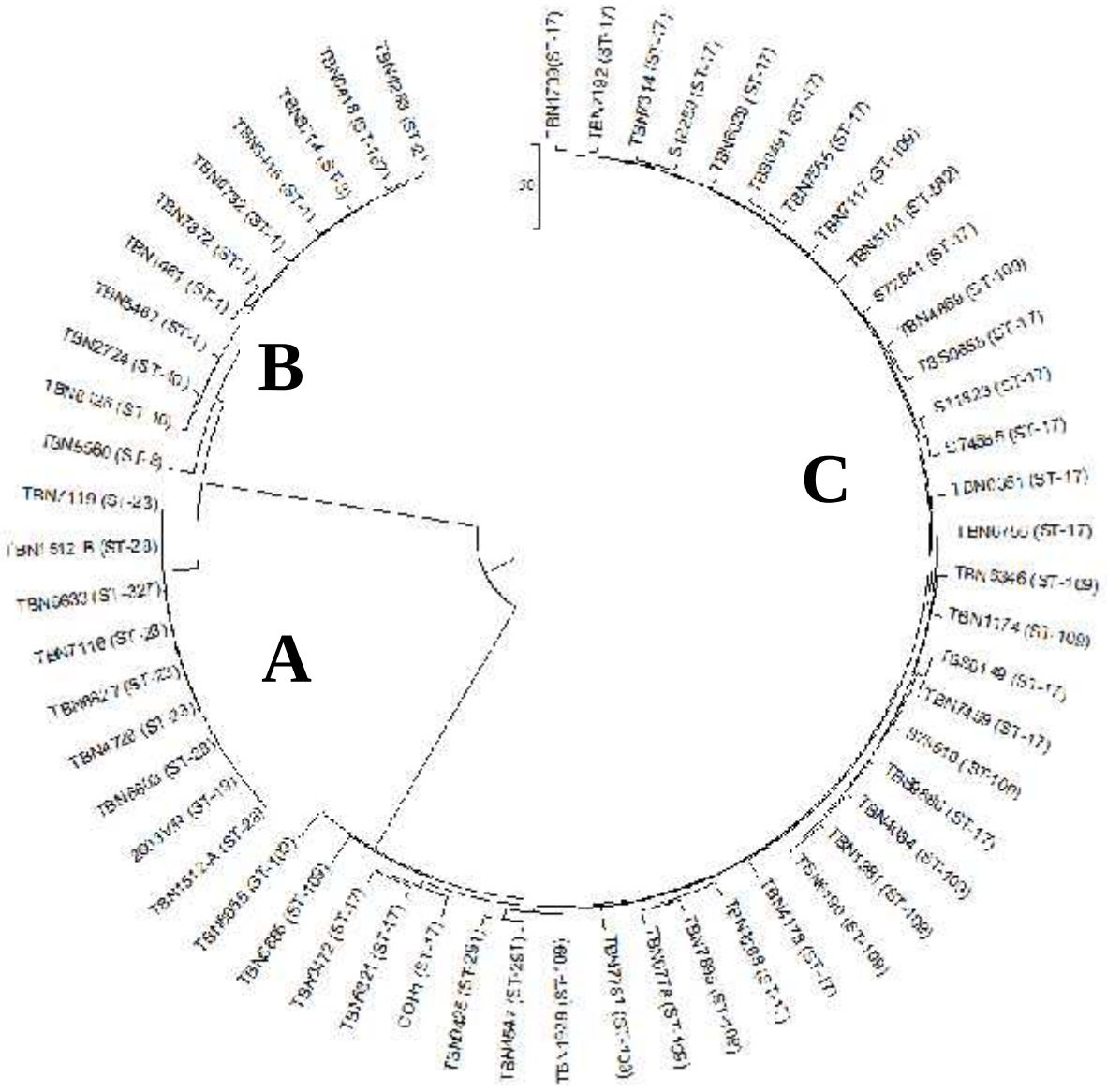


Figure 4.6: Phylogenetic analysis of genes encoding different chimeric forms of the surface proteins group B streptococcus 2018

Chapter 5 Discussion

5.1 Serotype distribution

To the best of our knowledge this study provides the most in-depth analysis of the serotype epidemiology of colonizing isolates in mother-newborn dyads and invasive GBS isolates in an industrializing country setting and particularly from Africa. Although there are extensive data on maternal colonizing serotype distribution from industrialized countries (Croak *et al.* 2003; Ippolito *et al.* 2010; Jones *et al.* 2006a; Kunze *et al.* 2011), there are limited data comparing serotype distribution from colonized mothers and their newborns from industrializing countries (Al-Sweih *et al.* 2005; Suara *et al.* 1994).

The findings from our study, are similar to those reported elsewhere, (Al-Sweih *et al.* 2005; Eren *et al.* 2005; Hakansson *et al.* 2008; Suara *et al.* 1994; Tsolia *et al.* 2003) and confirm that maternal GBS vaginal-colonization is commonly (52.5%) associated with infant colonization. The vertical transmission of GBS from mother to the newborn was corroborated by the high concordance of matching serotypes in the colonized mother-newborn dyads. Our study, however, also identified GBS in 113 (28.1%) of 402 colonized newborns born to mothers in whom GBS was not identified by vaginal swabbing.

The latter, as well as the incomplete, albeit high, concordance in serotypes distribution associated with colonization in mother-newborn dyads in our study may be due to the limitations of the study sampling methods in sensitivity of identifying multiple concurrent serotype colonization, other serotyping techniques such as iELISA may be ideal for

identification of multiple serotype colonization. The exclusion of rectal swabs to detect GBS colonization may have compromised the sensitivity in detecting GBS colonization (Quinlan *et al.* 2000). Other possibilities for discordant serotypes between mothers and newborns may include the possibility of GBS acquisition from non-maternal sources during or soon after birth (Ferrieri *et al.* 2004; Sensini *et al.* 1997; Strus *et al.* 2009).

Previous studies have reported that the sensitivity of detecting GBS colonization in pregnant women by undertaking both rectal and vaginal swabs is 18.5%-51.0% higher compared to taking vaginal swabs alone (Dillon, Jr. *et al.* 1982; Kovavisarach *et al.* 2007; Quinlan *et al.* 2000) and suggests that maternal rectal colonization may also contribute to acquisition of GBS in newborns. Additionally, the yield of GBS on vaginal swabbing undertaken after the onset of labor may have been affected by the draining liquor following the rupture of the placental membranes. Consequently, our study provides a minimal estimate as to the prevalence of GBS colonization in pregnant women during labor.

The serotype distribution identified in invasive disease isolates in our study are consistent with other smaller studies on invasive disease isolates elsewhere from Africa such as in Malawi (Gray *et al.* 2007), Zimbabwe (Moyo *et al.* 2002), and Morocco (AitMhand *et al.* 2000), as well as an earlier study at the same centre as this study from 1997-1998 (Madhi *et al.* 2003). The serotype data of invasive disease isolates from this study are nevertheless important as it confirms the absence of temporal variation in serotype distribution of invasive disease isolates over more than a decade in the study-setting (Madhi *et al.* 2003). This has positive implications for the design of serotype-specific polysaccharide vaccines for prevention of newborn GBS sepsis. Serotype III was responsible for 69.4% of invasive GBS disease among infants younger than 90 days age, which was consistent to other studies from

sub-Saharan Africa (56.0-58.3%) (Gray *et al.* 2007; Madhi *et al.* 2003). Serotype Ia was the second most frequently (18.8%) identified invasive serotype in infants younger than 90 days of age in our study and slightly less than previously reported from South Africa (28.7%) and Malawi (21%) (Gray *et al.* 2007; Madhi *et al.* 2003).

These findings are also consistent with serotype distribution data of invasive disease isolates in young infants from industrialized countries (44% to 65% for serotype III and 15% to 33% for serotype Ia) (Berg *et al.* 2000; Fluegge *et al.* 2005; Harrison *et al.* 1998; Martins *et al.* 2007). Overall, 85.4% and 98.2% of EOD and LOD invasive isolates respectively belonged to serotypes Ia, Ib or III. These serotypes are included in a tri-valent GBS conjugate vaccine currently under evaluation for safety and immunogenicity in pregnant women. Such a trivalent serotype-specific GBS vaccine would therefore provide potential cover against at least 80% of invasive disease serotypes in young infants in settings such as ours.

Our data indicated that serotype III (OR= 2.29) was the most invasive of all studied serotypes based on a method of analysis described by Brueggemann *et al*. (Brueggemann *et al.* 2003). These data are also consistent with findings from Portugal (OR=1.42) (Martins *et al.* 2007), Sweden (OR=2.61) (Berg *et al.* 2000), and Israel (OR=1.84) (Bisharat *et al.* 2005) where the same method was used. Due to this consistency we benchmarked invasive potential of other serotypes with type III as a referent serotype (Brueggemann *et al.* 2004); Table 5.1. The data consistently reports that serotype Ib, II and V, are less invasive than serotype III, however, the invasive potential of serotype Ia is variable across different sites. In South Africa, Taiwan and The Netherlands (van *et al.* 2009b), serotype Ia was less invasive than serotype III, although more invasive in Portugal (Martins *et al.* 2007), Israel (Bisharat *et al.* 2005) and Sweden (Berg *et al.* 2000). The reasons for variation in invasive potential of

serotype Ia reported at different sites are unclear, however, data from Portugal suggests that the high invasive index of this serotype can be attributed to a dominant clone (ST-23 and ST-24), suggesting that the underlying genotype can influence the invasive potential. In contrast, our study found no isolate either from colonizing or invasive disease isolates to be associated with ST-24 lineage.

Table 5.1: Estimation of invasive potential of group B streptococcus from different countries

Serotype	Country					
	South Africa	**Portugal (Martins *et al.* 2007)**	**Israel (Bisharat *et al.* 2005)**	**Sweden (Berg *et al.* 2000)**	**Netherlands (van *et al.* 2009b)**	**Taiwan (van *et al.* 2009b)**
Ia	163[a]/31[b] (0.49; 0.31-0.77)[c]	42/13 (1.52; 0.63-3.67)	12/10 (1.14; 0.41-3.18)	15/20 (1.37; 0.61-3.10)	24/6 (0.52; 0.63-3.67)	13/5 (0.22; 0.07-0.70)
Ib	36/7 (0.50; 0.22- 1.18)	14/1 (0.35; 0.04-2.93)	9/2 (0.30; 0.05-1.57)	15/2 (0.14 0.03-0.64)	ND	5/2 (0.22; 0.04-1.27)
II	61/7 (0.30 0.13- 0.67)	46/8 (0.85; 0.32-2.27)	23/14 (0.83; 0.34-2.03)	13/4 (0.32; 0.09-1.07)	ND	ND
III	202/78 (1.00)	59/12 (1.00)	26/19 1.00	36/35 (1.00)	20/33 (1.00)	19/34 (1.00)
IV	(20/5) (0.65; 0.23- 1.79)	6/1 (0.81; 0.09-7.44)	ND	3/2 (0.69; 0.11-4.36)	ND	ND
V	55/8 (0.38; 0.17- 0.83)	59/7 (0.58; 0.21-1.59)	18/9 (0.68; 0.25-1.85)	25/7 (0.29; 0.11-0.75)	14/4 (0.17; 0.05-0.60)	15/2 (0.07; 0.02-0.36)

In our data two isolates of the same serotype (i.e. III) were obtained from the same infant, and only one isolate was included in the analysis. [a] Value indicates colonizing isolates. [b] Value indicates neonatal invasive isolates.[c] Value in parenthesis indicates OR (relative to serotype III) and 95% CI. [d] ND: No data for either invasive, colonization or both.

Serotype V was identified in a low proportion of invasive disease isolates (5.6%) in our study, which is also consistent with earlier studies from South Africa (5.6%) and Malawi (4.0%) (Gray *et al.* 2007; Madhi *et al.* 2003). In the United States, serotype V is responsible for a high proportion of the invasive cases among infants (15.0%-28.0%) (Lin *et al.* 1998; Phares *et al.* 2008), similarly a higher prevalence of serotype V has also been reported in European studies including in England/Wales (13.0%) (Weisner *et al.* 2004) and Sweden (22.5%) (Persson *et al.* 2004). Recent identification of these historically uncommon circulating strains in both colonizing and invasive disease isolates raises questions as to whether introduction of a vaccine composed of only selected serotypes could cause a shift in serotype distribution in colonizing and subsequently invasive disease isolates to that of non-vaccine serotypes. This would require ongoing surveillance following the introduction of GBS conjugate vaccines.

Despite the success of intrapartum antibiotic prophylaxis in reducing GBS associated neonatal sepsis in industrialized countries, the high costs and resource intensiveness is prohibitive in industrializing countries (Black *et al.* 2010). In Sub-Saharan Africa GBS neonatal disease occurs in 3.06 per 1,000 live births in South Africa (Madhi *et al.* 2003), and 1.81 per 1,000 live births in Malawi (Gray *et al.* 2007). Further limitations to this intervention strategy include concerns about the emergence of GBS antimicrobial resistance and absence of reducing LOD (Moore *et al.* 2003). Although most industrialized countries have reduced the incidence of EOD, in other countries where IAP is being practiced the burden of EOD remains substantial, including in Finland (0.6-0.7), Spain (0.89) and Czech Republic (0.7-1.0) per 1000 live birth (Lyytikainen *et al.* 2003; Sastre *et al.* 2005; Strakova & Motlova 2004). These findings may be due to missing colonization when screening for GBS during pregnancy.

5.2 Pilus island distribution

Ours, to the best of our knowledge, is the first study describing the prevalence of pilus islands in isolates from vaginally colonized mothers and infants with invasive disease outside of Europe and the USA (Margarit *et al*., 2009; Martins *et al.*, 2010). There is no other published data on the pilus island distribution from sub-Saharan Africa where GBS remains the most frequently isolated pathogens in neonates (Berkley *et al.*, 2005; Gray *et al.*, 2007; Madhi *et al.*, 2003; Milledge *et al.*, 2005). The data from our study were consistent with those from industrialized countries in that all isolates were associated with the presence of PI-2a or PI-2b; Figure 5.1 . Pilus island-1 and PI-2b were almost always present together, and the presence of the pilus island was largely dependent on serotype (Margarit *et al*., 2009; Martins *et al*., 2010).

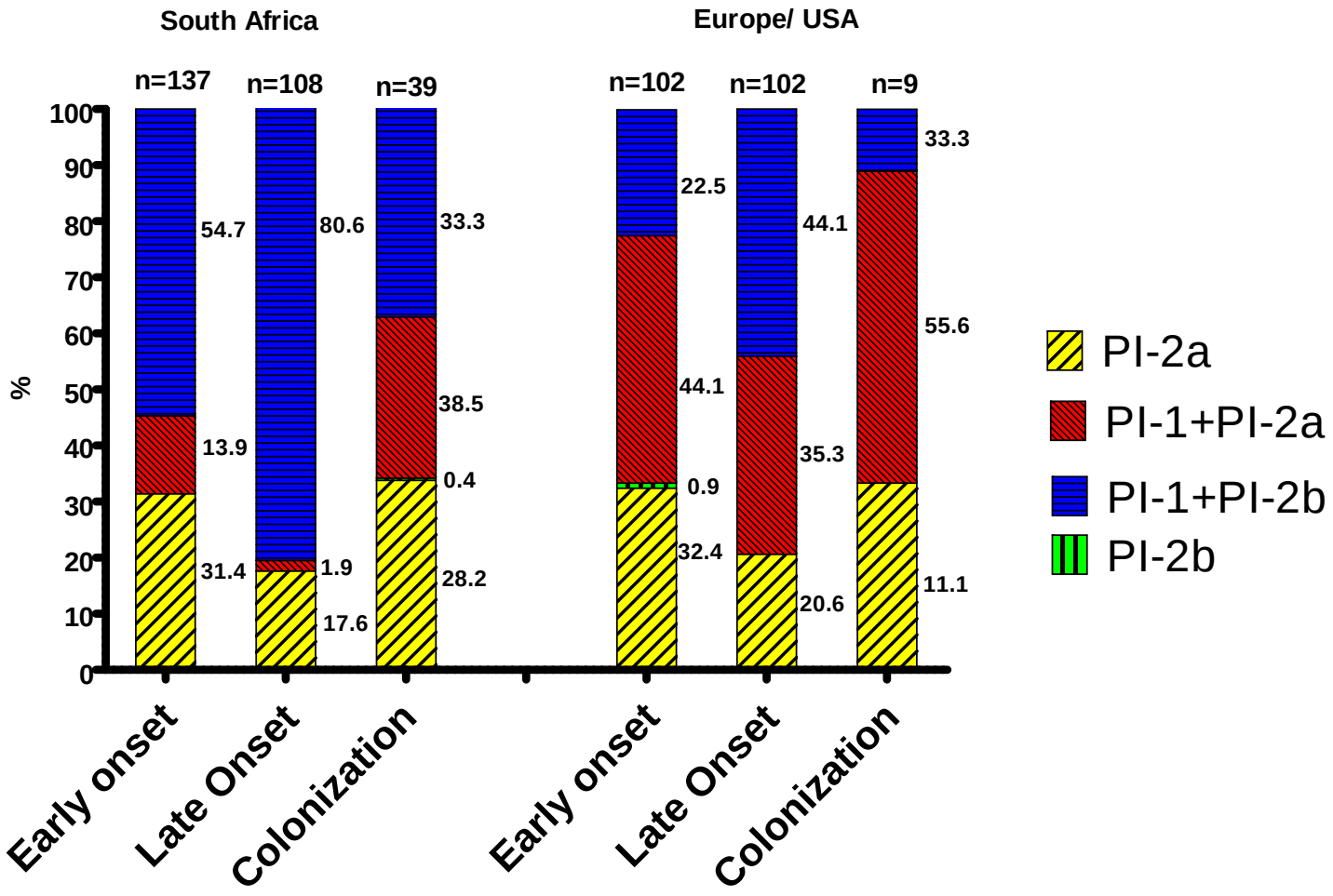


Figure 5.1: Distribution of group B streptococcus pilus island by region and source of isolates

There were differences in the pilus island distribution between colonizing and invasive disease isolates and with respect to timing of invasive disease onset. A combination of PI-1 and PI-2a was associated with the majority of EOD and colonizing isolates, whilst a combination of PI-1 and PI-2b was associated with the majority of LOD, this is in line with the distribution of pilus island in isolates from Europe and USA; Figure 5.1. There was, however, a difference in the frequency of pilus islands in EOD, in contrast to our finding, Margarit *et al*., 2009 observed that a combination of PI-1 and PI-2a was associated with EOD (44.1%) whilst this study reported 13.9% of EOD isolates to be associated with a combination of PI-1 and PI-2a. The differences in the pilus island distribution between colonizing and invasive disease isolates can be attributed to the stable relationship between serotype and pilus island combinations and indicates that differences in pilus island distribution between colonizing and invasive disease isolates was due to the differences in capsular related virulence factors rather than being associated with pilus island.

Our initial screening PCR which targets AP1, failed to detect either of the PI-2 islands in 15.0% of all isolates. Additional screening for the PI-2 islands with AP2 as a target significantly improved the sensitivity of detection, thus AP1 region of the PI-2 island is not highly conserved in GBS isolates. Variability in PI-2a was previously described by Margarit *et al.*, 2009, in a mice-experiment model vaccinated with the proteins of PI-2a which induced variant-specific protection. This observation had previously raised questions about the prospects of a pilus based vaccine that will include PI-2a proteins, however, a recent publication by Nuccitelli *et al*., 2011 applied a structural vaccinology approach to design a synthetic protein with multivalent activity.

Since a vaccine composed of multiple pilus island components has the potential to cover all serotypes, there is a need for in depth research into the global epidemiology of GBS pilus islands, to ensure that vaccines in development are broadly protective across all geographic locations. Despite minor inconsistency, our data on pilus island distribution is comparable to the previous study by Margarita *et al*., 2009, and the similarities are sufficient to confirm that the serotype and associated pilus island distribution in South Africa is similar to that reported in the USA and Europe. Based on this homogeneity of pilus island distribution, a pilus based vaccine developed for these industrialized countries, should provide similar coverage in South Africa. What remains to be determined is whether pressure from such a vaccine can result in the emergence of the strains which lack pilus islands as the dominant virulent types, as has been described for pathogen such as *S. pneumoniae* (Bogaert *et al.* 2005; Singleton *et al.* 2007).

5.3 Genetic population

We report for the first time the genetic characteristic of GBS isolates from colonization and invasive disease in sub-Saharan Africa. Our study showed that the GBS population structure in South Africa is similar to that from USA, Europe and few other African countries (Bergseng *et al.* 2009; Bohnsack *et al.* 2008; Brochet *et al.* 2009; Davies *et al.* 2004; Jones *et al.* 2006b; Luan *et al.* 2005; Manning *et al.* 2008; Manning *et al.* 2009; Sadowy *et al.* 2010; Salloum *et al.* 2011; Tien *et al.* 2011). There is a paucity of data regarding the genetic population of GBS in Africa and none in sub-Saharan Africa to establish whether there is clonal diversity in our region. Multilocus sequence characterized GBS invasive disease and

colonizing isolates into five (CC1, CC10, CC17, CC19 and CC23) and six (CC1, CC10, CC17, CC19, CC-22 and CC23) major clonal complexes respectively. This finding was consistent with other studies in industrialized countries, reaffirming that there is limited geographic genetic diversity among GBS isolates.

Multilocus sequence type characterization revealed 19 sequence types in colonizing isolates that were not associated with invasive disease isolates, while seven sequence types described in invasive disease isolates were not identified among colonizing isolates. This finding indicates that clones associated with invasive disease were less diverse compared to colonizing isolates. There was however a slight bias that was introduced in our study, in that full MLST was performed on selected isolates that were a representative of as many diverse alleles as possible based on a prescreening profile. This approach may have compromised identification of other clonal lineages that could have been missed during prescreening. Despite this limitation, our study provided a reliable insight in the genotypic characteristics of GBS isolates circulating in South Africa.

With the exception of CC-22, which was rarely detected elsewhere (Marchaim *et al.* 2006), the five major clonal complexes showed similar distribution to elsewhere; Table 5.2. Collectively, the five major clonal complexes accounted for 97.5% of all colonizing isolates in our study, which was similar to the 93.2% and 86.4% reported from the UK and six US academic centers respectively, Table 5.2, (Bohnsack *et al.* 2008; Jones *et al.* 2006b). This is also similar to a previous study that characterized maternal colonizing isolates from Poland and Canada, where the five major clonal complexes accounted for all colonizing isolates (Manning *et al.* 2008; Sadowy *et al.* 2010). Moreover our data is comparable to the data from the Central African Republic, where the five major clonal complex was responsible for 86.3%

of colonizing isolates (Brochet *et al.* 2009).

Although the majority of invasive disease and colonizing isolates in this study were distributed within the same clonal complex, only one rare clone comprising three colonizing isolates was not associated with invasive disease, which may however be due to the limited number of isolates analyzed. Clonal complex-22 comprised of two sequence type lineages, including ST-22 and ST-37, and was associated with only serotype II in colonizing isolates. Lineage CC-22 has previously been reported in colonizing isolates, and consistent with our findings it was expressed mainly by serotype II isolates (Bisharat *et al.* 2005; Marchaim *et al.* 2006). There was, however, differences in the frequency of the clonal complexes associated with invasive disease and colonization, for example, CC-17 was significantly associated with invasive disease whilst CC-10 and CC-19 were significantly associated with colonizing isolates. Moreover, within CC-23 was a ST-144/Ia combination, which appears to have reduced virulence potential, as no EOD isolate was found with this combination compared to 8.3% in colonizing isolates. The heterogeneous genetic feature from seven mother-newborn pairs suggests possible external transmission or failure to detect multiple colonization in the mothers.

Table 5.2: Global distribution of major clonal complexes among group B streptococcus colonizing isolates

Regions	Year	No of isolates	Clonal Complexes (%)						Comments
			CC-1	CC-10	CC-17	CC-19	CC-23	Other	
South Africa	2004-2007	121	19.8	23.1	19.8	15.8	19.0	2.5	
Africa									
Central African Republic (Brochet *et al.* 2009)	2005-2006	88	10.2	9.1	6.8	43.2	17.0	13.7	
Senegal (Brochet *et al.* 2009)	2005-2006	75	32.0		17.3	12.0	16.0	22.7	
Europe/Middle East									
Poland (Sadowy *et al.* 2010)	1996-2005	14	35.7		7.1	28.6	28.6		
Israel (Marchaim *et al.* 2006)	2000	72	58.3		9.7		13.9	18.1	
UK (Jones *et al.* 2006b)	2000-2003	190	20.5	17.4	12.1	20.0	23.2	7.1	
Germany (Fluegge *et al.* 2011)	2001-2003	46	4.3		30.4	50.0			MLST performed only in serotype III isolates
Americas									
USA (Bohnsack *et al.* 2008)	1995-1999	770	16.0		11.9	17.9	40.5	12.8	
Canada (Davies *et al.* 2004)	1995-1999	55	3.6		30.9	63.6	1.8		MLST performed only in serotype III isolates
Canada (Manning *et al.* 2008)	1998-2000	232	28.0		6.0	17.0	25.0	24.0	

The overall genetic characteristics of GBS in both EOD and LOD isolates in South Africa was similar to that described from the USA and Europe; Table 5.3. Similar to previous reports, CC-17 and CC-23 remained the major clonal complexes in both EOD (42.4% v/s 23.7%; respectively) and LOD (57.1% v/s 34.3%; respectively). These two major clonal complexes have over the years remained the dominant clones associated with invasive disease, in Italy CC-17 and CC-23 were responsible for at least 62.1% in EOD and 92.1% in LOD (Imperi *et al.* 2011). In Germany 92.3% of EOD and 86.1% of LOD were associated with CC-17 and CC-23 respectively (Fluegge *et al.* 2011). In contrast, studies from Canada reported CC-19 (57.1%) as the most common clonal complexes associated with invasive disease (Davies *et al.* 2004). This finding suggests whilst geographic variation exists, there appears to be limited genotypic diversity of invasive GBS isolates globally, which are mainly restricted to a limited number of clonal complexes.

Table 5.3: Global distribution of group B streptococcus major clonal complexes among early onset and late onset disease

Regions	Year	No of isolates	Clonal Complexes (%)						Comments
			CC-1	CC10	CC-17	CC-19	CC-23	Other	
South Africa	2004-2008	131	18.6[a]/5.7[b]	8.5/0	42.4/57.1	6.8/2.9	23.7/34.3		
Asia									
Taiwan (Tien *et al.* 2011)	2001-2004	17	5.9	9.1	41.2	17.6	23.5		
Europe/Middle East									
Sweden (Luan *et al.* 2005)	1988-1997	102	11.8		33.3	28.4	14.7	11.8	
UK (Jones *et al.* 2006b)	2000-2003	190	25.0	10.9	26.6	17.2	15.6	4.7	
Germany (Fluegge *et al.* 2011)	2001-2003	185			66.7/79.8	22.9/13.5	6.3/2.2	3.1/4.5	MLST performed only in serotype III isolates
France (Salloum *et al.* 2011)	2003	67	4.5		67.1	5.8	13.4	9.0	
Italy (Imperi *et al.* 2011)	2005-2008	75	13.5/0		48.6/81.6	10.8/5.3	13.5/2.6	13.5/10.5	
Norway (Bergseng *et al.* 2009)	2006-2007	96	31.5/21.4		25.9/38.1	20.4/14.3	3.7/14.3	18.5/11.9	
Americas									
USA (Bohnsack *et al.* 2008)	1995-1999	129	18.6		17.8	10.1	36.4	17.0	
Canada (Davies *et al.* 2004)	1995-1999	28			35.7	57.1	7.1		MLST performed only in serotype III isolates
Canada (Manning *et al.* 2009)	1998-2000	181	19.0/8.0		17.0/57.0	27.0/20.0	24.0/4.0	13.0/11.0	

[a]-Value indicates early onset disease, [b]-value indicates late onset disease

There was, however, no significant difference between invasive disease and colonizing-isolates within the clonal complex distribution when analyzed by the capsular serotype. The majority of colonizing and invasive-disease isolates within CC-17 (95.8 v/s 92.9%) and CC-23 (91.5 v/s 83.3%) were mainly serotype III and Ia, respectively. In addition, the identification of three (1%) serotype IV isolates within CC-17 may indicate capsular switching, since CC-17 was exclusively associated with serotype III (Bohnsack *et al.* 2008; Imperi *et al.* 2011; Lamy *et al.* 2006; Luan *et al.* 2005). Serotype IV isolates grouping within CC-17 were recently found to be genotypically similar to serotype III strains compared to non CC-17 serotype IV strains (Bellais *et al.* 2012). The likelihood of capsular switch from serotype III to serotype IV was evident at the molecular level because the flanking genes of serotype IV were found to be similar to that of serotype III isolates (Bellais *et al.* 2012). This finding indicates that the recent description of serotype IV invasive disease in the USA and Europe might be as a result of isolates belonging to the hypervirulent CC-17 (Bellais *et al.* 2012; Persson *et al.* 2004; Puopolo & Madoff 2007). Whether the capsular switch occurred independently in different regions or is due to spread of a single clone remains to be explored.

Other than one Portuguese study (Martins *et al.* 2010), there have been no other studies describing the relationship between clonal distribution and the distribution of pilus island within GBS isolates. In this study, we correlated the distribution of clonal complexes to pilus island, to establish if there is any molecular association. Our findings showed that 94.1% of colonizing and 89.3% of invasive disease within CC-17 were associated with a combination of PI-1 and PI-2b, while 80.0% of colonizing and 88.9% of invasive disease were associated with PI-2a. These findings were partly consistent with the study by Martins *et al (*2010). It can be

suggested that the association between clonal complexes and pilus islands might be influenced by the capsular serotypes.

The overrepresentation of ST-17 (41.0%-67.1%) has been confirmed among invasive disease GBS isolates (Fluegge *et al.* 2011; Imperi *et al.* 2010; Salloum *et al.* 2011; Tien *et al.* 2011). The enhanced invasiveness of ST-17 has been previously found to be associated with gbs2018 allele C (Lamy *et al.* 2006; Tazi *et al.* 2010). In agreement with previous studies that described the association of ST-17 and gbs2018C, all isolates of ST-17 and its single locus variants were grouped within the cluster C (Bellais *et al.* 2012; Lamy *et al.* 2006). Our findings confirms that there is a strong association between genes encoding HvgA (gbs2018C) and certain genetic markers, and that identification of sequence encoding the specific allelic forms of gbs2018 can be used to identify ST-17 specific GBS isolates. Isolates representing serotype IV carrying a ST-17 single locus variant were also grouped within cluster allele C of gbs2018. This finding illustrates the need for ongoing surveillance as efforts to develop serotype-specific polysaccharide-protein conjugate vaccine are underway. The evidence of capsular switch from serotype III and IV may be a concern on the effectiveness of future serotype-specific vaccines excluding serotype IV, as serotype IV associated with ST-17 may emerge as a serotype which causes “replacement disease” in the presence of vaccine effectiveness against the targeted serotypes.

The experience with other conjugate vaccine with regard to changes in incidence of non-vaccine serotype disease in the presence of vaccination has varied. This includes varying increases in non-vaccine serotypes disease following the introduction of pneumococcal

polysaccharide conjugated heptavalent vaccine (PCV7) (Barricarte *et al.* 2007; Singleton *et al.* 2007), while neither meningococcal serogroup C nor *Haemophilus* influenzae type b conjugated vaccines have resulted in any significant serotype replacement disease (Ladhani *et al.* 2008; Trotter *et al.* 2006).

Chapter 6 Conclusion

This study highlighted that the difference in serotype distribution between invasive disease and colonizing isolates is related to an increased invasive potential of certain GBS serotypes rather than due to increased risk of transmission of these from mother to newborns. Analysis of the prevalence of pilus island showed that the difference in the pilus island distribution between invasive disease and colonizing GBS isolates was influenced by the capsular serotype. Moreover, this study found that there was an overlap in the genetic characteristics of GBS associated with colonization and invasive disease, with certain clonal lineages appearing to have a propensity to colonize than causing invasive disease, and similarly there was a genetic relationship between the capsular serotype and clonal lineage.

Additionally, this study has provided valuable data on the serotype epidemiology in South Africa, and the potential efficacy of GBS conjugate vaccine against capsular serotypes Ia, Ib and III currently under clinical trial. We reported that the vaccine including serotype Ia, Ib and III has the potential of preventing 74.1%, 85.4% and 98.2% of GBS associated with maternal vaginal-colonization, invasive disease in EOD and LOD respectively. Equally as important, a vaccine containing the ancillary protein 1 and 2 of each pilus island can become the basis of a universal GBS vaccine. Furthermore, the study showed that emergence of serotype IV among the invasive associated clonal lineage may in the future limit the effectiveness of a trivalent GBS vaccine. Hence this study suggests that monitoring of serotype IV in colonizing and invasive disease isolates is warranted. The outcome of this study contributes to the research focusing on the development of an effective vaccine, either a pilus-based or polysaccharide-protein conjugate vaccine.

Above all, there are important gaps that still remain globally, including investigating the sequence conservation of different pilus island proteins, their expression in GBS isolates, and determining the specific antibodies against pilus proteins that are required to reduce the risk of GBS colonization in pregnant women. These issues are important in determining the optimal formulation of vaccine antigens, and moreover to predict the impact of a pilus based vaccine.

Chapter 7 Reference

Afshar, B., Broughton, K., Creti, R., Decheva, A., Hufnagel, M., Kriz, P., Lambertsen, L., Lovgren, M., Melin, P., Orefici, G., Poyart, C., Radtke, A., Rodriguez-Granger, J., Sorensen, U. B., Telford, J., Valinsky, L., Zachariadou, L. & Efstratiou, A. (2011). International external quality assurance for laboratory identification and typing of *Streptococcus agalactiae* (Group B streptococci). *J Clin.Microbiol,* 49, 1475-1482.

AitMhand, R., Moustaoui, N., Belabbes, H., Elmdaghri, N. & Benbachir, M. (2000). Serotypes and antimicrobial susceptibility of group B streptococcus isolated from neonates in Casablanca. *Scand.J Infect Dis,* 32, 339-340.

Al-Sweih, N., Hammoud, M., Al-Shimmiri, M., Jamal, M., Neil, L. & Rotimi, V. (2005). Serotype distribution and mother-to-baby transmission rate of Streptococcus agalactiae among expectant mothers in Kuwait. *Arch.Gynecol Obstet,* 272, 131-135.

Amin, A., Abdulrazzaq, Y. M. & Uduman, S. (2002). Group B streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. *J Infect,* 45, 42-46.

Arakere, G., Flores, A. E., Ferrieri, P. & Frasch, C. E. (1999). Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. *J Clin.Microbiol,* 37, 2564-2567.

Baker, C. J., Paoletti, L. C., Wessels, M. R., Guttormsen, H. K., Rench, M. A., Hickman, M. E. & Kasper, D. L. (1999). Safety and immunogenicity of capsular polysaccharide-tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. *J Infect Dis,* 179, 142-150.

Baker, C. J., Rench, M. A. & McInnes, P. (2003). Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. *Vaccine,* 21, 3468-3472.

Barocchi, M. A., Ries, J., Zogaj, X., Hemsley, C., Albiger, B., Kanth, A., Dahlberg, S., Fernebro, J., Moschioni, M., Masignani, V., Hultenby, K., Taddei, A. R., Beiter, K., Wartha, F., von, E. A., Covacci, A., Holden, D. W., Normark, S., Rappuoli, R. & Henriques-Normark, B. (2006). A pneumococcal pilus influences virulence and host inflammatory responses. *Proc.Natl Acad.Sci U.S.A,* 103, 2857-2862.

Barricarte, A., Castilla, J., Gil-Setas, A., Torroba, L., Navarro-Alonso, J. A., Irisarri, F. & Arriazu, M. (2007). Effectiveness of the 7-valent pneumococcal conjugate vaccine: a population-based case-control study. *Clin.Infect Dis,* 44, 1436-1441.

Beal, S. & Dancer, S. (2006). Antenatal prevention of neonatal group B streptococcal infection. *Gynaecological and Perinatal Practice,* 6, 218-225.

Bellais, S., Six, A., Fouet, A., Longo, M., Dmytruk, N., Glaser, P., Trieu-Cuot, P. & Poyart, C. (2012). Capsular switching in group B streptococcus CC17 hypervirulent clone: A future challenge for polysaccharide vaccine development. *J Infect Dis,* 206, 1745-1752.

Berg, S., Trollfors, B., Lagergard, T., Zackrisson, G. & Claesson, B. A. (2000). Serotypes and clinical manifestations of group B streptococcal infections in western Sweden. *Clin.Microbiol Infect,* 6, 9-13.

Bergseng, H., Afset, J. E., Radtke, A., Loeseth, K., Lyng, R. V., Rygg, M. & Bergh, K. (2009). Molecular and phenotypic characterization of invasive group B streptococcus strains from infants in Norway 2006-2007. *Clin.Microbiol Infect,* 15, 1182-1185.

Bergseng, H., Rygg, M., Bevanger, L. & Bergh, K. (2008). Invasive group B streptococcus (GBS) disease in Norway 1996-2006. *Eur.J Clin.Microbiol Infect Dis,* 27, 1193-1199.

Berkley, J. A., Lowe, B. S., Mwangi, I., Williams, T., Bauni, E., Mwarumba, S., Ngetsa, C., Slack, M. P., Njenga, S., Hart, C. A., Maitland, K., English, M., Marsh, K. & Scott, J. A. (2005). Bacteremia among children admitted to a rural hospital in Kenya. *N.Engl.J Med,* 352, 39-47.

Berkowitz, G. S. & Papiernik, E. (1993). Epidemiology of preterm birth. *Epidemiol.Rev,* 15, 414-443.

Bisharat, N., Crook, D. W., Leigh, J., Harding, R. M., Ward, P. N., Coffey, T. J., Maiden, M. C., Peto, T. & Jones, N. (2004). Hyperinvasive neonatal group B streptococcus has arisen from a bovine ancestor. *J Clin.Microbiol,* 42, 2161-2167.

Bisharat, N., Jones, N., Marchaim, D., Block, C., Harding, R. M., Yagupsky, P., Peto, T. & Crook, D. W. (2005). Population structure of group B streptococcus from a low-incidence region for invasive neonatal disease. *Microbiology,* 151, 1875-1881.

Black, R. E., Cousens, S., Johnson, H. L., Lawn, J. E., Rudan, I., Bassani, D. G., Jha, P., Campbell, H., Walker, C. F., Cibulskis, R., Eisele, T., Liu, L. & Mathers, C. (2010). Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet,* 375, 1969-1987.

Blumberg, H. M., Stephens, D. S., Modansky, M., Erwin, M., Elliot, J., Facklam, R. R., Schuchat, A., Baughman, W. & Farley, M. M. (1996). Invasive group B streptococcal disease: the emergence of serotype V. *J Infect Dis,* 173, 365-373.

Bogaert, D., Veenhoven, R. H., Sluijter, M., Wannet, W. J., Rijkers, G. T., Mitchell, T. J., Clarke, S. C., Goessens, W. H., Schilder, A. G., Sanders, E. A., de, G. R. & Hermans, P. W. (2005). Molecular epidemiology of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media. *J Clin.Microbiol,* 43, 74-83.

Bohnsack, J. F., Whiting, A., Gottschalk, M., Dunn, D. M., Weiss, R., Azimi, P. H., Philips, J. B., III, Weisman, L. E., Rhoads, G. G. & Lin, F. Y. (2008). Population structure of invasive and colonizing strains of Streptococcus agalactiae from neonates of six U.S. Academic Centers from 1995 to 1999. *J Clin.Microbiol,* 46, 1285-1291.

Brimil, N., Barthell, E., Heindrichs, U., Kuhn, M., Lutticken, R. & Spellerberg, B. (2006). Epidemiology of Streptococcus agalactiae colonization in Germany. *Int.J Med Microbiol,* 296, 39-44.

Brochet, M., Couve, E., Bercion, R., Sire, J. M. & Glaser, P. (2009). Population structure of human isolates of Streptococcus agalactiae from Dakar and Bangui. *J Clin.Microbiol,* 47, 800-803.

Bromberger, P. I., Chandler, B., Gezon, H. & Haddow, J. E. (1980). Rapid detection of neonatal group B streptococcal infections by latex agglutination. *J Pediatr.,* 96, 104-106.

Brueggemann, A. B., Griffiths, D. T., Meats, E., Peto, T., Crook, D. W. & Spratt, B. G. (2003). Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. *J Infect Dis,* 187, 1424-1432.

Brueggemann, A. B., Peto, T. E., Crook, D. W., Butler, J. C., Kristinsson, K. G. & Spratt, B. G. (2004). Temporal and geographic stability of the serogroup-specific invasive disease potential of Streptococcus pneumoniae in children. *J Infect Dis,* 190, 1203-1211.

Brueggemann, A. B. & Spratt, B. G. (2003). Geographic distribution and clonal diversity of Streptococcus pneumoniae serotype 1 isolates. *J Clin.Microbiol,* 41, 4966-4970.

Buttery, J. & Moxon, E. R. (2002). Capsulate bacteria and the lung. *Br.Med Bull.,* 61, 63-80.

CDC (1996). Prevention of perinatal group B streptococcal disease; a public health perspective. *MMWR Morb Mortal Wkly Rep,* 45, 1-24.

CDC (2005). Early-onset and late-onset neonatal group B streptococcal disease-United States, 1996-2004. *MMWR Morb Mortal Wkly Rep,* 54, 1205-8.

Chaffin, D. O., Mentele, L. M. & Rubens, C. E. (2005). Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. *J Bacteriol.,* 187, 4615-4626.

Chaudhry, B. Y., Akhtar, N. & Balouch, A. H. (2010). Vaginal carriage rate of group B streptococcus in pregnant women and its transmission to neonates. *J Ayub.Med Coll.Abbottabad.,* 22, 167-170.

Chen, K. T., Puopolo, K. M., Eichenwald, E. C., Onderdonk, A. B. & Lieberman, E. (2005). No increase in rates of early-onset neonatal sepsis by antibiotic-resistant group B streptococcus in the era of intrapartum antibiotic prophylaxis. *Am.J Obstet Gynecol,* 192, 1167-1171.

Cheng, Q., Debol, S., Lam, H., Eby, R., Edwards, L., Matsuka, Y., Olmsted, S. B. & Cleary, P. P. (2002). Immunization with C5a peptidase or peptidase-type III polysaccharide conjugate vaccines enhances clearance of group B streptococci from lungs of infected mice. *Infect Immun.,* 70, 6409-6415.

Chuard, C. & Reller, L. B. (1998). Bile-esculin test for presumptive identification of enterococci and streptococci: effects of bile concentration, inoculation technique, and incubation time. *J Clin.Microbiol,* 36, 1135-1136.

Cieslewicz, M. J., Chaffin, D., Glusman, G., Kasper, D., Madan, A., Rodrigues, S., Fahey, J., Wessels, M. R. & Rubens, C. E. (2005). Structural and genetic diversity of group B streptococcus capsular polysaccharides. *Infect Immun.,* 73, 3096-3103.

Cooper, J. E. & Feil, E. J. (2004). Multilocus sequence typing--what is resolved? *Trends Microbiol,* 12, 373-377.

Croak, A., Abate, G., Goodrum, K. & Modrzakowski, M. (2003). Predominance of serotype V and frequency of erythromycin resistance in Streptococcus agalactiae in Ohio. *Am.J Obstet Gynecol,* 188, 1148-1150.

Cutland, C. L., Madhi, S. A., Zell, E. R., Kuwanda, L., Laque, M., Groome, M., Gorwitz, R., Thigpen, M. C., Patel, R., Velaphi, S. C., Adrian, P., Klugman, K., Schuchat, A. & Schrag, S. J. (2009). Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised, controlled trial. *Lancet,* 374, 1909-1916.

Cutland, C. L., Schrag, S. J., Zell, E. R., Kuwanda, L., Buchmann, E., Velaphi, S. C., Groome, M. J., Adrian, P. V. & Madhi, S. A. (2012). Maternal HIV infection and vertical transmission of pathogenic bacteria. *Pediatrics,* 130, e581-e590.

Davies, H. D., Jones, N., Whittam, T. S., Elsayed, S., Bisharat, N. & Baker, C. J. (2004). Multilocus sequence typing of serotype III group B streptococcus and correlation with pathogenic potential. *J Infect Dis,* 189, 1097-1102.

Davies, H. D., Raj, S., Adair, C., Robinson, J. & McGeer, A. (2001). Population-based active surveillance for neonatal group B streptococcal infections in Alberta, Canada: implications for vaccine formulation. *Pediatr.Infect Dis J,* 20, 879-884.

de Steenwinkel, F. D., Tak, H. V., Muller, A. E., Nouwen, J. L., Oostvogel, P. M. & Mocumbi, S. M. (2008). Low carriage rate of group B streptococcus in pregnant women in Maputo, Mozambique. *Trop.Med Int.Health,* 13, 427-429.

Dhanoa, A., Karunakaran, R. & Puthucheary, S. D. (2010). Serotype distribution and antibiotic susceptibility of group B streptococci in pregnant women. *Epidemiol.Infect,* 138, 979-981.

Dillon, H. C., Jr., Gray, E., Pass, M. A. & Gray, B. M. (1982). Anorectal and vaginal carriage of group B streptococci during pregnancy. *J Infect Dis,* 145, 794-799.

Dore, N., Bennett, D., Kaliszer, M., Cafferkey, M. & Smyth, C. J. (2003). Molecular epidemiology of group B streptococci in Ireland: associations between serotype, invasive status and presence of genes encoding putative virulence factors. *Epidemiol.Infect,* 131, 823-833.

Dramsi, S., Caliot, E., Bonne, I., Guadagnini, S., Prevost, M. C., Kojadinovic, M., Lalioui, L., Poyart, C. & Trieu-Cuot, P. (2006). Assembly and role of pili in group B streptococci. *Mol.Microbiol,* 60, 1401-1413.

Dzowela, T., Komolafe, O. O. & Igbigbi, A. (2005). Prevalence of group B streptococcus colonization in ante-natal women at the Queen Elizabeth central hospital, Blantyre- a preliminary study. *Malawi Medical Journal,* 17, 97-99.

Edmond, K. M., Kortsalioudaki, C., Scott, S., Schrag, S. J., Zaidi, A. K., Cousens, S. & Heath, P. T. (2012). Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. *Lancet,* 379, 547-556.

Edwards, M. S., Kasper, D. L., Jennings, H. J., Baker, C. J. & Nicholson-Weller, A. (1982). Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. *J Immunol,* 128, 1278-1283.

Edwards, M. S., Wessels, M. R. & Baker, C. J. (1993). Capsular polysaccharide regulates neutrophil complement receptor interactions with type III group B streptococci. *Infect Immun.,* 61, 2866-2871.

Eickhoff, T. C., Klein, J., Daly, K., Ingall, D. & Finland, M. (1964). Neonatal sepsis and other infections due to group beta-hemolytic streptococcus. *N.Engl.J Med,* 271, 1221-1228.

Ekelund, K. & Konradsen, H. B. (2004). Invasive group B streptococcal disease in infants: a 19-year nationwide study. Serotype distribution, incidence and recurrent infection. *Epidemiol.Infect,* 132, 1083-1090.

El, B. P., Duarte, G., Maffei, C. M., Quintana, S. M., De Sa Rosa E Silva AC & Nogueira, A. A. (2006). Group B streptococcus carriers among HIV-1 infected pregnant women: prevalence and risk factors. *Eur.J Obstet Gynecol Reprod.Biol.*, 128, 54-58.

Elbaradie, S. M. Y., Mahmoud, M. & Farid, M. (2009). Maternal and neonatal screening for group B streptococci by *scpB* gene based PCR: A preliminary. *Indian J Med Res,* 27, 17-21.

Enright, M. C., Spratt, B. G., Kalia, A., Cross, J. H. & Bessen, D. E. (2001). Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone. *Infect Immun.*, 69, 2416-2427.

Epalza, C., Goetghebuer, T., Hainaut, M., Prayez, F., Barlow, P., Dediste, A., Marchant, A. & Levy, J. (2010). High incidence of invasive group B streptococcal infections in HIV-exposed uninfected infants. *Pediatrics,* 126, e631-e638.

Eren, A., Kucukercan, M., Oguzoglu, N., Unal, N. & Karateke, A. (2005). The carriage of group B streptococci in Turkish pregnant women and its transmission rate in newborns and serotype distribution. *Turk.J Pediatr.,* 47, 28-33.

Facklam, R. (2002). What happened to the streptococci: overview of taxonomic and nomenclature changes. *Clin.Microbiol Rev,* 15, 613-630.

Facklam, R. R. & Moody, M. D. (1970). Presumptive identification of group D streptococci: the bile-esculin test. *Appl.Microbiol,* 20, 245-250.

Faro, S., Brehm, B., Smith, F., Mouzoon, M., Greisinger, A., Wehmanen, O. & Turrentine, M. A. (2010). Screening for group B streptococcus: a private hospital's experience. *Infect Dis Obstet Gynecol,* 2010.

Ferrieri, P., Hillier, S. L., Krohn, M. A., Moore, D., Paoletti, L. C. & Flores, A. E. (2004). Characterization of vaginal & rectal colonization with multiple serotypes of group B streptococci using multiple colony picks. *Indian J Med Res,* 119 Suppl, 208-212.

Fluegge, K., Supper, S., Siedler, A. & Berner, R. (2005). Serotype distribution of invasive group B streptococcal isolates in infants: results from a nationwide active laboratory surveillance study over 2 years in Germany. *Clin.Infect Dis,* 40, 760-763.

Fluegge, K., Wons, J., Spellerberg, B., Swoboda, S., Siedler, A., Hufnagel, M. & Berner, R. (2011). Genetic differences between invasive and noninvasive neonatal group B streptococcal isolates. *Pediatr.Infect Dis J.*

Foxman, B., Gillespie, B. W., Manning, S. D. & Marrs, C. F. (2007). Risk factors for group B streptococcal colonization: potential for different transmission systems by capsular type. *Ann.Epidemiol.,* 17, 854-862.

Fuchs, P. C., Christy, C. & Jones, R. N. (1978). Multiple-inocula (replicator) CAMP test for presumptive identification of group B streptococci. *J Clin.Microbiol,* 7, 232-233.

Garland, S. M., Kelly, N. & Ugoni, A. M. (2000). Is antenatal group B streptococcal carriage a predictor of adverse obstetric outcome? *Infect Dis Obstet Gynecol,* 8, 138-142.

Gaspar, A. H. & Ton-That, H. (2006). Assembly of distinct pilus structures on the surface of Corynebacterium diphtheriae. *J Bacteriol.,* 188, 1526-1533.

Gilson, G. J., Christensen, F., Romero, H., Bekes, K., Silva, L. & Qualls, C. R. (2000). Prevention of group B streptococcus early-onset neonatal sepsis: comparison of the Center for Disease Control and prevention screening-based protocol to a risk-based protocol in infants at greater than 37 weeks' gestation. *J Perinatol.,* 20, 491-495.

Gonzalez Pedraza, A. A., Ortiz Zaragoza, M. C. & Mota, V. R. (2002). Serotypes and antimicrobial susceptibility of group B Streptococcus from pregnant women in Mexico. *Rev Latinoam.Microbiol,* 44, 133-136.

Gordillo, M. E., Singh, K. V., Baker, C. J. & Murray, B. E. (1993). Typing of group B streptococci: comparison of pulsed-field gel electrophoresis and conventional electrophoresis. *J Clin.Microbiol,* 31, 1430-1434.

Goyal, R., Singh, N. P., Lal, P. & Gupta, P. (2004). Group B Streptococcus colonisation in obstetric cases in a tertiary care hospital in Delhi, India. *Ann.Trop.Paediatr.,* 24, 189-190.

Gray, K. J., Bennett, S. L., French, N., Phiri, A. J. & Graham, S. M. (2007). Invasive group B streptococcal infection in infants, Malawi. *Emerg.Infect Dis,* 13, 223-229.

Gray, K. J., Kafulafula, G., Matemba, M., Kamdolozi, M., Membe, G. & French, N. (2011). Group B Streptococcus and HIV infection in pregnant women, Malawi, 2008-2010. *Emerg.Infect Dis,* 17, 1932-1935.

Grimwood, K., Stone, P. R., Gosling, I. A., Green, R., Darlow, B. A., Lennon, D. R. & Martin, D. R. (2002). Late antenatal carriage of group B streptococcus by New Zealand women. *Aust.N.Z.J Obstet Gynaecol.,* 42, 182-186.

Gupta, C. & Briski, L. E. (2004). Comparison of two culture media and three sampling techniques for sensitive and rapid screening of vaginal colonization by group B streptococcus in pregnant women. *J Clin.Microbiol,* 42, 3975-3977.

Hakansson, S., Axemo, P., Bremme, K., Bryngelsson, A. L., Wallin, M. C., Ekstrom, C. M., Granlund, M., Jacobsson, B., Kallen, K., Spetz, E. & Tessin, I. (2008). Group B streptococcal carriage in Sweden: a national study on risk factors for mother and infant colonisation. *Acta Obstet Gynecol Scand.,* 87, 50-58.

Hansen, S. M., Uldbjerg, N., Kilian, M. & Sorensen, U. B. (2004). Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. *J Clin.Microbiol*, 42, 83-89.

Harrison, L. H., Elliott, J. A., Dwyer, D. M., Libonati, J. P., Ferrieri, P., Billmann, L. & Schuchat, A. (1998). Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. *J Infect Dis,* 177, 998-1002.

Hickman, M. E., Rench, M. A., Ferrieri, P. & Baker, C. J. (1999). Changing epidemiology of group B streptococcal colonization. *Pediatrics,* 104, 203-209.

Hong, J. S., Choi, C. W., Park, K. U., Kim, S. N., Lee, H. J., Lee, H. R., Choi, E. H., Park, K. H., Suh, C. S., Kim, B. I., Choi, S. T. & Kim, S. S. (2010a). Genital group B streptococcus carrier rate and serotype distribution in Korean pregnant women: implications for group B streptococcal disease in Korean neonates. *J Perinat.Med,* 38, 373-377.

Hong, J. S., Choi, C. W., Park, K. U., Kim, S. N., Lee, H. J., Lee, H. R., Choi, E. H., Park, K. H., Suh, C. S., Kim, B. I., Choi, S. T. & Kim, S. S. (2010b). Genital group B streptococcus carrier rate and serotype distribution in Korean pregnant women: implications for group B streptococcal disease in Korean neonates. *J Perinat.Med,* 38, 373-377.

Hoshina, K., Suzuki, Y., Nishida, H., Kaneko, K., Matsuda, S., Kobayashi, M. & Kadoi, N. (2002). Trend of neonatal group B streptococcal infection during the last 15 years. *Pediatr.Int.,* 44, 641-646.

Imperi, M., Gherardi, G., Berardi, A., Baldassarri, L., Pataracchia, M., Dicuonzo, G., Orefici, G. & Creti, R. (2011). Invasive neonatal GBS infections from an area-based surveillance study in Italy. *Clin.Microbiol Infect,* 17, 1834-1839.

Imperi, M., Pataracchia, M., Alfarone, G., Baldassarri, L., Orefici, G. & Creti, R. (2010). A multiplex PCR assay for the direct identification of the capsular type (Ia to IX) of Streptococcus agalactiae. *J Microbiol Methods,* 80, 212-214.

Ingram, D. L., Suggs, D. M. & Pearson, A. W. (1982). Detection of group B streptococcal antigen in early-onset and late-onset group B streptococcal disease with the Wellcogen Strep B latex agglutination test. *J Clin.Microbiol,* 16, 656-658.

Ippolito, D. L., James, W. A., Tinnemore, D., Huang, R. R., Dehart, M. J., Williams, J., Wingerd, M. A. & Demons, S. T. (2010). Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. *BMC Infect Dis,* 10, 336.

Isaacs, D. (1998). Prevention of early onset group B streptococcal infection: screen, treat, or observe? *Arch.Dis Child Fetal Neonatal Ed,* 79, F81-F82.

Jamie, W. E., Edwards, R. K. & Duff, P. (2004). Vaginal-perianal compared with vaginal-rectal cultures for identification of group B streptococci. *Obstet Gynecol,* 104, 1058-1061.

Jennings, H. J., Katzenellenbogen, E., Lugowski, C. & Kasper, D. L. (1983a). Structure of native polysaccharide antigens of type Ia and type Ib group B Streptococcus. *Biochemistry,* 22, 1258-1264.

Jennings, H. J., Rosell, K. G., Katzenellenbogen, E. & Kasper, D. L. (1983b). Structural determination of the capsular polysaccharide antigen of type II group B Streptococcus. *J Biol.Chem.,* 258, 1793-1798.

Joachim, A., Matee, M. I., Massawe, F. A. & Lyamuya, E. F. (2009). Maternal and neonatal colonisation of group B streptococcus at Muhimbili National Hospital in Dar es Salaam, Tanzania: prevalence, risk factors and antimicrobial resistance. *BMC Public Health,* 9, 437.

Jones, N., Oliver, K. A., Jones, Y., Haines, A. & Crook, D. (2006a). Carriage of group B streptococcus in pregnant in pregnant women from Oxford, UK. *J Clin.Pathol,* 59, 363-366.

Jones, N., Bohnsack, J. F., Takahashi, S., Oliver, K. A., Chan, M. S., Kunst, F., Glaser, P., Rusniok, C., Crook, D. W., Harding, R. M., Bisharat, N. & Spratt, B. G. (2003). Multilocus sequence typing system for group B streptococcus. *J Clin.Microbiol,* 41, 2530-2536.

Jones, N., Oliver, K. A., Barry, J., Harding, R. M., Bisharat, N., Spratt, B. G., Peto, T. & Crook, D. W. (2006b). Enhanced invasiveness of bovine-derived neonatal sequence type 17 group B streptococcus is independent of capsular serotype. *Clin.Infect Dis,* 42, 915-924.

Jordan, H. T., Farley, M. M., Craig, A., Mohle-Boetani, J., Harrison, L. H., Petit, S., Lynfield, R., Thomas, A., Zansky, S., Gershman, K., Albanese, B. A., Schaffner, W. & Schrag, S. J. (2008). Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. *Pediatr.Infect Dis J,* 27, 1057-1064.

Kieran, E., Matheson, M., Mann, A. G., Efstratiou, A. A., Butler, K. & Gorman, W. (1998). Group B streptococcus (GBS) colonisation among expectant Irish mothers. *Ir.Med J,* 91, 21-22.

Kogan, G., Brisson, J. R., Kasper, D. L., von, H. C., Orefici, G. & Jennings, H. J. (1995). Structural elucidation of the novel type VII group B Streptococcus capsular polysaccharide by high resolution NMR spectroscopy. *Carbohydr.Res,* 277, 1-9.

Kogan, G., Uhrin, D., Brisson, J. R., Paoletti, L. C., Blodgett, A. E., Kasper, D. L. & Jennings, H. J. (1996). Structural and immunochemical characterization of the type VIII group B Streptococcus capsular polysaccharide. *J Biol.Chem.,* 271, 8786-8790.

Kotiw, M., Zhang, G. W., Daggard, G., Reiss-Levy, E., Tapsall, J. W. & Numa, A. (2003). Late-onset and recurrent neonatal group B streptococcal disease associated with breast milk transmission. *Pediatric.Dev.Pathol,* 6, 251-256.

Kovavisarach, E., Sa-adying, W. & Kanjanahareutai, S. (2007). Comparison of combined vaginal-anorectal, vaginal and anorectal cultures in detecting of group B streptococci in pregnant women in labor. *J Med Assoc Thai.,* 90, 1710-1714.

Krishnan, V., Gaspar, A. H., Ye, N., Mandlik, A., Ton-That, H. & Narayana, S. V. (2007). An IgG-like domain in the minor pilin GBS52 of Streptococcus agalactiae mediates lung epithelial cell adhesion. *Structure,* 15, 893-903.

Kunze, M., Ziegler, A., Fluegge, K., Hentschel, R., Proempeler, H. & Berner, R. (2011). Colonization, serotypes and transmission rates of group B streptococci in pregnant women and their infants born at a single University Center in Germany. *J Perinat.Med,* 39, 417-422.

Kuruvilla, K. A., Thomas, N., Jesudasan, M. V. & Jana, A. K. (1999a). Neonatal Group B Streptococcal bacteraemia in India: ten years' experience. *Acta Paediatr.,* 88, 1031-1032.

Lachenauer, C. S., Kasper, D. L., Shimada, J., Ichiman, Y., Ohtsuka, H., Kaku, M., Paoletti, L. C., Ferrieri, P. & Madoff, L. C. (1999). Serotypes VI and VIII predominate among group B streptococci isolated from pregnant Japanese women. *J Infect Dis,* 179, 1030-1033.

Ladhani, S., Ramsay, M. E., Chandra, M. & Slack, M. P. (2008). No evidence for *Haemophilus influenzae* serotype replacement in Europe after introduction of the Hib

conjugate vaccine. *Lancet Infect Dis,* 8, 275-276.

Lamy, M. C., Dramsi, S., Billoet, A., Reglier-Poupet, H., Tazi, A., Raymond, J., Guerin, F., Couve, E., Kunst, F., Glaser, P., Trieu-Cuot, P. & Poyart, C. (2006). Rapid detection of the "highly virulent" group B streptococcus ST-17 clone. *Microbes Infect,* 8, 1714-1722.

Lancefield, R. C. (1934). A serological differentiation of specific types of bovine hemolytic streptococci (group B). *J Exp Med,* 59, 441-458.

Larsson, C., Stalhammar-Carlemalm, M. & Lindahl, G. (1999). Protection against experimental infection with group B streptococcus by immunization with a bivalent protein vaccine. *Vaccine,* 17, 454-458.

Lauer, P., Rinaudo, C. D., Soriani, M., Margarit, I., Maione, D., Rosini, R., Taddei, A. R., Mora, M., Rappuoli, R., Grandi, G. & Telford, J. L. (2005). Genome analysis reveals pili in Group B streptococcus. *Science,* 309, 105.

Liddy, H. & Holliman, R. (2002). Group B streptococcus highly resistant to gentamicin. *J Antimicrob.Chemother,* 50, 142-143.

Lin, F. Y., Clemens, J. D., Azimi, P. H., Regan, J. A., Weisman, L. E., Philips, J. B., III, Rhoads, G. G., Clark, P., Brenner, R. A. & Ferrieri, P. (1998). Capsular polysaccharide types of group B streptococcal isolates from neonates with early-onset systemic infection. *J Infect Dis,* 177, 790-792.

Lin, F. Y., Philips, J. B., III, Azimi, P. H., Weisman, L. E., Clark, P., Rhoads, G. G., Regan, J., Concepcion, N. F., Frasch, C. E., Troendle, J., Brenner, R. A., Gray, B. M., Bhushan, R., Fitzgerald, G., Moyer, P. & Clemens, J. D. (2001). Level of maternal antibody required to protect neonates against early-onset disease caused by group B Streptococcus type Ia: a multicenter, seroepidemiology study. *J Infect Dis,* 184, 1022-1028.

Lin, F. Y., Weisman, L. E., Azimi, P. H., Philips, J. B., III, Clark, P., Regan, J., Rhoads, G. G., Frasch, C. E., Gray, B. M., Troendle, J., Brenner, R. A., Moyer, P. & Clemens, J. D. (2004).

Level of maternal IgG anti-group B streptococcus type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen. *J Infect Dis,* 190, 928-934.

Luan, S. L., Granlund, M., Sellin, M., Lagergard, T., Spratt, B. G. & Norgren, M. (2005). Multilocus sequence typing of Swedish invasive group B streptococcus isolates indicates a neonatally associated genetic lineage and capsule switching. *J Clin.Microbiol,* 43, 3727-3733.

Luck, S., Torny, M., d'Agapeyeff, K., Pitt, A., Heath, P., Breathnach, A. & Russell, A. B. (2003). Estimated early-onset group B streptococcal neonatal disease. *Lancet,* 361, 1953-1954.

Lyytikainen, O., Nuorti, J. P., Halmesmaki, E., Carlson, P., Uotila, J., Vuento, R., Ranta, T., Sarkkinen, H., Ammala, M., Kostiala, A. & Jarvenpaa, A. L. (2003). Invasive group B streptococcal infections in Finland: a population-based study. *Emerg.Infect Dis,* 9, 469-473.

Madhi, S. A., Kuwanda, L., Cutland, C. & Klugman, K. P. (2005). The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and -uninfected children. *Clin.Infect Dis,* 40, 1511-1518.

Madhi, S. A., Radebe, K., Crewe-Brown, H., Frasch, C. E., Arakere, G., Mokhachane, M. & Kimura, A. (2003). High burden of invasive Streptococcus agalactiae disease in South African infants. *Ann.Trop.Paediatr.,* 23, 15-23.

Maiden, M. C., Bygraves, J. A., Feil, E., Morelli, G., Russell, J. E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D. A., Feavers, I. M., Achtman, M. & Spratt, B. G. (1998). Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. *Proc.Natl Acad.Sci U.S.A,* 95, 3140-3145.

Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T., Rosini, R., D'Agostino, N., Miorin, L., Buccato, S., Mariani, M., Galli, G., Nogarotto, R., Nardi, D., V, Vegni, F., Fraser, C., Mancuso, G., Teti, G., Madoff, L. C., Paoletti, L. C., Rappuoli, R., Kasper, D. L., Telford, J. L. & Grandi, G. (2005). Identification of a universal Group B streptococcus vaccine by multiple genome screen. *Science,* 309, 148-150.

Maisey, H. C., Hensler, M., Nizet, V. & Doran, K. S. (2007). Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. *J Bacteriol.,* 189, 1464-1467.

Maniatis, A. N., Palermos, J., Kantzanou, M., Maniatis, N. A., Christodoulou, C. & Legakis, N. J. (1996). Streptococcus agalactiae: a vaginal pathogen? *J Med Microbiol,* 44, 199-202.

Manning, S. D., Lewis, M. A., Springman, A. C., Lehotzky, E., Whittam, T. S. & Davies, H. D. (2008). Genotypic diversity and serotype distribution of group B streptococcus isolated from women before and after delivery. *Clin.Infect Dis,* 46, 1829-1837.

Manning, S. D., Springman, A. C., Lehotzky, E., Lewis, M. A., Whittam, T. S. & Davies, H. D. (2009). Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. *J Clin.Microbiol,* 47, 1143-1148.

Marchaim, D., Efrati, S., Melamed, R., Gortzak-Uzan, L., Riesenberg, K., Zaidenstein, R. & Schlaeffer, F. (2006). Clonal variability of group B Streptococcus among different groups of carriers in southern Israel. *Eur.J Clin.Microbiol Infect Dis,* 25, 443-448.

Margarit, I., Rinaudo, C. D., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M., Buccato, S., Pagani, M., Tresoldi, E., Berardi, A., Creti, R., Baker, C. J., Telford, J. L. & Grandi, G. (2009). Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. *J Infect Dis,* 199, 108-115.

Martin, D., Rioux, S., Gagnon, E., Boyer, M., Hamel, J., Charland, N. & Brodeur, B. R. (2002). Protection from group B streptococcal infection in neonatal mice by maternal immunization with recombinant Sip protein. *Infect Immun.,* 70, 4897-4901.

Martins, E. R., Andreu, A., Correia, P., Juncosa, T., Bosch, J., Ramirez, M. & Melo-Cristino, J. (2011). Group B streptococci causing neonatal infections in Barcelona are a stable clonal population: 18-year surveillance. *J Clin.Microbiol,* 49, 2911-2918.

Martins, E. R., Melo-Cristino, J. & Ramirez, M. (2010). Evidence for rare capsular switching in Streptococcus agalactiae. *J Bacteriol.,* 192, 1361-1369.

Martins, E. R., Pessanha, M. A., Ramirez, M. & Melo-Cristino, J. (2007). Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. *J Clin.Microbiol,* 45, 3224-3229.

Matsubara, K., Katayama, K., Baba, K., Nigami, H., Harigaya, H. & Sugiyama, M. (2002). Seroepidemiologic studies of serotype VIII group B streptococcus in Japan. *J Infect Dis,* 186, 855-858.

Matsubara, K., Kawai, M., Nakahata, T., Kato, F., Tsukahara, H., Yamakawa, M., Hashimoto, K., Shimada, S., Maeda, S., Okumura, M. & Kanaoka, H. (2007). Procedures for prevention of perinatal group B streptococcal diseases: a multicenter questionnaire survey of hospitals in the Kyoto Neonatal Disease Study Group, Japan. *J Infect Chemother,* 13, 59-62.

Mavenyengwa, R. T., Afset, J. E., Schei, B., Berg, S., Caspersen, T., Bergseng, H. & Moyo, S. R. (2010). Group B Streptococcus colonization during pregnancy and maternal-fetal transmission in Zimbabwe. *Acta Obstet Gynecol Scand.,* 89, 250-255.

Mavenyengwa, R. T., Masunga, P., Meque, E., Kudinha, T., Moyo, S. R., Bevanger, L., Bergh, K., Nziramasanga, P. & Mapako, T. (2006). Streptococcus agalactiae (group B streptococcus (GBS)) colonisation and persistence, in pregnancy; a comparison of two diverse communities (rural and urban). *Cent.Afr.J Med,* 52, 38-43.

McKenna, D. S., Matson, S. & Northern, I. (2003). Maternal group B streptococcal (GBS) genital tract colonization at term in women who have asymptomatic GBS bacteriuria. *Infect Dis Obstet Gynecol,* 11, 203-207.

Moore, M. R., Schrag, S. J. & Schuchat, A. (2003). Effects of intrapartum antimicrobial prophylaxis for prevention of group-B-streptococcal disease on the incidence and ecology of early-onset neonatal sepsis. *Lancet Infect Dis,* 3, 201-213.

Mora, M., Bensi, G., Capo, S., Falugi, F., Zingaretti, C., Manetti, A. G., Maggi, T., Taddei, A. R., Grandi, G. & Telford, J. L. (2005). Group A Streptococcus produce pilus-like structures containing protective antigens and Lancefield T antigens. *Proc.Natl Acad.Sci U.S.A,* 102, 15641-15646.

Motlova, J., Strakova, L., Urbaskova, P., Sak, P. & Sever, T. (2004). Vaginal & rectal carriage of Streptococcus agalactiae in the Czech Republic: incidence, serotypes distribution & susceptibility to antibiotics. *Indian J Med Res,* 119 Suppl, 84-87.

Moyo, S. R., Maeland, J. A. & Bergh, K. (2002). Typing of human isolates of Streptococcus agalactiae (group B streptococcus, GBS) strains from Zimbabwe. *J Med Microbiol,* 51, 595-600.

Nagano, Y., Nagano, N., Takahashi, S., Murono, K., Fujita, K., Taguchi, F. & Okuwaki, Y. (1991). Restriction endonuclease digest patterns of chromosomal DNA from group B beta-haemolytic streptococci. *J Med Microbiol,* 35, 297-303.

Nahaei, M. R., Ghandchilar, N., Bilan, N. & Ghahramani, P. (2007). Maternal carriage and neonatal colonization of *Streptococcus agalactiae* in Tabriz, Northwest Iran. *Iran,Med.Sci,* 32, 177-181.

Nizet, V., Gibson, R. L., Chi, E. Y., Framson, P. E., Hulse, M. & Rubens, C. E. (1996). Group B streptococcal beta-hemolysin expression is associated with of lung epithelial cells. *Infection and Immunity,* 64, 3818-3826.

Nizet, V. (2002). Streptococcal beta-hemolysins: genetics and role in disease pathogenesis. *Trends Microbiol,* 10, 575-580.

Nobbs, A. H., Rosini, R., Rinaudo, C. D., Maione, D., Grandi, G. & Telford, J. L. (2008). Sortase A utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in Streptococcus agalactiae. *Infect Immun.,* 76, 3550-3560.

Nuccitelli, A., Cozzi, R., Gourlay, L. J., Donnarumma, D., Necchi, F., Norais, N., Telford, J. L., Rappuoli, R., Bolognesi, M., Maione, D., Grandi, G. & Rinaudo, C. D. (2011). Structure-based approach to rationally design a chimeric protein for an effective vaccine against Group B streptococcus infections. *Proc.Natl Acad.Sci U.S.A,* 108, 10278-10283.

Ojukwu, J. U., Abonyi, L. E., Ugwu, J. & Orji, I. K. (2006). Neonatal septicemia in high risk babies in South-Eastern Nigeria. *J Perinat.Med,* 34, 166-172.

Oladottir, G. L., Erlendsdottir, H., Palsson, G., Bjornsdottir, E. S., Kristinsson, K. G. & Haraldsson, A. (2011). Increasing incidence of late-onset neonatal invasive group B streptococcal infections in Iceland. *Pediatr.Infect Dis J,* 30, 661-663.

Orafu, C., Gill, P., Nelson, K., Hecht, B. & Hopkins, M. (2002). Perianal versus anorectal specimens: is there a difference in Group B streptococcal detection? *Obstet Gynecol,* 99, 1036-1039.

Osman, N. B., Folgosa, E. & Bergstrom, S. (1995). An incident case-referent study of threatening preterm birth and genital infection. *J Trop.Pediatr.,* 41, 267-272.

Palacios, G. C., Gonzalez, M. N., Beltran, M., Arredondo, J. L., Torres, J. & Solorzano, F. (2005). Serotypes of 286 group B streptococci isolated from asymptomatic carriers and invasive disease cases in Mexico. *Rev Latinoam.Microbiol,* 47, 21-24.

Palmeiro, J. K., De Carvalho, N. S., Botelho, A. C., Fracalanzza, S. E., Madeira, H. M. & la-Costa, L. M. (2011). Maternal group B streptococcal immunization: capsular polysaccharide (CPS)-based vaccines and their implications on prevention. *Vaccine,* 29, 3729-3730.

Perez-Ruiz, M., Rodriguez-Granger, J. M., Bautista-Marin, M. F., Romero-Noguera, J. & Rosa-Fraile, M. (2004). Genetic diversity of Streptococcus agalactiae strains colonizing the same pregnant woman. *Epidemiol.Infect,* 132, 375-378.

Persson, E., Berg, S., Trollfors, B., Larsson, P., Ek, E., Backhaus, E., Claesson, B. E., Jonsson, L., Radberg, G., Ripa, T. & Johansson, S. (2004). Serotypes and clinical manifestations of invasive group B streptococcal infections in western Sweden 1998-2001. *Clin.Microbiol Infect*, 10, 791-796.

Pezzicoli, A., Santi, I., Lauer, P., Rosini, R., Rinaudo, D., Grandi, G., Telford, J. L. & Soriani, M. (2008). Pilus backbone contributes to group B streptococcus paracellular translocation through epithelial cells. *J Infect Dis*, 198, 890-898.

Phares, C. R., Lynfield, R., Farley, M. M., Mohle-Boetani, J., Harrison, L. H., Petit, S., Craig, A. S., Schaffner, W., Zansky, S. M., Gershman, K., Stefonek, K. R., Albanese, B. A., Zell, E. R., Schuchat, A. & Schrag, S. J. (2008). Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. *JAMA*, 299, 2056-2065.

Poyart, C., Reglier-Poupet, H., Tazi, A., Billoet, A., Dmytruk, N., Bidet, P., Bingen, E., Raymond, J. & Trieu-Cuot, P. (2008). Invasive group B streptococcal infections in infants, France. *Emerg.Infect Dis*, 14, 1647-1649.

Poyart, C., Tazi, A., Reglier-Poupet, H., Billoet, A., Tavares, N., Raymond, J. & Trieu-Cuot, P. (2007). Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. *J Clin.Microbiol*, 45, 1985-1988.

Puopolo, K. M. & Madoff, L. C. (2007). Type IV neonatal early-onset group B streptococcal disease in a United States hospital. *J Clin.Microbiol*, 45, 1360-1362.

Puopolo, K. M., Madoff, L. C. & Eichenwald, E. C. (2005). Early-onset group B streptococcal disease in the era of maternal screening. *Pediatrics*, 115, 1240-1246.

Quinlan, J. D., Hill, D. A., Maxwell, B. D., Boone, S., Hoover, F. & Lense, J. J. (2000). The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. *J Fam.Pract.*, 49, 447-448.

Regan, J. A., Klebanoff, M. A. & Nugent, R. P. (1991). The epidemiology of group B streptococcal colonization in pregnancy. Vaginal Infections and Prematurity Study Group. *Obstet Gynecol,* 77, 604-610.

Rinaudo, C. D., Rosini, R., Galeotti, C. L., Berti, F., Necchi, F., Reguzzi, V., Ghezzo, C., Telford, J. L., Grandi, G. & Maione, D. (2010). Specific involvement of pilus type 2a in biofilm formation in group B Streptococcus. *Plos One,* 5, e9216.

Rosini, R., Rinaudo, C. D., Soriani, M., Lauer, P., Mora, M., Maione, D., Taddei, A., Santi, I., Ghezzo, C., Brettoni, C., Buccato, S., Margarit, I., Grandi, G. & Telford, J. L. (2006). Identification of novel genomic islands coding for antigenic pilus-like structures in Streptococcus agalactiae. *Mol.Microbiol,* 61, 126-141.

Sadowy, E., Matynia, B. & Hryniewicz, W. (2010). Population structure, virulence factors and resistance determinants of invasive, non-invasive and colonizing Streptococcus agalactiae in Poland. *J Antimicrob.Chemother,* 65, 1907-1914.

Salloum, M., van, d. M.-M., Valentin-Domelier, A. S. & Quentin, R. (2011). Diversity of prophage DNA regions of Streptococcus agalactiae clonal lineages from adults and neonates with invasive infectious disease. *Plos One,* 6, e20256.

Sastre, J. B., Colomer, B. F., Cotallo, G. D., Aparicio, A. R. & Grupo De hospitales Castrillo (2005). Trends in the epidemiology of neonatal sepsis of vertical transmission in the era of group B streptococcal prevention. *Acta Paediatr.,* 94, 451-457.

Sauer, F. G., Mulvey, M. A., Schilling, J. D., Martinez, J. J. & Hultgren, S. J. (2000). Bacterial pili: molecular mechanisms of pathogenesis. *Curr Opin.Microbiol,* 3, 65-72.

Savoia, D., Gottimer, C., Crocilla', C. & Zucca, M. (2008). Streptococcus agalactiae in pregnant women: phenotypic and genotypic characters. *J Infect,* 56, 120-125.

Schrag, S. J., Gorwitz, R., Fultz-Butts, K. & Schuchat, A. (2002a). Prevention of perinatal group B streptococcal disease. *MMWR Recomm Rep,* 51, 1-22.

Schrag, S. J., Zell, E. R., Lynfield, R., Roome, A., Arnold, K. E., Craig, A. S., Harrison, L. H., Reingold, A., Stefonek, K., Smith, G., Gamble, M. & Schuchat, A. (2002b). A population-based comparison of strategies to prevent early-onset group B streptococcal disease in neonates. *N.Engl.J Med,* 347, 233-239.

Schrag, S. J., Zywicki, S., Farley, M. M., Reingold, A. L., Harrison, L. H., Lefkowitz, L. B., Hadler, J. L., Danila, R., Cieslak, P. R. & Schuchat, A. (2000). Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. *N.Engl.J Med,* 342, 15-20.

Sensini, A., Tissi, L., Verducci, N., Orofino, M., von, H. C., Brunelli, B., Mala, G. L., Perocchi, F., Brunelli, R., Lauro, V., Ferrarese, R. & Gilardi, G. (1997). Carriage of group B streptococcus in pregnant women and newborns: a 2-year study at Perugia General Hospital. *Clin.Microbiol Infect,* 3, 324-328.

Seo, Y. S., Srinivasan, U., Oh, K. Y., Shin, J. H., Chae, J. D., Kim, M. Y., Yang, J. H., Yoon, H. R., Miller, B., Debusscher, J., Foxman, B. & Ki, M. (2010). Changing molecular epidemiology of group B streptococcus in Korea. *J Korean Med Sci,* 25, 817-823.

Seoud, M., Nassar, A. H., Zalloua, P., Boghossian, N., Ezeddine, J., Fakhoury, H., Abboud, J., Melki, I., Araj, G., Nacouzi, G., Sanyoura, M. & Yunis, K. (2010a). Prenatal and neonatal Group B streptococcus screening and serotyping in Lebanon: incidence and implications. *Acta Obstet Gynecol Scand.,* 89, 399-403.

Severin, W. P. (1972). Latex agglutination in the diagnosis of meningococcal meningitis. *J Clin.Pathol,* 25, 1079-1082.

Sharmila, V., Joseph, N. M., Arun, B. T., Chaturvedula, L. & Sistla, S. (2011). Genital tract group B streptococcal colonization in pregnant women: a South Indian perspective. *J Infect Dev.Ctries.,* 5, 592-595.

Shet, A. & Ferrieri, P. (2004). Neonatal & maternal group B streptococcal infections: a comprehensive review. *Indian J Med Res,* 120, 141-150.

Sigge, A., Schmid, M., Mauerer, S. & Spellerberg, B. (2008). Heterogeneity of hemolysin expression during neonatal Streptococcus agalactiae sepsis. *J Clin.Microbiol,* 46, 807-809.

Silverman, P. M. (1997). Towards a structural biology of bacterial conjugation. *Mol.Microbiol,* 23, 423-429.

Simoes, J. A., Alves, V. M., Fracalanzza, S. E., de Camargo, R. P., Mathias, L., Milanez, H. M. & Brolazo, E. M. (2007). Phenotypical characteristics of group B streptococcus in parturients. *Braz.J Infect Dis,* 11, 261-266.

Singleton, R. J., Hennessy, T. W., Bulkow, L. R., Hammitt, L. L., Zulz, T., Hurlburt, D. A., Butler, J. C., Rudolph, K. & Parkinson, A. (2007). Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA,* 297, 1784-1792.

Slotved, H. C., Elliott, J., Thompson, T. & Konradsen, H. B. (2003a). Latex assay for serotyping of group B streptococcus isolates. *J Clin.Microbiol,* 41, 4445-4447.

Slotved, H. C., Kong, F., Lambertsen, L., Sauer, S. & Gilbert, G. L. (2007). Serotype IX, a Proposed New Streptococcus agalactiae Serotype. *J Clin.Microbiol,* 45, 2929-2936.

Spellerberg, B. (2000). Pathogenesis of neonatal Streptococcus agalactiae infections. *Microbes Infect,* 2, 1733-1742.

Spratt, B. G., Hanage, W. P., Li, B., Aanensen, D. M. & Feil, E. J. (2004). Displaying the relatedness among isolates of bacterial species -- the eBURST approach. *FEMS Microbiol Lett.,* 241, 129-134.

Strakova, L. & Motlova, J. (2004). Active surveillance of early onset disease due to group B streptococci in newborns. *Indian J Med Res,* 119 Suppl, 205-207.

Strus, M., Pawlik, D., Brzychczy-Wloch, M., Gosiewski, T., Rytlewski, K., Lauterbach, R. & Heczko, P. B. (2009). Group B streptococcus colonization of pregnant women and their children observed on obstetric and neonatal wards of the University Hospital in Krakow, Poland. *J Med Microbiol,* 58, 228-233.

Suara, R. O., Adegbola, R. A., Baker, C. J., Secka, O., Mulholland, E. K. & Greenwood, B. M. (1994). Carriage of group B streptococci in pregnant Gambian mothers and their infants. *J Infect Dis,* 170, 1316-1319.

Suara, R. O., Adegbola, R. A., Mulholland, E. K., Greenwood, B. M. & Baker, C. J. (1998). Seroprevalence of antibodies to group B streptococcal polysaccharides in Gambian mothers and their newborns. *J Natl Med Assoc,* 90, 109-114.

Taha, M. K., Morand, P. C., Pereira, Y., Eugene, E., Giorgini, D., Larribe, M. & Nassif, X. (1998). Pilus-mediated adhesion of Neisseria meningitidis: the essential role of cell contact-dependent transcriptional upregulation of the PilC1 protein. *Mol.Microbiol,* 28, 1153-1163.

Tazi, A., Disson, O., Bellais, S., Bouaboud, A., Dmytruk, N., Dramsi, S., Mistou, M. Y., Khun, H., Mechler, C., Tardieux, I., Trieu-Cuot, P., Lecuit, M. & Poyart, C. (2010). The surface protein HvgA mediates group B streptococcus hypervirulence and meningeal tropism in neonates. *J Exp Med,* 207, 2313-2322.

Telford, J. L., Barocchi, M. A., Margarit, I., Rappuoli, R. & Grandi, G. (2006). Pili in gram-positive pathogens. *Nat Rev Microbiol,* 4, 509-519.

Terakubo, S., Ichiman, Y., Takemura, H., Yamamoto, H., Shimada, J. & Nakashima, H. (2003). [Serotypes and antibody levels of group B streptococci in pregnant women]. *Kansenshogaku Zasshi,* 77, 121-126.

Thigpen, M. C., Whitney, C. G., Messonnier, N. E., Zell, E. R., Lynfield, R., Hadler, J. L.,

Harrison, L. H., Farley, M. M., Reingold, A., Bennett, N. M., Craig, A. S., Schaffner, W., Thomas, A., Lewis, M. M., Scallan, E. & Schuchat, A. (2011). Bacterial meningitis in the United States, 1998-2007. *N.Engl.J Med,* 364, 2016-2025.

Tien, N., Ho, C. M., Lin, H. J., Shih, M. C., Ho, M. W., Lin, H. C., Lin, H. S., Chang, C. C. & Lu, J. J. (2011). Multilocus sequence typing of invasive group B streptococcus in central area of Taiwan. *J Microbiol Immunol Infect,* 44, 430-434.

Ton-That, H. & Schneewind, O. (2003). Assembly of pili on the surface of Corynebacterium diphtheriae. *Mol.Microbiol,* 50, 1429-1438.

Toresani, I., Limansky, A., Bogado, I., Guardati, M. C., Viale, A. & Sutich, E. G. (2001). Phenotypic and genotypic study of Streptococcus agalactiae in vagina of pregnant women in Argentina. *Medicina (B Aires),* 61, 295-300.

Trijbels-Smeulders, M. A., Kimpen, J. L., Kollee, L. A., Bakkers, J., Melchers, W., Spanjaard, L., Wannet, W. J. & Hoogkamp-Korstanje, M. A. (2006). Serotypes, genotypes, and antibiotic susceptibility profiles of group B streptococci causing neonatal sepsis and meningitis before and after introduction of antibiotic prophylaxis. *Pediatr.Infect Dis J,* 25, 945-948.

Trotter, C. L., Ramsay, M. E., Gray, S., Fox, A. & Kaczmarski, E. (2006). No evidence for capsule replacement following mass immunisation with meningococcal serogroup C conjugate vaccines in England and Wales. *Lancet Infect Dis,* 6, 616-617.

Tsolia, M., Psoma, M., Gavrili, S., Petrochilou, V., Michalas, S., Legakis, N. & Karpathios, T. (2003). Group B streptococcus colonization of Greek pregnant women and neonates: prevalence, risk factors and serotypes. *Clin.Microbiol Infect,* 9, 832-838.

Turner, C., Turner, P., Po, L., Maner, N., De, Z. A., Afshar, B., Efstratiou, A., Heath, P. T. & Nosten, F. (2012). Group B streptococcal carriage, serotype distribution and antibiotic susceptibilities in pregnant women at the time of delivery in a refugee population on the Thai-Myanmar border. *BMC Infect Dis,* 12, 34.

Uh, Y., Jang, I. H., Yoon, K. J., Lee, C. H., Kwon, J. Y. & Kim, M. C. (1997). Colonization rates and serotypes of group B streptococci isolated from pregnant women in a Korean tertiary hospital. *Eur.J Clin.Microbiol Infect Dis,* 16, 753-756.

Valkenburg-van den Berg AW, Sprij, A. J., Oostvogel, P. M., Mutsaers, J. A., Renes, W. B., Rosendaal, F. R. & Joep, D. P. (2006). Prevalence of colonisation with group B Streptococci in pregnant women of a multi-ethnic population in The Netherlands. *Eur.J Obstet Gynecol Reprod.Biol.,* 124, 178-183.

Vallejo, J. G., Baker, C. J. & Edwards, M. S. (1996). Roles of the bacterial cell wall and capsule in induction of tumor necrosis factor alpha by type III group B streptococci. *Infect Immun.,* 64, 5042-5046.

van, d. M.-M., Jouannet, C., Domelier, A. S., Arnault, L., Lartigue, M. F. & Quentin, R. (2009a). Genetic diversity of Streptococcus agalactiae strains and density of vaginal carriage. *J Med Microbiol,* 58, 169-173.

van, E. E., Yahiaoui, R., Visser, C., Oostvogel, P., Muller, A., Ho, Y. R., Wu, J. J. & van, B. A. (2009b). Epidemiology of and prenatal molecular distinction between invasive and colonizing group B streptococci in The Netherlands and Taiwan. *Eur.J Clin.Microbiol Infect Dis,* 28, 921-928.

Virji, M., Saunders, J. R., Sims, G., Makepeace, K., Maskell, D. & Ferguson, D. J. (1993). Pilus-facilitated adherence of Neisseria meningitidis to human epithelial and endothelial cells: modulation of adherence phenotype occurs concurrently with changes in primary amino acid sequence and the glycosylation status of pilin. *Mol.Microbiol,* 10, 1013-1028.

von, H. C., D'Ascenzi, S., Wagner, B., Jelinkova, J., Alfarone, G., Recchia, S., Wagner, M. & Orefici, G. (1993). Immunochemistry of capsular type polysaccharide and virulence properties of type VI Streptococcus agalactiae (group B streptococci). *Infect Immun.,* 61, 1272-1280.

Weiman, S., Uchiyama, S., Lin, F. Y., Chaffin, D., Varki, A., Nizet, V. & Lewis, A. L. (2010). O-Acetylation of sialic acid on Group B Streptococcus inhibits neutrophil suppression

and virulence. *Biochem.J,* 428, 163-168.

Weisner, A. M., Johnson, A. P., Lamagni, T. L., Arnold, E., Warner, M., Heath, P. T. & Efstratiou, A. (2004). Characterization of group B streptococci recovered from infants with invasive disease in England and Wales. *Clinical Infectious Disease,* 38, 1203-1208.

Wessels, M. R., Benedi, W. J., Jennings, H. J., Michon, F., DiFabio, J. L. & Kasper, D. L. (1989). Isolation and characterization of type IV group B streptococcus capsular polysaccharide. *Infect Immun.,* 57, 1089-1094.

Wessels, M. R., DiFabio, J. L., Benedi, V. J., Kasper, D. L., Michon, F., Brisson, J. R., Jelinkova, J. & Jennings, H. J. (1991). Structural determination and immunochemical characterization of the type V group B Streptococcus capsular polysaccharide. *J Biol.Chem.,* 266, 6714-6719.

Whitney, C. G., Daly, S., Limpongsanurak, S., Festin, M. R., Thinn, K. K., Chipato, T., Lumbiganon, P., Sauvarin, J., Andrews, W. & Tolosa, J. E. (2004). The international infections in pregnancy study: group B streptococcal colonization in pregnant women. *J Matern.Fetal Neonatal Med,* 15, 267-274.

Whitney, C. G., Farley, M. M., Hadler, J., Harrison, L. H., Bennett, N. M., Lynfield, R., Reingold, A., Cieslak, P. R., Pilishvili, T., Jackson, D., Facklam, R. R., Jorgensen, J. H. & Schuchat, A. (2003). Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N.Engl.J Med,* 348, 1737-1746.

WHO position paper (2006). Vaccine tetanus. *Weekly Epidemiol Rec,* 81, 198-208.

Yu, H. W., Lin, H. C., Yang, P. H., Hsu, C. H., Hsieh, W. S., Tsao, L. Y., Chen, C. H., Lin, H. C. & Tseng, Y. C. (2011). Group B Streptococcal Infection in Taiwan: Maternal Colonization and Neonatal Infection. *Pediatrics & Neonatology,* 52, 190-195.

Zaleznik, D. F., Rench, M. A., Hillier, S., Krohn, M. A., Platt, R., Lee, M. L., Flores, A. E., Ferrieri, P. & Baker, C. J. (2000a). Invasive disease due to group B streptococcus in pregnant

women and neonates from diverse population groups. *Clin.Infect Dis,* 30, 276-281.

Zaman, K., Roy, E., Arifeen, S. E., Rahman, M., Raqib, R., Wilson, E., Omer, S. B., Shahid, N. S., Breiman, R. F. & Steinhoff, M. C. (2008). Effectiveness of maternal influenza immunization in mothers and infants. *N.Engl.J Med,* 359, 1555-1564.

Zamzami, T. Y., Marzouki, A. M. & Nasrat, H. A. (2011). Prevalence rate of group B streptococcal colonization among women in labor at King Abdul-Aziz University Hospital. *Arch.Gynecol Obstet,* 284, 677-679.

Zhao, Z., Kong, F., Zeng, X., Gidding, H. F., Morgan, J. & Gilbert, G. L. (2008). Distribution of genotypes and antibiotic resistance genes among invasive Streptococcus agalactiae (group B streptococcus) isolates from Australasian patients belonging to different age groups. *Clin.Microbiol Infect,* 14, 260-267.

Appendices

Appendix A
Human researc ethics committe clearance certifcate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Madzivhandila

CLEARANCE CERTIFICATE	**PROTOCOL NUMBER M080366**
PROJECT	Serotype, Pilus Island Distribution and Molecular Epidemiology of Streptococcus agalactiae from Colonization and Invasive Disease
INVESTIGATORS	Mr M Madzivhandila
DEPARTMENT	School of Pathology
DATE CONSIDERED	08.03.25
DECISION OF THE COMMITTEE*	Approved unconditionally

Unless otherwise specified this ethical clearance is valid for 5 years and may be renewed upon application.

DATE 17/05/2010 **CHAIRPERSON** .....
(Professor P E Cleaton Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Dr P Adrian

DECLARATION OF INVESTIGATOR(S)

To be completed in duplicate and **ONE COPY** returned to the Secretary at Room 10004, 10th Floor, Senate House, University.
I/We fully understand the conditions under which I am/we are authorized to carry out the abovementioned research and I/we guarantee to ensure compliance with these conditions. Should any departure to be contemplated from the research procedure as approved I/we undertake to resubmit the protocol to the Committee. **I agree to a completion of a yearly progress report.**

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES...

Appendix B

Original Publications

OPEN ACCESS Freely available online

PLoS one

Serotype Distribution and Invasive Potential of Group B Streptococcus Isolates Causing Disease in Infants and Colonizing Maternal-Newborn Dyads

Mashudu Madzivhandila[1], Peter V. Adrian[1], Clare L. Cutland[1], Locadiah Kuwanda[1], Stephanie J. Schrag[2], Shabir A. Madhi[1]*

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Abstract

Background: Serotype-specific polysaccharide based group B streptococcus (GBS) vaccines are being developed. An understanding of the serotype epidemiology associated with maternal colonization and invasive disease in infants is necessary to determine the potential coverage of serotype-specific GBS vaccines.

Methods: Colonizing GBS isolates were identified by vaginal swabbing of mothers during active labor and from skin of their newborns post-delivery. Invasive GBS isolates from infants were identified through laboratory-based surveillance. GBS serotyping was done by latex agglutination. Serologically non-typeable isolates were typed by a serotype-specific PCR method. The invasive potential of GBS serotypes associated with sepsis within seven days of birth was evaluated in association to maternal colonizing serotypes.

Results: GBS was identified in 289 (52.4%) newborns born to 551 women with GBS-vaginal colonization and from 113 (5.6%) newborns born to 2,010 mothers in whom GBS was not cultured from vaginal swabs. The serotype distribution among vaginal-colonizing isolates was as follows: III (37.3%), Ia (30.1%), and II (11.3%), V (10.2%), Ib (6.7%) and IV (3.7%). There were no significant differences in serotype distribution between vaginal and newborn colonizing isolates (P = 0.77). Serotype distribution of invasive GBS isolates were significantly different to that of colonizing isolates (P<0.0001). Serotype III was the most common invasive serotype in newborns less than 7 days (57.7%) and in infants 7 to 90 days of age (84.3%; P<0.001). Relative to serotype III, other serotypes showed reduced invasive potential: Ia (0.49; 95%CI 0.31–0.77), II (0.30; 95%CI 0.13–0.67) and V (0.38; 95%CI 0.17–0.83).

Conclusion: In South Africa, an anti-GBS vaccine including serotypes Ia, Ib and III has the potential of preventing 74.1%, 85.4% and 98.2% of GBS associated with maternal vaginal-colonization, invasive disease in neonates less than 7 days and invasive disease in infants between 7–90 days of age, respectively.

Citation: Madzivhandila M, Adrian PV, Cutland CL, Kuwanda L, Schrag SJ, et al. (2011) Serotype Distribution and Invasive Potential of Group B Streptococcus Isolates Causing Disease in Infants and Colonizing Maternal-Newborn Dyads. PLoS ONE 6(3): e17861. doi:10.1371/journal.pone.0017861

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Competing Interests: S.A.M. is currently receiving research-grant support from Novartis in relation to GBS. All other authors declare that no competing interests exist.

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Introduction

Group B streptococcus (GBS) has been identified as a major cause of neonatal infection since the 1970s [1,2]. GBS acquisition by newborns from maternal recto-vaginal colonization is an established risk factor for GBS sepsis within the first 7 days of life [3]. Vertical acquisition of GBS, involving colonization of the skin or mucous membranes, occurs in 15% to 50% of newborns born to GBS colonized mothers. An estimated 1–2% of newborns colonized by GBS develop invasive disease [4,5].

Maternal vaccination against GBS, aimed at possibly reducing maternal colonization and enhancing transplacental transfer of anti-GBS antibody to the foetus, is being explored to prevent GBS associated sepsis during early infancy [6,7]. The success of maternal immunization in preventing young infant morbidity and mortality is best characterized by the success of tetanus immunization program and inactivated influenza vaccine studies during pregnancy [8,9].

GBS serotype specific polysaccharide-protein conjugate vaccine candidates, including serotypes Ia, Ib and III, have been evaluated for safety and immunogenicity [6,10]. These vaccines are expected to provide serotype specific protection, hence, the need to undertake serotype characterization of colonizing and invasive GBS isolates, and estimating the invasive potential of individual serotype. GBS serotyping is based on the identification of specific capsular polysaccharide (CPS) epitopes (Ia, Ib, II to IX) [11,12].

There are limited data on GBS serotype epidemiology associated with recto-genital maternal colonization or invasive disease in infants from industrializing countries.

We aimed to: i. study the serotype distribution of GBS isolates associated with colonization in mother-newborn dyads; ii. characterize the capsular serotype distribution of invasive isolates from infants; and iii. compare the relative invasive potential of GBS serotypes associated with early-onset disease.

Materials and Methods

Ethics Statement

Analysis of the GBS isolates for this study was approved by the Human Research Ethics Committee on Human Subjects (HREC) at the University of the Witwatersrand. Signed informed consent for collection of the isolates was obtained from study participants of the PoPS study, which was also approved by the HREC. The PoPS study was registered in ClinicalTrials.gov (Trial number: NCT00136370).

Study Population

The study was conducted at a secondary-tertiary care hospital (Chris Hani-Baragwanath Hospital; CHBH) which provides health care to an urban population of 1.5 million indigenous Africans living in Soweto, South Africa. There are approximately 28 000 births annually in Soweto, 75% are born at CHBH and the remainder at one of seven surrounding primary health care clinics. The majority of Sowetan children requiring hospitalization are admitted at CHBH.

GBS isolates from mother-newborn dyads were identified from participants involved in the Prevention of Perinatal Sepsis (PoPS) trial undertaken at CHBH between April 2004 to October 2007 as reported [13]. Briefly, mothers were randomized during active labor for intra-vaginal chlorhexidine washes (0.5%) or external genitalia wipes with water. Vaginal swabs were undertaken prior to initiation of any study procedure and swabs were obtained from the newborn's ears, nose and umbilicus shortly after birth. The swabs were transported in Amies medium and processed using standard microbiological assays as described [13]. The PoPS study indicated that intra-vaginal chlorhexidine wipes were not associated with any reduction in vertical acquisition of GBS in the newborns. [13] Serotyping in this study was, nevertheless, limited to mother-newborn dyads who had been randomized to the placebo arm of the PoPS trial.

In addition, prospective surveillance of early infant sepsis at CHBH was undertaken from January 2004 to December 2008. GBS isolates from blood and cerebrospinal fluid (CSF) from infants with invasive disease, identified as part of routine medical care, were retrieved from the laboratory. The isolates were stored at −70°C in a broth containing skim milk, tryptone, glucose and glycerol (STGG) at the Respiratory and Meningeal Pathogens Research Unit., Johannesburg, South Africa, where serotyping was subsequently undertaken.

Latex agglutination capsular serotyping

Capsular serotyping was performed with the latex agglutination method with specific antisera against types Ia, Ib and II to IX CPS antigens (Statens Serum Institute, SSI, Sweden) as previously described by Slotved *et al* [14]. Isolates that were reactive to sera against serotype III were further tested with sera against serotype VI to test for cross reactivity. Isolates that tested negative with all antisera were designated as serologically non-typeable and further typed by PCR. The GBS strains Ia (SSI-615), Ib (SS-618), II (SS-619), III (SS-620), IV (SS-1243), V (SS-1160), VI (SS-1354), VII (SS-1355), VIII (SS-1356), and IX (27412) were used as serotype specific control strains.

Molecular capsular typing

Isolates designated as non-typeable by latex agglutination were characterized by a molecular capsular typing assay. DNA was extracted with the QIAamp DNA mini kit (Qiagen GmbH, USA) as per manufacture's recommendation. Capsular typing was performed with a singleplex PCR method for serotypes Ia, Ib, II, III, IV and V using primer sequences reported by Poyart *et al* [15]. The gene encoding *dltb* was used as a positive control for GBS identification.

Statistical analysis

Data were analyzed using Graphpad Prism version 4.01 and STATA version 8.0. Serotype distribution between maternal and neonatal colonizing strains, and strains causing invasive disease was determined by a two tailed Fisher's exact test. Logistic regression was used to determine the association of frequency of serotypes in relation to the timing of onset of infant sepsis and disease syndrome.

As maternal colonization is a primarily risk factor for early onset (<7days of age) GBS disease, analyses of the invasive potential of GBS isolates was restricted to episodes within 7 days of birth and serotype associated with late onset (≥7 days of age) were excluded. Invasive potential of individual serotypes was estimated by calculating odds ratio (OR) using serotype III as a fixed reference serotype, since it has been consistently shown to be the most prevalent serotype in both colonizing and invasive disease isolates. This method has an advantage over calculating OR by reference to all other serotypes because the resulting estimate is a robust measure of invasive potential. The relative serotype-specific invasive potential was calculated based on the formula of odds ratio (OR) = (ad)/(bc) as described by Brueggemann *et al* [16]. Where "a" is the number of early onset isolates of a specific serotype; "b" is the number of early onset isolates of serotype III; "c" is the number of that specific serotype from maternal colonization isolates; and "d" is the number of serotype III from maternal colonization isolates. Associated 95% confidence intervals (95% CI) were estimated. Where two isolates of the same serotype were obtained from the same infant, only one isolate was included in the analysis.

Results

Overall, GBS was identified from the vagina of 551 (21.5%) of 2 561 mothers and from the skin/mucosal surface of 402 (15.8%) of 2 542 newborns born mothers who were swabbed. GBS was identified in 289 (52.5%) newborns of the 551 mothers who were identified to be vaginally colonized by GBS during labor. In addition, GBS was also identified in a further 113 (5.6%) newborns born to 2,010 mothers in whom GBS was not detected on vaginal swabbing.

A total of 284 GBS isolates were obtained from 282 infants with invasive disease. These included 222 from blood (77.5%), and 62 from CSF (21.1%). GBS isolates were obtained from blood and CSF from two infants. The age distribution of infants with invasive isolates included 137 (48.2%) under 7 days of age, 108 (38.0%) between 7 to 90 days of age and 39 (13.7%) from children older than 90 days of age.

Serotype distribution of maternal and newborn colonizing isolates

Serotyping was done on 541 (98.2%) of 551 available isolates obtained from colonized mothers and on 396 (98.5%) of 402

available isolates from colonized newborns. There were no differences in the serotype distribution (data not shown) between isolates from HIV-infected women (n = 119) compared to HIV-uninfected women (n = 418; P = 0.55). A total of 106 (19.6%) maternal and 76 (19.2%) newborn colonizing GBS isolates were serologically non-typeable (Table 1). Molecular capsular typing was successful in identifying a capsular serotype gene in 173 (95.1%) of the 182 serologically non-typable colonizing isolates (Table 1). The frequency of serotype distribution between serologically typeable isolates compared to those only typable by PCR differed as follows: Ia (32.4% vs. 14.8%, respectively; P<0.0001), Ib (2.4% vs. 23.1%, respectively; P<0.0001), II (13.3% vs. 4.4%, respectively; P = 0.0004), III (40.3% vs. 22.5%, respectively; P<0.0001); IV (1.3% vs. 15.4%, respectively; P<0.0001) and V (10.3% vs. 14.8%, respectively; P = 0.09).

Overall, of the colonizing isolates, serotype III was the most common in mothers (37.3%) and newborns (36.2%) (Table 1). Collectively, serotypes Ia, Ib and III accounted for 74.1% (401/541) of maternal and 69.6% (275/395) of newborn colonizing isolates. There were no differences in the relative frequencies of any of the serotypes between colonized mothers and colonized newborns. Isolates were available for serotyping from 280 (96.9%) of 289 mother-newborn pairs who were both colonized with GBS, among which there was 90.7% (254/280) concordance in serotype between the mother and newborn isolates. In addition, invasive GBS disease as a result of serotype III (n = 3) and Ia (n = 3) occurred in six newborns less than 7 days of age in whom colonizing isolates were available from the mother and the newborn, of which the invasive serotype was identical to the colonizing serotype in all of these newborns.

Serotype distribution of invasive GBS isolates

The dominant serotypes causing invasive disease in infants less than 7 days and in those aged between 7 to 90 days were serotype III (57.7% vs. 84.3%, respectively; P<0.0001) and serotype Ia (22.6% vs. 13.9%, respectively; P = 0.01). Together, these serotypes accounted for 80.3% of invasive GBS disease occurring in neonates less than 7 days of age and 98.2% of invasive disease occurring in infants aged 7 to 90 days. Serotypes III and Ia together accounted for 53.9% of invasive GBS isolates occurring in children older than 90 days of age. Individually, serotypes Ib, II, IV, and V accounted for less than 6% of invasive isolates in infants less than 7 days of age and in infants aged 7 to 90 days. No invasive isolate was reactive to sera against serotypes VI, VII, VIII and IX. After adjusting for the type of specimen from where the isolate was recovered (i.e. blood only vs. CSF) and age group, serotype III remained the dominant cause of invasive disease in infants aged 7 to 90 days (Adjusted odds ratio: 3.60; 95%CI 1.91–6.78; P<0.0001).

Serologically non-typeable isolates were less common from invasive GBS isolates (20/284; 7.0%) compared to maternal or newborn colonizing isolates. Genotypic serotyping by PCR was successful in attributing a serotype to all serologically non-typeable invasive isolates (Table 2).

Estimates of the relative invasive potential of serotypes

There were significant differences in the serotype distribution between invasive isolates from neonates younger than 7 days and maternal colonizing isolates. A higher proportion of newborn invasive isolates in those less than 7 days age were serotype III (79/137; 57.7%) compared to maternal colonizing isolates (202/537; 37.6%; P<0.0001). Conversely comparing invasive isolates in newborns under 7 days to maternal colonizing isolates, serotype II [7/137 (5.1%) *vs.* 61/537 (11.3%); respectively, P = 0.0008] and serotype V [8/137 (5.8%) *vs.* 55/537 (10.2%), respectively; P = 0.014] were less common as invasive isolates (Table 3).

Discussion

To our knowledge this study provides the most in-depth insight of the serotype epidemiology of colonizing isolates in mother-newborn dyads and invasive GBS isolates in an industrializing country setting, and particularly from Africa. Although there are extensive data on maternal colonizing serotype distribution from industrialized countries [17,18], there are limited data comparing serotype distribution from colonized mothers and their newborns from industrializing countries [19,20]. The findings from our study are similar to those reported elsewhere, [5,19,20] and confirm that maternal GBS vaginal-colonization is commonly (52.5%) associated with infant colonization. The vertical transmission of GBS from mother to the newborn was corroborated by the high concordance of matching serotypes in the colonized mother-

Table 1. Serotype distribution among maternal and newborn colonizing Group B streptococcus isolates.

Serotype	Maternal colonization isolates			Neonatal colonization isolates			P value Comparing total of maternal vs. newborn serotypes
	Latex no = 541 (%)	PCR[3] no = 106	Total no = 541	Latex no = 395	PCR no = 76	Total no = 395	
Ia	146 (27.0)[1]	17 (16.0)	163 (30.1)	98 (24.8)	10 (13.2)	108 (27.3)	P = 0.38
Ib	12 (2.2)	24 (22.6)	36 (6.7)	6 (1.5)	18 (23.7)	24 (6.1)	P = 0.79
II	55 (10.2)	6 (5.7)	61 (11.3)	45 (11.4)	2 (2.6)	47 (11.9)	P = 0.84
III	177 (32.7)	25 (23.6)	202 (37.3)	127 (32.2)	16 (21.1)	143 (36.2)	P = 0.73
IV	5 (0.9)	15 (14.2)	20 (3.7)	5 (1.3)	13 (17.1)	18 (4.6)	P = 0.51
V	40 (7.4)	15 (14.2)	55 (10.2)	38 (9.6)	12 (15.8)	50 (12.7)	P = 0.61
Non typeable	106 (19.6)	4 (3.8)		76 (19.2)	5 (6.6)		P = 0.22

[3]Molecular serotype identification by PCR was done by primers targeting serotypes Ia to V.

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Table 2. Serotype distribution among invasive disease isolates from early onset (EOS; <7 days of age), late onset diseases (LOD; 7 to 90 days of age) and in children older than 90 days.

Serotype	Early Onset no = 137	Late Onset no = 108	>90 days age no = 39	P value EOS vs LOD
Ia	31 [1][1] (22.6)[2]	15 [1] (13.9)	9 [0] (23.1)	P = 0.99
Ib	7 [2] (5.1)	0 [0] (0)	6 [2] (15.4)	P = 0.009
II	7 [2] (5.1)	0 [0] (0)	6 [2] (15.4)	P = 0.009
III	79 [3] (57.7)	91 [3] (84.3)	12 [1] (30.8)	P<0.0001
IV	5 [1] (3.6)	0 [0] (0)	0 [0] (0)	P = 0.07
V	8 [0] (5.8)	2 [1] (1.9)	6 [2] (15.4)	P = 0.19
NT	0	0	0	

[1]Figure in squared parenthesis indicates number of isolates which were serologically non-reactive but typed by PCR.
[2]Figure in rounded parenthesis is a percentage.
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newborn dyads. Our study, however, also identified GBS in 113 (28.1%) of 402 colonized newborns born to mothers in whom GBS was not identified by vaginal swabbing.

The latter, as well as the incomplete, albeit high, concordance in serotypes distribution associated with colonization in mother-newborn dyads in our study may be due to the limitations of the study in the sensitivity of identifying maternal colonization, limitations in detecting concurrent multiple colonizing serotypes, or possibly acquisition of GBS from non-maternal sources during or soon after birth [21]. The exclusion of rectal swabs to detect GBS colonization may have compromised the sensitivity in detecting GBS colonization [22]. Previous studies have reported that the sensitivity of detecting GBS colonization in pregnant women by undertaking both rectal and vaginal swabs is 18.5%–51% higher compared to taking vaginal swabs alone [23–27] and suggests that maternal rectal colonization may be important source for acquisition of GBS in newborns. Additionally, the yield of GBS on vaginal swabbing undertaken after the onset of labor may have been affected by draining liquor following the rupture of the placental membranes. Consequently, our study provides a minimal estimate as to the prevalence of GBS colonization in pregnant women during labor, as well as the proportion of colonized newborns who have acquired GBS from their mothers.

The serotype distribution identified in invasive isolates in our study are consistent with other smaller studies on invasive isolates elsewhere from Sub-Saharan Africa such as in Malawi [28], Zimbabwe [29], and an earlier study at the same centre as this study from 1997–1998. [30]) The serotype data of invasive isolates from this study are nevertheless important as it confirms the absence of temporal variation in serotype distribution of invasive isolates over more than a decade in the study-setting. [30] This has positive implications for the design of serotype-specific polysaccharide vaccines for prevention of newborn GBS sepsis.

Serotype III was responsible for 69.4% of invasive GBS disease among infants under the age of 90 days, which is in keeping with other studies from Sub Saharan Africa (56.0–58.3%) [28,30]. Serotype Ia was the second most frequently identified invasive serotype in infants under 90 days of age in our study (18.8%), consistent with previous studies from South Africa (28.7%) and Malawi (21%) [28,30]. These findings are also consistent with serotype distribution data of invasive isolates in young infants from industrialized countries (44% to 65% for serotype III and 33% to 15% for serotype Ia) [31–34]. Overall, 85.4% and 98.2% of invasive isolates in children less than 7 days and those aged 7 to 90 days of age, respectively belonged to serotypes Ia, Ib or III, which are currently being considered as serotypes to be included in a polysaccharide-protein conjugate vaccine against GBS [6,10]. Similarly a trivalent serotype-specific GBS vaccine including these serotypes would provide potential cover against at least 80% of invasive serotypes in young infants in USA and European countries [31–34].

Our data indicated that serotype III (OR = 2.29) was the most invasive of all studied serotypes based on a method of analysis described by Brueggemann *et al.* [35]. These data are also consistent with findings from Portugal (OR = 1.42) [34], Sweden (OR = 2.61) [31], and Israel (OR = 1.84) [36] where the same

Table 3. Estimation of invasive potential of Group B streptococcus (GBS) serotypes in different countries.

Serotype	Country					
	South Africa	Portugal [34]	Israel [36]	Sweden [31]	Netherlands [37]	Taiwan [37]
Ia	163[a]/31[b] (0.49; 0.31–0.77)[c]	42/13 (1.52; 0.63–3.67)	12/10 (1.14; 0.41–3.18)	15/20 (1.37; 0.61–3.10)	24/6 (0.52; 0.63–3.67)	13/5 (0.22; 0.07–0.70)
Ib	36/7 (0.50; 0.22–1.18)	14/1 (0.35; 0.04–2.93)	9/2 (0.30; 0.05–1.37)	15/2 (0.14 0.03–0.64)	ND	5/2 (0.22; 0.04–1.27)
II	61/7 (0.30 0.13–0.67)	46/8 (0.85; 0.32–2.27)	23/14 (0.83; 0.34–2.03)	13/4 (0.32; 0.09–1.07)	ND	ND
III	202/78 (1.00)	59/12 (1.00)	26/19 1.00	36/35 (1.00)	20/33 (1.00)	19/34 (1.00)
IV	(20/5) (0.65; 0.23–1.79)	6/1 (0.81; 0.09–7.44)	ND	3/2 (0.69; 0.11–4.36)	ND	ND
V	55/8 (0.38; 0.17–0.83)	59/7 (0.58; 0.21–1.59)	18/9 (0.68; 0.25–1.85)	25/7 (0.29; 0.11–0.75)	14/4 (0.17; 0.05–0.60)	15/2 (0.07; 0.02–0.36)

In our data two isolates of the same serotype (i.e III) were obtained from the same infant, and only one isolate was included in the analysis.
[a]Value indicates colonizing isolates.
[b]Value indicates neonatal invasive isolates.
[c]Value in parenthesis indicates OR and 95% CI.
[d]ND: No data for either invasive, colonization or both isolates.
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method was used. Due to this consistency we benchmarked invasive potential of other serotypes with type III as a referent serotype [16] as summarized in Table 3. The data consistently reports that serotype Ib, II and V, are less invasive than serotype III. However, the invasive potential of serotype Ia is variable across different sites. In South Africa, Taiwan and The Netherlands [37], serotype Ia was less invasive than type III, although more invasive in Portugal [34], Israel [36] and Sweden [31]. The reasons for variation in invasive potential of serotype Ia reported at different sites are unclear, however data from Portugal suggests that the high invasive index of this serotype can be attributed to a dominant clone (ST-23 and ST-24), suggesting that the underlying genotype can influence the invasive potential.

Serotype V was identified in a low proportion of invasive isolates (5.6%) in our study, which is consistent with earlier studies from South Africa (5.6%) and Malawi (4.0%) [28,30]. In the United States, serotype V is responsible for a high proportion of the invasive cases among infants (28%) [38]. Similarly, a higher prevalence of serotype V has also been reported in studies from England and Wales (13%) [39].Recent identification of these historically uncommon circulating strains in both colonization and invasive disease isolates raises questions as to whether introduction of a vaccine composed of the most common invasive serotypes could cause a shift in serotype distribution in colonization, and thus disease. This warrants the need for ongoing international surveillance of invasive GBS serotypes in order to optimize GBS vaccine formulations.

Our study demonstrated differences between serotype epidemiology of isolates associated with invasive disease in newborns compared to maternal colonizing isolates. These findings corroborate the finding of other studies from industrialized country settings [31,34,36,37]. Our study, however, clarified that the difference in serotype distribution between invasive and colonizing isolates is related to an increased invasive potential of certain GBS serotypes (i.e. III and Ia) rather than due to increased risk of acquisition of these by the newborn from the mother.

Despite the success of intrapartum antibiotic prophylaxis in reducing GBS associated neonatal sepsis in industrialized countries, the high costs and resource intensiveness is prohibitive in industrializing countries [40]. Further limitations to this intervention strategy include concerns about the emergence of antimicrobial resistance in GBS, and a lack of efficacy in reducing GBS sepsis in infants older than 7 days of age [41]. As a result, neonatal morbidity due to GBS remains a public-health problem. In Sub-Saharan Africa GBS neonatal disease occurs in 3.06 per 1,000 live births in South Africa [30], and 1.81 per 1,000 live births in Malawi [28]. Consequently, the agenda of maternal immunization with GBS vaccines aimed at either reducing maternal recto-anal colonization, or preventing disease in the newborns through transplacental antibody transfer, needs to be explored in settings with a high burden of GBS disease.

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Author Contributions

Conceived and designed the experiments: SAM PVA. Performed the experiments: MM PVA. Analyzed the data: MM PVA LK. Contributed reagents/materials/analysis tools: SJS. Undertook the serologic and molecular serotyping of isolates, contributed to the analyses and drafted the initial manuscript: MM PVA. Was involved in sample collection and contributed to the manuscript write-up: CLC. Assisted in data analysis and manuscript write-up: LK. Provided GBS control strains and contributed to the manuscript write-up: SJS. Conceived the study, supervised the overall project and contributed to data analysis and write-up of the manuscript: SAM PVA. All authors read and approved the final manuscript.

References

1. Behrman RE (1973) The new challenge in neonatal infections. Fetal and Neonatal Medicine 82: 705–706.
2. Lancefield RC (1934) Serological differentiation of specific types of bovine haemolytic streptococci (Group B). J Exp Med 59: 458.
3. CDC (2002) Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. Morb Mortal Recomm Rep 51: 1–22.
4. Beal S, Dancer S (2006) Antenatal prevention of neonatal group B streptococcal infection. Gynaecological and Perinatal Practice 6: 218–225.
5. Eren A, Kucukercan M, Oguzoglu N, Unal N, Karateke A (2005) The carriage of group B streptococci in Turkish pregnant women and its transmission rate in newborns and serotype distribution. The Turkish Journal of Pediatrics 13: 28–33.
6. Baker CJ, Rench MA, McInnes P (2003) Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. Vaccine 21: 3468–3472.
7. Baker CJ, Edwards MS (2003) Group B streptococcal conjugate vaccines. Archives of Disease in Childhood 88: 375–378.
8. WHO position paper (2006) Vaccine tetanus. Weekly Epidemiol Rec 81: 198–208.
9. Zaman K, Roy E, Arifeen SE, Rahman M, Raqib R, et al. (2008) Effectiveness of maternal influenza immunization in mothers and infants. New England Journal of Medicine 359: 1555–1564.
10. Baker CJ, Paoletti LC, Wessels MR, Guttormsen H-K, Rench MA, et al. (1999) Safety and immunogenicity of capsular polysaccharide-tetanus toxoid conjugate vaccine for group B streptococcal type Ia and Ib. Journal of Infectious Disease 179: 142–150.
11. Buttery J, Moxon R (2002) Capsulate bacteria and the lung. British Council 61: 63–80.
12. Slotved H-C, Kong F, Lambertsen L, Sauer S, Gilbert GL (2007) Serotype IX, a proposed new *Streptococcus agalactiae* serotype. Journal of Clinical Microbiology 45: 2929–2936.
13. Cutland CL, Madhi SA, Zell ER, Kuwanda L, Laque M, et al. (2009) Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised controlled trial. Lancet 6736: 1–8.
14. Slotved H-C, Elliot J, Thompson T, Konradsen HB (2003) Latex assay for serotyping of group B streptococcal isolates. Journal of Clinical Microbiology 41: 4445–4447.
15. Poyart C, Tazi A, Poupet-Reglier H, Billoet A, Tavares N, et al. (2007) Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. Journal of Clinical Microbiology 45: 1985–1988.
16. Brueggemann AB, Peto TEA, Crook DW, Butler JC, Kristinsson JG, et al. (2004) Temporal and geographic stability of the serogroup-specific invasive disease potential of *Streptococcus pneumoniae* in children. Journal of Infectious Disease 190: 1203–1211.
17. Amin A, Abdulrazzaq YM, Uduman S (2002) Group B streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. Journal of Infection 45: 42–46.
18. Brimil N, Barthell E, Heindrichs U, Kuhn M, Lutticken R, et al. (2006) Epidemiology of *Streptococcus agalactiae* colonization in Germany. International Journal of Medical Microbiology 296: 39–44.
19. Al-Sweih N, Hammoud M, Al-Shimmiri M, Jamal M, Neil L, et al. (2005) Serotype distribution and mother-to-baby transmission rate of *Streptococcus agalactiae* among expectant mother in Kuwait. Archives of Gynecology and Obstetrics 272: 131–135.

20. Sunan RO, Adegbola RA, Baker CJ, Secka O, Mulholland EK, et al. (1994) Carriage of group B streptococci in pregnant Gambian mothers and their infants. Journal of Infectious Disease 170: 1316–1319.
21. Szuta M, Pawlik D, Brzychczy-Włoch M, Gosiewski T, Rybiewski K, et al. (2009) Group B streptococcus colonization of pregnant women and their children observed on obstetric and neonatal wards of the University hospital in Krakow, Poland. Journal of Medical Microbiology 58: 228–233.
22. Quinn JD, Hill A, Maxwell BD, Boone S, Hoover F, et al. (2000) The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. Journal of Medical Microbiology 49: 447–448.
23. Baldi MS, Zawaneh S, Cruz AC, Manzilla G, Baer H, et al. (1977) Rectal colonization with group B streptococcus: relation to vaginal colonization of pregnant women. Journal of Infectious Disease 135: 308–312.
24. Dillon HC, Gray E, Pass MA, Gray BM (1982) Anorectal and vaginal carriage of group B streptococci during pregnancy. Journal of Infectious Disease 145: 794–799.
25. Hoogkam-Korstanje JA, Gerards LJ, Cats BP (1982) Maternal carriage and neonatal acquisition of group B streptococci. Journal of Infectious Disease 145: 800–805.
26. Kovavisarach E, Sa-adying W, Kanjanaharenai S (2007) Comparisons of combined vaginal-anorectal, vaginal and anorectal cultures in the detecting of group B streptococci in pregnant women in labor. Journal of the Medical Association of Thailand (Chotmaihet thangphaet) 90: 1710–4.
27. Quinlan JD, Hill A, Maxwell B, Boone S, Hoover F, et al. (2000) The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. The Journal of Family Practice 49: 448.
28. Gray KJ, Bennet SL, French N, Phiri AJ, Graham SM (2007) Invasive group B streptococcal infection in infants, Malawi. Emerging Infectious Disease 13: 223–229.
29. Moon SR, Macland JA, Bergh K (2002) Typing of human isolates of *Streptococcus agalactiae* (group B streptococcus, GBS) strains from Zimbabwe. Journal of Medical Microbiol 51: 595–600.
30. Madhi SA, Radebe K, Crewe-Brown H, Frasch CE, Arakere G, et al. (2003) High burden of invasive Streptococcus agalactiae disease in South African infants. Annals of Tropical Paediatrics 23: 13–23.
31. Berg S, Trollfors B, Lagergard T, Zackrisson G, Claesson BA (2000) Serotype and clinical manifestation of Group B streptococcal infections in Western Sweden. Clinical Microbiology and Infectious Disease 6: 9–13.
32. Flurgge K, Supper S, Siedler A, Berner R (2005) Serotype distribution of invasive group B streptococcal isolates in infants: Results from a nationwide active laboratory surveillance study over 2 years in Germany. Clinical Infectious Disease 40: 760–765.
33. Harrison LH, Elliot JA, Dwyer DM, Libonati JP, Ferrieri P, et al. The Maryland Emerging Infectious Program (1998) Serotype distribution of invasive group B streptococcal isolates in Maryland: Implications for vaccine formulation. Journal of Infectious Disease 177: 998–1002.
34. Martins ER, Pessanha MA, Ramirez M, Melo-Cristino J, The Portuguese group for the study of streptococcal infections (2007) Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. Journal of Clinical Microbiology 45: 3224–3229.
35. Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, et al. (2003) Clonal relationship between invasive and carriage *Streptococcus Pneumoniae* and serotype- and clone-specific differences in invasive disease potential. Journal of Infectious Disease 187: 1424–1432.
36. Bisharat N, Jones N, Marchaim D, Block C, Harding RM, et al. (2005) Population structure of group B streptococcus from a low incidence region for invasive neonatal disease. Microbiology 151: 1873–1881.
37. van Elzakker E, Yahiaoui R, Visser C, Oostvogel P, Muller A, et al. (2009) Epidemiology of and prenatal molecular distinction between invasive and colonizing group B streptococci in The Netherlands and Taiwan. Eur J Clin Microbiol Infect Dis 28: 923–928.
38. Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, et al. (2008) Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Journal of American Medical Association 229: 2056–2065.
39. Weisner AM, Johnson AP, Lamagni TL, Arnold E, Warner M, et al. (2004) Characterization of group B streptococci recovered from infants with invasive disease in England and Wales. Clinical Infectious Disease 38: 1203–1208.
40. Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, et al. (2010) Global, regional, and national causes of child mortality in 2008: a systemic analysis. Lancet 6736: 39–47.
41. Moore MR, Schrag SJ, Schuchat A (2003) Effects of intrapartum antimicrobial prophylaxis for prevention of group B streptococcal disease on the incidence and ecology of early-onset neonatal sepsis. Lancet Infectious Disease 2: 201–213.

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Distribution of pilus islands of group B streptococcus associated with maternal colonization and invasive disease in South Africa

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Group B streptococcus (GBS) is a leading cause of neonatal sepsis. Sortase-dependent pilus-like structures have been identified on the surface of GBS, and have been found to be important in the adhesion and attachment of GBS to host cells. Three pilus island alleles, PI-1, PI-2a and PI-2b, have been described, and their proteins are being explored as vaccine candidates. The pilus islands from 541 colonization isolates and 284 invasive isolates were characterized by PCR. All isolates carried at least one pilus island, and they were identified alone or in combinations at the following overall frequencies: PI-2a, 29.8 %; PI-2b, 0.2 %; PI-1 + PI-2a, 24.8 %; and PI-1 + PI-2b, 45.1 %. A combination of PI-1 + PI-2a (28.7 vs 17.6 %) was more common among colonizing compared with invasive isolates. Conversely, a combination of PI-1 + PI-2b (37.2 vs 60.2 %) was more frequently associated with invasive disease compared to colonization. There was a strong association between pilus islands when adjusted for serotype distribution, PI-2a was identified in 92.6 % of colonizing and 90.0 % of invasive serotype Ia isolates, whereas serotype III was associated with co-expression of a PI-1 and PI-2b among 84.6 % of colonizing and 96.5 % of invasive isolates. Based on this homogeneity of pilus island distribution, a pilus-based vaccine developed for Europe and the USA will have similar coverage in South Africa.

INTRODUCTION

Group B streptococcus (GBS) is a commensal bacterium of both the genito-urinary and gastrointestinal tract, and a leading cause of neonatal invasive disease (Ho *et al.*, 2006; Qinlan *et al.*, 2000; Schuchat, 1998). Development of a GBS vaccine requires identification and characterization of antigens from GBS isolates from geographically diverse settings to determine the optimal vaccine formulation candidate antigens (Lauer *et al.*, 2005; Maione *et al.*, 2005). The roles of external structures or surface molecules on GBS biology and pathogenesis, and their potential as vaccine candidates, are being explored (Baker & Edwards, 2003; Larsson *et al.*, 1999; Maione *et al.*, 2005; Margarit *et al.*, 2009).

Genome-wide analysis of eight GBS strains led to the identification of genomic regions whose genes encode pilus structures that extend beyond the bacterial cell-wall peptidoglycan and the capsular polysaccharide (Dramsi *et al.*, 2006; Lauer *et al.*, 2005; Rosini *et al.*, 2006). Pili are formed from polymerized proteins, which have an N-terminal conserved signal peptide and a C-terminal LPXTG motif; the LPXTG domain enabling covalent attachment of the pilus proteins to the cell-wall peptidoglycan (Lalioui *et al.*, 2005; Nobbs *et al.*, 2008; Ton-That & Schneewind, 2004). GBS pili have been described as contributing to GBS adhesiveness and supporting the transcytoses of the bacteria through differentiated human epithelial cells (Pezzicoli *et al.*, 2008).

Three pilus islands (PI-1, PI-2a and PI-2b) have been described (Lauer *et al.*, 2005; Rosini *et al.*, 2006). A published report of isolates collected from the USA and Europe suggested that all GBS isolates have either PI-2a or PI-2b present, and that many isolates carry the additional PI-1 (Margarit *et al.*, 2009). Identification of these surface exposed antigens that are conserved in the majority of GBS strains has raised optimism that a pilus-based vaccine can be developed as an alternative to or complement GBS polysaccharide-protein conjugate vaccines (Margarit *et al.*, 2009). The aim of this study was to determine the prevalence of pilus islands in GBS isolates associated with maternal vaginal colonization and invasive disease in infants from an African setting.

Abbreviations: 95 %CI, 95 % confidence interval; GBS, group B streptococcus; HREC, Human Research Ethics Committee; PoPS, prevention of perinatal sepsis.

METHODS

Bacterial isolates. The study involved characterization of 541 vaginal-colonizing GBS isolates identified in women during labour who had participated in a study on 'Prevention of Perinatal Sepsis (PoPS)' between 2005 and 2007 as described previously (Cutland *et al.*, 2009). In addition, 284 invasive GBS isolates including 222 (78.2 %) from blood and 62 (21.8 %) from cerebrospinal fluid were obtained from 282 infants with invasive disease hospitalized at Chris-Hani Baragwanath Hospital (CHBH), Soweto, South Africa, between 2004 and 2008. Of the invasive isolates 137 (48.2 %) were obtained from infants less than 7 days old (early onset), 108 (38.0 %) were from infants between 7 and 90 days old (late onset) and 39 (13.7 %) were from infants older than 90 days. All the isolates had been archived in skim milk, tryptone, glycerol and glucose broth (STGG) at −70 °C at the Respiratory and Meningeal Pathogens Research Unit (RMPRU), Johannesburg, South Africa. Serotyping of the isolates was determined as described previously (Madzivhandila *et al.*, 2011). GBS reference strains 2603 V/R (PI-1 and PI-2a) and COH1 (PI-2b) were obtained from the American Type Culture Collection (ATCC).

Primer and probe design. Nucleotide sequences of complete and incomplete GBS genomes were obtained from the open genome resources (OGeR) database (http://oger.tu-bs.de) (Klein *et al.*, 2009) and additional sequences of pilus island genes were obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/). Sequence identity measurements and alignments were performed with BioEdit software version 5.0.9. Ancillary protein 1 (AP1) was selected as a real-time PCR target to allow identification and discrimination between the pilus islands. Isolates that tested negative for all the AP1 genes, or isolates from which PI-2a and PI-2b could not be detected, were reamplified by conventional PCR with a second set of primers representing conserved regions of AP2. Primers and *Taq*-Man probes were designed with the Primer Express 3.0 software (Applied Biosystems). Primer and probe sequences and reaction concentrations are listed in Table 1.

PCR. Frozen bacterial isolates were subcultured on CNA (colistin nalidixic acid) agar and incubated overnight at 37 °C in 5% CO_2. One GBS colony was suspended in 300 µl nuclease-free distilled H_2O and the suspension was heated at 95 °C for 10 min. The tubes were centrifuged at 9000 *g* for 1 min to pellet the cell debris. Four microlitres of the supernatant was added to each PCR. PCRs were run on an AB 7500 instrument (Applied Biosystems) in a 25 µl reaction volume with TaqMan universal PCR master mix without AmpErase UNG (Applied Biosytems). The detection of PI-2b was performed as a singleplex reaction, and PI-1 and PI-2a were detected in duplex. AP2 was detected by standard PCR and PCR products were detected by agarose gel electrophoresis.

Statistical analysis. Data were analysed with GraphPad Prism version 4.01 and STATA version 8.0. Distribution of pilus islands in maternal colonizing isolates and invasive isolates was determined by a two-tailed Fisher's exact test. A logistic regression model was used to test for the association between pilus islands in relation to serotype and the disease onset. A X^2 test was used to compare pilus island distribution in infants aged younger than 7 days, between 7 and 90 days, and older than 90 days. A P value of <0.05 was considered significant.

Ethical considerations. Analysis of the GBS isolates for this study was approved by the Human Research Ethics Committee (HREC) on Human Subjects at the University of the Witwatersrand (HREC reference no. M080366). Signed informed consent for collection of the colonization isolates was obtained from study participants of the PoPS study (HREC reference no. 030207) and approval to archive neonatal sepsis isolates from a surveillance study (HREC reference no. M03-10-07).

RESULTS

The real-time PCR assay was able to detect the AP1 gene for PI-2a and PI-2b in 49 and 36 % of isolates, respectively. Additional screening with PCR assays for the AP2 gene of PI-2a and PI-2b negative isolates, revealed that all isolates harboured either PI-2a or PI-2b in 55 and 45 % of isolates, respectively. PI-1 was found in 70 % of all isolates, and was associated with PI-2a 45 % of the time. In contrast, the

Table 1. Oligonucleotide sequences of the primers and probes for detection AP1 and BP

Gene/pilus protein	Sequence (5′–3′)*	Primer concn (nM)
PI-1AP1	Forward GGTGAAACCCAAGATACCAATCA	110
	Reverse CTTTGCCTAATGGTGTAGCATTGT	40
	Probe 6-FAM CACTTGGAAAAGTAATTG-MGB	250
PI-2aAP1	Forward ACTGCGGTCCCAAGAGCTT	40
	Reverse GATGGCCCAAATTTTCAAAGG	110
	Probe VIC-AGCTTTCTTGGCTTTATT-MGB	250
PI-2bAP1	Forward TCTATGCAGGTTGGCAAAAGGTA	900
	Reverse TCGGCCTCCGTTTGGA	900
	Probe NED-CATATCGTGTCAATATTG-MGB	250
PI-1AP2	Forward CCGGGCTCATCCATGGGGACT	300
	Reverse ACAGCAGTCTCTGCCTGTCCGA	300
PI-2aAP2	Forward ATGGTTCGTACTTTGGTCGTGCTT	300
	Reverse TCGGCCTGTTTCAACTTTTCGCT	300
PI-2bAP2	Forward ACCGGCTGAAGGTATTGTTGCGA	300
	Reverse GCCACCATACTTGTCCAGTAAACGG	300

6-FAM, 6-Carboxyfluorescein; MGB, minor groove binder.
*FAM, NED and VIC are fluorescent dyes (Applied Biosystems).

Table 2. Pilus island distribution among invasive disease and colonization GBS isolates grouped by serotype

Serotype	Pilus island							
	PI-2a		PI-2b		PI-1+PI-2a		PI-1+PI-2b	
	Col	ID	Col	ID	Col	ID	Col	ID
Ia	151	50	0	0	7	4	5	1
Ib	0	6	0	0	31	6	5	1
II	14	2	1	0	41	11	5	0
III	0	0	0	0	32	17	170	165
IV	7	0	0	0	2	1	11	4
V	10	5	1	0	39	11	5	0
NT	1	0	0	0	3	0	0	0
Total	183	63	2	0	155	50	201	171

Col, Colonization isolates; ID, Invasive disease isolates; NT, nontypable.

majority (99.5 %) of PI-2b positive isolates were associated with PI-1 (Table 2).

Pilus islands in colonization isolates were identified alone or in combinations at the following overall hierarchical frequency: PI-1+PI-2b (n=201; 37.2 %), PI-2a (n=183; 33.8 %), PI-1+PI-2a (n=155; 28.7 %) and PI-2b (n=2; 0.4 %) (Fig. 1). More than one type of pilus island was identified in 356 (65.9 %) of colonizing isolates. Pilus islands in invasive disease isolates were identified at the following overall frequency PI-1+PI-2b (n=171; 60.2 %), PI-2a (n=63; 22.2 %), PI-1+PI-2a (n=50; 17.6 %). More than one type of pilus island was identified in 221 (77.8 %) of invasive disease isolates (Fig. 1).

There were significant differences when the pilus island distributions were stratified by colonization or invasive disease. A combination of PI-1 and PI-2b was more common among invasive disease isolates (171/284; 60.2 %) than colonizing isolates (201/541; 37.2 %; P<0.0001), and isolates with both PI-1 and PI-2a were more common in colonizing (155/541; 28.7 %) than invasive isolates (50/284; 17.6 %; P=0.0005). However when adjusted for serotype distribution, these differences were not significant.

When the pilus island distribution in invasive isolates was stratified according to age of onset, the differences were significant in infants aged younger than 7 days, and those between 7 and 90 days. A combination of PI-1 and PI-2b was significantly carried by infants aged between 7 and 90 days (54.7 vs 80.6 %; P<0.0001), whilst PI-2a alone or in combination with PI-1 were significantly associated with infants aged younger than 7 days (31.4 vs 17.6 % P=0.02 and 13.9 vs 1.9 % P=0.0008, respectively) (Fig. 1). The distribution of pilus islands alone or in combination from infants older than 90 days were as follows PI-2a (33.3 %), PI-1+PI-2a (38.5 %), PI-1+PI-2b (28.2 %) (Fig. 1).

When the differences in pilus island distribution were adjusted with respect to the differences in the serotype distribution, there were significant associations between the capsular serotype and pilus island combinations

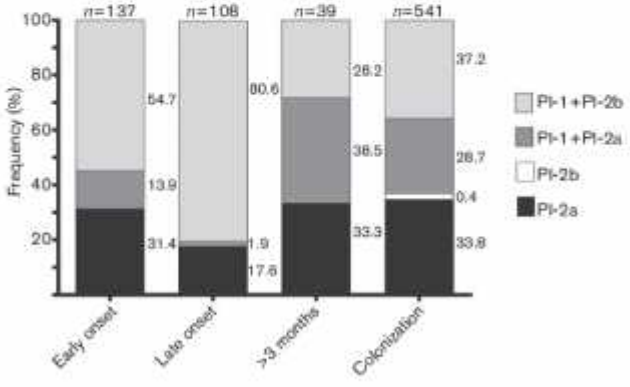

Fig. 1. Group B streptococcal pilus islands grouped by source of isolation.

irrespective of whether isolates were from a colonization or invasive group (Table 2). The majority of colonization (84.2 %, $P<0.0001$) and invasive disease (90.7 % $P<0.0001$) isolates representing capsular serotype III carried a combination of PI-1 and PI-2b. Similarly PI-2a alone was frequently carried by 92.6 and 90.9 % of colonization and invasive disease isolates representing serotype Ia, respectively. In isolates that carried a combination of PI-1 and PI-2a, serotype Ib, II and V accounted for 71.6 % of colonization and 56.0 % of invasive disease isolates. All but one non-typable colonization isolate carried a combination of PI-1 and PI-2a (Table 2).

When pilus island distribution was adjusted with respect to serotype and the disease onset, serotype was the dominant predictor in the distribution of PI-2a alone [adjusted odds ratio 0.38; 95 % confidence interval (95 %CI) 0.3–0.5; $P<0.0001$] or combination of PI-1 and PI-2b (adjusted odds ratio 2.55; 95 %CI 2.00–3.26; $P<0.0001$). A combination of PI and PI-2a showed no significant association with serotype when adjusted with respect to serotype and the disease onset (adjusted odds ratio 1.25; 95 %CI 0.94–1.66; $P=0.12$).

DISCUSSION

This is, to the best of our knowledge, the first study describing the prevalence of pilus islands in isolates from vaginally colonized mothers, and infants with invasive disease, collected outside of Europe and the USA (Margarit *et al.*, 2009; Martins *et al.*, 2010). There are currently no published data on the pilus island distribution from sub-Saharan Africa where GBS remains the most frequently isolated pathogens in neonates (Berkley *et al.*, 2005; Gray *et al.*, 2007; Madhi *et al.*, 2003; Milledge *et al.*, 2005). Our data were consistent with previous studies from industrialized countries in that all isolates were associated with the presence of either PI-2a or PI-2b. PI-1 and PI-2b were almost always present together, and the presence of PI-1 in PI-2a harbouring isolates was largely dependent on serotype, with the majority of serotype Ib, II and V isolates harbouring these two pilus islands together, whereas the majority of type Ia isolates were associated PI-2a on its own (Margarit *et al.*, 2009; Martins *et al.*, 2010). This is, to the best of our knowledge, the first study from a developing country wherein a substantial population of colonization and invasive disease isolates has been analysed, allowing better comparisons between strains from invasive disease and maternal colonization.

Although there were differences in the pilus island distribution between colonization and invasive disease isolates, and with respect to the age of onset of invasive disease, when the groups were adjusted for differences in serotype distribution within these groups there were no significant differences in the pilus island distribution. This can be attributed to the stable relationship between serotype and pilus island combinations, and suggests that the difference in pilus island distribution between colonizing and invasive disease isolates was due to the difference in the relative invasive potential of different serotypes (Madzivhandila *et al.*, 2011). This is similar to what has been reported by Margarit *et al.* (2009) where it was shown that the relationship between pilus island and serotype was conserved irrespective of whether isolates were from a colonizing or invasive group.

Our PCR, which targeted conserved regions of AP1, failed to detect either of the PI-2 islands in 15 % of all isolates. High levels of sequence variability have been reported in PI-2a and to a lesser extent in PI-2b by Margarit *et al.* (2009), and we demonstrated that screening for additional target genes, such as AP2 for the detection of PI-2, is essential to maximize the sensitivity of detection. Despite the high levels of variability that showed variant-specific protection in PI-2a backbone protein immunized mice, which raised questions on the prospects of this target, a recent publication by Nuccitelli *et al.* (2011) applied a structural vaccinology approach to design a synthetic protein with multivalent activity.

Since a vaccine composed of pilus island components has the potential to cover all serotypes, there is a need for in depth research into the global epidemiology of GBS pilus islands to ensure that vaccines in development are broadly protective across all geographical locations. Our data on pilus island distribution are comparable to the data in the published study by Margarit *et al.* (2009), and the similarities are sufficient to confirm that the serotype and associated pilus island distribution in South Africa is similar to that reported in the USA and Europe, and based on this homogeneity of pilus island distribution, a pilus-based vaccine developed for these industrialized countries will have similar coverage in South Africa. What remains to be determined is whether pressure from such a vaccine can result in the emergence of strains that lack pilus islands as the dominant virulent types as has been witnessed for other pathogens, such as *Streptococcus pneumoniae* (Bogaert *et al.*, 2005; Singleton *et al.*, 2007).

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REFERENCES

Baker, C. J. & Edwards, M. S. (2003). Group B streptococcal conjugate vaccines. *Arch Dis Child* **88**, 375–378.

Berkley, J. A., Lowe, B. S., Mwangi, I., Williams, T., Bauni, E., Mwarumba, S., Ngetsa, C., Slack, M. P., Njenga, S. & other authors (2005). Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med* **352**, 39–47.

Bogaert, D., Veenhoven, R. H., Sluijter, M., Wannet, W. J., Rijkers, G. T., Mitchell, T. J., Clarke, S. C., Goessens, W. H., Schilder, A. G. & other authors (2005). Molecular epidemiology of pneumococcal colonization in response to pneumococcal conjugate vaccination in children with recurrent acute otitis media. *J Clin Microbiol* **43**, 74–83.

Cutland, C. L., Madhi, S. A., Zell, E. R., Kuwanda, L., Laque, M., Groome, M., Gorwitz, R., Thigpen, M. C., Patel, R. & Velaphi, S. C. (2009). Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised, controlled trial. *Lancet* **374**, 1909–1916.

Dramsi, S., Caliot, E., Bonne, I., Guadagnini, S., Prévost, M. C., Kojadinovic, M., Lalioui, L., Poyart, C. & Trieu-Cuot, P. (2006). Assembly and role of pili in group B streptococci. *Mol Microbiol* **60**, 1401–1413.

Gray, K. J., Bennett, S. L., French, N., Phiri, A. J. & Graham, S. M. (2007). Invasive group B streptococcal infection in infants, Malawi. *Emerg Infect Dis* **13**, 223–229.

Ho, C. M., Ho, M. W., Chen, C. M., Liao, W. C., Liu, Y. M., Lin, P. C. & Wang, J. H. (2006). Clinical characteristics of group B streptococcus bacteremia in non-pregnant adults. *J Microbiol Immunol Infect* **39**, 396–401.

Klein, J., Munch, R., Biegler, I., Haddad, I., Retter, I. & Jahn, D. (2009). Strepto-DB, a database for comparative genomics of group A (GAS) and B (GBS) streptococci implemented with the novel database platform 'open genome resource' (OGeR). *Nucleic Acids Res* **37** (Database), D494–D498.

Lalioui, L., Pellegrini, E., Dramsi, S., Baptista, M., Bourgeois, N., Doucet-Populaire, F., Rusniok, C., Zouine, M., Glaser, P. & other authors (2005). The SrtA sortase of *Streptococcus agalactiae* is required for cell wall anchoring of proteins containing the LPXTG motif, for adhesion to epithelial cells, and for colonization of the mouse intestine. *Infect Immun* **73**, 3342–3350.

Larsson, C., Stålhammar-Carlemalm, M. & Lindahl, G. (1999). Protection against experimental infection with group B streptococcus by immunization with a bivalent protein vaccine. *Vaccine* **17**, 454–458.

Lauer, P., Rinaudo, C. D., Soriani, M., Margarit, I., Maione, D., Rosini, R., Taddei, A. R., Mora, M., Rappuoli, R. & other authors (2005). Genome analysis reveals pili in group B streptococcus. *Science* **309**, 105.

Madhi, S. A., Radebe, K., Crewe-Brown, H., Frasch, C. E., Arakere, G., Mokhachane, M. & Kimura, A. (2003). High burden of invasive *Streptococcus agalactiae* disease in South African infants. *Ann Trop Paediatr* **23**, 15–23.

Madzivhandila, M., Adrian, P. V., Cutland, C. L., Kuwanda, L., Schrag, S. J. & Madhi, S. A. (2011). Serotype distribution and invasive potential of group B streptococcus isolates causing disease in infants and colonizing maternal-newborn dyads. *PLoS ONE* **6**, e17861.

Maione, D., Margarit, I., Rinaudo, C. D., Masignani, V., Mora, M., Scarselli, M., Tettelin, H., Brettoni, C., Iacobini, E. T. & other authors (2005). Identification of a universal group B streptococcus vaccine by multiple genome screen. *Science* **309**, 148–150.

Margarit, I., Rinaudo, C. D., Galeotti, C. L., Maione, D., Ghezzo, C., Buttazzoni, E., Rosini, R., Runci, Y., Mora, M. & other authors (2009). Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. *J Infect Dis* **199**, 108–115.

Martins, E. R., Melo-Cristino, J. & Ramirez, M. (2010). Evidence for rare capsular switching in *Streptococcus agalactiae*. *J Bacteriol* **192**, 1361–1369.

Milledge, J., Calis, J. C., Graham, S. M., Phiri, A., Wilson, L. K., Soko, D., Mbvwinji, M., Walsh, A. L., Rogerson, S. R. & Molyneux, E. M. (2005). Aetiology of neonatal sepsis in Blantyre, Malawi 1996–2001. *Ann Trop Paediatr* **25**, 101–110.

Nobbs, A. H., Rosini, R., Rinaudo, C. D., Maione, D., Grandi, G. & Telford, J. L. (2008). Sortase A utilizes an ancillary protein anchor for efficient cell wall anchoring of pili in *Streptococcus agalactiae*. *Infect Immun* **76**, 3550–3560.

Nuccitelli, A., Cozzi, R., Gourlay, L. J., Donnarumma, D., Necchi, F., Norais, N., Telford, J. L., Rappuoli, R., Bolognesi, M. & other authors (2011). Structure-based approach to rationally design a chimeric protein for an effective vaccine against group B streptococcus infections. *Proc Natl Acad Sci U S A* **108**, 10278–10283.

Pezzicoli, A., Santi, I., Lauer, P., Rosini, R., Rinaudo, D., Grandi, G., Telford, J. L. & Soriani, M. (2008). Pilus backbone contributes to group B streptococcus paracellular translocation through epithelial cells. *J Infect Dis* **198**, 890–898.

Qinlan, J. D., Hill, A., Maxwell, B. D., Boone, S., Hoover, F. & Lense, J. J. (2000). The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. *J Fam Pract* **49**, 447–448.

Rosini, R., Rinaudo, C. D., Soriani, M., Lauer, P., Mora, M., Maione, D., Taddei, A., Santi, I., Ghezzo, C. & other authors (2006). Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. *Mol Microbiol* **61**, 126–141.

Schuchat, A. (1998). Epidemiology of group B streptococcal disease in the United States: shifting paradigms. *Clin Microbiol Rev* **11**, 497–513.

Singleton, R. J., Hennessy, T. W., Bulkow, L. R., Hammitt, L. L., Zulz, T., Hurlburt, D. A., Butler, J. C., Rudolph, K. & Parkinson, A. (2007). Invasive pneumococcal disease caused by nonvaccine serotypes among Alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA* **297**, 1784–1792.

Ton-That, H. & Schneewind, O. (2004). Assembly of pili in Gram-positive bacteria. *Trends Microbiol* **12**, 228–234.