Determining immunological correlates of protection against Group B *Streptococcus* colonization in pregnant women

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A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy

Johannesburg, 2016

DECLARATION

I, Gaurav Kwatra declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

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29th day of July 2016

DEDICATION

I dedicate this work to my Grand-Mother

Late Smt. Amarvati Kwatra

PUBLICATIONS AND PRESENTATIONS ARISING FROM THIS THESIS

Publications

1. **Kwatra G**, Madhi SA, Cutland CL, Buchmann EJ, Adrian PV. Evaluation of Trans-Vag broth, colistin-nalidixic agar, and CHROMagar StrepB for detection of group B Streptococcus in vaginal and rectal swabs from pregnant women in South Africa. *J Clin Microbiol* 2013; 51:2515-9. (Appendix 1)

2. **Kwatra G**, Adrian PV, Shiri T, Buchmann EJ, Cutland CL, Madhi SA. Serotype-specific acquisition and loss of group B streptococcus recto-vaginal colonization in late pregnancy. *PLoS One* 2014; 9:e98778. (Appendix 2)

3. **Kwatra G**, Adrian PV, Shiri T, Buchmann EJ, Cutland CL, Madhi SA. Natural acquired humoral immunity against serotype-specific Group B Streptococcus recto-vaginal colonization acquisition in pregnant women. *Clinical microbiology and infection* 2015; 21, 568 e13-21. (Appendix 3)

4. **Kwatra G**, Adrian PV, Shiri T, Izu A, Cutland CL, Buchmann EJ, Madhi SA: Serotype-Specific Cell-Mediated Immunity Associated With Clearance of Homotypic Group B Streptococcus Rectovaginal Colonization in Pregnant Women. *The Journal of infectious diseases* 2016, 213(12):1923-1926. (Appendix 4)

5. **Kwatra G**, Cunnington MC, Merrall E, Adrian PV, Ip M, Klugman KP, Tam WH, Madhi SA: Prevalence of maternal colonisation with group B streptococcus: a systematic review and meta-analysis. *The Lancet Infectious diseases* 2016. (Appendix 5)

6. **Kwatra G**, Adrian PV, Shiri T, Cutland CL, Buchmann EJ and Madhi SA. Association between mucosal immune response and Group B Streptococcus colonization in pregnant women. (Manuscript in preparation)

Presentations

1. **Kwatra G**, Madhi SA, Cutland CL, Buchmann EJ, Adrian PV. Comparison of CHROMagar Strep B, Columbia CNAagar and Lim broth for the isolation of Group B Streptococcus from vaginal and rectal swabs from South Africa. 22nd *European Congress of Clinical Microbiology and Infectious Diseases*, 31st March-02nd April 2012, London, UK (Poster presentation, no: 1761).

2. **Kwatra G**, Adrian PV, Shiri T, Cutland CL, Buchmann EJ and Madhi SA. Impact of cell-mediated immune response to Group B Streptococcus on host clearance of colonization. *International African Vaccine Conference*, November 2012, Cape Town, South Africa. (Poster presentation, no: 86)

3. **Kwatra G**, Cunnington MC, Valencia C, Adrian PV, Ip M, Klugman KP, Madhi SA, Tam WH and Slobod KS. Maternal Colonization with Group B Streptococcus: Do rates vary across regions? 8th *World Society for Paediatrics Infectious Diseases*,

November 2013, Cape Town, South Africa. (Poster presentation by Cunnington MC, no: 291)

ABSTRACT

Introduction: Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) is the major risk factor for invasive GBS disease in newborn's. Maternal vaccination against GBS during pregnancy may prevent or reduce subsequent recto-vaginal colonization in women, which could lower fetal/newborn exposure to GBS and contribute to reducing GBS associated infections during early infancy. In this study we determined the immunological correlates of protection against GBS colonization in black African pregnant women.

Methods: We compared GBS serotype-specific serum IgG, mucosal IgG, mucosal IgA and cellular immune responses in relation to GBS rectovaginal acquisition and clearance in pregnant women from 20 to 37+ weeks of gestational age. Furthermore, we also evaluated different media for isolation of GBS from vaginal and rectal swabs.

Results: The prevalence of recto-vaginal GBS colonization was 33.0%, 32.7%, 28.7% and 28.4% at 20-25 weeks, 26-30 weeks, 31-35 weeks and 37+ weeks of gestational age, respectively. The most common identified serotypes were Ia (39.2%), III (32.8%) and V (12.4%). The cumulative overall recto-vaginal acquisition rate of new serotypes during the study was 27.9%, including 11.2%, 8.2% and 4.3% for serotypes Ia, III and V, respectively. The recovery of GBS from rectal swabs was significantly higher from direct plating on chromogenic medium ($p<0.0001$) than from selective broth method.

New-acquisition of GBS was inversely correlated with serotype-specific serum IgG concentration for serotype III ($p=0.009$) and OPA titer for serotype Ia and III ($p<0.001$ for both) at time of enrolment. Serum IgG concentration significantly associated with

protection against recto-vaginal acquisition of the homotypic serotype was ≥1 μg/ml for serotype V (p=0.039), ≥3 μg/ml for serotype Ia (p=0.043) and III (p=0.023). Mucosal IgG correlated significantly with serum IgG with Rho values of 0.839, 0.621 and 0.426 (all p<0.001) for serotype Ia, III and V, respectively. The clearance of serotype-specific GBS recto-vaginal colonization during pregnancy was positively associated with presence of homotypic capsular ELISpot IFN-γ positivity for serotype III (p=0.008)

Conclusion: Maternal GBS colonization could be used as end point to evaluate efficacy of GBS vaccine. A serotype-specific capsular polysaccharide based GBS vaccine able to elicit both humoral and cell-mediated capsular immune responses could confer protection against EOD by reducing the exposure of the newborn's to GBS colonization during the peri-partum period.

ACKNOWLEDGEMENTS

I would like to sincerely thank:

- My Supervisors Prof. Shabir A. Madhi, Dr Peter V. Adrian and Prof. Eckhart J. Buchmann for their continuous guidance, support and encouragement throughout the project.
- Dr Clare L. Cutland for kindly supervising the sample collection at community clinics and Dr Tinevimbo Shiri for helping me in statistical analysis.
- The Respiratory and Meningeal Pathogens Research Unit clinical study staff for assisting with the enrolments and follow-up of the participants.
- The Respiratory and Meningeal Pathogens Research Unit laboratory staff for assisting with lab assays: Bianca Smith, Ntombi Florence Miya, Palesa Morailane, Makhosazana Sibeko, Lendewe Maphalla.
- Dr Marriane Cunnington, Novartis vaccines for her contribution and pertinent advice on meta-analysis and systematic review.
- Prof. Carol. J. Baker, Bayer College of Medicine, Texas for kindly providing standard GBS reference serums
- Novartis Vaccines, Italy for kindly providing CPS antigens.
- Antenatal staff of Lillian Ngoyi, Mofolo, Diepkloof and Michael Maponya community clinics in Soweto for all their help and support.
- Participating pregnant women for selflessly volunteering their participation.
- Dr Ziyaad Dangor and Dr Mashudu Madzivhandila for their continuous support and encouragement throughout my project.

- My family, my father Harish Kumar Kwatra, my mother Lata Kwatra, my brother Gagan Deep, my babhi Dr Rashi, my lovely nephew Sanath and niece Sanaa and my in parents in law Mr C.L. Dhar and Veena Dhar for their never ending support.
- My best friend and now my wife Nisha for believing in me and for her continuous support.
- Funders
    - This work was funded by research supported by the South African Research Chairs Initiative in Vaccine Preventable Diseases of the Department of Science and Technology and National Research Foundation.
    - Medical Research Council: Respiratory and Meningeal Pathogen Research Unit
    - Novartis Vaccines, Italy.

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ABBREVIATIONS

AP: Ancillary protein

BP: Backbone protein

BPS: Group B protective surface protein

CA: CHROMagar Strep B

CAMP: Christie Atkinson Munch-Petersen

CDC: Centre for Ddiseases Ccontrol

CI: Confidence interval

CNA: Colistin nalidixic agar

CPS: Capsular polysaccharide

DPSM: Direct plating on selective media

ELISA: Enzyme Linked Immunosorbent Assay

ELISpot: Enzyme Llinked immuno spot Immunospot

EOD: Early onset disease

GBS: Group B *Streptococcus*

GMC: Geometric mean concentration

GMT: Geometric mean titre

IAP: Intrapartum antibiotic prophylaxis

ICF: Informed consent form

IFN-γ: interferon-γ

IQR: Inter quartile range

LLD: Lower limit of detection

LMP: Last normal menstrual period

LOD: Late onset disease

MESH: Medical Subject Heading

MFI: Mean fluorescent intensity

NPV: Negative predictive value

NT: Non typeable

OPA: Opsonophagocytic assay

PBMC: Peripheral blood mononuclear cells

PCR: Polymerase chain reaction

PCV: Pneumococcal conjugate vaccine

PHS: Pooled human serum

PI: Pilus island

PPV: Positive predictive value

RMPRU: Respiratory and meningeal pathogen research unit

SBA: Sheep blood agar

SBSBA: Selective broth subcultured on blood agar

SBSSM: Selective broth subcultured on selective media

SFU: Spot forming unit

SIP: Surface immunogenic protein

PREFACE

This thesis is presented to the reader in the University of the Witwatersrand's recommended "divided block" format. In this format, the thesis consists of two parts: the first part includes the Introduction and Methods chapters that are written in the traditional thesis format, and second part in which the Results and Discussion of the study's objectives are presented as individual chapters.

This thesis aimed to identify GBS serotype-specific capsular antibody thresholds that correlate with protection against colonization in pregnant women, which may assist in the licensure of the maternal GBS polysaccharide-protein conjugate vaccine undergoing development.

In the Introduction chapter, I will briefly describe the epidemiology of invasive GBS disease, risk factors and the strategies to prevent it. I will further briefly describe prevalence of maternal GBS colonization and methods for its detection. Thereafter, I will discuss the concept of vaccination to prevent or reduce maternal GBS colonization as an alternative strategy to prevent GBS disease in young infants. In Chapter 2, material and methods are described.

Chapter's 3-8 presents the results and discussion of my work. In Chapter three, meta-analysis and systematic review on prevalence of maternal GBS colonization is reported. In chapter four, the comparison of different media for isolation and detection of GBS from maternal specimens is described. In chapter 5, dynamics of GBS colonization in later half of the pregnancy is presented. Chapter 6, details the association between natural occurring

serotype-specific serum IgG and risk of new acquisition of GBS. Chapter 7, details the association between natural occurring serotype-specific cellular immune responses and clearance of existing GBS colonization. Chapter 8, details the association between natural occurring serotype-specific serum IgG and mucosal IgG as well as mucosal IgG and IgA association with maternal GBS colonization.

The thesis concludes with chapter 9, with a summary of the main findings of my research in the context of maternal GBS colonization as surrogate marker for evaluating GBS vaccine.

Chapter 1 Introduction

1.1 Epidemiology of Group B Streptococcus disease

Streptococcus agalactiae, also known as Group B *Streptococcus* (GBS), is a leading cause of neonatal invasive bacterial disease (Stoll et al., 2011). It is a common commensal bacterium colonizing the gastrointestinal and genitourinary tracts of women (Stoll and Schuchat, 1998). Historically GBS was associated with bovine mastitis and only reported as a human pathogen in the 1960's, following which it became recognized as the leading cause of neonatal invasive disease (Eickhoff et al., 1964). Invasive GBS disease in neonates manifest primarily as bacteremia, pneumonia or meningitis (Burman et al., 1992). Developmental abnormalities such mental retardness, blindness and deafness have also been reported in half of GBS meningitis survivors (Bedford et al., 2001, Libster et al., 2012, Edwards et al., 1985).

The burden of invasive GBS disease is highest in infants less than three months of age and the incidence is substantially higher in developing countries with limited or no preventive strategies. The incidence (per 1000 live births) of invasive GBS disease globally in infants less than 90 days was reviewed by Edmond et al, with a reported overall incidence of 0.53 globally (Edmond et al., 2012). However, variability in incidence of GBS disease between regions as well as within regions was observed. Among the regions, Africa region reported highest incidence (1.21), followed by America (0.67) and Europe (0.57); whereas Southeast Asia reported the lowest incidence (0.02). The low incidence in Southeast Asia may be an underestimate due to large number of deliveries occurring outside health care facilities which coupled with a high proportion of babies

dying within 24 hours of birth, results in many newborn's deaths not being investigated for bacterial sepsis including in epidemiological studies (Montagu et al., 2011). Furthermore, it should be noted that only five studies from low-income countries were reported in the review, accounting for 5% of the total studies of those included in the review. In a separate review on studies conducted in developing countries, Africa region reported the highest incidence, including an incidence of 3.0 in South Africa (Dagnew et al., 2012).

In neonates, GBS disease is stratified into cases which occur within the first seven days of life (0-6 days) that are defined as early-onset disease (EOD) and account for 70-80% of total GBS disease cases. Of the EOD, 80% cases present within first 24 hours of life (Madhi et al., 2013, Schuchat et al., 2000). In the remaining cases among infants, invasive GBS disease primarily occurs from seven days to three months of life, these are defined as late-onset disease(Chan SHS, 2000).

The global incidence (per 1000 live births) of EOD has been reported to be 0.43, with Africa region reporting the highest incidence (0.53), followed by America (0.50) and Europe (0.45) (Edmond et al., 2012); whereas the incidence in Southeast Asia was reportedly only 0.11 (95%CI: 0.012-0.220). The incidence of EOD in South African infants has been reported in three previous studies (Haffejee et al., 1991, Madhi et al., 2003, Cutland et al., 2015). The first study was conducted from 1986 to 1989 and reported an incidence of 2.09/1000 for EOD in women of Indian descent (Haffejee et al., 1991), and similar incidences for EOD (2.06/1000) was reported ten years later in Black-African women (Madhi et al., 2003). Between 2004 and 2008, the incidence of EOD among Black-African Sowetan women in South Africa has declined marginally to 1.5 (95%CI:

1.31-1.71) (Cutland et al., 2015). In developed countries such as the USA, the incidence (per 1000 live births) of EOD in full-term newborns declined by approximately 90% (1.7in 1993 to 0.26/in 2010) due to routine screening of pregnant women for GBS recto-vaginal colonization at 35-37 weeks of gestational age, which was coupled with intrapartum antibiotic prophylaxis (IAP) provided to colonized women 4 hours prior to delivery (Schrag and Schuchat, 2004). Nevertheless, GBS is the main cause of EOD (38-43%) in USA, followed by *E.coli* (24-29%) (Stoll et al., 2011, Weston et al., 2011).

The global incidence (per 1000 live births) of LOD has been reported to be 0.24, with highest in Africa (0.7) (Edmond et al., 2012). There has been an increase in the incidence of LOD in South Africa from 0.67 to 1.22 in the past 20 years (Madhi et al., 2003, Cutland et al., 2015, Haffejee et al., 1991). The increase in LOD infection might be due to increase in HIV infection rate among pregnant women from 1.4% in 1991 to 29.5% in 2011(Health, 2011). In developed countries despite implementation of IAP strategy, the incidence of LOD unlike EOD has remained unchanged (0.3-0.4/1000 live births) over the past 20 years (Schrag and Verani, 2013). Furthermore, whilst GBS is the leading cause of invasive disease in newborn infants, the incidence of GBS related disease in elderly adults also appears to be on the increase (Edwards and Baker, 2005).

1.2 Risk factors for GBS neonatal disease

Group B *Streptococcus* is a normal commensal of gastrointestinal and genitourinary tracts of pregnant women and vertical acquisition of GBS by the fetus *in-utero* or newborn (during labor) from recto-vaginally colonized mothers is the major risk factor for EOD in infants (Verani et al., 2010). Approximately 10% to 40% of pregnant women are colonized

with GBS in the vagina or rectum (Picard and Bergeron, 2004). Vertical transmission of GBS occurs in approximately 50% of newborn's of GBS-colonized mothers, of whom 1–2% develop EOD (Beal and Dancer, 2006, Heath and Feldman, 2005). In comparison to other microorganisms, GBS bind very efficiently to human vaginal epithelium (Sobel et al., 1982). It is however, unclear how asymptomatic colonization in mothers, become invasive and pathogenic in the infant. Mechanisms such as bacterial overgrowth and changes in gene expression have been proposed that could increase the invasive potential of GBS in the infants (Rajagopal, 2009).

GBS colonization in the infant may ascend in-utero by penetrating into amniotic cavity regardless of whether membrane has been ruptured or not (Whidbey et al., 2013). Studies have also shown that GBS can grow rapidly in amniotic fluid both *in vivo* and *in vitro* experiments (Hemming et al., 1985). Furthermore, rapid onset of EOD in neonates, particularly cases presenting at the time of delivery or a few hours after birth, suggests that the GBS infection process possibly started in-utero rather than during delivery (Madhi et al., 2013). Studies have demonstrated concordance of 90.7% in serotypes between the mother and newborn pairs who were colonized by GBS whereas 100% of infants with EOD had the same serotype at presentation as the maternal colonizing serotype (Dillon et al., 1987, Madzivhandila et al., 2011).

The risk factors for the development of LOD as compared to EOD are poorly understood with maternal colonization is still thought to be primary source. Studies have demonstrated that almost half of infants with LOD had the same serotype at presentation as the mother was colonized with at birth, (Dillon et al., 1987) and the same genotype was

isolated from the mother-infant pairs as late as 8 weeks post-partum (Berardi et al., 2013). These studies suggest that delayed vertically transmission could be a source of infection resulting in LOD. It has also been shown that breast-milk feeding may be a source of transmission of GBS resulting in either late onset or recurrence of GBS disease (Kotiw et al., 2003). Nosocomial and community acquired transmission is also thought common source of GBS colonization of neonates, which too could result in LOD (Jordan et al., 2008).

1.3 Microbiology, Identifications and Growth conditions

Streptococcus agalactiae (GBS) is a Gram-positive pathogen and belongs to genus streptococcus. The classification of group B is based upon specific cell wall polysaccharide antigens reacting to hyperimmune serum under the Lancefield grouping (Lancefield, 1934). GBS are facultative anaerobes and exhibit β hemolytic pattern of growth when grown on blood agar, producing a clear zone around colonies (Nizet, 2002). Detection of GBS on blood agar is often superimposed by normal gram negative microflora. The selectivity of GBS on blood agar or other media is increased by supplementing the medium with antibiotics to maximize the recovery of GBS and minimizing the likelihood of culture of gram negative pathogens. The current CDC recommendation for the isolation of GBS from vaginal and rectal or recto-vaginal swabs is by growth in a selective broth medium (Todd-Hewitt broth with gentamicin and colistin or nalidixic acid), followed by subculture on blood agar or selective media (Verani et al., 2010). These GBS colonies are further confirmed by Christie Atkinson Munch-Petersen (CAMP) test (Fuchs et al., 1978), Bile esculin test (Chuard and Reller, 1998), catalase test and group B antigen latex agglutination (Elliott et al., 2004). Verification of GBS colonies can also be done by using fluorescent antibodies (Boyer et al., 1981) or by genetic probes (Kong et al., 2005).

Christie Atkinson Munch-Petersen (CAMP) test is used for presumptive identification of GBS and involves the haemolytic activity of staphylococcal B-lysin on red blood cells which is enhanced by the factor produced only by GBS called CAMP factor. GBS produces a clear arrow-head shaped haemolysis at the junction with the line of staphylococcus (Fuchs et al., 1978). Occasionally GBS on blood agar may be mistaken for *Enterococcus faecalis*, and in order to discriminate between the two, bile esculin test is performed (Chuard and Reller, 1998). Bile esculin medium favours growth of GBS and enterococcus both, however, by-products produced as a result of enterococcus growth react with the iron salts in the medium causing the medium to blacken.

The selective broth method recommended by CDC has been reported to have sensitivity ranging from 82% to 99%. (Busetti et al., 2007, Craven et al., 2010, Gupta and Briski, 2004, Jones et al., 2006). There are, however, limitations to this approach including that the procedure requires at least 48-72 hours of culture time and identification of GBS-like colonies on blood agar requires laboratory expertise, particularly when they are mixed with other microflora (Poisson et al., 2010). Studies have shown that direct plating on colistin and nalidixic agar (CNA) is a low-cost alternative for GBS recovery, albeit with a lower sensitivity (59%-83%) (Bosch-Mestres et al., 2003, El Aila et al., 2010, Louie et al., 2010). In recent years, several commercial chromogenic media have been tested for their suitability for detecting GBS such as Granada medium, CHROMagar Strep B (CA) and Chrom ID Strepto B agar (El Aila et al., 2010, Poisson et al., 2011). CHROMagar Strep B is a commercially available selective chromogenic medium that inhibits most saprophytic bacteria and yeasts, and produces mauve-coloured GBS colonies in aerobic conditions irrespective of their haemolytic properties, allowing direct visual identification. The clinical sensitivity of CA ranges from 93%-98% (Charron, 2009, Poisson et al., 2010),

40%-91% for Granada Medium (El Aila et al., 2010, Gupta and Briski, 2004, Overman et al., 2002) and 88%-95% for Chrom ID Strepto B agar (El Aila et al., 2010, Craven et al., 2010). In order to select suitable media for the study, we evaluated direct plating on CA, CNA and selective broth enrichment method for the isolation of GBS in swabs from pregnant women.

1.4 Pathogenesis and virulence factors

The development of GBS disease is complex process. Several virulence factors of GBS have been identified that are critical for its ability to cause disease (Rajagopal, 2009). The pathogenesis of invasive GBS disease has been broadly divided into following steps (Rajagopal, 2009).

1. **Adherence**: GBS is a part of the common flora colonizing at the vagina, rectum, and gastrointestinal mucosa. The initial step in the pathogenesis of GBS is adherence to host cells. Surface proteins such as pili, C5a peptidase, α-C protein, Lmb, FbsA, BibA and Rib are linked to the firm adherence of GBS to host cells.
2. **Penetration of host cellular barriers**: Penetration of GBS into host cells and/or tissues is the key step of GBS neonatal disease. Surface proteins such as Alpha C protein and FbsB can promote entry into host cells. Pore forming toxins such as CAMP factor and hemolysin are also a critical component of GBS pathogenesis promoting its entry into host cells and facilitating their intracellular survival and dissemination. Hyaluronatelyase a secreted protease is also thought to facilitate the spread of GBS during infection.
3. **Immune evasion:** GBS escape from host immune clearance is an important step of GBS neonatal disease. GBS encodes a number of virulence factors which help them

to avoid host defence mechanisms such capsular polysaccharides (CPS), C5a peptidase and superoxide dismutase.

4. **Activate inflammatory reactions:** GBS infection can induce host inflammatory reactions. Experiments showed that a GBS infection of human respiratory epithelial cell lines is associated with increased production of proinflammatory cytokines such as tumour necrosis factor alpha, interleukin (IL)-8 and IL-10 than for uninfected cells, suggesting inflammatory reaction of phagocytes to GBS infection (Henneke et al., 2005). Hemolysin also induces host inflammatory responses by inducing macrophage production of nitric oxide synthase and generation of nitric oxide which further contributes to neonatal sepsis (Ring et al., 2000).

1.4.1 Capsular polysaccharides

Of all virulence factors, the role of capsular polysaccharide has been extensively studied. Capsular polysaccharide is one of the factors that enable GBS to survive in the host by preventing the activation of complement pathways involved in opsonophagocytosis (Edwards et al., 1982). Due to its importance in GBS pathogenesis, CPS is considered as a major epitope for vaccine development. Group B *Streptococcus* is classified into ten serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII and IX) based on the structure of the CPS (Arakere et al., 1999, Slotved et al., 2007). Structure analysis of CPS's are now complete for all GBS serotypes. Despite being composed of only few sugars: glucose, galactose, *N*-acetylglucosamine, and sialic acid, there is nevertheless structural and antigenic heterogenicity between the CPS of serotypes (Kogan et al., 1996). The capsular polysaccharide for serotype Ia, Ib, II, III, IV,V, and VII consists of glucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid (Jennings et al., 1983a, Jennings et al.,

1983b, Kogan et al., 1995, Wessels et al., 1991, Wessels et al., 1987), while serotype VI and VIII contains rhamnose instead of N-acetylglucosamine (Kogan et al., 1996, von Hunolstein et al., 1993). Sialic acid which is present as a terminal side chain residue in the repeating structure of each GBS CPS, enhances GBS pathogenic abilities by interfering with the host innate immune recognition, specifically with opsonic components of the complement system(Campbell et al., 1991, Marques et al., 1992). Despite the degree of similarity between the serotype-specific CPS repeating unit, significant antigenic variation was observed at both the nucleotide and amino acids sequences (Cieslewicz et al., 2005). There are a number of GBS isolates which do not express a polysaccharide capsule on their surface and hence are nontypeable (NT) (Slotved et al., 2007). The loss of capsule expression has been linked to mutations in genes responsible for capsule expression (Sellin et al., 2000). Molecular based typing has been developed to overcome the limitations of serologically non-typeable techniques (Poyart et al., 2007).

1.4.2 Surface proteins

It is now apparent that most GBS strains express antigenic surface proteins that are critical for its viability and ability to cause invasive disease (Rajagopal, 2009). A number of surface protein antigens have been identified and evaluated as vaccine candidate, that include α and β antigen of the c protein complex (Madoff et al., 1991), R proteins (Flores and Ferrieri, 1989), Rib protein (Stalhammar-Carlemalm et al., 1993), C5a peptidase (Cheng et al., 2002), surface immunogenic protein (Sip) (Brodeur et al., 2000) and Group B protective surface protein (BPS) (Erdogan et al., 2002). Some of these surface proteins like C5a peptidase, SIP and BPS are present in the majority of strains regardless of GBS

serotype and have been evaluated for the immunogenicity, and likewise showed to elicit protective immunity in animal models (Brodeur et al., 2000, Cheng et al., 2002).

1.4.3 Pili

The presence of cell-surface appendages known as pili has been discovered on the surface of GBS (Lauer et al., 2005). Pili are long proteinaceous filaments strands attached to the bacterial surface that helps in colonization and invasion by facilitating the attachment and adherence of the pathogen to host cells (Rosini et al., 2006). Pili also facilitate the penetration of GBS through the blood-brain barrier and play a role in biofilm formation (Banerjee et al., 2011, Borges et al., 2012). In GBS two genomic units have been identified : pilus island-1 (PI-1) and pilus island-2 (PI-2) that are responsible for pilus like structures. Pilus island-2 further exists in two variants PI-2a and PI-2b (Rosini et al., 2006). Each pilus island genomic region consists of 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). In figure 1.1, for PI-1, GBS80 represents the BP, while the other two pili structure, GBS104 and GBS52 are AP1 and AP2 respectively. In PI-2a, GBS59 represents BP whereas GBS67 and GBS 150 are AP1 and AP2 respectively. In PI-2b, SAN1518 represents BP and SAN1519 and SAN1516 are AP1 and AP2 respectively (Rosini et al., 2006).

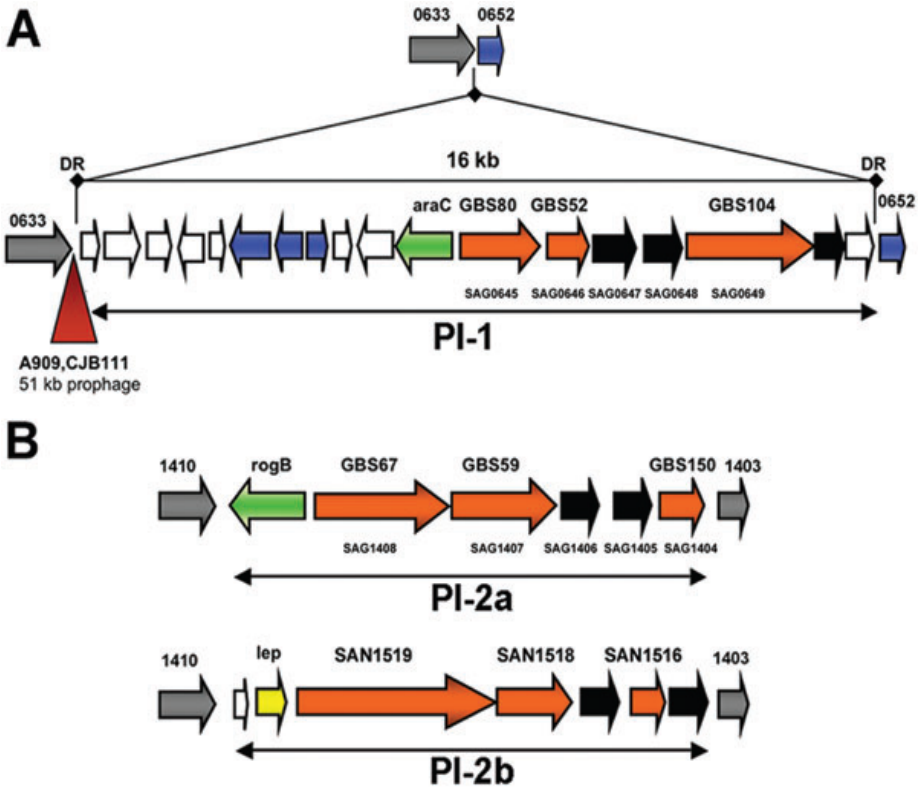


Figure 1.1: Schematic representation of GBS pilus island regions.

Genes coding for LPXTG-containing proteins are represented with orange arrows, sortases presented as black arrows (taken from Rosini et al, 2006 (Rosini et al., 2006)

1.5 Prevalence of maternal GBS colonization

Rectal and/or vaginal colonization with GBS among pregnant women varies worldwide (Picard and Bergeron, 2004). A systematic review of maternal GBS colonization from low and middle income countries undertaken in 1998 reported a regional prevalence range of 8% to 18% by geographic region. The microbiological method of GBS isolation was identified as a key driver of this variation (Stoll and Schuchat, 1998).

GBS colonization rates in pregnant women in Sub-Saharan Africa have been reported to vary from 1.8% to 32% (de Steenwinkel et al., 2008, Moyo et al., 2000). The investigation from South Africa has found 21% of pregnant women to be vaginally culture positive (Cutland et al., 2009). Lower colonization rates have been reported in other African countries such as in Nigeria (11.3%) (Onipede, 2012), Malawi (16.5%) (Dzowela T, 2005), Central African republic (17.5%) (Brochet et al., 2009) while high rates have been reported from the Gambia (22%) (Suara et al., 1994), Ethiopia (20.9%) (Musa Mohammed, 2012), Senegal (20%) (Brochet et al., 2009), Tanzania (23%) (Joachim et al., 2009), Egypt (25.3%) (Shabayek et al., 2009) and Zimbabwe (31.5%) (Moyo et al., 2000, Onipede, 2012). Mozambique indicated a very low prevalence of GBS colonization of 1.8% (de Steenwinkel et al., 2008). The variations between countries could possibly be due to differences in sampling sites. For instance some investigators have used vaginal swabs only (Onipede, 2012, Shabayek et al., 2009), high vaginal and rectal swabs (Joachim et al., 2009) or rectovaginal swab (de Steenwinkel et al., 2008). Other variations in isolation frequency could be due to differences in culture methods.

In recent years the sensitivity of culture methods has increased and there is now published guidance on recommended methods for specimen collection, processing and GBS isolation (Verani et al., 2010). This should allow a clearer interpretation of any population differences in colonization. I co-led a systematic review and meta-analysis with Marianne Cunnington (GSK) of prospective epidemiological studies that detected GBS carriage which used recommended microbiology methods (Verani et al., 2010), to assess the prevalence of maternal GBS colonization across geographic regions (Chapter 3).

1.6 Dynamics of GBS colonization during pregnancy

GBS colonization is highly dynamic during pregnancy and can be transient, intermittent or persistent (Verani et al., 2010), only few studies have reported on the dynamics of GBS colonization by way of multiple sampling (four or more time points) during pregnancy (Hansen et al., 2004, Goodman et al., 1997, Mavenyengwa et al., 2010). The overall GBS colonization found in these studies, showed half of the pregnant women enrolled in these studies are colonized with GBS at some point during pregnancy. A trend of decrease in colonization with increasing gestational age has also been reported (Goodman et al., 1997, Gilbert et al., 2002) whereas others have reported an increase in colonization with gestational age (Towers et al., 2010, Zamzami et al., 2011, Baker et al., 1975). As one of my thesis objectives, I evaluated the serotype-specific dynamics of GBS colonization among pregnant women from 20 to 37+ weeks of gestational age.

1.7 Serotype distribution associated with maternal GBS colonization

Vaccination against GBS has been investigated as a tool for reducing maternal colonization as well as early-infancy GBS sepsis (Verani et al., 2010). Current vaccine strategies focus on antigens presented on the surface of GBS which includes CPS and surface proteins, therefore epidemiological studies on the serotype and surface antigen distribution among GBS colonizing strains are important for vaccine development as this could differ by geographic region (Johri et al., 2006).

Serotypes Ia (6.8-28.5%), Ib (0-14.6%), II (0-28.1%), III (6.2-45.3%) and V (4.1-44.0%) remains the major serotypes responsible for colonization in different geographical regions (Table 1.1) with Ia and III being the most common . In South Africa, serotype III

has been the dominant colonizing (37.3%) and invasive serotype followed by Ia (Madzivhandila et al., 2011). Recently, there has been the emergence of serotype V as major (10.2-44.0%) colonizing serotype in pregnant women from African countries like the Gambia, Senegal, Zimbabwe and Central African Republic (Brochet et al., 2009, Moyo et al., 2000, Suara et al., 1998). Collectively the major serotypes in Africa are Ia, III, V and II, except in The Gambia where serotypes III (6.2%) and Zimbabwe where serotype II (4.3%) are in minority. In contrast, a study from United Arab Emirates reported serotype IV as a predominant serotype (26%), followed by Ia (24.6%) and III (17.5%) (Amin et al., 2002). In Japan, serotype VI and VIII were found to be responsible for over 60% of GBS serotypes colonizing pregnant women (Lachenauer et al., 1999). Due to inconsistency on the prevalence of GBS serotypes in different geographic region, continuous surveillance is necessary as the colonizing serotypes could inform serotype formulation included in capsular polysaccharide based vaccines.

Studies have also reported difference in serotype distribution between maternal colonizing isolates and invasive isolates from neonates (Madzivhandila et al., 2011, Martins et al., 2007) with serotype III and Ia has been reported to be more invasive than serotype Ib, II and V in Africa as well as in Europe. However, in USA serotype V is responsible for high number of invasive cases among infants. The reason for variation in invasive potential between serotypes is unclear, however it has been suggested differences at the genotype level can influence invasive potential. Furthermore, identification of serotype Ia, Ib, II, III and V in both colonizing and invasive isolates supports formulation of a pentavalent vaccine, as including only common serotypes in a vaccine could cause shift in serotype distribution in colonizing and subsequently in invasive disease isolates.

Table 1. 1: Distribution of serotypes among GBS isolates from pregnant women

Location	Total number of isolates	Serotype distribution										
		Ia	Ib	II	III	IV	V	VI	VII	VIII	IX	NT
USA, (Ippolito et al., 2010)	207	59 (28.5)	25 (12.1)	25 (12.1)	56 (27.1)	4 (1.9)	35 (16.9)	2 (1.0)	0	0	0	0
Germany (Brimil et al., 2006)	75	13 (17.3)	11 (14.6)	16 (21.3)	21 (28.0)	2 (2.7)	12 (16.0)	0	0	0	0	0
Italy (Savoia et al., 2008)	73	16 (20.5)	5 (6.8)	4 (5.5)	23 (32.9)	6 (8.2)	19 (26.1)	0	0	0	0	0
Sweden (Hakansson et al., 2008)	69	16 (23.2)	2 (2.9)	6 (8.7)	22 (31.9)	1 (1.4)	5 (7.2)	0	0	0	0	17 (24.6)
Kuwait (Al-Sweih et al., 2005)	124	11 (8.9)	3 (2.4)	10 (8.1)	33 (26.6)	1 (0.8)	27 (21.8)	11 (8.9)	6 (4.8)	0	0	22 (17.7)
United Arab Emirates (Amin et al., 2002)	57	14 (24.6)	0	2 (3.5)	10 (17.5)	15 (26.3)	7 (12.3)	0	0	0	0	9 (15.8)
Iran (Jannati et al., 2012)	56	4 (7.1)	5 (8.9)	7 (12.5)	6 (10.7)	7 (12.5)	11 (19.6)	6 (10.7)	3 (5.3)	3 (5.3)	0	4 (7.1)
Japan (Lachenauer et al., 1999)	73	5 (6.8)	6 (8.2)	0	8 (11.1)	0	3 (4.1)	18 (24.6)	0	26 (35.6)	0	7 (9.6)
Gambia (Suara et al., 1998)	32	6 (18.7)	0	9 (28.1)	2 (6.2)	1 (3.1)	12 (37.5)	0	0	0	0	2 (6.2)
Zimbabwe (Moyo et al., 2002)	117	17 (14.5)	6 (5.1)	5 (4.3)	53 (45.3)	6 (5.1)	28 (23.9)	0	0	0	0	2 (1.7)
Senegal (Brochet et al., 2009)	75	13 (17.3)	4 (5.3)	9 (12.0)	16 (21.3)	0	33 (44.0)	0	0	0	0	0
Central African Republic (Brochet et al., 2009)	88	24 (27.3)	7 (7.9)	20 (22.7)	15 (17.1)	0	22 (25.0)	0	0	0	0	0
South Africa (Madzivhandila et al., 2011)	541	163 (30.1)	36 (6.6)	61 (11.3)	202 (37.3)	20 (3.7)	55 (10.2)	0	0	0	0	4 (0.7)

1.8 Relationship between GBS serotypes and pilus islands

Pilus sequences are conserved with all GBS strains and all clinical GBS isolates carrying at least 1 of the 3 pilus islands and was associated with the presence of either PI-2a or PI-2b identified alone or in combination with PI-1 (Madzivhandila et al., 2013, Margarit et al., 2009). Association between specific serotypes and pilus islands was also observed, with the majority of serotype III carried in combination of PI-1 and PI-2b, serotype Ia were associated with PI-2a and most of the serotype Ib and V were associated with combination of PI-1 and PI-2a (Margarit *et al.* 2009). Difference in pilus island distribution with respect to source of isolates was also observed with majority of colonizing isolates from the adults carried both PI-1 and PI-2a, while invasive isolates from infant carried both PI-1 and PI-2b.

1.9 Prevention of invasive GBS disease

The preventative strategies against invasive GBS disease in newborn's include intra-partum antibiotic prophylaxis (IAP), local antiseptics and immunoprophylaxis.

1.9.1 Intrapartum antibiotic prophylaxis

To date only maternal IAP has been shown to be effective against invasive GBS disease (Schrag and Verani, 2013). In the USA, IAP for colonized mothers reduced the burden (per 1000 live births) of EOD from 1.7 to 0.26 between 1993 and 2010. The incidence of LOD, however, remains unchanged for20 years since this strategy has been implemented (0.3-0.4/1000 live births) (Schrag and Verani, 2013). Other developed countries such as Spain and Australia have also adopted IAP strategies and documented a

decline in the EOD (Isaacs and Royle, 1999, Lopez Sastre et al., 2005). The implementation of IAP in developing and in low resource countries is, however, likely to be constrained because of the large number of deliveries occurring outside of health care facilities (Montagu et al., 2011), logistical and cost issues related to screening for GBS colonization and timeous administration of intravenous antibiotics (Schrag and Verani, 2013). Additionally, IAP using intravenous antibiotic administration has been associated with an increased rate of illness from antibiotic non-susceptible bacteria in some settings (Verani et al., 2010). Furthermore, IAP does not prevent EOD in premature newborn's, GBS associated stillbirths or premature births (Edwards and Gonik, 2013, Patten et al., 2006).

1.9.2 Local Antiseptic Agents

A novel approach to prevention of neonatal invasive GBS disease is reduction of maternal vaginal colonization with antiseptic agents such as chlorohexidine during labor. A recent review of four studies that used vaginal chlorohexidine washes/douches during labour to prevent EOD, suggested that whilst it might reduce colonization of the newborn's, it was not effective in preventing EOD (Ohlsson et al., 2014).

1.9.3 Maternal immunization

Another potential strategy for prevention of invasive GBS disease in young infants, is maternal vaccination against GBS aimed at enhancing transplacental transfer of GBS antibody to the foetus and possibly through reducing maternal colonization in the women (Baker and Edwards, 2003, Verani et al., 2010). A protective role for GBS serotype III

antibodies was described in the 1970s by Baker and Kasper (Baker and Kasper, 1976). These investigators showed that infants with invasive early-onset or late-onset GBS disease were born to mothers with very low levels of serum type III CPS specific antibodies at delivery. Furthermore, findings such as human sera containing sufficient concentration of CPS specific IgG promoted efficient opsonization and phagocytosis (Edwards et al., 1979) and protection from lethal experimental infection in animal models, (Gotoff et al., 1986, Klegerman et al., 1983) confirmed the importance of capsular serotype specific antibodies. This information, coupled with the success of maternal tetanus vaccination during pregnancy in reducing neonatal tetanus (Steinhoff, 2013) suggested that active immunization of pregnant women with GBS vaccine in the 2^{nd} -3^{rd} trimester could potentially protect young infants against invasive GBS disease (Baker et al., 2003, Edwards et al., 2012).

1.10 GBS Vaccines

The "first generation" GBS vaccines consisted of purified CPS of serotypes III (Baker et al., 1978). In contrast to the excellent immunogenicity observed in adults immunized with pneumococcal polysaccharide vaccine, GBS polysaccharide vaccine, while well tolerated, had variable immunogenicity (Baker and Kasper, 1985). The safety of GBS CPS vaccines enabled progress to phase 1 trials in pregnant women, which supported the feasibility of maternal immunization as an approach to prevent invasive GBS disease in infants; and subsequently led to the development of polysaccharide-protein conjugate GBS vaccines with enhanced immunogenicity (Baker et al., 1988). The "second generation" GBS vaccines consisted of purified serotype specific CPS covalently coupled

to carrier protein such as tetanus toxoid or CRM197 carrier protein (Baker et al., 1999, Baker et al., 2004, Baker et al., 2003, Kasper et al., 1996).

Most of the polysaccharide-protein conjugate vaccines have been administrated safely to healthy adults, including administration of serotype III-TT vaccine in pregnant women during the third trimester. The study demonstrated excellent immunogenicity with a maternal GMC of type-III specific of 9.8µg/ml at delivery and correlated well with infant cord values (rho = 0.91). Additionally antibody from the infants of vaccinated individuals promoted opsonophagocytosis and persisted up to 2 months of age (Baker et al., 2003). In order to provide sufficient coverage against dominant serotypes, a CPS conjugate vaccine will need to be multivalent. In murine models up to four serotypes have been combined (Paoletti et al., 1994) whereas in pregnant women a trivalent vaccine composed of capsular serotypes Ia, Ib and III has recently completed phase I and II trials in South Africa (Madhi et al., 2013).

The "third generation" of GBS vaccines currently under development targets the GBS surface protein antigens, which contribute to the pathogenesis of GBS disease. Although the importance of surface proteins in immunity to *Streptococcus pyogenes* has long been appreciated, their role in immunity against GBS was only recognized in the 1970s, when Lancefield described the cross serotype protection afforded by rabbit antiserum to protease sensitive epitopes (Lancefield et al., 1975). The intervening years have witnessed considerable research exploring diversity of GBS surface proteins and increased awareness of their roles in immunity and pathogenicity. Much of the interest in these proteins arises from a desire to identify protective antigens for inclusion in GBS

vaccines as vaccine candidates or carrier proteins for specific GBS polysaccharides. Recently, three variants of pilus like structures has been discovered on the GBS, each encoded by a distinct pathogenicity island (Lauer et al., 2005). It has been shown that pilus sequences are conserved with all GBS strains carrying at least 1 of the 3 pilus island (PI) and a combination of the components from three PI conferred protection against all tested GBS challenge strains. This indicates that pilus based vaccine can be developed to prevent GBS infection (Margarit et al., 2009).

1.11 Licensure of GBS vaccine

Since 1996 conjugate vaccines to each of the clinically significant GBS serotypes have been developed (Baker et al., 2004, Baker et al., 1999, Baker et al., 2003). One of the biggest challenges in the licensure of a GBS vaccine is to demonstrate efficacy. Due to low incidence of invasive disease, a sample size of approximately 60,000 pregnant women would be required to demonstrate modest efficacy, such a trial would be large and costly (Madhi et al., 2013). Moreover, with the introduction of IAP in some developed countries, efficacy trials become even more challenging as vaccine would need to prove effective in a group already receiving IAP, which would require even larger sample size in the efficacy trials. Therefore, there are limited localities where such a study could be undertaken to measure vaccine efficacy against the clinical endpoint of invasive GBS disease (Madhi et al., 2013). Additionally, undertaking studies of GBS vaccine in pregnant women is complicated by concerns about the potential liability issues of vaccinating pregnant women where birth defects could be falsely attributed to vaccination (Robbins et al., 1995)

Consequently, for licensure of GBS vaccine targeted at pregnant women, alternate strategies need to be explored. This could include immunogenicity studies that measure protective levels of antibodies, as it is the major mechanism by which vaccines provide protection (Siber, 1997). It is theoretically possible to establish protective antibody concentration in an immunized population and such licensure-pathway strategy is accepted for vaccines including meningococcal vaccines, inactivated influenza vaccine and newer formulations of pneumococcal conjugate vaccine (Frasch et al., 2009, World Health Organization Immunization Vaccines and Biologicals, 2012, The European Agency for the Evaluation of Medicinal Products, 1997).

1.12 Immunologic correlates of protection

Because of the difficulty of conducting efficacy trials for GBS vaccine, identification of surrogate immunologic measures of efficacy is necessary. Maternal colonization with GBS is believed to be the principal source of infection for neonatal early-onset GBS disease and occurs in approximately 50% of newborn's born to GBS-colonized mothers (Cutland et al., 2009). Therefore by targeting the removal of vaginal colonization with GBS prior to childbirth, the source of infection can be eliminated, and thus at least early-onset neonatal sepsis caused by this organism can be prevented (Heath and Feldman, 2005). The concept of vaccine preventing acquisition of target organisms was first demonstrated with Hib conjugate vaccine (Takala et al., 1991) and later with pneumococcal conjugate vaccines (O'Brien and Dagan, 2003). A similar phenomenon will be expected for GBS vaccines and vaccination may be used as an approach for reducing the prevalence of GBS colonization in healthy women, possibly by inducing high levels of type-specific serum and subsequent reduction in the transmission of GBS to the newborn. Furthermore, should vaccination prevent or reduce recto-vaginal colonization in the

women, this could also have indirect protective effect on community as a whole by reducing transmission of GBS within the community. Already, a monovalent GBS serotype III conjugate vaccine has been reported to significantly reduce the rate of recto-vaginal GBS acquisition among non-pregnant women (Hillier, 2009).

The proof of principle of the effect of reduction of colonization upon subsequent indirect protection of non-vaccinated individuals has been demonstrated through the introduction of polysaccharide conjugate vaccines, against bacterial pathogens, such as *Streptococcus pneumoniae*. Whilst a putative serological correlate of direct protection against invasive pneumococcal disease has been established (Siber et al., 2007), the indirect effects among unvaccinated portions of the population is thought to be due to the effect of the polysaccharide-protein conjugate vaccines against colonization in the vaccinated child and subsequent interruption of transmission of the bacteria in the community (Isaacman et al., 2007). This highlights the importance of targeting colonization which is the reservoir of infection.

1.12.1 Humoral immune response

Although spontaneous clearance and acquisition of GBS has been reported in pregnant women with new acquisition rate of 8.5% to 17.6% between the first trimester and birth, there is a paucity of data on host immune mediators affecting GBS colonization (Hansen et al., 2004, Goodman et al., 1997). There are limited, mainly cross sectional studies, which have evaluated the association between serum serotype specific capsular antibody and GBS recto-vaginal colonization in women. (Beachler et al., 1979, Cleat et al.,

1980, Linden et al., 1982, Campbell et al., 2000, Davies et al., 2001, Hordnes et al., 1996, Baker et al., 1980, Anthony et al., 1984, Skidmore et al., 1985, Ratei et al., 1990, Matsubara et al., 2002, Matsubara et al., 2003). These studies reported higher concentration of serotype-specific antibodies among colonized as compared to uncolonized to the homotypic serotype. These studies were not designed to address whether CPS antibody reduces the risk of GBS colonization during pregnancy. We are, however, unaware of any longitudinal cohort study which evaluated the association between serotype-specific CPS antibody and subsequent risk of GBS recto-vaginal acquisition during the latter half of pregnancy. The likely efficacy of GBS polysaccharide-protein conjugate vaccines against maternal recto-vaginal colonization could be inferred through study of whether there is any association of capsular antibody titers and new acquisition of homotypic GBS serotypes in pregnant women.

The antibodies to GBS CPS protect the host by opsonising and thus facilitating phagocytosis. The ability of antibodies in serum sample to opsonize bacteria can be measured by in vitro opsonophagocytosis assays (OPA) (Romero-Steiner et al., 2006). These assays are designed to quantify functional antibodies in human serum samples that enable phagocytic effector cells (such differentiated HL-60 cells) to recognize and destroy bacteria in the presence of a complement source (Guttormsen et al., 2008). As the host's protection is mediated mainly by phagocytosis, the functional activity of antibodies rather than only the concentration, may correlate better with reduced risk of colonization (Johnson et al., 1999). For pneumococcal conjugate vaccine (PCV), OPA titers have been shown to be the best functional correlate of protection against invasive disease at a threshold of ≥1:8 (Jodar et al., 2003). A good correlation between an OPA assay and the IgG antibodies measured by ELISA was also observed (Nurkka et al., 2001).

The GBS vaccine elicits functional antibodies measured by OPA assay and persists for up to 2 years post-vaccination (Edwards et al., 2012). There is limited data on presence of functional antibody and GBS colonization in pregnant women. As part of my research, I investigated the association between serotype-specific functional antibody measured by OPA assay and risk of new acquisition by homotypic serotype during pregnancy. We also assessed the correlation between serum IgG and OPA activity.

1.12.2 Mucosal immune response

Haemophilus influenzae type b conjugate vaccines (Takala et al., 1991) and PCV (Dagan et al., 2002) has been shown to reduce nasopharyngeal colonization by the vaccine-serotypes. The mechanisms of protection against colonization by conjugate vaccines are not completely understood. Conjugate vaccines elicit IgA and IgG in saliva, suggesting a possible role of mucosal antibodies (Choo et al., 2000, Kauppi et al., 1995, Nurkka et al., 2001). A strong positive correlation was also observed between serum IgG and salivary IgG (Nurkka et al., 2001), suggests transudation of serum IgG onto the nasopharyngeal mucosal surface, which may prevent colonization by creating a steric inhibition of the pneumococcal surface proteins with binding sites on host cells (Dagan et al., 2005).

Hordes et al studied the association between maternal GBS colonization and mucosal IgG and IgA antibodies (Hordnes et al., 1996). The study demonstrated colonization with serotype-specific GBS in rectum or cervix had pronounced effect on the local immune responses in terms of IgG and IgA in the genital tract. In my research, I investigated the association between serotype-specific mucosal IgG and IgA and new

acquisition of homotypic serotype GBS colonization as well as correlation between serum IgG and mucosal IgG.

1.12.3 Cellular Immune Response

Host immune factors that affect duration of colonization are poorly understood. Studies were conducted that showed a limited role of antibodies in clearance of *Streptococcus pneumoniae* colonization existing at the time of vaccination, suggesting involvement of other mechanisms (McCool and Weiser, 2004). In the murine model it was demonstrated by Van Rossum et al, that innate and CD4+ lymphocyte cell mediated immunity rather than a humoral immune response is important for clearance of *Streptococcus pneumoniae* colonization from the murine nasopharynx (van Rossum et al., 2005). It has been suggested that exposure to microbial antigens leads to induction of effector T-cells that migrate to peripheral tissues resulting in the pathogen elimination (Reiner et al., 2007). Identification of cell-mediated immune factors that influence GBS colonization is likely to affect the GBS transmission and overall incidence of GBS disease.

There is no data on association of cellular immune response and GBS colonization among pregnant women. In my research, I investigated the role cellular immunity in clearance of existing GBS colonization. In order to access effector T-cell responses freshly isolated PBMC were assayed for cells that produce interferon gamma using ELISpot assay in response to GBS CPS antigens.

1.13 Aims and Objectives

The overall theme of this research was to determine the immunological correlates of protection for vaginal and/or rectal GBS colonization in pregnant women.

Study Objectives:

i. To undertake a systematic review and meta-analysis on prevalence of maternal GBS colonization across geographic regions.

ii. To compare different media for the isolation of GBS from vaginal and rectal swabs from pregnant women in our clinical setting.

iii. To determine the dynamics of GBS colonization in pregnant women between the 20^{th} and 37+ weeks, in terms of new acquisition and clearance of serotype-specific colonization rates.

iv. To determine the concentration of GBS serotype-specific capsular polysaccharide IgG antibodies in serum associated with risk of recto-vaginal GBS acquisition.

v. To determine the association between GBS serotype-specific cellular immunity and acquisition and clearance of existing GBS colonization in pregnant women during the latter half of pregnancy.

vi. To determine the association between GBS serotype-specific capsular polysaccharide IgG and IgA antibodies in vaginal mucosal secretions and GBS colonization in pregnant women.

Chapter 2 Material and methods for GBS colonization studies

2.1 Participant demographics

Participant recruitment and follow up was conducted at the local community ante-natal clinics in Soweto, a peri-urban black African suburb of Johannesburg. These community clinics were mainly utilized by black African pregnant women who represent 80% of the South African population group (Statistics, 2015). Pregnant women normally visit antenatal clinics close to their place of residence. Evaluation and screening of the pregnant women at study enrolment included documenting the health of the mother and foetus, gestational age of the foetus estimated based on last normal menstrual period (LMP) or sonar if available, HIV infection status (HIV status was determined by rapid ELISA screening test as a standard of care in the clinics or with HIV ELISA test undertaken if the rapid test was positive or indeterminate). The mother then followed up 5-6 weeks later depending on the timing of her gestation.

2.2 Ethics Approval

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (IRB/Protocol-M090937; Appendix 4) and informed written consent was obtained from all participants. This, observational study was registered with South African National Clinical Trials Register, number DOH-27-0210-3012.

2.3 Participant enrolment

Community ante-natal clinics involved in the study were Pimville (MichealMaponya), Lillian Ngoyi, Diepkloof and Mofolo. Pregnant women were approached in these ante-natal clinics when they come for their routine ante-natal visit at 20-25 weeks gestational age. Pregnant women were informed regarding the study, interested women were given informed consent form (ICF) to read and screened for eligibility criteria. Consenting and eligible women were allocated a unique identification number and thereafter demographic and pregnancy related data was collected.

2.4 Inclusion and exclusion criteria

All pregnant women were eligible for inclusion in the study if they meet the following criteria:

2.4.1 **Inclusion criteria**:

- Are in their 20^{th} - 25^{th} week of gestation.
- Are able to provide informed consent.
- Are confirmed to be HIV-uninfected as part of standard of care

2.4.2 **Exclusion Criteria**:

- Acute illness or a symptomatic vaginal discharge.
- Antibiotic treatment in the previous two weeks.
- Have a known or suspected condition in which vaginal exams are contraindicated, e.g. placenta previa.

2.5 Procedures conducted at enrolment (visit-1) and follow-up visits

- Maternal case report forms were completed by the study nurse.
- The gestation age of the mother was calculated based upon normal LMP or sonar, if available. In cases, where there was discordance between LMP and sonar results, the sonar results were used for estimation of gestational age.
- Maternal pregnancy and medical history was obtained from the mothers available clinical records.
- Blood was taken from the mother (5mL) to measure antibodies to the common CPS serotypes (Ia, ,III and V)
- Vaginal secretion was collected from the mother to measure antibodies to the common CPS serotypes (Ia, III and V)
- Blood was taken from the mother (5ml) in heparinsed tubes to measure cellular immune response against CPS serotypes (Ia, III and V).
- The mother was swabbed (rectal and lower vaginal) for GBS culture.
- Duplicate vaginal and rectal swabs were collected from 130 participants to compare media for isolation of GBS.
- The mother was given a copy of the informed consent form with a follow-up date to return at the next visit.

2.5.1 Participant Follow-up

All participants were followed-up at 5-6 weeks interval till 37+ weeks and the following samples were collected at follow-up visits (Table 2.1).

Visit-2: Lower vaginal and rectal swab.

Visit-3: Lower vaginal and rectal swab.

Visit-4: Lower vaginal swab, rectal swab, blood (5ml) and vaginal secretions.

Sample collections were done by study nurses of RMPRU and were trained on sample collection using standard operating procedures available at RMPRU. Recruitment of participants, sample collection and follow up was overseen by Gaurav Kwatra and Dr Clare L. Cutland.

Table 2.1: Sample collection at different study visits.

Samples	Gestation Period			
	20-25 weeks	26-30 weeks	31-35 weeks	37+ weeks
Clotted Blood [a]	√	X	X	√
Heparinised Blood [b]	√	X	X	X
Vaginal and rectal swab*[c]	√	√	√	√
Vaginal mucosal secretion swab [d]	√	X	X	√

*For 130 participants duplicate vaginal and rectal swabs were collected at visit-1 to compare different media for isolation of GBS.

a. Clotted blood was used for serum collection to measure serum IgG for CPS (Ia, III and V) and OPA assay for CPS (Ia and III).
b. Heparinised blood was used to perform ELISpot assay for GBS CPS antigens (Ia, III and V).
c. Vaginal and Rectal swabs were processed to detect GBS colonization. GBS isolates were serotyped and pilus typed.
d. Mucosal secretions of the vaginal tract were collected with an ORACOL swab to measure mucosal IgA and IgG for GBS CPS (Ia, III and V).

2.6 Sample size

It was estimated that a sample size of 600 was required, (adjusted to 661 to allow for a 10% loss of follow up) to obtain sufficient numbers of mothers who became colonized during the course of the study. These numbers were based on the following assumptions. From previous studies, the number of new GBS acquisitions during pregnancy ranged from 9.9%-20%, with an average of 10% (Goodman et al., 1997, Towers et al., 2010, Chua et al., 1995). In a past study conducted by our unit, vaginal colonization prevalence of pregnant women during labor was 20.9% (Cutland et al., 2009), and was estimated to be about 26% if recto-vaginal swabbing is performed based on the increase in sensitivity of swabbing as compared to vaginal swabbing only.

With a sample size of 600, and an assumed acquisition rate of 10%, approximately 60 cases of new acquisition were expected, and assuming a colonization rate of 26%, 384 participants will remain un-colonized throughout the study. Hence, we anticipated a new acquisition and non-colonized ratio of 1:6.4. In order to compare the geometric mean antibody titres between the new acquisition and non-colonized by student t-test with natural log transformed data, the following sample size calculation was performed.

From previously published antibody data (Davies et al., 2001), the standard deviation for all serotypes was log=2.5. If one were to power the study to detect a difference of log=1.2, (translates to new acquisition having a median titres of 0.1µg/ml and the non-colonized control group having a median titre of 0.35 µg/ml) with p = 0.05 and 80 % power, the minimum number of new acquisition cases (irrespective of GBS serotype) required was 40. Similarly, sample size required for similar analysis for single serotypes

was undertaken, This was premised on data from our setting which previously reported that serotype III accounted for 50 % of all clinical cases of invasive GBS disease and 33% of colonizing isolates in pregnant women at birth. Thus, if a third of all acquisitions were serotype III, the case to control ratio would be 1:19.2. From previously published antibody data of serotype III, the standard deviation for antibody titres was significantly lower than for multiple serotypes, and estimated at log=1.85. Using a similar probability, power, and difference between means, 20 cases of single serotype colonization were required. Thus, with the above assumptions, a sample size of 600 was expected to yield approximately 60 acquisitions of which 20 were the major serotype. This sample size was unlikely to be powered to evaluate the less common serotypes individually.

2.7 Lab Methods

2.7.1 Processing of Specimens

2.7.1.1 Whole blood

Whole blood was collected from mothers at 20-25 weeks of gestation and 37+ weeks. All blood samples were kept at room temperature to allow clotting, following which it was transported within 4-6 hours to the RMPRU lab for processing. Blood was centrifuged for 15 min at a 3220g relative centrifugal force. The serum was then aliquoted into 2 pre-labelled tubes and stored at –70ºC.

2.7.1.2 Vaginal and rectal swabs

Vaginal and rectal swabs were collected from the mothers, for isolation, identification and serotyping of Group B *Streptococcus*. Both vaginal and rectal specimens

were collected using rayon tipped swabs that were placed into Amies transport medium without charcoal (Transwab Amies, Medical wire, U.K.). The rectal swab was inserted approximately 2 cm passed the anal verge and rotated against the rectal mucosa. The vaginal swab was inserted approximately 2 cm passed the introitus towards the lower vagina mucosal wall and rotated. Once swabs are received in laboratory if not processed immediately, they were stored at 2-8˚C and were processed within 24 hours of collection.

2.7.1.3 Heparinized blood

Heparinized blood was collected from mothers at 20-25 weeks of gestation and was transported within 4-6 hours to the RMPRU lab for processing. In order to access effector T-cell responses freshly isolated PBMC from heparinized blood were assayed for cells that produce interferon-γ (IFN-γ) using ELISpot assay in response to GBS CPS antigens.

2.7.1.4 Vaginal mucosal secretion

Vaginal mucosal secretion was collected from mothers at 20-25 weeks of gestation and 37+ weeks. All vaginal secretion samples were collected using ORACOL swabs and were kept at 2-8˚C after collection and transported on ice within 4-6 hours to the lab for processing. Mucosal secretions were extracted by adding 300µl of extraction buffer (10% FBS in PBS) and was centrifuged for 15 min at a 3220g relative centrifugal force at 4˚C. The mucosal secretion was then aliquoted into pre-labelled tubes and stored immediately at –70ºC.

2.8 Isolation of GBS

For GBS isolation, swabs were inoculated onto CHROMagar Strep B (CA; Media Mage, Johannesburg, South Africa) according to the following procedure.

- CHROMagar surface was dried in the 37°C ambient incubator for about 15 minutes, labelled with the participant number and divided into quadrants.
- Swabs from the Amies transport medium were rubbed in a small circle onto the first quadrant of the agar plate. The swab was rotated whilst plating, to ensure that the entire surface (which could potentially contain GBS) comes into contact with the agar.
- The CA plate was streaked as per standard plating out technique Divide the plate into four quadrants. Streak from the one quadrant to the next quadrant with a single line. Once in the new quadrant spread the growth by creating a zigzag line with 3-5 folds without re-entering the initial quadrant. Repeat the process across all 4 quadrants and change the loop after the completion of each quadrant.
- After streaking, the CA plate was incubated at 37°C in the ambient incubator.
- After 18-24 hours the plate was inspected for growth of GBS. If no GBS-like growth was present, it was incubated for an additional 24 hours and the plate was inspected again. Typical GBS colonies presented as mauve coloured colonies on CA (Figure 2.1). If at 48 hours, the plate yielded no GBS colonies, no further testing was done and NO GBS isolated was recorded.
- The total amount of GBS isolated was quantified using the standard microbiology colony quantification method (Table 2.2) and secondary identification testing for GBS was done.

Table 2. 2: colony quantification method

Scant growth	< 25 colonies in 1st quadrant only
1+	≥ 25 colonies in quadrant 1 plus < 25 colonies in quadrant 2nd
2+	≥ 25 colonies in quadrant 2nd plus < 25 colonies in quadrant 3rd
3+	≥ 25 colonies in quadrant 3rd plus < 25 colonies in quadrant 4th
4+	≥ 25 colonies in 4th quadrant

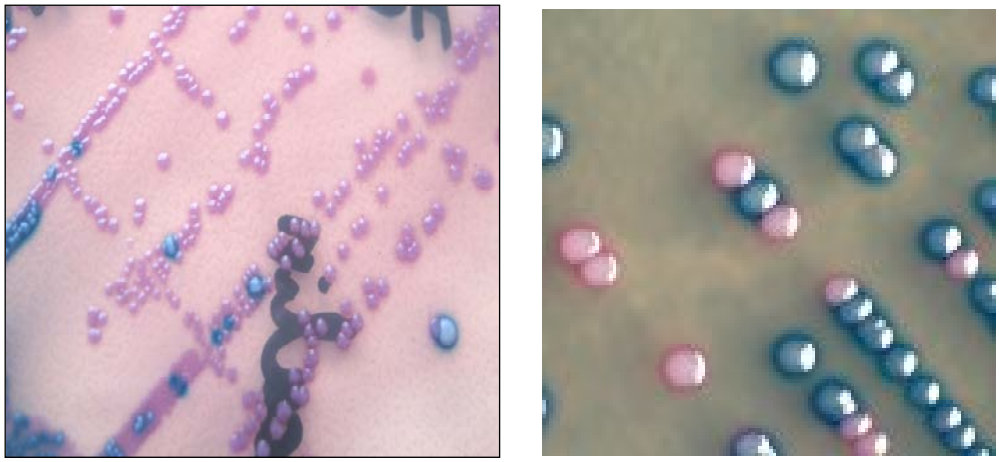


Figure 2.1: Mauve coloured colonies of Group B *Streptococcus* on CHROMagar Strep B

2.9 Secondary Identification of Group B *Streptococcus*

Some other microorganisms like Groups A, C, F and G streptococci and some strains of staphylococcus may also appear mauve, violet or pinkish and some colonies of enterococci may appear purplish, therefore mauve colonies were further tested by secondary tests (CAMP test, Bile esculin test, Catalase test and Group B antigen test) for further confirmation.

2.9.1 CAMP Test

GBS produces a positive CAMP test result with clear arrow-head shaped hemolysis at the junction with the line of staphylococcus (Figure 2.2). All non-group B streptococci are CAMP negative although some strains of group A may produce a positive result if incubated in at increased CO_2 concentration.

Procedure:

Pre-warmed 5% sheep blood agar plate was divided and labelled as indicated by the diagram below. A single streak of *Staphylococcus aureus* (ATCC 25923) was made down the middle of the plate and positive and negative controls on either side and perpendicular to the *Staphylococcus aureus* streak. GBS colonies were streaked from the edge of the plate toward the middle. Multiple isolates to be tested were added onto the plate. The plate was incubated at 37˚C in 5% CO_2 for 18-24 hours.

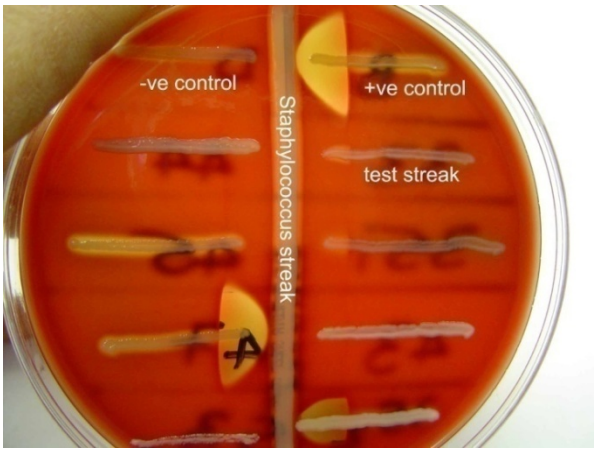


Figure 2.2: CAMP test results with clear arrow head hemolysis

Reading and interpretation:

GBS produces a positive CAMP test result with clear arrow-head shaped haemolysis at the junction with the line of *Staphylococcus aureus*. Some Staphylococcus strains can produce a small arrow-head of lysis. If the arrow-head shaped haemolysis is not clear, the results were recorded as indeterminate.

2.9.2 Bile aesculin test:

Bile aesculin agar is used primarily to differentiate Enterococcus spp. (Group D *Streptococcus*) from GBS. Group B *Streptococcus* grows as clear, mucoid colonies on Bile esculin agar with no hydrolysis, resulting no blackening of the growth medium. Enterococcus species also grows well on bile aesculin agar, but cause blackening of the medium through aesculin hydrolysis.

Procedure

Pre-warmed bile esculin agar plates were divided into quadrants and labelled. The positive control (GBS) and negative control (*E. faecalis*) were streaked along with the test isolates onto different quadrants of the bile esculin plate. The same colonies that have been streaked on the CAMP plate were streaked in the corresponding positions on the bile aesculin plate and incubated at 37˚C in 5% CO_2 for 18-24 hours.

Reading and interpretation

GBS grows as clear, mucoid colonies with no blackening of the growth medium.

Interpretation of CAMP and Bile-esculin tests (Figure 2.3)

- If the CAMP test was negative and bile esculin hydrolysis was positive: no GBS isolated.

- If the CAMP test was positive and bile esculin hydrolysis is negative: Proceed with the catalase and group B streptex tests.
- If the CAMP test and bile esculin hydrolysis is indeterminate: Proceed further with the catalase, group B streptex test and PCR test

2.9.3 Catalase test:

Catalase test was used to distinguish between Staphylococcus spp and GBS.

Procedure

A drop of the catalase reagent (hydrogen peroxide) was added to a glass slide. The GBS culture on the CAMP plate was touched with a sterile loop and mixed into the reagent. Immediate production of bubbles was interpreted as positive reaction. The absence of bubbling was interpreted as negative.

Reading and Interpretation

GBS isolates are catalase negative when tested with catalase reagent.

2.9.4 Group B antigen agglutination test:

Colonies which were CAMP positive, Bile esculin negative and catalase negative were further tested for Group B antigen agglutination test using STREPTEX agglutination test kits as per kit instructions.

Procedure

- All the reagents of the kit were warmed up to room temperature prior to use.

- Two to three single colonies of the GBS culture were picked off and mixed in a tube containing 400µl of the extraction enzyme.
- The suspension was incubated in a water bath or incubator at 37 ˚C for 10 minutes, with shaking after 5 minutes.
- Reading card was labelled with the relevant information.
- 50µl of the bacterial extract was added to the circle on the reading card.
- One drop of the pre-warmed latex reagent was added to the circle of the test card (already containing the drop of the bacterial extract) and was mixed with a clean mixing stick or pipette tip.
- The card was gently rock for one minute and observed for agglutination.

Reading and Interpretation

Group B *Streptococcus* produce clear agglutination.

2.9.5 Storage of GBS isolate:

All confirmed GBS isolates were stored in STGG storage medium at -70°C.

2.10 Group B *Streptococcus* serotyping by Latex agglutination

All GBS isolates were serotyped with a latex agglutination method using specific antisera against type Ia, Ib, II to IX CPS antigens as described (Afshar et al., 2011).

Procedure

- GBS isolates in the STGG medium were thawed and 20µl were plated on a labelled, pre-warmed blood agar plate and streaked out for single colonies as per standard technique.

- The plates were incubated for 18-24 hours at 37°C in 5% CO2.
- 50µl of sterile PBS was pipetted into a sterile tube and a heavy suspension from the pure growth was made in the PBS. No clumps in the suspension were insured.
- 5µL of the suspension was pipetted onto the glass slide
- 5µL of the serotype-specific latex bead suspensions was added aseptically onto the corresponding slide and mixed with the bacterial suspension using a new clean mixing stick or sterile pipette tip.
- The slide was gently rocked for 5 to 10 seconds to aid mixing. A positive reaction was scored when the sample agglutinated with a specific serum within 30 seconds.

Interpretation and reporting

Positive agglutination reaction with latex anti-sera of a particular serotype indicated the serotype of a GBS isolate. Any agglutination after 30 seconds was considered as indeterminate and further confirmation was done by PCR method. GBS Isolates that test negative by latex agglutination for all serotypes were further typed by a PCR method

2.11 PCR method for the detection of Group B Streptococcus serotype

GBS isolates that tested negative by latex agglutination for all serotypes were further typed by a single plex PCR method for serotypes Ia, Ib, II, III, IV and V using primer sequences described by Poyart*et al* (Table 2.3). Briefly, DNA was extracted from GBS isolates with the Easy Mag DNA Extraction Machine and added to the PCR reaction mixture (Table 2.4). The gene encoding dlts was used as a positive control for GBS identification. Negative control contained filtered dH_2O (sterile) instead of DNA to give an indication of any false positive results.

Table 2.3: Primer and probe sequences

Primer name	Sequence (5'-3')
Ia	Forward 5'- GGTCAGACTGGATTAATGGTATGC-3' Reverse 5' GTAGAAATAGCCTATATACGTTGAATGC- 3'
Ib	Forward 5'- TAAACGAGAATGGAATATCACAAACC-3' Reverse 5' GAATTAACTTCAATCCCTAAACAATATCG- 3'
II	Forward 5'- GCTTCAGTAAGTATTGTAAGACGATAG-3' Reverse 5' TTCTCTAGGAAATCAAATAATTCTATAGGG-3'
III	Forward TCCGTACTACAACAGACTCATCC-3' Reverse 5' AGTAACCGTCCATACATTCTATAAGC-3'
IV	Forward GGTGGTAATCCTAAGAGTGAACTGT-3' Reverse 5' CCTCCCCAATTTCGTCCATAATGGT-3'
V	Forward GAGGCCAATCAGTTGCACGTAA-3' Reverse 5' AACCTTCTCCTTCACACTAATCCT-3'
Dlts	Forward AGGAATACCAGGCGATGAACCGAT-3' Reverse 5' TGCTCTAATTCTCCCCTTATGGC-3'

Table 2.4: Reaction mixture

Reagents	Stock concentration	1 reaction (25 µl)	Final Concentration
EconoTaq	2X	12.5	1X
Primer F	10µM	1	250 nM
Primer R	10µM	1	300 nM
DNA template		5	
dH2O (Sterile)		5.5	

Cycle Conditions

The amplification of a target DNA was achieved through following 5 stages.

- Stage 1: Activation of Taq DNA polymerase at 94°C for 2 min
- Stage 2: Denaturing process of the DNA template for 30 sec at 94°C.
- Stage 3: Annealing of the complementary primers at 55.1°C for 30 sec.
- Stage 4: Extension period for 1min and 30 sec at 72°C.

- Repeat stage 2 to stage 4 for 35 PCR cycles
- Stage 5: Final extension for 10 min at 72°C.

Interpretation of results

The serotype of the GBS isolate was determined on the basis of matching band size of DNA fragment for capsular polysaccharide by agarose gel electrophoresis.

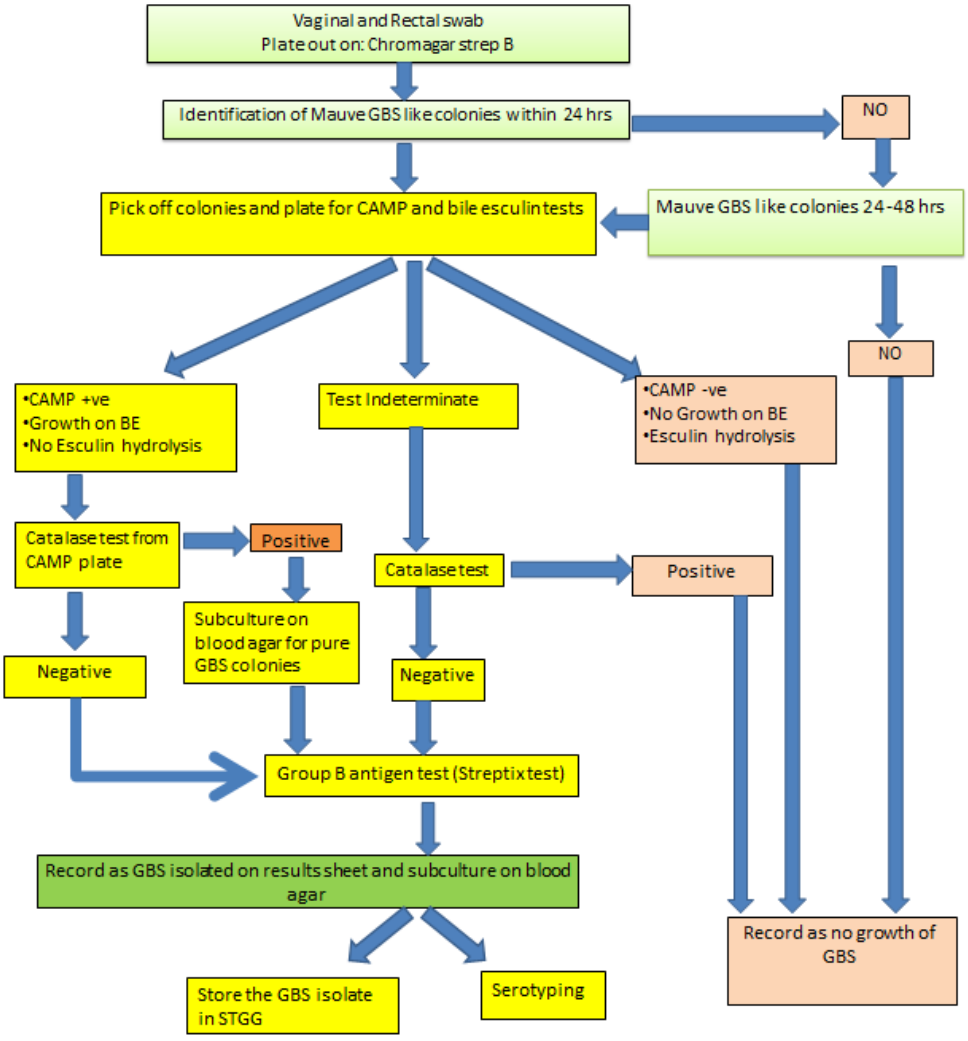


Figure 2.3: Algorithm for Group B *Streptococcus* isolation from vaginal and rectal swabs

2.12 Pilus typing

Pilus island (PI) typing of GBS isolates were done by real time PCR with Taqman probes for PI-1, PI-2a and PI-2b, with primers that target the genomic regions coding for the ancillary protein (AP)-1 of each PI as described previously (Madzivhandila et al., 2013). Briefly, GBS isolates were sub-cultured and three GBS colonies were suspended in 300 µl nuclease-free distilled water and the suspension was heated at 95°C for 10 minutes.

The suspension was centrifuged at 9000 g for 1 min to pellet the cell debris. Four microliters of the supernatant was added to each PCR. The PCRs were run on an AB 7500 instrument (Applied Biosystems) in a 25 µl reaction volume with TaqMan universal PCR master (Applied Biosytems). The detection PI-1 and PI-2a were performed as a duplex reaction and PI-2b was performed as single-plex . GBS strains 2603 V/R (PI-1 and PI- 2a) and COH1 (PI-2b) were used as reference strains. The reaction mixture (25µl) consisted 250nM of probe, 12.5µl of master mix, 4µl of DNA, primer (900 nM for PI-2b and 300nM and 110nM for forward and reverse primer for PI-1 and PI-2a, respectively). Cycle conditions included AmpErase activation at 30°C for 2 minutes, Taq DNA polymerase activation at 95°C for 10 minutes followed by 40 cycles of denaturation at 94°C for 15 seconds and extension for 1 minute at 60°C. Positive C_T value was defined as the point where the amplification curve crosses a manually set threshold.

2.13 Antibody measurement by Luminex assay

We developed a multiplex bead-based immunoassay method for the simultaneous measurement of antibodies to 4 different GBS antigens present in the samples. With this assay, antibodies to four capsular polysaccharides can be quantified simultaneously. The Luminex assay was found to be less labour intensive and require less sample volume as compared to ELISA (Pang et al., 2005).

2.13.1 GBS capsular polysaccharides and reference serum

Group B *Streptococcus* serotype-specific capsular polysaccharides were kindly provided by Novartis Vaccines, Italy. Polygam (pooled gamma globulin; National Bioproducts, South Africa) was used as in-house reference serum for IgG measurement

whereas pooled human serum was used for IgA measurement. Antibody concentrations were assigned to the reference serum by calibrating them with the standard serotype-specific GBS reference serum kindly provided by Prof. Carol J. Baker. Serotype-specific antibody concentrations in the reference sera are shown in table 2.5

Table 2.5: Antibody concentration in reference serum

Reference Serum	Serotype	IgG	IgA
	Ia	11.72	0.51
	Ib	4.33	0.18
	III	8.78	1.99
	V	6.41	5.58

2.13.2 Validation of Luminex assay

The Luminex multiplex assay for antibody analysis (both IgG and IgA) was validated using standard validation steps such as linearity of the assay, a multiplex versus single-plex comparison, determining lower limits of detection and the specificity of the in-house reference serum.

2.13.2.1 Linearity

The first step of validation was to detect linearity as well as range of the assay. The standard curves were developed using Polygam (for IgG) or pooled serum (for IgA) as reference at different dilutions for each of the serotype-specific antibodies (Figure 2.4.1-2.4.8). The dilutions in the figures below are represented by a standard (Std) and were as follows: Std1- 1:100, Std2- 1:400, Std3- 1:1600, Std4- 1:6400, Std5- 1:25600, Std6- 1:102400 and Std7- 1:409600.

Figure 2.4.1: Standard linearity curve for Serotype Ia, IgG

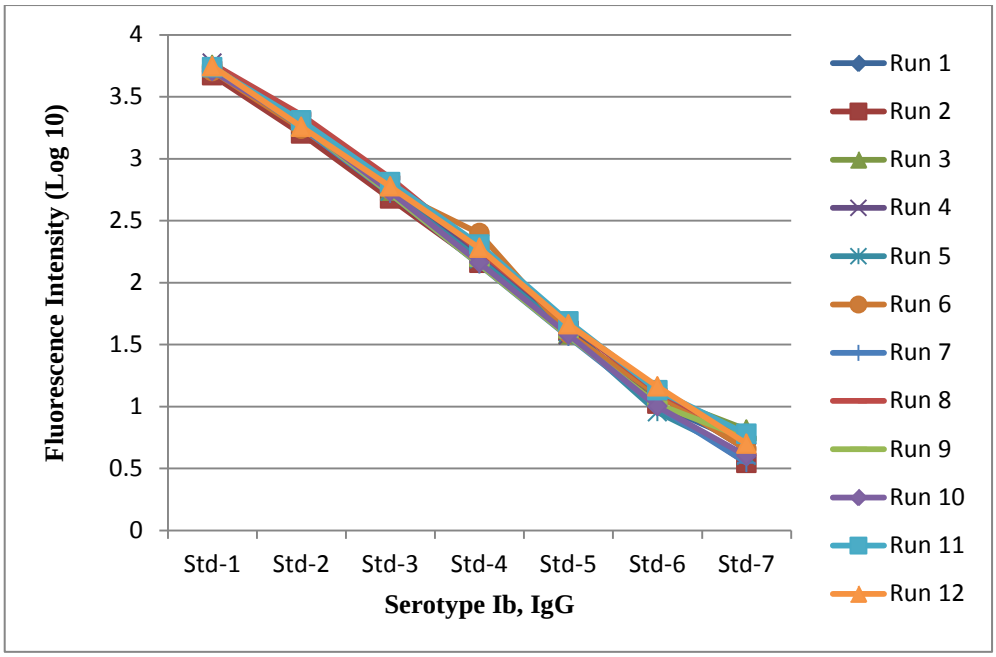


Figure 2.4.2: Standard linearity curve for Serotype Ib, IgG

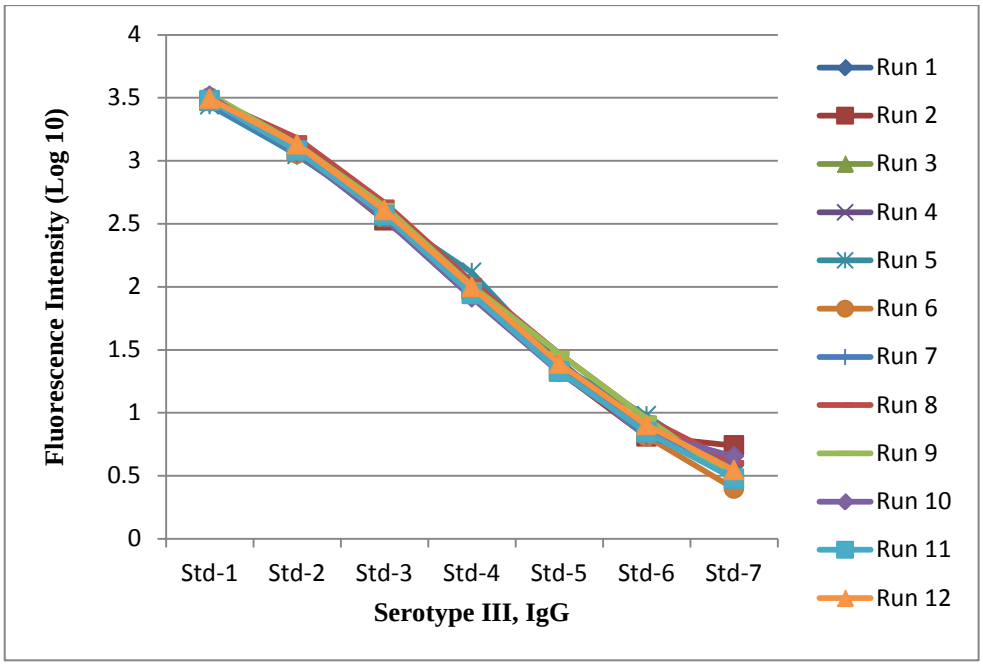


Figure 2.4.3: Standard linearity curve for Serotype III, IgG

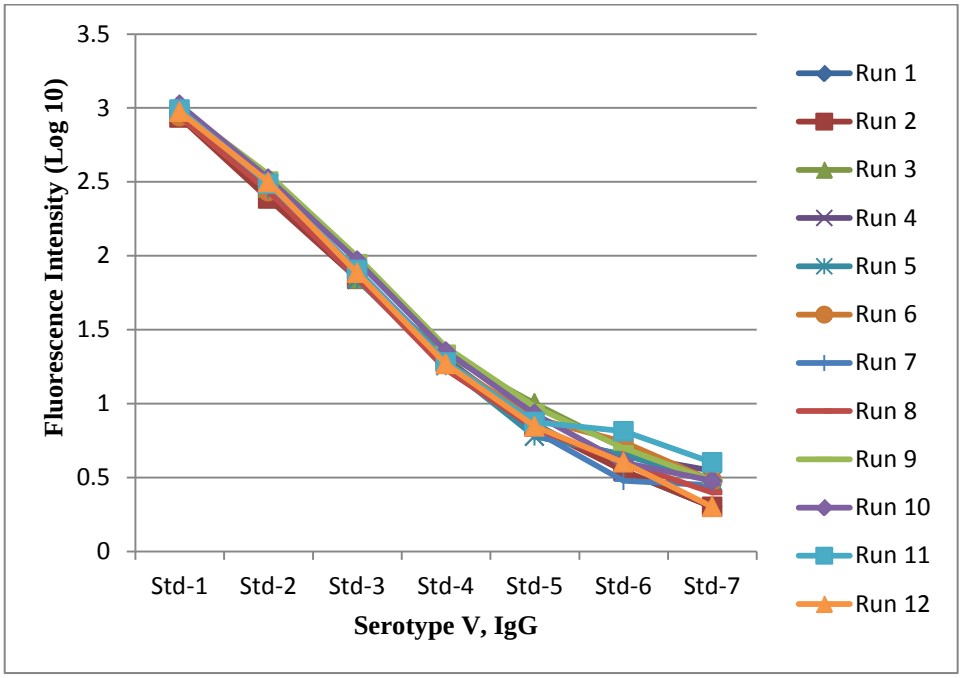


Figure 2.4.4: Standard linearity curve for Serotype V, IgG

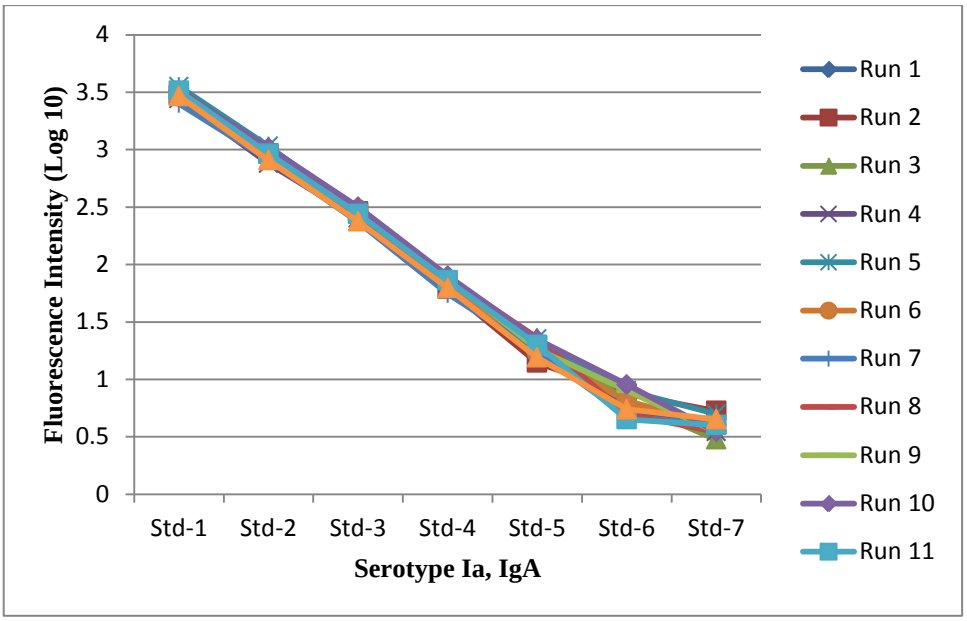


Figure 2.4.5: Standard linearity curve for Serotype Ia, IgA

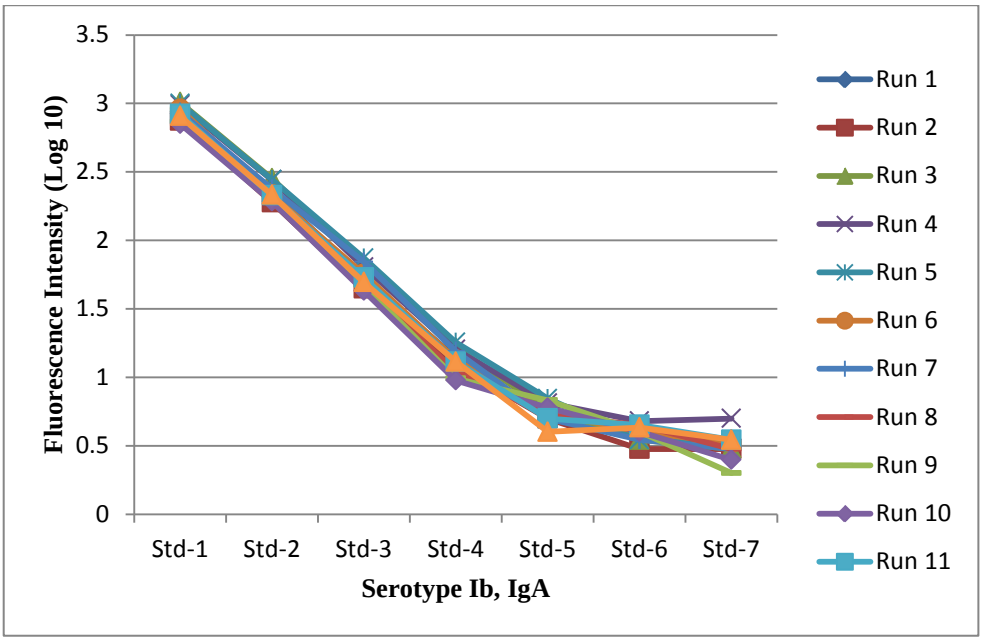


Figure 2.4.6: Standard linearity curve for Serotype Ib, IgA

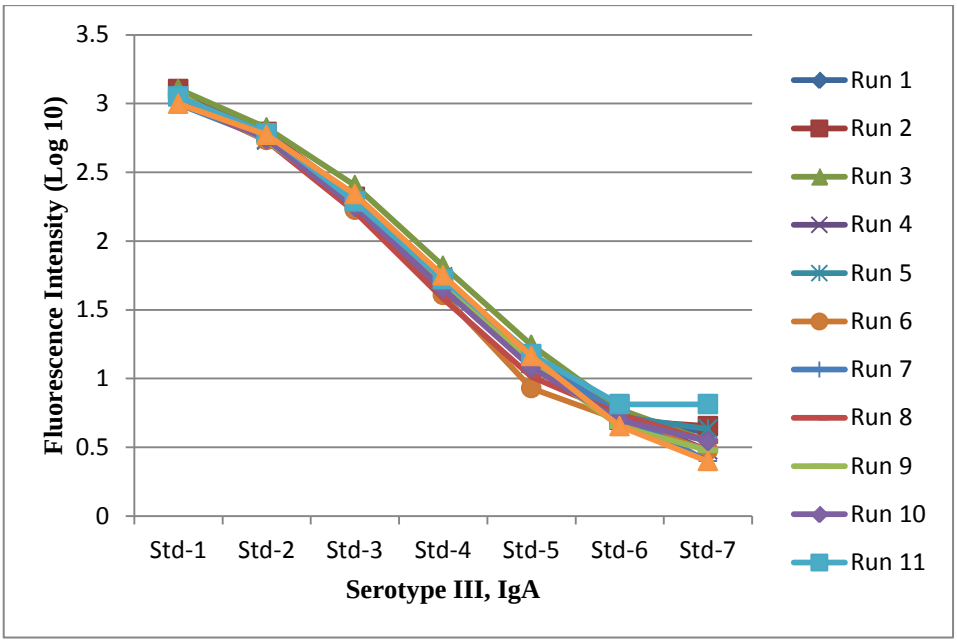


Figure 2.4.7: Standard linearity curve for Serotype III, IgA

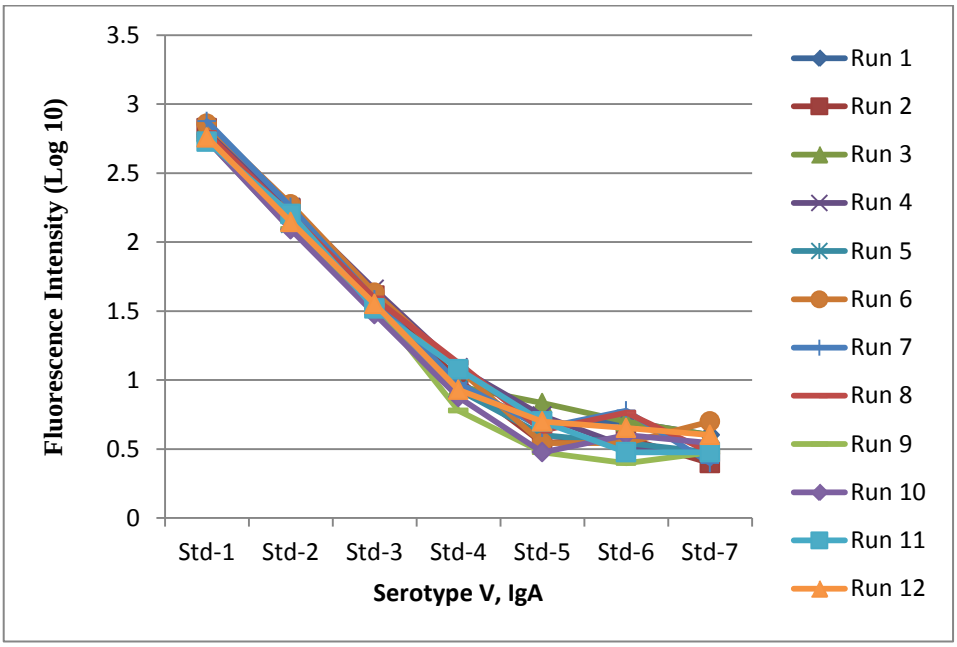


Figure 2.4.8: Standard linearity curve for Serotype V, IgA

2.13.2.2 The multiplex vs singleplex assay

The multiplex assay was validated by comparing the Mean fluorescence intensity (MFI) values for reference serum (1:100 dilution) obtained with the multiplex assay as compared to those obtained by singleplex assays. We observed a maximum of 20% variation in with multiplex assay as compared to singleplex.

Table 2.6: The MFI values with the multiplex assay as compared to singleplex assay for IgG

Serotype	Median %	Min %	Max %
Ia	100	86	111
Ib	85	79	97
III	101	84	110
V	103	82	112

Table 2.7: The MFI values with the multiplex assay as compared to singleplex assay for IgA

Serotype	Median %	Min %	Max %
Ia	106	91	119
Ib	99	83	120
III	99	73	119
V	110	80	122

2.13.2.3 Detection limits

Lower Limits of detection (LLD) for the multiplex assay were calculated from the fluorescence of the mean blank value plus 3 standard deviations. The LLD values were converted to antibody concentration from an "averaged" reference curve consisting of the mean fluorescence values at each concentration and the concentrations for the cut off were determined relative to the reference. For statistical purposes, any value that falls below the LLD was assigned a value of half of the LLD.

Table 2.8: Detection limits for Serum IgG, Mucosal IgG and Mucosal IgA

	Serotype			
	Ia	Ib	III	V
LLD (Serum IgG), µg/ml	0.0003	0.0016	0.005	0.009
½ of LLD (Serum IgG)	0.00015	0.0008	0.0025	0.0045
LLD (Mucosal IgG), ng/ml	0.007	0.033	0.091	0.22
½ of LLD (Mucosal IgG)	0.003	0.016	0.045	0.11
LLD (Mucosal IgA), ng/ml	0.010	0.018	0.032	0.64
½ of LLD (Mucosal IgA)	0.005	0.009	0.016	0.32

LLD- Lower Limit of detection

2.13.2.4 Specificity assay

To assess analytical specificity and to verify antigenic integrity of each GBS CPS antigen–microsphere set, each GBS CPS antigen (100 µg/ml) was incubated at 37˚C for 2 hours as an inhibitor to different wells containing the multiplexed GBS–microsphere mix and reference serum added at 1:100 dilution. Following incubation, the serology assay was performed. Specificity results for each GBS antigen–microsphere using homologous and heterologous inhibition were determined by calculating the percent inhibition in MFI

signal in the presence of the GBS inhibitor relative to the MFI signal in the absence of the inhibitor using the following equation (Table 2.9 and Table 2.10)

$$\% \text{ inhibition} = 100 \times \frac{(\text{MFI of reference serum}) - (\text{MFI of reference serum} + \text{inhibitor antigen})}{\text{MFI of reference serum}}$$

Table 2.9: Specificity of the multiplexed GBS immunoassay for IgG

Specificity assay for IgG using Polygam as reference				
	Antigen			
Inhibitor Antigen	IA	IB	III	V
IA	99.20	40.90	11.16	9.91
IB	0.00	97.05	1.69	4.40
III	-3.81	-9.61	94.77	18.30
V	-4.23	-9.22	-4.52	84.60

For IgG, homologous inhibition was >90% for all serotypes, with the exception of serotype V (85%). Heterologous inhibition across serotypes was <10%, except for serotypes Ib and III, which were inhibited by 41% and 11% with type Ia CPS respectively, and serotype V (18%) was inhibited by serotype-III CPS.

Table 2.10: Specificity of the multiplexed GBS immunoassay for IgA

Specificity assay for IgA using pooled human sera as reference				
	Antigen			
Inhibitor Antigen	IA	IB	III	V
IA	99.68	80.88	21.58	-13.60
IB	9.25	99.12	-4.63	3.73
III	1.85	-2.30	99.22	6.95
V	-3.93	0.41	-8.65	98.44

For IgA homologous inhibition was >95% for all serotypes. Heterologous inhibition across serotypes was <10%, except for serotypes Ib which was inhibited by 81% with type Ia CPS, along with 22% inhibition of serotype III.

2.13.2.5 Non-specific binding

In each experiment, for serum IgG, control beads were included to determine non-specific binding. For control beads, the coupling procedure was followed, except that no GBS antigen was added. In case of non-specific binding, the mean fluorescence intensity (MFI) values were subtracted from the antigen-specific results.

2.13.2.6 Sample and Secondary antibody Dilutions

The optimal serum dilutions were 1:100 for the measurement of antigen-specific IgG and 1:5 for the measurement of antigen-specific mucosal IgG and IgA. The optimal secondary antibody dilutions were 1:100 for IgG and IgA.

2.13.3 Conjugation of antigens to the beads

Capsular polysaccharides were coupled to the microsphere beads (Biorad, CA, U.S.A) with the crosslinking agent4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium(DMTMM) as described (Schlottmann et al., 2006). Briefly, 40 mg of DMTMM was weighed out in a 5ml tube and 2.7 ml of GBS serotype-specific CPS solution (1mg/ml) was added, vortexed and incubated at RT on rotating shaker for 60 min. DMTMM modified CPS was purified using Sephadex G25 PD-10 columns and eluted in 3.5 ml of PBS. 1ml of beads (centrifuged at 12000 rpm) were suspended in 500ul of DMTMM modified CPS and incubated overnight at RT on a shaker. After incubation beads were washed twice with wash buffer (Phosphate buffer solution (PBS)-Tween) and stored in assay buffer (PBS 7.2, 10% Fetal Bovine serum and 0.05% Sodium Azide) at 4°C.

2.13.4 Luminex assay Procedure

The following steps were taken to run the assay

- All samples, standards and controls were prepared in true duplicates in assay buffer
- The 1.2 µm filter membrane plate (Pall) was saturated with 100µl of assay buffer.
- GBS Antigen–microspheres (3500 per antigen) in a volume of 50 µl in assay buffer were added to the wells of a filter plate and aspirated by use of a vacuum manifold filtration system (Bio-Rad).
- The plate was then washed twice with wash buffer (Phosphate buffer solution 7.2, 0.5% Tween, 0.02% Sodium Azide).
- Standards prepared from reference serum were serially diluted in four-fold dilutions beginning at1:100.

- The reference standard, controls and serum samples (50ul) were added to the GBS–microspheres and incubated for 60 min at RT, with shaking.
- After the incubation, the plate was washed three times with wash buffer.
- Fifty microlitres of goat anti-human, phycoerythrin conjugated IgG or IgA (1:100 dilution in wash buffer) (Jackson immunoresearch) was added into each well and incubated for 30 min at RT, with shaking.
- After the incubation, the plate was washed three times with wash buffer and the contents of the plate were resuspended in 130µl of wash buffer.
- Thereafter, 100-110 µl of wash buffer containing the beads was transferred to the reading plate.
- Bead fluorescence was read with the Bioplex 200 instrument (Bio-Rad, Texas, U.S.A) using Bio-Plex manager 5.0, software (Bio-Rad) and data was analyzed using a 5 Parameter Logistic curve fit.
- All over range and low range samples were re-tested with higher or lower dilutions (1:50-1:1000 for IgG and 1:2-1:10 for IgA).

2.14 Serum IgG opsonophagocytic activity (OPA) assay

The functional activity of serum IgG was determined by OPA assay for serotype Ia and III using the HL-60 cell line as described previously (Michon et al., 2006).

2.14.1 HL-60 cells for OPA assay

Undifferentiated HL-60 cells were purchased from the American Type Culture Collection (ATCC) and were grown in 50 ml of growth media (500ml RPMI 1640, 100ml inactivated FBS , 6.2 ml of L-glutamine (200mM), 5ml of penicillin-streptomycin

(10000 IU/ml) in 300 ml tissue culture flasks. The cells were counted, viability was determined and divided three times a week (Mon, Wed and Fri) into a cell density of 3×10^5 cells/ml (Monday and Wednesday) and 2×10^5 cells/ml (Friday) and fed twice a week (Tuesday and Thursday). All culture flasks were incubated at 37°C in 5% CO_2 atmosphere in the upright position with loose caps.

Undifferentiated cells grown to a cell density of $4\text{-}10 \times 10^5$ cells/ml with a viability of > 90% were used for differentiation. Differentiation was carried out in cultures with a 200 ml volume at 2×10^5 cells/ml (600 ml flasks) of growth medium by the addition of 100 mM N,N-dimethylformamide (DMF) as the chemical inducer (1550 µl of DMF/ 200 ml growth medium) and incubated for a period of 5 days at 37 °C, 5% CO_2 in the slanted position with the cap loose. HL-60 cells were 90-95% differentiated into granulocytes by 5 days, and were ready to be used as phagocytes with a typical yield of 6-8 $\times 10^5$ cells/ml. After 5 days, a viable count was performed and cells with a viability of less than 70% were not used in the assay. Differentiated HL-60 cells were pre-washed twice with Hank's Balanced Salt solution w/o Ca^{2+} and Mg^{2+} ($HBSS^-$) + 0.2% BSA buffer (10 ml/tube) and resuspended in Hank's Balanced Salt solution w/o phenol red ($HBSS^+$) + 0.2% BSA (1 ml/tube). Total cell count and viability was determined and the cell suspension was diluted to a concentration of 1×10^7 cells/ml.

2.14.2 Bacterial stocks

Standard GBS strains (serotype Ia (A909) and serotype III (COH-1) from ATCC were used for the preparation of frozen stocks. Viable counts on random samples were performed to determine the concentration of GBS after freezing protocol and the dilution

factor required for the concentration of 1000 cfu/20ul ($5x10^5$cfu/ml) was calculated. Purity of bacterial suspension was also performed.

2.14.3 Quality control sera

Polygam with known reproducible OPA titre was included in each assay for quality control.

2.14.4 Serum dilutions

Starting serum dilutions were calculated on the basis of the antibody concentration of the sample as per table 2.11

Table 2.11: Serum dilutions for OPA assay

IgG concentration μg/ml	Sample dilution in OPA
0–4	1/1
4-7	1/2
7-10	1/4
10-15	1/8
15-30	1/16
30-50	1/32
> 50	1/64

2.14.5 Procedure for OPA assay

- 10 µl of Opsono buffer ($HBSS^+$ + 0.1% gelatin) was added to each well of a 96-well plate except for row A.

- 20 µl of diluted or undiluted test serum was added to the wells of row A. (Test serum was heat inactivated at 56^{o}C for 30 min prior to testing). QC sera (Polygam) was added to wells A11 and A12.
- Test and QC serum was serially diluted (2 fold) (rows A through H), except wells H9 through H12, which are reserved for the complement (C') controls.
- 20 µl of bacterial suspension was added to each well (1000 cfu/well). Frozen stock of bacteria was serially diluted in Opsono buffer to obtain 1000 cfu in 20 µl (5X 10^4/ml). The bacterial suspension was kept on ice until ready to use.
- The plate containing test serum and bacteria was incubated at 37°C for 15 min, 5% CO_2.
- After incubation, to each well 10 µl of complement source (Baby Rabbit Serum) and 40 µl of differentiated HL-60 cells was added (4 x 10^5 cells/well).
- The plate was incubated at 37°C for 45 minutes with horizontal shaking (220 rpm). After incubation the plate was cooled on ice for one minute to stop the reaction or kept on ice thereafter if more than two plates were run.
- From each well 5µl was platted onto Todd Hewitt Yeast plates and incubated at 37C and 5% CO_2.
- The colonies were counted after 18 hours after to avoid overgrowth and compared to the count in control wells which corresponds to the number of bacteria present in the each well after 45 minutes of incubation period in the presence of complement source.
- Opsonophagocytic activity was expressed as the titer at which the serum dilution yielded 50% killing compared with the bacterial growth in the complement controls. The limit of detection was 8. For statistical analysis, samples below the detection limit were assigned an arbitrary titer of 4.

2.15 ELISpot assay for measure of cellular immune response

Cellular immune response was determined at Visit-1 (enrolment) by Interferon-γ (IFN-γ) release assays, performed by Enzyme-linked Immunospot (ELISpot) assay kit for human IFN-γ as per manufacturer instructions (Cat no: 3420-2A, Mabtech, Sweden). Briefly, Peripheral blood mononuclear cells (PBMC) were isolated from the heparinised blood using ficoll gradient centrifugation and re-suspended in AIM-V medium (Gibco, Invitrogen, USA). Approximately, 0.25-0.3 x 10^6 PBMS's were added per well onto ELISpot plates (MAIP S4510; Millipore, Ireland) coated with IFN-γ capture antibody (Mabtech, Sweden). PBMC's were stimulated with GBS serotype-specific capsular polysaccharide antigens separately (CPS-Ia, III and V) for 18-24 hours at 37°C in 5% CO_2 with a final concentration of 40µg/ml. After incubation, ELISpot plates were developed using biotinylated anti-IFN-γ detection antibody (Mabtech, Sweden) which was conjugated subsequently to streptavidin-alkaline phosphatase (Mabtech, Sweden), and visualized using an AP conjugate substrate kit (Bio-Rad, CA, USA). Checker board titrations were performed to optimize PBMC's and antigen concentrations per well. AIM-V medium was used as negative control to assess background levels and phytohaemagglutinin (Sigma, USA) was used as a positive control. Cellular immune response against each antigen was detected in single well only. Spot forming units (SFU) were counted using the magnifying lens. Background (SFU in negative control) was subtracted and results were expressed as number of SFU/10^6 PBMC. An ELISpot response was considered as positive if the number of antigen-specific spots was ≥7 SFU/10^6 PBMC and at least double the number of spots in the negative control well.

Chapter 3 Prevalence of maternal Colonization with Group B *Streptococcus*: a systematic review and meta-analysis

3.1 Abstract

Background: The most important risk factor for early-onset (babies younger than 7 days) invasive group B streptococcal disease is rectovaginal colonisation of the mother at delivery. We aimed to assess whether differences in colonisation drive regional differences in the incidence of early-onset invasive disease.

Methods: We did a systematic review of maternal group B streptococcus colonisation studies by searching MEDLINE, Embase, Pascal Biomed, WHOLIS, and African Index Medicus databases for studies published between January, 1997, and March 31, 2015, that reported the prevalence of group B streptococcus colonisation in pregnant women. We also reviewed reference lists of selected studies and contacted experts to identify additional studies. Prospective studies in which swabs were collected from pregnant women according to US Centers for Disease Control and Prevention guidelines that used selective culture methods were included in the analyses. We calculated mean prevalence estimates (with 95% CIs) of maternal colonisation across studies, by WHO region. We assessed heterogeneity using the *I*2 statistic and the Cochran Q test.

Results: 221 full-text articles were assessed, of which 78 studies that included 73791 pregnant women across 37 countries met prespecified inclusion criteria. The estimated mean prevalence of rectovaginal group B streptococcus colonisation was 17.9% (95% CI 16.2‑19.7) overall and was highest in Africa (22.4, 18.1‑26.7) followed by the Americas

(19.7, 16.7‑22.7) and Europe (19.0, 16.1‑22.0). Studies from southeast Asia had the lowest estimated mean prevalence (11.1%, 95% CI 6.8‑15.3). Significant heterogeneity was noted across and within regions (all p≤0.005). Differences in the timing of specimen collection in pregnancy, selective culture methods, and study sample size did not explain the heterogeneity.

Conclusion: The country and regional heterogeneity in maternal group B streptococcus colonisation is unlikely to completely explain geographical variation in early-onset invasive disease incidence. The contribution of sociodemographic, clinical risk factor, and population differences in natural immunity need further investigation to understand these regional differences in group B streptococcus maternal colonisation and early-onset disease.

3.2 Introduction

Group B Streptococcus is a significant cause of severe infection in infants aged < 3 months, most often manifesting as sepsis, pneumonia and meningitis. Infant invasive GBS disease occurs as a continuum over the first 3 months of life, but is commonly divided into early onset disease (EOD), occurring within 0-6 days of birth, or late onset disease (LOD), occurring within 7-90 days of birth. Early onset disease is vertically acquired from the mother, whereas LOD can also be acquired nosocomially or in the community (Verani et al., 2010). Case fatality ratios remain as high as 36% even in high income settings such as Norway and USA (Hajdu et al., 2006, Jordan et al., 2008). Moreover, among survivors of GBS meningitis, 46-50 % develop moderate to severe neurological impairment (Bedford et al., 2001, Edwards et al., 1985, Libster et al., 2012).

Substantial variation in invasive disease incidence is reported both within and across geographic regions, ranging from 0.02 per 1000 live births (LB) in Southeast Asia to 1.21 per 1000 LB in Africa (Edmond et al., 2012). Some variation may be attributable to the introduction of antibiotic based interventions: Intrapartum antibiotic prophylaxis (IAP), given to mothers rectovaginally colonized with GBS in late pregnancy, has resulted in substantial decreases in incidence of EOD (Schrag et al., 2000). However, substantial variation in incidence of EOD remains in low and middle income countries, where the logistic and cost challenges associated with routine culture based screening and IAP have limited its implementation. Factors underlying this variation may include differences in case ascertainment and laboratory diagnostics, or population differences in exposure and susceptibility (Dagnew et al., 2012).

The most significant risk factor for invasive GBS EOD is rectovaginal colonization of the mother at the time of birth (Stoll et al., 2011). Vertical transmission of GBS occurs to approximately 50% of the newborns of GBS colonized mothers of whom 1-2% develops EOD. If population-based differences in exposure are driving the observed differences in EOD incidence, one would expect to observe similar disparities in the prevalence of maternal colonization during pregnancy. A previous systematic review of maternal GBS colonization data from low and middle income countries undertaken in 1998 reported a regional prevalence range of 8% to 18% by geographic region. The microbiological method of GBS isolation was identified as a key driver of this variation (Stoll and Schuchat, 1998). In recent years the sensitivity of culture methods has increased and there is now published guidance on recommended methods for specimen collection, processing and GBS isolation (Verani et al., 2010). This should allow a clearer interpretation of any population differences in colonization.

We conducted a systematic review and meta-analysis of prospective epidemiological studies that detected GBS carriage and used recommended microbiology methods (Verani et al., 2010), to assess whether the prevalence of maternal GBS colonization matched the patterns of invasive disease incidence across geographic regions.

3.3 Methods

3.3.1 Literature search strategy and selection criteria

We searched five literature databases (Medline, Embase, Pascal Biomed, WHOLIS and African Index Medicus) to identify studies reporting the prevalence of GBS colonization in pregnant women. Searches were completed with the following terms: [‘Streptococcus agalactiae (MeSH/keyword) OR ‘Group B Streptococcus’ OR ‘Streptococcus Group B’] AND [Colonization (MeSH/keyword) OR ‘Colonisation’]. The WHOLIS and African Index Medicus searches were completed using the following text: Group B *Streptococcus* colonization or *Streptococcus agalactiae* colonization. Searches were restricted to human studies published between January 1997 and March 2015 (inclusive); the lower limit reflecting the dates of a previous systematic review on the same topic (Stoll and Schuchat, 1998). No language restrictions were applied. After an initial selection, based on review of the publication titles and abstracts, a full-text review of remaining studies was completed against specific inclusion/exclusion criteria (Table 3.1). These criteria aimed to focus the review on studies with robust methods and to increase comparability across studies. Furthermore, we also screened bibliographies of selected studies and contacted experts for any potentially relevant studies that had not been identified through the search strategies.

Table 3.1: Inclusion criteria applied to literature search

- Sufficient methodological detail to allow an assessment of specimen collection and microbiological methods
- Consistent with guidelines from the Centers for Disease Control and Prevention (CDC) for specimen collection and microbiological identification of GBS: Collection of rectovaginal or vaginal and rectal swabs
- Use of selective culture methods for GBS isolation and identification
- Prospective data collection
- Pregnant population
- Original research article describing data not previously published

3.3.2 Data Extraction

The Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) checklist and flow diagram were used to identify, screen and exclude studies. Two authors (Gaurav Kwatra and Marianne C Cunnington) independently examined titles, abstracts, full text articles and abstracted data using the same data abstraction forms and selection criteria. Disagreements were resolved by consensus among the authorship group. Data were abstracted on key study characteristics including geographic region (as defined by World Health Organization), study period, study population, timing (late pregnancy (35+ weeks of gestation) versus at delivery versus other) and site (single rectovaginal swab versus separate vaginal and rectal swabs) of specimen collection, method of GBS isolation (selective broth followed by subculture on blood agar or selective agar versus direct plating on selective media) sample size and the proportion of women colonized with GBS. Culture methods could include those recommended by the CDC (GBS growth on selective broth

followed by subculture on blood agar or selective media) or those outside of the CDC recommendations using some form of selectivity in the medium for GBS isolation (Verani et al., 2010).

3.3.4 Statistical analyses

A random effects meta-analysis was used to calculate the mean prevalence estimate of maternal colonization across studies and by region. Ninety-five percent confidence intervals were calculated around prevalence estimates. Studies were categorized by region, sorted by study size and heterogeneity between studies was assessed, first visually by the overlap of confidence intervals and then quantitatively by the I^2 statistic (Higgins and Thompson, 2002) and the Cochran Q-test for heterogeneity with a significance level of 5% applied(WG, 1954). The I^2 statistic is interpreted as the proportion of the total variation in the estimates of prevalence that is due to the heterogeneity between studies. Potential drivers of statistical variation (heterogeneity), including timing of specimen collection and culture method, were explored using descriptive stratified analyses. A funnel plot was used to explore how prevalence estimates varied by study size, in case of "small study effects"(Sterne et al., 2000, Sterne et al., 2001) . Additionally for studies with available data on the distribution of serotypes, a random effects meta-analysis was used to calculate the mean prevalence of serotypes Ia, Ib or III; and serotypes Ia, Ib, II, III or V. Statistical analyses were completed using R software (version 2.15.2) and SAS (version 9.2).

3.3.5 Sensitivity analysis

The CDC recommendation for rectovaginal swab collection meant that studies in which lower vaginal swabs only were collected were excluded. To investigate any potential bias introduced, studies meeting all pre-defined criteria, but reporting estimates

for lower vaginal colonization only were identified and a separate meta-analysis was completed. Studies were categorized by region, as classified by World Health Organization (WHO) criteria. As the Western Pacific region included an ethnically diverse range of countries (within this meta-analysis, Australia, China, including Hong Kong, Japan, Korea and New Zealand), an alternative categorization excluding Australia and New Zealand was also considered in a separate sensitivity analysis.

3.4 Results

3.4.1 Systematic Review

A total of 1852 papers were identified from the literature search and 30 papers were identified through a search of paper bibliographies and expert opinion. Two hundred and twenty one papers were retained after an initial review of titles and abstracts and the removal of duplicates. Seventy eight papers were included in the meta-analysis after full text review and application of pre-defined inclusion/exclusion criteria to the 221 papers (Figure 3.1).

The majority of reports identified were from Europe and the Americas, whilst Africa had the fewest number (Table 3.2). The proportion of studies meeting inclusion criteria was lowest for studies from Africa and Eastern Mediterranean regions (Table 3.2). The most common reasons for exclusion were failure to follow CDC guidelines for specimen collection (lower vaginal and rectal swabbing) and selective culture methods or insufficient methodological detail (69.2% of total exclusions) (Verani et al., 2010).

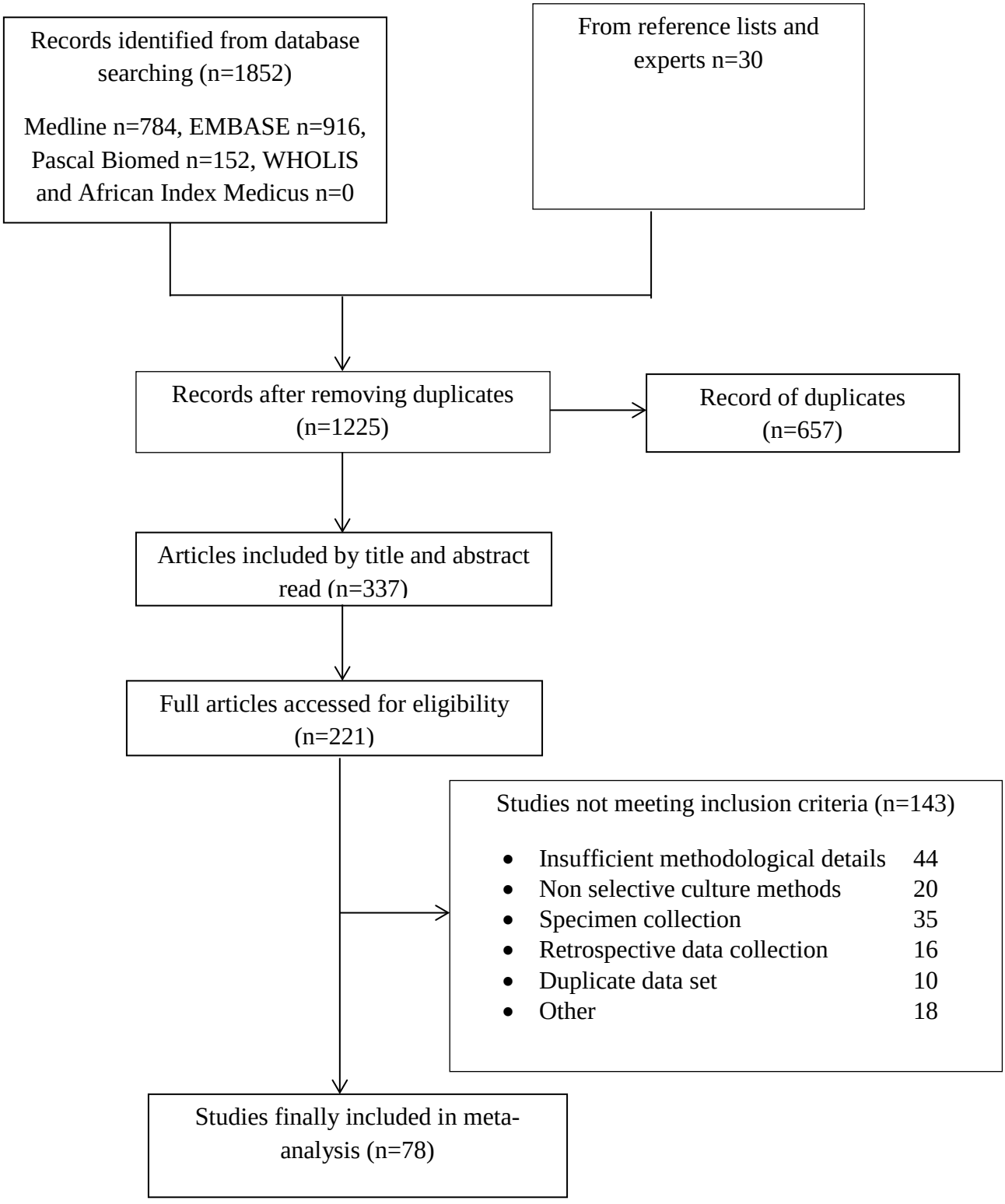


Figure 3.1: Flow diagram of selected studies reporting on GBS colonization

Table 3.2: Inclusion/exclusion of studies by region

Region	Total Identified	No. Included (%)	No. Excluded* (%)
Africa	14	4 (29)	10 (71)
Americas	76	29 (38)	47 (62)
Eastern Mediterranean	32	7 (22)	25 (78)
Europe	59	23 (39)	36 (61)
Southeast Asia	20	7 (35)	13 (65)
Western Pacific	19	8 (42)	11 (58)
International	1	0 (0)	1 (100)
Total	221	78 (35)	143 (65)

3.4.2 Prevalence of maternal colonization Meta-Analysis

Overall, 73791 pregnant women were captured in 78 studies across 37 countries (median number of subjects per study of 391). A total of 13 100 women were positive for rectovaginal colonization with GBS. Twenty three studies from Europe captured the highest number of total subjects (n=31 642) while 7 studies from the Eastern Mediterranean region captured the lowest number (n= 2 729). The highest median number of subjects per study was in Western Pacific (n=1 049), and the lowest in the Eastern Mediterranean (n=300). Study details are summarized table 3.3. The study-specific prevalence estimates ranged from 0.5% (95% CI, 0.0, 1.1) in Mexico (Romero Gutierrez et al., 2005) to 37.9% (95% CI, 25.4, 50.4) in Denmark (Hansen et al., 2004). Table 3.4 summarizes the random-effects meta-analyses by region. Regional prevalence estimates ranged from 11.1% (95% CI, 6.8, 15.3) in Southeast Asia to 22.4% (95% CI, 18.1, 26.7) in Africa. The overall global prevalence estimate of 17.9% (95% CI, 16.2, 19.7) aligned with the two most studied regions: Europe and the Americas.

Table 3.3: Characteristics of Included Studies by region and study size [1]

Region	Author	Year	Country	Sample timing	Study setting	Study size	Number of GBS positive cases
Africa				TOTAL		2735	619
	Gray et al.(Gray et al., 2011)	2012	Malawi	Labour	Pregnant women (≥16 years of age) in their third trimester of pregnancy were recruited from the labor ward of Queen Elizabeth Central Hospital, Blantyre (QECH), during October 2008–March 2010.	1441	313
	Mavenyengwa et al.(Mavenyengwa et al., 2010)	2010	Zimbabwe	Delivery or Labour	Between 2003 and 2005 at a maternity clinic and two district hospitals covering urban, urban-rural and rural communities	676	142
	Kwatra et al.(Kwatra et al., 2014)	2014	South Africa	Late Pregnancy	Between August 2010 and August 2011 at community antenatal clinics in Soweto	521	148
	Dzowela et al.(Dzowela T, 2005)	2005	Malawi	Not Indicated	June/July 2004, all women attending antenatal clinic and tertiary urban hospital without antibiotic use in previous 2 weeks	97	16
Americas				TOTAL		24892	4877
	Lin et al.(Lin et al., 2011)	2011	US	Delivery or Labour	Between February 5, 2008 and February 4, 2009 at 3 hospitals in Houston, TX and Oakland, CA.	5497	1031
	Campbell et al.(Campbell et al., 2000)	2000	US	Delivery or Labour	October 1992 and January 1995 from three hospitals in Houston (St. Luke's Episcopal, the Methodist Hospital, and Ben Taub General Hospital) and from one hospital in Seattle (University of Washington Medical Center)	3307	856
	Gupta and Briski (Gupta and Briski, 2004)	2004	US	Late Pregnancy	St. John Hospital and Medical Center Laboratory, Detroit, Mich., for screening for GBS colonization were included in this prospective study. Specimens were received over 6 months, from July 2003 through December 2003.	1635	385
	Towers et al.(Towers et al., 2010)	2010	US	Delivery or Labour	Long Beach Memorial Women's Hospital over a 3-year period	1472	296
	Larcher et al.(Larcher et al., 2005)	2005	Argentina	Late Pregnancy	Servicio de Ginecología y Obstetricia del Hospital Privado Centro Médico de Córdoba, Argentina, July 1st 2001 and December 31st 2002	1228	17
	Davies et al.(Davies et al., 2001)	2001	Canada	Late Pregnancy	From November 1998 through May 2000, from 16 representative offices of obstetric care practitioners across Calgary	1207	235

[1] Sample timing = Late pregnancy defined as ≥35 weeks gestation; Not indicated when not readily categorizable (e.g. including subjects <35 weeks gestation or a combination of subjects during late pregnancy and at delivery or labour); Other when swabs collected at <35 weeks of gestation.

	Di Bartolomeo et al.(Di Bartolomeo et al., 2005)	2005	Argentina	Not applicable	Posadas Hospital, April 2003 - May 2004	1203	113
	Montibello et al.(Montibello et al., 2011)	2011	Argentina	Late Pregnancy	In the services of Bacteriology Hospital "Juan A. Fernandez" and the Naval Hospital "Pedro Mallo," both of the Autonomous City of Buenos Aires, and Hospital Piñeyro the town of Junin, Province of Buenos Aires	962	168
	Ocampo-Torres et al.(Ocampo-Torres et al., 2000)	2000	Mexico	Delivery or Labour	three public hospitals of San Cristobal de Las Casas, Chiapas, Between February and September 1999	910	78
	Gonzalez et al.(Alberto Gonzalez Padraza Aviles, 2002)	2002	Mexico	Other	Primary Health Center in Tlalpan, México, March 1999 and December 2001	691	97
Region	Author	Year	Country	Sample timing	Study setting	Study size	Number of GBS positive cases
…							
	Hickman et al.(Hickman et al., 1999)	1999	US	Delivery or Labour	January 26, 1994 through February 11, 1995 at four Houston hospitals.	546	153
	Gutierrez et al.(Romero Gutierrez et al., 2005)	2005	Mexico	Late Pregnancy	Hospital de Gineco-Pediatria num. 48, Leon, Guanajuato, November 2000 - March 2003	433	2
	Rocchetti et al.(Rocchetti et al., 2011)	2011	Brazil	Late Pregnancy	Botucatu Medical School, Sao Paulo State University, UNESP, between February 2006 and January 2007.	405	103
	Price et al.(Price et al., 2006)	2006	Canada	Late Pregnancy	Consecutive patients presenting between October 2003 and April 2005 to a maternity centre in Hamilton	330	62
	Simoes et al.(Simoes et al., 2007)	2011	Brazil	Delivery or Labour	November 2003 to May 2004, at the Jundiaí Teaching Hospital	316	46
	Jordan et al.(Jordan et al., 2010)	2010	US	Late Pregnancy	2 May 2006 through 14 August 2006, Washington, Pittsburgh and...	306	75
	Laufer et al.(Laufer et al., 2009)	2009	Uruguay	Delivery or Labour	Pereira Rossell Hospital, 2-month period in 2008	300	52
	Church et al.(Church et al., 2008)	2008	Canada	Late Pregnancy	large maternity clinic in the CHR (Maternity Care Clinic, Sunridge Professional Building, Calgary, Alberta, Canada)	279	50
	Madani et al.(Madani et al., 1998)	1998	Canada	Other	Pregnant women who attended the antenatal clinic at St. Boniface General Hospital in Winnipeg, Canada, from May 1995 through April 1996	264	64
	Garcia et al.	2003	Argentina	Other	Hospital de Clinicas Jose de San Martin de la universidad de Buenos Aires, April 2000 - March 2002	259	47
	Feuerschuette et al.(Feuerschuette et al., 2012)	2012	Brazil	Late Pregnancy	Feuerschuette's clinic, Tubara, Brazil, December 2010 to August 2011	254	73
	Quinlan et al.(Quinlan et al., 2000)	2000	US	Late Pregnancy	Faculty practice of the Florida Hospital Family Practice Residency Program from April 1998 through April 1999	222	54

	Nomura et al.(Nomura et al., 2005)	2005	Brazil	Delivery or Labour	Maternity of the State, Univeristy of Campinas, Brazil, Feb 2003 - Jan 2004.	203	56
	Costa et al.(Costa et al., 2008)	2008	Brazil	Delivery or Labour	public maternity center in the northeast region of Brazil (São Luís, Maranhão), Novenber 2005-March 2006.	201	41
	Orrett et al.(Orrett, 2003)	2003	Trinidad	Late Pregnancy	Between 2000 and 2001 in antenatal clinics of general hospitals in north and south of the island	201	66
	Jamie et al.(Jamie et al., 2004)	2004	US	Other	University of Florida clinic system, July 2003 to October 2003	200	71
	Orsello et al.(Orsello and Dommermuth, 2003)	2003	US	35-37	Naval Hospital Bremerton, Wash. 152 consecutive patients at 35-37 weeks' gestation (7 excluded as only a single swab used)	145	35
	El Beitune et al.(El Beitune et al., 2006)	2006	Brazil	Late Pregnancy	Medicine School of Ribeirao Preto, University of Sao Paulo, Ribeirao Preto, SP, Brazil, November 2002 to April 2004	106	15
	Daimaru-Enoki et al.(Daimaru-Enoki et al., 2005)	2005	US	Late Pregnancy	March 2001 through September 2002 involving routine obstetric patients who delivered at Cedars-Sinai Medical Center (CSMC),	81	19
Eastern Mediterranean				TOTAL		2729	443
	Hammoud et al.(Hammoud et al., 2003)	2003	Kuwait	Delivery or Labour	Maternity Hospital, was conducted between July 2000 and January 2001	1120	159

Region	Author	Year	Country	Sample timing	Study setting	Study size	Number of GBS positive cases
…							
	Zamzami et al.(Zamzami et al., 2011)	2011	Saudi Arabia	Delivery or Labour	Delivery room at King Abdul-Aziz University Hospital (KAUH), Jeddah, Saudi Arabia; May 1 2009 - May 30 2010	326	103
	Hassanzadeh et al.(Parvin Hassanzedah, 2011)	2011	Iran	Delivery or Labour	three teaching hospitals in Shiraz, southwest of Iran, April 2006 to March 2007,	310	43
	Ferjani et al.(Ferjani et al., 2006)	2006	Tunisia	Other	maternité hospitalo-universitaire de la région de Sousse (centre tunisien). 1er janvier au 31 juillet 2003	300	39
	Jerbi et al.(Jerbi et al., 2007)	2007	Tunisia	Delivery or Labour	Mar - May 2005	294	38
	Moghaddam(Moghaddam, 2010)	2010	Iran	Other	Emam-Reza Hospital in Mashhad (Khorasan-Razavi Province - Iran) July 2005 - January 2007	201	25
	Seyyed et al.(Seyyed et al., 2013)	2013	Iran	Delivery or Labour	Two referral university hospitals (Al-Zahra and Shaheed Beheshty), Isfahan University of Medical Sciences, isfahan, Iran, March 2010 - May 2011	178	36
Europe				TOTAL		30462	5925
	Cantoni et al.(Cantoni et	2013	Italy	Late Pregnancy	July 1, 2004-June 30, 2006, public hospitals of the region Friuli-Venezia Giulia in north-eastern Italy.	15239	3010

	al., 2013)						
	Busetti et al.(Busetti et al., 2007)	2007	Italy	Late Pregnancy	1 January 2002 to 31 December 2005, Burlo Garofolo Institute, the mother-and-child hospital of Trieste, (North-Eastern Italy)	5020	901
	Hakansson et al.(Hakansson et al., 2008)	2008	Sweden	Delivery or Labour	National population based cohort of women giving birth in a single calendar week in 2005	1569	400
	Rausch et al.(Rausch et al., 2009)	2009	Switzerland	Late Pregnancy	Between March 2005 and September 2006 women at study hospital in Bern	1316	276
	Liebana-Martos(Maria del Carmen Liebana-Martos, 2015)	2015	Spain	Late Pregnancy	Between 2009 and 2011 in antenatal clinics in Granada	1180	188
	Tsolia et al.(Tsolia et al., 2003)	2003	Greece	Not Indicated	Between January 2000 and May 2001 at tertiary public hospital in Athens and 4 private hospitals in Athens or Drama in Northern Greece	1014	67
	Kunze et al.(Kunze et al., 2011)	2011	Germany	Late Pregnancy	In 2005 at single tertiary hospital in Freiburg	869	183
	Valkenburg van den Berg et al.(Valkenburg-van den Berg et al., 2006)	2006	The Netherlands	Delivery or Labour	Between July 2000 and December 2002 convenience sampling from 3 obstetric outpatient hospital centers and 6 midwifery practices in The Hague	761	174
	Jones et al.(Jones et al., 2006)	2005	UK	Other	Between 2001 and 2003, women attending clinics attached to two hospitals in Oxford and Banbury	748	147
	Motlova et al.(Motlova et al., 2004)	2004	Czech Republic	Delivery or Labour	Sep 2001 to May 2002, women at childbirth were screened for GBS carriage in two hospitals (Prague, Ceske Budejovice) of different regions	586	172
	Kieran et al. (Kieran et al., 1998)	1998	Ireland	Late Pregnancy		504	129
	Eren et al.(Eren et al., 2005)	2005	Turkey	Delivery or Labour	May 2000 to January 2001,Zeynep Kamil Women's and Children's Diseases Training and Research Hospital.	500	46
	Prosniewska et al.(Prosniewska M., 2014)	2014	Poland	Other	2010-2012, Clinic of Perinatology of the Faculty of Gynaecology and Obstetrics of the Medical University in Ludz, Poland	377	89
Region	Author	Year	Country	Sample timing	Study setting	Study size	Number of GBS positive cases
…							
	Jaureguy et al.(Jaureguy et al., 2003)	2004	France	Other		370	57
	Votava et al.(Votava et al., 2001)	2001	Czech Republic	Delivery or Labour	Jan-july 2000, women in labor attending the Department of Gynecology and Obstetrics at Masaryk University	319	87
	Arisoy et al.(Arisoy et al., 2003)	2003	Turkey	Late Pregnancy	March to Dec 2000, Social Security Hospital of Izmir	310	33
	Barbaros et al.(Barbaros et al., 2005)	2005	Turkey	Delivery or Labour	January 2002 to March 2003, Maternity Department of Cerrahpasa Medical Faculty and Bakirkoy SSK Hospital, Istanbul, Turkey.	300	24

	Strus et al.(Strus et al., 2009)	2009	Poland	Delivery or Labour	2004-2006, University Hospital in Cracow	250	43
	Tejerizo Lopez et al.(L.C. Tejerizo, 1998)	1998	Spain	Delivery or Labour	Hospital Virgen de la Vega, Salamanca	112	13
	El Aila et al.(El Aila et al., 2010)	2010	Belgium	Late Pregnancy	Between June 2009 and January 2010 from women at university hospital in Ghent	100	22
	Romanik et al.(Romanik et al., 2011)	2011	Poland	Late Pregnancy	Department of gynocology and obstetrics, medical university of silesia, Tychy, Poland.	80	23
	Roccasalva et al.(L.S. Roccasalva, 2008)	2008	Italy	Late Pregnancy	Il Dipartimento de Scienze Microbiologiche e Ginecologiche dell'Universita di Catania, Feb 2007-May 2008	60	7
	Hansen et al.(Hansen et al., 2004)	2004	Denmark	Late Pregnancy	Between May 1999 and June 2001 longitudinal cohort of women followed during pregnancy at Aarhus University Hospital	58	22
Southeast Asia				TOTAL		3749	406
	Chan et al.(Chan et al., 2013)	2013	Bangladesh	Delivery or Labour	Between January and October 2011, women attending maternity center in Dhaka	1219	94
	Patil et al.(Patil KP, 2013)	2013	India	Delivery or Labour	June 2007 to May 2008, tertiary care centre, pregnant women at labor	905	110
	Turner et al.(Turner et al., 2012)	2012	Thailand	Delivery or Labour	Shoklo Malaria Research Unit (SMRU) clinic between April 2009 and May 2010. All women who attended the antenatal clinic and were between 28 and 30 weeks gestation	549	47
	Tor-Udom et al.(Tor-Udom et al., 2006)	2006	Thailand	Late Pregnancy	From September 2004 to February 2005, pregnant women at the 35 to 37 weeks of gestation, receiving antenatal care at Thammasart Hospital	406	65
	Kovavisarach et al.(Kovavisarach et al., 2007)	2007	Thailand	Delivery or Labour	The study was carried out in the labor room (LR) of the Rajavithi Hospital between October 1and 30, 2004.	320	58
	Sharmila et al.(Sharmila et al., 2011)	2011	India	Late Pregnancy	September 2006 to June 2008, at Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), a tertiary care teaching institute located at Puducherry, South India, Pregnant women with a gestational age of 35-37 weeks, attending the antenatal out-patient department	300	7
	KonikKara et al.(Konikkara et al., 2014)	2014	India	Late Pregnancy	January to June 2008 in the Diagnostic Microbiology Laboratory of a tertiary care referral hospital in South Kanara District of Karnataka,pregnant women who had come for antenatal check-up at 35 to 37 weeks	50	8

Region	Author	Year	Country	Sample timing	Study setting	Study size	Number of GBS positive cases
…							
Western Pacific				TOTAL		9773	1049
	Lu et al.(Lu et al., 2014)	2013	China	Late Pregnancy	Between September 2011 and February 2013, women attending tertiary teaching hospital in Beijing	2850	201
	Lee et al.(Lee et al., 2010)	2010	South Korea	Late Pregnancy	Between January 2006 and May 2008 women attending for routine prenatal care at 2 hospitals in Daejeon and 2 hospitals in Seoul	2526	204
	Morita et al.(Morita et al., 2014)	2014	Japan	Late Pregnancy	Between November 2010 and October 2011 pregnant women attending Red Cross Medical Center for routine screening	1425	319
	Gilbert et al.(Gilbert et al., 2002)	2002	Australia	Delivery or Labour	Women attending one community and one tertiary hospital in Sydney at delivery	1096	268
	Tsui et al.(Tsui et al., 2009)	2009	Hong Kong	Other	Between January and May 2002 in tertiary obstetric centers across Hong Kong (serving population of 1 million). First 20 consecutive women attending antenatal booking clinics each day were enrolled.	1002	91
	Uh et al.(Uh et al., 1997)	1997	Korea	Other	Between May 1995 and January 1996 women attending tertiary hospital for delivery in Wonju	459	26
	Grimwood et al.(Grimwood et al., 2002)	2002	New Zealand	Late Pregnancy	October-December 1998 and July-December 1999 volunteer midwives in Auckland hospitals recruited convenience sample of pregnant women	240	52
	Park et al.(Park et al., 2013)	2013	South Korea	Late Pregnancy	Women attending teaching and general hospitals in Seoul for routine prenatal care	175	15

Table 3.4: Meta-analysis of studies reporting prevalence of maternal colonization by region

Region	No. studies	No. subjects	Median subjects per study	Number of GBS positive cases	Estimated Prevalence (95% CI)	Within region test for heterogeneity I^2, p value
Africa	4	2735	**598.5**	**619**	**22.4** (18.1, 26.7)	82.7%, p =0.005
Americas	29	23163	**316**	**4360**	**19.7** (16.7, 22.7)	98.4%, p < 0.0001
Eastern Mediterranean	7	2729	**300**	**443**	**16.7** (11.7, 21.7)	91.9%, p < 0.0001
Europe	23	31642	**500**	**6113**	**19.0** (16.1, 22.0)	97.2%, p < 0.0001
Southeast Asia	7	3749	**406**	**389**	**11.1** (6.8, 15.3)	95.1%, p < 0.0001
Western Pacific	8	9773	**1049**	**1176**	**13.3** (7.8, 18.8)	98.7%, p < 0.0001
Overall	**78**	**73791**	**391**	**13100**	**17.9** (16.2, 19.7)	**98.1%, p < 0.0001**

The colonization prevalence estimates are summarized in more detail in the forest plot (Figure 3.2-3.7), by region, sorted by study size and further categorized by timing of specimen collection (delivery, late pregnancy (35wks-delivery) or other). The heterogeneity across estimates is immediately evident, with overall I^2=98.1%. The level of heterogeneity remained significant within each region (lowest I^2=82.7%, p=0.005 for Africa, with just four studies) and while prevalence estimates from the same country often coincided (e.g. Tunisia(Jerbi et al., 2007, Ferjani et al., 2006)) this was not always the case (e.g. India (Konikkara et al., 2014, Sharmila et al., 2011)).

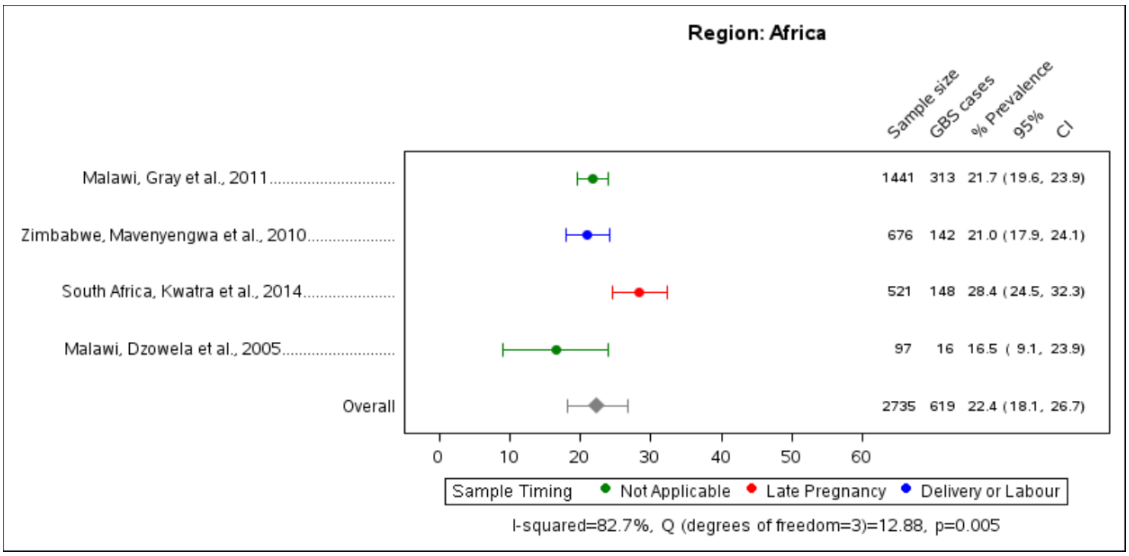


Figure 3.2: Meta-analysis of studies that reported maternal GBS colonization in Africa

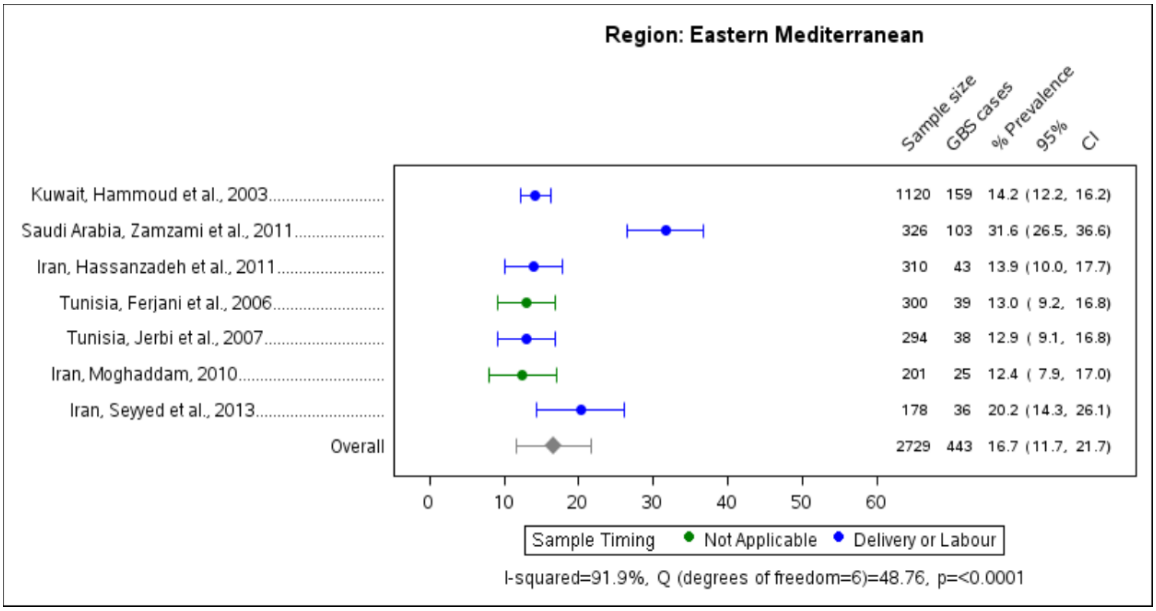


Figure 3.3: Meta-analysis of studies that reported maternal GBS colonization in Eastern Mediterranean

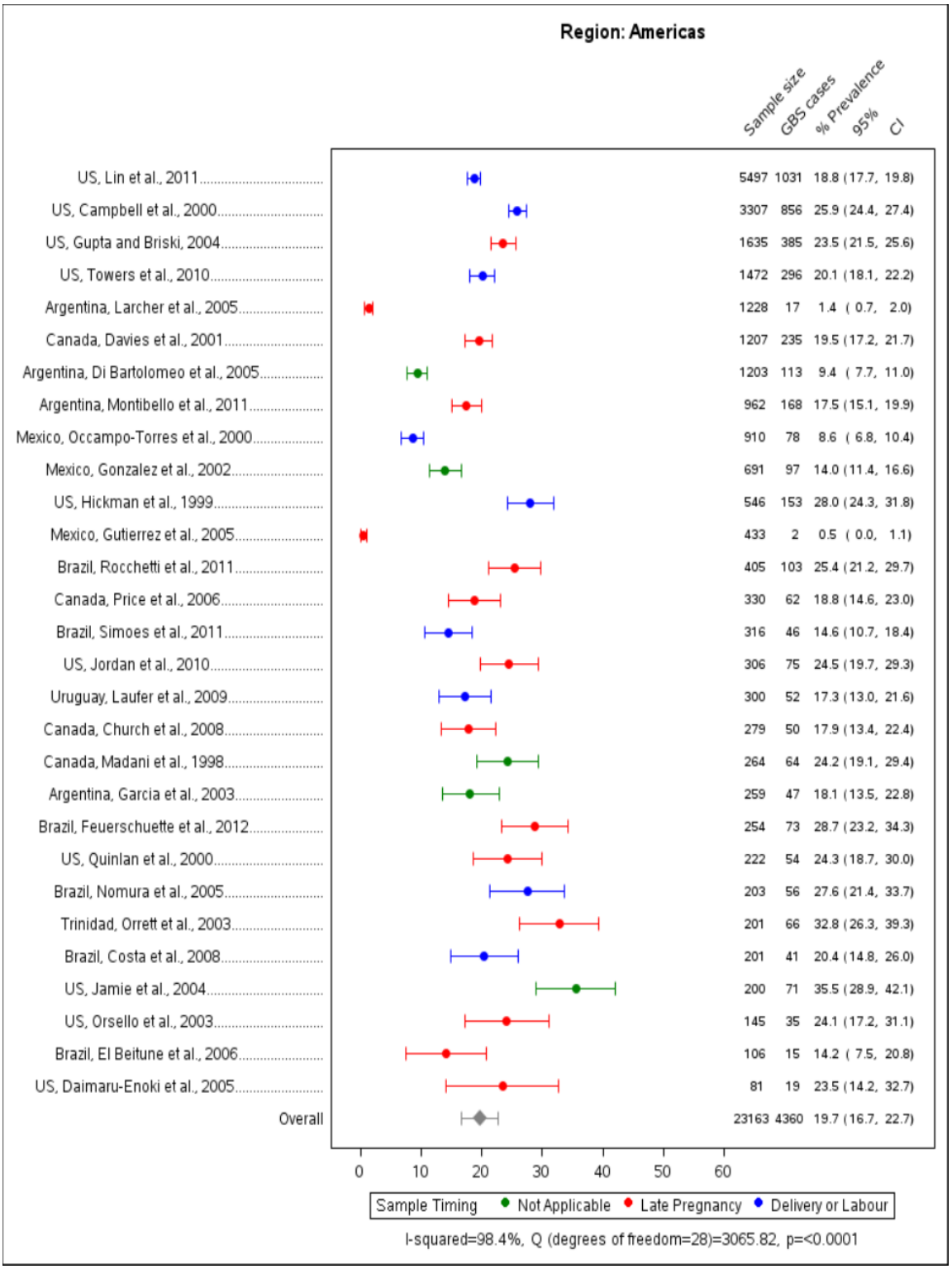


Figure 3.4: Meta-analysis of studies that reported maternal GBS colonization in America

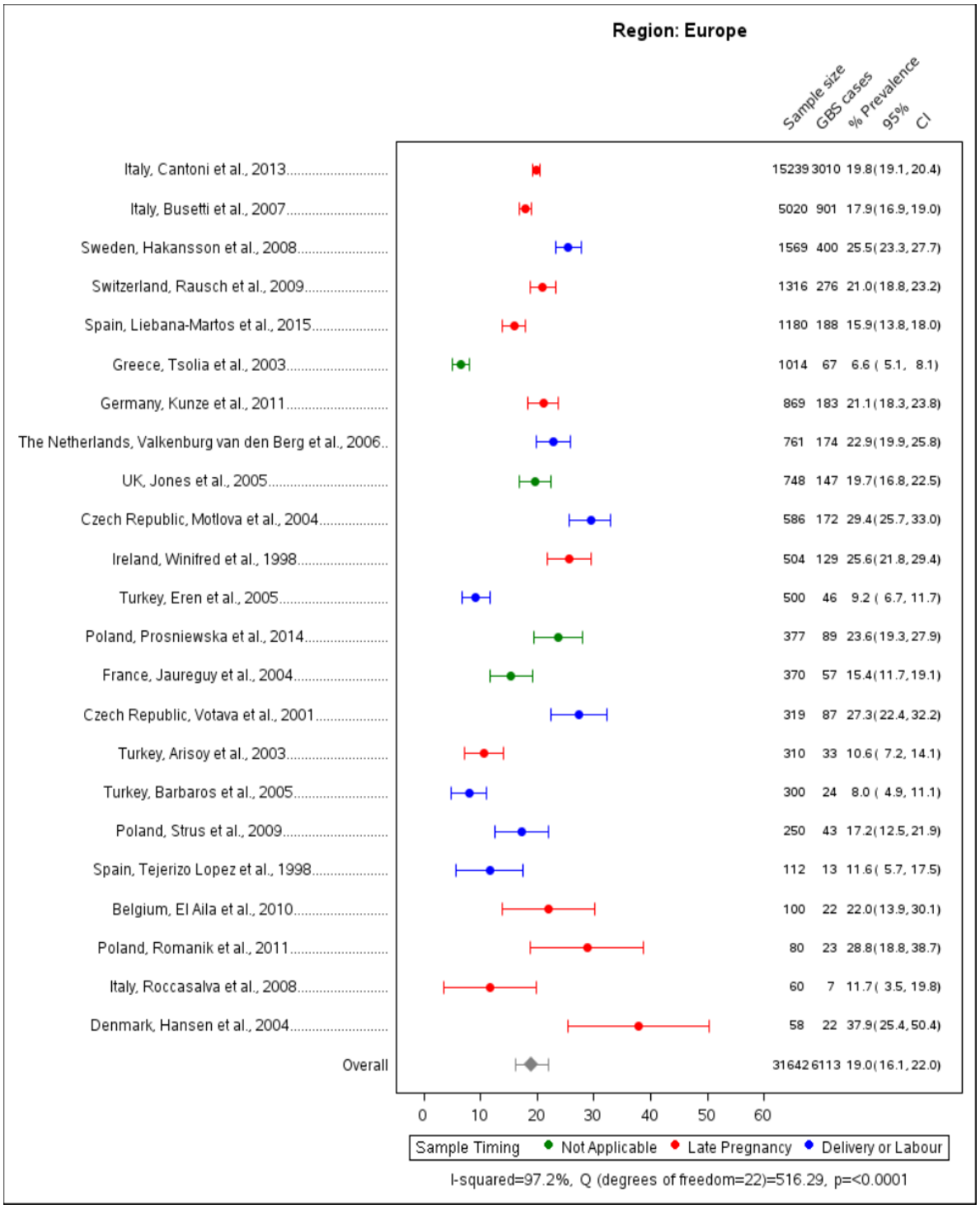


Figure 3.5: Meta-analysis of studies that reported maternal GBS colonization in Europe

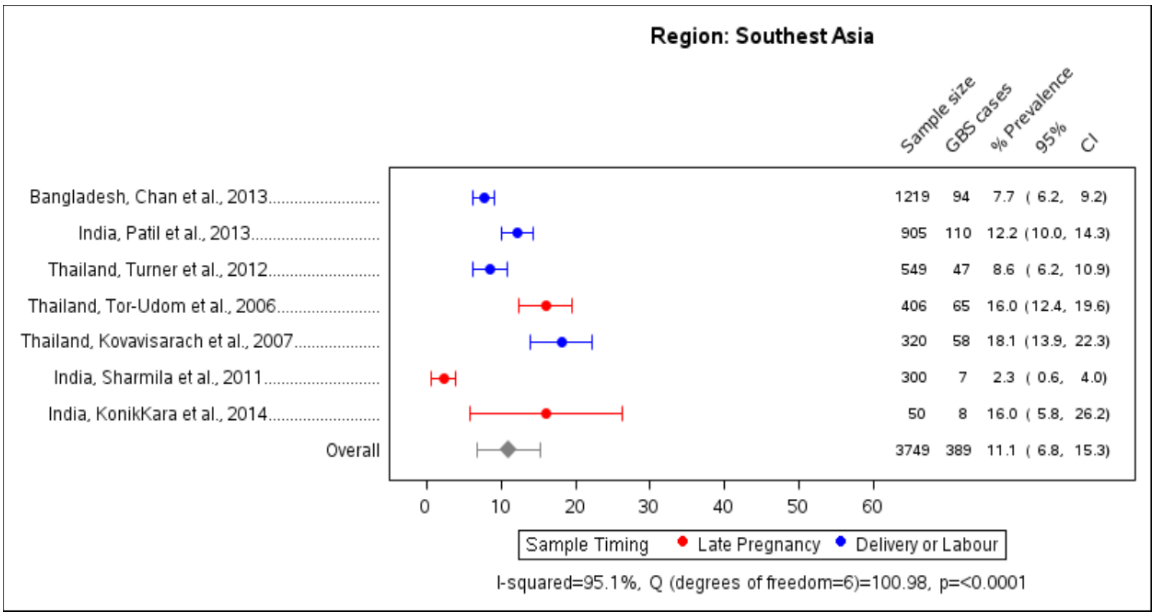


Figure 3.6: Meta-analysis of studies that reported maternal GBS colonization in Southeast Asia

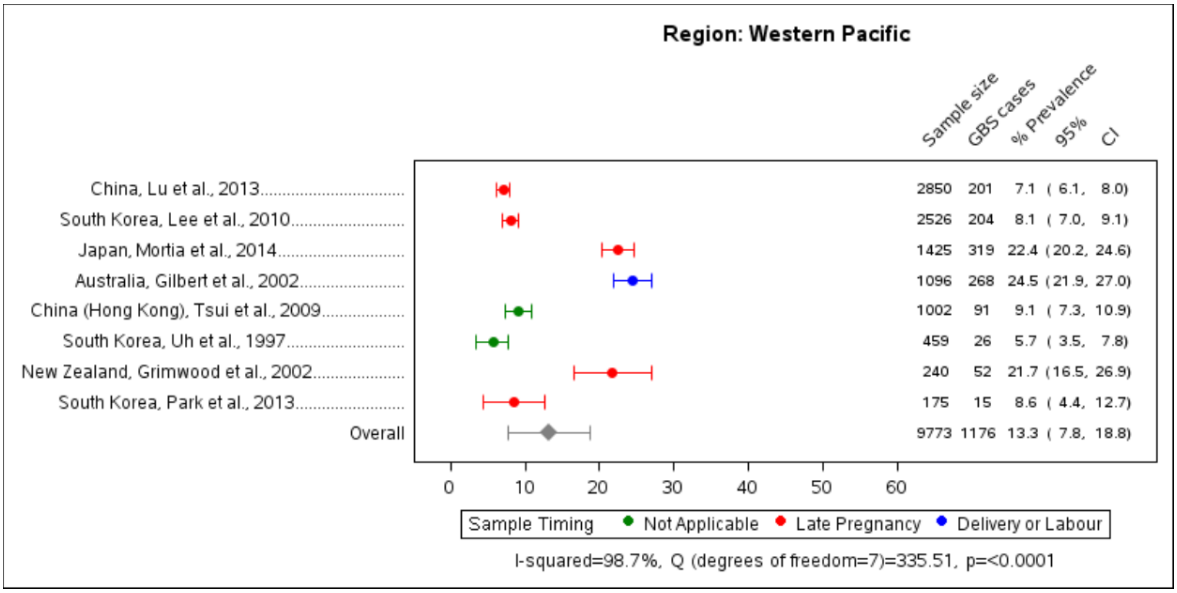


Figure 3.7: Meta-analysis of studies that reported maternal GBS colonization in Western Pacific

Additional sources of heterogeneity were investigated: several different selective culture methods were reported including selective broth subcultured on sheep blood agar (SBSBA), selective broth subcultured on selective media (SBSSM) and direct plating on selective agar media (DPSM) (Table 3.5). To explore the contribution of these methods to the observed heterogeneity, an analysis was restricted to studies with ≥400 subjects using selective broth subcultured on sheep blood agar culture (SBSBA) as the most common method (Table 3.6) However, the patterns of mean prevalence across regions remained similar to the primary analysis with heterogeneity estimates high (I^2 ≥90% across all regions).

Table 3. 5: For studies ≥ 400 subjects, details of the culture method used

Region	Author,	Country	Study size	Culture method	Swabs	comments
Africa						
	Mavenyengwa et al., 2010(Mavenyengwa et al., 2010)	Zimbabwe	767	DPSM and SBSBA	vaginal and rectal	both DPSM and SBSBA was used for colonization rate
	Kwatra et al., 2014(Kwatra et al., 2014)	South Africa	521	DPSM	Vaginal and Rectal	Direct plating on selective media only
Americas						
	Lin et al., 2011(Lin et al., 2011)	USA	5497	SBSSM	Vaginal and Rectal	no comments
	Campbell et al., 2000(Campbell et al., 2000)	USA	3307	SBSBA	Vaginal and Rectal	no comments
	Gupta, 2004(Gupta and Briski, 2004)	USA	1635	SB (23.5%) and DPSM (21.5%)	combined recto-vaginal	SB the yield is high. Need to use only recto-vaginal data
	Towers et al.,2010(Towers et al., 2010)	USA	1472	SBSBA	vginal and rectal	no comments
	Larcher et al.,2005(Larcher et al., 2005)	Argentina	1228	SBSBA	Vaginal and Rectal	no comments
	Davies et al.,2001(Davies et al., 2001)	Canada	1207	SB	combined vaginal and rectal	subculture plate not mentioned
	Di Bartolomeo et al., 2005(Di Bartolomeo et al., 2005)	Argentina	1203	SBSBA	Vaginal and Rectal	no comments
	Montibello et al., 2011	Argentina	962	DPSM, SBABA,SBSSM	vaginal and rectal	DPSM was inferior to SBSBA and SBSBA was inferior to SBSSM
	Occampo-Torres et al., 2000(Ocampo-Torres et al., 2000)	Mexico	910	SBSBA	vaginal and perianal	no comments
	Gonzalez et al., 2002(Alberto Gonzalez Padraza Aviles, 2002)	Mexico	691	DPSM	vaginal, rectal and urine	rectal (2.7%) colonization is low as compared to vaginal colonization (11.1%)
	Hickman et al., 1999(Hickman et al., 1999)	USA	546	SBSBA	Vaginal and Rectal	no comments
	Gutierrez et al., 2005(Romero Gutierrez et al., 2005)	Mexico	433	DPSM and SB	Vaginal and Rectal	In different language can't understand the complete paper
	Rocchetti et al., 2011(Rocchetti et al., 2011)	Brazil	405	SBSBA	Vaginal introitus, vaginal (high) and perianal	Extra high vaginal swab was used
Eastern Mediterranean						
	Hammoud et al., 2003	Kuwait	1120	DPSM and SBSBA	combined recto-vaginal	both DPSM and SBSBA was used for colonization rate
Europe						
	Cantoni et al., 2013(Cantoni et al., 2013)	Italy	15239	SBSBA	combined recto-vaginal	no comments
	Busetti et al., 2007(Busetti et al., 2007)	Italy	5020	DPSM and SBSBA	Vaginal and rectal	DPSM was inferior to SBSBA.
	Hakansson et al., 2008(Hakansson et al., 2008)	Sweden	1569	SBSBA	combined recto-vaginal	no comments
	Rausch et al., 2009(Rausch et al., 2009)	Switzerland	1316	SBSBA	vaginal and rectal	no comments
	Tsolia et al., 2003(Tsolia et al., 2003)	Greece	1014	SBSBA	vaginal and rectal	no comments

	Kunze et al., 2011(Kunze et al., 2011)	Germany	869	SBSBA	combined recto-vaginal	no comments
	Valkenburg van den Berg et al., 2006(Valkenburg-van den Berg et al., 2006)	The Netherlands	761	SBSBA	combined recto-vaginal	no comments
	Jones et al., 2005(Jones et al., 2006)	UK	748	SBSBA	combined recto-vaginal	DPSM was inferior to SBSBA.
	Motlova et al., 2004(Motlova et al., 2004)	Czech Republic	586	SBSBA	vaginal and rectal	no comments
	Kieran et al., 1998(Kieran et al., 1998)	Ireland	504	SBSBA and SBSSM	vaginal and peranal	SBSSM (129/504-25.6%)5 has higer sensitivity than SBSBA (112/504-22.2%)
	Eren et al., 2005(Eren et al., 2005)	Turkey	500	SBSBA	vaginal and rectal	no comments
Southeast Asia						
	Chan et al., 2013(Chan et al., 2013)	Bangladesh	1219	SBSBA	vaginal and rectal	no comments
	Patil et al., 2013(Patil KP, 2013)	India	905	SBSBA	vaginal and rectal	no comments
	Turner et al., 2012(Turner et al., 2012)	Thailand	549	SBSBA	combined recto-vaginal	no comments
	Tor-Udom et al., 2006(Tor-Udom et al., 2006)	Thailand	406	SBSBA	vaginal and rectal (self collected)	no comments
Western Pacific						
	Lu et al., 2013(Lu et al., 2014)	China	2850	Unclear	combined recto-vaginal	CDC method mentioned in reference only(no description in the methods)
	Lee et al., 2010(Lee et al., 2010)	South Korea	2526	SBSBA	Vaginal and Rectal	no comments
	Mortia et al., 2014(Morita et al., 2014)	Japan	1425	SBSBA and SBSSM	combined recto-vaginal	SBSSM (318/142-22.3%)5 has higer sensitivity than SBSBA (299/1425-21.0%)
	Gilbert et al., 2002(Gilbert et al., 2002)	Australia	1096	SBSBA	vaginal and rectal	no comments
	Tsui et al., 2009(Tsui et al., 2009)	Hong Kong	1002	SBSBA	High vaginal, low vaginal and rectal	For consistency, we can remove high vaginal swab results, in that case colonization rate will become 91/1004-9.1%
	Uh et al., 1997(Uh et al., 1997)	South Korea	459	DPSM and SBSBA	Vaginal and Rectal	DPSM was inferior to SBSBA.

Table 3. 6: Meta-analysis of studies using SBSBA culture method with studies ≥ 400 subjects

Region**	Author,	Country	Study size	Number of GBS positive cases	Estimated prevalence (95% CI)
Americas	**7 studies, I^2=99.4%**		**9071**	**1616**	**16.8 (9.1, 24.6)**
	(Campbell et al., 2000)	USA	3307	856	25.9 (24.4, 27.4)
	(Towers et al., 2010)	USA	1472	296	20.1 (18.1, 22.2)
	(Larcher et al., 2005)	Argentina	1228	17	1.4 (0.7, 2.0)
	(Di Bartolomeo et al., 2005)	Argentina	1203	113	9.4 (7.7, 11.0)
	(Ocampo-Torres et al., 2000)	Mexico	910	78	8.6 (6.8, 10.4)
	(Hickman et al., 1999)	USA	546	153	28.0 (24.3, 31.8)
	(Rocchetti et al., 2011)	Brazil	405	103	25.4 (21.2, 29.7)
Europe	**9 studies, I^2=98.2%**		**22602**	**4475**	**19.4 (14.6, 24.1)**
	(Cantoni et al., 2013)	Italy	15239	3010	19.8 (19.1, 20.4)
	(Hakansson et al., 2008)	Sweden	1569	400	25.5 (23.3, 27.7)
	(Rausch et al., 2009)	Switzerland	1316	276	21.0 (18.8, 23.2)
	(Tsolia et al., 2003)	Greece	1014	67	6.6 (5.1, 8.1)
	(Kunze et al., 2011)	Germany	869	183	21.1 (18.3, 23.8)
	(Valkenburg-van den Berg et al., 2006)	The Netherlands	761	174	22.9 (19.9, 25.8)
	(Jones et al., 2006)	UK	748	147	19.7 (16.8, 22.5)
	(Motlova et al., 2004)	Czech Republic	586	172	29.4 (25.7, 33.0)
	(Eren et al., 2005)	Turkey	500	46	9.2 (6.7, 11.7)
Southeast Asia	**4 studies, , I^2=90.2%**		**3079**	**316**	**10.9 (7.3, 14.4)**
	(Chan et al., 2013)	Bangladesh	1219	94	7.7 (6.2, 9.2)
	(Patil KP, 2013)	India	905	110	12.2 (10.0, 14.3)
	(Turner et al., 2012)	Thailand	549	47	8.6 (6.2, 10.9)
	(Tor-Udom et al., 2006)	Thailand	406	65	16.0 (12.4, 19.6)
Western Pacific	**3 studies, I^2=99.1%**		**4624**	**563**	**13.8 (3.5, 24.2)**
	(Lee et al., 2010)	South Korea	2526	204	8.1 (7.0, 9.1)
	(Gilbert et al., 2002)	Australia	1096	268	24.5 (21.9, 27.0)
	(Tsui et al., 2009)	China (Hong Kong)	1002	91	9.1 (7.3, 10.9)
Overall	**23 studies, I^2=99.0%**		**39376**	**6970**	**16.4 (13.1, 19.8)**

*For all five I^2 statistics presented, the associated test of heterogeneity p<0.0001
** No studies meeting the relevant criteria were identified for Africa

Stratification according to maternal demographic characteristics was explored but analyses were limited by variation in the ways studies reported this information. There were relatively few studies with adequate subject numbers that allowed stratification by

maternal age (<20 years versus ≥20 years) which precluded heterogeneity estimates for most regions (Table 3.7). There was no difference in observed heterogeneity by study period (Table 3.8)

Table 3.7: Studies with ≥ 400 subjects and meta-analysis as stratified by age

				<20 yrs*			>=20 yrs*		
Author	Year	Country	Timing	Study size	Number of GBS positive cases	**% (95% CI)**	Study size	Number of GBS positive cases	**% (95% CI)**
Africa									
(Gray et al., 2011)	2011	Malawi	Other	345	68	20 (16-24)	1494	322	22 (19-24)
(Kwatra et al., 2014)	2014	South Africa	Late Pregnancy	92	27	29 (20-39)	569	191	34 (30-37)
Americas									
(Campbell et al., 2000)	2000	US	Delivery or Labour	575	149	26 (22-29)	2732	707	26 (24-28)
(Larcher et al., 2005)	2005	Argentina	Late Pregnancy	47	1	2 (0-6)	1197	16	1.3 (0.7-2.0)
(Ocampo-Torres et al., 2000)	2000	Mexico	Delivery or Labour	212	16	8 (4-11)	698	62	9 (7-11)
Europe									
(Valkenburg-van den Berg et al., 2006)	2006	The Netherlands	Delivery or Labour	41	13	32 (17-46)	1660	353	21 (19-23)
(Hakansson et al., 2008)	2008	Sweden	Delivery or Labour	17	4	24 (3-44)	1556	396	25 (23-28)
(Tsolia et al., 2003)	2003	Greece	Other	148	8	5 (2-9)	866	59	7 (5-8)
SE Asia									
(Chan et al., 2013)	2013	Bangladesh	Delivery or Labour	601	217	36 (32-40)	618	237	38 (35-42)

*<19yrs vs >=19 yrs for Occampo-Torres et al.; <=22 yrs vs >22 yrs for Chan et al. Late pregnancy: ≥35 weeks of gestation. Other: swabs collected at <35 weeks of gestation.

Table 3. 8: Meta-analysis of studies stratified by year of publication after 2005

Region	Author	Year	Country	Study Size	Number of GBS positive cases	Estimated prevalence (95% CI)
2006-07	**8 studies, I^2=77.7%**			**7537**	**1352**	**17.0 (14.6, 19.4)**
Americas	(Price et al., 2006)	2006	Canada	330	62	18.8 (14.6, 23.0)
Americas	(El Beitune et al., 2006)	2006	Brazil	106	15	14.2 (7.5, 20.8)
E Med	(Ferjani et al., 2006)	2006	Tunisia	300	39	13.0 (9.2, 16.8)
Europe	(Valkenburg-van den Berg et al., 2006)	2006	The Netherlands	761	174	22.9 (19.9, 25.8)
SE Asia	(Tor-Udom et al., 2006)	2006	Thailand	406	65	16.0 (12.4, 19.6)
E Med	(Jerbi et al., 2007)	2007	Tunisia	294	38	12.9 (9.1, 16.8)
Europe	(Busetti et al., 2007)	2007	Italy	5020	901	17.9 (16.9, 19.0)
SE Asia	(Kovavisarach et al., 2007)	2007	Thailand	320	58	18.1 (13.9, 22.3)
2008-09	**8 studies, I^2=91.2%**			**4977**	**960**	**17.7 (13.9, 21.6)**
Americas	(Church et al., 2008)	2008	Canada	279	50	17.9 (13.4, 22.4)
Americas	(Costa et al., 2008)	2008	Brazil	201	41	20.4 (14.8, 26.0)
Europe	(Hakansson et al., 2008)	2008	Sweden	1569	400	25.5 (23.3, 27.7)
Europe	(L.S. Roccasalva, 2008)	2008	Italy	60	7	11.7 (3.5, 19.8)
Americas	(Laufer et al., 2009)	2009	Uruguay	300	52	17.3 (13.0, 21.6)
Europe	(Rausch et al., 2009)	2009	Switzerland	1316	276	21.0 (18.8, 23.2)
Europe	(Strus et al., 2009)	2009	Poland	250	43	17.2 (12.5, 21.9)
W Pacific	(Tsui et al., 2009)	2009	China (Hong Kong)	1002	91	9.1 (7.3, 10.9)
2010-11	**15 studies, I^2=97.4%**			**15787**	**2784**	**18.6 (15.0, 22.3)**
Africa	(Mavenyengwa et al., 2010).	2010	Zimbabwe	676	142	21.0 (17.9, 24.1)
Americas	(Towers et al., 2010)	2010	US	1472	296	20.1 (18.1, 22.2

Americas	(Jordan et al., 2010)	2010	US	306	75	24.5 (19.7, 29.3)
E Med	(Moghaddam, 2010)	2010	Iran	201	25	12.4 (7.9, 17.0)
Europe	(El Aila et al., 2010)	2010	Belgium	100	22	22.0 (13.9, 30.1)
W Pacific	(Lee et al., 2010)	2010	South Korea	2526	204	8.1 (7.0, 9.1)
Africa	(Gray et al., 2011)	2011	Malawi	1441	313	21.7 (19.6, 23.9)
Americas	(Lin et al., 2011)	2011	US	5497	1,031	18.8 (17.7, 19.8)
Americas	(Montibello et al., 2011)	2011	Argentina	962	168	17.5 (15.1, 19.9)
Americas	(Rocchetti et al., 2011)	2011	Brazil	405	103	25.4 (21.2, 29.7)
Americas	(Simoes et al., 2007)	2011	Brazil	316	46	14.6 (10.7, 18.4)
E Med	(Zamzami et al., 2011)	2011	Saudi Arabia	326	103	31.6 (26.5, 36.6)
E Med	(Parvin Hassanzedah, 2011)	2011	Iran	310	43	13.9 (10.0, 17.7)
Europe	(Kunze et al., 2011)	2011	Germany	869	183	21.1 (18.3, 23.8)
Europe	(Romanik et al., 2011)	2011	Poland	80	23	28.8 (18.8, 38.7)
SE Asia	(Sharmila et al., 2011)	2011	India	300	7	2.3 (0.6, 4.0)
2012-13	**8 studies, I^2=98.9%**			**21369**	**3,586**	**13.8 (8.5, 19.1)**
Americas	(Feuerschuette et al., 2012)	2012	Brazil	254	73	28.7 (23.2, 34.3)
SE Asia	(Turner et al., 2012)	2012	Thailand	549	47	8.6 (6.2, 10.9)
E Med	(Seyyed et al., 2013)	2013	Iran	178	36	20.2 (14.3, 26.1)
Europe	(Cantoni et al., 2013)	2013	Italy	15239	3,010	19.8 (19.1, 20.4)
W Pacific	(Lu et al., 2014)	2013	China	2850	201	7.1 (6.1, 8.0)
SE Asia	(Chan et al., 2013)	2013	Bangladesh	1219	94	7.7 (6.2, 9.2)
SE Asia	(Patil KP, 2013)	2013	India	905	110	12.2 (10.0, 14.3)
W Pacific	(Park et al., 2013)	2013	South Korea	175	15	8.6 (4.4, 12.7)
2014-15	**5 studies, I^2=89.5%**			**3553**	**752**	**20.3 (13.7, 26.9)**
Africa	(Kwatra et al., 2014)(Chapter 5)	2014	South Africa	521	148	28.4 (24.5, 32.3)

Europe	(Prosniewska M., 2014)	2014	Poland	377	89	23.6 (19.3, 27.9)
SE Asia	(Konikkara et al., 2014)	2014	India	50	8	16.0 (5.8, 26.2)
W Pacific	(Morita et al., 2014)	2014	Japan	1425	319	22.4 (20.2, 24.6)
Europe	(Maria del Carmen Liebana-Martos, 2015)	2015	Spain	1880	188	9.6 (5.4, 13.8)
Overall	**44 studies, I^2=97.1%**			**53223**	**9434**	**17.5 (15.6, 19.4)**

3.4.3 Sensitivity Analysis:

To investigate any potential bias introduced by the exclusion of studies reporting colonization from lower vaginal swabs only, a separate meta-analysis was completed. Seventeen studies were identified capturing 18,495 subjects with a mean prevalence estimate was 14.2% (95% CI: 10.7, 17.6). A similar heterogeneity to the primary meta-analyses was observed (I^2= 97.5%) (Table 3.9). Secondly, the ethnic diversity of the Western Pacific region was revisited. For this region, there were eight studies originally included, from China (including Hong Kong), Japan, Korea, Australia and New Zealand (Table 3.10). The two Australian and New Zealand studies accounted for 14% of subjects included in the original analysis (1336/9773). The exclusion of these two studies led to a non-significant reduction in the mean prevalence estimate from 13.3% (95% CI, 7.8, 18.8) to 10.1% (95% CI, 5.2, 15.1), aligning more closely with the Southeast Asian estimate of 11.1% (95% CI, 6.8, 15.3).

3.4.4 Serotype Distribution Meta-Analysis

In total, 22 studies reported serotype distribution, but 5 were excluded due to reporting of combined serotypes only (n=2), combined maternal and neonatal isolate data (n=1), or failure to report raw data (n=2). The remaining 17 studies are summarized in Table 3.11. Studies from the Americas and Europe reported lower proportions of serotypes Ia, Ib or III isolates: 55.0% (95% CI: 52.3, 57.7) and 58.3% (95% CI: 52.2, 64.5) respectively, mainly reflecting a higher proportion of serotype II isolates in those regions. Serotypes VI, VII and VIII were rare everywhere, though one study from South Korea, (Lee et al., 2010) reported these for 7.3% (14/192) of vaginal and rectal GBS isolates.

3.4.5 Publication Bias

To investigate how prevalence estimates varied by study size, a funnel plot was produced and showed little asymmetry on either side of the overall prevalence estimate (Figure 3.8, regression test with study size as a predictor, p=0.89).

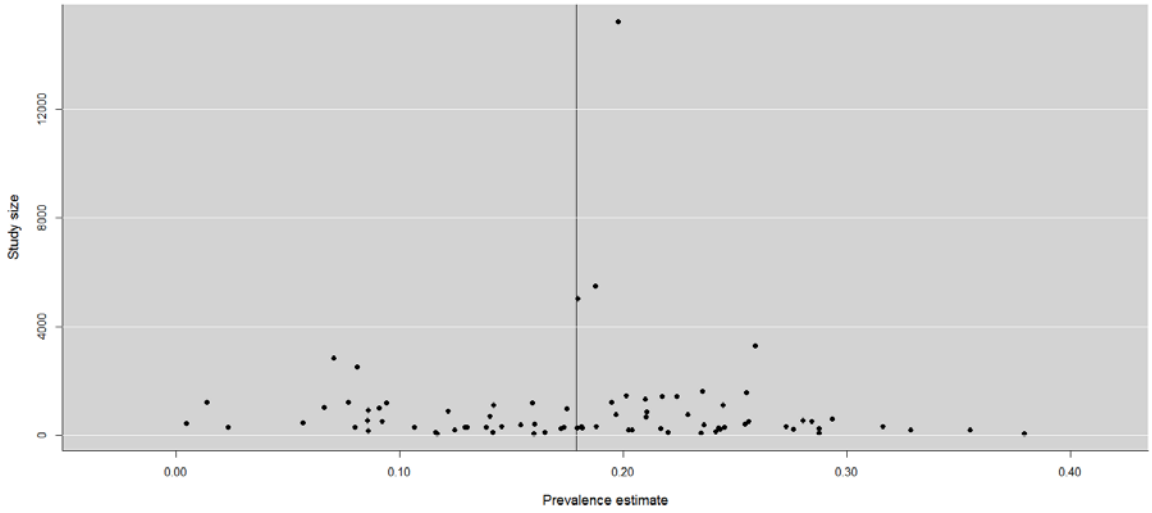


Figure 3.8: Funnel plot to explore how prevalence estimates vary by study size

Table 3.9: Meta-analysis of studies estimating prevalence of vaginal-only GBS colonization

Region	Author	Year	Country	Study Size	Number of GBS positive cases	Estimated prevalence (95% CI)
Americas	(Krohn et al., 1999)	1999	US	7806	1689	21.6 (20.7, 22.6)
Americas	(Zusman et al., 2006)	2006	Brazil	598	107	17.9 (14.8, 21.0)
SE Asia	(Das et al., 2003)	2003	India	200	15	7.5 (3.8, 11.2
SE Asia	(Goyal et al., 2004)	2004	India	304	4	1.3 (0.0, 2.6)
SE Asia	(Chaudhry et al., 2010)	2010	Pakistan	200	17	8.5 (4.6, 12.4)
SE Asia	(Sobhana Surya Pradeep M, 2013)	2013	India	83	3	3.6 (0.0, 7.6)
Africa	(Cutland et al., 2009)	2009	South Africa	3964	825	20.8 (19.5, 22.1)
Africa	(Okon, 2013)	2013	Nigeria	97	13	13.4 (6.6, 20.2)
Africa	(Mitima et al., 2014)	2014	Democratic Republic Congo	509	103	20.2 (16.7, 23.7)
E Med	(Ben Hamouda et al., 2008)	2008	Tunisia	207	27	13.0 (8.5, 17.6)
E Med	(Farnaz Fatemi, 2009)	2009	Iran	330	68	20.6 (16.2, 25.0)
E Med	(S. Mansouri, 2008)	2008	Iran	602	55	9.1 (6.8, 11.4)
E Med	(Shabayek et al., 2009)	2009	Egypt	150	45	30.0 (22.7, 37.3)
E Med	(Tajbakhsh et al., 2013)	2013	Iran	285	27	9.5 (6.1, 12.9)
Europe	Krasmianin et al.	2009	Poland	100	19	19.0 (11.3, 26.7)
Europe	(Sensini et al., 1997)	1997	Italy	2300	260	11.3 (10.0, 12.6)
Europe	(Verhoeven et al., 2014)	2014	France	760	127	16.7 (14.1, 19.4)
Overall	**17 studies, I^2=98%**			**18495**	**3404**	**14.2 (10.7, 17.6)**

Table 3.10: Sensitivity analyses – impact of Australia and New Zealand within Western Pacific region

Region	No. studies	No. subjects	Median subjects per study (IQR)	Number of GBS positive cases	Estimated Prevalence (95% CI)	Within region test for heterogeneity I^2, p value
Europe - original	23	31642	**500** (275-942)	**6113**	**19.0** (16.1, 22.0)	97.2%, p < 0.0001
Europe including Australia and New Zealand	25	32978	**500** (250-1014)	**6433**	**19.3** (16.6, 22.1)	96.9%, p<0.0001
Western Pacific – original	8	9773	**1049** (404-1700)	**1176**	**13.3** (7.8, 18.8)	98.7%, p < 0.0001
Western Pacific excluding Australia and New Zealand	6	8437	**1213.5** (595-2251)	**856**	**10.1** (5.2, 15.1)	98.3%, p<0.0001
Australia and New Zealand	2	1336	**668** (454-882)	**320**	**23.9** (21.6, 26.2)	No. of studies too small

Table 3. 11: Meta-analysis of serotype isolated

Region	Author	Year	Country	Total serotypes isolated	Serotype Ia, Ib or III				Serotype Ia, Ib, II, III or V			
					#	%	95% CI	I^2	#	%	95% CI	I^2
OVERALL, 17 studies				**3391**	**2029**	**63.3**	**(57.7, 68.8)**	**90.4%**	**3105**	**91.5**	**(88.9, 94.0)**	**88.0%**
Africa	(Kwatra et al., 2014)	2014	South Africa	152	115	75.7	(68.8, 82.5)		144	94.7	(91.2, 98.3)	
Africa	(Gray et al., 2011)	2012	Malawi	390	247	63.3	(58.6, 68.1)		380	97.4	(95.9, 99.0)	
AFRICA, 2 studies				**542**	**362**	**69.2**	**(57.2, 81.3)**	**88.1%**	**524**	**96.6**	**(94.1, 99.0)**	**46.2%**
Americas	(Campbell et al., 2000)	2000	US	856	472	55.1	(51.8, 58.5)		806	94.2	(92.6, 95.7)	
Americas	(Davies et al., 2001)	2001	Canada	233	126	54.1	(47.7, 60.5)		201	86.3	(81.8, 90.7)	
Americas	(Hickman et al., 1999)	1999	US	153	88	57.5	(49.7, 65.3)		146	95.4	(92.1, 98.7)	
Americas	(Simoes et al., 2007)	2011	Brazil	47	23	48.9	(34.6, 63.2)		36	76.6	(64.5, 88.7)	
AMERICAS, 4 studies				**1289**	**709**	**55.0**	**(52.3, 57.7)**	**0.0%**	**1189**	**89.6**	**(82.6, 96.6)**	**92.9%**
Europe	(Hakansson et al., 2008)	2008	Sweden	356	170	47.8	(42.6, 52.9)		295	82.9	(79.0, 86.8)	
Europe	(Tsolia et al., 2003)	2003	Greece	67	36	53.7	(41.8, 65.7)		60	89.6	(82.2, 96.9)	
Europe	(Kunze et al., 2011)	2011	Germany	155	98	63.2	(55.6, 70.8)		140	90.3	(85.7, 95.0)	
Europe	(Jones et al., 2006)	2005	UK	159	108	67.9	(60.7, 75.2)		153	96.2	(93.3, 99.2)	
Europe	(Winifred and Hasker, 1972)	1998	Ireland	129	78	60.5	(52.0, 68.9)		116	89.9	(84.7, 95.1)	
Europe	(L.S. Roccasalva, 2008)	2008	Italy	7	5	71.4	(38.0, 100.0)		7	93.8	(77.0, 100.0)	
Europe	(Maria del Carmen Liebana-Martos, 2015)	2015	Spain	212	117	55.2	(48.5, 61.9)		175	82.5	(77.4, 87.7)	
EUROPE, 7 studies				**1085**	**612**	**58.3**	**(52.2, 64.5)**	**72.6%**	**946**	**88.9**	**(84.7, 93.2)**	**79.2%**
W Pacific	(Lu et al., 2014)	2013	China	201	151	75.1	(69.1, 81.1)		195	97.0	(94.7, 99.4)	
W Pacific	(Uh et al., 1997)	1997	South Korea	193	132	68.4	(61.8, 75.0)		175	90.7	(86.6, 94.8)	
W Pacific	(Grimwood et al., 2002)	2002	New Zealand	29	27	93.1	(83.9, 100.0)		27	93.1	(83.9, 100.0)	
W Pacific	(Lee et al., 2010)	2010	South Korea	52	36	69.2	(56.7, 81.8)		49	94.2	(87.9, 100.0)	
W PACIFIC, 4 studies				**475**	**346**	**76.5**	**(65.4, 87.5)**	**86.8%**	**446**	**94.2**	**(90.6, 97.7)**	**56.1%**

1. For Jones et al. (ref 39), serotyping was performed on isolates that were positive between two different culture methods with overall GBS positive only shown for CDC method

3.5 Discussion

This systematic review and meta-analysis identified 78 studies reporting the prevalence of maternal recto-vaginal colonization with GBS in pregnancy. The overall mean prevalence globally was 17.9% (95% CI: 16.2, 19.7) with the highest prevalence reported among African studies (22.4%, 95% CI 18.1, 26.7%). The mean prevalence reported from studies in the Americas, Europe and the Eastern Mediterranean regions was very similar and the lowest prevalence was reported from SouthEast Asia and Western Pacific studies.

The criteria used to select studies for this meta-analysis, primarily based on CDC recommendations for GBS screening and identification using selective culture methods (Verani et al., 2010), aimed to focus on robust data while limiting between study heterogeneity due to methodological variation. However, statistically significant heterogeneity was still observed across all studies and across studies within individual regions. A sensitivity analysis considering studies with lower vaginal swab collection reflected the same pattern of regional heterogeneity. Further stratification by variables poorly controlled for by inclusion criteria (e.g. timing of specimen collection and type of selective culture method) did not lead to a reduction in heterogeneity of prevalence estimates across studies. Additional variables, such as demographics, were difficult to explore as few studies reported these with sufficient detail. The colonization prevalence range reported in the previous review was 8 to 18%, (Stoll and Schuchat, 1998) which was similar to the 11% to 22% observed in the current meta-analysis. This, despite greater standardization in culture methods used in studies included in the current meta-analysis, and which was considered to be an important driver for the variability in colonization

between studies in the earlier meta-analysis. However, as these studies were not undertaken in the same places, a direct comparison of these estimates needs to be viewed with caution, considering even within country differences observed in colonization in the current meta-analysis. Nevertheless, these data suggest that other factors are likely contributing towards regional variations in maternal GBS colonization and consequently risk for early-onset disease in their newborns.

Furthermore, the heterogeneity may have been driven by unmeasured variables: differences in study population selection may have led to higher or lower risk populations being represented which was difficult to assess across studies. Several obstetric variables, such as parity and premature rupture of membranes, have been associated with higher risk of GBS colonization (Kwatra et al., 2014) ; but without access to individual level subject data this was difficult to explore.

There have been reports of differential sensitivity of culture methods between populations. For example, in South Africa one study reported that selective broth was not suitable for the recovery of GBS from rectal swabs due to masking of the GBS colonies by persistent microflora. In that population, direct plating on selective agar was reported as the most sensitive method of isolation (Kwatra et al., 2013). Studies from Brazil, Denmark and Spain have also reported increased GBS recovery using direct plating on selective media as compared to selective broth (Hansen and Sorensen, 2003, Gil et al., 1999, Chaves et al., 2010). Further optimization of microbiological techniques in different populations will contribute to a better understanding of potential ascertainment differences and their impact on GBS colonization prevalence estimates.

The pattern of GBS colonization prevalence, with the highest estimates from Africa and lowest from Southeast Asia and Western Pacific, matches the pattern of GBS neonatal disease incidence (Edmond et al., 2012). An algorithm to predict EOD incidence based on maternal colonization prevalence can be used to explore these patterns further: 50% of infants born to women colonized with GBS will themselves be colonized and 2% of those colonized infants will develop GBS EOD (Madhi et al., 2013, Schuchat et al., 2000). Data from South Africa and the United States, prior to the introduction of routine screening and IAP, where approximately 20% of pregnant women had GBS vaginal colonization, confirmed the expected 2 per 1000 live births EOD incidence (Madhi et al., 2013, Cutland et al., 2012, Cutland et al., 2009). Based on this algorithm, and regional prevalence estimates from this meta-analysis, one would expect the incidence of EOD GBS in Southeast Asian and Western Pacific studies to be half that observed in African studies. However, the overall invasive disease incidence those regions is commonly reported to be much lower than the expected 1 per 1000 live births (Edmond et al., 2012). While differences in colonization prevalence may explain some of the regional differences in disease incidence, other factors including natural immunity, serotype distribution with varying virulence between serotypes and surveillance methods may contribute and need to be further investigated.

This systematic review and meta-analysis aimed to identify studies from all geographic regions by searching five different literature databases and by allowing all language publications. However, few studies from Africa, which has got high proportion of low income countries, were identified from initial searches and even fewer were eligible for inclusion. Thus, some publication is likely to remain.

To ensure quality and to guard against bias in the identified studies, this meta-analysis had very stringent inclusion and exclusion criteria based upon rigorous methodology and aiming to standardise studies with respect to factors known to influence the estimate of colonisation prevalence (for example culture method for GBS isolation and identification). Nevertheless, this meta-analysis observed substantial variation in the prevalence of maternal colonization both within and across geographic regions. The within region difference were not explained by differences in specimen collection and microbiological culture techniques; though additional analyses with subject level data are recommended to better understand the role of population selection, obstetric and sociodemographic risk factors. A further limitation of the available data includes the limited serotype distribution by region, and we were only able to include 17 studies in this analysis with no studies from Southeast Asia and Eastern Mediterranean.

Underlying population differences in natural immunity through transplacental acquisition of serotype-specific capsular antibody by the fetus remain an alternative explanation that should be explored in future studies to interrogate the disparity between exposure and incidence rates across geographic regions. A clearer understanding of the factors underlying this variation will be critical to the more efficient design of screening and prevention programs for maternal and infant GBS disease.

Chapter 4 Evaluation of Trans-Vag broth, CNA agar and CHROMagar StrepB for the detection of Group B *Streptococcus* in vaginal and rectal swabs from pregnant women in South Africa

4.1 Abstract

Background: Maternal vaginal colonization with Group B *Streptococcus* (GBS) is a major risk factor for invasive GBS infection in newborns. The CDC recommended method for detecting GBS colonization is to culture vaginal and rectal swabs in selective broth followed by subculture on blood agar or selective media. A high incidence of antimicrobial resistance in faecal microflora can compromise the recovery of GBS from selective broth.

Methods: We compared CHROMagar StrepB (CA), Columbia colistin and nalidixic agar (CNA) and Trans-Vag selective broth enrichment for the isolation of GBS from 130 vaginal and 130 rectal swabs from pregnant women. Swabs were randomized to be plated first on either CA or CNA, following which the swab was inoculated in Trans-Vag broth.

Results: GBS was cultured from 37.7% of vaginal swabs and 33.1% of rectal swabs. There were no differences in the detection rate from vaginal swabs between CA (31.5%), CNA (26.2%) and selective broth (30.0%). The sensitivities in relation to a composite score were 83.7%, 69.4% and 79.6% respectively. Recovery of GBS from rectal swabs was, however, significantly higher from CA (29.2%; $p<0.0001$) and CNA (23.8%; $p=0.002$) than from selective broth (9.2%). The sensitivities were 88.4%, 72.1% and 27.9%, respectively. The order of plating on solid medium was significant ($p=0.003$), with GBS detection rates of 30.8% and 24.6% when plated first and second respectively.

Conclusion: These findings show that selective broth is not suitable for the recovery of GBS from rectal swabs in settings such as ours, due to masking of GBS colonies by other microflora.

4.2 Introduction

Infection by Group B *Streptococcus* (GBS) is one of the most common infections in newborns (Van Dyke et al., 2009). Maternal colonization has been found to be major risk factor for invasive GBS disease within six days of birth. Approximately 10-40% of pregnant women are colonized with GBS either in the vagina or rectum or both (Picard and Bergeron, 2004). The rate of peripartum transmission of GBS to newborns of colonized women is approximately 50%, following which 1-3% of these newborns develop invasive GBS disease in the first week of life (Beal and Dancer, 2006, Heath and Feldman, 2005). The Centres for Disease Control and Prevention (CDC) has recommended that all pregnant women be screened for recto-vaginal carriage by GBS at 35-37 weeks gestation, to identify women who should receive intrapartum antimicrobial prophylaxis (IAP). The use of IAP targeted at GBS-colonized pregnant women has reduced the incidence of invasive GBS disease by 86-89% where successfully implemented (Brozanski et al., 2000, Lin et al., 2001, Schrag et al., 2002). The sensitivity of screening methods for identification of maternal carriage of GBS depends on the timing of specimen collection, the source of the specimen and the culture technique used. Optimally, specimens should be collected as close to delivery as possible. The use of vaginal and rectal swab specimens has been shown to yield higher GBS culture-positivity rates compared to vaginal swabs alone or cervical specimens (Platt et al., 1995, Chan SHS, 2000, Quinlan et al., 2000). The current CDC recommendation for the isolation of GBS from vaginal and rectal or recto-vaginal swabs is

by growth in a selective broth medium (Todd-Hewitt broth with gentamicin and colistin or nalidixic acid), followed by subculture on blood agar or selective media (Verani et al., 2010). The reported sensitivity of selective broth for culture of GBS is 82%-99% (Gupta and Briski, 2004, Craven et al., 2010, Jones et al., 2006, Busetti et al., 2007). There are however limitations to this approach.

The procedure requires at least an additional 24 hours of culture time compared to direct plating on selective agar, and isolated GBS-like colonies require further identification with ancillary tests e.g. CAMP factor or B-antigen (Verani et al., 2010). Furthermore, identification of GBS-like colonies on blood agar requires laboratory expertise, particularly when they are mixed with other microflora (Poisson et al., 2010). Considering methods to decrease the GBS detection time, studies have shown that direct plating on colistin and nalidixic agar (CNA) is a low-cost alternative for GBS recovery, albeit with a lower sensitivity, ranging from 59%-83% (Bosch-Mestres et al., 2003, El Aila et al., 2010, Louie et al., 2010). In recent years, several commercial chromogenic media have been tested for their suitability for detecting GBS such as Granada medium, CHROMagar StrepB (CA) and Chrom ID Strepto B agar (El Aila et al., 2010, Poisson et al., 2011).

CA is a commercially available selective chromogenic medium that inhibits most saprophytic bacteria and yeasts, and produces mauve-coloured GBS colonies in aerobic conditions irrespective of their haemolytic properties, allowing direct visual identification. The use of chromogenic media may improve the yield of GBS, while reducing labor costs and turnaround time. The clinical sensitivity of these media vary from study to study, with most studies reporting 93%-98% for CA (Charron, 2009, Poisson et al., 2010), 40%-91%

for Granada Medium (El Aila et al., 2010, Gupta and Briski, 2004, Overman et al., 2002) and 88%-95% for Chrom ID Strepto B agar (El Aila et al., 2010, Craven et al., 2010). The aim of this study was to evaluate direct plating on CA, CNA and selective broth enrichment for the isolation of GBS in swabs from pregnant women.

4.3 Materials and Methods

4.3.1 Study Design

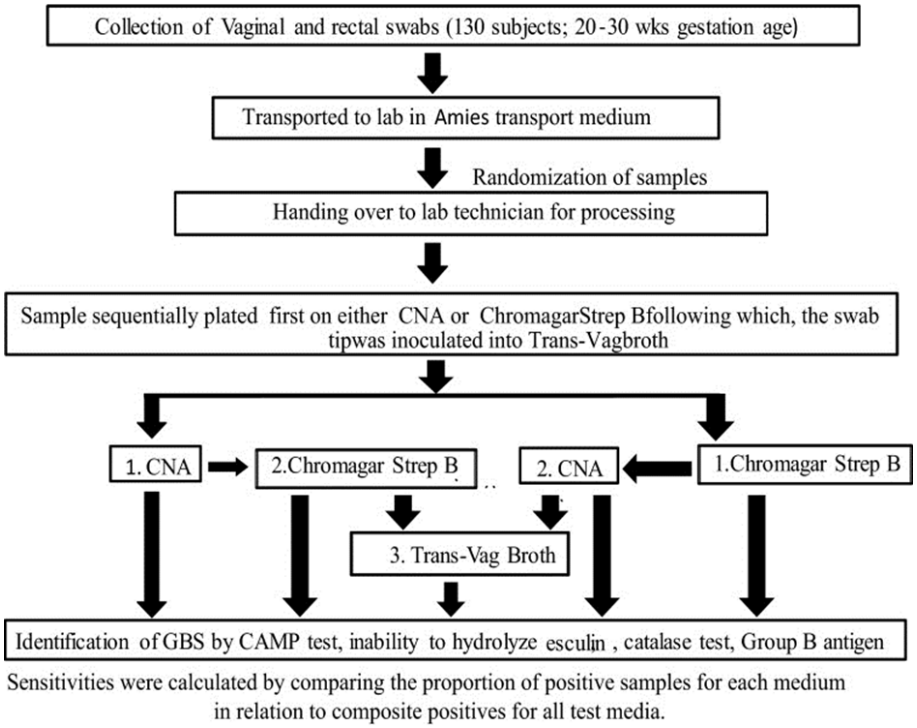
The study methods have been detailed in chapter 2. Briefly, the study was conducted at prenatal community clinics in Soweto, Johannesburg. Inclusion criteria were HIV-uninfected pregnant women above 20 weeks of gestational age, able to provide informed consent for participation. Exclusion criteria were concurrent antibiotic use, acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examinations were contraindicated. A convenience sampling of lower vaginal and rectal swabs was collected between Jan 2011 and April 2011, by trained study-nurses from 130 pregnant women. Samples were collected with rayon tip swabs that were placed into 5ml of Amies transport medium without charcoal. All samples were transported to the laboratory within 4 hours of collection, where they were processed within 2 hours.

4.3.2 Randomization and processing of samples

All vaginal and rectal swabs were randomized with a randomization log. The swab number was linked to three different random numbered labels, one for each culture medium type. Each sample was randomized to be plated first on either CNA or CA, following which the swab tip was inoculated into selective (Trans-Vag) broth (Todd Hewitt broth with gentamicin 8µg/ml and nalidixic acid 15µg/ml) and incubated for 24

hours before being plated on 5% sheep blood agar (SBA) (Figure 4.1). The randomization process ensured that the technician reading the plates and entering the data was blinded with respect to the participant, type of swab (rectal or vaginal), the order of plating, and the swab/plate pairs. The CNA and SBA plates were incubated at 37°C in 5% CO_2 for 18-24 hours and the CA plates were incubated at 37°C for 18-24 hours in aerobic conditions. If the expected colonies were not visible after 24 hours of incubation the plates were re-incubated for an additional 24 hours and re-examined for growth. Up to four GBS-like colonies were isolated from the solid culture media on the basis of colony morphology and mauve colour on CA and colony morphology and beta-haemolysis on CNA and blood agar. Isolates were confirmed as GBS by testing for CAMP factor, inability to hydrolyze esculin, catalase negativity and group B antigen latex agglutination positivity. CA and CNA pre-poured plates were purchased from Media Mage, Johannesburg, South Africa.

Figure 4.1: Collection, randomization and laboratory handling of vaginal and rectal swabs for the isolation of Group B *Streptococcus*



4.3.4 Statistical methods

Descriptive statistics included statements of frequency with percentages, means ± standard deviations (SDs), and medians with interquartile ranges (IQRs). Sensitivities (detection rates) were calculated by comparing the proportion of positive samples for each medium in relation to a gold standard which was a composite positive for all test media. The McNemar test for correlated percentages and Chi-Square test with Yates' correction were used to compare the culture methods with $p<0.05$ considered statistically significant.

4.4 Results

4.4.1 Study population

The mean age of the women at swabbing was 25.7±5.9 years, and the mean gestational age was 23.8±3.2 weeks. The median parity and gravidity were 1 (IQR 0-1) and 2 (IQR 1-2) respectively**.**

4.4.2 CA and CNA vs Trans-Vag selective broth enrichment

Overall, a total of 37.7% of vaginal swabs and 33.1% of rectal swabs tested positive for GBS (Table 4.1). There was no statistically significant difference in the detection rate for vaginal swabs between CA (31.5%), CNA (26.2%) and selective broth (30.0%). The sensitivities with respect to composite scores were 83.7%, 69.4% and 79.6% respectively. The recovery of GBS for rectal swabs was, however, significantly higher from CA (29.2%; $p<0.0001$) and CNA (23.8%; $p=0.002$) than with selective broth media (9.2%). The sensitivities were 88.4%, 72.1% and 27.9% respectively. Despite the higher sensitivity reported for CA over CNA for both vaginal and rectal swabs, these differences were not statistically significant ($p>0.05$).

4.4.3 Accuracy

The accuracy of a medium was defined as the percentage of isolated colonies that were identified as GBS by confirmatory testing, divided by the total number of GBS-like colonies that were isolated from a plate and subjected to confirmatory tests. The denominator includes both confirmed GBS (true positives) and colonies having similar colony appearances to GBS, but failed the confirmatory tests (false positives). For vaginal swabs, CA was the most accurate at discriminating between true positive and false positive

isolates (92.8%) followed by selective broth enrichment (90.6%) and CNA (84.3%). There were no significant differences in the accuracy of primary identification between CA and selective broth, and between CNA agar and selective broth. After direct plating colonies were, however, significantly more likely to be GBS following isolation with CA, than with CNA ($p=0.026$).

The accuracy of GBS identification from rectal swabs was significantly better with CA (83.0%; $p<0.0001$) and CNA (77.3%; $p=0.0004$) than with selective broth enrichment (54.5%). CA was found to be more accurate than CNA, but these differences were not statistically significant ($p=0.26$). The number of GBS-like colonies that could be identified from cultured vaginal and rectal swabs was similar between CA (167 vaginal; 159 rectal) and CNA (153 vaginal; 150 rectal). Approximately half the number of GBS-like colonies could, however, be identified among the other flora following selective broth enrichment from rectal swabs (171 vaginal; 88 rectal). A higher percentage of false positive GBS isolates was reported from rectal swabs compared to vaginal swabs with CA (17.0% vs 7.2%; $p=0.01$), CNA (22.7% vs 15.7%; $p=0.16$) and selective broth (45.5% vs 9.4%; $p<0.0001$) (Table 4.1)

Table 4. 1: Performance characteristics of CHROMagar Strep B, Colistin and Nalidixic agar and selective broth

Medium	Swab					
	Vaginal, n=130			Rectal, n=130		
	No. (%) positive for GBS	Sensitivity * (%)	Accuracy† (%)	No. (%) positive for GBS	Sensitivity * (%)	Accuracy† (%)
CA	41(31.5%)	83.7%	155/167 (92.8%)	38(29.2%)	88.4%	132/159 (83.0%)
CNA	34(26.2%)	69.4%	129/153 (84.3%)	31(23.8%)	72.1%	116/150 (77.3%)
Selective Broth	39(30.0%)	79.6%	155/171 (90.6%)	12(9.2%)	27.9%	48/88 (54.5%)
Total GBS isolated	**49(37.7%)**			**43(33.1%)**		

*Sensitivity was calculated in comparison to composite scores of all media.

† Accuracy was calculated as the number of confirmed GBS colonies isolated / total number of GBS like colonies that were confirmed as either positive or negative for GBS.

4.4.4 Impact of randomization and the order of plating

The order in which swabs were plated onto the two selective media had a significant effect on the culture-positivity rate. Out of 260 swabs tested directly on CNA and CA, a total of 86 swabs (33.1%) were positive for GBS. Direct culture on CNA and CA yielded GBS from 80 (30.8%) swabs when plated first compared to 64 (24.6%) when plated second (p=0.003). GBS was isolated from both media in 58 (22.3%) of the swabs. The number of swabs from which GBS was isolated from a single medium was 22 (8.5%), and 6 (2.3%) from the first and second plating respectively. When CNA and CA were plated first, the sensitivities were 86.4% and 87.5% respectively, and when CNA and CA were plated second, the sensitivities were 57.4% and 82.2% respectively (Table 4.2).

Table 4. 2: Performance characteristics of CNA and CA with respect to the order of plating

Specimen	Plated first on CNA						
	CNA		CA		Trans-Vag*		
	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	Total no. (%) positive GBS
Vaginal, n=65	20 (30.8%)	80%	21 (32.3%)	84%	23 (35.4%)	92%	25 (38.5%)
Rectal, n=65	18 (27.7%)	94.7%	16 (24.6%)	84.2%	8 (12.3%)	42.1%	19 (29.2%)
Total, n=130	**38 (29.2%)**	**86.4%**	**37 (28.5%)**	**84.1%**	**31 (23.8%)**	**70.4%**	**44 (33.8%)**
	Plated first on CA						
	CNA		CA		Trans-Vag*		
	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	Total no. (%) positive GBS
Vaginal, n=65	14 (21.5%)	58.3%	20 (30.8%)	83.3%	16 (24.6%)	66.7%	24 (36.9%)
Rectal, n=65	13 (20%)	54.2%	22 (33.8%)	91.7%	4 (6.2%)	16.7%	24 (36.9%)
Total, n=130	**27 (20.8%)**	**56.2%**	**42 (32.3%)**	**87.5%**	**20 (15.4%)**	**41.7%**	**48 (36.9%)**

*After direct plating the swab was placed in Trans-Vag broth.

4.5 Discussion

Current CDC guidelines for prenatal GBS screening recommends the collection of a single recto-vaginal swab or separate vaginal and rectal swabs that are enriched overnight in Todd-Hewitt broth with antibiotics, followed by culture on blood agar or selective media (Verani et al., 2010). Whilst these guidelines have been developed within the context of high-income countries, there is very little data on the validity of this method in low to middle-income countries where there are differences in the distribution of saprophytic organisms and antimicrobial resistance is common (Nys et al., 2004, Newman and Seidu, 2002, Shanahan et al., 1993, Pal, 2010). In this study we compared the recovery rate of GBS by standard methods with direct plating onto either CNA agar or CA from either vaginal or rectal swabs from pregnant South African women to determine whether these alternatives would provide better recovery rates. One of the main reasons for this comparison was the presence of vaginal flora that are resistant to antimicrobial agents, in particular Proteus sp., that occur in a small percentage of swabs and are able to persist in Trans-Vag broth and swarm the subcultured blood agar plate, rendering it unreadable (P.V. Adrian, personal communication). This necessitated the use of both Trans-Vag broth and direct plating on CNA in a previous study of vaginal swabs from South Africa (Cutland et al., 2009).

In our study there were no statistically significant differences in the recovery of GBS from vaginal swabs between the different media, suggesting that any of these three options are valid in our clinical setting, and can be decided upon, based on cost, availability, and desired turnaround time. In contrast, the recovery of GBS from rectal swabs following selective broth enrichment was inferior to either of the direct plating

methods. The main reason for the poor recovery of GBS, was survival and overgrowth of non-GBS organisms in the Trans-Vag broth, which masked the presence of GBS on the subculture plates. The results of our study differ from several previously published reports, in which it had been shown that the selective broth method is more sensitive than direct plating on selective media for recovering GBS from rectal or recto-vaginal swabs (Jones et al., 2006, Gupta and Briski, 2004, Craven et al., 2010, Busetti et al., 2007). While these results may be valid in settings such as those where the studies were conducted, other studies from Brazil, Denmark and Spain have demonstrated better recoveries of GBS from rectal swabs with a solid selective medium compared to selective broth (Hansen and Sorensen, 2003, Chaves et al., 2010, Gil et al., 1999). The use of pigmented enrichment broth such as Strep B carrot broth has been shown to increase the sensitivity of GBS detection by culture over LIM broth (Church et al., 2008), and when used as an enrichment step prior to PCR screening (Munson et al., 2010), however, GBS growth and pigment development can still be suppressed by the overgrowth of fecal bacteria and mask the presence of GBS on culture (Munson et al., 2010). Since the mechanism behind the poor recovery of GBS from rectal swabs is related to persistence and overgrowth of resistant faecal bacteria (Gil et al., 1999), it is likely that the collection of a single recto-vaginal swab or the co-processing of vaginal and rectal swabs may be severely compromised by the use of selective broth in our setting.

Overall, CA produced the highest recovery of GBS from both vaginal and rectal swabs. One advantage of the chromogenic medium was that it was significantly easier to discriminate between GBS and Group D *Streptococcus* (GDS) in co-colonised samples due to differences in the colour development of the colonies. GBS turns mauve whereas GDS turns blue. This is particularly important in comparison with samples plated on blood agar,

where the GBS colonies are in the minority, and single GBS colonies can be overlooked amid large numbers of morphologically similar GDS. Despite CA being relatively more expensive than CNA or a selective broth blood agar combination, CA offers cost savings in terms of the reduced processing time, and the ease with which GBS colonies can be identified on the plates. Moreover, due to the increased accuracy of colony identification through pigment formation, the number of confirmatory tests required is reduced, as the need for additional tests to rule out the morphologically similar colonies of Group D streptococci is eliminated. While the calculated values of accuracy will vary with the skill and experience of the user, CA offers an easier learning curve compared to a blood-based isolation medium. In comparing the two selective solid agars, CA had a higher sensitivity and accuracy than CNA for both vaginal and rectal swabs, although these values were not statistically significant, most likely due to a limited sample size.

From a review of the literature, no study in a clinical setting has considered the order of plating when comparing and reporting the sensitivity of culture media to isolate GBS. In this study, the order of plating had a significant effect on the recovery of GBS, and suggests that future studies where solid media are compared should follow randomised protocols to prevent detection bias based on plating order. Possible reasons for the lower recovery of GBS from swabs plated for a second time include removal of the organisms from the surface of the swab in the first plating, or blockage of the swab surface by sterile agar from the previous plating, preventing transfer of organisms. The sensitivity of direct plating on CNA when plated first in this study was similar to that described by Louie *et al* (Louie et al., 2010) in which swabs were inoculated first on CNA.

In conclusion, selective broth enrichment showed significantly lower sensitivity for the recovery of GBS from rectal swabs. This method is not suitable for recovery of GBS from rectal swabs or recto-vaginal swabs in regions with a high prevalence of antimicrobial resistant flora. The recovery of GBS from vaginal swabs plated on CA appears equal in sensitivity to selective broth, and offers a less time-consuming and less labour-intensive process.

Chapter 5 Serotype-Specific Acquisition and Loss of Group B *Streptococcus* Recto-vaginal Colonization in Late Pregnancy

5.1 Abstract

Background: Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) and consequent vertical transmission to the newborn predisposes neonates to early-onset invasive GBS disease. This study aimed to determine the acquisition and loss of serotype-specific recto-vaginal GBS colonization from 20-37+ weeks of gestational age.

Methods: Vaginal and rectal swabs were collected from HIV-uninfected women at 20-25 weeks of gestation age and at 5-6 weekly intervals thereafter. Swabs were cultured for GBS and isolates were serotyped by latex agglutination. Serologically non-typable isolates and pilus islands were characterized by PCR.

Results: The prevalence of recto-vaginal GBS colonization was 33.0%, 32.7%, 28.7% and 28.4% at 20-25 weeks, 26-30 weeks, 31-35 weeks and 37 + weeks of gestational age, respectively. The most common identified serotypes were Ia (39.2%), III (32.8%) and V (12.4%). Of 507 participants who completed all four study visits, the cumulative overall recto-vaginal acquisition rate of new serotypes during the study was 27.9%, including 11.2%, 8.2% and 4.3% for serotypes Ia, III and V, respectively. Comparing the common colonizing serotypes, serotype III was more likely to be associated with persistent colonization throughout the study (29%) than Ia (18%; p=0.045) or V (6%; p=0.002). The median duration of recto-vaginal GBS colonization for serotype III was 6.35 weeks, which was longer than other serotypes. Pilus island proteins were detected in all GBS isolates and their subtype distribution was associated with specific serotypes.

Conclusion: South African pregnant women have a high prevalence of GBS recto-vaginal colonization from 20 weeks of gestational age onwards, including high GBS acquisition rates in the last pregnancy-trimesters. There are differences in specific-serotype colonization patterns during pregnancy.

5.2 Introduction

Maternal vaginal colonization with Group B *Streptococcus* (GBS) is the major risk factor for early onset invasive GBS disease (EOD) in newborns (Verani et al., 2010, Chan SHS, 2000). Screening of pregnant women for GBS colonization during the third trimester, coupled with targeted intrapartum antibiotic prophylaxis (IAP) of colonized women during labor, has reduced the incidence of invasive GBS disease in industrialized countries(Schrag and Schuchat, 2004).

An alternate preventive strategy against EOD is vaccination of pregnant women, which could enhance transplacental transfer of GBS antibody to the fetus. Studies have identified an association between high maternal serotype-specific capsular polysaccharide (CPS) antibody concentrations with reduced risk of recto-vaginal colonization and reduced risk of newborns developing EOD (Baker and Kasper, 1976, Baker et al., 1981). Since GBS CPS-protein conjugate vaccines are serotype-specific, it is important to characterize the serotype distribution of GBS in different regions of the world as well as understand the changes which occur in GBS colonization during pregnancy (Ippolito et al., 2010). Other potential vaccine candidates include GBS surface protein antigens such as pilus island (PI) proteins that are present in all GBS isolates(Madzivhandila et al., 2013). Although it has

been shown that maternal GBS colonization during pregnancy may fluctuate (Hansen et al., 2004, Goodman et al., 1997, Mavenyengwa et al., 2010), there are limited longitudinal studies on the rate of serotype-specific GBS acquisition and duration of colonization during pregnancy.

We aimed to determine the acquisition and loss of GBS recto-vaginal colonization, including serotype-specific changes, among South African pregnant women from 20 weeks to at least 37 weeks of gestational age. We also studied the PI distribution of recto-vaginal colonizing GBS isolates and their association with capsular serotype.

5.3 Material and Methods

The detailed methods of the study have been described in Chapter 2. Briefly, pregnant HIV-uninfected women aged 18-45 years and who were 20-25 weeks gestational age were enrolled at antenatal community clinics in Soweto, Johannesburg from August 2010 to August 2011. Exclusion criteria included antibiotic treatment in the previous two weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examination was contraindicated. Study procedures included lower vaginal and rectal swabs collection for GBS culture starting at 20-25 weeks (visit-1), followed by three subsequent visits (visits 2-4) at 5-6 weekly intervals, up until 37-40 weeks (visit-4) of gestational age. For GBS isolation, swabs were inoculated onto CA. Serotyping was performed by the latex agglutination method as described (Afshar et al., 2011). Isolates that tested negative by latex agglutination for all serotypes were further typed by a PCR method using primer sequences described by Poyart *et al* (Poyart et al., 2007). Pilus island proteins of all GBS isolates were detected by PCR for PI-1, PI-2a and

PI-2b, with primers that target the genomic regions coding for the ancillary protein (AP)-1 of each PI as described previously (Madzivhandila et al., 2013).

5.3.1 Statistical analysis

Data were analyzed using SAS version 9.2 software (SAS Institute, Inc., NC, USA). A visit sample pair of vaginal and rectal swabs was considered negative if no GBS growth was evident on either swab, and positive if GBS was grown from either swab. The pregnant women were grouped into transient, intermittent and persistent carriers according to the presence of GBS colonization and to individual serotypes at the four sampling time points. Transient carriers were defined as women who were colonized at only one of the four visits, intermittent carriers as those who were colonized at two or three of the visits and persistent carriers as those colonized at all four study visits.

Descriptive statistics included the prevalence of colonization at individual time points and changes of recto-vaginal colonization status. Analysis of the changes in recto-vaginal colonization over time was restricted to the 507 participants who completed all four study visits. New acquisition of GBS was defined as positive culture of a new serotype which was not previously present. The new acquisition rate was defined as the number of new serotype acquisitions divided by the number of participants who were at a risk of acquiring the new serotype. Thus, women who were already previously colonized by a particular serotype were excluded subsequently from the denominator for estimating acquisition rate for the homotypic serotype. The rate of new acquisitions by all GBS serotypes were calculated from the sum of acquisition rates for the individual serotypes,

and by using the above methods for GBS acquisition rates in a serotype independent manner. Clearance of colonization was defined as a negative GBS culture for a specific serotype following a positive sample at the previous visit for the homotypic serotype. The rate of colonization clearance was defined as the number of GBS-negative participants at the analyzed time point divided by the number of participants at the previous visit who were positive for that serotype, and was also calculated in a serotype independent manner.

Survival analysis methods were used to estimate the duration of colonization of specific serotypes. A colonizing event was defined as the period of time between acquisition and clearance of a GBS serotype. Date of acquisition was calculated as the midpoint between the last visit without serotype-specific colonization and the first visit at which a positive sample was obtained for the homotypic serotype, while date for termination of serotype-specific colonization was calculated as the midpoint between the last visit with colonization and the subsequent negative visit for that serotype. In this analysis, if colonization occurred at the first visit, this was taken as the start of colonization, and if colonization occurred at the last visit, a right censoring approach was applied. We used the Kaplan-Meier method to estimate the duration of GBS colonization. The log-rank test was used to examine differences in duration of carriage between serotypes.

Positive predictive value (PPV) and negative predictive value (NPV) were calculated for the culture results at different sampling points with the 37-40 week visit as the reference standard. For participants who were colonized with same serotype on multiple visits, only one serotype specific isolate was used to study PI association with capsular serotype. The chi-square test was used to compare proportions. Logistic regression analysis was used to

determine the association between GBS colonization and demographic characteristics at enrolment. A p-value of < 0.05 was considered significant.

5.4 Results

5.4.1 Demographic characteristics

Of the 661 enrolled participants, 621 (93.9%), 595 (90.0%) and 521(78.8%) completed visits 2, 3 and 4, respectively. Five-hundred and seven (76.7%) women completed all four study visits. A detailed trial profile is indicated in Figure 5.1. The main reason for women not attending all four visits was birth of the baby (13%; 86/661) before the final visit. The demographic characteristics are displayed in Table 5.1. The mean age of the participants at enrolment was 25.9 (standard deviation; S.D±5.6) years. Only 5(0.76%) pregnant women have taken antibiotic treatment during the study.

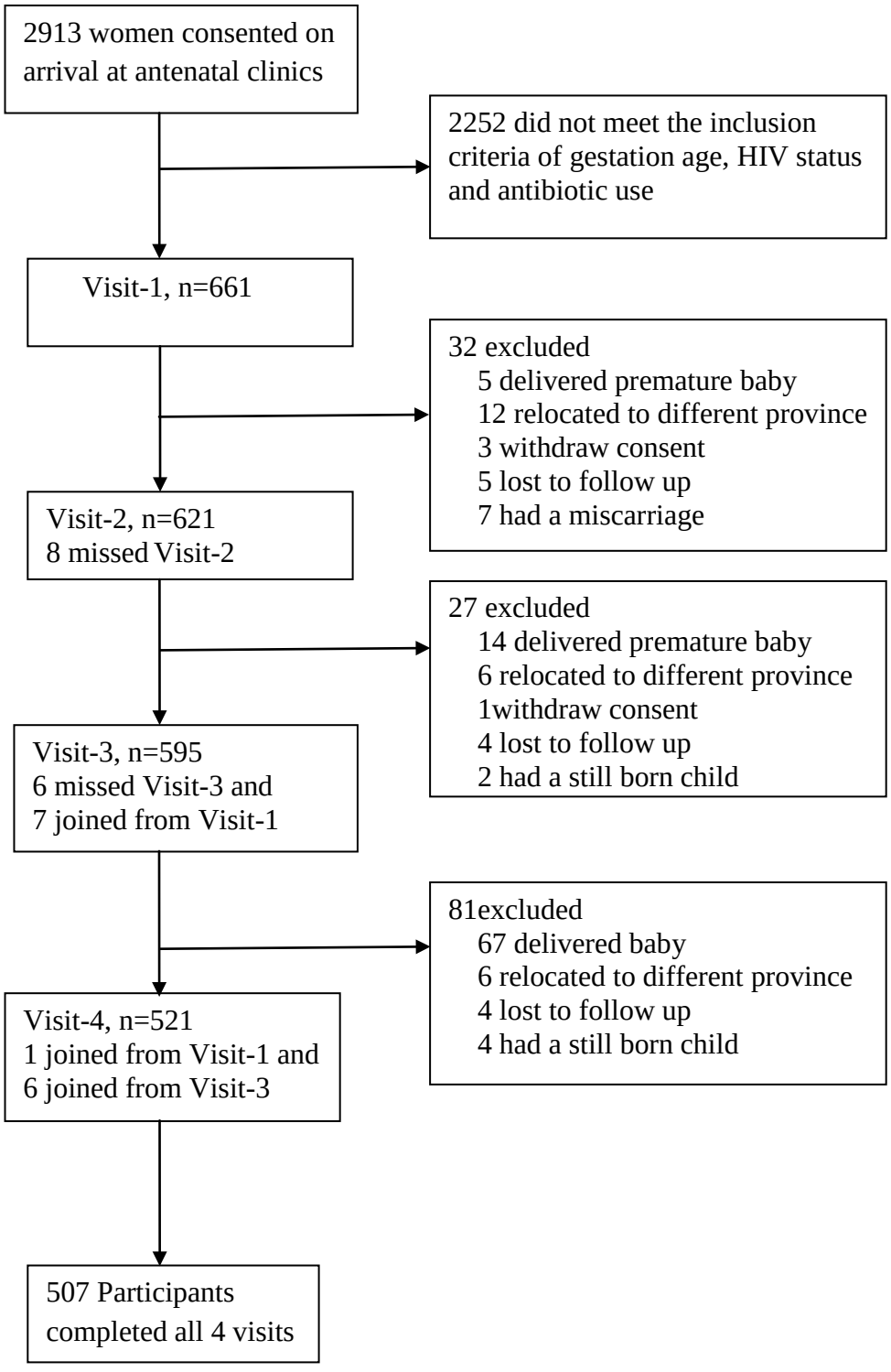


Figure 5.1: Trial Profile

Table 5. 1: Demographics of the study population at time of enrolment (n=661)

Demographic characteristic		Overall (n=661)	GBS Colonized	GBS Uncolonized
Age(years)	<20	92 (13.9%)[a]	27 (29.3%)[b]	65 (70.7%)[c]
Mean age:25.9(S.D±5.6)	20-24	234 (35.4%)	78 (33.3%)	156 (66.6%)
	25-28	161 (24.4%)	53 (32.9%)	108 (67.1%)
	29-32	95 (14.4%)	29 (30.5%)	66 (69.5%)
	33-35	40 (6.1%)	20 (50.0%)	20 (50.0%)
	36+	39 (5.9%)	11 (28.2%)	28 (71.8%)
Parity	0	338 (51.1%)	97 (28.7%)	241 (71.3%)
Median parity:0 (range; 0-5)	1-2	304 (46.0%)	113 (37.2%)	191 (62.8%)
	3-5	19 (2.9%)	8 (42.1%)	11 (57.9%)
Gravidity	1	286 (43.3%)	80 (28.0%)	206 (72.0%)
Median gravidity:2 (range; 1-8)	2	221(33.4%)	80 (36.2%)	141 (63.8%)
	3	106 (16.0%)	38 (35.8%)	68 (64.2%)
	≥ 4	48 (7.3%)	20 (41.7%)	28(58.3%)
Previous Abortion (spontaneous)	0	553 (83.7%)	176 (31.8%)	377 (68.2%)
Median abortion:0 (range; 0-3)	1	88 (13.3%)	36 (40.9%)	52 (59.1%)
	2	19 (2.9%)	6 (31.6%)	13 (68.4%)
	3	1 (0.2%)	0 (0.0%)	1 (100%)
Stillborn	0	651 (98.5%)	214 (32.9%)	437 (67.1%)
Median stillbirths:0 (range; 0-1)	1	10 (1.5%)	4 (40.0%)	6 (60.0%)

[a] Data are no (%) of total participants, [b,c] Data are row %

5.4.2 Prevalence of GBS colonization

The overall prevalence of recto-vaginal GBS colonization was 33.0% (218), 32.7% (203), 28.7% (171) and 28.4% (148) at 20-25 weeks, 26-30 weeks, 31-35 weeks and 37+ weeks of gestational age, respectively. The lower prevalence of colonization associated with 31-35 weeks and 37+ weeks compared to 20-25 weeks and 26-30 weeks was specifically associated with a decrease in prevalence of vaginal colonization, table 5.2 (23.3% to 19.0%). In the 86 women who gave birth before the final visit, vaginal GBS colonization was detected in 17(19.8%) at the last attended visit compared to 99/521 (19.0%) who gave birth after visit-4 (p=0.867). The inclusion of rectal swab GBS-culture increased the overall detection of GBS colonization by approximately 10% across the four study time-points (p<0.0001) and the prevalence of rectal colonization remained similar at each study time-point.

Table 5.2: Prevalence of Group B *Streptococcus* colonization during the study visits

Site of colonization	Visit-1 (20-25 weeks) n=661 Mean gestation age: 22.7 weeks	Visit-2 (26-30 weeks) n=621 Mean gestation age: 27.9 weeks	Visit-3 (31-35 weeks) n=595 Mean gestation age: 32.5 weeks	Visit-4 37+ weeks) n=521 Mean gestation age: 37.5 weeks
Vaginal only (%; 95% CI)	62 (9.4%; 7.2-11.6)	65 (10.5%; 8.1-12.9)	47 (7.9%; 5.7-10.1)	31 (5.9%; 3.9-7.9)
Rectal only (%; 95% CI)	64 (9.7%; 7.5-12.0)	62 (10%; 7.6-12.4)	56 (9.4%; 7.1-12.0)	49 (9.4%; 6.9-11.9)
Both vaginal and rectal (%; 95% CI)	92 (13.9%; 11.3-16.5)	76 (12.2%; 9.63-14.8)	68 (11.4%; 8.9-14.0)	68 (13.1%; 10.2-16.0)
Vaginal and/or rectal (%; 95% CI)	218 (33.0%; 29.4-36.6)	203 (32.7%; 29.0-36.4)	171 (28.7%; 25.1-32.3)	148 (28.4%; 24.5-32.3)

CI- Confidence interval, n=number of participants

Of several demographic characteristics evaluated at enrolment independently by univariate analysis, parity (OR: 1.22; 95% CI: 1.02-1.47; p=0.030) and gravidity (OR: 1.17; 95% CI: 1.01-1.36; p=0.046), were positively associated with GBS recto-vaginal colonization, with the highest colonization prevalence among women with parity ≥3 (42.1%) and gravidity of ≥4 (41.7%), (Table 5.3). In a multivariate analysis, none of the demographic characteristics were associated with GBS recto-vaginal colonization, Table 5.3. In a serotype-specific univariate analysis at enrolment, multiparity was associated with a higher prevalence of serotype III colonization (OR: 1.37; 95% CI: 1.07-1.76; p=0.012), gravidity also showed possible association with serotype III colonization (p=0.068). In the multivariate analysis, higher parity was found to be positively associated with serotype III colonization (Adjusted OR: 6.69; 95% CI: 1.47-30.4; P=0.014). Gravidity (p=0.053) and abortions (p=0.057) also showed a possible association with serotype III colonization at enrolment, Table 5.3. None of the demographic characteristics were found to be associated with serotype Ia colonization in the univariate or multivariate analysis. There were no identifiable factors associated with a higher prevalence of colonization with GBS at visit-4 alone.

Table 5.3: Univariate and multivariate association between serotype-specific colonization at enrolment and observed demographic characteristics

Characteristic	Overall GBS colonization at enrolment		Serotype III colonization at enrolment		Serotype Ia colonization at enrolment	
	Univariate OR (95% CI), p	Multivariate AOR (95% CI), p	Univariate OR (95% CI), p	Multivariate AOR (95% CI), p	Univariate OR (95% CI), p	Multivariate AOR (95% CI), p
Age	1.01(0.98-1.04), 0.408	0.98 (0.95-1.02), 0.536	1.01 (0.97-1.06), 0.527	0.98 (0.92-1.03), 0.45	1.00 (0.96-1.04), 0.96	0.99 (0.94-1.04), 0.656
Parity	1.22 (1.02-1.47), 0.03	1.99 (0.83-4.79), 0.121	1.37 (1.07-1.76), 0.012	6.69 (1.47-30.4), 0.014	1.05 (0.82-1.34), 0.69	0.71(0.27-1.90), 0.499
Gravidity	1.17 (1.01-1.36), 0.046	0.63 (0.27-1.50), 0.296	1.22 (0.99-1.51), 0.068	0.22 (0.05-1.02), 0.053	1.11 (0.91-1.35), 0.317	1.48 (0.57-3.81), 0.422
Abortion	1.18 (0.84-1.64), 0.341	1.77 (0.73-4.30), 0.207	1.10 (0.66-1.81), 0.721	4.14 (0.96-18.0), 0.057	1.25 (0.82-1.91), 0.298	0.86 (0.31-2.38), 0.774
Stillborn	1.36 (0.38-4.88), 0.635	1.25 (0.32-4.94), 0.741	0.94 (0.12-7.51), 0.951	0.76 (0.08-7.30), 0.813	2.60 (0.66-10.3), 0.171	2.14 (0.49- 9.29), 0.309

OR: odds ratio; AOR: adjusted OR; CI: confidence interval

5.4.3 Serotype and pilus island distribution

The proportional representation of serotypes remained consistent at each of the consecutive sampling time-points. Of women colonized, the proportional representation of the major serotypes were 36.2% to 41.4% for Ia, 31.3% to 34.9% for III, 10.3% to 15.6% for V, 7.2% to 7.5% for II, 3.5% to 4.6% for Ib, 2.0% to 4.0% for IV and 0.0% to 3.3% for IX (Table 5.4). The concordance of serotypes for GBS cultured concurrently from vaginal and rectal swabs was 91.3%, 89.5%, 94.1% and 94.1% for the four consecutive visits, respectively. Only 1.6% of GBS isolates were serologically non-typeable by latex agglutination and were serotyped by PCR.

All GBS isolates harbored one or more PIs, either PI-2a on its own or with a combination of PI-2a or PI-2b in combination with PI-1.The most common PI arrangement was PI-2a on its own, which occurred in 103/227 (45.4%), 92/211 (43.6%), 79/175 (45.1%) and 63/152 (41.5%) of isolates at visits 1-4, respectively, followed by PI combination PI-2b and PI-1, which occurred in 75/227(33.0%), 69/211(32.7%), 68/175 (38.9%) and 58/152 (38.2%) of isolates at visits 1-4, respectively. The least common PI arrangement was a combination of PI-2a and PI-1 which occurred in 49/227 (21.6%), 50/211 (23.7%), 29/175 (16.0%) and 31/152 (20.4%) of isolates at visits 1-4, respectively. There were no significant changes in the prevalence of PI distribution with respect to different visits, with the exception of PI-2a which was less common at visit-4 (18.0%, 94/521) compared to visit-1 (23.0%, 152/661; p=0.007), and which was attributable to a lower prevalence of serotype Ia at visit-4 (10.6%, 55/521) compared to visit-1 (14.2%, 94/661).

There was a strong correlation between the presence of particular combinations of PI and the serotype; Figure 5.2. Most serotype Ia isolates were associated with PI-2a (94.9%; 148/156), whereas the majority of serotype III isolates were associated with the combination of PI-1 and PI-2b (88.2%; 105/119). The association between PIs and serotype V was more variable, with a PI-1+PI-2a combination occurring in 64.7% (33/51) and PI-2a alone occurring in 29.4% (15/51) of isolates.

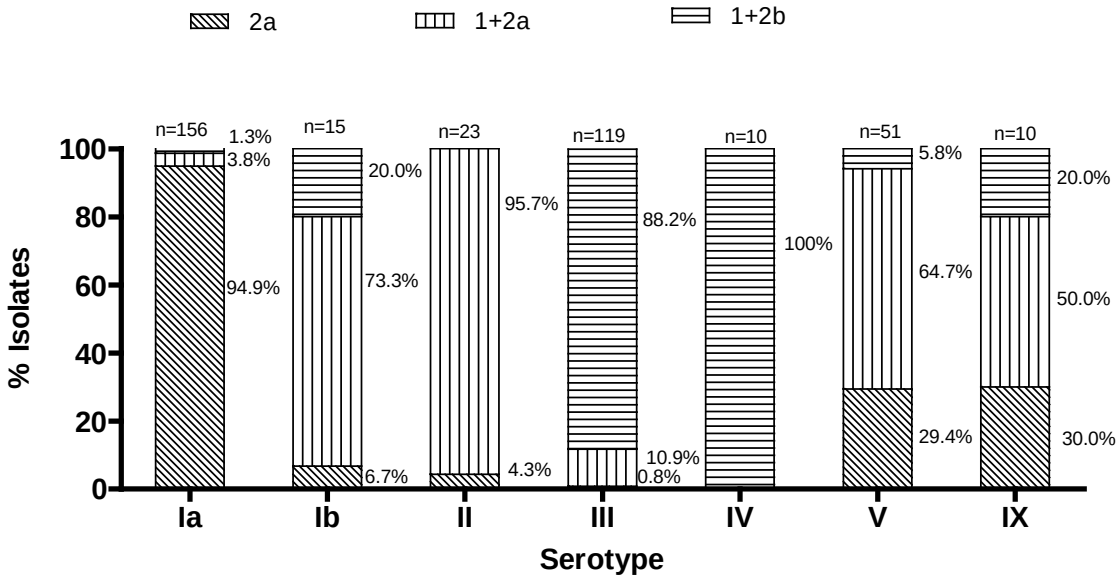


Figure 5.2: Association of pilus island proteins and serotypes among Group B *Streptococcus* isolates

Table 5.4: Distribution of serotype associated with GBS recto-vaginal colonization

Serotype	Site	Number of isolates per visit [a]			
		Visit-1	Visit-2	Visit-3	Visit-4
		n=227	n=211	n=175	n=152
Ia	Vaginal only	36 (15.9%)	31 (14.7%)	29 (16.6%)	19 (12.5%)
	Rectal only	29 (12.8%)	26 (12.3%)	18 (10.3%)	18 (11.8%)
	Rectal and vaginal	29 (12.8%)	23 (10.9%)	24 (13.7%)	18 (11.8%)
	Overall	94 (41.4%)	80 (37.9%)	71 (40.6%)	55 (36.2%)
Ib	Vaginal only	1 (0.4%)	0 (0.0%)	2 (1.1%)	1 (0.7%)
	Rectal only	4 (1.8%)	4 (1.9%)	0 (0.0%)	3 (2.0%)
	Rectal and vaginal	3 (1.3%)	2 (0.9%)	2 (1.1%)	3 (2.0%)
	Overall	8 (3.5%)	6 (2.8%)	4 (2.3%)	7 (4.6%)
II	Vaginal only	6 (2.6%)	5 (2.4%)	3 (1.7%)	1 (0.7%)
	Rectal only	4 (1.8%)	4 (1.9%)	6 (3.4%)	6 (3.9%)
	Rectal and vaginal	7 (3.1%)	4 (1.9%)	4 (2.3%)	4 (2.6%)
	Overall	17 (7.5%)	13 (6.2%)	13 (7.4%)	11 (7.2%)
III	Vaginal only	17 (7.5%)	19 (9.0%)	12 (6.9%)	11 (7.2%)
	Rectal only	21 (9.3%)	24 (11.4%)	24 (13.7%)	17 (11.2%)
	Rectal and vaginal	33 (14.5%)	23 (10.9%)	25 (14.3%)	25 (16.4%)
	Overall	71 (31.3%)	66 (31.3%)	61 (34.9%)	53 (34.9%)
IV	Vaginal only	3 (1.3%)	2 (0.9%)	1 (0.6%)	0 (0.0%)
	Rectal only	3 (1.3%)	2 (0.9%)	3 (1.7%)	2 (1.3%)
	Rectal and vaginal	3 (1.3%)	3 (1.4%)	1 (0.6%)	1 (0.7%)
	Overall	9 (4.0%)	7 (3.3%)	5 (2.9%)	3 (2.0%)
V	Vaginal only	7 (3.1%)	11 (5.2%)	3 (1.7%)	4 (2.6%)
	Rectal only	10 (4.4%)	9 (4.3%)	7 (4.0%)	5 (3.3%)
	Rectal and vaginal	9 (4.0%)	13 (6.2%)	8 (4.6%)	9 (5.9%)
	Overall	26 (11.5%)	33 (15.6%)	18 (10.3%)	18 (11.8%)
IX	Vaginal only	1 (0.4%)	4 (1.9%)	0 (0.0%)	0 (0.0%)
	Rectal only	1 (0.4%)	1 (0.5%)	1 (0.6%)	0 (0.0%)
	Rectal and vaginal	0 (0.0%)	1 (0.5%)	2 (1.1%)	5 (3.3%)
	Overall	2 (0.9%)	6 (2.8%)	3 (1.7%)	5 (3.3%)

[a]Percent serotype distribution was calculated using the total number of isolates at each visit as the denominator

5.4.4 Changes in GBS colonization overtime

Five hundred and seven participants who completed all four study visits were similar in their demographic characteristics compared to the 154 participants not included in this analysis. In the analyzed subset, the prevalence of recto-vaginal GBS colonization

was 32.1% (163), 30.4% (154) , 29.0% (147) and 27.8% (141) at 20-25 weeks, 26-30 weeks, 31-35 weeks and 37 + weeks of gestational age, respectively. Two hundred and fifty-two (49.7%) women were colonized at least once during the study period, of whom 70 (27.8%) were persistent carriers, 83 (32.9%) transient carriers and 99 (39.3%) were intermittently colonized for any serotype (Table 5.5)

The cumulative serotype-specific prevalence across the study period was 23.7% (120/507) for Ia, 18.3% (93/507) for III, 7.1% (36/507) for V, 4.3% (22/507) for II and 2.8% (14/507) for Ib. All GBS serotypes were variable in their colonization patterns. Comparing the three most common colonizing serotype carriers, 29% (27/93) of serotype III carriers were associated with persistent colonization compared to serotype Ia (18%; 21/120; p=0.045) or V (6%; 2/36; p=0.002). Serotype V was the most dynamic, with 94.4% (34/36) of colonized women either being transient or intermittent carriers compared to 82.5% (99/120; p=0.106) for Ia and 71.0% (66/93; p=0.004) for III. Only one serotype was detected in 83.3% (210/252) of GBS carriers during the study period, with 85.7% (60/70) of women persistently colonized being associated with the same serotype.

Of the 16.7% (42/252) women in whom multiple serotypes were detected over the study period, two serotypes were detected in 9.5% (24/252) and three serotypes in 7.1% (18/252) participants. Among women in whom multiple serotypes were detected, a new serotype was observed at the immediate next visit in 85.7% (36/42) of cases, while a new serotype was detected following a period of no colonization by the preceding serotype in six women.

Table 5.5: Patterns of GBS recto-vaginal colonization overall, and by individual serotypes (n=507)

Code*	Any Serotype	Serotypes						
		Ia	Ib	II	III	IV	V	IX
-,-,-,-	255(50.3%)	387(76.3%)	493(97.2%)	485(95.7%)	414(81.7%)	500(98.6%)	471(92.9%)	500(98.6%)
-,+,-,-	14(2.8%)	10(2.0%)	0(0.0%)	2(0.4%0	7(1.4%)	0(0.0%)	5(1.0%)	3(0.6%)
-,-,+,-	13(2.6%)	11(2.2%)	1(0.2%)	1(0.2%)	9(1.8%)	0(0.0%)	2(0.4%)	0(0.0%)
-,-,-,+	27(5.3%)	12(2.4%)	3(0.6%)	2(0.4%)	10(2%)	0(0.0%)	7(1.4%)	1(0.2%)
-,+,+,-	8(1.6%)	4(0.8%)	0(0.0%)	0(0.0%)	2(0.4%)	1(0.2%)	0(0.0%)	0(0.0%)
-,+,+,+	14(2.8%)	6(1.2%)	2(0.4%)	2(0.4%)	1(0.2%)	0(0.0%)	5(1.0%)	1(0.2%)
-,-,+,+	9(1.8%)	4(0.8%)	0(0.0%)	2(0.4%)	6(1.2%)	0(0.0%)	1(0.2%)	0(0.0%)
-,+,-,+	4(0.8%)	2(0.4%)	0(0.0%)	0(0.0%)	2(0.4%)	0(0.0%)	1(0.2%)	0(0.0%)
+,-,-,-	29(5.7%)	23(4.5%)	4(0.8%)	3(0.6%)	13(2.6%)	2(0.4%)	4(0.8%)	0(0.0%)
+,+,-,-	20(3.9%)	10(2.0%)	2(0.4%)	3(0.6%)	4(0.8%)	1(0.2%)	3(0.6%)	0(0.0%)
+,+,+,-	17(3.4%)	7(1.4%)	0(0.0%)	0(0.0%)	6(1.2%)	0(0.0%)	3(0.6%)	0(0.0%)
+,+,+,+	70(13.8%)	21(4.1%)	1(0.2%)	4(0.8%)	27(5.3%)	3(0.6%)	2(0.4%)	2(0.4%)
+,-,+,-	10(2.0%)	5(1.0%)	0(0.0%)	2(0.4%)	0(0.0%)	0(0.0%)	1(0.2%)	0(0.0%)
+,+,-,+	7(1.4%)	1(0.2%)	1(0.2%)	0(0.0%)	2(0.4%)	0(0.0%)	2(0.4%)	0(0.0%)
+,-,-,+	4(0.8%)	2(0.4%0	0(0.0%)	0(0.0%)	2(0.4%)	0(0.0%)	0(0.0%)	0(0.0%)
+,-,+,+	6(1.2%)	2(0.4%)	0(0.0%)	1(0.2%)	2(0.4%)	0(0.0%)	0(0.0%)	0(0.0%)

Code*: visit-1,visit-2,visit-3,visit-4

Groups: Noncarrier: (-,-,-,-),

Transient carrier: (-,+,-,-) (-,-,+,-) (-,-,-,+) (+,-,-,-)

Intermitent carrier: (-,+,+,-) (-,+,+,+) (-,-,+,+) (-,+,-,+) (+,+,-,-) (+,+,+,-) (+,-,+,-) (+,+,-,+) (+,-,-,+) (+,-,+,+)

Persistent carrier: (+,+,+,+)

"-" Negative for GBS," +"Positive for GBS

5.4.5 New acquisition and clearance of colonization

Three hundred and forty-four participants who completed all four study visits were not colonized at visit-1, of whom 89 (25.9%) became colonized at one of the subsequent three visits. When including new serotype acquisition in those previously colonized by a heterotypic serotype (n=39), the cumulative overall recto-vaginal acquisition rate of new serotypes during the study, calculated from the sum of acquisition rates for the individual serotypes was 27.9%. The number of new acquisitions was highest for serotypes Ia (11.2%, 49/436), III (8.2%, 37/451) and V (4.3%, 21/492); Table 5.5. The mean new acquisition rate of GBS was 11.4% (S.D ±0.5%) at 5-6 week visit intervals, including 11.6% between visit-1 and visit-2, and 10.8% and 11.7% in the intervals of subsequent consecutive visits. Of 163 participants who were colonized at visit-1, 76(46.6%) were no longer colonized by visit-4. The rate of colonization-clearance was 75% (6/8) for serotype Ib, 73.3% (11/15) for V and 63.4% (45/71) for Ia. The overall clearance of any GBS colonization was 30.1% (49/163), 29.2% (45/154) and 32.7% (48/147) between visits-1 and -2, visits-2 and -3, and visits-3 and -4, respectively. No demographic characteristics were identified that were associated with either new acquisition or clearance of colonization.

5.4.6 Duration of GBS colonization

The median duration of recto-vaginal GBS colonization was 6.35 weeks for serotype III, which tended to be longer than other serotypes, including serotype Ia (median: 5.21 weeks; p=0.02; Table 5.6) which was the second most common colonizing serotype. The difference in duration of colonization between serotype III and less prevalent serotypes was generally longer, but not statistically significant.

Table 5.6: Estimated duration of Group B *Streptococcus* recto-vaginal colonization

Serotype	Colonization duration (weeks) *		
	Mean (95% CI)	Median	p-value†
Ia	7.52(6.6-8.4)	5.21	0.026
Ib	5.22(4.03-6.42)	3.62	0.358
II	6.11(5.07-7.16)	4.24	0.736
III	9.15(8.1-10.2)	6.35	Reference
IV	6.94(3.8-10.0)	4.81	0.998
V	8.60 (6.80-10.39)	5.96	0.332
IX	6.21(4.7-7.7)	4.31	0.651

* Time from enrolment, CI- Confidence interval, † compared to serotype III

5.4.7 Predictive values for each visit culture with respect culture status at visit-4

Positive and negative predictive values of serotype-specific culture at 20-25 weeks, 26-30 weeks and 31-35 weeks of gestational age compared to 37 + weeks colonization status are presented in Table 5.7. The overall positive predictive values were 53.4%, 61.7% and 67.4% for GBS-positive cultures at 20-25 weeks, 26-30 weeks and 31-35 weeks, respectively, relative to positivity at 37+ weeks, while the negative predictive values for 20-25 weeks, 26-30 weeks and 31-35 weeks ranged from 84.3% to 88.3%. Serotypes Ia and V had lower PPVs compared with serotype III at each time-point. The observed PPVs at 31-35 weeks, were 55-70% for the three commonest serotype.

Table 5.7: Predictive value for 20-25, 26-30 and 31-35 weeks cultures in relation to culture status at 37+ weeks

Serotype	20-25 weeks		26-30 weeks		31-35 weeks	
	PPV % (95% CI)	NPV % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Overall GBS	53.4(45.4-61.2)	84.3(80.0-88.0)	61.7(53.5-69.4)	87.0(83.0-90.3)	67.4(59.1-74.9)	88.3(84.6-91.5)
Ia	36.6(25.5-48.9)	94.5(91.9-96.4)	49.2(36.1-62.3)	95.5(93.2-97.2)	55.0(41.7-67.9)	96.2(94.0-97.8)
Ib	25.0(3.9-65.0)	99.0(97.7-99.7)	66.7(22.7-94.7)	99.4(98.3-99.9)	75.0(20.3-95.9)	99.2(98.0-99.8)
II	38.5(14.0-68.4)	98.8(97.4-99.6)	54.6(23.5-83.1)	99.0(97.7-99.7)	75.0(42.8-94.2)	99.6(98.5-99.9)
III	58.9(45.0-71.9)	95.8(93.5-97.4)	62.8(48.1-75.9)	95.6(93.3-97.3)	67.9(53.7-80.1)	96.5(94.3-98.0)
IV	50.0(12.4-87.6)	100(99.2-100)	60.0(15.4-93.5)	100(99.2-100)	75.0(20.3-95.9)	100(99.3-100)
V	26.7(8.0-55.1)	97.2(95.3-98.4)	47.6(25.8-70.2)	98.4(96.8-99.3)	57.1(28.9-82.2)	98.0(96.3-99.0)
IX	100(19.3-100)	99.6(98.6-99.9)	50.0(12.4-87.6)	99.8(98.9-100)	100(30.5-100)	99.8(98.9-100)

PPV: Positive predictive value, NPV: Negative predictive value, CI- Confidence interval

5.5 Discussion

To our knowledge this is the first serotype-specific longitudinal study conducted of recto-vaginal GBS colonization in pregnant women, in whom we demonstrated a high prevalence and acquisition rate of GBS recto-vaginal colonization. The overall rate of new acquisition at 5-6 week interval is in agreement with a previous study of non-pregnant women, although, the serotype-specific rates differed (Foxman et al., 2006). All GBS serotypes were variable in their colonization patterns, possibly due to the complex interaction between immunity and specific GBS serotypes, which is still incompletely understood. It may be that the higher frequency of persistent colonization and longer overall duration of colonization by serotype III, is related to a weaker natural immune response associated with its colonization compared to other serotypes (Davies et al., 2001). Consequently, there is a higher risk of exposure at birth to serotype III in our population, which corroborates with it being responsible for 49.2% to 57.7% of EOD in our setting (Madzivhandila et al., 2011, Madhi et al., 2003). The higher acquisition rate of serotype Ia may result in there being inadequate time for natural immunity to this serotype developing in the pregnant woman, which consequently increases the newborns' risk of developing EOD from serotype Ia, which is associated with 22.6% to 31% of EOD cases in our setting (Madhi et al., 2003, Madzivhandila et al., 2011).

The high incidence of new acquisition and loss of colonization during pregnancy highlights why screening is required as late as 35-37 weeks' gestational age for the IAP strategy to be effective, which is concordant with another study in pregnant women(Manning et al., 2008). In our study, if women had been screened at 31-35 weeks, 29.1% (42/141) of those who were colonized at 37+ would not have had IAP offered to

them and a lesser proportion (13.3%; 48/366) may have unnecessarily received IAP as they were no longer colonized at 37+ weeks. Although we did not identify any demographic characteristics associated with new acquisition or clearance of GBS, additional risk factors such as sexual activity during pregnancy were not fully explored (Meyn et al., 2002). The PPV of GBS cultures obtained from 20-35 weeks varied in serotype distribution compared to that at 37+ weeks. The prevalence of different GBS serotypes in a particular population can affect the PPV of late antenatal GBS cultures.

The high cumulative prevalence of GBS colonization (49.7%) found in our study is comparable to longitudinal studies (which were not serotype specific) from Denmark and Zimbabwe (Hansen et al., 2004, Mavenyengwa et al., 2010). Furthermore, the prevalence of colonization observed by us at 37+ weeks of gestational age was 28.4% (148/521), which was similar to that reported in cross-sectional studies from Europe (Motlova et al., 2004) and USA (Campbell et al., 2000). The prevalence of GBS colonization from African countries ranges from 16.5% in Malawi, 21-23% in The Gambia, Ethiopia and Tanzania and 31.6% in Zimbabwe (Suara et al., 1994, Joachim et al., 2009, Musa Mohammed, 2012, Dzowela T, 2005, Moyo et al., 2000). Our results also showed a decrease in the prevalence of GBS colonization with respect to increase in gestational age. This finding agrees with studies from the USA and Australia (Goodman et al., 1997, Gilbert et al., 2002), but contrasts with others that reported an increase in colonization with increasing gestational age (Zamzami et al., 2011, Baker et al., 1975).

Our findings on the dominant serotypes are comparable with serotype distribution data of maternal colonizing isolates from high-income countries, including 13% to 35% for

serotype Ia and 15% to 44% for serotype III (Ippolito et al., 2010). The identification of serotype IX in our study was notable in that it is rarely reported in colonizing studies and not previously described in Africa. To our knowledge, only 8 GBS colonizing isolates have been identified as serotype IX, including three from Denmark, two from Germany and one each from Canada, Hong Kong and Australia (Slotved et al., 2007). Our data on PI distribution is comparable to earlier published studies (Madzivhandila et al., 2013, Margarit et al., 2009) showing that all GBS isolates carried at least one PI, and were associated with the presence of either PI-2a or PI-2b identified alone or in combination with PI-1.

Our study is limited by the sensitivity of detection of GBS on selective media which is estimated at 85% (Chapter 4) and by the fact that in most cases only the dominant serotype was determined. This can lead to an underestimation of persistent colonization, an overestimation of new acquisitions and an underestimation of the duration of carriage.

Recent developments in the clinical evaluation of a tri-valent GBS polysaccharide-protein conjugate vaccine has renewed interest in the potential of this vaccine to protect neonates against invasive GBS disease by reducing recto-vaginal colonization during pregnancy (Edwards and Gonik, 2013). The findings of this study will be important in considering study design when evaluating the efficacy of maternal GBS vaccination protecting against GBS recto-vaginal acquisition and colonization during pregnancy as surrogate information on clinical vaccine efficacy may be gained by determining the immune responses that correlate with protection against serotype-specific GBS acquisition and colonization during pregnancy.

Chapter 6 Natural acquired humoral immunity against serotype-specific Group B *Streptococcus* recto-vaginal colonization acquisition in pregnant women.

6.1 Abstract

Background: Group B *Streptococcus* (GBS) recto-vaginal colonization in pregnant women is associated with invasive GBS disease in newborns, preterm delivery and stillbirths. We studied the association of GBS serotype-specific capsular polysaccharide (CPS) antibody on new acquisition and clearance of recto-vaginal GBS colonization in pregnant women from 20 weeks until 37-40 weeks of gestational age.

Methods: Serum serotype-specific CPS IgG antibody concentration was measured by multiplex-ELISA and opsonophagocytic activity (OPA) titers. Recto-vaginal swabs were evaluated for GBS colonization, using standard culture methods and serotyping by latex agglutination, at 5-6 weekly intervals.

Results: Higher serotype III CPS antibody concentration was associated with lower risk of recto-vaginal acquisition of serotype III during pregnancy ($p=0.009$). Furthermore, serotype-specific OPA titers to Ia and III were higher in women who remained free of GBS colonization throughout the study compared to those who acquired the homotypic serotype ($p<0.001$ for both serotypes). Serum CPS IgG of ≥1 µg/ml for serotype V, ≥3 µg/ml for serotype Ia and III were significantly associated with protection against recto-vaginal acquisition of the homotypic serotype.

Conclusion: A GBS vaccine that induces sufficient capsular antibody in pregnant women, including high OPA titers, could protect against recto-vaginal colonization during the latter half of pregnancy.

6.2 Introduction

Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) is the major risk factor for GBS invasive disease in newborns' under seven days of age (early-onset disease; EOD) (Verani et al., 2010, Chan SHS, 2000). Vertical transmission of GBS occurs to approximately 50% of babies born to GBS-colonized pregnant women, of whom 1–3% develop EOD in the absence of intrapartum antibiotic prophylaxis (IAP) (Beal and Dancer, 2006, Heath and Feldman, 2005). Recto-vaginal GBS colonization of pregnant women has also been associated with preterm birth and stillbirths (Edwards and Gonik, 2013). Screening for GBS recto-vaginal colonization in pregnant women at 35-37 weeks of gestational age, coupled with IAP during labor, has reduced the incidence of EOD in high-income settings(Schrag and Schuchat, 2004). Implementation of IAP would, however, be costly and logistically challenging for most resource-constrained countries (Colbourn et al., 2007, Schrag, 2011).

An alternate preventive strategy against invasive GBS disease in newborns' and young infants, could be vaccination of pregnant women with a GBS vaccine to induce humoral immunity, with protective antibodies transplacentally transferred to the fetus (Robbins et al., 1995). An association between low maternal serotype-specific capsular antibody concentration and increased risk of invasive GBS disease among newborns has been observed (Baker et al., 1981, Baker et al., 2013, Lin et al., 2004). Furthermore, should

GBS vaccination prior or during early stages of pregnancy prevent or reduce subsequent recto-vaginal colonization in women, this could lower fetal/newborn exposure to GBS and contribute to reducing EOD among term and preterm births; and potentially also reduce GBS-associated LOD, premature labor and stillbirths.

Although spontaneous clearance and acquisition of GBS has been reported in pregnant women, the association of host immune mediators and GBS colonization during pregnancy remains unclear (Hansen et al., 2004, Goodman et al., 1997). A few predominantly cross sectional studies, reported higher serotype-specific capsular polysaccharide (CPS) antibody in colonized compared to non-colonized women (Campbell et al., 2000, Davies et al., 2001, Anthony et al., 1984, Matsubara et al., 2003, Matsubara et al., 2002, Skidmore et al., 1985, Baker et al., 1980, Beachler et al., 1979). We are, however, unaware of any longitudinal cohort study which evaluated the association between serotype-specific CPS antibody and subsequent risk of GBS recto-vaginal acquisition during the latter half of pregnancy.

The primary objective of our study was to evaluate the association between natural serotype-specific serum CPS IgG antibody and opsonophagocytic activity in relation to GBS recto-vaginal acquisition in pregnant women from 20 to 37+ weeks of gestational age. Furthermore, we evaluated the association between CPS antibody and clearance of GBS colonization.

6.3 Material and Methods

The detailed methods of the study have been described in Chapter 2. Briefly, pregnant HIV-uninfected women aged 18-45 years and who were 20-25 weeks gestational age were enrolled at antenatal community clinics in Soweto, Johannesburg from August 2010 to August 2011. Exclusion criteria included antibiotic treatment in the previous two weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examination was contraindicated. Study procedures included lower vaginal and rectal swabs collection for GBS culture starting at 20-25 weeks (visit-1), followed by three subsequent visits (visits 2-4) at 5-6 weekly intervals, up until 37-40 weeks (visit-4) of gestational age. For GBS isolation, swabs were inoculated onto CA. Serotyping was performed by the latex agglutination method as described (Afshar et al., 2011). Isolates that tested negative by latex agglutination for all serotypes were further typed by a PCR method using primer sequences described by Poyart *et al* (Poyart et al., 2007).

6.3.1 Measurement of serotype-specific anti-capsular serum IgG

Quantitative serum serotype-specific CPS IgG antibody concentrations were measured with multiplex Luminex platform and have been detailed in chapter 2. Briefly, blood was collected at enrolment and visit-4. Serum was separated by centrifugation and stored at −70°C until analyzed. All GBS CPS antigens were kindly provided by Novartis Vaccines, Italy, and were coupled to the microsphere beads (Biorad, CA, U.S.A) with the crosslinking agent 4-(4,6-dimethoxy[1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) as described (Schlottmann et al., 2006). An in-house reference serum composed of purified pooled human IgG (Polygam, National Bioproducts, South Africa)

was calibrated with the standard serotype-specific GBS reference serum kindly provided by Prof. Carol J. Baker. Assays were performed in true duplicate and each plate included high and low control sera. Bead fluorescence was read with the Bioplex 200 instrument (Bio-Rad, Texas, U.S.A) using Bio-Plex manager 5.0, software (Bio-Rad). In each assay, an uncoated control bead was included to determine non-specific binding. For control beads, the coupling procedure was followed, except that no GBS CPS was added. In case of non-specific binding, the fluorescence intensity (FI) values were subtracted from the antigen-specific results. The optimal serum and secondary antibody dilution was 1:100. The results for serum CPS IgG are given in µg per ml with lower detection limits of 0.0003, 0.005 and 0.009 for serotype Ia, III and V, respectively. For statistical analysis, samples with values below these limits were assigned values of half of lower limit of detection.

The multiplex assay was validated by comparing the serotype-specific mean FI values for Polygam obtained with the multiplex assay with those for each serotype run in singleplex. The FI values obtained for Polygam with the multiplex assay were between 79% and 112% (median 103%) of those obtained with the single-plex assays. To assess analytical specificity of each GBS–microsphere set, Polygam was incubated at 1:100 dilution with each GBS CPS (100µg/ml) and incubated at 37˚C for 2 hours. The specificity was recorded as the difference in reactivity between the absorbed and unabsorbed sera in a multiplex assay. Homologous inhibition was >95% for all serotypes, with the exception of serotype V (85%). Heterologous inhibition across serotypes was <10%, except for serotypes Ib and III, which were inhibited by 41% and 11% with type Ia CPS respectively, and serotype V (18%) was inhibited by serotype-III CPS.

6.3.2 Serum IgG opsonophagocytic activity (OPA) assay

The functional activity of serum IgG was determined by opsonophagocytic activity (OPA) assay for serotype Ia and III using the HL-60 cell line and has been detailed in chapter 2. Briefly, 1000 cfu (20 µl of $5x10^4$cfu/ml) of GBS type Ia strain(A909) or type III strain(COH-1) cells were mixed with an appropriate dilution of heat inactivated serum sample (10µl), then mixed and incubated for 15 min at 37°C in a 5% CO_2 incubator. Baby rabbit complement (10µl) and HL-60 cells (40 µl of $1x10^7$, cultured for 5 days in the presence of dimethyl formamide) were added to the mixture and incubated at 37°C with 5% CO_2 for 1 hour with gentle agitation. Aliquots were removed and plated on Todd-Hewitt agar plates for quantitative culture. Opsonophagocytic activity was expressed as the titer at which the serum dilution yielded 50% killing compared with the bacterial growth in the complement controls. The limit of detection was 8. For statistical analysis, samples below the detection were assigned an arbitrary titer of 4.

6.3.3 Study definitions

A participant was considered to be colonized at a visit if GBS was cultured on the vaginal and/or rectal swab, and non-colonized if no growth was detected from either swab. A new episode of serotype-specific GBS recto-vaginal colonization (i.e. “new-acquisition group”) was defined as a participant not colonized by a specific GBS serotype at visit-1, who was subsequently colonized by that specific-serotype (irrespective of the number of study-visits completed). Concurrent identification of different serotypes from the vaginal and rectal swabs at a visit among participants with new acquisitions were treated as individual events. Participants not colonized with a specific-serotype at any study visit were categorized as being the “non-colonized group” for that serotype; and used as the

comparator group when evaluating the association between CPS antibody and risk for serotype-specific recto-vaginal acquisition.

The association of CPS antibody with clearance of serotype-specific GBS colonization was evaluated in participants who were colonized by the specific-serotype at visit-1 and who completed all study visits. The participants were stratified into those who remained colonized throughout (i.e. “persistently-colonized group”) and those in whom colonization of specific-serotype was cleared by visit-4 (i.e. “intermittent-colonized group”). Due to limitations associated with detecting multiple serotype carriage using traditional culture methods (Ferrieri et al., 2004), participants with GBS serotype colonization at visit-1 and who acquired different GBS serotype at subsequent visits were excluded from the analysis on CPS antibody and serotype-specific GBS acquisition or clearance.

6.3.4 Statistical analysis

Differences in geometric mean concentration (GMC) of serotype-specific CPS antibody and geometric mean OPA titer (GMOPT) between groups (new-acquisition versus non-colonized groups; and persistently-colonized versus intermittent-colonized groups) were analyzed on log transformed data by Student’s t-test. GMC’s at visit-1 and visit-4 for each group were analyzed by paired Student’s t-test. Evaluation of whether the serotype-specific CPS antibody was associated with new homotypic GBS acquisition, was undertaken using a univariate logistic regression analysis reporting the Odds ratio (OR) for the association between the serological variable and GBS acquisition, with logarithmic IgG

or OPA titer as a covariate. Univariate logistic regression analysis was also used for comparison between groups by demographics and obstetrical factors at visit-1. For categorical variables, groups were compared with either chi-square test or Fisher's two tailed exact test as appropriate. Correlations between serum IgG and OPA titers were determined on $\log_{10}$ transformed data by Spearman rank correlation. Data was analyzed using SAS version 9.2 software (SAS Institute, Inc., NC, USA). A p-value of <0.05 was considered significant.

6.4 Results

Of 661 enrolled participants, 95.1% (n=629) completed at least two and 76.7% (n=507) all four study-visits. The main reasons for not completing all study visits were related to obstetric outcomes such as premature birth (n=86; 13.0%) and miscarriages (n=13; 2.0%; Figure 6.1). A detailed profile of subject follow-up, serotype distribution and dynamics of GBS colonization has been detailed in chapter 5.

The overall prevalence of any-serotype GBS colonization at enrolment was 33.0% (218/661). Of those not colonized by GBS serotypes Ia, III or V at enrolment, 9.6% (52/541), 7.0% (39/560) and 2.8% (17/603) subsequently became colonized by the respective serotypes (i.e. new-acquisition group). This included 6 participants who cultured different serotypes from the vaginal and rectal site at the same visit and excluded 15 participants who were colonized at visit-1 and acquired different serotypes at subsequent visit. Of 507 participants who completed all scheduled visits, participants not colonized with a specific-serotype at any visit were 387 (76.3%), 414(81.6%) and 471(92.9%) for serotypes Ia, III and V, respectively (i.e. non-colonized group). There were no differences

in the demographic characteristics between the new-acquisition and non-colonized groups (Table 6.1).

Of the 507 participants who completed all scheduled visits, the overall GBS colonization prevalence at enrolment was 32.1% (163/507), including 14.0% (n=71), 11.1% (n=56) and 2.9% (n=15) for serotypes Ia, III and V, respectively. The prevalence of serotypes Ia, III and V colonization among the persistently-colonized group were 4.1% (21/507), 5.3% (27/507) and 0.4% (2/507), respectively; and 8.5% (43/507), 3.6% (18/507) and 1.8% (9/507), respectively, in the intermittent-colonized group. This excluded 22 intermittently colonized participants in whom colonization was not cleared by visit-4 or acquired new serotype at subsequent visits and remained colonized with that serotype thereafter. There were no differences in the demographic characteristics between the persistently-colonized and intermittent-colonized groups.

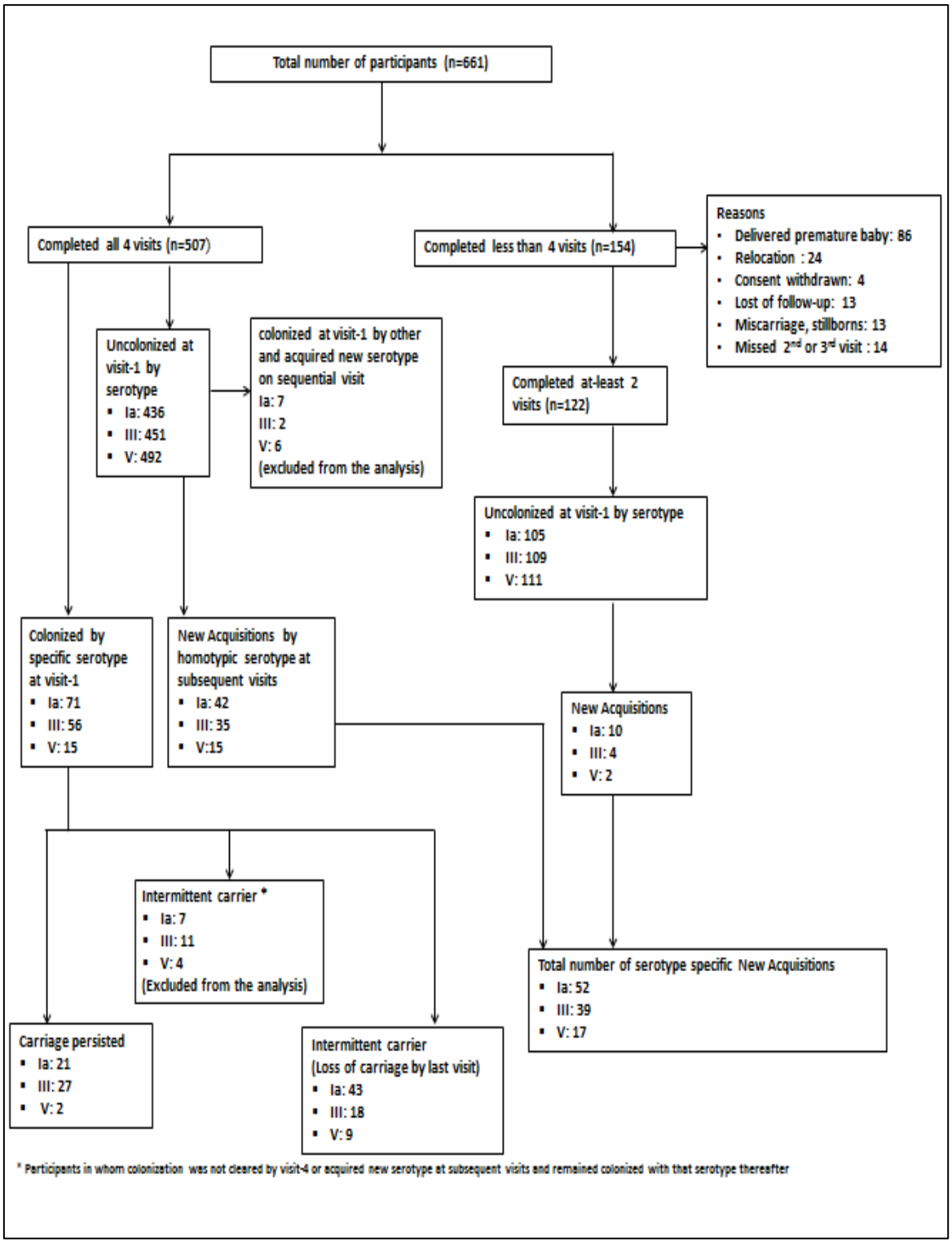


Figure 6.1: Selection of Groups

Table 6.1: Demographic characteristics new acquisition group compared to non-colonized group

Demographics	Serotype Ia			Serotype III			Serotype V		
	New-acquisition [a] n=52[d]	Non-colonized[b] n=387	p-value[c]	New-acquisition n=39	Non-colonized n=414	p-value	New-acquisition n=17	Non-colonized n=471	p-value
Age (years) Mean± SD[e]	25.65 ±5.76	26.06 ± 5.54	0.619	26.13 ± 6.2	26.10 ± 5.6	0.969	26.01 ±4.77	26.06 ±5.66	0.955
Parity median(range)	1(0-5)	0(0-5)	0.582	0(0-3)	0(0-5)	0.556	1(0-1)	0(0-5)	0.751
Gravidity median(range)	2(1-6)	2(1-8)	0.891	1(1-4)	2(1-8)	0.353	2(1-3)	2(1-8)	0.500
Previous spontaneous abortion [f]	6 (11.5%)	64 (16.5%)	0.358	4 (10.2%)	70 (16.9%)	0.289	1 (5.9%)	80 (17.0%)	0.253
Previous Stillbirths [g]	2 (3.8%)	4 (1.0%)	0.127	0 (0.0%)	6 (1.4%)	0.449	0 (0.0%)	7 (1.5%)	0.612

[a] New acquisition: women who acquired serotype-specific new acquisition; [b] Non-colonized: women who remain uncolonized by specific-serotype at all four study visits; [c] p-value by univariate logistic regression analysis [d] n: number of participants; [e] SD: Standard deviation; [f] Spontaneous abortion: The spontaneous abortion of pregnancy occurring before 28 gestational weeks; [g] Stillbirth: Fetal death occurring at 28 gestational weeks or later.

6.4.1 Association between serotype-specific CPS IgG and new acquisition of the homotypic serotype

The logistic regression analysis for the association between homotypic serotype acquisition and serotype-specific IgG concentration at enrolment demonstrated OR <1.00, indicating that the probability of a new acquisition decreased with increasing serotype-specific IgG concentrations; Table 6.2. Homotypic serotype antibody GMC was higher at enrolment among the non-colonized than new-acquisition group for serotype III (0.33µg/ml versus 0.17µg/ml, respectively; p=0.009), with a similar trend for serotype V (0.75µg/ml versus 0.42µg/ml, respectively; p=0.057) (Table 6.2).

Table 6. 2: Comparison of serum geometric mean concentration of serotype-specific CPS antibodies (GMC; µg/ml) and geometric mean OPA titers (GMOPT) at visit-1 for pregnant women who acquired (new-acquisition) compared to those who remain uncolonized (non-colonized group) for specific serotypes

Serotype	New-acquisition [a]	Non-colonized [b]	p-value [c]	Odds Ratio [d] (95 % CI)*
	GMC[e] (95% CI)[a]	GMC (95% CI)[b]		
Ia	0.28 (0.17, 0.47); n=52[f]	0.35 (0.27, 0.44); n=387	0.529	0.91 (0.68, 1.21)
III	0.17 (0.11, 0.26); n=39	0.33 (0.28, 0.38); n=414	0.009	0.47 (0.26, 0.84)
V	0.42 (0.26, 0.65); n=17	0.75 (0.66, 0.84); n=471	0.057	0.39 (0.15, 1.01)
OPA	GMOPT[g] (95% CI)	GMOPT (95% CI)		
Ia	5 (4, 6); n=52	14 (11, 17); n=387	<0.001	0.28 (0.12, 0.63)
III	20 (11, 36); n=39	132 (105, 164); n=414	<0.001	0.43 (0.30, 0.62)

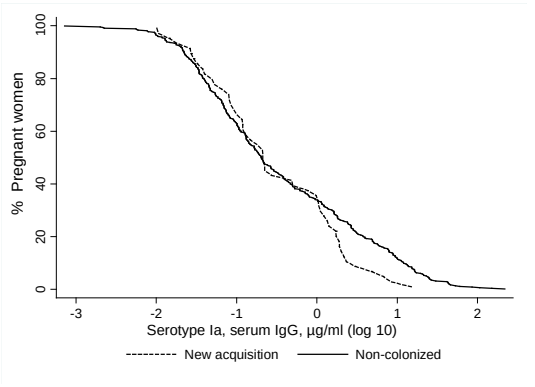
[a] New acquisition: women who acquired serotype-specific new acquisition; [b] Non-colonized: women who remained uncolonized by specific serotype; [c] p-value calculated by students-t test; [d] Odds ratio by logistic regression analysis; [e] GMC : geometric mean concentration; [g] GMOPT: geometric mean OPA titers; [f] n: number of participants.

Reverse cumulative distribution plots of serotype-specific CPS antibody concentrations of the non-colonized and new-acquisition groups are shown in Figures 5.2 a-c. The lowest threshold of CPS antibody concentration associated with lower odds of acquisition of the homotypic serotype varied between serotypes. The lowest antibody threshold significantly associated with remaining non-colonized throughout the study was ≥3 µg/ml for serotype Ia (22.0% in non-colonized versus 9.6% in new-acquisition group,

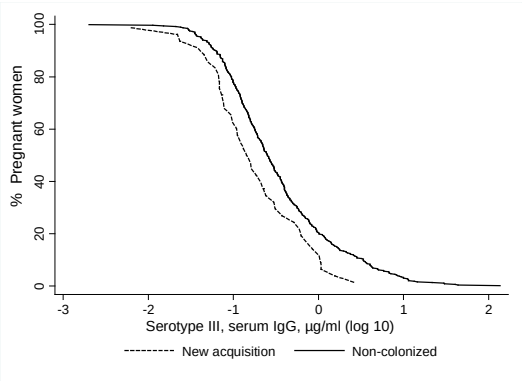
OR:0.37; 95%CI: 0.14,0.98), ≥3 μg/ml for serotype III (10.6% in non-colonized versus 0.0% in new-acquisition group, OR:0.11; 95%CI: 0.01-1.75) and ≥1μg/ml for serotype V (36.5% in non-colonized versus 11.8% in new-acquisition group, OR:0.23; 95%CI: 0.05-1.02; Table 6.3). This strength of association increased at higher CPS antibody thresholds for serotype Ia, and III (Table 6.3). None of the women who acquired serotype Ia had antibody concentration ≥15μg/ml (compared to 8.5% of non-colonized women, p=0.025) at enrolment; and none with new-acquisition of serotypes III had homotypic CPS antibody ≥3μg/ml (compared to 10.6% (p=0.023) of those who remained non-colonized; Table 6.3).

6.4.2 Association between serotype-specific opsonophagocytic activity and new acquisition of GBS

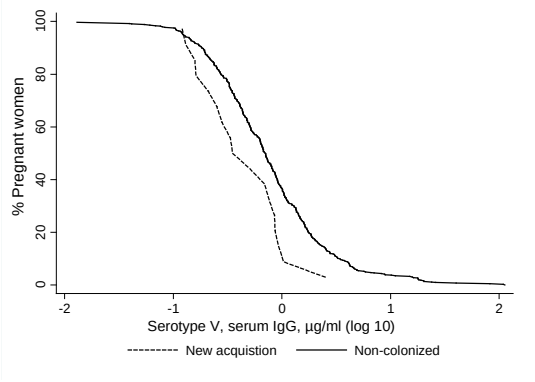
Reverse cumulative distribution plots of serotype-specific OPA titers of the non-colonized and new-acquisition for serotype Ia and III are shown in Figures 6.2 d-e. The logistic regression analysis for the probability of acquisition of GBS serotype Ia and III as a function of homotypic serotype OPA titer at enrolment had OR <1.00, indicating that the probability of a new acquisition decreased with increasing serotype-specific OPA titer; Table 6.2. Non-colonized women had higher serotype-specific GMOPT than the new-acquisition group for serotypes Ia (GMOPT: 14 versus 5, respectively; p<0.001) and III (GMOPT: 132 versus 20, respectively, p<0.001; Table 6.2). The presence of detectable OPA titers (≥8) was significantly associated with lower odds of acquiring the homotypic serotype for Ia (p=0.002) and III (<0.001). The strength of association between OPA titers and odds of new-acquisitions trended to be stronger at higher OPA threshold for serotypes Ia and III; Table 6.3.



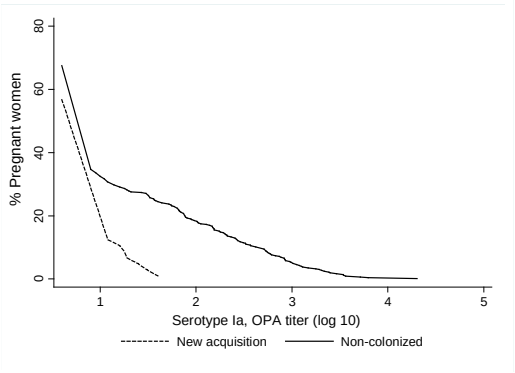
(a)



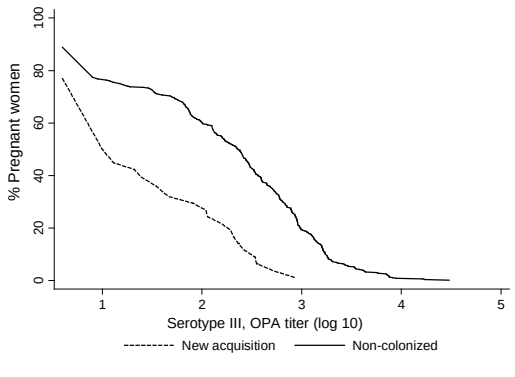
(b)



(c)



(d)



(e)

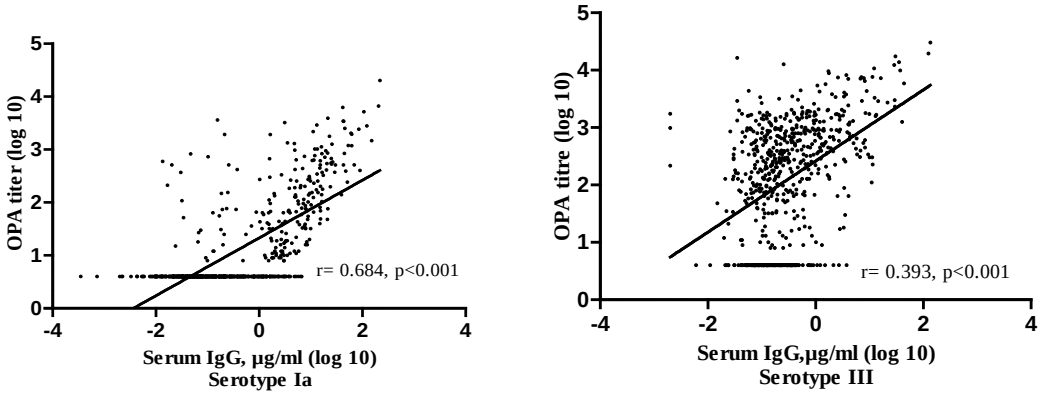
Figure 6.2: Reverse cumulative distribution curves of serotype-specific anticapsular serum IgG (a-c) and OPA titer (d-e) in participants who acquired GBS serotype (new acquisition) and those who remained uncolonized by the specific-serotype (Non-colonized)

Table 6. 3 : Association between serotype-specific capsular IgG antibody and acquisition of homotypic Group B *Streptococcus* in pregnant women

	Serotype Ia				Serotype III				Serotype V			
Antibody levels, µg/ml	New acquisition n=52[a]	Non-colonized n=387	Odds ratio (95%CI)	p-value[b]	New acquisition n=39	Non-colonized n=414	Odds ratio (95%CI)	p-value	New acquisition n=17	Non-colonized n=471	Odds ratio (95%CI)	p-value
< 0.5	32 (61.5 %)	235 (60.7%)	1.04 (0.57, 1.88)	0.910	29 (74.4%)	283 (68.4%)	1.34, (0.64, 2.84)	0.439	9 (52.9%)	184 (39.3%)	1.75 (0.66, 4.61)	0.25
≥ 0.5	20 (38.5%)	152 (39.3%)	0.98 (0.54, 1.78)	0.910	10 (25.6%)	131 (31.6%)	0.74 (0.35, 11.57)	0.439	8 (47.1%)	287 (60.9%)	0.57 (0.22, 1.51)	0.25
≥ 1	18 (34.6%)	131 (33.9%)	1.03 (0.56, 1.90)	0.91	5 (12.8%)	84 (20.3%)	0.57 (0.22, 1.52)	0.396	2 (11.8%)	172 (36.5%)	0.23 (0.05, 1.02)	0.039
≥ 2	8 (15.4%)	101 (26.1%)	0.51 (0.23, 1.13)	0.093	1 (2.6%)	55 (13.3%)	0.17 (0.02, 1.27)	0.070	1 (5.9%)	81 (17.2 %)	0.30 (0.04, 2.30)	0.328
≥ 3	5 (9.6%)	85 (22.0%)	0.37 (0.14, 0.98)	0.043	0 (0%)	44 (10.6%)	0.11 (0.01, 1.75)	0.023	0 (0%)	55 (11.7 %)	0.21 (0.01, 3.62)	0.239
≥ 5	3 (5.8%)	70 (18.1%)	0.28 (0.01, 0.91)	0.027	0 (0%)	26 (6.3%)	0.19 (0.01, 3.11)	0.151	0 (0%)	26 (5.5 %)	0.48 (0.02, 8.21)	1.000
≥ 10	1 (1.9%)	45 (11.6%)	0.15 (0.02, 1.11)	0.028	0 (0%)	13 (3.1%)	0.38 (0.02, 6.46)	0.615	0 (0%)	18 (3.8%)	0.70 (0.04, 12.1)	1.000
≥ 15	0 (0%)	33 (8.5%)	0.10 (0.01,1.67)	0.025	0 (0%)	6 (1.4%)	0.79 (0.04, 14.39)	1.000	0 (0%)	16 (3.4%)	0.78 (0.04, 13.7)	1.000
OPA Titers												
< 8	45 (86.5%)	251 (64.9%)	3.46 (0.64, 2.84)	0.002	18 (46.2%)	92 (22.2%)	3.00 (1.53, 5.87)	<0.001	Not done			
≥ 8	7 (13.5%)	135 (34.9%)	0.29 (0.13, 0.66)	0.002	21 (53.8%)	322 (77.8%)	0.33 (0.17, 0.65)	<0.001				
≥ 16	6 (11.5%)	114 (29.5%)	0.31 (0.13, 0.75)	0.006	17 (43.6%)	310 (74.9%)	0.26 (0.13, 0.51)	<0.001				
≥ 32	1 (1.9%)	104 (26.9%)	0.05 (0.01, 0.39)	<0.001	15 (38.5%)	302 (72.3%)	0.23 (0.12, 0.46)	<0.001				
≥ 64	0 (0%)	88 (22.7%)	0.03 (0.00, 0.52)	<0.001	12 (30.8%)	283 (68.4%)	0.21 (0.10, 0.42)	<0.001				
≥ 128	0 (0%)	67 (17.3%)	0.05 (0.00, 0.74)	<0.001	9 (23.1%)	244 (58.9%)	0.21 (0.10, 0.45)	<0.001				
≥ 256	0 (0%)	51 (13.2%)	0.06 (0.00, 1.02)	0.002	5 (12.8%)	202 (48.8%)	0.15 (0.06, 0.40)	<0.001				
≥ 512	0 (0%)	38 (9.8%)	0.09 (0.00, 1.43)	0.014	2 (5.1%)	147 (35.5%)	0.01 (0.02, 0.41)	<0.001				

[a] n: number of participants; [b] p-value calculated by either chi-square test or fisher exact test as appropriate.

There were significant correlations between serum IgG concentration and OPA titer with rho-values of 0.684 for serotype Ia and 0.393 for III, p<0.001 for both (Figure 6.3 a-b). OPA activity (titer ≥8) was detectable to the homotypic serotype in 92% (138/150) of serum samples with IgG concentration ≥3µg/ml for serotype Ia and 94.1% (192/204) of samples with IgG concentration ≥0.5µg/ml for serotype III.



(a) (b)

Figure 6.3: Correlation between serotype-specific serum IgG concentration and OPA titers at visit-1 for serotype Ia (a) and serotype III (b) irrespective of colonization status

6.4.3 Association between serotype-specific CPS IgG and clearance of colonization at enrolment

Serotype specific CPS antibody concentration was greater among women colonized compared to those not colonized by the homotypic serotype at visit-1 for serotypes Ia

(p=0.025) and III (p=0.018); and at visit-4 for serotype-Ia (p<0.001) with similar trend for serotype-III (p=0.060); albeit not statistically significant for OPA at visit-1 (Table 6.4). Among women colonized at enrolment, higher CPS GMC (1.49 versus 0.44 µg/ml, p=0.049) and GMOPT (41 versus 12; p=0.025) were observed for serotype Ia in those categorised as persistently-colonized than those with intermittent-colonization, respectively, but not so for serotypes III and V (Table 6.5).

Table 6.4: Cross-sectional comparison of geometric mean concentration (GMC) of serotype-specific CPS antibody (µg/ml) in pregnant women who were colonized and not colonized by the homotypic serotype at visit-1 or visit-4

Serotype	Visit-1			Visit-4		
Serum IgG	Colonized GMC [a] (95% CI)	Not-colonized GMC (95% CI)	p-value[b]	Colonized GMC (95% CI)	Not colonized GMC (95% CI)	p-value
Ia	0.71 (0.40, 1.27); n=71[c]	0.36 (0.28, 0.45); n=436	0.025	1.52 (0.80, 2.89); n=50	0.43 (0.12, 0.45); n=457	<0.001
III	0.51 (0.33, 0.79); n=56	0.30 (0.26, 0.35); n=451	0.018	0.47 (0.30,0.76); n=52	0.30 (0.26, 0.35); n=455	0.060
V	1.02 (0.52, 2.00); n=15	0.72 (0.65, 0.82); n=492	0.31	1.07 (0.66, 1.75); n=18	0.83 (0.74, 0.92); n=489	0.372
OPA	GMOPT [d] (95% CI)	GMOPT (95% CI)		Not done		
Ia	19 (12, 32); n=71	13 (10, 15); n=436	0.081			
III	178 (88, 359); n=56	111 (89, 138); n=451	0.166			

[a] GMC : geometric mean concentration; [b] p-value by students-t test; [c] n: number of participants; [d] GMOPT: geometric mean OPA titers.
*OPA was done at visit-1 for GBS serotype Ia and III

Table 6.5: Comparisons of geometric means of serum IgG (µg/ml) concentrations and OPA geometric mean titers at baseline in women who remained colonized throughout by particular serotype (persistent carriers) compared to those in whom colonization was cleared (intermittent carriers)

Variable and Serotype	Persistent carriers	Intermittent carriers	p-value [a]
Serum IgG	GMC[b] (95% CI)	GMC (95% CI)	
Ia	1.49 (0.51-4.3); n=21[c]	0.44 (0.22, 0.88); n=43	0.049
III	0.54 (0.28, 1.04); n=27	0.47 (0.19, 1.20); n=18	0.807
V	1.67;* n=2	0.71 (0.25, 1.99); n=9	0.437
OPA	GMOPT[d] (95% CI)	GMOPT (95% CI)	
Ia	41 (14, 116); n=21	12 (7, 21); n=43	0.025
III	221 (74, 656); n=27	216 (65, 712); n=18	0.979

[a] p-value by student-t test; [b] GMC : geometric mean concentration; [c] number of participants;
[d] GMOPT: geometric mean OPA titers; * not presented due to very wide CI intervals.

6.4.4 Effect of GBS colonization on kinetics of serotype-specific antibody

Analyzing women who completed all four study visits and were not colonized by a specific-serotype at enrolment, serotype-specific new-acquisition was associated with increase in GMC's and GMOPT between enrolment and visit-4. These increases were evident for GMCs for serotype Ia (p=0.001), III (p=0.004) and V (p=0.034); as well as GMOPT for serotypes Ia and III (p<0.001 for both; Table 6.6). For serotype Ia, a new acquisition event was also associated with an increase in correlation between serotype-specific serum IgG concentration and OPA titers, with rho-values of 0.524 at enrollment (p<0.001) and 0.792 at visit-4 (p<0.001) (Figure 4.3 a-b). In contrast, for serotype III a decrease in correlation between enrollment (rho=0.354, p=0.037) and visit-4 (rho=0.266,

p=0.122) was observed, despite significant increases in OPA titres (Figure 6.4 c-d). No change was observed in GMC between visit-1 and visit-4 among women who remained non-colonized by the specific-serotype throughout the study for serotypes Ia or III, although an increase was observed for serotype V (0.83 versus 0.75 µg/ml, p=0.011).

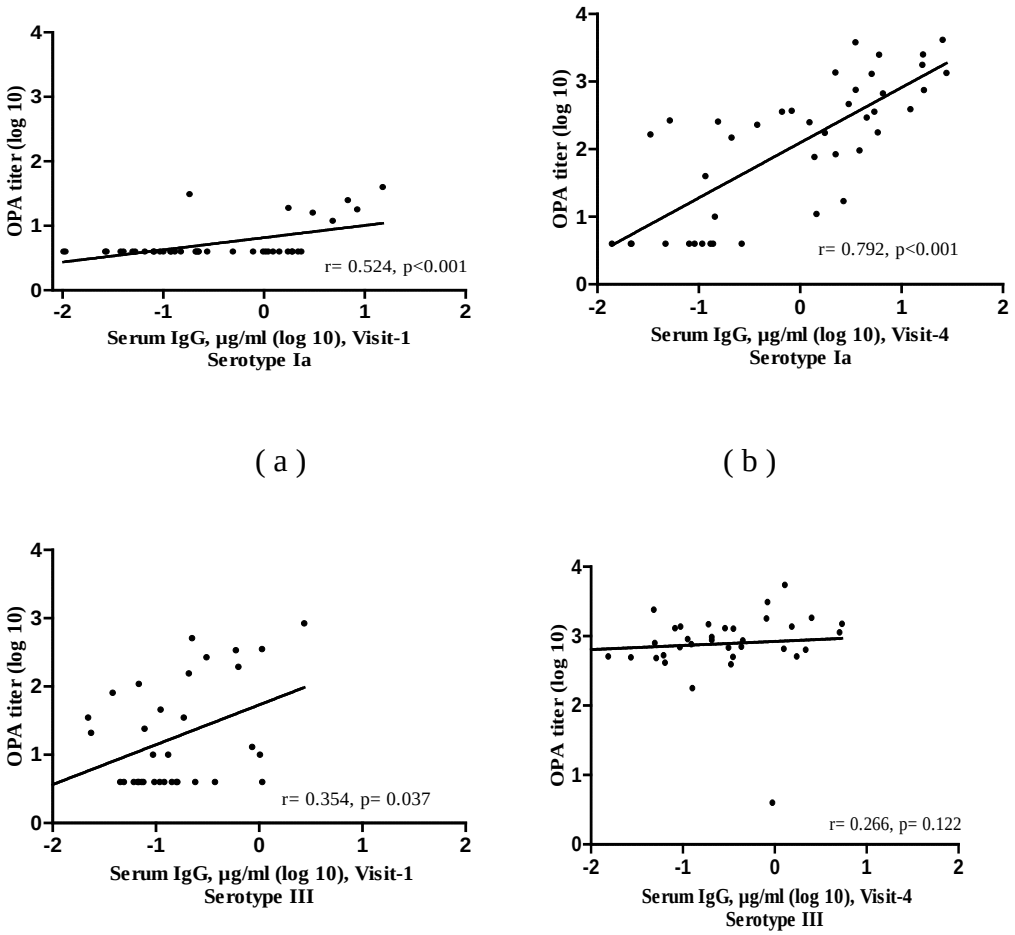


Figure 6.4: Correlation between serotype-specific serum IgG concentration and OPA titers at visit-1 and after new acquisition event (visit-4) for serotype Ia (a-b) and for serotype III (c-d)

Table 6.6: Comparisons of serum geometric mean concentration (GMC) serotype-specific capsular IgG antibody (µg/ml) and geometric mean opsonophagocytic titer (GMOPT) at enrolment and visit 4 in pregnant women who acquired GBS and those who remained uncolonized throughout

Participants with serotype-specific new acquisition				Participants who remain uncolonized by specific serotype		
Serotype	Visit 1	Visit 4	p-value[a]	Visit 1	Visit 4	p-value
Serum IgG	GMC[b] (95% CI)	GMC (95% CI)		GMC (95% CI)	GMC (95% CI)	
Ia	0.32 (0.18, 0.57); n=42[c]	0.86 (0.44, 1.70)	0.001	0.36 (0.28, 0.46); n=387	0.40 (0.32, 0.51)	0.169
III	0.14 (0.09, 0.22); n=35	0.26 (0.15, 0.46)	0.004	0.32 (0.28, 0.38); n=414	0.30 (0.26, 0.36)	0.272
V	0.38 (0.23, 0.61); n=15	0.76(0.43, 1.33)	0.034	0.75 (0.66, 0.84); n=471	0.83 (0.74, 0.93)	0.011
OPA	GMOPT[c] (95% CI)	GMOPT (95% CI)				
Ia	5 (4, 6); n=42	110 (53, 227)	<0.0001	Not done*		
III	17 (9, 32); n=35	776 (528, 1143)	<0.0001			

[a] p-value by paired students-t test; [b] GMC : geometric mean concentration; [c] n: number of participants; [d] GMOPT: geometric mean OPA titers

* OPA at visit-4 was done only for participants who acquired GBS serotype

6.5 Discussion

Our study demonstrated an association between natural-acquired serum serotype-specific CPS antibody and the risk of subsequent recto-vaginal colonization by the homotypic- serotype in pregnant women not colonized at enrolment. New-acquisition of GBS was inversely correlated with serotype-specific CPS IgG concentration (serotype III and V) and OPA titer (serotype Ia and III) at time of enrolment. These data suggest that strategies such as vaccination of women with GBS capsular polysaccharide-protein conjugate vaccine which induces antibody responses could reduce the risk of GBS recto-vaginal acquisition of vaccine-serotypes. The potential benefit to the women and their newborn would, however, be dependent on the timing of vaccination in the women and durability of persistence of sufficiently high antibody concentrations or OPA titers to prevent GBS acquisition during pregnancy. The potential of a protein-polysaccharide conjugate vaccine to induce mucosal immunity has been demonstrated for vaccines against *Haemophilus influenzae* type b, *Streptococcus pneumoniae* and *Neisseria meningitidis* in which reduced risks of nasopharyngeal mucosal-colonization acquisition of the targeted serotypes are evident (Barbour et al., 1995, Dagan et al., 2005, Maiden and Stuart, 2002).

In our study, the CPS antibody, including functional antibody measured by OPA, was most likely induced by recto-vaginal colonization, as indicated by the rise in serotype-specific GMCs and GMOPT among those with new-acquisitions of homotypic-serotypes. It is this increase in CPS antibody, which likely reduces the risk of these women becoming colonized by the same serotype. Although our study was not designed to establish the durability of this natural-acquired humoral-mediated immunity, the strengthening of association between higher serotype-specific antibody thresholds and OPA titers and the

reduced odds of becoming colonized by the homotypic serotype, indicates that this protection is likely to be transient with waning of antibody over time. Also, the paradoxically higher GMC and GMOPT in women colonized with serotype Ia at enrolment and who remained persistently-colonized thereafter compared to those who were only intermittently-colonized, indicates that the antibody which was likely induced by colonization, might not clear existing colonization. This clearance of established colonization may be due to other immune-mediators, including cell-mediated immunity.

Our study indicated that the threshold of CPS antibody associated with significantly reduced odds of acquisition of GBS was ≥3 µg/ml for serotypes Ia and III, and ≥1µg/ml for serotype-V. Furthermore, serotype-specific OPA titers of ≥ 8 were associated with similarly reduced odds of new-acquisition of the homotypic serotype for serotypes Ia and III. The OPA titers correlated more strongly than IgG concentrations in relation to new-acquisition of serotype Ia and III. This shows the importance of measuring functional antibody in the evaluation of immune responses to GBS conjugate vaccine. Also, the correlation between OPA titers and CPS IgG concentration was modest, suggesting either impaired functional activity of antibody or limitations of the serology assay with respect to purity of antigens used or possible role of IgM antibodies in opsonophagocytosis (Simell et al., 2012).

Previous studies on the association of GBS colonization and serum IgG antibody have mainly been cross-sectional studies and have generally reported higher serotype-specific CPS antibody in colonized compared to non-colonized pregnant women for the homotypic serotype (Campbell et al., 2000, Davies et al., 2001, Anthony et al., 1984,

Baker et al., 1980, Beachler et al., 1979, Matsubara et al., 2003, Matsubara et al., 2002, Skidmore et al., 1985). These studies were, however, not designed to address whether CPS antibody reduces the risk of GBS colonization during pregnancy, as shown by us. Nevertheless, the findings of the previous studies are confirmed in our study, where higher serotype-specific CPS IgG concentrations were also observed in women colonized by the homotypic-serotype compared to those not colonized when analyzed cross-sectionally at either visit-1 or visit-4 for serotypes Ia and III. This higher antibody concentration in colonized women in such studies likely reflect recent acquisition of that serotype, which induced the higher antibody levels compared to women not colonized, as demonstrated in kinetics of antibody response to new acquisitions in our study.

Our study has certain limitations. First, although we explored for thresholds of serotype-specific CPS IgG and OPA titers associated with reduced odds of new acquisition by homotypic serotypes, the findings need to be interpreted with caution in the absence of a standardized reference assay to measure either CPS antibody or OPA titers (Madhi et al., 2013). We tried to optimize the comparability of our assay to that of other laboratories by calibrating our in-house reference (Polygam) with reference sera from Dr Baker who has published widely in the field. Second, the sensitivity of detection of GBS on selective media is estimated at 85% and mainly identifies the dominant colonizing serotype, with lower-density co-colonizing serotypes likely being missed (Chapter 4). Therefore, it is possible that we overestimated new-acquisition through missing colonization episodes not detectable by standard culture methods at visit-1; and similarly may also have missed some new-acquisitions.

In summary, our results indicate that the effect of GBS conjugate vaccine on recto-vaginal GBS colonization warrants further investigation. This could provide recto-vaginal colonization as surrogate endpoint to evaluate the potential effect of GBS conjugate vaccine in protecting newborns (including those born prematurely) from exposure to GBS at birth and hence reduce their risk of EOD. Already, a monovalent GBS serotype III conjugate vaccine has been reported to significantly reduce the rate of recto-vaginal GBS acquisition among non-pregnant women (Hillier, 2009). This potential effect, of vaccination with GBS vaccine targeted at women, against recto-vaginal colonization may be over and above the additional protection of vaccination against invasive GBS disease in young infants born at full-term, which is likely to be conferred through transplacental acquisition of protective serotype-specific CPS antibody. Furthermore, it is plausible that reducing the risk of GBS acquisition during pregnancy, could reduce the incidence of GBS-associated chorio-amnionitis, premature labor and stillbirths (Edwards and Gonik, 2013).

Chapter 7 Serotype specific cellular-mediated immunity associated with clearance of homotypic Group B *Streptococcus* recto-vaginal colonization in pregnant women

7.1 Abstract

Background: We investigated the association between Group B *Streptococcus* serotype-specific capsular polysaccharide cellular immunity at 20 weeks gestational age in pregnant women, and effect thereof on recto-vaginal serotype-specific GBS colonization up to 37 weeks of gestational age.

Methods: Group B *Streptococcus* serotype-specific capsular polysaccharide cellular immunity was measured by ELISpot Interferon-γ (IFN-γ) release assay. Recto-vaginal swabs were evaluated for GBS colonization, using standard culture methods and serotyping by latex agglutination, at 5-6 weekly intervals.

Results: Among women colonized by serotype III at enrolment, IFN-γ ELISpot positivity was more common in those in whom colonization was cleared (44.4%) than in those in whom colonization persisted (7.4%; p=0.008) with a similar trend observed for serotype Ia.

Conclusion: Presence of serotype-specific capsular polysaccharide cell-mediated immunity contributes to the clearance of GBS recto-vaginal colonization.

7.2 Introduction

Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) and its vertical transmission to newborns is the primary source of early-onset (<7 days age; EOD) invasive GBS disease (Heath and Feldman, 2005), and has also been associated with stillbirths and premature deliveries (Edwards and Gonik, 2013). Spontaneous clearance and acquisition of GBS has been reported in pregnant women (Hansen et al., 2004, Goodman et al., 1997), however, the host immune mediators influencing the dynamics of GBS colonization remain to be fully elucidated. We recently established that capsular specific antibody and opsonophagocytic activity reduced the risk of new homotypic serotype acquisition between 20 to 37+ weeks of pregnancy, however, neither was associated with clearance of GBS colonization (Chapter 6).

The aim of this study was to determine the association of presence of GBS cellular immunity as assessed by Interferon-γ (IFN-γ) release assays, performed by Enzyme-linked Immunospot (ELISpot) assay on the dynamics of GBS colonization in women during the latter half of pregnancy.

7.3 Material and Methods

The detailed methods of the study have been described in Chapter 2. Briefly, pregnant HIV-uninfected women aged 18-45 years and who were 20-25 weeks gestational age were enrolled at antenatal community clinics in Soweto, Johannesburg from August 2010 to August 2011. Exclusion criteria included antibiotic treatment in the previous two weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examination was contraindicated.

Study procedures included lower vaginal and rectal swabs collection for GBS culture starting at 20-25 weeks (visit-1), followed by three subsequent visits (visits 2-4) at 5-6 weekly intervals, up until 37-40 weeks (visit-4) of gestational age. For GBS isolation, swabs were inoculated onto CHROMagar StrepB (CA; Media Mage, Johannesburg, South Africa). Serotyping was performed by the latex agglutination method as described (Afshar et al., 2011). Isolates that tested negative by latex agglutination for all serotypes were further typed by a PCR method using primer sequences described by Poyart *et al* (Poyart et al., 2007).

7.3.1 Measure of cellular immune response

Cellular immune response was determined at Visit-1 (enrolment) by Interferon-γ (IFN-γ) release assays, performed by Enzyme-linked Immunospot (ELISpot) assay kit for human IFN-γ as per manufacturer instructions (Cat no: 3420-2A, Mabtech, Sweden). Briefly, peripheral blood mononuclear cells (PBMC) were isolated from the heparinised blood using ficoll gradient centrifugation and re-suspended in AIM-V medium (Gibco, Invitrogen, USA). Around, 0.25-0.3 x 10^6 PBMC's were added per well onto ELISpot plates (MAIP S4510; Millipore, Ireland) coated with IFN-γ capture antibody (Mabtech, Sweden). PBMC's were stimulated with GBS serotype-specific capsular polysaccharide (CPS) antigens separately (CPS-Ia, III and V) for 18-24 hours at 37°C in 5% CO_2 with a final concentration of 40µg/ml (GBS CPS antigens were kindly provided by Novartis Vaccines, Italy). After incubation, ELISpot plates were developed using biotinylated anti-IFN-γ detection antibody (Mabtech, Sweden) which detected with streptavidin-alkaline phosphatase (Mabtech, Sweden), and visualized with an AP conjugate substrate kit (Bio-Rad, CA, USA). Checker board titrations were performed to optimize PBMC's and antigen concentrations per well. AIM-V medium was used as negative control to assess

background levels and phytohaemagglutinin (Sigma, USA) was used as a positive control. Cellular immune responses against each antigen were detected in single well only. Spot forming units (SFU) were counted with a magnifying lens. Background (SFU in negative control) was subtracted and results were expressed as number of SFU/10^6 PBMC. An ELISpot response was considered as positive if the number of antigen-specific spots was ≥7 SFU/10^6 PBMC and at least double the number of spots in the negative control well.

In addition, serotype-specific serum CPS IgG antibody concentrations were measured by multiplex Luminex (serotypes Ia, III and V) and opsonophagocytic activity (OPA) assay (Ia and III) (Chapter 6)

7.3.2 Study definition

A participant was considered to be colonized at a visit if GBS was cultured on either the vaginal or rectal swab; and non-colonized if no growth was detected from either site. The association of cellular immunity with clearance of serotype-specific GBS colonization was evaluated in women who were colonized by the specific serotype at visit-1 and who completed all four study visits. The women were stratified into those in whom colonization of the specific serotypes was cleared by visit-4 (i.e. “cleared-colonization group”) and those who remained colonized throughout the study (i.e. “persistently-colonized group”). Due to limitation associated with detecting multiple serotype carriage using traditional culture methods, participants colonized with any GBS serotype at visit-1 and who acquired a different GBS serotype at a subsequent visit and remained colonized with that serotype thereafter were excluded from the analysis.

We also evaluated the association of cellular immunity on acquisition of serotype-specific GBS colonization in women not colonized by a specific GBS serotype at Visit-1, who were subsequently colonized by that specific-serotype (irrespective of the number of study-visits completed) ("new-acquisition group") to those not colonized with that specific-serotype at any study visit ("non-colonized group").

7.3.3 Statistical analysis

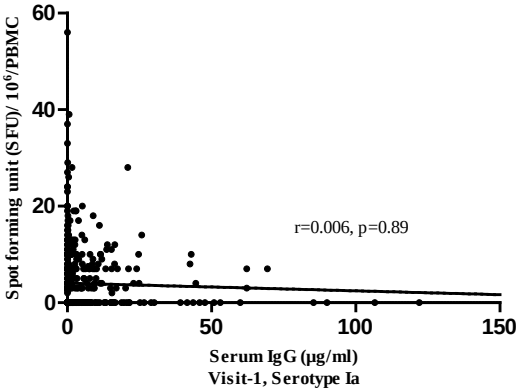
For categorical variables, groups were compared with either chi-square test or Fisher's two tailed exact test as appropriate. The Mann-Whitney test was used to detect differences in SFU/10^6 PBMC between groups ("persistently-colonized" versus "cleared-colonization" groups). Correlation between serotype-specific serum IgG and serotype-specific OPA titers in relation to number of SFU/10^6 PBMC by ELISpot at visit-1 were calculated using Spearman's correlation coefficient. To explore the association between SFU/10^6 PBMC's and clearance of GBS colonization, we used a Bayesian model to estimate the posterior distribution of the probability that a woman with a GBS homotypic SFU/10^6 PBMC's greater than or equal to *c*, would clear GBS colonization, denoted by P(D=1|SFU>=*c*) (Carey et al., 2001). For each value *c*, we graphically display the 25%, 50%, and 75% quantiles of the posterior distribution of P(D=1|SFU>=*c*). Data were analyzed using Graphpad version 5.0 software (Graphpad software, California, USA). R version 2.15 (Vienna, Austria)(Jags), A p-value of <0.05 was considered significant.

7.4 Results

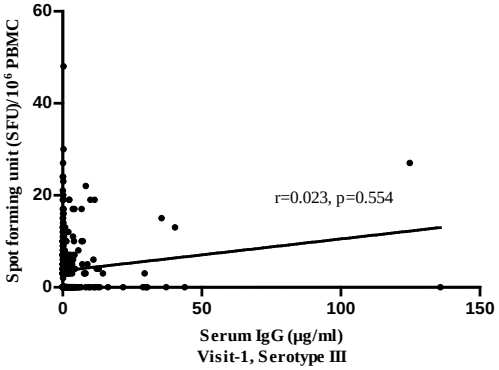
A detailed profile of participant demographics and follow up has been detailed in chapter 6. Briefly, of 661 participants, 76.7% (507/661) completed all four study-visits and 95.1% (629/661) completed at least two study-visits. Of the 507 women who completed all scheduled visits, 71 (14.0%), 56 (11.1%) and 15 (2.9%) were colonized by serotypes Ia, III and V, respectively at enrolment. Of the women already colonized at enrolment, 21/71 (29.6%), 27/56 (48.2%) and 2/15 (13.3%) were categorized as being "persistently-colonized"; and 43 (60.6%), 18 (32.1%) and 9 (60.0%) were in the "cleared-colonization" groups for serotypes Ia, III and V, respectively. This excluded 22 participants colonized at enrolment who cleared the initial serotype (Ia (7/71; 9.8%), III (11/56; 19.6%) and V (4/15; 26.7%) but who acquired a new serotype at subsequent visit and remained colonized with subsequent serotype thereafter. There were no differences in the demographic characteristics between the persistently-colonized and those in the cleared-colonization groups.

Of those not colonized by specific GBS serotype Ia, III and V at enrolment and who had at least one subsequent study visit, 9.6% (52/541), 7.0% (39/560) and 2.8% (17/603) subsequently become colonized by the respective serotypes (i.e. "new-acquisition" group). Of the participants who completed all scheduled visits, 387 (76.3%), 414 (81.6%) and 471 (92.9%) for serotype Ia, III and V, respectively (i.e. "non-colonized group) were never colonized by that specific serotype. There were no differences in the demographic characteristics between the new-acquisition and non-colonized group (chapter 6).

Of the 661 enrolled participants in whom cellular immune response was determined at visit-1, six participants were excluded from the analysis due to contamination detected in the assay. Of the remaining 655 participants, serotype-specific ELISpot responses were prevalent in 191(29.2%), 154(23.5%) and 143 (21.8%) for serotypes Ia, III and V respectively at visit-1. There was no correlation between serotype-specific serum IgG concentrations and number of SFU/10^6 PBMC from ELISpot assay for serotypes Ia (Rho=0.006; p=0.89), III (Rho=0.023; p=0.55) or V (Rho=0.021; p=0.59) (Figure 7.1). Similarly, there was no correlation observed between serotype-specific OPA titers and SFU/10^6 by ELISpot assay for either serotype Ia (Rho= 0.017; p=0.66) or III (Rho=0.042; p=0.28) (Figure 7.2).



(a)



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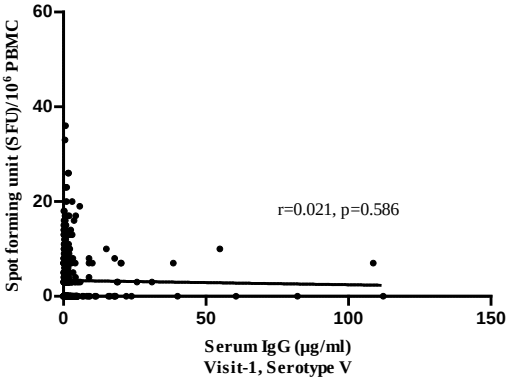
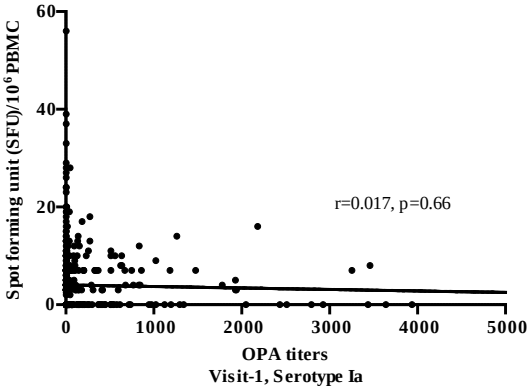
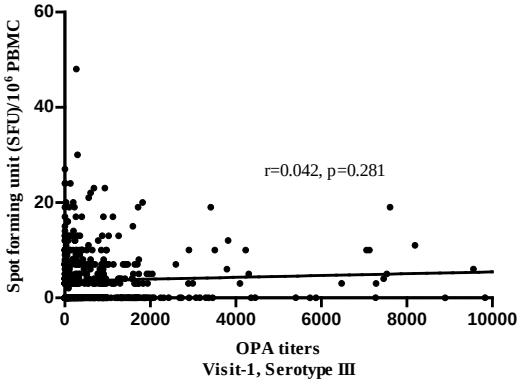


(c)

Figure 7.1: Correlation between serotype-specific serum IgG concentration and spot forming units for IFN-γ at visit-1 for serotype Ia (a), III (b) and V(c)



(d)



(e)

Figure 7.2: Correlation between serotype-specific OPA titers and spot forming units for IFN-γ at visit-1 for serotype Ia (a) and III (b)

Among women colonized with serotype III at enrolment, ELISpot positivity was found in higher percentage of women who cleared colonization (44.4%) compared with those who remained persistently-colonized (7.4%; p=0.008), with a similar trend for serotype Ia (44.2% versus 19.0%, respectively; p=0.06) and serotype V (22.9% versus 0.0%, respectively); Figure 7.2. Serotype-specific median SFU tended to be higher among those who cleared colonization than persistently-colonized for serotypes III (p=0.055) and Ia (p=0.144), albeit not significant; Figure 7.3.

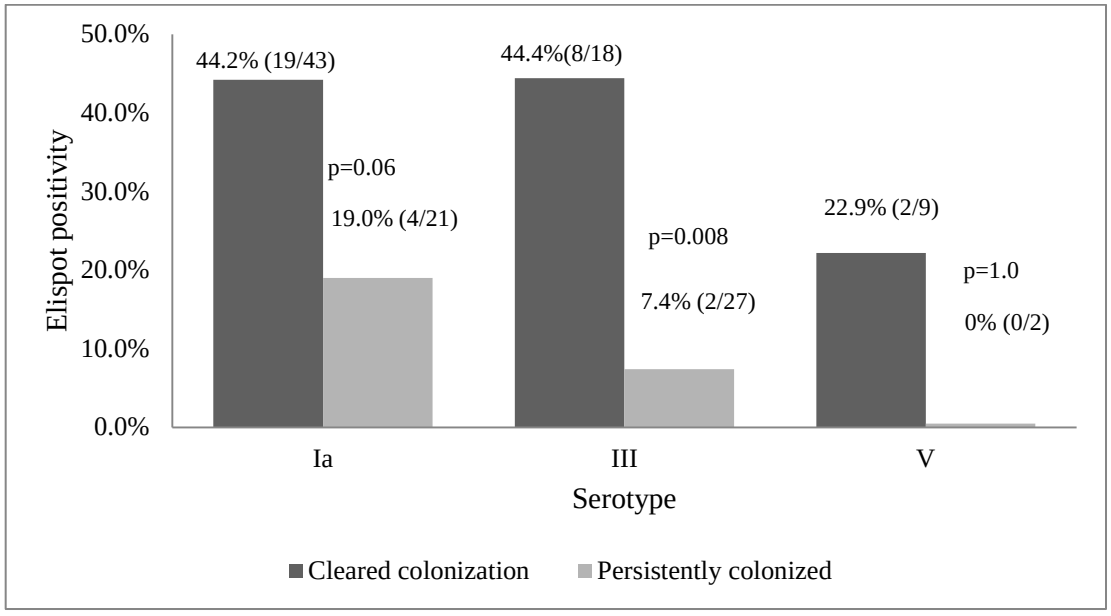
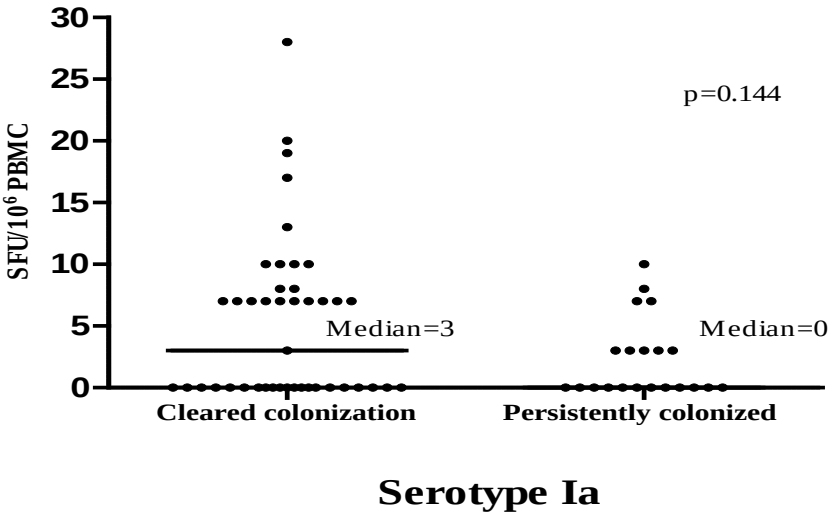
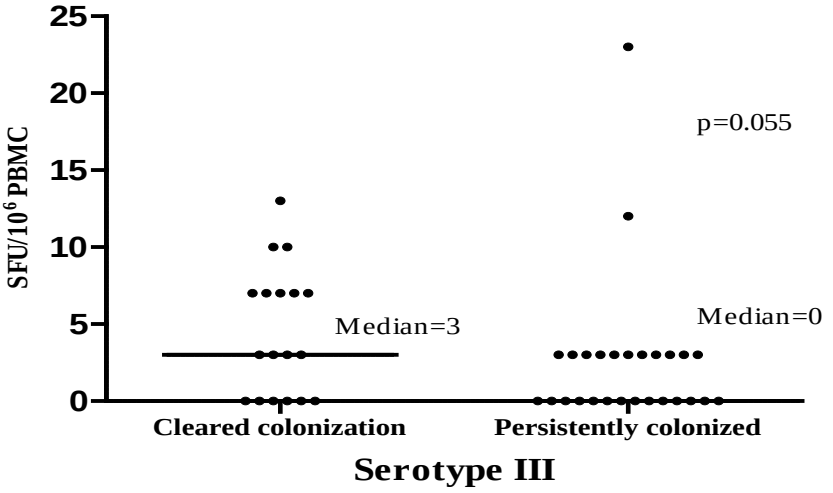


Figure 7.3: Comparisons of ELISpot positivity at enrolment in pregnant women who has cleared colonization (Cleared colonization group) to those who remain colonized throughout by that serotype (Persistently colonized group)



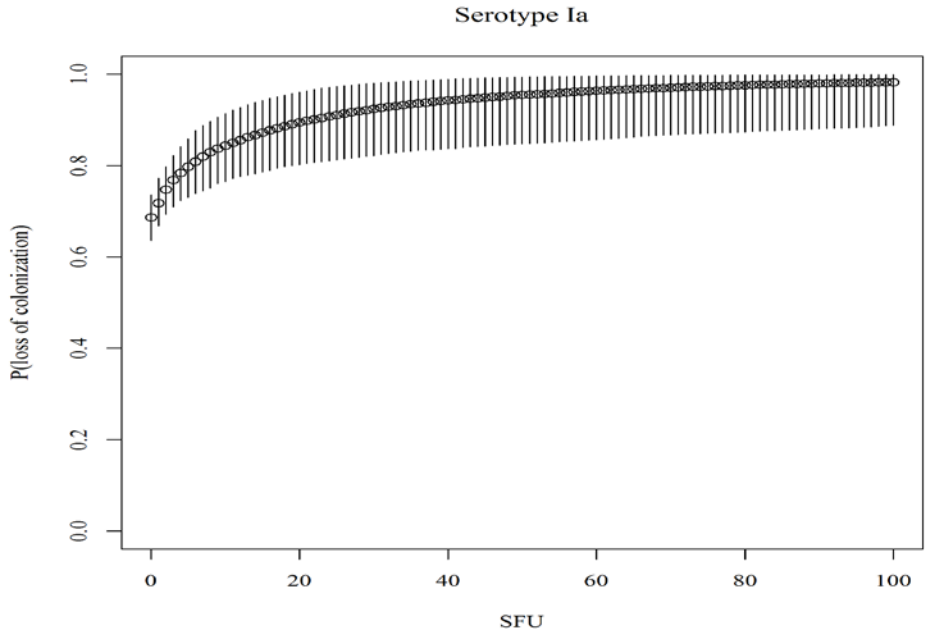
(a)



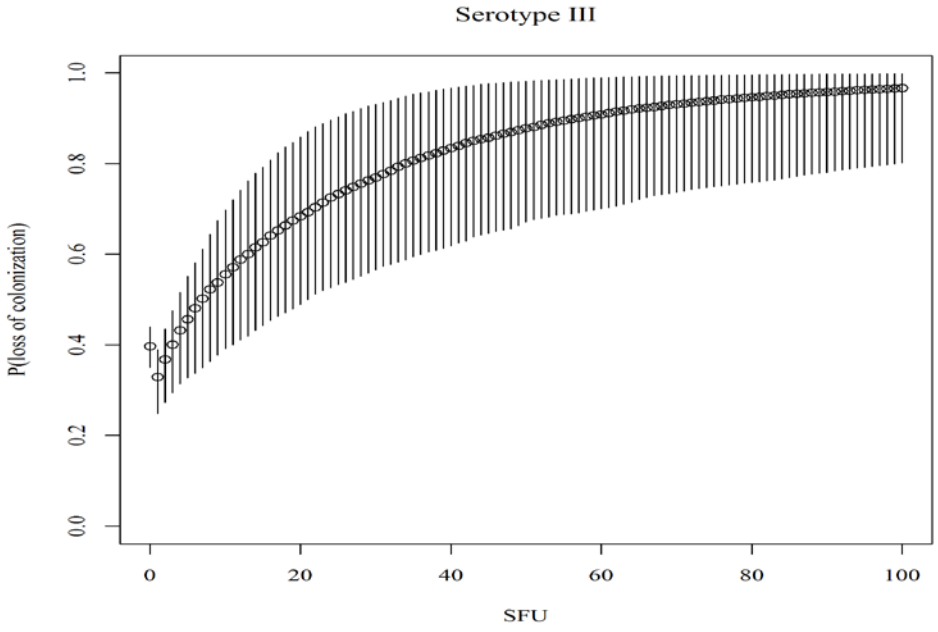
(b)

Figure 7.4: Comparison of median SFU/10^6 at visit-1 for pregnant women who lost colonization by visit-4 (cleared colonization group) compared to those who remain colonized at all visits (persistently colonized group) for serotypes Ia (a) and III (b)

Using a Bayesian framework the probability of losing colonization increased with higher SFU; Figure 7.4. We observed a 50% and 70% probability of losing colonization with ≥7 SFU/10^6 and ≥ 22 SFU/10^6 PBMC respectively for serotype III. For serotype Ia, we observed 82% probability of losing colonization with ≥7 SFU/10^6 PBMC. There was no correlation between serotype-specific ELISpot positivity and clearance of non-homotypic serotype. The lowest threshold associated with clearance of serotype III colonization was ≥7 SFU/10^6; being prevalent among 44.4% (8/18) in "cleared-colonization" versus 7.4% (2/27) in "persistent-carrier" group (OR: 10.00, 95% CI: 1.80-55.55; p=0.008); and 48.8% (21/43) and 19% (4/21) in the respective group for serotype Ia (OR: 4.06, 95% CI: 1.17-14.06; p=0.03); table 7.1.



(a)



(b)

Figure 7.5: Probability of clearance GBS serotype Ia (a) and serotype III (b) colonization at varying SFU/10^6 PBMC's using a Bayesian model

Table 7.1: Association between quantitative serotype-specific spot forming unit (SFU) by ELISpot assay and clearance of homotypic GBS in pregnant women

	Serotype Ia [a]				**Serotype III**			
SFU/10^6 PBMC	Cleared-colonization group (n=43)	Persistent-colonized group (n=21)	Odds ratio	p-value	Cleared colonization group (n=18)	Persistent-colonized group (n=27)	Odds ratio	p-value
≥ 3	22 (51.2%)	9 (42.9%)	1.39(0.48-3.99)	0.53	12 (66.6%)	13 (48.1%)	2.15 (0.62-7.42)	0.22
≥ 7	21 (48.8%)	4 (19.0%)	4.06 (1.17-14.06)	0.03	8 (44.4%)	2 (7.4%)	10.0 (1.80-55.55)	0.008
≥ 10	9 (20.9%)	1 (4.8%)	5.29 (0.62-44.96)	0.15	3 (16.7%)	2 (7.4%)	2.50 (0.37-16.73)	0.37

[a] For Serotype Ia, two participants in the cleared colonization group who had ≥7 SFU/10^6 PBMC at baseline but failed in criteria of having double number of spots as compared to negative control for being ELISpot positive were included in the quantitative analysis.

*Serotype V was not analysed due to insufficient numbers for comparison.

Furthermore, whereas we previously reported significant associations between serotype-specific antibody concentration and OPA in relation to subsequent new acquisition of GBS during pregnancy (Chapter 6), there was no significant difference in ELISpot positivity among women who became colonized (new-acquisition group) compared to those who remained uncolonized (non-colonized group) for serotypes Ia (21.6% (11/51) vs 29.4% (113/384); p=0.24), III (17.9% (7/39) vs 23.9% (98/410); p=0.40) or V (11.8%(2/17) vs 21.8% (102/467); p=0.546). From the non-colonized group three participants for serotype Ia and four each for serotype III and V, and from the new-acquisition group one participant for serotype Ia, were excluded from the analysis due to contamination.

7.5 Discussion

We have shown that clearance of serotype-specific GBS recto-vaginal colonization during pregnancy was associated with presence of homotypic capsular ELISpot IFN-γ positivity, whereas no such association was observed for serotype specific capsular antibody or OPA as reported for the same cohort (Chapter 6). Conversely, ELIspot positivity was not associated with a significantly reduced risk of GBS acquisition which was, however, positively associated with serotype specific capsular antibody and OPA for serotypes Ia and III as reported. These data suggest that presence of cell mediated immune response contributes to the clearance of recto-vaginal GBS colonization, whereas we have previously reported that humoral immunity is required to reduce the risk of recto-vaginal GBS acquisition during pregnancy (Chapter 6).

The results of the study are in accordance with studies on other capsular bacteria such as *Streptococcus pneumoniae* that shows cell-mediated immune response being

important for mucosal clearance of *Streptococcus pneumoniae* in animal models (McCool and Weiser, 2004, van Rossum et al., 2005). A serotype-specific capsular based GBS vaccine able to elicit both humoral and cell-mediated capsular immune responses could therefore confer protection against EOD by reducing the exposure of the newborn's to GBS colonization during the peri-partum period. This could offer an additional mode of protection against invasive GBS disease especially for preterm newborn's, who might not fully benefit from transplacental acquisition of maternal serotype specific capsular antibody which mainly occurs beyond 34 weeks gestational age. Also, clearance of GBS recto-vaginal colonization during pregnancy, coupled with prevention of new acquisition mediated by presence of humoral immunity, could theoretically mitigate against other GBS associated illness such as chorioamnionitis and adverse birth outcomes of GBS-associated stillbirths and premature labour. This would, however, require for vaccination to occur early in the second trimester of pregnancy and to induce serotype-specific functional antibody and cellular immunity for it to impact wholly on the full spectrum of GBS associated morbidity and mortality in the women, fetus and newborn's.

Chapter 8 Association between mucosal immune response and Group B *Streptococcus* colonization in pregnant women

8.1 Abstract

Background: Establishing association between mucosal immunity and Group B *Streptococcus* (GBS) colonization in pregnant women could contribute to the evaluation of GBS vaccines. We studied the association of serotype-specific mucosal antibodies on new acquisition and clearance of recto-vaginal GBS colonization in pregnant women and association between serum IgG and mucosal IgG.

Methods: Serotype-specific GBS capsular mucosal IgG and IgA concentrations were measured by multiplex Luminex assay in vaginal mucosal secretions. Recto-vaginal swabs for GBS colonization was undertaken at 20-25 weeks of gestation and thereafter at 5-6 weekly intervals. Standard microbiological methods were used for GBS culture and serotyping undertaken by latex agglutination and PCR if non-typeable by latex agglutination.

Results: Serotype-specific capsular mucosal IgG trended to be higher in pregnant women who remained uncolonized compared to those who acquired homotypic GBS serotype. Mucosal IgG correlated significantly with serum IgG with rho values of 0.839, 0.621 and 0.426 (all $p<0.001$) for serotypes Ia, III and V, respectively.

Conclusion: The results suggest that IgG in mucosal secretions is derived from serum IgG and could be responsible for the reduction of GBS colonization by preventing new acquisition.

8.2 Introduction

Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) is the major risk factor for GBS invasive disease in newborn (Verani et al., 2010, Chan SHS, 2000). Vaccination may be used as an approach for reducing the prevalence of GBS colonization, possibly by inducing high levels of type-specific serum antibody and subsequent reduction in the transmission of GBS to the newborn (Chapter 6). The ability of vaccines to induce mucosal immunity and to reduce colonization has been demonstrated for other bacterial polysaccharide-protein conjugate vaccines, including against *Haemophilus influenzae* type b, *Streptococcus pneumoniae* and *Neisseria meningitides,* where these conjugate vaccines not only decreases invasive disease, but also nasopharyngeal colonization (Barbour et al., 1995, Dagan et al., 2002, Maiden and Stuart, 2002).

Spontaneous clearance and acquisition of GBS are evident in pregnant women, however, the association of mucosal immune response GBS colonization during pregnancy remains unclear (Hansen et al., 2004, Goodman et al., 1997). A cross sectional study, reported higher mucosal serotype-specific capsular polysaccharide (CPS) antibody in colonized compared to non-colonized women (Hordnes et al., 1996).

The objective of our study was to evaluate the association between naturally induced serotype-specific mucosal IgG and IgA antibody to susceptibility for recto-vaginal acquisition and clearance of homotypic serotypes in pregnant women. Association between serotype- specific serum IgG and mucosal IgG was also investigated.

8.3 Material and Methods

The detailed methods of the study have been described in Chapter 2. Briefly, pregnant HIV-uninfected women aged 18-45 years and who were 20-25 weeks gestational age were enrolled at antenatal community clinics in Soweto, Johannesburg from August 2010 to August 2011. Exclusion criteria included antibiotic treatment in the previous two weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examination was contraindicated. Study procedures included lower vaginal and rectal swabs collection for GBS culture starting at 20-25 weeks (visit-1), followed by three subsequent visits (visits 2-4) at 5-6 weekly intervals, up until 37-40 weeks (visit-4) of gestational age. For GBS isolation, swabs were inoculated onto CHROMagar StrepB (CA; Media Mage, Johannesburg, South Africa). Serotyping was performed by the latex agglutination method as described (Afshar et al., 2011). Isolates that tested negative by latex agglutination for all serotypes were further typed by a PCR method using primer sequences described by Poyart *et al* (Poyart et al., 2007). Vaginal mucosal secretions were collected at 20-25 weeks of gestation and at the last visit. Mucosal secretions were collected with Oracol swabs and were extracted by centrifugation by adding 300ul of extraction buffer (10% FBS in PBS) and stored at −70 °C.

8.3.1 Measure of serotype-specific anti-capsular mucosal IgG and IgA

Quantitative mucosal IgG and IgA, immunoassay was performed on the multiplex luminex platform using Polygam (pooled gamma globulin) and pooled human serum (PHS) as reference for mucosal IgG and IgA, respectively and has been detailed in chapter 2. Serotype specific IgG and IgA values for reference serums were derived by calibrating to the standard serotype-specific GBS reference serums kindly provided by Prof. Carol J. Baker. All GBS capsular polysaccharide (CPS) antigens for assay were kindly provided by Novartis Vaccine, Italy. For the measurement of serotype-specific mucosal IgG and IgA, the optimal dilution was 1:2. The optimal secondary antibody dilutions were 1:100. The results for mucosal anti-GBS CPS IgG are given in ng per ml with lower detection limits of 0.007, 0.091 and 0.224 for mucosal IgG and 0.01, 0.032 and 0.64 for mucosal IgA for serotype Ia, III and V, respectively. For statistical analysis, samples with values below these limits were assigned values of half of lower limit of detection.

For IgG all serotypes showed 90% or higher homologous inhibition except serotype V where it was 85% and more than 10% heterologous inhibition was only observed in case of serotype Ib and III, when inhibited by serotype Ia CPS with 40% and 11%, respectively and 17% for serotype V, when inhibited by serotype III CPS. For IgA all serotypes showed 95% or higher homologous inhibition, however, very high heterologous inhibition of 82% was observed in case of serotype Ib antibodies when inhibited by serotype Ia CPS. As serotype Ia and Ib are structural isomers cross reactivity with heterologous CPS could be due to sharing of common isotopes that are identified by cross reacting antibodies. Furthermore, less than 10% cross reactivity was observed in between serotype Ia antibodies and serotype Ib CPS, therefore, it could be the presence of high Group B antigen contamination in serotype Ib CPS used for coating of beads that could have

overestimated the serotype Ib specific antibodies in reference serum which were further adsorbed by group B antigen present in the Ia CPS used for inhibition. More than 10% heterologous inhibition was also observed in case of serotype III (22%), when inhibited by serotype Ia CPS.

8.3.2 Study definitions and statistical analysis

The study definitions and statistical analysis has been detailed in chapter 6. Briefly, A new episode of GBS recto-vaginal colonization (New acquisition group) was defined as a subject not colonized by the specific serotype at Visit-1 and who became colonized at any subsequent visit (vaginal or rectal or both) by that serotype. Participants not colonized with a specific serotype at Visit-1 and at all subsequent visits, were regarded as having been free of colonization for that serotype (non-colonized group) and used as the comparator group when evaluating the association between CPS antibody and risk for serotype-specific recto-vaginal acquisition. Furthermore, the association of mucosal IgG or IgA and clearance of serotype specific GBS colonization was evaluated in participants colonized by a specific serotype at Visit-1 who remained colonized throughout the study (persistent carriers) and compared them to those in whom colonization was cleared by Visit-4 (Intermittent carriers).

Differences in GMC's of serotype-specific anti-capsular antibodies between groups were analyzed on log transformed data by Student-t test. Evaluation of whether the serotype specific mucosal IgG or IgA were associated with new homotypic GBS acquisition, was undertaken using logistic regression analysis reporting the Odds ratio (OR) for the association between a serological variable and GBS acquisition (with

logarithmic mucosal IgG or IgA as a covariate). Correlations between serum IgG and mucosal IgG were determined on log transformed data by Spearman rank correlation. A p-value of <0.05 was considered significant.

8.4 Results

Of 661 participants, 76.7% (507/661) completed all four study-visits and 95.1% (629/661) completed at least two study-visits. A detailed profile of participant demographics and follow-up has been reported (Chapter 6)

8.4.1 Association between serotype-specific anti-CPS mucosal IgG and IgA and new acquisition of the homotypic serotype

The logistic regression analysis for the association between homotypic serotype acquisition and serotype-specific mucosal IgG concentration at enrolment demonstrated OR<1.00, indicating that the probability of a new acquisition decreased with increasing serotype-specific mucosal IgG concentrations; Table 8.1. Participants who remain uncolonized throughout study trended toward higher GMCs of serotype-specific GBS CPS mucosal IgG concentrations than women who acquired the homotypic serotype; Table 8.1. The distributions of serotype-specific mucosal IgG antibody levels for women who acquired GBS and those who remained un-colonized are summarized in Figure 8.1. For mucosal IgA, pregnant women who remained uncolonized trended to have higher GMC's than those who became colonized for serotype V (2.15 vs 1.03, p=0.06), but not for any of the other serotypes.

Analysing women who completed all four study visits and were not colonized by a specific serotype at enrolment, serotype-specific new-acquisition was associated with increase in GMC's between enrolment and visit-4 (Table 8.2). These increases were evident for mucosal IgG GMC's for serotype Ia ($p=0.003$) and III ($p<0.001$); For serotype III and V, new acquisition event was also associated with increase in correlation between serotype-specific mucosal IgG and serum IgG with Rho values of 0.477 ($p=0.004$) and 0.210 ($p=0.45$) at enrolment to Rho values of 0.764 ($p<0.001$) and 0.500 ($p=0.58$) at visit-4 for serotype III and V, respectively. For mucosal IgA new acquisition event was associated with increase in GMC's for serotypes V only ($p=0.023$, Table 8.2).

Table 8.1: Association between serotype specific anti-capsular mucosal IgG and mucosal IgA concentrations on acquisition of serotype-specific Group B streptococcus recto-vaginal colonization in pregnant women

Variable and serotype	Non-colonized[a] GMC [e], ng/ml (95% CI)	New acquisitions [b] GMC, ng/ml (95% CI)	p-value [c]	Odds Ratio [d] (95% CI)
Mucosal IgG				
Ia	0.90 (0.68, 1.18) n=52[g]	0.47 (0.22, 1.00) n=387	0.10	0.81 (0.63, 1.05)
III	0.97 (0.81, 1.15) n=39	0.72 (0.40, 1.29) n=414	0.33	0.80 (0.52, 1.25)
V	1.91 (1.66, 2.19) n=17	1.21 (0.65, 2.24) n=471	0.21	0.60 (0.27, 1.34)
Mucosal IgA				
Ia	0.11 (0.09, 0.13)	0.09 (0.05, 0.17)	0.61	0.92 (0.68, 1.26)
III	0.20 (0.16, 0.24)	0.14 (0.07, 0.27)	0.29	0.80 (0.52, 1.21)
V	2.15 (1.85, 2.49)	1.03 (0.51, 2.05)	0.06	0.46 (0.20, 1.04)

[a] Non-colonized: women who remained uncolonized by specific serotype; [b] New acquisition: women who acquired serotype-specific new acquisition; [c] p-value calculated by students-t test; [d] Odds ratio by logistic regression analysis; [e] GMC : geometric mean concentration; [g] n: number of participants.

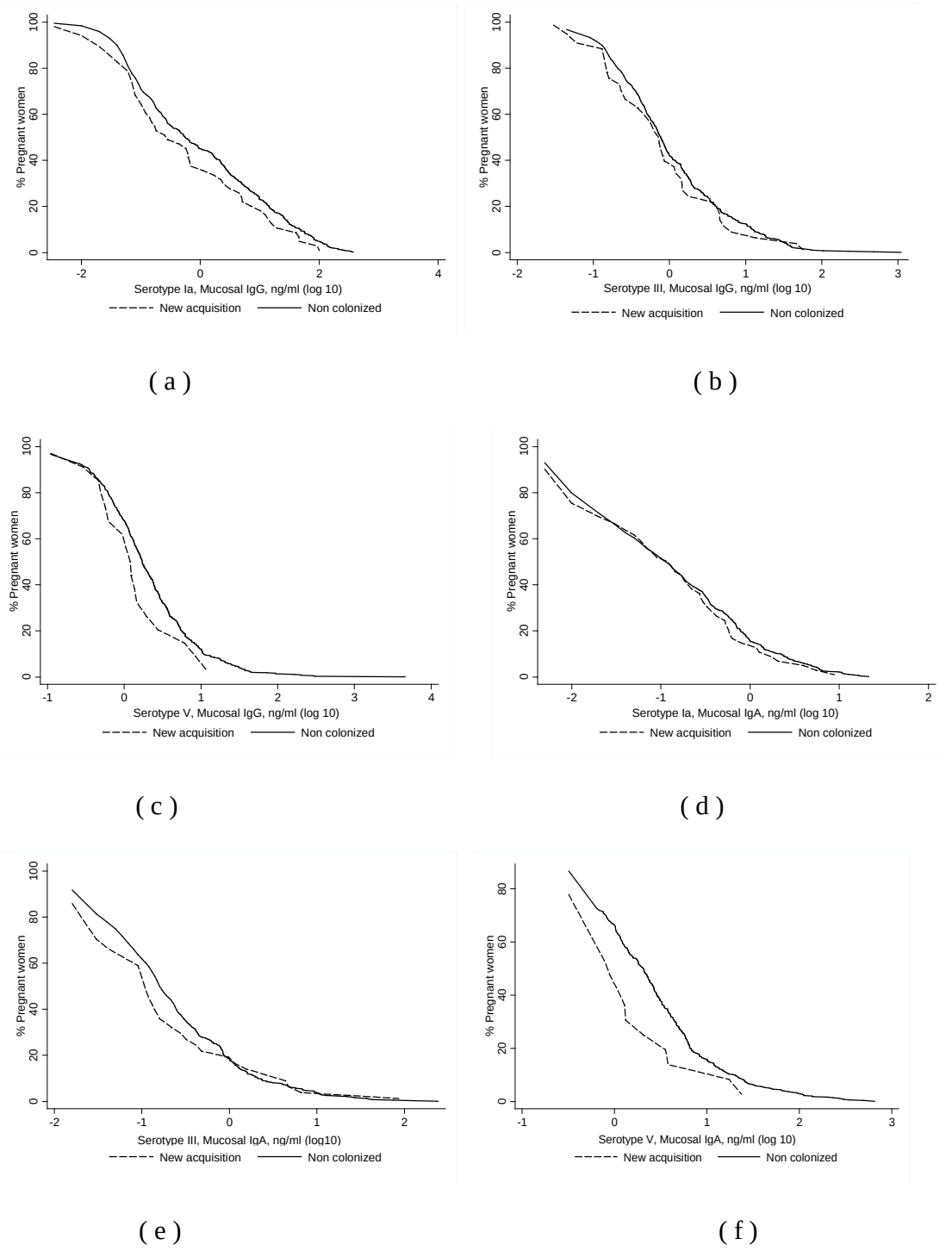


Figure 8.1: Reverse cumulative distribution curves of serotype-specific anti-capsular mucosal IgG (Fig 8.1 a-c) and mucosal IgA (Fig 8.1 d-f) in participants who acquired GBS serotype (New acquisition) and those who remained uncolonized by that serotype (Non-colonized)

Table 8.2: Comparisons of serum geometric mean concentration (GMC) serotype-specific capsular IgG antibody (µg/ml) and geometric mean opsonophagocytic titer (GMOPT) at enrolment and end of study in pregnant women who acquired GBS and those who remained uncolonized throughout

Participants with serotype-specific new acquisition				Correlation between serum IgG and Mucosal IgG	
Serotype	Visit 1	Visit 4	p-value[a]	Visit 1	Visit 4
Mucosal IgG	GMC[b] (95% CI)	GMC (95% CI)		Rho, p-value [d]	Rho, p-value
Ia	0.49 (0.20, 1.18); n=42[c]	1.88 (0.73, 4.87)	0.003	0.724, p<0.001	0.708, p<0.001
III	0.68 (0.34, 1.33); n=35	1.18 (0.61, 2.26)	0.16	0.477, p=0.004	0.764, p<0.001
V	0.95 (0.53, 1.71); n=15	3.16(1.61, 6.18)	0.003	0.211, p=0.45	0.500, p=0.06
Mucosal IgA					
Ia	0.09 (0.05, 0.18)	0.18 (0.08, 0.38)	0.06		
III	0.14 (0.07, 0.30)	0.28 (0.14, 0.53)	0.12		
V	0.83 (0.43, 1.58)	2.56 (0.98, 6.66)	0.012		

[a] p-value by paired students-t test; [b] GMC : geometric mean concentration; [c] n: number of participants; [d] Rho value by Spearman correlation test

8.4.2 Correlation between serum IgG and mucosal IgG concentrations

The concentration of GBS serotype specific CPS IgG in vaginal mucosal secretion correlated with the serum IgG concentrations with Rho values of 0.839, 0.621 and 0.418; all $p < 0.001$, for serotype Ia (figure 8.2), III (figure 8.3) and V (figure 8.4), respectively. The number of positive samples for CPS IgG in mucosal secretion were 99.6% (234/235), 99.2% (133/134) and 97.8% (227/232) for serotype Ia, III and V, respectively, when CPS IgG concentration in serum exceeded 1µg/ml for the respective serotypes. Correspondingly, the number of IgG positive samples were 69.7% (297/426), 92.2% (486/527) and 91.8% (394/429) when concentration in serum was <1µg/ml.

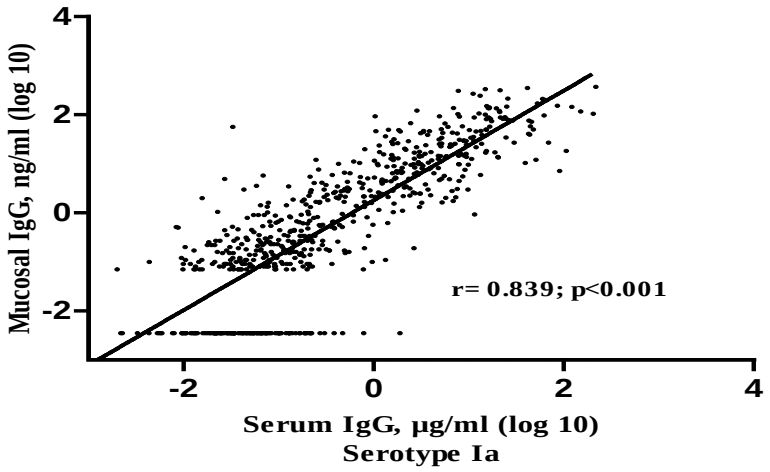


Figure 8.2: Correlation between serum IgG and mucosal IgG for serotype Ia

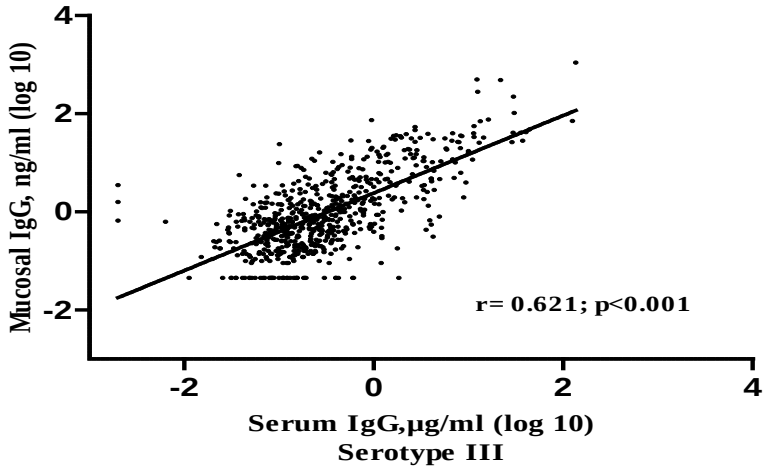


Figure 8.3: Correlation between serum IgG and mucosal IgG for serotype III

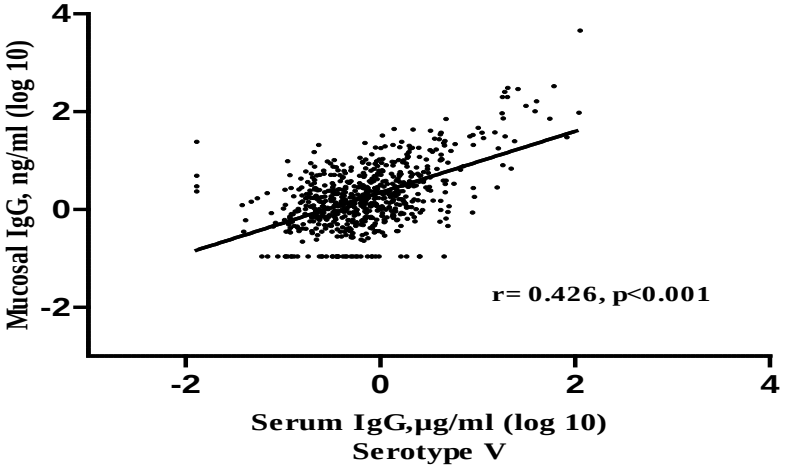


Figure 8.4: Correlation between serum IgG and mucosal IgG for serotype V

8.4.3 Association between serotype-specific CPS IgG and clearance of colonization evident at enrolment

Serotype specific mucosal IgG antibody concentration was higher among women colonized compared to those not colonized by the homotypic serotype at visit-1 for

serotypes III (p=0.005) and at visit-4 for serotype-Ia (p=0.001), with similar trends observed for other serotypes; Table 8.3. For mucosal IgA, no association was observed between colonization and IgA concentration except for serotype Ia at visit-4 (p=0.004). There was no association between mucosal IgG or IgA and clearance of serotype-specific GBS colonization among women already colonized at Visit-1; Table 8.3. Women colonized at Visit-1 in whom carriage persisted, paradoxically, had higher IgG GMC compared to those in whom colonization was cleared; Table 8.4.

Table 8.3: Cross-sectional comparison of geometric mean concentration (GMC) of serotype-specific CPS antibody (ng/mL) in pregnant women who were colonized and not colonized by the homotypic serotype at visit-1 or visit-4

Serotype	Visit-1			Visit-4		
Mucosal IgG	Colonized GMC[a](95% CI)	Not-colonized GMC(95% CI)	p-value[b]	Colonized GMC(95% CI)	Not colonized GMC (95% CI)	p-value
Ia	1.39 (0.75, 2.56); n=71[c]	0.87 (0.67, 1.12); n=436	0.17	4.79 (2.24, 10.21); n=50	1.23 (0.95, 1.60); n=457	0.001
III	1.90 (1.15, 3.14); n=56	0.94 (0.79, 1.10); n=451	0.005	1.54 (0.86, 2.73); n=52	1.41 (1.21, 1.64); n=455	0.73
V	2.20 (1.35, 3.60); n=15	1.85 (1.77, 2.17); n=492	0.66	3.59 (2.07, 6.21); n=18	2.47 (2.19, 2.80); n=489	0.26
Mucosal IgA	GMC[a](95% CI)	GMC(95% CI)		GMC(95% CI)	GMC (95% CI)	
Ia	0.12 (0.07, 0.20); n=71	0.11 (0.90, 0.16); n=436	0.75	0.38 (0.20, 0.70); n=50	0.14 (0.11, 0.17); n=457	0.004
III	0.29 (0.17, 0.49); n=56	0.19 (0.16, 0.22); n=451	0.11	0.30 (0.16, 0.54); n=52	0.29 (0.24, 0.35); n=455	0.89
V	2.17 (1.01, 4.63); n=15	2.05 (1.77, 2.37); n=492	0.89	2.55 (1.20, 5.40); n=18	2.52 (2.14, 2.96); n=489	0.97

[a]GMC: geometric mean concentration;[b] p-value by students-t test; [c]n: number of participants

Table 8.4: Comparisons of geometric means of Mucosal IgG and Mucosal IgA (ng/ml) concentrations at baseline (visit 1) for pregnant women who were colonized throughout by particular serotype (Persistent carriers) compared to those who lost colonization by 4th visit (Intermittent carriers)

Variable and Serotype	Persistent carriers GMC [b] (95% CI)	Intermittent carriers GMC (95% CI)	[a] p-value
Mucosal IgG			
Ia	4.21(1.11, 16.0), n=21[c]	0.77(0.39, 1.55),n=43	0.01
III	1.63(0.87, 3.08), n=27	2.32(0.71, 7.51), n=18	0.56
V	4.02 (0.34, 4.65), n=2	2.96 (0.86, 5.44), n=9	0.53
Mucosal IgA			
Ia	0.37(0.12, 1.10)	0.08(0.04, 0.15)	0.01
III	0.31(0.13, 0.70)	0.34(0.14, 0.78)	0.86
V	6.39 [d]	1.21(0.53, 2.76)	0.10

[a] p-value by student-t test; [b]GMC : geometric mean concentration; [c] number of participants; [d] not presented due to very wide CI

8.5 Discussion

Our study demonstrated a correlation between mucosal IgG and serum IgG and supports the notion that mucosal IgG is derived from serum (Nurkka et al., 2001). On the same cohort, we established that serum capsular specific antibody reduced the risk of new homotypic serotype acquisition between 20 to 37+ weeks of pregnancy (Chapter 6). As measurable amount of CPS IgG antibodies have been found in vaginal mucosal secretions and correlated with serum IgG concentrations measured in the same cohort, this suggesting that higher amount of diffused IgG on mucosal surface may have contributed to the reduced risk of colonization. For *Streptococcus pneumoniae* it has been suggested that, new acquisition of a serotype is reduced due to serum IgG of respective serotype diffused on the mucosal surface that binds to the polysaccharide capsule of the *Streptococcus pneumoniae* and create a steric inhibition of the interaction of pneumococcal surface proteins with binding sites on the mammalian epithelial cell surface (Dagan et al., 2005).

When the effect of naturally acquired mucosal IgG and IgA on new acquisition of GBS was analyzed, the coefficient of the logarithm antibody concentrations was negative in each of the regression analyses, which suggests that there is a lower probability of new acquisition of GBS with increasing mucosal IgG and IgA concentrations. No statistical significance was, however, identified for any of the studied serotypes. Also, the paradoxically higher GMC in women colonized with GBS serotype at enrolment and who remained persistently-colonized thereafter compared to those who lost colonization, indicates that the antibody which was likely induced by colonization, might not clear existing colonization. This further supports the previous finding on the same cohort that

cell-mediated immunity rather than humoral immune response is required for clearance of established GBS colonization (Chapter 7).

In summary, our results indicate that mucosal IgG is derived from serum and high concentrations of pre-existing mucosal GBS CPS-specific IgG in maternal sera during pregnancy may protect against colonization by the homotypic serotype.

Our study has few limitations. Firstly, our study is limited by the sensitivity of detection of GBS on selective media which is estimated at 85% and by the fact that in most cases only the dominant serotype was determined and the serotypes carried in minority may be missed (Chapter 4). Therefore, it is possible that we missed continued colonization and potentially overestimated new acquisition or missed colonization altogether and underestimated new acquisition. Second limitation is lack of precise knowledge about the dilution factor introduced during extraction of vaginal mucous from the Oracol swab and furthermore, there might be an adsorption of antibodies in mucosal secretions of colonized women due to extraction of GBS along with secretions.

Chapter 9 Conclusion

The series of studies undertaken in this PhD resulted in several novel findings. Foremost, the prevalence of GBS recto-vaginal colonization among pregnant women from South Africa was very high, including a high rate (25.9%; 89/344) of new acquisitions during the last half of pregnancy. We also observed a dynamic state in clearance of GBS during the latter half of pregnancy, with 46.6% (76/163) of women clearing GBS between 26 to 37+ weeks of gestational age. The risk of new acquisition by the GBS serotype among pregnant women was inversely correlated with maternal serotype-specific antibodies, whereas loss of GBS colonization was positively correlated with serotype-specific cellular immune responses. Furthermore, we also demonstrated chromogenic medium CHROMagar Strep B produced highest recovery of GBS from vaginal and rectal swabs as compared to the selective broth method recommended by CDC.

These are the first findings that established sero-correlate of protection against GBS colonization among pregnant women and provides the foundation against which the efficacy of polysaccharide-protein GBS conjugate vaccines can be evaluated to determine whether it will protect against new GBS acquisition and/or clearance thereof during pregnancy. The thresholds of serum CPS serotype-specific antibody significantly associated with reduced odds of acquisition of GBS were ≥ 3µg/ml for serotypes Ia and III, and ≥1 µg/ml for serotype-V. Furthermore, we also demonstrated functional antibody titers determined by OPA assay correlated more strongly than serum IgG concentrations in relation to new-acquisition of serotype-Ia and III with serotype-specific OPA titers of ≥8

were significantly associated with reduction of new-acquisition of the homotypic serotype for serotypes Ia and III.

The loss of serotype-specific GBS colonization during pregnancy was associated with presence of homotypic capsular ELISpot IFN-γ positivity, whereas no such association was observed for serotype specific capsular antibody. Conversely, ELISpot positivity was not associated with a significantly reduced risk of GBS acquisition, suggesting that the immune response required for clearance of recto-vaginal GBS colonization is cell-mediated, whereas humoral immunity is required to reduce the risk of recto-vaginal GBS acquisition during pregnancy. Our study demonstrated a correlation between mucosal IgG and serum IgG and supports the concept that mucosal IgG is derived from serum.

The high incidence of new acquisition and loss of colonization during pregnancy supports late screening at 35-37 weeks' gestational age for the IAP strategy to be effective. Our findings on the dominant serotypes in South Africa are comparable with serotype distribution data of maternal colonizing isolates from industrialized countries, which could further guide in the formulation of GBS polysaccharide based vaccines. Furthermore, identification of serotype IX in our population was notable in that it is rarely reported in colonization studies and not previously described in Africa. All GBS serotypes were variable in their colonization patterns, possibly due to the complex interaction between immunity and specific GBS serotypes or due to differences in pathogenesis, which is still incompletely understood and needs further investigation.

The prevalence of GBS colonization among pregnant women in this setting is very high and requires attention as maternal colonization is a major risk factor for EOD. Even though CDC has recommended universal screening for GBS recto-vaginal colonization in pregnant women at 35-37 weeks of gestational age, coupled with IAP during labor to reduce the incidence of EOD, the implementation of IAP would be costly and logistically challenging in our setting. Therefore, vaccination of pregnant women with GBS vaccine to induce protective antibody can be an alternate preventive strategy.

In this study we also demonstrated the limitations of the selective broth enrichment method for the isolation of GBS from rectal swabs, and the advantage of using chromogenic medium for GBS isolation in terms of reduced processing time, better sensitivity and the ease with which GBS colonies can be identified on the plates. Based on the study results we recommend the use of chromogenic medium in regions with a high prevalence of antimicrobial resistant flora.

In conclusion, high serotype-specific CPS antibody and more specifically presence of functional antibody measured by opsonophagocytic activity assay, was associated with a reduced risk of subsequent acquisition of serotype-specific GBS colonization during pregnancy. A serotype-specific capsular based GBS vaccine able to elicit both humoral and cell-mediated capsular immune responses in the mother may result in decreased colonization, therefore could confer protection against EOD by reducing the exposure of the newborn's to GBS colonization during the peri-partum period. This could offer an additional mode of protection against invasive GBS disease especially for preterm newborn's who might not fully benefit from transplacental acquisition of maternal serotype specific capsular antibody which mainly occurs beyond 34 weeks gestational age.

Moreover, reduction in colonization could result in lower fetal exposure to GBS and consequently lower the risk for GBS-associated premature labor and/or stillbirths.

Recent developments in the clinical evaluation of a GBS polysaccharide-protein conjugate vaccine has renewed interest in the potential of this vaccine to protect neonates against invasive GBS disease by reducing recto-vaginal colonization during pregnancy. A trivalent GBS vaccine for pregnant women aimed at protecting newborns from invasive disease has recently completed phase-II evaluations. Further clinical evaluation of the vaccine is, however, challenging as large sample size of 60,000 would be required to demonstrate vaccine efficacy. An alternate pathway to licensure could be based on immunologic correlates of protection and by selecting surrogate markers for vaccine efficacy. Also, as maternal colonization is the prerequisite for invasive disease in infant, vaccine efficacy against colonization may be accepted as surrogate for reduction in especially EOD. If an efficacy end point is maternal GBS colonization rather than invasive disease, the sample size required for such a study would be much smaller compared to an infant invasive disease trial endpoint. The findings of our studies could inform the study design when evaluating the efficacy of maternal GBS vaccine in protecting against GBS recto-vaginal acquisition and colonization during pregnancy.

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Appendices

Appendix 1: Kwatra G, Madhi SA, Cutland CL, Buchmann EJ, Adrian PV. Evaluation of Trans-Vag broth, colistin-nalidixic agar, and CHROMagar StrepB for detection of group B Streptococcus in vaginal and rectal swabs from pregnant women in South Africa. *J Clin Microbiol* 2013; 51:2515-9.

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Appendix 5: Kwatra G, Cunnington MC, Merrall E, Adrian PV, Ip M, Klugman KP, Tam WH, Madhi SA: Prevalence of maternal colonisation with group B streptococcus: a systematic review and meta-analysis. *The Lancet Infectious diseases* 2016.

Appendix 6: Clearance Certificate granted by University of the Witwatersrand Human Research Ethics committee.

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Evaluation of Trans-Vag Broth, Colistin-Nalidixic Agar, and CHROMagar StrepB for Detection of Group B Streptococcus in Vaginal and Rectal Swabs from Pregnant Women in South Africa

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Maternal vaginal colonization with group B streptococcus (GBS) is a major risk factor for invasive GBS infection in newborns. The CDC-recommended method for detecting GBS colonization is to culture vaginal and rectal swabs in a selective broth followed by subculture on blood agar or a selective medium. A high incidence of antimicrobial resistance in the fecal microflora can compromise the recovery of GBS from the selective broth. Here, we compared CHROMagar StrepB (CA), Columbia colistin-nalidixic agar (CNA), and Trans-Vag selective broth enrichment for the isolation of GBS from 130 vaginal and 130 rectal swabs from pregnant women. The swabs were randomized for plating first on either CA or CNA, and they then were inoculated in Trans-Vag broth. GBS was cultured from 37.7% of the vaginal swabs and 33.1% of the rectal swabs. There were no differences in the detection rates for the vaginal swabs between CA (31.5%), CNA (26.2%), and the selective broth (30.0%). The sensitivities in relation to a composite score were 83.7%, 69.4%, and 79.6%, respectively. However, recovery of GBS from the rectal swabs was significantly higher from CA (29.2%; $P < 0.0001$) and CNA (23.8%; $P = 0.002$) than from the selective broth (9.2%). The sensitivities were 88.4%, 72.1%, and 27.9%, respectively. The order of plating on the solid medium was significant ($P = 0.003$), with GBS detection rates of 30.8% and 24.6% when swabs were plated first and second, respectively. These findings show that a selective broth is not suitable for the recovery of GBS from rectal swabs in settings such as ours, due to masking of the GBS colonies by persistent microflora.

Infection by group B streptococcus (GBS) is one of the most common infections in newborns (1). Maternal colonization has been found to be a major risk factor for invasive GBS disease within 6 days of birth. Approximately 10 to 40% of pregnant women are colonized with GBS in either the vagina, the rectum, or both areas (2). The rate of peripartum transmission of GBS to newborns of colonized women is approximately 50%, after which 1 to 2% of these newborns develop invasive GBS infection in the first week of life (3, 4). The Centers for Disease Control and Prevention (CDC) has recommended that all pregnant women be screened for rectovaginal carriage of GBS at 35 to 37 weeks' gestation to identify women who should receive intrapartum antimicrobial prophylaxis (IAP). When successfully implemented, IAP targeted at GBS-colonized pregnant women has reduced the incidence of invasive GBS disease by 86 to 89% (5–7). The sensitivities of screening methods for the identification of maternal carriage of GBS depends on the timing of specimen collection, the source of the specimen, and the culture technique used. Optimally, specimens should be collected as close to delivery as possible. The use of vaginal and rectal swab specimens has been shown to yield higher GBS culture-positivity rates than vaginal swabs alone or cervical specimens (8–10). The current CDC recommendation for the isolation of GBS from vaginal and rectal or rectovaginal swabs is growth in a selective broth medium (Todd-Hewitt broth with gentamicin and colistin or nalidixic acid), followed by subculture on blood agar or a selective medium (11). The reported sensitivity of selective broth for the culture of GBS is 82% to 99% (12–15). There are, however, limitations to this approach. The procedure requires at least an additional 24 h of culture time compared to that for direct plating on selective agar, and isolated GBS-like colonies require further identification with ancillary tests, e.g., the CAMP factor or B antigen test (11). Furthermore, identification of GBS-like colonies on blood agar requires laboratory expertise, particularly when they are mixed with other microflora (16). Considering methods to decrease the GBS detection time, studies have shown that direct plating on colistin-nalidixic agar (CNA) is a low-cost alternative for GBS recovery, albeit with a lower sensitivity that has ranged from 59% to 83% (17–19). In recent years, several commercial chromogenic media, such as Granada medium, CHROMagar StrepB (CA), and chromID Strepto B agar, have been tested for their suitability for detecting GBS (18, 20). CA is a commercially available selective chromogenic medium that inhibits most saprophytic bacteria and yeasts and produces mauve-colored GBS colonies under aerobic conditions irrespective of their hemolytic properties, allowing direct visual identification. The use of chromogenic media may improve the yield of GBS while reducing labor costs and turnaround time. The clinical sensitivities of these media vary from study to study, with most studies reporting 93% to 98% for CA (16, 21), 40% to 91% for

Received 30 January 2013 Returned for modification 6 April 2013
Accepted 3 May 2013
Published ahead of print 22 May 2013
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doi:10.1128/JCM.00251-13

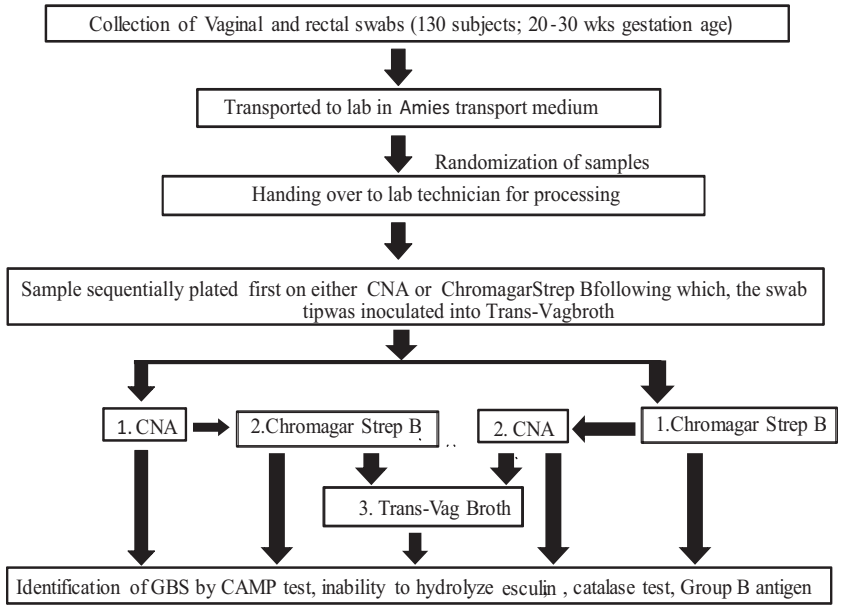

FIG 1 Procedure for the collection, randomization, and laboratory handling of the vaginal and rectal swabs for the isolation of group B streptococcus.

Granada medium (12, 18, 22), and 88% to 95% for chromID Strepto B agar (13, 18). The aim of this study was to evaluate direct plating on CA and CNA and selective broth enrichment for the isolation of GBS in swabs from pregnant women.

MATERIALS AND METHODS

Study design. The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (IRB/Protocol-M090937) and conducted at prenatal community clinics in Soweto, Johannesburg. Included were HIV-uninfected pregnant women at more than 20 weeks of gestation who were able to provide informed consent for participation. Exclusion criteria were concurrent antibiotic use, an acute illness, a symptomatic vaginal discharge, and a known or suspected condition in which clinical vaginal examinations were contraindicated. A convenience sampling of lower vaginal and rectal swabs was collected from 130 pregnant women between January 2011 and April 2011 by trained study nurses.

Sample collection. Samples were collected with rayon-tipped swabs that were placed into 5 ml of Amies transport medium without charcoal (catalog no. MW170, Transwab Amies; Medical Wire, UK). All samples were transported to the laboratory within 4 h of collection, where they were processed within 2 h.

Randomization and processing of samples. All vaginal and rectal swabs were randomized using a randomization log. The swab number was linked to three different randomly numbered labels, one for each culture medium type. Each sample was randomized to be plated first on either CNA or CA, after which the swab tip was inoculated into the selective (Trans-Vag) broth (Todd-Hewitt broth with 8 μg/ml gentamicin and 15 μg/ml nalidixic acid) and incubated for 24 h before being plated on 5% sheep blood agar (SBA) (Fig. 1). The randomization process ensured that the technicians reading the plates and entering the data were blinded with respect to the participant, the type of swab (rectal or vaginal), the order of plating, and the swab/plate pairs. The CNA and SBA plates were incubated at 37°C in 5% CO_2 for 18 to 24 h, and the CA plates were incubated at 37°C for 18 to 24 h under aerobic conditions. If the expected colonies were not visible after 24 h of incubation, the plates were reincubated for an additional 24 h and reexamined for growth. Up to four GBS-like colonies were isolated from the solid culture medium on the basis of the colony morphology and mauve color on CA and the colony morphology and beta-hemolysis on CNA and blood agar. Isolates were confirmed to be GBS by testing for CAMP factor, an inability to hydrolyze esculin, catalase negativity, and group B antigen latex agglutination positivity (Omega Diagnostics, Scotland, UK). The CA and CNA prepoured plates were purchased from Media Mage (Johannesburg, South Africa).

Statistical methods. Descriptive statistics included statements of frequency with percentages, means ± standard deviations (SDs), and medians with interquartile ranges (IQRs). Sensitivities (detection rates) were calculated by comparing the proportion of positive samples for each medium in relation to a gold standard, which was a composite positive for all test media. The McNemar test for correlated percentages and the chi-square test with Yates' correction were used to compare the culture methods, with P values of <0.05 being considered statistically significant.

RESULTS

Study population. The mean age (SD) of the women at swabbing was 25.7 years (±5.9 years), and the mean gestational age (SD) was 23.8 weeks (±3.2 weeks). The median parity and gravidity were 1 (IQR, 0 to 1) and 2 (IQR, 1 to 2), respectively.

CA and CNA versus Trans-Vag selective broth enrichment. Overall, 37.7% of the vaginal swabs and 33.1% of the rectal swabs tested positive for GBS (Table 1). There were no statistically significant differences in the detection rates for the vaginal swabs between CA (31.5%), CNA (26.2%), and the selective broth (30.0%). The sensitivities with respect to the composite scores were 83.7%, 69.4%, and 79.6%, respectively. The recovery of GBS from the rectal swabs, however, was significantly higher from CA (29.2%; $P < 0.0001$) and CNA (23.8%; $P = 0.002$) than from the selective broth medium (9.2%). The sensitivities were 88.4%, 72.1%, and 27.9%, respectively. Despite the higher sensitivity reported for CA than for CNA for the vaginal and rectal swabs, these differences were not statistically significant ($P > 0.05$).

Accuracy. The accuracy of a medium was defined as the per-

TABLE 1 Performance characteristics of CA, CNA, and the selective broth for detection of GBS from vaginal and rectal swabs

Medium	Vaginal swabs ($n = 130$)			Rectal swabs ($n = 130$)		
	No. (%) positive for GBS	Sensitivity (%)[a]	Accuracy (%)[b]	No. (%) positive for GBS	Sensitivity (%)[a]	Accuracy (%)[b]
CA	41 (31.5)	83.7	155/167 (92.8)	38 (29.2)	88.4	132/159 (83.0)
CNA	34 (26.2)	69.4	129/153 (84.3)	31 (23.8)	72.1	116/150 (77.3)
Selective broth	39 (30.0)	79.6	155/171 (90.6)	12 (9.2)	27.9	48/88 (54.5)
Total GBS isolated	49 (37.7)			43 (33.1)		

[a] Sensitivity was calculated in comparison to composite scores of all media.
[b] Accuracy was calculated as the number of confirmed GBS colonies isolated/total number of GBS-like colonies that were confirmed as either positive or negative for GBS.

centage of isolated colonies that were identified as GBS by confirmatory testing divided by the total number of GBS-like colonies that were isolated from a plate and subjected to confirmatory tests. The denominator includes both confirmed GBS colonies (true positives) and colonies having appearances similar to those of GBS but failing the confirmatory tests (false positives). For the vaginal swabs, CA was the most accurate for discriminating between true-positive and false-positive isolates (92.8%), followed by selective broth enrichment (90.6%) and CNA (84.3%). There were no significant differences in the accuracies of primary identification between CA and the selective broth or between CNA agar and the selective broth. However, after direct plating, colonies were significantly more likely to be GBS following isolation with CA than with CNA ($P = 0.026$).

The accuracy of GBS identification from the rectal swabs was significantly better with CA (83.0%; $P < 0.0001$) and CNA (77.3%; $P = 0.0004$) than with selective broth enrichment (54.5%). CA was found to be more accurate than CNA, but these differences were not statistically significant ($P = 0.26$). The numbers of GBS-like colonies that were identified from the cultured vaginal and rectal swabs were similar between CA (167 vaginal, 159 rectal) and CNA (153 vaginal, 150 rectal). However, about half the numbers of GBS-like colonies were identified among the other flora following selective broth enrichment from the rectal swabs (171 vaginal, 88 rectal). A higher percentage of false-positive GBS isolates was reported from the rectal swabs than from the vaginal swabs with CA (17.0% versus 7.2%; $P = 0.01$), CNA (22.7% versus 15.7%; $P = 0.16$), and the selective broth (45.5% versus 9.4%; $P < 0.0001$) (Table 1).

Impact of randomization and the order of plating. The order in which the swabs were plated onto the two selective media had a significant effect on the culture positivity rate. Out of 260 swabs tested directly on CNA and CA, a total of 86 (33.1%) swabs were positive for GBS. Direct culture on CNA and CA yielded GBS from 80 (30.8%) swabs when plated first compared to 64 (24.6%) when plated second ($P = 0.003$). GBS was isolated from both media in 58 (22.3%) of the swabs. The numbers of swabs from which GBS was isolated from a single medium were 22 (8.5%) and 6 (2.3%) from the first and second platings, respectively. When CNA and CA were plated first, the sensitivities were 86.4% and 87.5%, respectively, and when CNA and CA were plated second, the sensitivities were 57.4% and 82.2%, respectively (Table 2).

DISCUSSION

Current CDC guidelines for prenatal GBS screening recommend the collection of a single rectovaginal swab or separate vaginal and rectal swabs that are enriched overnight in Todd-Hewitt broth with antibiotics, followed by culture on blood agar or a selective medium (11). While these guidelines were developed within the context of high-income countries, there are few data on the validity of this method in low- to middle-income countries where there are differences in the distributions of saprophytic organisms and antimicrobial resistance is common (23–26). In this study, we compared the recovery rates of GBS by standard methods with direct plating onto either CNA or CA from either the vaginal or rectal swabs from pregnant South African women to determine whether these alternatives would provide better recovery rates. One of the main reasons for this comparison was the presence of vaginal flora that are resistant to antimicrobial agents, particularly *Proteus* spp., which occur in a small percentage of swabs and are able to persist in Trans-Vag broth and swarm the subcultured blood agar plate, rendering it unreadable (P. V. Adrian, personal communication). This necessitated the use of both Trans-Vag

TABLE 2 Performance characteristics of CNA and CA with respect to the order of plating

Specimen	CNA		CA		Trans-Vag[a]		
	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	No. (%) positive for GBS	Sensitivity (%)	Total no. (%) positive for GBS
Plated first on CAN							
Vaginal ($n = 65$)	20 (30.8)	80	21 (32.3)	84	23 (35.4)	92	25 (38.5)
Rectal ($n = 65$)	18 (27.7)	94.7	16 (24.6)	84.2	8 (12.3)	42.1	19 (29.2)
Total ($n = 130$)	38 (29.2)	86.4	37 (28.5)	84.1	31 (23.8)	70.4	44 (33.8)
Plated first on CA							
Vaginal ($n = 65$)	14 (21.5)	58.3	20 (30.8)	83.3	16 (24.6)	66.7	24 (36.9)
Rectal ($n = 65$)	13 (20)	54.2	22 (33.8)	91.7	4 (6.2)	16.7	24 (36.9)
Total ($n = 130$)	27 (20.8)	56.2	42 (32.3)	87.5	20 (15.4)	41.7	48 (36.9)

[a] After direct plating, the swab was placed in Trans-Vag broth.

broth and direct plating on CNA in a previous study of vaginal swabs from South Africa (27).

In our study, there were no statistically significant differences in the recoveries of GBS from vaginal swabs between the different media, suggesting that any of these three options is valid in our clinical setting and the choice can be made based on cost, availability, and desired turnaround time. In contrast, the recovery of GBS from rectal swabs following selective broth enrichment was inferior to that with either of the direct plating methods. The main reasons for the poor recovery of GBS were survival and overgrowth of non-GBS organisms in the Trans-Vag broth, which masked the presence of GBS on the subculture plates. The results of our study differ from those of several previously published reports, in which it was shown that the selective broth method is more sensitive than direct plating on a selective medium for recovering GBS from rectal or rectovaginal swabs (12–15). While these results may be valid in settings such as those where the studies were conducted, other studies from Brazil, Denmark, and Spain demonstrated better recoveries of GBS from rectal swabs with a solid selective medium than with a selective broth (28–30). Pigmented enrichment broth, such as StrepB carrot broth, has been shown to increase the sensitivity of GBS detection by culture over that with LIM broth (Todd-Hewitt broth with colistin and nalidixic acid) (31) when used as an enrichment step prior to PCR screening (32); however, GBS growth and pigment development can still be suppressed by the overgrowth of fecal bacteria and mask the presence of GBS on culture (32). Since the mechanism behind the poor recovery of GBS from rectal swabs is related to persistence and overgrowth of resistant fecal bacteria (30), it is likely that the collection of a single rectovaginal swab or the coprocessing of vaginal and rectal swabs may be severely compromised by the use of a selective broth in our setting.

Overall, CA produced the highest recovery rate of GBS from the vaginal and rectal swabs. One advantage of the chromogenic medium was that it was significantly easier to discriminate between GBS and group D streptococcus (GDS) in cocolonized samples due to differences in the color development of the colonies. GBS turns mauve, whereas GDS turns blue. This is particularly important in comparisons with samples plated on blood agar, where the GBS colonies are in the minority and single GBS colonies can be overlooked amid large numbers of morphologically similar GDS. Despite CA being more expensive than CNA or a selective broth-blood agar combination, CA offers cost savings in terms of reduced processing time and the ease with which GBS colonies can be identified on the plates. Moreover, due to the increased accuracy of colony identification through pigment formation, the number of confirmatory tests required is reduced, as the need for additional tests to rule out the morphologically similar colonies of group D streptococci is eliminated. While the calculated values of accuracy will vary with the skill and experience of the users, CA offers an easier learning curve compared to that for a blood-based isolation medium. In a comparison of the two selective solid agars, CA had higher sensitivity and accuracy than CNA for the vaginal and rectal swabs, although these values were not statistically significant, most likely due to a limited sample size.

From a review of the literature, no study in a clinical setting had considered the order of plating when comparing and reporting the sensitivities of culture media to isolate GBS. In this study, the order of plating had a significant effect on the recovery of GBS, which suggests that future studies where solid media are compared should follow randomized protocols to prevent detection bias based on plating order. Possible reasons for the lower recovery rate of GBS from the swabs plated for a second time include removal of the organisms from the surface of the swab in the first plating and blockage of the swab surface by sterile agar from the previous plating, preventing transfer of organisms. The sensitivity of direct plating on CNA when plated first in this study was similar to that described by Louie et al. (19), who also inoculated swabs on CNA first.

In conclusion, selective broth enrichment showed significantly lower sensitivity for the recovery of GBS from rectal swabs. This method is not suitable for the recovery of GBS from rectal swabs or rectovaginal swabs in regions with a high prevalence of antimicrobial-resistant flora. The recovery of GBS from the vaginal swabs plated on CA appears to have sensitivity equal to that of the selective broth and offers a less time-consuming and less labor-intensive process. For these reasons, our laboratory has started to use CA for current and future studies.

ACKNOWLEDGMENTS

This project was funded by the South African National Research Foundation (Vaccine Preventable Diseases Research Chair Grant). The laboratory has received funding from Novartis for clinical trials of GBS vaccines. The funders had no role in study design, data collection or analysis, the decision to publish, or preparation of the manuscript.

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Serotype-Specific Acquisition and Loss of Group B *Streptococcus* Recto-Vaginal Colonization in Late Pregnancy

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Abstract

Background: Maternal recto-vaginal colonization with Group B *Streptococcus* (GBS) and consequent vertical transmission to the newborn predisposes neonates to early-onset invasive GBS disease. This study aimed to determine the acquisition and loss of serotype-specific recto-vaginal GBS colonization from 20–37+ weeks of gestational age.

Methods: Vaginal and rectal swabs were collected from HIV-uninfected women at 20–25 weeks of gestation age and at 5–6 weekly intervals thereafter. Swabs were cultured for GBS and isolates were serotyped by latex agglutination. Serologically non-typable isolates and pilus islands were characterized by PCR.

Results: The prevalence of recto-vaginal GBS colonization was 33.0%, 32.7%, 28.7% and 28.4% at 20–25 weeks, 26–30 weeks, 31–35 weeks and 37+ weeks of gestational age, respectively. The most common identified serotypes were Ia (39.2%), III (32.8%) and V (12.4%). Of 507 participants who completed all four study visits, the cumulative overall recto-vaginal acquisition rate of new serotypes during the study was 27.9%, including 11.2%, 8.2% and 4.3% for serotypes Ia, III and V, respectively. Comparing the common colonizing serotypes, serotype III was more likely to be associated with persistent colonization throughout the study (29%) than Ia (18%; $p = 0.045$) or V (6%; $p = 0.002$). The median duration of recto-vaginal GBS colonization for serotype III was 6.35 weeks, which was longer than other serotypes. Pilus island proteins were detected in all GBS isolates and their subtype distribution was associated with specific serotypes.

Conclusion: South African pregnant women have a high prevalence of GBS recto-vaginal colonization from 20 weeks of gestational age onwards, including high GBS acquisition rates in the last pregnancy-trimesters. There are differences in specific-serotype colonization patterns during pregnancy.

Citation: Kwatra G, Adrian PV, Shiri T, Buchmann EJ, Cutland CL, et al. (2014) Serotype-Specific Acquisition and Loss of Group B *Streptococcus* Recto-Vaginal Colonization in Late Pregnancy. PLOS ONE 9(6): e98778. doi:10.1371/journal.pone.0098778

Editor: Bernard Beall, Centers for Disease Control & Prevention, United States of America

Received January 18, 2014; **Accepted** May 6, 2014; **Published** June 30, 2014

Funding: This work was funded by research supported by the South African Research Chairs Initiative in Vaccine Preventable Diseases of the Department of Science and Technology and National Research Foundation. Part-funding was also granted by Novartis Vaccines, Italy. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript.

Competing Interests: RMPRU Laboratory has received partial funding for this study from a commercial source (Novartis Vaccines). However, Novartis vaccines did not play any role in the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript. Further, this does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

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Introduction

Maternal vaginal colonization with Group B *Streptococcus* (GBS) is the major risk factor for early onset invasive GBS disease (EOD) in newborns [1,2]. Screening of pregnant women for GBS colonization during the third trimester, coupled with targeted intrapartum antibiotic prophylaxis (IAP) of colonized women during labor, has reduced the incidence of invasive GBS disease in industrialized countries [3].

An alternate preventive strategy against EOD is vaccination of pregnant women, which could enhance transplacental transfer of anti-GBS antibody to the fetus. Studies have identified an association between high maternal serotype-specific anti-capsular polysaccharide (CPS) antibody concentrations with reduced risk of recto-vaginal colonization and reduced risk of newborns developing EOD [4,5]. Since GBS CPS-protein conjugate vaccines are serotype-specific, it is important to characterize the serotype distribution of GBS in different regions of the world as well as understand the changes which occur in GBS colonization during pregnancy [6]. Other potential vaccine candidates include GBS surface protein antigens such as pilus island (PI) proteins that are present in all GBS isolates [7]. Although it has been shown that maternal GBS colonization during pregnancy may fluctuate [8,9,10], there are limited longitudinal studies on the rate of

serotype-specific GBS acquisition and duration of colonization during pregnancy.

We aimed to determine the acquisition and loss of GBS recto-vaginal colonization, including serotype-specific changes, among South African pregnant women from 20 weeks to at least 37 weeks of gestational age. We also studied the PI distribution of recto-vaginal colonizing GBS isolates and their association with capsular serotype.

Materials and Methods

Study Population

The study was conducted at prenatal community clinics in Soweto (Lillian Ngoyi, Diepkloof, Mofolo and Michael Maponya), Johannesburg from August 2010 to August 2011. Inclusion criteria were HIV-uninfected pregnant women confirmed by HIV ELISA test non-reactivity at enrolment, from 20–25 weeks of gestational age based on last menstrual cycle and who consented to study participation. Exclusion criteria at enrolment included antibiotic treatment in the previous two weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition in which clinical vaginal examinations were contradicted. If antibiotics were taken after the first visit, the collection of specimens was delayed for at least two weeks after the last antibiotic dose.

Swab collection and culture of GBS

Lower vaginal and rectal swabs were collected for GBS culture starting at 20–25 weeks (Visit-1), followed by three subsequent visits (Visits 2–4) at 5–6 weekly intervals, up to 37–40 weeks (Visit-4) of gestational age. Demographic and pregnancy-related data were collected at the first visit. All samples were collected by trained study nurses with rayon-tipped swabs that were placed into Amies transport medium without charcoal (cat #MW170, Transwab Amies, Medical wire, U.K.). Swabs were transported to the lab within 4 hours of collection, and processed within 2 hours. For GBS isolation, swabs were inoculated onto CHRO-Magar StrepB (CA; Media Mage, Johannesburg, South Africa) and the CA plates were incubated at 37°C for 18–24 hours in aerobic conditions [11]. If GBS-like colonies were not visible within 24 hours after incubation, the plates were incubated for a further 24 hours and re-examined for growth. Up to four GBS-like colonies were isolated and confirmed as GBS by testing for CAMP factor, inability to hydrolyze esculin, catalase negativity and group B antigen.

Capsular serotyping

Serotyping was performed by the latex agglutination method with specific antisera against types Ia, Ib and II to IX CPS antigens (Statens Serum Institute, SSI, Sweden) as described [12]. Isolates that tested negative by latex agglutination for all serotypes were further typed by a PCR method for serotypes Ia, Ib, II, III, IV and V using primer sequences described by Poyart *et al* [13]. The gene encoding dlts was used as a PCR positive control for GBS identification.

Pilus typing

Pilus island proteins of all GBS isolates were detected by PCR for PI-1, PI-2a and PI-2b, with primers that target the genomic regions coding for the ancillary protein (AP)-1 of each PI. Isolates that tested negative for all the AP1 genes, or isolates from which neither PI-2a or PI-2b could be detected, were amplified by a second set of primers representing conserved regions of AP-2 as described previously [7].

Statistical analysis

Data were analyzed using SAS version 9.2 software (SAS Institute, Inc., NC, USA). A visit sample pair of vaginal and rectal swabs was considered negative if no GBS growth was evident on either swab, and positive if GBS was grown from either swab. The pregnant women were grouped into transient, intermittent and persistent carriers according to the presence of GBS colonization and to individual serotypes at the four sampling time points. Transient carriers were defined as women who were colonized at only one of the four visits, intermittent carriers as those who were colonized at two or three of the visits and persistent carriers as those colonized at all four study visits.

Descriptive statistics included the prevalence of colonization at individual time points and changes of recto-vaginal colonization status. Analysis of the changes in recto-vaginal colonization over time was restricted to the 507 participants who completed all four study visits. New acquisition of GBS was defined as positive culture of a new serotype which was not previously present. The new acquisition rate was defined as the number of new serotype acquisitions divided by the number of participants who were at a risk of acquiring the new serotype. Thus, women who were already previously colonized by a particular serotype were excluded subsequently from the denominator for estimating acquisition rate for the homotypic serotype. The rate of new acquisitions by all GBS serotypes were calculated from the sum of acquisition rates for the individual serotypes, and by using the above methods for GBS acquisition rates in a serotype independent manner. Clearance of colonization was defined as a negative GBS culture for a specific serotype following a positive sample at the previous visit for the homotypic serotype. The rate of colonization clearance was defined as the number of GBS-negative participants at the analyzed time point divided by the number of participants at the previous visit who were positive for that serotype, and was also calculated in a serotype independent manner.

Survival analysis methods were used to estimate the duration of colonization of specific serotypes. A colonizing event was defined as the period of time between acquisition and clearance of a GBS serotype. Date of acquisition was calculated as the midpoint between the last visit without serotype-specific colonization and the first visit at which a positive sample was obtained for the homotypic serotype, while date for termination of serotype-specific colonization was calculated as the midpoint between the last visit with colonization and the subsequent negative visit for that serotype. In this analysis, if colonization occurred at the first visit, this was taken as the start of colonization, and if colonization occurred at the last visit, a right censoring approach was applied. We used the Kaplan-Meier method to estimate the duration of GBS colonization. The log-rank test was used to examine differences in duration of carriage between serotypes.

Positive predictive value (PPV) and negative predictive value (NPV) were calculated for the culture results at different sampling points with the 37–40 week visit as the reference standard. For participants who were colonized with same serotype on multiple visits, only one serotype specific isolate was used to study PI association with capsular serotype.

The chi-square test was used to compare proportions. Logistic regression analysis was used to determine the association between GBS colonization and demographic characteristics at enrolment. A p-value of <0.05 was considered significant.

Ethics statement

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (IRB/Protocol-

M090937) and informed written consent was obtained from all participating mothers. The trial is registered with South African National Clinical Trials Register, number DOH-27-0210-3012.

Results

Demographic characteristics

Of the 661 enrolled participants, 621 (93.9%), 595 (90.0%) and 521 (78.8%) completed visits 2, 3 and 4, respectively. Five-hundred and seven (76.7%) women completed all four study visits. A detailed trial profile is indicated in figure 1. The main reason for women not attending all four visits was birth of the baby (13%; 86/661) before the final visit. The demographic characteristics are displayed in table 1. The mean age of the participants at enrolment was 25.9 (standard deviation; S.D±5.6) years. Only 5 (0.76%) pregnant women have taken antibiotic treatment during the study.

Prevalence of GBS colonization

The overall prevalence of recto-vaginal GBS colonization was 33.0% (218), 32.7% (203), 28.7% (171) and 28.4% (148) at 20–25 weeks, 26–30 weeks, 31–35 weeks and 37+ weeks of gestational age, respectively. The lower prevalence of colonization associated with 31–35 weeks and 37+ weeks compared to 20–25 weeks and 26–30 weeks was specifically associated with a decrease in prevalence of vaginal colonization, table 2 (23.3% to 19.0%). In the 86 women who gave birth before the final visit, vaginal GBS colonization was detected in 17(19.8%) at the last attended visit compared to 99/521 (19.0%) who gave birth after visit-4 ($p = 0.867$). The inclusion of rectal swab GBS-culture increased the overall detection of GBS colonization by approximately 10% across the four study time-points ($p<0.0001$) and the prevalence of rectal colonization remained similar at each study time-point.

Of several demographic characteristics evaluated at enrolment independently by univariate analysis, parity (OR: 1.22; 95% CI: 1.02–1.47; $p = 0.030$) and gravidity (OR: 1.17; 95% CI: 1.01–1.36; $p = 0.046$), (Table 3) were significantly associated with GBS recto-vaginal colonization, with the highest colonization prevalence observed among women with parity ≥3 (42.1%) and gravidity of ≥4 (41.7%), (Table 1). In a multivariate analysis, none of the demographic characteristics were found to be associated with GBS recto-vaginal colonization, (Table 3). In a serotype-specific univariate analysis at enrolment, multiparity was associated with a higher prevalence of serotype III colonization (OR: 1.37; 95% CI: 1.07–1.76; $p = 0.012$), gravidity also showed possible association with serotype III colonization ($p = 0.068$). In the multivariate analysis parity was found to be associated with serotype III colonization (Adjusted OR: 6.69; 95% CI: 1.47–30.4; $P = 0.014$). Gravidity ($p = 0.053$) and abortions ($p = 0.057$) also showed a possible association with serotype III colonization, (Table 3). None of the demographic characteristics were found to be associated with serotype Ia colonization in the univariate or multivariate analysis. There were no identifiable factors associated with a higher prevalence of colonization with GBS at visit-4 alone.

Serotype and pilus island distribution

The proportional representation of serotypes remained consistent at each of the consecutive sampling time-points. Of women colonized, the proportional representation of the major serotypes were 36.2% to 41.4% for Ia, 31.3% to 34.9% for III, 10.3% to 15.6% for V, 7.2% to 7.5% for II, 3.5% to 4.6% for Ib, 2.0% to 4.0% for IV and 0.0% to 3.3% for IX (Table S1 in file S1). The concordance of serotypes for GBS cultured concurrently from vaginal and rectal swabs was 91.3%, 89.5%, 94.1% and 94.1% for the four consecutive visits, respectively. Only 1.6% of GBS isolates were serologically non-typable by latex agglutination and were serotyped by PCR.

All GBS isolates harbored one or more PIs, either PI-2a on its own or with a combination of PI-2a or PI-2b in combination with PI-1.The most common PI arrangement was PI-2a on its own, which occurred in 103/227 (45.4%), 92/211 (43.6%), 79/175 (45.1%) and 63/152 (41.5%) of isolates at visits 1–4, respectively, followed by PI combination PI-2b and PI-1, which occurred in 75/227 (33.0%), 69/211 (32.7%), 68/175 (38.9%) and 58/152 (38.2%) of isolates at visits 1–4, respectively. The least common PI arrangement was a combination of PI-2a and PI-1 which occurred in 49/227 (21.6%), 50/211 (23.7%), 29/175 (16.0%) and 31/152 (20.4%) of isolates at visits 1–4, respectively. There were no significant changes in the prevalence of PI distribution with respect to different visits, with the exception of PI-2a which was less common at visit-4 (18.0%, 94/521) compared to visit-1 (23.0%, 152/661; $p = 0.007$), and which was attributable to a lower prevalence of serotype Ia at visit-4 (10.6%, 55/521) compared to visit-1 (14.2%, 94/661).

There was a strong correlation between the presence of particular combinations of PI and the serotype; Figure 2. Most serotype Ia isolates were associated with PI-2a (94.9%; 148/156), whereas the majority of serotype III isolates were associated with the combination of PI-1 and PI-2b (88.2%; 105/119). The association between PIs and serotype V was more variable, with a PI-1+PI-2a combination occurring in 64.7% (33/51) and PI-2a alone occurring in 29.4% (15/51) of isolates.

Changes in GBS colonization overtime

Five hundred and seven participants who completed all four study visits were similar in their demographic characteristics compared to the 154 participants not included in this analysis (data not shown). In the analyzed subset, the prevalence of recto-vaginal GBS colonization was 32.1% (163), 30.4% (154), 29.0% (147) and 27.8% (141) at 20–25 weeks, 26–30 weeks, 31–35 weeks and 37+ weeks of gestational age, respectively. Two hundred and fifty-two (49.7%) women were colonized at least once during the study period, of whom 70 (27.8%) were persistent carriers, 83 (32.9%) transient carriers and 99 (39.3%) were intermittently colonized for any serotype (Table S2 in file S1).

The cumulative serotype-specific prevalence across the study period was 23.7% (120/507) for Ia, 18.3% (93/507) for III, 7.1% (36/507) for V, 4.3% (22/507) for II and 2.8% (14/507) for Ib. All GBS serotypes were variable in their colonization patterns. Comparing the three most common colonizing serotype carriers, 29% (27/93) of serotype III carriers were associated with persistent colonization compared to serotype Ia (18%; 21/120; $p = 0.045$) or V (6%; 2/36; $p = 0.002$). Serotype V was the most dynamic, with 94.4% (34/36) of colonized women either being transient or intermittent carriers compared to 82.5% (99/120; $p = 0.106$) for Ia and 71.0% (66/93; $p = 0.004$) for III. Only one serotype was detected in 83.3% (210/252) of GBS carriers during the study period, with 85.7% (60/70) of women persistently colonized being associated with the same serotype.

Of the 16.7% (42/252) women in whom multiple serotypes were detected over the study period, two serotypes were detected in 9.5% (24/252) and three serotypes in 7.1% (18/252) participants. Among women in whom multiple serotypes were detected, a new serotype was observed at the immediate next visit in 85.7% (36/42) of cases, while a new serotype was detected following a period of no colonization by the preceding serotype in six women.

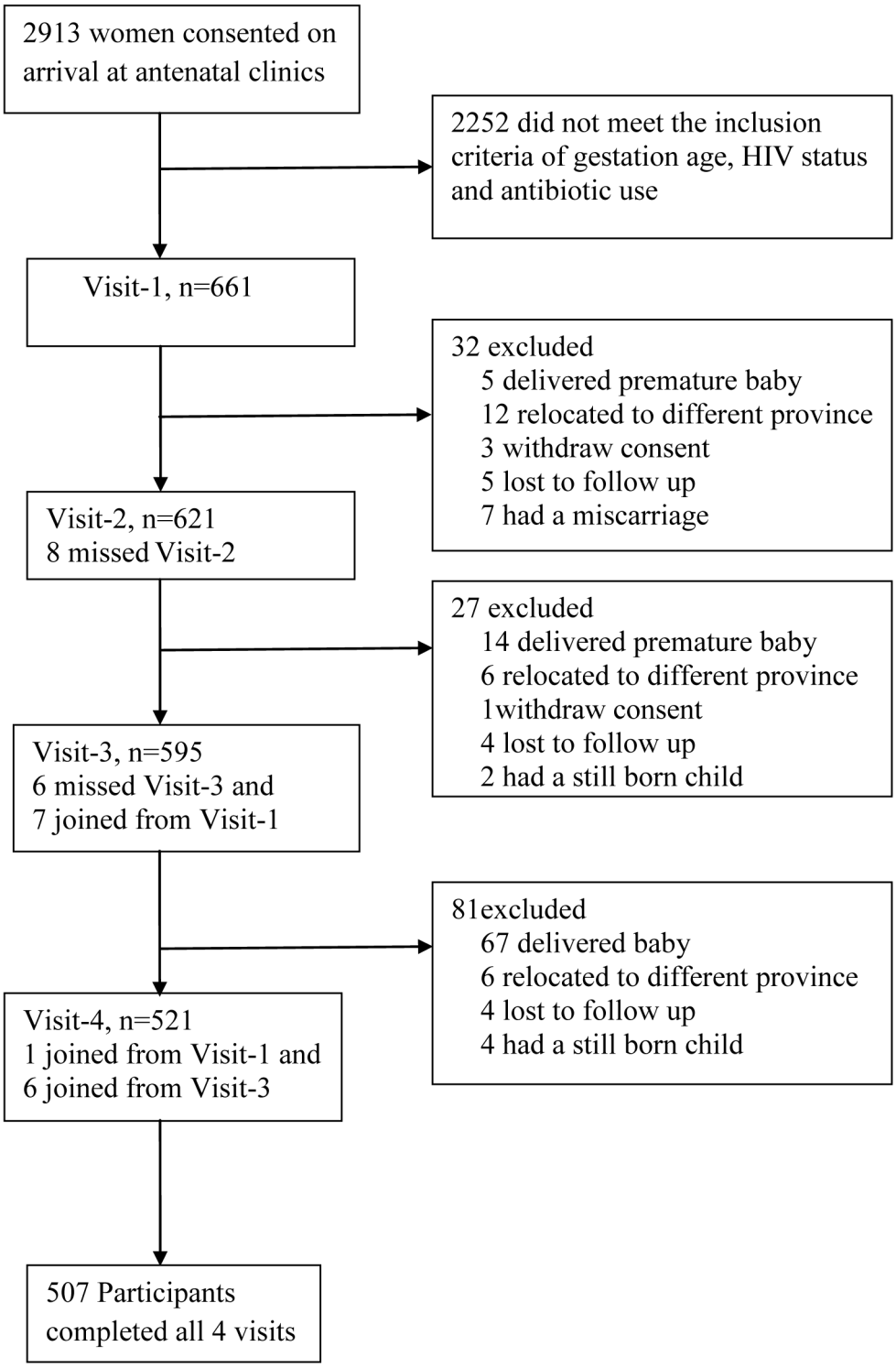

Figure 1. Trial Profile.
doi:10.1371/journal.pone.0098778.g001

New acquisition and clearance of colonization

Three hundred and forty-four participants who completed all four study visits were not colonized at visit-1, of whom 89 (25.9%) became colonized at one of the subsequent three visits. When including new serotype acquisition in those previously colonized by a heterotypic serotype (n = 39), the cumulative overall recto-vaginal acquisition rate of new serotypes during the study, calculated from the sum of acquisition rates for the individual serotypes was 27.9%. The number of new acquisitions was highest for serotypes Ia (11.2%, 49/436), III (8.2%, 37/451) and V (4.3%, 21/492); table S2 in file S1. The mean new acquisition rate of GBS was 11.4% (S.D ±0.5%) at 5–6 week visit intervals, including 11.6% between visit-1 and visit-2, and 10.8% and 11.7% in the

Table 1. Demographics of the study population at time of enrolment (n = 661).

Demographic characteristic		Overall (n = 661)	GBS Colonized	GBS Uncolonized
Age (years)	<20	92 (13.9%)[a]	27 (29.3%)[b]	65 (70.7%)[c]
Mean age: 25.9 (S.D±5.6)	20–24	234 (35.4%)	78 (33.3%)	156 (66.6%)
	25–28	161 (24.4%)	53 (32.9%)	108 (67.1%)
	29–32	95 (14.4%)	29 (30.5%)	66 (69.5%)
	33–35	40 (6.1%)	20 (50.0%)	20 (50.0%)
	36+	39 (5.9%)	11 (28.2%)	28 (71.8%)
Parity	0	338 (51.1%)	97 (28.7%)	241 (71.3%)
Median parity: 0 (range; 0–5)	1–2	304 (46.0%)	113 (37.2%)	191 (62.8%)
	3–5	19 (2.9%)	8 (42.1%)	11 (57.9%)
Gravidity	1	286 (43.3%)	80 (28.0%)	206 (72.0%)
Median gravidity: 2 (range; 1–8)	2	221 (33.4%)	80 (36.2%)	141 (63.8%)
	3	106 (16.0%)	38 (35.8%)	68 (64.2%)
	≥4	48 (7.3%)	20 (41.7%)	28 (58.3%)
Previous Abortion (spontaneous)	0	553 (83.7%)	176 (31.8%)	377 (68.2%)
Median abortion: 0 (range; 0–3)	1	88 (13.3%)	36 (40.9%)	52 (59.1%)
	2	19 (2.9%)	6 (31.6%)	13 (68.4%)
	3	1 (0.2%)	0 (0.0%)	1 (100%)
Stillborn	0	651 (98.5%)	214 (32.9%)	437 (67.1%)
Median stillbirths: 0 (range; 0–1)	1	10 (1.5%)	4 (40.0%)	6 (60.0%)

[a]Data are no (%) of total participants, [b,c]Data are row %.
doi:10.1371/journal.pone.0098778.t001

intervals of subsequent consecutive visits. Of 163 participants who were colonized at visit-1, 76 (46.6%) were no longer colonized by visit-4. The rate of colonization-clearance was 75% (6/8) for serotype Ib, 73.3% (11/15) for V and 63.4% (45/71) for Ia. The overall clearance of any GBS colonization was 30.1% (49/163), 29.2% (45/154) and 32.7% (48/147) between visits-1 and -2, visits-2 and -3, and visits-3 and -4, respectively. No demographic characteristics were identified that were associated with either new acquisition or clearance of colonization.

Duration of GBS colonization

The median duration of recto-vaginal GBS colonization was 6.35 weeks for serotype III, which tended to be longer than other serotypes, including serotype Ia (median: 5.21 weeks; p = 0.02; table 4) which was the second most common colonizing serotype. The difference in duration of colonization between serotype III and less prevalent serotypes was not statistically significant.

Table 2. Prevalence of Group B *Streptococcus* colonization during the study visits.

Site of colonization	Visit-1	Visit-2	Visit-3	Visit-4
	(20–25 weeks)	(26–30 weeks)	(31–35 weeks)	37+weeks)
	n = 661	n = 621	n = 595	n = 521
	Mean gestation age: 22.7 weeks	Mean gestation age: 27.9 weeks	Mean gestation age: 32.5 weeks	Mean gestation age: 37.5 weeks
Vaginal only (%; 95% CI)	62	65	47	31
	(9.4%; 7.2–11.6)	(10.5%; 8.1–12.9)	(7.9%; 5.7–10.1)	(5.9%; 3.9–7.9)
Rectal only (%; 95% CI)	64	62	56	49
	(9.7%; 7.5–12.0)	(10%; 7.6–12.4)	(9.4%; 7.1–12.0)	(9.4%; 6.9–11.9)
Both vaginal and rectal (%; 95% CI)	92	76	68	68
	(13.9%; 11.3–16.5)	(12.2%; 9.63–14.8)	(11.4%; 8.9–14.0)	(13.1%; 10.2–16.0)
Vaginal and/or rectal (%; 95% CI)	218	203	171	148
	(33.0%; 29.4–36.6)	(32.7%; 29.0–36.4)	(28.7%; 25.1–32.3)	(28.4%; 24.5–32.3)

CI-Confidence interval, n = number of participants.
doi:10.1371/journal.pone.0098778.t002

Table 3. Univariate and multivariate association between serotype-specific colonization and observed demographic characteristics at enrolment.

Characteristic	Overall GBS colonization at enrolment		Serotype III colonization at enrolment		Serotype Ia colonization at enrolment	
	Univariate	Multivariate	Univariate	Multivariate	Univariate	Multivariate
	OR (95% CI), p	AOR (95% CI), p	OR (95% CI), p	AOR (95% CI), p	OR (95% CI), p	AOR (95% CI), p
Age	0.98 (0.95–1.02),	1.01 (0.97–1.06),	0.98 (0.92–1.03),	1.00 (0.96–1.04),	0.99 (0.94–1.04),	
	0.536	0.527	0.45	0.96	0.656	
Parity	1.99 (0.83–4.79),	1.37 (1.07–1.76),	6.69 (1.47–30.4),	1.05 (0.82–1.34),	0.71 (0.27–1.90),	
	0.121	0.012	0.014	0.69	0.499	
Gravidity	0.63 (0.27–1.50),	1.22 (0.99–1.51),	0.22 (0.05–1.02),	1.11 (0.91–1.35),	1.48 (0.57–3.81),	
	0.296	0.068	0.053	0.317	0.422	
Abortion	1.77 (0.73–4.30),	1.10 (0.66–1.81),	4.14 (0.96–18.0),	1.25 (0.82–1.91),	0.86 (0.31–2.38),	
	0.207	0.721	0.057	0.298	0.774	
Stillborn	1.25 (0.32–4.94),	0.94 (0.12–7.51),	0.76 (0.08–7.30),	2.60 (0.66–10.3),	2.14 (0.49–9.29),	
	0.741	0.951	0.813	0.171	0.309	

OR: odds ratio; AOR: adjusted OR; CI: confidence interval.
doi:10.1371/journal.pone.0098778.t003

Predictive values for each visit culture with respect culture status at visit-4

Positive and negative predictive values of serotype-specific culture at 20–25 weeks, 26–30 weeks and 31–35 weeks of gestational age compared to 37+ weeks colonization status are presented in table 5. The overall positive predictive values were 53.4%, 61.7% and 67.4% for GBS-positive cultures at 20–25 weeks, 26–30 weeks and 31–35 weeks, respectively, relative to positivity at 37+ weeks, while the negative predictive values for 20–25 weeks, 26–30 weeks and 31–35 weeks ranged from 84.3% to 88.3%. Serotypes Ia and V had lower PPVs compared with serotype III at each time-point. The observed PPVs at 31–35 weeks, were 55–70% for the three commonest serotypes.

Discussion

To our knowledge this is the first serotype-specific longitudinal study conducted of recto-vaginal GBS colonization in pregnant women, in whom we demonstrated a high prevalence and acquisition rate of GBS recto-vaginal colonization. The overall rate of new acquisition at 5–6 week interval is in agreement with a previous study of non-pregnant women, although, the serotype-specific rates differed [14]. All GBS serotypes were variable in their colonization patterns, possibly due to the complex interaction between immunity and specific GBS serotypes, which is still incompletely understood. It may be that the higher frequency of persistent colonization and longer overall duration of colonization by serotype III, is related to a weaker natural immune response

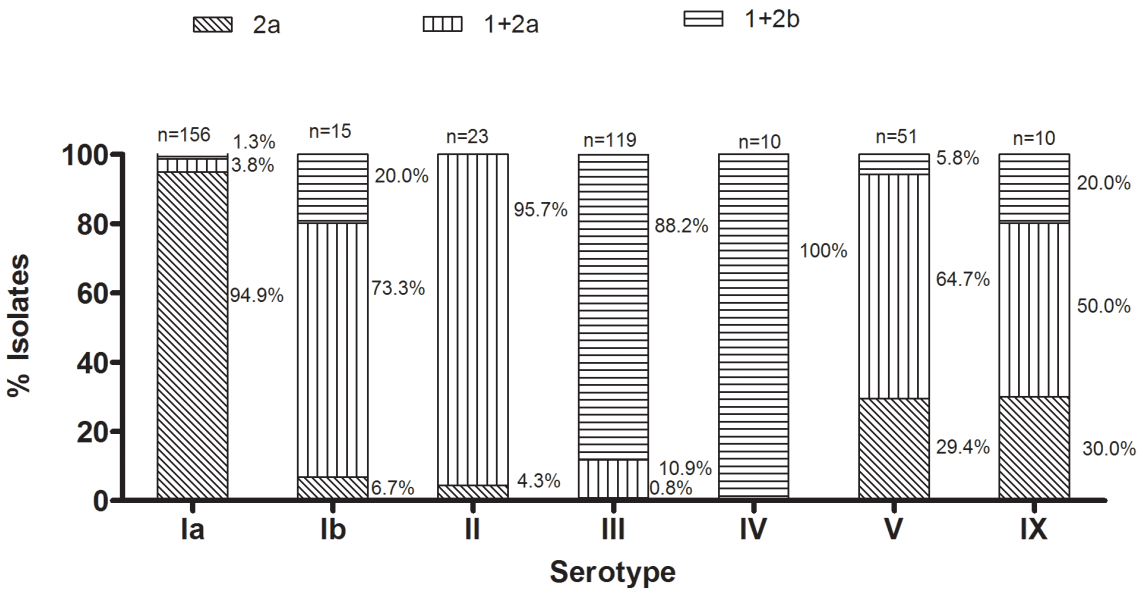

Figure 2. Association of pilus island proteins and serotypes among Group B *Streptococcus* isolates.
doi:10.1371/journal.pone.0098778.g002

Table 4. Estimated duration of Group B *Streptococcus* recto-vaginal colonization.

Serotype	Colonization duration (weeks)*		
	Mean (95% CI)	Median	p-value†
Ia	7.52 (6.6–8.4)	5.21	0.026
Ib	5.22 (4.03–6.42)	3.62	0.358
II	6.11 (5.07–7.16)	4.24	0.736
III	9.15 (8.1–10.2)	6.35	Reference
IV	6.94 (3.8–10.0)	4.81	0.998
V	8.60 (6.80–10.39)	5.96	0.332
IX	6.21 (4.7–7.7)	4.31	0.651

*Time from enrolment, CI-Confidence interval,
†compared to serotype III.
doi:10.1371/journal.pone.0098778.t004

associated with its colonization compared to other serotypes [15]. Consequently, there is a higher risk of exposure at birth to serotype III in our population, which corroborates with it being responsible for 49.2% to 57.7% of EOD in our setting [16,17]. The higher acquisition rate of serotype Ia may result in there being inadequate time for natural immunity to this serotype developing in the pregnant woman, which consequently increases the newborn's risk of developing EOD from serotype Ia, associated with 22.6% to 31% of EOD cases in our setting [16,17].

The high incidence of new acquisition and loss of colonization during pregnancy highlights why screening is required as late as 35–37 weeks' gestational age for the IAP strategy to be effective, which is concordant with another study in pregnant women [18]. In our study, if women had been screened at 31–35 weeks, 29.1% (42/141) of those who were colonized at 37+ would not have had IAP offered to them and a lesser proportion (13.3%; 48/366) may have unnecessarily received IAP as they were no longer colonized at 37+ weeks. Although we did not identify any demographic characteristics associated with new acquisition or clearance of GBS, additional risk factors such as sexual activity during pregnancy were not fully explored [19]. The PPV of GBS cultures obtained from 20–35 weeks varied in serotype distribution compared to that at 37+ weeks. The prevalence of different GBS serotypes in a particular population can affect the PPV of late antenatal GBS cultures.

The high cumulative prevalence of GBS colonization (49.7%) found in our study is comparable to longitudinal studies from Denmark and Zimbabwe [8,10]. Furthermore, the prevalence of colonization observed by us at 37+ weeks of gestational age was 28.4% (148/521), which was similar to that reported in cross-sectional studies from Europe [20] and USA [21]. The prevalence of GBS colonization from African countries ranges from 16.5% in Malawi, 21–23% in The Gambia, Ethiopia and Tanzania and 31.6% in Zimbabwe [22,23,24,25,26]. Our results also showed a decrease in the prevalence of GBS colonization with respect to increase in gestational age. This finding agrees with studies from the USA and Australia [9,27] but contrasts with others that reported an increase in colonization with increasing gestational age [28,29].

Our findings on the dominant serotypes are comparable with serotype distribution data of maternal colonizing isolates from industrialized countries, including 13% to 35% for serotype Ia and 15% to 44% for serotype III [6]. The identification of serotype IX in our study was notable in that it is rarely reported in colonizing studies and not previously described in Africa. To our knowledge, only 8 GBS colonizing isolates have been identified as serotype IX, including three from Denmark, two from Germany and one each from Canada, Hong Kong and Australia [30]. Our data on PI distribution is comparable to earlier published studies [7,31] showing that all GBS isolates carried at least one PI, and were

Table 5. Predictive value for 20–25, 26–30 and 31–35 weeks cultures in relation to culture status at 37+ weeks.

Serotype	20–25 weeks		26–30 weeks		31–35 weeks	
	PPV % (95% CI)	NPV % (95% CI)	PPV % (95% CI)	NPV % (95% CI)	PPV % (95% CI)	NPV % (95% CI)
Overall GBS	53.4 (45.4–61.2)	84.3 (80.0–88.0)	61.7 (53.5–69.4)	87.0 (83.0–90.3)	67.4 (59.1–74.9)	88.3 (84.6–91.5)
Ia	36.6 (25.5–48.9)	94.5 (91.9–96.4)	49.2 (36.1–62.3)	95.5 (93.2–97.2)	55.0 (41.7–67.9)	96.2 (94.0–97.8)
Ib	25.0 (3.9–65.0)	99.0 (97.7–99.7)	66.7 (22.7–94.7)	99.4 (98.3–99.9)	75.0 (20.3–95.9)	99.2 (98.0–99.8)
II	38.5 (14.0–68.4)	98.8 (97.4–99.6)	54.6 (23.5–83.1)	99.0 (97.7–99.7)	75.0 (42.8–94.2)	99.6 (98.5–99.9)
III	58.9 (45.0–71.9)	95.8 (93.5–97.4)	62.8 (48.1–75.9)	95.6 (93.3–97.3)	67.9 (53.7–80.1)	96.5 (94.3–98.0)
IV	50.0 (12.4–87.6)	100 (99.2–100)	60.0 (15.4–93.5)	100 (99.2–100)	75.0 (20.3–95.9)	100 (99.3–100)
V	26.7 (8.0–55.1)	97.2 (95.3–98.4)	47.6 (25.8–70.2)	98.4 (96.8–99.3)	57.1 (28.9–82.2)	98.0 (96.3–99.0)
IX	100 (19.3–100)	99.6 (98.6–99.9)	50.0 (12.4–87.6)	99.8 (98.9–100)	100 (30.5–100)	99.8 (98.9–100)

PPV: Positive predictive value, NPV: Negative predictive value, CI-Confidence interval.
doi:10.1371/journal.pone.0098778.t005

associated with the presence of either PI-2a or PI-2b identified alone or in combination with PI-1.

Our study is limited by the sensitivity of detection of GBS on selective media which is estimated at 85% [11] and by the fact that in most cases only the dominant serotype was determined. This can lead to an underestimation of persistent colonization, an overestimation of new acquisitions, and an underestimation of the duration of carriage.

Recent developments in the clinical evaluation of a tri-valent GBS polysaccharide-protein conjugate vaccine has renewed interest in the potential of this vaccine to protect neonates against invasive GBS disease by reducing recto-vaginal colonization during pregnancy [32]. The findings of this study will be important in considering study design when evaluating the efficacy of maternal GBS vaccination protecting against GBS recto-vaginal acquisition and colonization during pregnancy as surrogate information on clinical vaccine efficacy may be gained by determining the immune responses that correlate with protection against serotype-specific GBS acquisition and colonization during pregnancy.

Supporting Information

File S1
(DOCX)

Acknowledgments

We acknowledge the contribution of the lab staff Bianca Smith and Makhosazana Sibeko.

We thank the antenatal staff of Lillian Ngoyi, Mofolo, Diepkloof and Michael Maponya community clinics in Soweto for all their help and support and the participating pregnant women for selflessly volunteering their participation.

Author Contributions

Conceived and designed the experiments: GK PVA EJB CLC SAM. Performed the experiments: GK PVA. Analyzed the data: GK PVA TS. Contributed reagents/materials/analysis tools: TS CLC. Wrote the paper: GK PVA EJB CLC SAM.

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ORIGINAL ARTICLE BACTERIOLOGY

Natural acquired humoral immunity against serotype-specific group B *Streptococcus* rectovaginal colonization acquisition in pregnant women

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Abstract

Group B *Streptococcus* (GBS) rectovaginal colonization in pregnant women is associated with invasive GBS disease in newborns, preterm delivery and stillbirths. We studied the association of GBS serotype-specific capsular polysaccharide (CPS) antibody on new acquisition and clearance of rectovaginal GBS colonization in pregnant women from 20 weeks until 37 to 40 weeks' gestation. Serum serotype-specific CPS IgG antibody concentration was measured by multiplex enzyme-linked immunosorbent assay and opsonophagocytic activity (OPA) titres. Rectovaginal swabs were evaluated for GBS colonization, using standard culture methods and serotyping by latex agglutination, at five to six weekly intervals. Higher serotype III CPS antibody concentration was associated with lower risk of rectovaginal acquisition of serotype III during pregnancy (p 0.009). Furthermore, serotype-specific OPA titres to Ia and III were higher in women who remained free of GBS colonization throughout the study compared to those who acquired the homotypic serotype (p <0.001 for both serotypes). Serum CPS IgG values of $\geq$1 μg/mL for serotype V and $\geq$3 μg/mL for serotypes Ia and III were significantly associated with protection against rectovaginal acquisition of the homotypic serotype. A GBS vaccine that induces sufficient capsular antibody in pregnant women, including high OPA titres, could protect against rectovaginal colonization during the latter half of pregnancy.

Keywords: Antibody, colonization, group B *Streptococcus*, opsonophagocytic activity, pregnant women
Original Submission: 9 September 2014; **Revised Submission:** 24 December 2014; **Accepted:** 31 January 2015
Editor: I. Gyssens
Article published online: 10 February 2015

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Introduction

Maternal rectovaginal colonization with group B *Streptococcus* (GBS) is the major risk factor for GBS invasive disease in newborns under 7 days of age (early-onset disease; EOD) [1,2]. Vertical transmission of GBS occurs to approximately 50% of newborns of GBS-colonized pregnant women, of whom 1% to 2% develop EOD in the absence of intrapartum antibiotic prophylaxis (IAP) [3,4]. Rectovaginal GBS colonization of pregnant women has also been associated with preterm birth and stillbirth [5]. Screening for GBS rectovaginal colonization in pregnant women at 35 to 37 weeks' gestation, coupled with IAP during labor, has reduced the incidence of EOD in high-income settings [6]. However, implementation of IAP would be costly and logistically challenging for most resource-constrained countries [7,8].

An alternate preventive strategy against invasive GBS disease in newborns and young infants could be vaccination of pregnant women with a GBS vaccine to induce humoral immunity, with protective antibodies transplacentally transferred to the fetus [9]. An association between low maternal serotype-specific

http://dx.doi.org/10.1016/j.cmi.2015.01.030

capsular antibody concentration and increased risk of invasive GBS disease among newborns has been observed [10–12]. Furthermore, if GBS vaccination before or during the early stages of pregnancy could prevent or reduce subsequent rectovaginal colonization in women, this could lower fetal/newborn exposure to GBS and contribute to reducing EOD among term and preterm births as well as potentially reduce GBS-associated premature labor and stillbirth.

Although spontaneous clearance and acquisition of GBS has been reported in pregnant women, the association of host immune mediators and GBS colonization during pregnancy remains unclear [13,14]. A few predominantly cross-sectional studies have reported higher serotype-specific capsular polysaccharide (CPS) antibody in colonized compared to non-colonized women [15–22]. We are, however, unaware of any longitudinal cohort study which evaluated the association between serotype-specific CPS antibody and subsequent risk of GBS rectovaginal acquisition during the latter half of pregnancy.

The primary objective of our study was to evaluate the association between natural serotype-specific serum CPS IgG antibody and opsonophagocytic activity (OPA) in relation to GBS rectovaginal acquisition in pregnant women from 20 to 37 + weeks' gestation. Furthermore, we evaluated the association between CPS antibody and clearance of GBS colonization.

Methods

Study population

The study was conducted at four antenatal community clinics in Soweto, Johannesburg, from August 2010 to August 2011. Inclusion criteria were HIV-uninfected pregnant women (confirmed by HIV enzyme-linked immunosorbent assay non-reactivity) at 20 to 25 weeks' gestation. Exclusion criteria included antibiotic treatment in the previous 2 weeks, any acute illness, symptomatic vaginal discharge and a known or suspected condition contraindicating a vaginal examination. If antibiotics were taken after the first visit, the collection of study specimens was delayed for at least 2 weeks after the last dose.

Swab collection for GBS culture and serotyping

Lower vaginal and rectal swabs were collected for GBS culture at 20 to 25 weeks' gestation (visit 1), followed at five to six weekly intervals (visits 2 and 3), and finally at 37 to 40 weeks' gestation (visit 4). Samples were collected by trained study nurses with rayon-tipped swabs that were placed into Amies transport medium without charcoal (Transwab Amies; Medical Wire, UK). For GBS isolation, swabs were inoculated onto CHROMagar StrepB (CA; Media Mage, South Africa) as previously described [23]. Serotyping was performed by the latex agglutination method with specific antisera (Statens Serum Institute, Sweden) to serotypes Ia, Ib and II to IX CPS antigens, as previously described [24]. Isolates testing negative by latex agglutination were further typed by PCR using primer sequences described by Poyart et al. [25].

Measurement of serotype-specific anticapsular serum IgG

Blood was collected at enrolment and visit 4. Serum was separated by centrifugation and stored at −70 °C until analysis. Quantitative serum serotype-specific CPS IgG antibody concentrations were measured with the multiplex Luminex platform. All GBS CPS antigens were kindly provided by Novartis Vaccines, Italy, and were coupled to the microsphere beads (Bio-Rad, USA) with the cross-linking agent 4-(4,6-dimethoxy [1,3,5]triazin-2-yl)-4-methyl-morpholinium (DMTMM) as previously described [26]. An in-house reference serum composed of purified pooled human IgG (Polygam; National Bioproducts, South Africa) was calibrated with the standard serotype-specific GBS reference serum kindly provided by C. J. Baker (Section of Infectious Diseases, Department of Pediatrics, Baylor College of Medicine, AHouston, TX, USA). Assays were performed in true duplicate and each plate included high and low control sera. Bead fluorescence was read with the Bio-Plex 200 instrument (Bio-Rad) using Bio-Plex manager 5.0 software (Bio-Rad). In each assay, an uncoated control bead was included to determine nonspecific binding. For control beads, the coupling procedure was followed, except that no GBS CPS was added. In case of nonspecific binding, the fluorescence intensity values were subtracted from the antigen-specific results. The optimal serum and secondary antibody dilution was 1:100. The results for serum CPS IgG are given in μg per mL with lower detection limits of 0.0003, 0.005 and 0.009 for serotype Ia, III and V, respectively. For statistical analysis, samples with values below these limits were assigned values of half of lower limit of detection.

The multiplex assay was validated by comparing the serotype-specific mean fluorescence intensity values for Polygam obtained with the multiplex assay with those for each serotype run in single plex. The fluorescence intensity values obtained for Polygam with the multiplex assay were between 79% and 112% (median 103%) of those obtained with the single-plex assays. To assess analytical specificity of each GBS–microsphere set, Polygam was incubated at 1:100 dilution with each GBS CPS (100 μg/mL) and incubated at 37°C for 2 hours. The specificity was recorded as the difference in reactivity between the absorbed and unabsorbed sera in a multiplex assay. Homologous inhibition was >95% for all serotypes, with the exception of serotype V (85%). Heterologous inhibition across serotypes was <10%, except for serotypes Ib and III,

which were inhibited by 41% and 11% with type Ia CPS, respectively, and serotype V (18%) was inhibited by serotype III CPS.

Serum IgG OPA assay

The functional activity of serum IgG was determined by OPA assay for serotypes Ia and III using the HL-60 cell line as described [27]. Briefly, 1000 CFU (20 μL of 5×10^4 CFU/mL) of GBS type Ia strain (A909) or type III strain (COH-1) cells were mixed with an appropriate dilution of heat-inactivated serum sample (10 μL), then mixed and incubated for 15 minutes at 37°C in a 5% CO_2 incubator. Baby rabbit complement (10 μL) and HL-60 cells (40 μL of 1×10^7, cultured for 5 days in the presence of dimethyl formamide) were added to the mixture and incubated at 37°C with 5% CO_2 for 1 hour with gentle agitation. Aliquots were removed and plated on Todd-Hewitt agar plates for quantitative culture. OPA was expressed as the titre at which the serum dilution yielded 50% killing compared to the bacterial growth in the complement controls. The limit of detection was 8. For statistical analysis, samples below the detection were assigned an arbitrary titre of 4.

Study definitions

A participant was considered to be colonized at a visit if GBS was cultured on the vaginal and/or rectal swab, and non-colonized if no growth was detected from either swab. A new episode of serotype-specific GBS rectovaginal colonization (i.e. new acquisition group) was defined as a participant not colonized by a specific GBS serotype at visit 1 who was subsequently colonized by that specific serotype (irrespective of the number of study visits completed). Concurrent identification of different serotypes from the vaginal and rectal swabs at a visit among participants with new acquisitions were treated as individual events. Participants not colonized with a specific serotype at any study visit were categorized as being the non-colonized group for that serotype and were used as the comparator group when evaluating the association between CPS antibody and risk for serotype-specific rectovaginal acquisition.

The association of CPS antibody with clearance of serotype-specific GBS colonization was evaluated in participants who were colonized by the specific serotype at visit 1 and who completed all study visits. The participants were stratified into those who remained colonized throughout (i.e. persistently colonized group) and those in whom colonization of specific serotype was cleared by visit 4 (i.e. intermittently colonized group). As a result of limitations associated with detecting multiple serotype carriage using traditional culture methods [28], participants with GBS serotype colonization at visit 1 who acquired a different GBS serotype at a subsequent visit were excluded from the analysis on CPS antibody and serotype-specific GBS acquisition or clearance.

Statistical analysis

Differences in geometric mean concentration (GMC) of serotype-specific CPS antibody and geometric mean OPA titre (GMOPT) between groups (new acquisition vs. noncolonized groups; and persistently colonized vs. intermittent colonized groups) were analysed on log-transformed data by Student's *t* test. GMC values at visits 1 and 4 for each group were analysed by paired Student's *t* test. Evaluation of whether the serotype-specific CPS antibody was associated with new homotypic GBS acquisition was undertaken using univariate logistic regression analysis reporting the odds ratio (OR) for the association between the serological variable and GBS acquisition, with logarithmic IgG or OPA titre as a covariate. Univariate logistic regression analysis was also used for comparison between groups by demographic and obstetric factors at visit 1.

For categorical variables, groups were compared by either the chi-square test or Fisher's two-tailed exact test, as appropriate. Correlations between serum IgG and OPA titres were determined on log-transformed data by Spearman rank correlation. Data were analysed using SAS software, version 9.2 (SAS Institute, USA). A p value of <0.05 was considered significant.

Ethics Statement

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (IRB/Protocol-M090937), and informed written consent was obtained from all participants. This observational study was registered with the South African National Clinical Trials Register (DOH-27-0210-3012).

Results

Of 661 enrolled participants, 95.1% (n = 629) completed at least two and 76.7% (n = 507) all four study visits. The main reasons for not completing all study visits were related to obstetric outcomes such as premature birth (n = 86; 13.0%) and miscarriage (n = 13; 2.0%; Supplementary Fig. 1). A detailed profile of subject follow-up, serotype distribution and dynamics of GBS colonization has been previously published [29].

The overall prevalence of any serotype GBS colonization at enrolment was 33.0% (218/661).

Of those not colonized by GBS serotypes Ia, III or V at enrolment, 9.6% (52/541), 7.0% (39/560) and 2.8% (17/603) subsequently become colonized by the respective serotypes (i.e. new acquisition group). This included six participants for

whom different serotypes were cultured from the vaginal and rectal site at the same visit and excluded 15 participants who were colonized at visit 1 and acquired a different serotype at a subsequent visit. Of 507 participants who completed all scheduled visits, participants not colonized with a specific serotype at any visit were 387 (76.3%), 414 (81.6%) and 471 (92.9%) for serotypes Ia, III and V, respectively (i.e. non-colonized group). There were no differences in the demographic characteristics between the new acquisition and noncolonized groups (Table 1).

Of the 507 participants who completed all scheduled visits, the overall GBS colonization prevalence at enrolment was 32.1% (163/507), including 14.0% (n = 71), 11.1% (n = 56) and 2.9% (n = 15) for serotypes Ia, III and V, respectively. The prevalence of serotypes Ia, III and V colonization among the persistently colonized group were 4.1% (21/507), 5.3% (27/507) and 0.4% (2/507), respectively; and 8.5% (43/507), 3.6% (18/507) and 1.8% (9/507), respectively, in the intermittent colonized group. This excluded 22 intermittently colonized participants in whom colonization was not cleared by visit 4 or who acquired a new serotype at subsequent visits and who remained colonized with that serotype thereafter. There were no differences in the demographic characteristics between the persistently colonized and intermittent colonized groups (data not shown).

Association between serotype-specific CPS IgG and new acquisition of the homotypic serotype

The logistic regression analysis for the association between homotypic serotype acquisition and serotype-specific IgG concentration at enrolment demonstrated an OR of <1.00, indicating that the probability of a new acquisition decreased with increasing serotype-specific IgG concentrations (Table 2). Homotypic serotype antibody GMC was higher at enrolment among the noncolonized than the new acquisition group for serotype III (0.33 μg/mL vs. 0.17 μg/mL, respectively; p 0.009), with a similar trend for serotype V (0.75 μg/mL vs. 0.42 μg/mL, respectively; p 0.057) (Table 2).

Reverse cumulative distribution plots of serotype-specific CPS antibody concentrations of the noncolonized and new acquisition groups are shown in Fig. 1a–c. The lowest threshold of CPS antibody concentration associated with lower odds of acquisition of the homotypic serotype varied between serotypes. The lowest antibody threshold significantly associated with remaining noncolonized throughout the study was ≥3 μg/mL for serotype Ia (22.0% in noncolonized vs. 9.6% in new acquisition group, OR 0.37; 95% confidence interval (CI) 0.14,0.98), ≥3 μg/mL for serotype III (10.6% in noncolonized vs. 0.0% in new acquisition group, OR 0.11; 95% CI 0.01–1.75) and ≥1 μg/mL for serotype V (36.5% in noncolonized vs. 11.8% in new acquisition group, OR 0.23; 95% CI 0.05–1.02; Table 3). This strength of association increased at higher CPS antibody thresholds for serotypes Ia and III (Table 3). None of the women who acquired serotype Ia had antibody concentration of ≥15 μg/mL (compared to 8.5% of noncolonized women, p 0.025) at enrolment; and none with new acquisition of serotypes III had homotypic CPS antibody ≥3 μg/mL (compared to 10.6% (p 0.023) of those who remained noncolonized; Table 3).

Association between serotype-specific OPA and new acquisition of GBS

Reverse cumulative distribution plots of serotype-specific OPA titres of the noncolonized and new acquisition groups for serotypes Ia and III are shown in Fig. 1d, e. The logistic regression analysis for the probability of acquisition of GBS serotypes Ia and III as a function of homotypic serotype OPA titre at enrolment had an OR of <1.00, indicating that the probability of a new acquisition decreased with increasing serotype-specific OPA titre (Table 2). Noncolonized women had higher serotype-specific GMOPT than the new acquisition group for serotypes Ia (GMOPT: 14 vs. 5, respectively; p < 0.001) and III (GMOPT: 132 vs. 20, respectively, p < 0.001; Table 2). The

TABLE 1. Demographic characteristics of women who acquired a specific group B *Streptococcus* serotype (new acquisition) compared to those who remained noncolonized by that serotype throughout the study

	Serotype Ia			Serotype III			Serotype V		
	New acquisition	Not colonized		New acquisition	Not colonized		New acquisition	Not colonized	
Characteristic	(n = 52)	(n = 387)	p	(n = 39)	(n = 414)	p	(n = 17)	(n = 471)	p
Age (years), mean ± SD	25.65 ± 5.76	26.06 ± 5.54	0.619	26.13 ± 6.2	26.10 ± 5.6	0.969	26.01 ± 4.77	26.06 ± 5.66	0.955
Parity, median (range)	1 (0–5)	0 (0–5)	0.582	0 (0–3)	0 (0–5)	0.556	1 (0–1)	0 (0–5)	0.751
Gravidity, median (range)	2 (1–6)	2 (1–8)	0.891	1 (1–4)	2 (1–8)	0.353	2 (1–3)	2 (1–8)	0.500
Previous spontaneous abortion, n (%)	6 (11.5)	64 (16.5)	0.358	4 (10.2)	70 (16.9)	0.289	1 (5.9)	80 (17.0)	0.253
Previous stillbirth, n (%)	2 (3.8)	4 (1.0)	0.127	0 (0.0)	6 (1.4)	0.449	0 (0.0)	7 (1.5)	0.612

New acquisition was defined as women who acquired serotype-specific new acquisition; noncolonized was defined as women who remained uncolonized by specific serotype at all four study visits; spontaneous abortion was defined as the spontaneous abortion of pregnancy occurring before 28 weeks' gestation; and stillbirth was defined as fetal death occurring at 28 weeks' gestation or later. p values were calculated by univariate logistic regression analysis.

TABLE 2. Comparison of mean titres (μg/mL) at visit 1 for pregnant women for specific serotypes

	New acquisition	Noncolonized		
Serotype	**GMC (95% CI)**	**GMC (95% CI)**	**p[a]**	**Odds ratio (95% CI)[b]**
Ia	0.28 (0.17, 0.47); *n* = 52	0.35 (0.27, 0.44); *n* = 387	0.529	0.91 (0.68, 1.21)
III	0.17 (0.11, 0.26); *n* = 39	0.33 (0.28, 0.38); *n* = 414	0.009	0.47 (0.26, 0.84)
V	0.42 (0.26, 0.65); *n* = 17	0.75 (0.66, 0.84); *n* = 471	0.057	0.39 (0.15, 1.01)
OPA	**GMOPT (95% CI)**	**GMOPT (95% CI)**		
Ia	5 (4, 6); *n* = 52	14 (11, 17); *n* = 387	<0.001	0.28 (0.12, 0.63)
III	20 (11, 36); *n* = 39	132 (105, 164); *n* = 414	<0.001	0.43 (0.30, 0.62)

New acquisition indicates women who acquired serotype-specific new acquisition; noncolonized indicates women who remained uncolonized by specific serotype.
CI, confidence interval; GMC, geometric mean concentration; GMOPT, geometric mean opsonophagocytic activity titre; OPA, opsonophagocytic activity.
[a]p value calculated by Student's *t* test.
[b]Odds ratio calculated by logistic regression analysis.

presence of detectable OPA titres (≥8) was significantly associated with lower odds of acquiring the homotypic serotype for Ia (p 0.002) and III (<0.001). The strength of association between OPA titres and odds of new acquisitions trended to be stronger at higher OPA threshold for serotypes Ia and III (Table 3).

There were significant correlations between serum IgG concentration and OPA titre with *r* values of 0.684 for serotype Ia and 0.393 for serotype III (p <0.001 for both) (Supplementary Fig. 2a, b). OPA activity (titre ≥8) was detectable to the homotypic serotype in 92% (138/150) of serum samples with IgG concentration ≥3 μg/mL for serotype Ia and 94.1% (192/204) of samples with IgG concentration ≥0.5 μg/mL for serotype III.

Association between serotype-specific CPS IgG and clearance of colonization at enrolment

Serotype-specific CPS antibody concentration was greater among women colonized compared to those not colonized by the homotypic serotype at visit 1 for serotype Ia (p 0.025) and serotype III (p 0.018); and at visit 4 for serotype Ia (p < 0.001) with similar trend for serotype III (p 0.060), although this was not statistically significant for OPA at visit 1 (Supplementary Table 1). Among women colonized at enrolment, higher CPS GMC (1.49 vs. 0.44 μg/mL, p 0.049) and GMOPT (41 vs. 12; p 0.025) were observed for serotype Ia in those categorized as persistently colonized than those with intermittent colonization, respectively, but not so for serotypes III and V (Supplementary Table 2).

Effect of GBS colonization on kinetics of serotype-specific antibody

When we analysed women who completed all four study visits and who were not colonized by a specific serotype at enrolment, serotype-specific new acquisition was associated with an increase in GMC and GMOPT values between enrolment and visit 4. These increases were evident for GMC for serotype Ia (p 0.001), III (p 0.004) and V (p 0.034), as well as GMOPT for serotypes Ia and III (p < 0.001 for both; Table 4). For serotype Ia, a new acquisition event was also associated with an increase in correlation between serotype-specific serum IgG concentration and OPA titres, with *r* values of 0.524 at enrollment (p < 0.001) and 0.792 at visit 4 (p < 0.001) (Supplementary Fig. 2c, d). In contrast, for serotype III, a decrease in correlation between enrollment (*r* = 0.354, p 0.037) and visit 4 (*r* = 0.266, p 0.122) was observed, despite significant increases in OPA titres (Supplementary Fig. 2e, f). No change was observed in GMC between visits 1 and 4 among women who remained noncolonized by the specific serotype throughout the study for serotypes Ia or III, although an increase was observed for serotype V (0.83 vs. 0.75 μg/mL, p 0.011).

Discussion

Our study demonstrated an association between naturally acquired serum serotype-specific CPS antibody and the risk of subsequent rectovaginal colonization by the homotypic serotype in pregnant women not colonized at enrolment. New acquisition of GBS was inversely correlated with serotype-specific CPS IgG concentration (serotypes III and V) and OPA titre (serotypes Ia and III) at time of enrolment. These data suggest that strategies such as vaccination of women with GBS CPS–protein conjugate vaccine which induces antibody responses (Hyderman RF, et al. Program and Abstracts of the 32nd European Society for Paediatric Infectious Diseases) could reduce the risk of GBS rectovaginal acquisition of vaccine serotypes. The potential benefit to the women and their newborns would, however, be dependent on the timing of vaccination in the women and durability of persistence of sufficiently high antibody concentrations or OPA titres to prevent GBS acquisition during pregnancy. The potential of protein–polysaccharide conjugate vaccine to induce mucosal immunity has been demonstrated for vaccines against *Haemophilus*

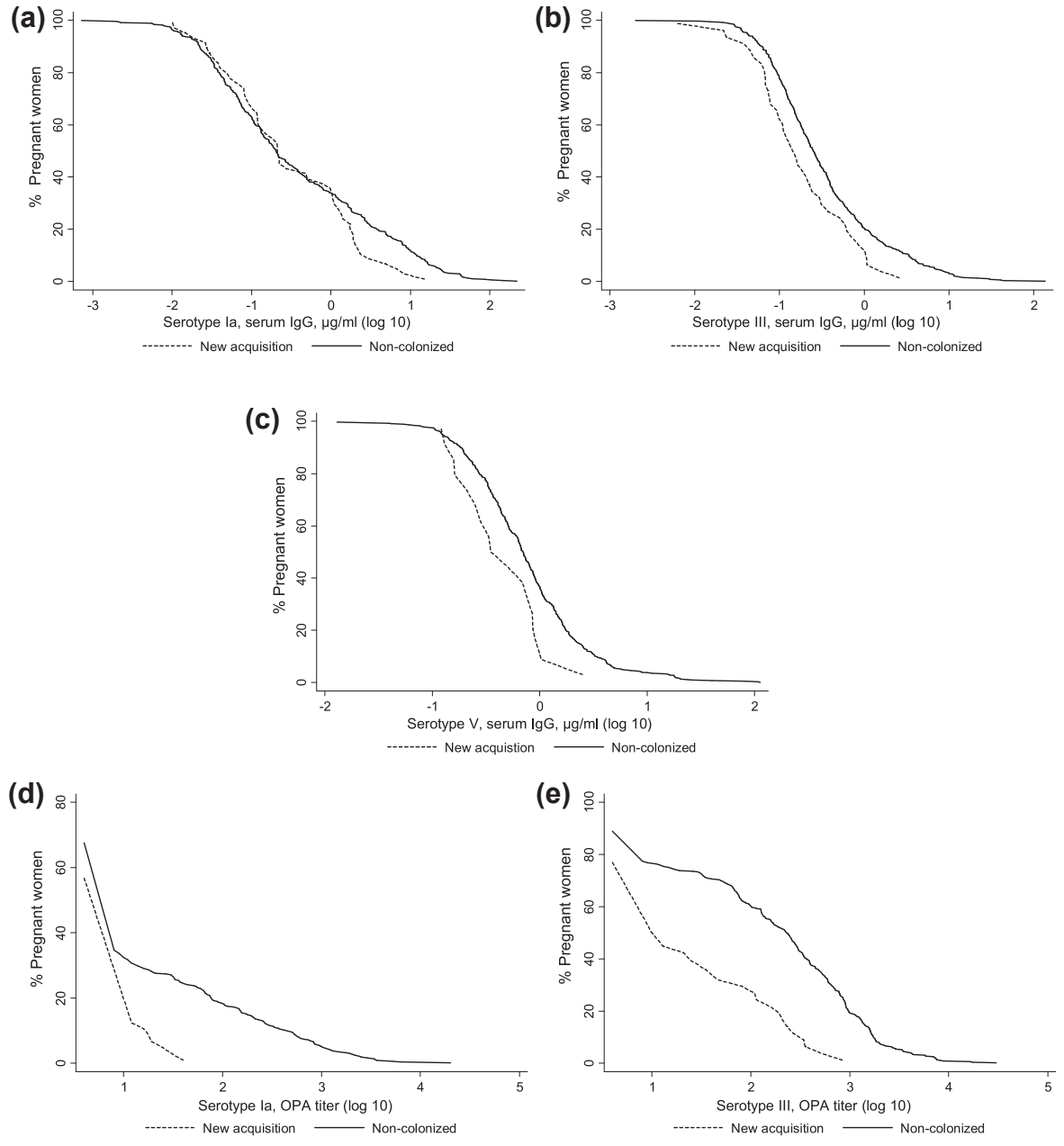

FIG. 1. Reverse cumulative distribution curves of serotype-specific anticapsular serum IgG (**a–c**) and OPA titre (**d–e**) in participants who acquired GBS serotype (new acquisition) and those who remained uncolonized by the specific serotype (noncolonized).

influenzae type b, *Streptococcus pneumoniae* and *Neisseria meningitidis* in which reduced risks of nasopharyngeal mucosal colonization acquisition of the targeted serotypes are evident [30–32].

In our study, the CPS antibody, including functional antibody measured by OPA, was most likely induced by rectovaginal colonization, as indicated by the rise in serotype-specific GMC and GMOPT among those with new acquisitions of homotypic serotypes. It is this increase in CPS antibody which likely reduces the risk of these women becoming colonized by the same serotype. Although our study was not designed to establish the durability of this natural-acquired humoral-mediated immunity, the strengthening of association between higher serotype-specific antibody thresholds and OPA titres and the reduced odds of becoming colonized by the homotypic serotype indicates that this protection is likely to be transient, with waning of antibody over time. Also, the paradoxically higher GMC and GMOPT in women colonized with serotype Ia at

TABLE 3. Association between serotype-specific capsular IgG antibody and acquisition of homotypic group B *Streptococcus* in pregnant women

Characteristic	Serotype Ia				Serotype III				Serotype V			
	New acquisition (n = 52)	Noncolonized (n = 387)	Odds ratio (95% CI)	p[a]	New acquisition (n = 39)	Noncolonized (n = 414)	Odds ratio (95% CI)	p	New acquisition (n = 17)	Noncolonized (n = 471)	Odds ratio (95% CI)	p
Antibody level (μg/mL)												
<0.5	32 (61.5%)	235 (60.7%)	1.04 (0.57, 1.88)	0.910	29 (74.4%)	283 (68.4%)	1.34, (0.64, 2.84)	0.439	9 (52.9%)	184 (39.3%)	1.75 (0.66, 4.61)	0.25
≥0.5	20 (38.5%)	152 (39.3%)	0.98 (0.54, 1.78)	0.910	10 (25.6%)	131 (31.6%)	0.74 (0.35, 11.57)	0.439	8 (47.1%)	287 (60.9%)	0.57 (0.22, 1.51)	0.25
≥1	18 (34.6%)	131 (33.9%)	1.03 (0.56, 1.90)	0.91	5 (12.8%)	84 (20.3%)	0.57 (0.22, 1.52)	0.396	2 (11.8%)	172 (36.5%)	0.23 (0.05, 1.02)	0.039
≥2	8 (15.4%)	101 (26.1%)	0.51 (0.23, 1.13)	0.093	1 (2.6%)	55 (13.3%)	0.17 (0.02, 1.27)	0.070	1 (5.9%)	81 (17.2%)	0.30 (0.04, 2.30)	0.328
≥3	5 (9.6%)	85 (22.0%)	0.37 (0.14, 0.98)	0.043	0 (0%)	44 (10.6%)	0.11 (0.01, 1.75)	0.023	0 (0%)	55 (11.7%)	0.21 (0.01, 3.62)	0.239
≥5	3 (5.8%)	70 (18.1%)	0.28 (0.01, 0.91)	0.027	0 (0%)	26 (6.3%)	0.19 (0.01, 3.11)	0.151	0 (0%)	26 (5.5%)	0.48 (0.02, 8.21)	1.000
≥10	1 (1.9%)	45 (11.6%)	0.15 (0.02, 1.11)	0.028	0 (0%)	13 (3.1%)	0.38 (0.02, 6.46)	0.615	0 (0%)	18 (3.8%)	0.70 (0.04, 12.1)	1.000
≥15	0 (0%)	33 (8.5%)	0.10 (0.01,1.67)	0.025	0 (0%)	6 (1.4%)	0.79 (0.04, 14.39)	1.000	0 (0%)	16 (3.4%)	0.78 (0.04, 13.7)	1.000
OPA titres												
<8	45 (86.5%)	251 (64.9%)	3.46 (0.64, 2.84)	0.002	18 (46.2%)	92 (22.2%)	3.00 (1.53, 5.87)	<0.001	ND	ND		
≥8	7 (13.5%)	135 (34.9%)	0.29 (0.13, 0.66)	0.002	21 (53.8%)	322 (77.8%)	0.33 (0.17, 0.65)	<0.001	ND	ND		
≥16	6 (11.5%)	114 (29.5%)	0.31 (0.13, 0.75)	0.006	17 (43.6%)	310 (74.9%)	0.26 (0.13, 0.51)	<0.001	ND	ND		
≥32	1 (1.9%)	104 (26.9%)	0.05 (0.01, 0.39)	<0.001	15 (38.5%)	302 (72.3%)	0.23 (0.12, 0.46)	<0.001	ND	ND		
≥64	0 (0%)	88 (22.7%)	0.03 (0.00, 0.52)	<0.001	12 (30.8%)	283 (68.4%)	0.21 (0.10, 0.42)	<0.001	ND	ND		
≥128	0 (0%)	67 (17.3%)	0.05 (0.00, 0.74)	<0.001	9 (23.1%)	244 (58.9%)	0.21 (0.10, 0.45)	<0.001	ND	ND		
≥256	0 (0%)	51 (13.2%)	0.06 (0.00, 1.02)	0.002	5 (12.8%)	202 (48.8%)	0.15 (0.06, 0.40)	<0.001	ND	ND		
≥512	0 (0%)	38 (9.8%)	0.09 (0.00, 1.43)	0.014	2 (5.1%)	147 (35.5%)	0.01 (0.02, 0.41)	<0.001	ND	ND		

CI, confidence interval; ND, not done; OPA, opsonophagocytic activity.
[a]p value calculated by either chi-square test or Fisher's exact test, as appropriate.

enrolment who remained persistently colonized thereafter, compared to those who were only intermittently colonized, indicates that the antibody which was likely induced by colonization might not clear existing colonization. This clearance of established colonization may be due to other immune mediators, including cell-mediated immunity.

Our study indicated that the threshold of CPS antibody associated with significantly reduced odds of acquisition of GBS was ≥3 μg/mL for serotypes Ia and III and ≥1 μg/mL for serotype V. Furthermore, serotype-specific OPA titres of ≥8 were associated with similarly reduced odds of new acquisition of the homotypic serotype for serotypes Ia and III. The OPA titres correlated more strongly than IgG concentrations in relation to new acquisition of serotypes Ia and III. This shows the importance of measuring functional antibody in the evaluation of immune responses to GBS conjugate vaccine. Also, the correlation between OPA titres and CPS IgG concentration was modest, suggesting either impaired functional activity of antibody or limitations of the serology assay with respect to purity of antigens used or possible role of IgM antibodies in opsonophagocytosis [33].

Previous studies on the association of GBS colonization and serum IgG antibody have mainly been cross-sectional studies and have generally reported higher serotype-specific CPS antibody in colonized compared to noncolonized pregnant women for the homotypic serotype [15–22]. These studies were, however, not designed to address whether CPS antibody reduces the risk of GBS colonization during pregnancy, as shown by us. Nevertheless, the findings of the previous studies are confirmed in our study, where higher serotype-specific CPS IgG concentrations were also observed in women colonized by the homotypic serotype compared to those not colonized when analysed cross-sectionally at either visit 1 or visit 4 for serotypes Ia and III. This higher antibody concentration in colonized women in such studies likely reflect recent acquisition of that serotype, which induced the higher antibody levels compared to women not colonized, as demonstrated by the kinetics of the antibody response to new acquisitions in our study.

Our study has certain limitations. Firstly, although we explored for thresholds of serotype-specific CPS IgG and OPA titres associated with reduced odds of new acquisition by homotypic serotypes, the findings need to be interpreted with caution in the absence of a standardized reference assay to measure either CPS antibody or OPA titres [34]. We tried to optimize the comparability of our assay to that of other laboratories by calibrating our in-house reference (Polygam) with reference sera from C. J. Baker, who has published widely in the field. Secondly, the sensitivity of detection of GBS on selective media is estimated at 85% and mainly identifies the dominant

TABLE 4. Comparison of mean titres (μg/mL) at visits 1 and 4 for pregnant women for specific serotypes who acquired GBS and those who remained uncolonized

Serotype	Serotype-specific new acquisition			Uncolonized by specific serotype		
	Visit 1	Visit 4		Visit 1	Visit 4	
Serum IgG	GMC (95% CI)	GMC (95% CI)	p[a]	GMC (95% CI)	GMC (95% CI)	p[a]
Ia	0.32 (0.18, 0.57); *n* = 42	0.86 (0.44, 1.70)	0.001	0.36 (0.28, 0.46); *n* = 387	0.40 (0.32, 0.51)	0.169
III	0.14 (0.09, 0.22); *n* = 35	0.26 (0.15, 0.46)	0.004	0.32 (0.28, 0.38); *n* = 414	0.30 (0.26, 0.36)	0.272
V	0.38 (0.23, 0.61); *n* = 15	0.76 (0.43, 1.33)	0.034	0.75 (0.66, 0.84); *n* = 471	0.83 (0.74, 0.93)	0.011
OPA	**GMOPT (95% CI)**	**GMOPT (95% CI)**				
Ia	5 (4, 6); *n* = 42	110 (53, 227)	<0.0001	Not done[b]		
III	17 (9, 32); *n* = 35	776 (528, 1143)	<0.0001			

Comparisons of serum GMC serotype-specific capsular IgG antibody (μg/mL) and GMOPT at enrolment and end of study in pregnant women who acquired GBS and those who remained uncolonized throughout.
CI, confidence interval; GBS, group B *Streptococcus*; GMC, geometric mean concentration; GMOPT, geometric mean opsonophagocytic activity titre; OPA, opsonophagocytic activity.
[a]p value by paired Student's *t* test.
[b]OPA at visit 4 was done only for participants who acquired GBS serotype.

colonizing serotype, with lower-density co-colonizing serotypes likely being missed [23]. Therefore, it is possible that we overestimated new acquisition through missing colonization episodes not detectable by standard culture methods at visit 1, and we similarly may also have missed some new acquisitions.

In summary, our results indicate that the effect of GBS conjugate vaccine on rectovaginal GBS colonization warrants further investigation. This could provide rectovaginal colonization as a surrogate end point to evaluate the potential effect of GBS conjugate vaccine in protecting newborns (including those born prematurely) from exposure to GBS at birth and hence reduce their risk of EOD. A monovalent GBS serotype III conjugate vaccine has already been reported to significantly reduce the rate of rectovaginal GBS acquisition among nonpregnant women (Hillier SL, et al. Program and Abstracts of the 47th Infectious Diseases Society of America annual meeting, abstract 186). This potential effect of vaccination with GBS vaccine targeted at women against rectovaginal colonization may be over and above the additional protection of vaccination against invasive GBS disease in young infants born at full term, which is likely to be conferred through transplacental acquisition of protective serotype-specific CPS antibody. Furthermore, it is plausible that reducing the risk of GBS acquisition during pregnancy could reduce the incidence of GBS-associated chorioamnionitis, premature labor and stillbirth [5].

Transparency declaration

This work was funded by research supported by the South African Research Chairs Initiative in Vaccine Preventable Diseases of the Department of Science and Technology (64797) and National Research Foundation. Part-funding was also granted by Novartis Vaccines, Italy. GK was supported by South African Research Chairs Initiative (SARChI) grant. Any opinion, findings and conclusions or recommendations expressed in this material are those of the author(s) and therefore the NRF and DST do not accept any liability with regard thereto. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript. All authors report no conflicts of interest relevant to this article.

Acknowledgements.

We thank C. J. Baker for kindly providing standard GBS reference serums; Novartis Vaccines, Italy, for kindly providing CPS antigens; M. Madzivhandila, P. Morailane, N. F. Miya and L. Maphalla for assistance with laboratory work; the antenatal staff of Lillian Ngoyi, Mofolo, Diepkloof and Michael Maponya community clinics in Soweto for all their help and support; and the participating pregnant women for selflessly volunteering their participation.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.cmi.2015.01.030.

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The Journal of Infectious Diseases

BRIEF REPORT

Appendix 4

Serotype-Specific Cell-Mediated Immunity Associated With Clearance of Homotypic Group B *Streptococcus* Rectovaginal Colonization in Pregnant Women

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We investigated the association between group B *Streptococcus* (GBS) serotype-specific capsular polysaccharide cellular immunity, measured with enzyme-linked immunospot (ELISPOT) interferon γ release assay at 20 weeks gestation in pregnant women, and its effect on rectovaginal serotype-specific GBS colonization up to 37 weeks gestation. Among women colonized by serotype III at enrollment, interferon γ ELISPOT positivity was more common in those in whom colonization was cleared (44.4%) than in those in whom colonization persisted (7.4%; $P = .008$), with a similar trend observed for serotype Ia. Presence of serotype-specific capsular polysaccharide cell-mediated immunity contributes to the clearance of GBS rectovaginal colonization.

Keywords. group B *Streptococcus*; cellular immunity; colonization.

Maternal rectovaginal colonization with group B *Streptococcus* (GBS) and its vertical transmission to newborns is the primary source of early-onset (<7 days age) invasive GBS disease [1] and has also been associated with stillbirths and premature deliveries [2]. Spontaneous clearance and acquisition of GBS has been reported in pregnant women [3, 4]; however, the host immune mediators influencing the dynamics of GBS colonization remain to be fully elucidated. We established in an earlier study that capsular-specific antibody and opsonophagocytic activity reduced the risk of new homotypic serotype acquisition between 20 and 37+ weeks of pregnancy, but neither was associated with clearance of GBS colonization [5]. The aim of this study was to determine the association between the presence of GBS cellular immunity, as assessed by enzyme-linked immunospot (ELISPOT) interferon (IFN) γ release assays, and the dynamics of GBS colonization in women during the latter half of pregnancy.

Received 15 October 2015; accepted 31 January 2016; published online 29 March 2016.

Presented in part: International African Vaccine Conference, Cape Town, South Africa, 8–11 November 2012. Abstract 86.

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The Journal of Infectious Diseases® 2016;213:1923–6

DOI: 10.1093/infdis/jiw056

MATERIAL AND METHODS

Study Participants and Design

Detailed study methods have been published elsewhere [6]. Briefly, pregnant women aged 18–45 years, who were without human immunodeficiency virus infection and at 20–25 weeks gestation, were enrolled at antenatal community clinics in Soweto, Johannesburg, from August 2010 to August 2011. Exclusion criteria included antibiotic treatment in the previous 2 weeks, any acute illness, symptomatic vaginal discharge, and a known or suspected condition in which clinical vaginal examination was contraindicated.

Study procedures included lower vaginal and rectal swab sample collection for GBS culture starting at 20–25 weeks (visit 1), followed by 3 subsequent visits (visits 2–4) at 5–6 weekly intervals, until 37–40 weeks gestation (visit 4). For GBS isolation, swab samples were inoculated onto CHROMagar StrepB agar (CA; Media Mage), as described elsewhere [7]. Serotyping was performed using the latex agglutination method, as described elsewhere [8]. Isolates that tested negative at latex agglutination for all serotypes were further typed with a polymerase chain reaction method, using primer sequences described by Poyart et al [9].

Measure of Cellular Immune Response

Cellular immune response was determined at visit 1 (enrollment) by IFN-γ release assays, with an ELISPOT assay kit for human IFN-γ according to the manufacturer's instructions (catalog No. 3420-2A; Mabtech). Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from the heparinized blood using Ficoll gradient centrifugation and resuspended in AIM-V medium (Gibco, Invitrogen). About $0.25–0.3 \times 10^6$ PBMCs per well were added to ELISPOT plates (MAIP S4510; Millipore) coated with IFN-γ capture antibody (Mabtech). PBMCs were stimulated with GBS serotype-specific capsular polysaccharide (CPS) antigens separately (CPS Ia, III, and V) for 18–24 hours at 37°C in 5% carbon dioxide, with a final concentration of 40 μg/mL. (GBS CPS antigens were provided by Novartis Vaccines).

After incubation, ELISPOT plates were developed using biotinylated anti–IFN-γ detection antibody (Mabtech), detected with streptavidin–alkaline phosphatase (Mabtech) and visualized with an alkaline phosphatase conjugate substrate kit

(Bio-Rad). Checkerboard titrations were performed to optimize PBMCs and antigen concentrations per well. AIM-V medium was used as a negative control to assess background levels, and phytohaemagglutinin (Sigma) was used as a positive control. Cellular immune responses against each antigen were detected in single wells only. Spot-forming units (SFUs) were counted with a magnifying lens. Background (SFUs in negative control) was subtracted, and results were expressed as SFUs per 10^6 PBMCs. An ELISPOT response was considered positive if the number of antigen-specific spots was ≥7 SFUs/10^6 PBMCs and at least double the number in the negative control well. In addition, serotype-specific serum CPS immunoglobulin (Ig) G antibody concentrations were measured by means multiplex Luminex (serotypes Ia, III, and V) and opsonophagocytic activity (OPA) (serotypes Ia and III) assay, as described elsewhere [5].

Study Definition

A participant was considered colonized at a visit if GBS was cultured on either the vaginal or rectal swab sample and noncolonized if no growth was detected from either site. The association of cellular immunity with clearance of serotype-specific GBS colonization was evaluated in women who were colonized by the specific serotype at visit 1 and who completed all 4 study visits. The women were stratified into those in whom colonization of the specific serotypes was cleared by visit 4 (the "cleared-colonization" group) and those who remained colonized throughout the study (the "persistently colonized" group). Because of limitations associated with detecting multiple serotype carriage using standard culture methods, we excluded from analysis participants colonized with any GBS serotype at visit 1 who acquired a different GBS serotype at a subsequent visit and remained colonized with that serotype thereafter. We also evaluated the association of cellular immunity and acquisition of serotype-specific GBS colonization in women not colonized by a specific GBS serotype at visit 1, who were subsequently colonized by that specific serotype (irrespective of the number of study visits completed) ("new-acquisition" group), compared with that in women not colonized with that specific serotype at any study visit ("noncolonized" group).

Statistical Analysis

For categorical variables, groups were compared with either χ^2 or Fisher 2-tailed exact tests, as appropriate. The Mann–Whitney test was used to detect differences in SFU counts between groups (persistently colonized vs cleared colonization). The correlations between serotype-specific serum IgG and serotype-specific OPA titers and the ELISPOT SFU count (per 10^6 PBMCs) at visit 1 were calculated using Spearman correlation coefficients. To explore the association between SFU counts and clearance of GBS colonization, we used a Bayesian model to estimate the posterior distribution of the probability that a woman with a GBS homotypic SFU count (per 10^6 PBMCs) greater or equal to c would clear GBS colonization (D), denoted by Probability (P) (clearance of GBS colonization (D) = 1|SFU ≥ c) [10]. For each value c, we graphically display the 25%, 50%, and 75% quantiles of the posterior distribution of $P(D = 1|\text{SFU} \geq c)$. Data were analyzed using GraphPad (version 5.0; GraphPad Software) and R (version 2.15) software [11]. Differences were considered significant at $P < .05$.

Ethics Statement

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (institutional review board protocol M090937), and informed written consent was obtained from all participants.

RESULTS

Of 661 participants, 76.7% (507 of 661) completed all 4 study visits and 95.1% (629 of 661) completed at least 2 study visits. A detailed profile of participant demographics (Supplementary Table 1) and follow-up has been reported elsewhere [6]. Of the 507 women who completed all scheduled visits, 71 (14.0%), 56 (11.1%), and 15 (2.9%) were colonized by serotypes Ia, III, and V, respectively at enrollment. Of the women already colonized at enrollment, 21 of 71 (29.6%), 27 of 56 (48.2%), and 2 of 15 (13.3%) were categorized as being persistently colonized; and 43 (60.6%), 18 (32.1%), and 9 (60.0%) were in the cleared-colonization groups for serotypes Ia, III, and V, respectively. This excluded 22 participants colonized at enrollment who cleared the initial serotype (Ia (7 of 71; 9.8%), III (11 of 56; 19.6%), and V (4 of 15; 26.7%) but who acquired a new serotype at a subsequent visit and remained colonized with the subsequent serotype thereafter. There were no differences in demographic characteristics between the persistently colonized and cleared-colonization groups (data not shown).

Of those not colonized by specific GBS serotype Ia, III, or V at enrollment and who had ≥1 subsequent study visit, 9.6% (52 of 541), 7.0% (39 of 560), and 2.8% (17 of 603) subsequently become colonized by the respective serotypes (ie, new-acquisition group). Of participants who completed all scheduled visits, 387 (76.3%), 414 (81.6%), and 471 (92.9%) for serotype Ia, III, and V, respectively (ie, noncolonized group), were never colonized by that specific serotype. There were no differences in demographic characteristics between the new-acquisition and noncolonized group [5].

Of the 661 enrolled participants in whom cellular immune response was determined at the first visit, 6 participants were excluded from the analysis owing to contamination detected in the assay. Of the remaining 655 participants, serotype-specific ELISPOT responses were prevalent in 191(29.2%), 154(23.5%), and 143 (21.8%) for serotypes Ia, III, and V, respectively, at visit 1. There was no correlation between serotype-specific serum IgG concentrations and SFU counts at ELISPOT assay for serotypes Ia ($\rho = 0.006$; $P = .89$), III ($\rho = 0.023$; $P = .55$), or V ($\rho = 0.021$; $P = .59$) (Supplementary Figure 1*A*–*C*). Similarly,

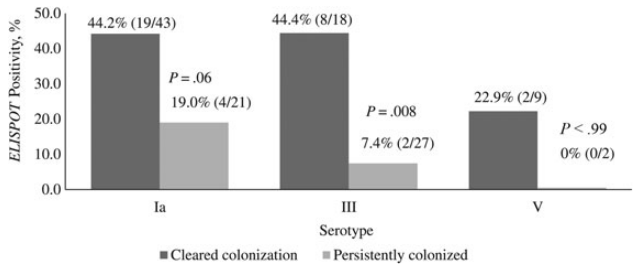

Figure 1. Comparisons of enzyme-linked immunospot (ELISPOT) positivity at enrollment in pregnant women who has cleared colonization (Cleared colonization group) to those who remain colonized throughout by that serotype (Persistently colonized group).

no correlation was observed between serotype-specific OPA titers and SFU count for either serotype Ia ($\rho = 0.017$; $P = .66$) or III ($\rho = 0.042$; $P = .28$) (Supplementary Figure 1*D* and 1*E*).

Among women colonized with serotype III at enrollment, ELISPOT positivity was found in higher percentage of those who cleared colonization (44.4%) than of those who remained persistently colonized (7.4%; $P = .008$), with a similar trend for serotype Ia (44.2% vs 19.0%, respectively; $P = .06$) and serotype V (22.9% vs 0.0%, respectively; Figure 1). Serotype-specific median SFU counts tended to be higher among those who cleared colonization than among those persistently colonized for serotypes III ($P = .055$) and Ia ($P = .14$), albeit not significant (Supplementary Figure 2).

In a Bayesian framework, the probability of losing colonization increased with higher SFU counts (Supplementary Figure 3). For serotype III, we observed 50% and 70% probability of losing colonization with SFU counts $\geq 7/10^6$ and $\geq 22/10^6$ PBMCs, respectively. For serotype Ia, we observed 82% probability of losing colonization with SFU counts $\geq 7/10^6$ PBMCs. There was no correlation between serotype-specific ELISPOT positivity and clearance of nonhomotypic serotype. The lowest threshold associated with clearance of serotype III colonization was ≥ 7 SFUs/10^6, with prevalences of 44.4% (8 of 18) in the cleared-colonization versus 7.4% (2 of 27) in the persistently colonized group (odds ratio, 10.00; 95% confidence interval, 1.80–55.55; $P = .008$) and 48.8% (21 of 43) and 19% (4 of 21) in the respective groups for serotype Ia (4.06; 1.17–14.06; $P = .03$) (Table 1).

Furthermore, whereas we previously reported significant associations between serotype-specific antibody concentration and OPA in relation to subsequent new acquisition of GBS during pregnancy [5], there was no significant difference in ELISPOT positivity between women who became colonized (new-acquisition group) and those who remained uncolonized (noncolonized group) for serotypes Ia (21.6% [11 of 51] vs 29.4% [113 of 384]; $P = .24$), III (17.9% [7 of 39] vs 23.9% [98 of 410]; $P = .40$) or V (11.8% [2 of 17] vs 21.8% [102 of 467]; $P = .55$). Excluded from analysis owing to contamination were 3 participants for serotype Ia and 4 each for serotypes III and V from the noncolonized group and 1 participant for serotype Ia from the new-acquisition group.

DISCUSSION

We have shown that clearance of serotype-specific GBS rectovaginal colonization during pregnancy was associated with presence of homotypic capsular ELISPOT IFN-γ positivity, whereas no such association was observed for serotype-specific capsular antibody or OPA, as reported for the same cohort [5]. Conversely, ELISPOT positivity was not associated with a significantly reduced risk of GBS acquisition, which was, however, positively associated with serotype-specific capsular antibody and OPA for serotypes Ia and III, as reported. These data suggest that presence of cell-mediated immune response contributes to the clearance of rectovaginal GBS colonization, whereas we have reported elsewhere that humoral immunity is required to reduce the risk of rectovaginal GBS acquisition during pregnancy [5].

The results of the current study are in accordance with findings of studies on other capsular bacteria, such as *Streptococcus pneumoniae,* for which cell-mediated immune response is important for mucosal clearance in animal models [12, 13]. A serotype-specific capsular-based GBS vaccine able to elicit

Table 1. Association Between Serotype-Specific SFU Counts by ELISPOT Assay and Clearance of Homotypic GBS in Pregnant Women[a]

	Serotype Ia[b]				Serotype III			
SFU Count, SFUs/10^6 PBMCs	Cleared-Colonization Group, No. (%) (n = 43)	Persistently Colonized Group, No. (%) (n = 21)	OR (95% CI)	*P*-Value	Cleared-Colonization Group, No. (%) (n = 18)	Persistently Colonized Group, No. (%) (n = 27)	OR (95% CI)	*P*-Value
≥3	22 (51.2)	9 (42.9)	1.39 (.48–3.99)	.53	12 (66.6)	13 (48.1)	2.15 (.62–7.42)	.22
≥7	21 (48.8)	4 (19.0)	4.06 (1.17–14.06)	.03	8 (44.4)	2 (7.4)	10.0 (1.80–55.55)	.008
≥10	9 (20.9)	1 (4.8)	5.29 (.62–44.96)	.15	3 (16.7)	2 (7.4)	2.50 (.37–16.73)	.37

Abbreviations: CI, confidence interval; ELISPOT, enzyme-linked immunospot; GBS, group B *Streptococcus*; OR, odds ratio; PBMCs, peripheral blood mononuclear cells; SFU, spot-forming unit.

[a] Serotype V was not analyzed because the numbers were insufficient for comparison.

[b] For serotype Ia, we included in the quantitative analysis 2 participants in the cleared-colonization group who had SFU counts ≥7 SFU/10^6 PBMCs at baseline but failed to meet the criterion for ELISPOT positivity of having at least double the spots of the negative control.

both humoral and cell-mediated capsular immune responses could therefore confer protection against early-onset disease by reducing the exposure of newborns to GBS colonization during the peripartum period. This could offer an additional mode of protection against invasive GBS disease, especially for preterm newborns, who might not fully benefit from transplacental acquisition of maternal serotype-specific capsular antibody, which mainly occurs beyond 34 weeks of gestation. Moreover, clearance of GBS rectovaginal colonization during pregnancy, coupled with prevention of new acquisition mediated by the presence of humoral immunity, could theoretically mitigate against other GBS-associated illness, such as chorioamnionitis, and adverse birth outcomes, such as stillbirths and premature labor. However, to affect the full spectrum of GBS-associated morbidity and mortality in women, fetuses, and newborns, vaccination would need to occur early in the second trimester of pregnancy and induce serotype-specific functional antibody and cellular immunity.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

Acknowledgments. We thank Novartis Vaccines, Italy, for kindly providing capsular polysaccharide antigens.

Disclaimer. Any opinion, findings and conclusions or recommendations expressed in this material are those of the authors, and the National Research Foundation and the Department of Science and Technology do not accept any liability with regard thereto. The funders had no role in study design, data collection, analysis, decision to publish, or preparation of the manuscript. Novartis Vaccines did not have any role in the conduct, analysis of data, writing of the manuscript, or decision to submit it for publication.

Financial support. This work was supported by the South African Research Chairs Initiative in Vaccine Preventable Diseases of the Department of Science and Technology and National Research Foundation, Novartis Vaccines, Italy, and the South African Research Chairs Initiative (grant to G. K.).

Potential conflicts of interest. The institution of G. K., P. V. A., A. I., C. L. C., and S. A. M. has received grant support from Novartis Vaccines related to epidemiological studies on group B *Streptococcus* (GBS). C. L. C. and S. A. M. are also clinical trialists for Novartis Vaccines GBS vaccine program; this study was funded in part by a grant from Novartis Vaccines. All other authors report no potential conflicts. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Prevalence of maternal colonisation with group B streptococcus: a systematic review and meta-analysis

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Summary

Background **The most important risk factor for early-onset (babies younger than 7 days) invasive group B streptococcal disease is rectovaginal colonisation of the mother at delivery. We aimed to assess whether differences in colonisation drive regional differences in the incidence of early-onset invasive disease.**

Methods **We did a systematic review of maternal group B streptococcus colonisation studies by searching MEDLINE, Embase, Pascal Biomed, WHOLIS, and African Index Medicus databases for studies published between January, 1997, and March 31, 2015, that reported the prevalence of group B streptococcus colonisation in pregnant women. We also reviewed reference lists of selected studies and contacted experts to identify additional studies. Prospective studies in which swabs were collected from pregnant women according to US Centers for Disease Control and Prevention guidelines that used selective culture methods were included in the analyses. We calculated mean prevalence estimates (with 95% CIs) of maternal colonisation across studies, by WHO region. We assessed heterogeneity using the I^2 statistic and the Cochran Q test.**

Findings **221 full-text articles were assessed, of which 78 studies that included 73 791 pregnant women across 37 countries met prespecified inclusion criteria. The estimated mean prevalence of rectovaginal group B streptococcus colonisation was 17·9% (95% CI 16·2–19·7) overall and was highest in Africa (22·4, 18·1–26·7) followed by the Americas (19·7, 16·7–22·7) and Europe (19·0, 16·1–22·0). Studies from southeast Asia had the lowest estimated mean prevalence (11·1%, 95% CI 6·8–15·3). Significant heterogeneity was noted across and within regions (all p≤0·005). Differences in the timing of specimen collection in pregnancy, selective culture methods, and study sample size did not explain the heterogeneity.**

Interpretation **The country and regional heterogeneity in maternal group B streptococcus colonisation is unlikely to completely explain geographical variation in early-onset invasive disease incidence. The contribution of socio-demographic, clinical risk factor, and population differences in natural immunity need further investigation to understand these regional differences in group B streptococcus maternal colonisation and early-onset disease.**

Funding **None.**

Lancet Infect Dis 2016

Published Online
May 25, 2016
http://dx.doi.org/10.1016/S1473-3099(16)30055-X

See Online/Comment
http://dx.doi.org/10.1016/S1473-3099(16)30072-X

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Introduction

Group B streptococcus is a substantial cause of severe infection in infants younger than 3 months, most often manifesting as sepsis, pneumonia, and meningitis. Infant invasive group B streptococcal disease occurs as a continuum over the first 3 months of life, but is commonly divided into early-onset disease, occurring within 0–6 days of birth, or late-onset disease, occurring within 7–90 days of birth. Early-onset disease is vertically acquired from the mother, whereas late-onset disease can also be acquired nosocomially or in the community.[1] Case fatality ratios remain as high as 36% even in high-income settings such as Norway and the USA.[2,3] Moreover, among survivors of group B streptococcal meningitis, 46–50% develop moderate-to-severe neurological impairment.[4–6]

Substantial variation exists in invasive disease incidence both within and across geographical regions, ranging from 0·02 per 1000 livebirths in southeast Asia to 1·21 per 1000 livebirths in Africa.[7] Some variation is probably attributable to the introduction of antibiotic-based interventions. Intrapartum antibiotic prophylaxis (IAP), given to mothers with rectovaginal colonisation with group B streptococcus in late pregnancy, has resulted in a substantial decrease in the incidence of early-onset disease.[8] However, substantial variation in incidence of early-onset disease remains in low-income and middle-income countries, where the logistic and cost challenges associated with routine culture-based screening and IAP have limited its implementation. Factors underlying this variation might include differences in case ascertainment and laboratory diagnostics, or population differences in exposure and susceptibility.[9]

The most important risk factor for invasive early-onset group B streptococcal disease is rectovaginal colonisation of the mother at the time of birth.[10] Vertical transmission of group B streptococcus occurs in about 50% of newborn babies of mothers with group B streptococcus colonisation, of whom 1–2% develop early-onset disease.[11] If population-based differences in exposure are driving the reported differences in early-onset disease incidence, one would expect to find similar disparities in the prevalence of maternal colonisation during pregnancy. In a 1998 systematic review[12] of maternal group B

Research in context

Evidence before this study

Group B streptococcus rectovaginal colonisation of the mother at the time of birth is the most important risk factor for early-onset (babies younger than 7 days) invasive group B streptococcal disease. Marked differences in prevalence of invasive group B streptococcal disease exist across geographical regions, ranging from 0·02 per 1000 livebirths in southeast Asia to 1·21 per 1000 livebirths in Africa. In a 1998 systematic review of maternal group B streptococcus colonisation, prevalence ranged from 8% to 18% by geographical region. This variability was partly attributed to differences in the microbiological methods used for group B streptococcus isolation between studies. Since then, the US Centers for Disease Control and Prevention and others have recommended methods for rectovaginal sample collection and culture of group B streptococcus, aimed at improving sensitivity of detection of maternal colonisation. We did a systematic review and meta-analysis of prospective, epidemiological studies to assess the prevalence of rectovaginal group B streptococcus colonisation globally, and explore whether this was associated with the reported geographical variation in incidence of invasive disease. We searched five databases (MEDLINE, Embase, Pascal Biomed, WHOLIS, and African Index Medicus) to identify studies that reported the prevalence of group B streptococcus colonisation in pregnant women. Searches were restricted to those published between January, 1997, and March 31, 2015. No language restrictions were applied. Searches were done using the following terms: "*Streptococcus agalactiae*" or "group B streptococcus" or "streptococcus group B" and "colonization" or "colonisation". For WHO databases, searches were done using the following terms: "group B streptococcus colonization" or "*Streptococcus agalactiae* colonization". The inclusion criteria were limited to prospective studies in pregnant women that used robust recommended methods of sample collection and culture methods, to enhance comparability across studies.

Added value of this study

The global mean prevalence estimate of rectovaginal group B streptococcus colonisation was 17·9% (95% CI 16·2–19·7). Significant heterogeneity was noted both within and across geographical regions. The mean prevalence estimate of maternal rectovaginal group B streptococcus colonisation was highest in studies from Africa (22·4%, 95% CI 18·1–26·7) and lowest in southeast Asia (11·1%, 6·8–15·3). Stratification by timing of specimen collection, type of selective culture method, and sample size did not explain the heterogeneity in mean prevalence of group B streptococcus colonisation between studies or geographical regions. Among 17 studies reporting serotype distribution of colonising strains, none of which were from southeast Asia, serotypes Ia, Ib, II, III, and V accounted for more than 91% of isolates. A group B streptococcus vaccine that targets these five serotypes would provide coverage against most serotypes to which newborn babies are exposed; however, similar data are needed for those from low-income and middle-income countries.

Implications of all the available evidence

In this meta-analysis, we identified significant variation in the prevalence of maternal colonisation both within and across geographical regions, which does not seem to be due to differences in sample collection or culture methods as previously proposed. Nevertheless, the difference in prevalence of maternal group B streptococcus colonisation between studies from Africa and southeast Asia, regions where intrapartum antibiotic prophylaxis for pregnant women with group B streptococcus colonisation is not done, was less marked than the difference in incidence of invasive group B streptococcal disease in young infants reported between these regions. This finding suggests that other factors, including sociodemographic indices, threshold for detection of invasive disease, clinical risk factors, population differences in natural immunity, and possibly differences in serotypes and strain virulence of colonising isolates, need further investigation to understand the regional differences in group B streptococcus maternal colonisation and early-onset disease. An improved understanding of these factors would be informative for the selection of serotypes to be included in a group B streptococcus serotype-specific polysaccharide vaccine. Such a vaccine would be developed for immunisation of pregnant women, as well as her fetus and offspring, against group B streptococcus.

streptococcus colonisation data from low-income and middle-income countries, mean prevalence ranged from 8% in the Asia–Pacific region to 18% in sub-Saharan Africa. The microbiological method of group B streptococcus isolation was identified as a key driver of this variation.[12] In recent years, the sensitivity of culture methods has increased and published guidance now exists for recommended methods of specimen collection, processing, and group B streptococcus isolation,[1] which should allow a clearer interpretation of any population differences in colonisation.

We did a systematic review and meta-analysis of prospective epidemiological studies in which group B streptococcus carriage was investigated by recommended microbiological methods[1] to assess whether the prevalence of maternal group B streptococcus colonisation matched the patterns of invasive disease incidence across geographical regions.

Methods

Search strategy and selection criteria

We searched five databases (MEDLINE, Embase, Pascal Biomed, WHOLIS, and African Index Medicus) to identify studies reporting the prevalence of group B streptococcus colonisation in pregnant women. We used the following search terms: "*Streptococcus agalactiae*" or "group B streptococcus" or "streptococcus group B" and "colonization" or "colonisation". The WHOLIS and

 www.thelancet.com/infection Published online May 25, 2016 http://dx.doi.org/10.1016/S1473-3099(16)30055-X

African Index Medicus searches were completed using the following terms: "group B streptococcus colonization" or "*Streptococcus agalactiae* colonization". We restricted searches to human studies published between January, 1997, and March 31, 2015. No language restrictions were applied. We also screened reference lists of selected studies and contacted experts in group B streptococcus epidemiology for any potentially relevant studies that had not been identified through the searches.

After an initial selection, based on review of the publication titles and abstracts, we did a full-text review of remaining studies. Studies were included if they met the following criteria: sufficient methodological detail to allow an assessment of specimen collection and microbiological methods; collection of rectovaginal or vaginal and rectal swabs done according to guidelines from the US Centers for Disease Control and Prevention (CDC) for specimen collection and microbiological identification of group B streptococcus; use of selective culture methods for isolation and identification of group B streptococcus; prospective data collection; pregnant population; and original research article describing data not published previously. We used these criteria to focus the systematic review on studies with robust methods and to increase comparability across studies.

Data analysis

We identified, screened, and excluded studies according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA). Two investigators (GK and MCC) independently examined titles, abstracts, full-text articles, and abstracted data using the same data abstraction forms and selection criteria. Disagreements were resolved by consensus among the investigators. Data were abstracted for the following study characteristics: geographical region (as defined by WHO: Africa, the Americas, southeast Asia, Europe, eastern Mediterranean, and western Pacific); study period; study population; timing (late pregnancy [≥35 weeks of gestation] *vs* at delivery *vs* <35 weeks) and site of specimen collection (single rectovaginal swab *vs* separate vaginal and rectal swabs); method of group B streptococcus isolation (selective broth followed by subculture on blood agar or selective agar *vs* direct plating on selective media); sample size; and proportion of women colonised with group B streptococcus. Culture methods could include those recommended by the CDC (group B streptococcus growth on selective broth followed by subculture on blood agar or selective media) or those outside of the CDC recommendations using some form of selectivity in the medium for group B streptococcus isolation.[1]

We did a random-effects meta-analysis to calculate mean prevalence estimates (with 95% CIs) of maternal colonisation across studies and by region. Studies were categorised by region and sorted by study size, and heterogeneity between studies was assessed, first visually by the overlap of 95% CIs and then quantitatively by the I^2 statistic[13] and the Cochran Q test for heterogeneity with a significance level of 5%.[14] The I^2 statistic is interpreted as the proportion of the total variation in the estimates of mean prevalence that is due to the heterogeneity between studies. We explored potential drivers of statistical variation (ie, heterogeneity), including timing of specimen collection and culture method, using descriptive stratified analyses. We used funnel plots to investigate how estimated mean prevalence varied by study size, in case of small study effects.[15,16] Additionally, for studies with available data on the distribution of serotypes, we did a random-effects meta-analysis to calculate the mean prevalence of women with serotypes

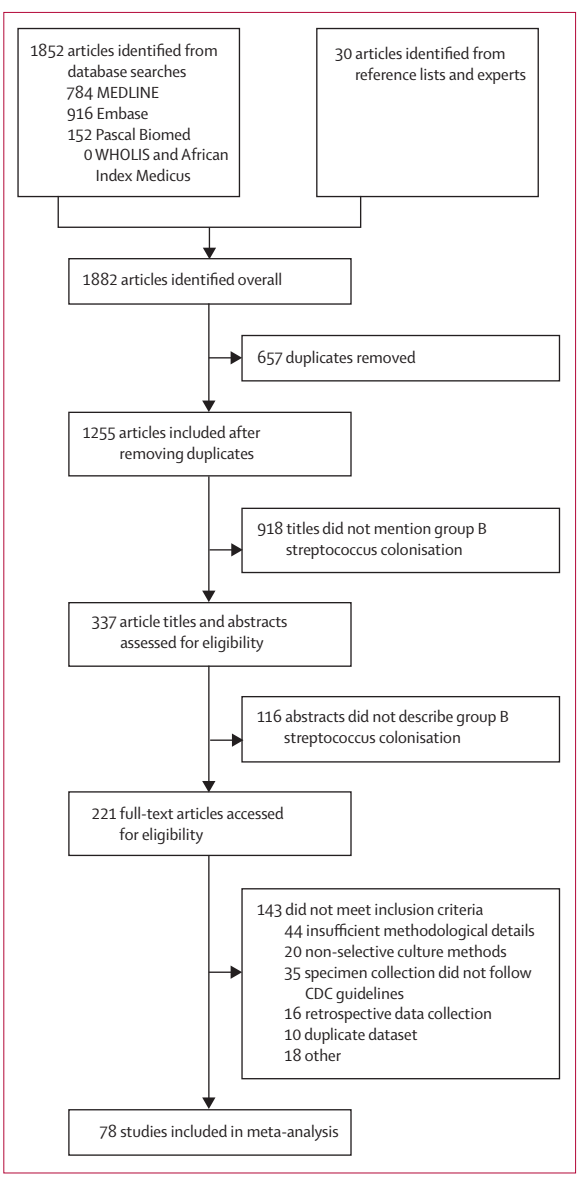

Figure: **Selection of group B streptococcus colonisation studies**
CDC=US Centers for Disease Control and Prevention.

For the **WHO regions** see http://www.who.int/about/regions/en/

Ia, Ib, or III, and serotypes Ia, Ib, II, III, or V. Statistical analyses were completed using R software (version 2.15.2) and SAS (version 9.2).

The CDC recommendation for rectovaginal swab collection meant that studies in which lower vaginal swabs only were collected were excluded. To investigate any potential bias introduced, we did a separate sensitivity analysis of studies that met all predefined criteria, but that reported estimates for lower vaginal colonisation only. Studies were categorised by region, as classified by WHO criteria. Because the western Pacific region includes an ethnically diverse range of countries (within this meta-analysis: Australia, China [including Hong Kong], Japan, South Korea, and New Zealand), we did a separate sensitivity analysis that excluded Australia and New Zealand.

Role of the funding source

There was no funding source for this study. The corresponding author had full access to all the data in the study and all authors had final responsibility for the decision to submit for publication.

See Online for appendix

Results

We identified 1852 articles from the literature search and 30 through a search of reference lists and expert opinion (figure). After an initial review of titles and abstracts and the removal of duplicates, we assessed the full text of 221 articles for eligibility, of which 78 met the meta-analysis inclusion criteria.[17–94]

Most identified reports were from Europe and the Americas, whereas the fewest reports were from Africa (table 1). The proportion of studies meeting inclusion criteria was lowest for studies from Africa and the eastern Mediterranean (table 1). The most common reasons for exclusion were did not follow CDC guidelines for specimen collection (lower vaginal and rectal swabbing) in 35 (24%) of 143 studies, non-selective culture methods in 20 (14%), and insufficient methodological detail in 44 (31%).[1]

73 791 pregnant women were included in 78 studies across 37 countries (median number of women per study 391, IQR 251–1011), of whom 13 100 were positive for rectovaginal colonisation with group B streptococcus (table 2). The 23 studies from Europe had the highest number of women (n=31 642), whereas the seven studies from the eastern Mediterranean region had the lowest number (n=2729). The highest median number of women per study was in the western Pacific (1049, IQR 404–1700) and the lowest was in the eastern Mediterranean (300, 248–318). The appendix (pp 1–5) provides further details of the included studies.

The study-specific mean prevalence estimates ranged from 0·5% (95% CI 0·0–1·1) in Mexico[17] to 37·9% (25·4–50·4) in Denmark.[18] Table 2 summarises the random-effects meta-analysis by region. Regional mean prevalence estimates ranged from 11·1% (95% CI 6·8–15·3) in southeast Asia to 22·4% (18·1–26·7) in Africa. The global mean prevalence estimate of 17·9% (95% CI 16·2–19·7) was similar to the estimates for the two most studied regions: Europe and the Americas.

The appendix (pp 6–11) summarises the colonisation mean prevalence estimates for all studies included in the meta-analysis, by geographical region.[17–94] The overall heterogeneity was 98·1% and remained significant within each region (lowest I^2 82·7%, p=0·005 for the four studies from Africa), and although mean prevalence estimates from the same country were often similar (eg, Tunisia[19,20]) this was not always the case (eg, India[21,22]).

We also investigated other sources of heterogeneity. Several different selective culture methods were reported,

	Number identified	Included	Excluded
Africa	14	4 (29%)	10 (71%)
Americas	76	29 (38%)	47 (62%)
Eastern Mediterranean	32	7 (22%)	25 (78%)
Europe	59	23 (39%)	36 (61%)
Southeast Asia	20	7 (35%)	13 (65%)
Western Pacific	19	8 (42%)	11 (58%)
International*	1	0 (0%)	1 (100%)
Total	221	78 (35%)	143 (65%)

*USA, Ireland, Thailand, Myanmar, Philippines, and Zimbabwe.

Table 1: **Inclusion and exclusion of studies by region**

	Number of studies	Number of women	Median number of women per study (IQR)	Number of women with GBS	Estimated mean prevalence (95% CI)	I^2*	p value
Africa	4	2735	599 (415–867)	619	22·4 (18·1–26·7)	82·7%	0·005
Americas	29	23 163	316 (222–962)	4360	19·7 (16·7–22·7)	98·4%	<0·0001
Eastern Mediterranean	7	2729	300 (248–318)	443	16·7 (11·7–21·7)	91·9%	<0·0001
Europe	23	31 642	500 (275–942)	6113	19·0 (16·1–22·0)	97·2%	<0·0001
Southeast Asia	7	3749	406 (310–727)	389	11·1 (6·8–15·3)	95·1%	<0·0001
Western Pacific	8	9773	1049 (404–1700)	1176	13·3 (7·8–18·8)	98·7%	<0·0001
Total	78	73 791	391 (251–1011)	13 100	17·9 (16·2–19·7)	98·1%	<0·0001

GBS=group B streptococcus. *Within-region test for heterogeneity.

Table 2: **Meta-analysis of studies reporting prevalence of maternal colonisation by region**

including selective broth subcultured on sheep blood agar, selective broth subcultured on selective media, and direct plating on selective agar media (appendix pp 12–13). To explore the contribution of these methods to the reported heterogeneity, we did a restricted analysis of studies with at least 400 women that used selective broth subcultured on sheep blood agar culture as the most common method (appendix p 14). However, the patterns of mean prevalence estimates across regions remained similar to the primary analysis, with high heterogeneity estimates (I^2 ≥90% across all regions).

We also investigated stratification according to maternal demographic characteristics, but analyses were limited by variation in the ways studies reported this information. Few studies had sufficient numbers of women to allow stratification by maternal age (<20 years *vs* ≥20 years), which precluded heterogeneity estimates for most regions (appendix p 15). We identified no difference in heterogeneity by study period (using year of publication as a proxy; appendix pp 16–17).

To investigate any potential bias introduced by the exclusion of studies reporting colonisation from lower vaginal swabs only, we did a separate meta-analysis. 17 studies were identified, including 18 495 women, with a mean prevalence estimate of 14·2% (95% CI 10·7–17·6) and a similar heterogeneity to the primary meta-analysis (I^2 97·5%; appendix p 18).

We also reassessed the ethnic diversity of the western Pacific region. For this region, eight studies were included originally, from China (including Hong Kong), Japan, South Korea, Australia, and New Zealand. The two Australian and New Zealand studies accounted for 1336 (14%) of 9773 women included in the original analysis. The exclusion of these two studies led to a numerical but non-significant reduction in the mean prevalence estimate from 13·3% (95% CI 7·8–18·8) to 10·1% (5·2–15·1), which is similar to the southeast Asian estimate of 11·1% (6·8–15·3; appendix p 19).

Serotype distribution was reported in 22 studies, but five were excluded because they reported combined serotype data only (n=2),[51,52] combined maternal and neonatal isolate data (n=1),[84] or did not report raw data (n=2).[75,82] Table 3 summarises data from the remaining 17 studies. Lower proportions of women with serotypes Ia, Ib, or III were reported in studies from the Americas (mean prevalence estimate 55·0%, 95% CI 52·3–57·7) and Europe (58·3%, 52·2–64·5) than for other regions, mainly as a result of a higher proportion of serotype II isolates in

	Country	Number of serotypes isolated	Serotypes Ia, Ib, or III			Serotypes Ia, Ib, II, III, or V		
			Number of women with at least one of these serotypes	Estimated mean prevalence (%, 95% CI)	I^2	Number of women with at least one of these serotypes	Estimated mean prevalence (%, 95% CI)	I^2
Overall		3391	2029	63·3% (57·7–68·8)	90·4%	3105	91·5% (88·9–94·0)	88·0%
Africa		542	362	69·2% (57·2–81·3)	88·1%	524	96·6% (94·1–99·0)	46·2%
Kwatra et al (2014)[24]	South Africa	152	115	75·7% (68·8–82·5)	..	144	94·7% (91·2–98·3)	..
Gray et al (2011)[25]	Malawi	390	247	63·3% (58·6–68·1)	..	380	97·4% (95·9–99·0)	..
Americas		1289	709	55·0% (52·3–57·7)	0·0%	1189	89·6% (82·6–96·6)	92·9%
Campbell et al (2000)[26]	USA	856	472	55·1% (51·8–58·5)	..	806	94·2% (92·6–95·7)	..
Davies et al (2001)[27]	Canada	233	126	54·1% (47·7–60·5)	..	201	86·3% (81·8–90·7)	..
Hickman et al (1999)[28]	USA	153	88	57·5% (49·7–65·3)	..	146	95·4% (92·1–98·7)	..
Simoes et al (2007)[29]	Brazil	47	23	48·9% (34·6–63·2)	..	36	76·6% (64·5–88·7)	..
Europe		1085	612	58·3% (52·2–64·5)	72·6%	946	88·9% (84·7–93·2)	79·2%
Hakansson et al (2008)[30]	Sweden	356	170	47·8% (42·6–52·9)	..	295	82·9% (79·0–86·8)	..
Tsolia et al (2003)[31]	Greece	67	36	53·7% (41·8–65·7)	..	60	89·6% (82·2–96·9)	..
Kunze et al (2011)[32]	Germany	155	98	63·2% (55·6–70·8)	..	140	90·3% (85·7–95·0)	..
Jones et al (2006)[33]*	UK	159	108	67·9% (60·7–75·2)	..	153	96·2% (93·3–99·2)	..
Kieran et al (1998)[83]	Ireland	129	78	60·5% (52·0–68·9)	..	116	89·9% (84·7–95·1)	..
Roccasalva et al (2008)[34]	Italy	7	5	71·4% (38·0–100·0)	..	7	93·8% (77·0–100·0)	..
Liébana-Martos Mdel et al (2015)[91]	Spain	212	117	55·2% (48·5–61·9)	..	175	82·5% (77·4–87·7)	..
Western Pacific		475	346	76·5% (65·4–87·5)	86·8%	446	94·2% (90·6–97·7)	56·1%
Lu et al (2014)[35]	China	201	151	75·1% (69·1–81·1)	..	195	97·0% (94·7–99·4)	..
Uh et al (1997)[36]	South Korea	193	132	68·4% (61·8–75·0)	..	175	90·7% (86·6–94·8)	..
Grimwood et al (2002)[37]	New Zealand	29	27	93·1% (83·9–100·0)	..	27	93·1% (83·9–100·0)	..
Lee et al (2010)[23]	South Korea	52	36	69·2% (56·7–81·8)	..	49	94·2% (87·9–100·0)	..

*Serotyping was done on isolates that were positive between two different culture methods with overall group B streptococcus positive only shown for US Centers for Disease Control and Prevention method.

Table 3: **Meta-analysis of serotypes isolated**

those regions (appendix pp 20–21). In a post-hoc analysis, serotypes VI, VII, and VIII were rare everywhere, although in one study from South Korea,[23] these serotypes were present in 14 (7%) of 192 vaginal and rectal group B streptococcus isolates (appendix pp 20–21).

To investigate how mean prevalence estimates varied by study size, we produced a funnel plot, which showed little asymmetry on either side of the overall mean prevalence estimate (appendix p 23; regression test with study size as a predictor p=0·89).

Discussion

In this systematic review and meta-analysis, the overall mean prevalence estimate of group B streptococcus in pregnant women was almost 20%, with the highest prevalence reported among African studies. The mean prevalence estimates reported from studies in the Americas, Europe, and the eastern Mediterranean regions were similar and the lowest prevalence estimates were from studies from southeast Asia and the western Pacific.

We selected studies for this meta-analysis, primarily based on CDC recommendations for group B streptococcus screening and identification using selective culture methods,[1] with the aim of focusing on robust data and limiting between-study heterogeneity due to methodological variation. However, we still identified significant heterogeneity across all studies and across studies within individual regions. A sensitivity analysis of studies with lower vaginal swab collection showed a similar pattern of regional heterogeneity. Further stratification by variables poorly controlled for by inclusion criteria (eg, timing of specimen collection and type of selective culture method) did not lead to a reduction in heterogeneity of mean prevalence estimates across studies. Additional variables, such as demographics, were difficult to explore because few studies reported these with sufficient detail. The colonisation prevalence range reported in the previous review[12] was 8–18%, which was similar to the 11–22% in this meta-analysis, despite greater standardisation in culture methods used in studies included in this meta-analysis, which was judged to be an important driver for the variability in colonisation between studies in the earlier meta-analysis. However, because the studies included in these two meta-analyses were not done in the same places, a direct comparison of these estimates must be interpreted with caution, since even within-country differences in colonisation were noted in the present meta-analysis. Nevertheless, these data suggest that other factors probably contribute towards regional variations in group B streptococcus colonisation in pregnant women and consequently risk for early-onset disease in their newborn babies.

Furthermore, the heterogeneity might have been driven by unmeasured variables; differences in study population selection might have led to higher or lower risk populations being represented, which was difficult to assess across studies. Several obstetric variables, such as parity and premature rupture of membranes, have been associated with higher risk of group B streptococcus colonisation,[24] but without access to individual-level data this was difficult to explore.

Differential sensitivity of culture methods between populations has been reported. For example, in South Africa, in one study[95] selective broth was not suitable for the recovery of group B streptococcus from rectal swabs because of masking of the group B streptococcus colonies by persistent microflora. In that population, direct plating on selective agar was the most sensitive method of isolation. Findings from studies from Denmark,[96] Spain,[97] and Brazil[98] have also shown increased group B streptococcus recovery using direct plating on selective media compared with selective broth. Further optimisation of microbiological techniques in different populations will contribute to a better understanding of potential ascertainment differences and their effect on group B streptococcus colonisation prevalence estimates.

The pattern of prevalence of group B streptococcus colonisation, with the highest estimates from Africa and lowest from southeast Asia and the western Pacific, matches the pattern of incidence of group B streptococcus neonatal disease.[7] An algorithm to predict early-onset disease incidence on the basis of maternal colonisation prevalence can be used to explore these patterns further; 50% of infants born to women colonised with group B streptococcus will themselves be colonised and 2% of those colonised infants will develop early-onset group B streptococcal disease.[11,99] Data from South Africa and the USA, before the introduction of routine screening and IAP, where about 20% of pregnant women had group B streptococcus vaginal colonisation, confirmed the expected prevalence of early-onset disease of two per 1000 livebirths.[11,100,101] Based on this algorithm, and regional prevalence estimates from the present meta-analysis, one would expect the prevalence of early-onset group B streptococcal disease in the southeast Asian and western Pacific studies to be half that in the African studies. However, the overall prevalence of invasive disease in those regions is commonly reported to be lower than the expected one per 1000 livebirths.[7] Although differences in colonisation prevalence might explain some of the regional differences in disease incidence, other factors including natural immunity and serotype distribution with varying virulence between serotypes and surveillance methods might also contribute and need to be investigated further.

In this systematic review and meta-analysis, we aimed to identify studies from all geographical regions by searching five different literature databases and by assessing publications in all languages. However, few studies from Africa, which has a high proportion of low-income countries, were identified from initial searches, and even fewer were eligible for inclusion. Thus, some publication bias is likely to remain.

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To ensure quality and to avoid bias in the identified studies, we used stringent inclusion criteria on the basis of rigorous methods and aimed to standardise studies with respect to factors known to affect the estimated prevalence of colonisation (eg, culture method for group B streptococcus isolation and identification). Nevertheless, we noted substantial variation in the prevalence of maternal colonisation both within and across geographical regions. The within-region differences were not explained by differences in specimen collection or microbiological culture techniques, although additional analyses with individual-level data are recommended to better understand the role of population selection, obstetric, and sociodemographic risk factors. A further limitation of the available data was that few data on serotype distribution by region were available, with only 17 studies contributing to this analysis, none of which were from southeast Asia or the eastern Mediterranean.

Underlying population differences in natural immunity through transplacental acquisition of serotype-specific capsular antibodies by the fetus remain an alternative explanation that should be explored in future studies to establish the disparity between rates of exposure to and prevalence of group B streptococcus across geographical regions. A clearer understanding of the factors underlying this variation will be crucial to enable more efficient design of screening and prevention programmes for maternal and infant group B streptococcal disease.

Contributors

GK, MCC, PVA, MI, KPK, WHT, and SAM participated in the conception, design, and implementation of the study. GK and MCC extracted data. GK, MCC, EM, and SAM wrote the first draft. EM did the statistical analysis. All authors interpreted the analysed data.

Declaration of interests

MCC and EM are permanent employees of GSK (formerly Novartis Vaccines). GK, PVA, and SAM's institution has received grant funds from Novartis for research into group B streptococcus epidemiology. SAM has also received travel support to attend a Novartis consultative meeting on group B streptococcus vaccines and is an adviser to Pfizer on group B streptococcus vaccines. All other authors declare no competing interests.

Acknowledgments

This work was initiated by Novartis Vaccines (now GSK Vaccines) in collaboration with investigators from the Respiratory and Meningeal Pathogens Research Unit and the authors. The authors are grateful to Francesca Dall'Acqua for SAS programming support.

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UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Division of the Deputy Registrar (Research)

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
R14/49 Mr Guarav Kwatra

CLEARANCE CERTIFICATE	**M090937**
PROJECT	Determining Immunological Correlates of Protection against Group B Streptococcus colonization in Women
INVESTIGATORS	Mr Guarav Kwatra.
DEPARTMENT	Respiratory & Meningeal Pathogens Res. Unit
DATE CONSIDERED	2009/10/02
DECISION OF THE COMMITTEE*	Approved unconditionally

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

DATE 2009/10/02 **CHAIRPERSON** .....
pp (Professor PE Cleaton-Jones)

*Guidelines for written 'informed consent' attached where applicable

cc: Supervisor : Prof SA Madhi

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.**

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