Vaginal colonization by Group B *Streptococcus* in different populations of African women:

risk factors and relation with the vaginal microbiome and the cervicovaginal immune system

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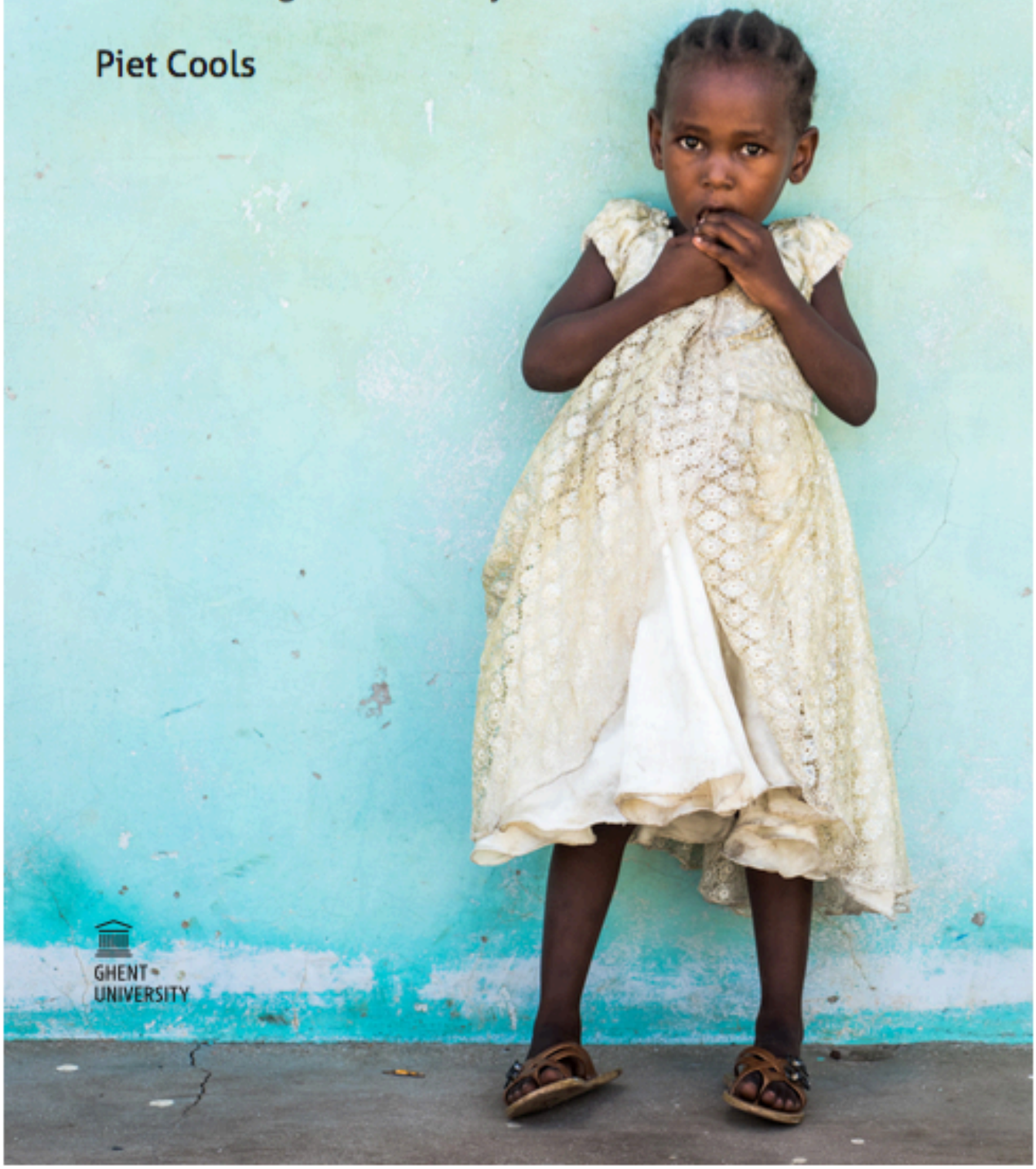

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Vaginal colonization by Group B *Streptococcus* in different populations of African women:

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Voor mijn Pa, die er niet meer kan bij zijn
Voor Moetie, voor Sigridje
Voor Augustje, die op komst is

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Abbreviations

AAP	American Academy of Pediatrics
ACOG	American Congress of Obstetricians and Gynecologists
AIDS	Acquired immunodeficiency syndrome
ANOVA	Analysis of variance
AOR	Adjusted odds ratio
ART	Antiretroviral therapy
ARV	Antiretroviral
BV	Bacterial vaginosis
CDC	Centers for Disease Control and Prevention
CI	Confidence interval
CoNS	Coagulase-negative staphylococci
Cpn60	Chaperonin 60
CPS	Capsular polysaccharide
CT	*Chlamydia trachomatis*
CVL	Cervicovaginal lavage
DNA	Deoxyribonucleic acid
E. coli	*Escherichia coli*
ECS	Elective caesarean section
EOD	Early-onset disease
EOS	Early-onset sepsis
FRT	Female reproductive tract
FSW	Female sex workers
GALT	Gut-associated lymphoid tissue
GBS	Group B *Streptococcus*
hCG	Human chorionic gonadotropin
HIC	High-income countries
HIV	Human immunodeficiency virus
HPLC	High-performance liquid chromatography
HSV-2	Herpes simplex virus type 2
IAP	Intrapartum antibiotic prophylaxis
ICRHK	International Centre for Reproductive Health Kenya
IFN-γ	Interferon gamma
IgG	Immunoglobulin
IL	Interleukin
ITM	Institute of Tropical Medicine
ITS-1	Internal transcribed spacer 1
IUD	Intrauterine device
KOH	Potassium hydroxide
LD_{50}	Lethal dose, 50%
LMIC	Low-and-middle-income countries
LOD	Late-onset disease
LOS	Late-onset sepsis
MANOVA	Multivariate analysis of variance
mRNA	Messenger ribonucleic acid
NG	*Neisseria gonorrhoeae*
NGS	Next-generation sequencing
NK cells	Natural killer cells
NOS	Newcastle-Ottawa scale
NRTI	Nucleoside reverse transcriptase inhibitors
OR	Odds ratio
PCR	Polymerase chain reaction
PMTCT	Prevention of mother-to-child transmission
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta- Analyses
PSA	Prostate-specific antigen
PTB	Preterm birth
qPCR	Quantitative polymerase chain reaction
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
RTI	Reproductive tract infection
SA	South Africa
SSA	Sub-Saharan Africa
ST	Serotype
T_h1	T-helper cell type 1
T_h2	T-helper cell type 2
T_h17	T-helper cell type 17
TT	Tetanus toxoid
TV	*Trichomonas vaginalis*
UTI	Urinary tract infection
UGent	Ghent University
VLBW	Very-low birth weight
WHO	World Health Organization

Content

Outline and objectives of the thesis

— CHAPTER 1

Sub-Saharan Africa (SSA) has the highest rates of neonatal sepsis mortality worldwide, and Group B *Streptococcus* (GBS) and *Escherichia coli* are considered the main pathogens. These bacteria infect the neonate ascending from the vagina. Chapters 2 to 5 provide background information on (GBS) neonatal sepsis. Chapters 5 and 6 introduce the female reproductive tract and the vaginal microbiota.

Objective 1 In SSA, epidemiological data on vaginal carriage of Group B *Streptococcus* (GBS) and *Escherichia coli*, the most important risk factors of neonatal sepsis, are very limited, although they are necessary to design and implement neonatal sepsis prevention strategies. Therefore, the first objective of this thesis was to assess the prevalence of vaginal GBS and *E. coli* in different populations from SSA. This objective is addressed in Chapter 7.

Objective 2 Prevention measures to reduce neonatal GBS sepsis are based on two main strategies: intrapartum antibiotic prophylaxis (IAP) and vaccination. Most GBS vaccines under development aim at eliciting protective antibodies against capsular polysaccharides (CPS), of which ten antigenically distinct CPS are known. Vaccines under development are serotype-specific but might not be effective in SSA populations because of different serotype distribution. Only a few studies have documented on GBS serotype distributions of vaginal isolates in SSA. Therefore, the second objective of this thesis was to assess the GBS serotype distribution in different populations of SSA. This objective is addressed in Chapter 7.

Objective 3 Vaccines are still under development and both IAP and vaccination strategies have their drawbacks. IAP is not effective against *E. coli* EOS and other adverse perinatal outcomes related to GBS. Furthermore, national guidelines for IAP are lacking in SSA countries and are difficult to implement. Therefore, the third objective of this thesis was to identify modifiable risk factors for

vaginal GBS and *E. coli* carriage that can be considered as prevention measures in public health recommendations to reduce neonatal sepsis. This objective is addressed in Chapter 7 and 8.

Objective 4 SSA remains the most heavily HIV-affected region with 67% of the global burden. HIV infection in women has been associated with increased rates of genital tract infections. Therefore, our fourth objective was to clarify to which extent HIV infection in women is associated with rectovaginal GBS. This objective is addressed in Chapter 8.

Objective 5 Infants born to HIV-infected mothers have increased rates of infectious morbidity and mortality compared to non-exposed infants, even if they remain HIV-uninfected. Since the first study reporting an increased risk of GBS neonatal sepsis in HIV-exposed neonates, evidence for this association has been accumulating. Therefore, the fifth objective of this thesis was to assess to which extent neonates born to HIV-infected mothers were at increased risk for GBS neonatal disease by means of systematic review and meta-analysis. This objective is addressed in Chapter 8.

Objective 6 Most current GBS vaccines aim to protect the fetus or neonate against neonatal GBS disease by immunoglobuline G (IgG) transferred transplacentally from maternal serum. However, fetal IgG concentrations increase with gestational age and might not be high enough to protect premature newborns, at high risk for EOS. Vaginal mucosal immunity to GBS is an important first-line defense against GBS EOS and some GBS-related adverse perinatal outcomes, as vaginal colonization is the first step in pathogenesis. Therefore, an optimal vaccine should also induce vaginal mucosal immunity. However, the human vaginal mucosal immune response to GBS colonization of the vaginal tract remains to be elucidated. Therefore, our sixth objective was to characterize the vaginal mucosal cytokine pattern associated with vaginal GBS colonization in populations at the highest need for better prevention measures against neonatal GBS disease. This objective is addressed in Chapter 9.

Chapter 10 summarizes the objectives and main findings of this thesis.

This doctoral thesis is based on previously published as well as on unpublished data.
(bold: will be discussed in this dissertation).

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Neonatal sepsis

— CHAPTER 2

2.4 Introduction

The incidence of overall infection is the highest in the first four weeks of life [Isaacs *et al.*,1991]. The WHO estimates that every year four million neonatal deaths occur, of which three-quarters take place in the first week of life [Lawn *et al.*, 2005]. Although more than 99% of these deaths arise in low- and middle-income countries, most research focuses on the 1% deaths in high-income countries [Lawn *et al.*, 2005]. Countries with the highest absolute numbers of neonatal deaths are mainly in South Asia, because of the large populations in this region, but the highest rates of neonatal mortality are found in sub-Saharan Africa.

The direct causes of these neonatal deaths are asphyxia (23%), preterm birth (28%) and severe infections (36%), including neonatal sepsis (26%), tetanus (7%) and diarrhoea (3%) (Figure 1).

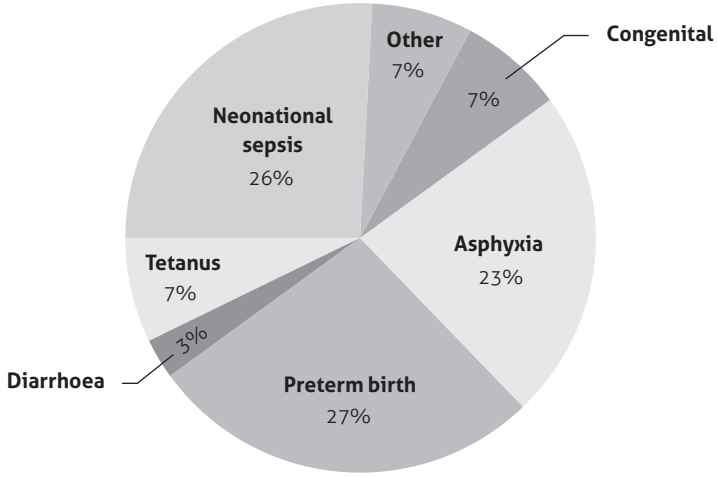

FIGURE 1 Direct causes of neonatal deaths.
Adapted from [Lawn, *et al.* 2005].

2.5 Pathophysiology of neonatal sepsis

Neonatal sepsis (neonatal septicemia) is a clinical syndrome caused by bacteria accessing the blood circulation (bacteremia), leading to systemic signs and symptoms of infection without specific localization (sepsis) or with a predominant manifestation in the lungs (pneumonia) or the meninges (meningitis).

Neonatal sepsis has been categoriszed in early-onset sepsis (EOS) and late-onset sepsis (LOS), with EOS occurring in the first seven days of life [Mukhopadhyay *et al.*, 2012]. Among very-low birth weight infants (VLBW, birth weight < 1500 grams), the cut-off for EOS is restricted to infection occurring in the first 72 hours of life [Mukhopadhyay *et al.*, 2012].

EOS is typically caused by bacterial pathogens transmitted vertically from mother to infant before or during childbirth (vertical transmission), while in LOS, bacterial pathogens are acquired from the community or nosocomially (horizontal transmisison) or vertically [Nizet *et al.*, 2011].

In EOS, the usual clinical presentation is pneumonia, and mortality rates vary from 3% to as high as 50% [Nizet *et al.*, 2011]. In LOS, meningitis is the most frequent clinical presentation and the mortality rate is usually lower, varying from 2% to 40% [Nizet *et al.*, 2011].

2.6 Etiology of neonatal sepsis

Early-onset sepsis The spectrum of bacterial pathogens causing EOS is a changing landscape. In the 1970s, *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) emerged in the US as an important cause of EOS [Stoll *et al.*, 2011] and has since then been considered a leading cause of EOS. The organisms most frequently involved in EOS of term and preterm infants are GBS and *Escherichia coli*, accounting for 70% of infections together [Simonsen *et al.*, 2014].

Other pathogens to consider are other streptococci (most commonly viridans group streptococci), *Staphylococcus aureus*, *Enterococcus* spp., Gram-negative bacilli such as *Enterobacter* spp., *Haemophilus influenzae* and *Listeria monocytogenes* [Simonsen *et al.*, 2014].

Although intrapartum chemoprophylaxis has been shown to reduce GBS EOS in high-income countries [Figure 2] (see 3.2), in these populations, GBS remains the most frequent pathogen in term infants, and *E. coli* the most significant pathogen in preterm infants [Stoll *et al.*, 2011; Simonsen *et al.*, 2014]. Data on causative agents of EOS in low-income countries, especially SSA, are scarce. However, a recent study of the WHO documented that also in SSA, GBS

and *E. coli* were the most common Gram-positive and Gram-negative bacteria isolated [Seale *et al.*, 2009].

Late-onset sepsis LOS can be divided in two distinct entities: disease occurring in the otherwise healthy term infants in the community, and disease affecting premature infants in the neonatal intensive care unit, often referred to as hospital-acquired sepsis, as the risk factors for LOS in these neonates are related to the necessities of their care such as the presence of catheters [Puopolo *et al.*, 2008]. In otherwise healthy term and near-term infants, LOS is largely caused by GBS and Gram-negatives such as *E. coli* and *Klebsiella* spp. [Puopolo *et al.*, 2008]. In premature infants, coagulase-negative staphylococci (CoNS) are the predominant pathogens of LOS [Puopolo *et al.*, 2008]. In high-income countries and middle- and low-income countries, studies attribute 53–78% and 36–47% of all LOS cases to CoNS, respectively [Dong *et al.*, 2015]. *Candida* spp. are increasingly important causes of LOS, occurring in 12 to 18% of extremely-low birth weight infants (birth weight < 1000 grams) [Puopolo *et al.*, 2008; Dong *et al.*, 2015].

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Neonatal GBS sepsis

— CHAPTER 3

3.1 Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a non-motile, facultative anaerobic, Gram-positive coccus that forms small, grey-white colonies on solid medium and diplococci or chains in broth. The gastrointestinal tract is the natural reservoir for GBS and is likely the source for vaginal colonization [Schuchat *et al.*, 1999; El Aila *et al.*, 2011]. Among pregnant women, GBS carriage rate in the vagina and rectum ranges from 10% to 40% [Gray *et al.*, 2007]. Colonization can be transient, intermittent or persistent and is commonly asymptomatic [Schuchat *et al.*, 1999; Verani *et al.*, 2010].

3.2 Prevention strategies for GBS EOS and/or LOS

To prevent GBS disease in infants and pregnant women, huge efforts have been made since the late 1980s. These are based on two strategies, intrapartum antibiotic prophylaxis (IAP) and vaccination [Melin *et al.*, 2013].

Intrapartum antibiotic prophylaxis IAP, usually with intravenous penicillin G or ampicillin [Verani *et al.*, 2010], aims at reducing maternal GBS colonization and subsequent transmission to neonates. Several clinical trials have demonstrated the efficacy of intravenous or intramuscular injection of antimicrobioal agents after the onset of labor or rupture of the membranes in reducing neonatal GBS colonization [Schuchat *et al.*, 1999]. In populations from high-income countries, IAP has been shown to reduce the incidence of EOS from 1.7 in 1993 to 0.6 per 1000 live births in 1998 [Schrag *et al.*, 2000].

There are two strategies to identify eligible women for IAP: a screening-based strategy and a risk-based strategy. According to a screening-based approach, all women are screened for the rectovaginal carriage of GBS during late pregnancy, and IAP is offered to carriers [Schrag *et al.*, 2000]. According to a risk-based approach, women who present at the time of labor with risk factors such as prolonged ruptures of the membranes, prematurity or maternal fever are offered IAP [Schrag *et al.*, 2000].

Most high-income countries have guidelines for the prevention of GBS perinatal disease, which, according to countries, are issued by public health authorities or by professional societies [Melin *et al.*, 2013]. In the US, the guidelines issued by the CDC recommend universal screening at 35–37 weeks' gestation and the use of IAP [Verani *et al.*, 2010].

The Belgian guidelines, issued in 2003, recommend screening of all pregnant women at 35–37 weeks of gestation [Melin *et al.*, 2011]. Women found positive for GBS or women with a previous infant with GBS invasive disease, or with GBS bacteriuria during current pregnancy, or delivering before 37 weeks of gestation, should receive IAP [Melin *et al.*, 2011]. If case of no screening or incomplete/unknown GBS results, the presence of intrapartum fever (≥ 38 °C) or the rupture of membranes for more than 18 h is indicative for IAP [Melin *et al.*, 2011]. If amnionitis is suspected, GBS prophylaxis is recommended to be replaced by a broad-spectrum antibiotic therapy that includes an agent known to be active against GBS [Melin *et al.*, 2011]. Similar screening based approaches are also recommended in France, Germany, Italy, Japan, Poland, Spain, Switzerland, whereas a risk-based approach is recommended in guidelines from the Netherlands, Australia, Denmark, UK and New Zealand [Melin *et al.*, 2011]. In SSA, no screening- or risk-based guidelines exist or are implemented in most countries, if they exist in these countries, these guidelines are hardly followed.

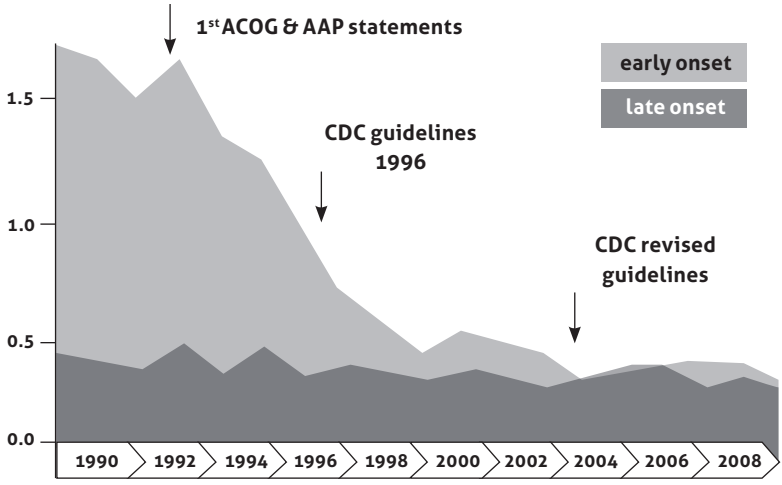

FIGURE 2 Incidence of early- and late-onset GBS disease. ACOG, American College of Obstetricians and Gynecologists; AAP, American Academy of Pediatrics. On the Y-axis, the incidence per 1000 live births is shown. Adapted from [Jordan *et al.*, 2008].

Screening for GBS carriage Crucial elements in the screening are **(i)** the time of collection, **(ii)** the type of specimen, **(iii)** the transport conditions of the specimen and **(iv)** the microbiological procedures for detection of GBS [Melin *et al.*, 2013].

(i) Rectovaginal GBS colonization is dynamic. Although early screening can be advantageous for prevention of GBS EOS in premature deliveries, the predictive value of early screenings has been shown to be very low [Verani *et al.*, 2010]. Late third trimester colonization has been suggested as a proxy for intrapartum colonization at birth [Verani *et al.*, 2010]. Currently, most guidelines recommend screening at 35–37 weeks of gestation [Melin *et al.*, 2013]. However, it is important to realize that up to 30% of mothers that are positive for rectovaginal GBS carriage at delivery, have a negative screening, and that up to 25% of women identified as GBS carriers during screening are no longer positive at time of delivery [Schrag *et al.*, 2002; Melin *et al.*, 2013].

(ii) A rectovaginal swab increases the predictive value substantially compared to a vaginal swab alone [Verani *et al.*, 2010; Dillon *et al.*, 1982; El Aila *et al.*, 2010].

(iii) After obtaining the specimens, Amies or Stuart transport medium has been widely recommended for maintaining GBS viability [Melin *et al.*, 2013], and it has been shown that prolonged time between collection and inoculation has a negative impact on the recovery of GBS [Rosa-Fraile *et al.*, 2005].

(iv) The use of a selective enrichment broth improves GBS detection substantially, as it has been shown that, when direct plating is used, as many as 50% of women who are GBS carriers have false-negative culture results [Baker *et al.*, 1977; Platt *et al.*, 1995; El Aila *et al.*, 2011]. Widely used enrichment broths are Todd-Hewitt broth supplemented with gentamicin (8 µg/ml) and nalidix acid (15µg/ml) (known as TransVag broth), or supplemented with colistin (10 µg/ml) and nalidixic acid (15 µg/ml) (known as Lim Broth) [Fenton *et al.*, 1979]. Addition of 5% sheep blood to these selective broths can further increase GBS recovery [Verani *et al.*, 2010].

After enrichment, the broth is inoculated onto blood agar plates, and ß-haemolyic colonies are presumptively identified by conventional tests such as the CAMP test or are identified using latex agglutination test with GBS antisera [Verani *et al.*, 2010], or more recently by means of matrix-assisted laser desorption/ionization – time-of-flight (Maldi-TOF). On Granada medium (known as Carrot medium in the US), β-hemolytic GBS strains are orange due to the expression produce an orange pigment. Expression of pigment and the β-haemolysin are genetically linked – both genes lie in the *cyl* operon (Rosa-Fraile *et al.*, 2008)

– making Granada medium not 100% sensitive (a small percentage of GBS strains are non-haemolytic), but however, 100% specific. In cintrast, on chromogenic agars, the selected characteristic is not related to β-hemolysin, but can be found in other species, making these agars possible more sensitive but less specific [Melin *et al.*, 2008]. Besides these culture-based techniques, molecular methods for the detection of GBS, such as polymerase chain reaction (PCR), have been described. Currently, quantitative PCR (qPCR) assays for the detection of GBS have been shown to be as sensitive or more sensitive compared to culture-based methods [El Aila *et al.*, 2011; Bergeron *et al.*, 2000; Davies *et al.*, 2004]. Despite their availability and proven sensitivity, their use is currently not widespread, even not in high-income countries. An important improvement of this technique would be the combined detection of GBS and genes associated with clindamycin resistance, to guide appropriate IAP for penicillin-allergic women at high risk of anaphylaxis [Melin *et al.*, 2013].

IAP has it drawbacks Although IAP for women with heavy GBS colonization may prevent part of the cases of LOS, the stable incidence of LOS during a period when use of IAP was increasing suggests that this intervention is not effective against LOS (see Figure 2). Furthermore, IAP is not effective against adverse perinatal outcomes related to GBS such as stillbirth or miscarriage. IAP has reduced the burden of GBS EOS in high-income countries (see Figure 2). However, it has been shown that up to 82% of term infants with EOS were born to women who had negative GBS cultures at screening [Van Dyke *et al.*, 2009; Puopolo *et al.*, 2005], suggesting that inaccurate screening, lack of communiation of results, improper implementation of IAP and antibiotic resistance contribute to ongoing disease [Melin *et al.*, 2011].

IAP has been related to the increase of the incidence of *E. coli* neonatal disease [Joseph *et al.*, 1998] and ampicillin-resistance isolates in *E. coli* neonatal disease [Bizzarro *et al.*, 2008], but this increase has not been confirmed in one other study [Bauserman *et al.*, 2013] and an increase in ampicillin-resistant *E. coli* might merely reflect the increased communitywide resistance [Al-Hasan *et al.*, 2009].

Finally, neonatal antibiotic exposure through IAP has been shown to strongly influence the development of the neonatal intestinal microbiota [Aloisio *et al.*, 2014; Tanaka *et al.*, 2016], which is essential for the differentiation of epithelium and the gut-associated lymphoid tissue (GALT) [Gritz *et al.*, 2015]. This effect has been shown to pertain even at 12 months of age [Azad *et al.*, 2015].

Vaccination Some of the above-mentioned IAP-related problems may be circumvented by maternal vaccination against GBS. Given the rapid onset and progression within hours of birth as well as the deficiencies in IAP strategies and absence of a solution for preventing LOS, it is clear that administration of a suitable vaccine in pregnancy could provide a better solution in all settings [Heath *et al.*, 2016].

The observations that laboratory animals were passively protected from bacterial challenge with GBS by antibodies against GBS CPS, and that infants with EOS and LOS were more likely than healthy infants (born to GBS colonized mothers) to have low levels of CPS specific IgG, prompted research on GBS CPS antigens and their potential use as targets for vaccination [Schuchat *et al.*, 1999].

In contrast to IAP, vaccination might prevent a broad range of GBS associated diseases besides EOS, such as GBS LOS, miscarriage, stillbirth and maternal infection.

During the last two decades, the first generations of vaccines targeting CPS have been extensively studied and phase I and phase II clinical trials in healthy adults have demonstrated safety and immunogenicity of monovalent or multivalent, uncoupled CPS or conjugated vaccines against serotypes Ia, Ib, II, III and V, but these are not yet licensed [Heath *et al.*, 2016].

Because these vaccines are serotype specific and serotype distribution differs according to population, a vaccine suitable for high-income populations might not be suitable for populations in SSA [Johri *et al.*, 2006]. To overcome this problem, new generations of vaccines that target conserved surface proteins are considered [Melin *et al.*, 2013]. C5a peptidase and Sip, expressed at the surface of all GBS strains were identified as possible candidates [Melin *et al.*, 2013]. Reverse vaccinology, where antigen candidates are identified using genomic analysis, bioinformatics and molecular biology [Johri *et al.*, 2006], has identified pilus-like structures on GBS as potential candidates [Maione *et al.*, 2005], which could be useful in preventing colonization by inhibiting bacterial adhesion [Pezzicoli *et al.*, 2008]. Table 1 gives an overview of the status of the current vaccine candidates.

Other strategies A less invasive approach to the interruption of perinatal transmission of GBS is the use of vaginal disinfectants when labor begins. The use of vaginal wipes with the antibacterial chlorhexidine has been explored during labor but failed to show a beneficial effect [Cutland *et al.*, 2009].

Developer	Candidate name/identifier	Preclinical	Phase I	Phase II	POC	Phase III
NIH	TT-CPS conjugates (M, B) CRM197–CPS-conjugate (M)				P	
Novartis/GSK	CRM197-CPS conjugates (M, T)				P	
Minervax	Rib surface proteins and AlphaC surface proteins					
Novartis/GSK	Pilus proteins					
Various academic groups	Other proteins and/or Protein-CPS conjugates					

TABLE 1 Development status of current vaccine candidates.
Adapted from [Heath *et al.*, 2016].

POC, proof-of-concept; M, monovalent; B, bivalent; T, trivalent; P, trials in pregnant women; dark grey, progress of clinical trials of the candidate vaccine. CRM197 is a non-toxic mutant of diphtheria toxin, currently used as a carrier protein for polysaccharides and haptens to make them immunogenic.

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Pathogenesis of GBS EOS

— CHAPTER 4

4.1 GBS virulence factors

Capsular polysaccharide (CPS) GBS strains are typically encapsulated by capsular polysaccharides belonging to one of the ten capsular serotypes (Ia, Ib, II to IX) [Rajagopal *et al.*, 2009]. CPS is considered a major virulence factor and the overwhelming majority of GBS isolated from invasive disease are encapsulated [Spellerberg *et al.*, 2000]. CPS are polymers which in GBS are composed of different arrangements of four monosaccharides, ie. glucose, galactose, N-acetylglucosamine and sialic acid (N-acetylneuraminic acid), except for serotype III that has rhamnose instead of N-acetylglucosamine and for serotype VI that lacks N-acetylglucosamine [Spellerberg *et al.*, 2000].

Because GBS strains are decorated with sialic acid, which is present as the most distant sugar moiety on all GBS CPS but also on glycans of vertebrate cells, GBS cells are masked (molecular mimicry) and impair the host to recognize GBS as nonself. Consequently, the sialylated CPS prevents binding of factor C3b of the complement system to the capsule of GBS, thereby preventing the activation of the alternative complement pathway [Marques *et al.*, 1992]. The complement pathway is a crucial element of the host innate immune system and consists of serum proteins that can be activated by an enzymatic cascade mechanism. Activation of the complement system leads to the production of opsonins, such as C3b, which attach to bacteria, tagging them for destruction, and to the production of chemokines that recruit immune cells to sites of infection [Mitchell *et al.*, 2003].

The importance of CPS in adaptive immunity against GBS has been first described by Baker and coworkers [Baker *et al.*, 1976], who demonstrated that placental transfer of maternal antibodies against CPS was associated with protection from invasive GBS infection.

β-hemolysin/cytolysin (β-H/C) (CylE) The β-H/C is responsible for the β-hemolytic phenotype of GBS, a characteristic zone of clearing around the colonies of GBS when grown on blood agar plates (β-hemolysis), which is a useful diagnostic characteristic in the clinical microbiology laboratory [Rajagopal *et al.*, 2009; Pritzlaff *et al.*, 2001].

The GBS β-H/C was recognized as early as the 1930s [Todd *et al.*, 1934], whereas early 1980s research provided insights in its mechanism of erythrocyte lysis and 1990s research showed a virulence factor with a broad spectrum of cytolytic properties [Liu *et al.*, 2006].

The surface-associated β-H/C is expressed by the vast majority of strains [Rajagopal *et al.*, 2009] and promotes bacterial invasion across epithelial and endothial walls, including the blood-brain barrier [Landwehr-Kenzel *et al.*, 2014]. Furthermore, β-H/C is associated with damage to lung epithelial and endothelial cells, brain epithelial cells and macrophages [Mitchell *et al.*, 2003]. GBS strains that are β-H/C deficient have shown impaired virulence in various *in vivo* models of pneumonia, sepsis and meningitis [Margarit *et al.*, 2009; Doran *et al.*, 2002a; Bebien *et al.*, 2012]. At sublytic concentrations, β-H/C has been shown to induce the expression of the anti-inflammatory cytokine interleukin-10 (IL-10) and inhibits both IL-12 and nitric oxide synthase (NOS2) expression in GBS-infected macrophages [Bebien *et al.*, 2012].

Christie, Atkins, Munch and Peterson (CAMP) factor The CAMP factor is a secreted protein that oligomerizes and forms discrete pores in susceptible target membranes. Besides its membrane-damaging activity, the CAMP factor has been shown to interact with the Fc portion of human IgG and IgM [Jurgens *et al.*, 1987]. *In vivo* studies have shown that injected purified CAMP factor was lethal to rabbits, and that co-administration of the CAMP factor with a sublethal dose of GBS induced septicemia and death in murine models [Jurgens *et al.*, 1987; Skalka *et al.*, 1981], stressing the importance of the CAMP factor in pathogenesis.

C5a peptidase (ScpB) The C5a peptidase is a serine protease and surface protein present on all strains of GBS [Johri *et al.*, 2006] and inactivates the complement factor C5a by cleavage. As C5a is an anafylatoxine that is important for the inflammatory response, including the recruitment of neutrophils, C5a peptidase induces an impaired innate immune reaction at the site of infection [Rajagopal *et al.*, 2009; Chmouryguina *et al.*, 1996; Takahashi *et al.*, 1995]. Furthermore, C5a peptidase has been shown to be involved in host colonization. Many GBS-host-cell interactions involve binding of GBS to the extracellular matrix of these host cells. By binding to molecules of this extracellular matrix, such as fibronectin, laminin and fibrinogen, which in turn bind host cell-surface proteins such as integrin, GBS can enhance colonization. Independent of its peptidase activity, C5a peptidase has been shown to bind to fibronectin [Maisey *et al.*, 2008; Rajagopal *et al.*, 2009].

Immunization of mice with C5a peptidase alone or conjugated with CPS showed an enhanced clearing of GBS from the lung, emphasizing the importance of C5a peptidase in the pathogenesis of GBS infection [Cheng *et al.*, 2002].

C antigen The C antigen of GBS is a protein complex consisting of two distinct and independently expressed components, the α C protein and the β C protein [Spellerberg *et al.*, 2000]. C proteins are expressed in 40–60% of clinical isolates [Hickman *et al.*, 1999; Johnson *et al.*, 1984] and in different animal models, vaccination with both the α C protein and the β C protein have been shown to elicit protective immunity [Spellerberg *et al.*, 2000].

The α C protein is commonly expressed on the surface of GBS serotypes Ia, Ib and II [Rajagopal *et al.*, 2009]. Equivalent proteins have been described in serotypes III and V [Stalhammar-Carlemalm *et al.*, 1993; Lachenauer *et al.*, 1996]. The surface-anchored α C protein promotes invasion of human cervical epithelial cells through α1β1-integrin binding or glycosaminoglycan binding in an actin-dependent manner [Bolduc *et al.*, 2007; Glaser *et al.*, 2002]. The importance of the α C protein as a virulene factor has been documented in a murine neonatal model of GBS infection, where GBS mutants lacking the protein were less virulent [Bolduc *et al.*, 2007; Li *et al.*, 1997].

By binding to IgA antibodies, the β C protein is thought to interfere with opsonophagocytosis of GBS [Spellerberg *et al.*, 2000; Jerlstrom *et al.*, 1991]. Furthermore, β C protein binds to the human serum protein factor H, which degrades complement factor C3b [Mitchell *et al.*, 2003]. Finally, the β C protein has been shown to bind sialic-acid-recognising immunogloblulin superfamily lectins (Siglecs), a family of cell-surface receptors with intracellular domains that send inhibitory signals to limit host cell activation. GBS binds Siglec-5 on macrophages and neutrophils, thereby downregaluting their immune function (see Figure 3) [Carlin *et al.*, 2009].

Pili Pili are filamentous cell-surface appendages better characterized in Gram-negative bacteria, where they facilitate adhesion and colonization of the host or other bacteria [Sauer *et al.*, 2000]. Pili were only recently described in GBS [Lauer *et al.*, 2005] and have been shown to promote colonization of epithelial cell surfaces, support biofilm formation, mediate GBS resistance to antimicrobial proteins and enhance translocation across the blood-brain barrier [Konto-Ghiorghi *et al.*, 2009; Dramsi *et al.*, 2006; Pezzicoli *et al.*, 2008; Maisey *et al.*, 2007; Maisey *et al.*, 2008b]. Interestingly, antibodies raised against immunogenic pilin subunits have been shown not only to elicit opsonophagocytic killing and protection from sepsis, but also to inhibit adherence, suggesting the

promise of a pilus-based vaccine that workins at two different levels [Nuccitelli *et al.*, 2011; Margarit *et al.*, 2009].

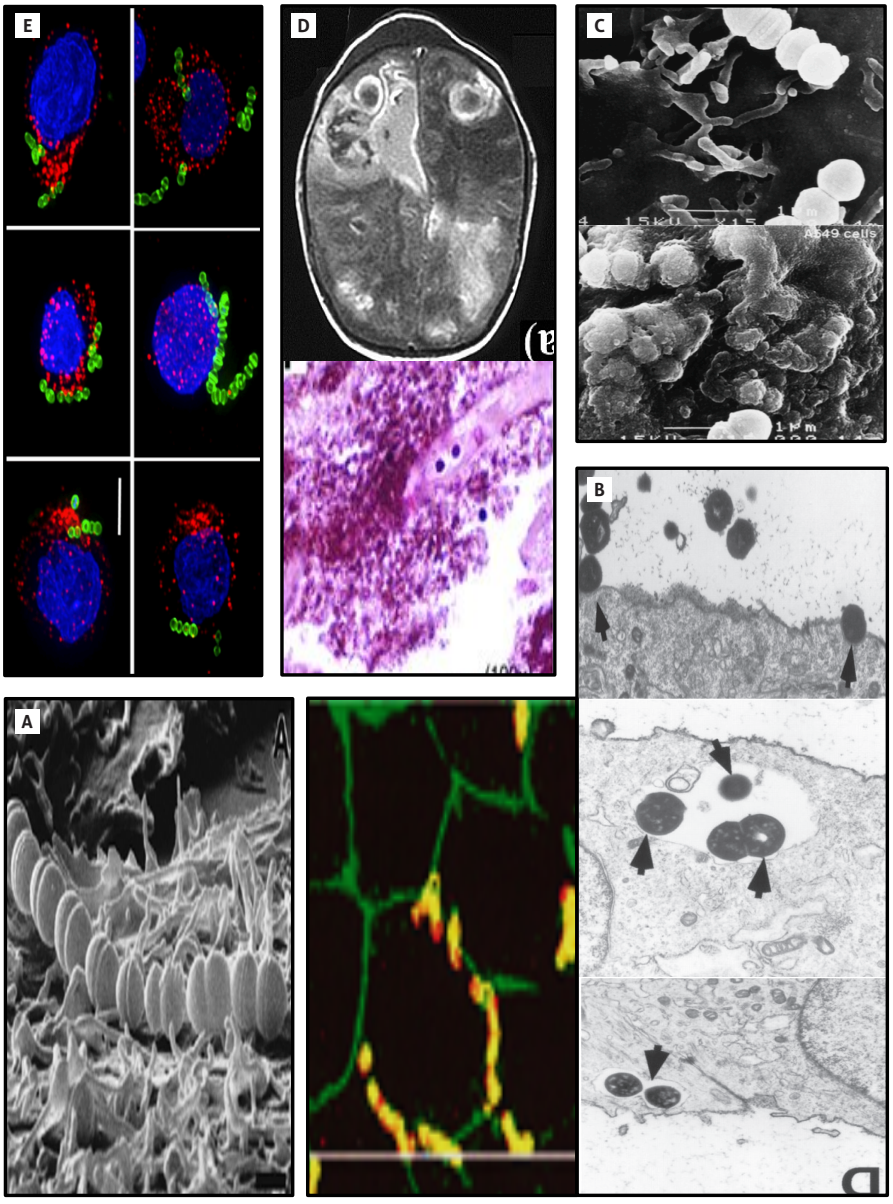

FIGURE 3

◂ **FIGURE 3 Pathogenesis of GBS EOS.**

Panel A. Colonization. Interactions of GBS with epithelial cells as viewed by scanning electron microscopy. Streptococcal chain adhering to the epithelial cell surface [Valentin-Weigand *et al.*, 1997].

Panel B. Invasion. *Left image*: association of group B *Streptococcus* (GBS) with the perimeter of epithelial cervical cells. Red, GBS stained with mouse polyclonal antisera against serotype V; green, tight junctions visualised with polyclonal anti-ZO-1 antibodies; yellow, colocalisation of GBS and tight juntions. GBS preferentially transverses cervical epithelial cells via the paracellular route [Soriani *et al.*, 2006]. *Right images*: transcytosis of GBS through a monolayer of chorion cells. Top image, apical surface of chorion cell engulfing two GBS cells; middle image, several GBS cells within a single intracellular vacuole; bottom image, two intracellular GBS cells near the apical surface of a chorion cell. Adherent and intracellular GBS cells are indicated by the arrows [Winram *et al.*, 1998].

Panel C. Pneumonia. Ultrastructural of GBS hemolysin-associated epithelial cell injury: scanning electron micrographs shows pneumocytes exposed to wild-type GBS (top image) and a hyperhemolytic mutant strain (bottom image) shows loss of microvillus architecture and bleb formation on the cell surface [Nizet *et al.*, 1996].

Panel D. Meningitis. *Top image*: neuroimaging of fatal GBS meningitis, transverse view of magnetic resonance imaging showing diffuse necrotizing encephalomalacia and formation of parenchymal abscesses or cysts [Doran, 2002b]. *Bottom image*: Gram stain of murine brain tissue infected with WT GBS [Doran *et al.*, 2005].

Panel E. Immune evasion. Green, GBS; blue, nuclei of macrophages; red, siglec-5 on cell surface; bar, 5 µm. *Left images*: GBS cells expressing β protein colocalize with siglec-5 on macrophages, increasing bacterial attachment but impairing phagocytosis. *Right images*: mutant GBS strain not expressing β protein does not colocalize [Carlin *et al.*, 2009].

Extracellular-matrix binding proteins GBS is able to enhance its colonization by binding to the host extracellular matrix. FbsA and FbsB are two fibrinogen-binding proteins expressed by GBS [Schubert *et al.*, 2002; Jacobsson *et al.*, 2003]. While FbsA promotes adhesion, FbsB facilitates invasion into host cells [Landwehr-Kenzel *et al.*, 2014]. Furthermore, GBS is able to bind host-cell laminin by means of its laminin-binding protein Lmb. GBS strains lacking Lmb showed decreased adherence to human brain microvascular endothelial cells [Spellerberg *et al.*, 1999a; Tenenbaum *et al.*, 2007].

Hyaluronate lyase GBS is able to secrete a protease known as hyaluronate lyase (HlyB), important for GBS pathogenesis [Lin *et al.*, 1994; Gase *et al.*, 1998]. Hyalurons, chief components of the extracellular matrix of human tissue, are found at higher concentrations in placental and lung tissue and in amniotic fluid [Lindahl *et al.*, 1978; Li *et al.*, 2001]. As hyaluronate lyase is able to cleave hyaluron [Li *et al.*, 2001; Pritchard *et al.*, 1994; Akhtar *et al.*, 2006], hyaluronate lyase is thought to engage in pathogenesis by promoting tissue invasion of GBS.

Superoxide dismutase Phagocytes such as neutrophils and macrophages kill engulfed bacterial pathogens by the production of reactive oxygen species (ROS). To counteract this attack, GBS is able to produce a superoxide dismutase, SodA [Rajagopal *et al.*, 2009]. Superoxide dismutases convert harmful singlet oxygen or superoxide anions (O^{2-}) into harmless molecular oxygen (O_2) and harmful H_2O_2, which, in turn, is metabolized by catalases and/or peroxidases [Rajagopal *et al.*, 2009].

In vitro, a GBS *sodA* mutant was found to be significantly more susceptible to oxidative stress and in a murine model, this mutant showed increased susceptibility to bacterial killing by macrophages and impaired survival in the bloodstream and brain [Poyart *et al.*, 2001].

4.2 Pathogenesis of GBS EOS

Using its plethora of virulence factors, GBS has to undertake several crucial steps for neonatal GBS disease to occur (see Figure 3).

(i) Colonization of the vaginal mucosa

The presence of GBS in the maternal genital tract at delivery determines whether or not a newborn is at risk for invasive disease. Among infants born to colonized women, the risk of EOS is 30-fold compared to infants born to women with a negative culture-based screening result [Boyer *et al.*, 1985]. A direct relationship has been shown between vaginal GBS load, the risk of vertical transmission and the likelihood of serious disease in neonates [Ancona *et al.*, 1980; Pass *et al.*, 1979]. Therefore, a crucial step, if not, a prerequisite, in the pathogenesis is the colonization of the vaginal mucosa.

To establish a successful colonization of the vagina, GBS must adhere to the vaginal epithelium. GBS has been shown to bind efficiently to exfoliated human vaginal epithelial cells or to vaginal tissue culture cells [Sobel *et al.*, 1982;

Jelinkova *et al.*, 1986] with maximal adherence at acidic pH characteristic of the vagina [Zawaneh *et al.*, 1979; Tamura *et al.*, 1994]. High-affinity interactions of GBS are facilitated by binding of bacterial FbsA, Lmb, C5a peptidase and pili to host extracellular matrix components such as fibrinogen, fibronectin and laminin, which in turn interact with host cell-anchored proteins such as integrins.

(ii) Invasion across host epithelial barriers to infect amniotic fluid

GBS can reach the fetus *in utero* through ascending infection of the amniotic fluid, or alternatively, the newborn may become contaminated with GBS on passage through the birth canal. The pivotal role of the ascending route in EOS has been underpinned by several observations. A direct relationship exists between the duration of membrane rupture before delivery and infection rate for EOS [Stewardson-Krieger *et al.*, 1978]. Stewardson-Krieger and coworkers demonstrated that when duration of membrane rupture was 18 hours or less, the infection rate was 0.7 per 1000 live births, when it was more than 30 hours, it increased to 18.3 per 1000 live births [Stewardson-Krieger *et al.*, 1978]. Furthermore, most neonates with EOS GBS disease show symptoms of pneumonia or sepsis at birth, suggesting that infection did take place *in utero* and not during passage through the birth canal. Histological examination of placentas from women with GBS chorioamnionitis showed bacterial infiltration along a choriodecidual route, implying that ascending infection may be a primary trigger in many instances of premature rupture of the membranes [Evaldson *et al.*, 1982].

GBS is thought to infect the amniotic fluid after the rupture of the membranes, but has also been shown to cross intact chorioamnionitic membranes [Galask *et al.*, 1984], for which crossing of chorion cells, amnion cells and the placental basement membrane is necessary. Transversion of chorion cell monolayers by GBS has been shown without disruption of intracellular junctions [Winram *et al.*, 1998]. Hyaluronic acid, an important component of the extracellular matrix that is abundant in placental tissues, can be degraded by the GBS hyaluron lyase to facilitate invasion of the amniotic fluid.

Although amniotic fluid has antimicrobial properties [Espinoza *et al.*, 2003], it supports the growth and proliferation of GBS [Hemming *et al.*, 1985], and a high GBS inoculum is thought to be delivered to the fetal lung, resulting in a continuum of intrapartum (stillbirth) to early postpartum infant death [Abbasi *et al.*, 1987; Baker *et al.*, 1978; Vollman *et al.*, 1976; Ablow *et al.*, 1976; Bergqvist

et al., 1978]. In contrast, when GBS is encountered on passage through the birth canal, a lesser inoculum is thought to be delivered to the neonate.

(iii) Pulmonary entry, pneumonia and bloodstream entry

Through inhalation of contaminated amniotic fluid by the fetus, GBS can enter the fetal lung. Subsequently, entry into the bloodstream occurs when GBS crosses the pulmonary epithelial layer, basement membrane and the vascular endothelial layer.

Intracellular invasion of both alveolar epithelial and pulmonary endothelial cells by GBS was demonstrated in newborn macaques following intra-amniotic challenge [Rubens *et al.*, 1991] and confirmed in human tissue culture lines for both cellular barriers [Rubens *et al.*, 1992; Gibson *et al.*, 1993], where GBS triggers host cytoskeleton changes, leading to endocytotic uptake within a membrane bound vacuole [Valentin-Weigand *et al.*, 1997; Nizet *et al.*, 1996]. Although cellular invasion may play a principal role in bloodstream penetration, cellular damage to the lung barrier, largely due to the actions of the β-H/C, contributes to the pathogenesis. Autopsies in fatal EOS cases reveal that 80% have histological evidence of lobar or multilobar pneumonia [Vollman *et al.*, 1976; Hemming *et al.*, 1985], characterized by dense bacterial infiltration, epithelial cell damage, alveolar hemorrhage and interstitial inflammatory exudate [Ablow *et al.*, 1976; Katzenstein *et al.*, 1976]. GBS disease is rarely limited to the initial pulmonary focus, but spreads to the bloodstream and thereby to other organs and tissues.

(iv) Inflammatory activation and sepsis

When failure of the pulmonary epithelial and endothelial barrier function and of immunological clearance allows GBS to enter the bloodstream causing bacteremia, development of sepis may occur. In the acute phase, there is an increase in inflammatory markers such as TNF-α, IL-1 and IL-6 [Maisey *et al.*, 2008a]. IL-12 has been shown to play an important role in regulating the systemic response to GBS infection. The proinflammatory effects of the GBS β-H/C also contribute to sepsis pathophysiology. Intravenous infusion of GBS in animal models produces similar pathophysiologic changes to those seen in human newborn infection, such as hypotension, persistent pulmonary hypertension, acidosis, tissue hypoxemia and multiple organ failure.

(v) Crossing blood-brain barrier and meningitis

To induce meningitis, the predominant clinical finding in case of GBS LOS, GBS must penetrate human microvascular endothelial cells, the single-cell layer constituting the blood-brain barrier. Intracellular invasion and transcytosis of human brain microvascular endothelial cell tissue culture monolayers have been shown *in vitro* [Nizet *et al.*, 1997], and serotype III strains, accounting for most of the isolates causing meningitis, invade more efficiently than strains of other serotypes. When GBS crosses the blood-brain barrier to enter the cerebrospinal fluid, it initiates an inflammatory meningeal reaction in the subarachnoid space, triggered through the sentinel function of the blood-brain barrier endothelium. Animal models suggest TNF-α has a pivotal role in the initial proinflammatory reaction [Ling *et al.*, 1995], which contributes significantly to the pathogenesis of meningitis and the central nerve system injury. Following initial inflammation, the cerebral fluid flow is altered, and cerebral hyperemia and ischemia occur.

(vi) Immune evasion

Following the entry of GBS into the lungs, blood and brain, normally considered sterile sites, the host attempts to clear the invading GBS mainly by the phagocytotic actions of neutrophils and macrophages [Spellerberg *et al.*, 2000]. Effective phagocytosis of GBS relies upon opsonization through complement and serotype-specific antibodies [Remington *et al.*, 2010]. Neonates are particularly prone to invasive disease due to their deficiencies in phagocytic cell function, specific antibody development, and/or classic and alternate complement pathways [Remington *et al.*, 2010].

The protective effect of maternal antibodies was first recognized in 1976 [Baker *et al.*, 1976] and the complement system appears to play a central role in the host defense against GBS. The LD50 of mice deficient in either complement C3 or C4 has been shown to be reduced 23–30-fold in comparison to controls [Wessels *et al.*, 1995], suggesting that both the classical and alternative pathways contribute to immunity.

GBS is equipped with virulence factors that thwart key components of effective opsonophagocytosis. Chief among these is the sialylated CPS that inhibits deposition of complement component C3b and thereby the activation of the alternative complement pathway [Platt *et al.*, 1994; Edwards *et al.*, 1993].

Furthermore, the CAMP factor and the β-C protein presumably render antibodies ineffective for opsonization by binding to the Fc portion of immunoglobulins and C5a peptidase impairs recruitment of neutrophils to sites of infection through cleavage of the chemotactic complement component C5a.

After phagocytosis, neutrophils and macrophages seek to kill the engulfed bacteria by the production of reactive oxygen species and other antimicrobial substances [Remington *et al.*, 2010]. Although streptococci are often thought of as extracellular pathogens, they can survive for prolonged periods within the phagolysosome of macrophages [Cornacchione *et al.*, 1998]. GBS superoxide dismutase can neutralize the actions of the reactive oxygen species thereby enhancing intracellular survival. Furthermore, the GBS β-H/C can induce direct cytolytic injury to macrophages. With highly hemolytic strains or with a large bacterial inoculum, killing of the phagocyte seems to outpace the phagocyte's microbicidal mechanisms, allowing bacterial proliferation, as was shown in an *in vitro* murine model [Remington *et al.*, 2010].

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Histology and anatomy of the vagina and cervix

— CHAPTER 5

The female reproductive tract (FRT) can be divided into external genitalia (vulva) and internal genitalia, located in the pelvic cavity. The internal genitalia consist of the vagina, the cervix, the uterus, the Fallopian tubes and the ovaries (Figure 4).

The vagina is an elastic, muscular canal lined with a mucosa and has an important role in reproduction as the penile recipient, the conduit for the menstrual flow and the birth channel during delivery. The vagina connects the uterus – the organ receiving the embryo, sheltering the fetus during pregnancy and delivering the newborn – to the outside world: the vulva forms the entrance and the cervix, that protrudes into the vagina, forms the interior end.

The cervix forms a mucosa-lined canal, the endocervix, which opens in the vagina and the uterine cavity via the external and internal os, respectively. The ectocervix is the part of the cervical channel that protrudes into the vagina.

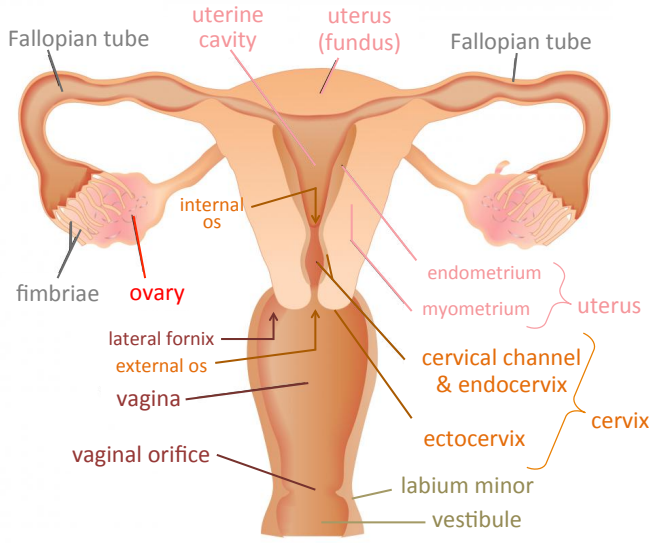

FIGURE 4 **Anatomy of the female reproductive tract.**

The vaginal mucosa consists of a stratified squamous non-keratinizing epithelium, beneath which is a vascular aglandular connective tissue layer, the lamina propria. Under the lamina propria, a layer of smooth muscle and an adventitia are located.

The stratified vaginal epithelium is made up of a basal layer of a single row of columnar cells, a parabasal layer of two to five cuboidal cells, an intermediate layer of variable thickness that contains flattened cells and a superficial layer that consists of flat epithelial cells, which are constantly exfoliated (Figure 5). The vaginal mucosa is continuous with the ectocervical stratified squamous epithelium.

The endocervix is lined by a single cell layer of columnar epithelial cells (some of which contain cilia) that secerete mucus under estrogen regulation. The surface of the endocervical epithelium is increased by the deep invaginations of the tubular glands, thereby increasing the mucus-secreting capacity. The secreted mucus covers the external os and fills the cervical canal, acting as a protective barrier against ascending infections from the vagina into the uterine cavity.

The area where the squamous multi-layered epithelium of the ectocervix meets the single-layered columnar epithelium of the endocervix is called the squamocolumnar junction (SCJ, or transformation zone) and its location on the cervix is variable and dependent on age.

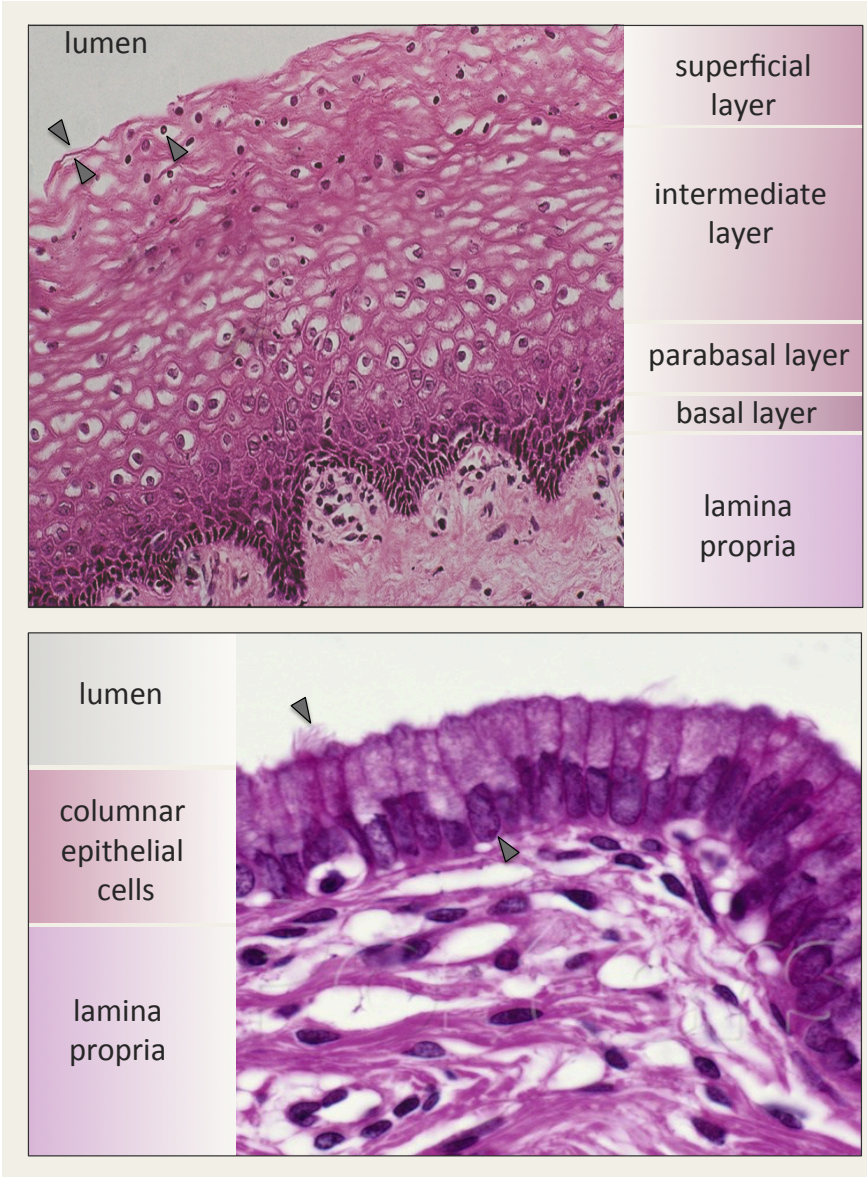

FIGURE 5 Histology of the vaginal and cervical mucosa.

Top panel: the four layers of the stratified squamous epithelium, located on top of the lamina propria. The left arrows indicate an exfoliated squamous epithelial cell; the right arrow indicates a nucleus of an epithelial cell.

Bottom panel: the single layered columnar epithelium of the endocervix. The left arrow indicates cilia of an epithelial cell; the right arrow indicates the nucleus of an epithelial cell.

The vaginal microbiota

— CHAPTER 6

6.1 Introduction

We are not just single individuals walking the planet, we are walking ecosystems. And like our planet, the human body harbors many different ecosystems containing microorganisms.

A human body hosts more bacterial cells than it has human cells, and encodes a 100-fold more unique genes than our own genome [Ley *et al.*, 2006]. Different sites in our body harbor different microbial communities, termed microbiota, that differ in composition and dynamics.

Microbiota exist in a mutualistic relationship with their human host [Dethlefsen *et al.*, 2007; Turnbaugh *et al.*, 2007; Ley *et al.*, 2006]. Disease might be caused by opportunistic pathogens, members of the microbiota that only under certain circumstances – such as a compromised immune system – cause disease; by true pathogens that are normally not part of microbiota; or by dysbiosis, the disturbance of the delicate balance of the microbiota.

The complex and dynamic nature of our microbiota only became clear during the last decade thanks to the advances and affordability of high-throughput DNA sequencing technologies, and we are only starting to understand the enormous impact of our microbiota on human health and disease [Eloe-Fadrosh *et al.*, 2013].

The human vagina and the associated vaginal microbiota are an example of a finely balanced mutualistic association, and disturbances of this balance have a major impact on reproductive health and disease [Ma *et al.*, 2012].

6.2 The vaginal microbiota from Döderlein to DGGE – the classical paradigm

In 1892, German gynecologist Döderlein was the first to describe an organism named Döderlein's bacillus, later renamed *Lactobacillus*, isolated from a vaginal specimen of healthy pregnant women [Martin *et al.*, 2012]. In 1899, Menge and Kronig described, in addition to *Lactobacillus*, the isolation of other anaerobic organisms from the human vagina [Menge *et al.*, 1899]. In the 1920s, a

vaginal discharge syndrome described by Curtis, termed 'white discharge syndrome', was linked with a microscopy- and culture-proven deprivation of *Lactobacillus* and with the presence of curved anaerobic motile rods, anaerobic cocci, and Gram-variable diphtheroidal rods [Martin *et al.*, 2012].

Thus, nearly 100 years ago, a symptomatic vaginal discharge syndrome was already known to be associated with significant shifts in the vaginal microbiota. At that time, investigators believed that bacterial-associated diseases or syndromes were caused by a single organism, providing fertile ground for the strong association found by Gardner and Duke between a Gram-variable coccobacillus, named *Haemophilus vaginalis* and later renamed *Gardnerella vaginalis*, and the white-discharge syndrome, at that time renamed nonspecific vaginitis [Gardner *et al.*, 1955]. Despite the fact that Koch's postulates were not fulfilled, *G. vaginalis* was believed to be the cause of nonspecific vaginitis, reflected in the term '*Gardnerella* vaginitis', frequently used to describe the syndrome.

In the 1980s, a group of researchers from the University of Washington revitalized the research on nonspecific vaginitis. They reported quantitative culture-based findings that nonspecific vaginitis was not only associated with increased abundance of *G. vaginalis* but also with striking increases of genital mycoplasmas and a number of anaerobic species such as *Bacteroides* spp. and *Peptococcus* spp. [Spiegel *et al.*, 1980; Spiegel *et al.*, 1983a; Spiegel *et al.*, 1983b; Hillier *et al.*, 1993]. Based on this seminal work, nonspecific vaginitis was renamed 'bacterial vaginitis'. Later the designation 'bacterial vaginosis' (BV) was proposed because of the microscopical observation of only few inflammatory cells in the vaginal fluids of women with BV. The same group described clinical markers to diagnose BV, now known as the Amsel criteria [Amsel *et al.*, 1983] (see further) and Nugent and co-workers revised the Gram-stain criteria for BV described by Spiegel, and this revised scoring system became known as the Nugent score (see further) [Nugent *et al.*, 1991; Spiegel *et al.*, 1983b]. Over the years, the Amsel criteria and Nugent score became the standard for diagnosing BV in routine clinical care and clinical research, respectively.

Until the revolution introduced by molecular studies, the vaginal microbiota was further studied using culture-dependent and microscopical techniques. The vaginal microbiota paradigm (Figure 6) was that lactobacilli are associated with a healthy vaginal microbiota and that a deprivation of lactobacilli paralleled with the presence of other (facultative) anaerobes is the hallmark of bacterial vaginosis (BV).

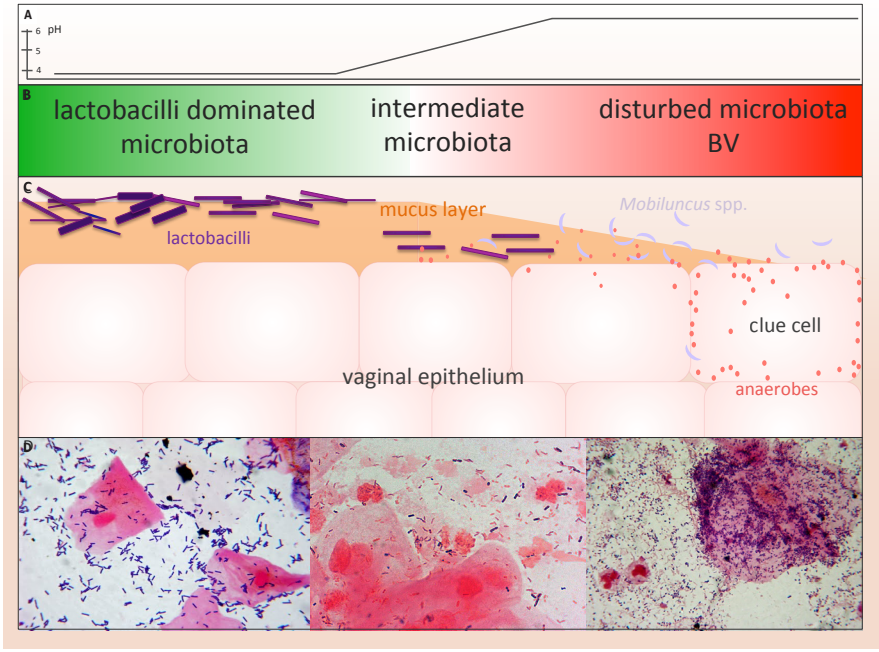

FIGURE 6 The vaginal microbiota of women of reproductive age – the classical paradigm

Panel A. Lactobacilli are thought to acidify the vaginal mucosa by production of lactic acid and deprivation of lactobacilli is associated with higher pH.

Panel B. In women of reproductive age, the spectrum of the vaginal microbiota goes from one dominated by lactobacilli, generally associated with health, to a microbiota lacking lactobacilli and dominated with anaerobes such as the BV associated bacteria. In between, an intermediate microbiota is thought to be the transition between the latter two.

Panel C. In the disturbed BV-associated microbiota, anaerobes break down the protective mucus layer and attach in large numbers to the vaginal epithelial cells, which are then called clue cells.

Panel D. Gram-stained vaginal smears from women with normal vaginal microbiota *(left)*, intermediate microbiota *(middle)*, and BV *(right)*.

Lactobacilli-dominated microbiota *Lactobacillus* spp. are keystone species of a healthy vaginal microbiota – with *L. crispatus* as the most prevalent amongst the vaginal lactobacilli – [Gupta *et al.*, 1998; Martin *et al.*, 1999; Sobel, 1999; Antonio *et al.*, 1999] and play a major role in protecting the vaginal environment from nonindigenous and potentially pathogenic microorganisms, including those responsible for BV, sexual transmitted infections and urinary tract infections by the production of lactic acid, H_2O_2 and bacteriocines [Gupta *et al.*, 1998; Hillier *et al.*, 1992; Sewankambo *et al.*, 1997; Sobel, 1999; van De Wijgert *et al.*, 2000].

- **Production of lactic acid** Lactobacilli benefit the host by producing lactic acid as a fermentation product that lowers the vaginal pH to 3.5–4.5, which is thought to be protective [Alakomi *et al.*, 2000; Boskey *et al.*, 1999].
- **Production of H_2O_2** Production of H_2O_2 is a well-known mechanism for bacterial antagonism, inhibiting growth of microorganisms via direct interaction or via human myeloperoxidase [Dahiya *et al.*, 1968; Eschenbach *et al.*, 1989]. *In vitro* studies have shown that H_2O_2 producing lactobacilli were able to inactivate genital pathogens like HIV-1, herpes simplex virus type 2 (HSV-2), *Trichomonas vaginalis*, *G. vaginalis*, *P. bivia* and *E. coli* and clinical studies have associated H_2O_2-producing lactobacilli with a decrease in BV and inflammatory markers [Hawes *et al.*, 1996; Mitchell *et al.*, 2013]. However, based on observations that cervicovaginal fluid has H_2O_2 blocking activity and the hypothesis that lactobacilli *in vivo* probably do not produce enough H_2O_2 to be effective, the *in vivo* beneficial effects of H_2O_2 producing strains are still a matter of discussion [O'Hanlon *et al.*, 2010; O'Hanlon *et al.*, 2011].
- **Bacteriocines** Several *Lactobacillus* species produce antimicrobial compouds named bacteriocines, low-molecular weight proteins that can inhibit the growth of a variety of bacteria including *G. vaginalis*, reinforcing the protective role of the lactobacilli [Alpay Karaoglu *et al.*, 2002; Aroutcheva *et al.*, 2001; Klaenhammer *et al.*, 1988].

Bacterial vaginosis Bacterial vaginosis is the most common vaginal condition of reproductive-age women and is (vaguely) characterized by a disruption of the equilibrium of the normal vaginal microbiota [Ma *et al.*, 2012]. Clinically, symptomatic women complain of vaginal discharge, vaginal and/or perineal pruritus, a fishy odor and dyspareunia.

Clinical studies defining BV based on Amsel criteria and/or Nugent score have shown that BV is an independent risk factor for a plethora of adverse health

outcomes like the acquisition of *Neisseria gonorrheae*, *Chlamydia trachomatis*, *T. vaginalis* [Brotman *et al.*, 2010a], HSV-2 [Cherpes *et al.*, 2005], the acquisition and transmission of HIV [Cohen *et al.*, 1995; Coleman *et al.*, 2007; Cu-Uvin *et al.*, 2001; Martin *et al.*, 1999; Spear *et al.*, 2007; Taha *et al.*, 1998], urinary tract infections [Harmanli *et al.*, 2000; Hillebrand *et al.*, 2002], pelvic inflammatory disease [Srinivasan & Fredricks, 2008], preterm labor [Leitich *et al.*, 2003], low birth weight [Hauth *et al.*, 2003], preterm rupture of membranes [Benedetto *et al.*, 2004], late miscarriage, spontaneous abortion [Hay *et al.*, 1992], chorioamnionitis [Rezeberga *et al.*, 2008], intra-amniotic infections [Gravett *et al.*, 1986], preterm birth [Hauth *et al.*, 2003] and postpartum maternal infections [Leitich *et al.*, 2003]. Preterm birth is the leading cause of neonatal mortality, and Lawn and coworkers have estimated that 27% of the direct causes of the 4 million neonatal deaths in 2000 can be attributed to preterm birth [Lawn *et al.*, 2005] (Figure 1).

Among the strongest risk factors for BV are the vaginal presence of menstrual blood, a new sexual partner, vaginal douching and smoking [Ma *et al.*, 2012].

Common species traditionally isolated from women with BV by culture-dependent techniques are anaerobes such as *G. vaginalis*, *Prevotella* spp., *Mobiluncus* spp., *Mycoplasma hominis* and *Ureaplasma urealyticum*. Historically, *G. vaginalis* has been considered the most important contributor to BV.

Clinically, BV is diagnosed when at least three of the four Amsel criteria are present, i.e. **(i)** a rate of at least 20% clue cells observed on wet mount microscopy, **(ii)** a 'fishy' odour elicited after mixing vaginal secretions with 10% KOH, **(iii)** vaginal secretions with an elevated pH of > 4.5 and **(iv)** a thin, white, skim-milk-like homogeneous vaginal discharge.

In research settings, BV is usually diagnosed by Nugent scoring of Gram-stained vaginal smears. Upon microscopic examination, the presence and semi-quantification of three bacterial cell morphology types (*Lactobacillus* morphotypes, small Gram-variable *Gardnerella* and *Bacteroides* spp. cell morphology types, and curved Gram-variable rods) is assessed (Figure 6). A score of 0–3 is considered as normal, a score of 4–6 as an intermediate vaginal microbiota, and a score of 7–10 as BV-positive. Further refinements of the Nugent score have been described and have improved our understanding of the vaginal microbiota [Ison *et al.*, 2002; Verhelst *et al.*, 2005]. The sensitivity and specificity of the Amsel criteria are 70% and 94%, respectively, when compared to the Nugent score [Schwebke *et al.*, 1996]. Compared to the Amsel criteria, the Nugent score allows for assessment of the alteration in the vaginal microbiota in a continuous way rather than a dichotomous way.

In summary, classical bacterial culture methods and the use of a rather simple microscopical assessment of the vaginal microbiota have resulted in knowledge on the role of the vaginal microbiota in (reproductive) health and disease. Much has been learned about the epidemiology of BV and its plethora of adverse health outcomes. However, issues such as the etiology of BV remained unresolved. In addition, observations such as **(i)** responders versus non-responders to treatment in symptomatic BV, **(ii)** the failure of preventive trials that aim to reduce preterm birth incidence in pregnant women by treating BV, **(iii)** symptomatic versus asymptomatic women with BV defined by Nugent score and **(iv)** asymptomatic women without lactobacilli remained unexplained. Better definitions of the vaginal microbiota were needed to try to resolve these enigmas.

6.3 The composition of the vaginal microflora – the molecular era

During the last two decades, our knowledge of diversity of the vaginal microbiota has expanded enormously through the use of molecular approaches that circumvented the need to cultivate. Indeed, it was found possible to extract DNA from microbiota and to amplify specific regions of the bacterial 16S rRNA genes containing phylogenetic information by means of polymerase-chain reaction (PCR). After PCR, the amplified fragments – amplicons – represent all bacterial members of a sampled microbiota. Individual amplicons can be separated using different techniques, such as the construction of clone libraries, denaturating gradient gel electrophoresis (DGGE), and terminal restriction fragment length polymorphism (T-RFLP). Sequencing of the individual amplicons yield identification, resulting in accurate descriptions of the bacterial composition of microbiota.

Pioneer molecular research Using above-mentioned approaches, important new insights soon emerged [Verhelst *et al.*, 2004; Burton *et al.*, 2003]. Detection of *Lactobacillus iners* in vaginal microbiota was a novel finding [Burton *et al.*, 2003; Verhelst *et al.*, 2004]. *L. iners*, a species that is difficult to cultivate, does not grow on traditional media nor has the typical characteristics of lactobacilli, appeared to be present in vaginal microbiota of women with and without BV. Importantly, a 16S cloning study showed that *Atopobium vaginae*, previously an unknown member of the vaginal microbiota, was strongly associated with BV [Verhelst *et al.*, 2004]. *A. vaginae* now appears to be a hallmark of BV and even stronger associated with BV than *G. vaginalis*. Other species detected

in BV-associated microbiota were *Megasphaera* spp., *Leptotrichia* spp., and undesignated bacteria named BV-associated bacterium-1 (BVAB-1) and BVAB-3, belonging to the phylum *Clostridium* [Fredricks *et al.*, 2005]. Of interest, it was shown that women with BV had different DGGE profiles indicating heterogeneity in the composition of vaginal microbiota of women with BV [Burton *et al.*, 2003].

Undoubtedly, these pioneer molecular studies were of utmost importance for our further understanding of the vaginal microbiota, but still they were limited in that the number of amplicons that could be sequenced by traditional Sanger sequencing was too low to allow all member species of the vaginal microbiota, especially the low abundant species, to be detected and identified.

Next-generation sequencing technologies The call for a high-throughput sequencing technology that could provide thousands of sequences per specimen at costs affordable in clinical research was answered by Roche, introducing its pyrosequencing technology on the 454 sequencing platform. Costs further tumbled by the rise of competing technologies. Collectively, these new technologies are referred to as next-generation sequencing (NGS).

Several NGS studies have analysed vaginal microbiota in different study populations in both cross-sectional and longitudinal design [Ravel *et al.*, 2013; Schellenberg *et al.*, 2009; Gajer *et al.*, 2012] and revealed compositions of the vaginal microbiota with unprecedented taxonomic resolution. Species shown previously to be associated with a healthy microbiota respectively with a disturbed BV-associated microbiota are consistenly found together with a long list of minority species, previously unknown to be present in the vaginal microbiota [van de Wijgert *et al.*, 2014].

Some authors introduced the name *community state*, referring to the composition of the individual vaginal microbiota. Bioinformatic analyses of community states of different women allows to group women with similar community states into different clusters, named community state types (CST), each with a markedly different bacterial species composition [Ravel *et al.*, 2011].

Although *L. crispatus* was long thought to be the most common *Lactobacillus* spp. [Antonio *et al.*, 1999], there is now emerging consensus that *L. iners* is the most prevalent vaginal species, including in women with BV and that *L. crispatus* is mostly present in women with a healthy vaginal microbiota [Zhou *et al.*, 2004; van de Wijgert *et al.*, 2014].

6.4 The classic vaginal microbiota paradigm revisited

Numerous studies have shown that women with high vaginal concentrations of lactobacilli such as *L. crispatus, L. gasseri, L. jensenii* and/or *L. vaginalis* species do not have symptomatic BV, but the corollary that women whose vaginal microbiota are deprived in lactobacilli also have BV or an 'unhealthy' microbiota is faulty logic [Santiago *et al.*, 2012; Jespers *et al.*, 2015; Ravel *et al.*, 2011; Zhou *et al.*, 2004; Ma *et al.*, 2012]. As much as 20–30% of asymptomatic and healthy women harbor vaginal microbiota reduced in lactobacilli and composed of a diverse array of facultative/strictly anaerobic bacteria that are associated with a somewhat higher pH (5.3–5.5), including members of the genera *Atopobium*, *Corynebacterium*, *Anaerococcus*, *Peptoniphilus*, *Prevotella*, *Gardnerella*, *Sneathia*, *Eggerthella*, *Mobiluncus* and *Finegoldia* [Ravel *et al.*, 2011; Zhou *et al.*, 2004; Zhou *et al.*, 2007; Zhou *et al.*, 2010; Hyman *et al.*, 2005].

Indeed, although NGS studies undeniable revealed valuable information on the composition of the vaginal microbiota, there is still a big gap of knowledge to be filled on the functional characteristics of these vaginal microbiota that drive the interactions with the host in health and disease. Of interest, in the field studying gut microbiota, *enterotypes* – the counterparts of CSTs – are not defined by the species composition or the presence of a core set of species, but by a core set of genes that are involved in critical metabolic pathways [Turnbaugh & Gordon, 2009].

6.5 The vaginal microbiota is highly dynamic

A lifetime of vaginal bacteria The composition of the vaginal microbiota changes profoundly during lifetime driven by estrogen levels [Cribby *et al.*, 2008]. After birth, the neonatal vagina is normally sterile but is very soon colonized with corynebacteria, staphylococci, and nonhemolytic streptococci mostly derived from the maternal vaginal microbiota [Mandar *et al.*, 1996]. Of interest, the maternal vaginal microbiota has recently been shown to play in a key rol in the colonization of neonates, as vaginally delivered infants harbored bacteria resembling those of the maternal vaginal microbiota, whereas neonates delivered by caesarean section harbored bacteria resembling those of the maternal skin microbiota [Dominguez-Bello *et al.*, 2010].

During the early stage of infancy, maternal residual estrogen is still present in the neonatal circulation and induces thickening of the vaginal epithelium

and deposition of glycogen in epithelial cells. Glycogen – a polysaccharide of glucose – is released upon exfoliation of these epithelial cells and is fermented by glucose-fermenting bacteria [Boskey *et al.*, 1999]. The presence of glycogen in the vaginal mucosa favors the colonization glucose-fermenting bacteria, mainly lactobacilli. The fermentation of glycogen by lactobacilli and the production of lactic acid as it's end product is thought to be the main mechanism for lowering the vaginal pH.

When the maternal estrogen levels in infants drop, the reverse cascade takes place: the vaginal mucosa thins, the glycogen levels drop, the lactobacilli disappear and the vaginal pH increases. This changed physiology is associated with a vaginal microbiota composed of species of the genera *Actinomyces*, *Bacteroides*, *Bifidobacterium*, *Fusobacterium*, *Peptococcus*, *PeptoStreptococcus*, *Propionibacterium*, *Staphylococcus* and *Veillonella*. Importantly, no bacteria associated with a normal or BV-associated vaginal microbiota of women of reproductive age, such as lactobacilli, *G. vaginalis*, *P. bivia* or *M. hominis* are present in prepubertal girls [Hill *et al.*, 1995; Dei *et al.*, 2010; Randelovic *et al.*, 2012].

At menarche, the production of estrogen leads to a thickened mucosa and a glycogen deposit in the vaginal mucosa, and the vaginal microbiota changes to one typical of women of reproductive age, dominated by lactobacilli [Hillier *et al.*, 1997; Marrie *et al.*, 1980]. The composition of the vaginal microbiota and its correlates has been most studied during this stage of life and is discussed above.

When menopause sets in, women develop a vaginal microbiota deprived in lactobacilli that resembles that of BV but without symptoms [Cauci *et al.*, 2002; Gustafsson *et al.*, 2011; Petricevic *et al.*, 2012].

Temporal dynamics of the vaginal microbiota Most studies have assessed the composition of the vaginal microbiota in a cross-sectional study designs in which samples are obtained from individuals at a single time point, or with weeks to months between sampling points. Although of major importance for our knowledge of the species composition of the vaginal microbiota and its correlates, these studies yielded little insight into the temporal dynamics of these vaginal microbiota.

Microscopic studies documented daily or two-weekly fluctuations in the composition of the vaginal microbiota [Brotman *et al.*, 2010b; Schwebke *et al.*, 1999], but it's truly dynamic composition became only fully clear after pioneering studies that assessed the diversity of the vaginal microbiota on a daily basis using molecular techniques [Santiago *et al.*, 2012; Ravel *et al.*, 2013]. Some

women were shown to harbor vaginal microbiota that changed markedly even on a day time, eg. some species with a nearly 100% relative abundance disappeared completely 'overnight' in order to reappear a day later [Santiago *et al.*, 2012; Ravel *et al.*, 2013]. In contrast to these highly dynamic microbiota, some women were found to have relatively stable microbiota, even if in the absence of *Lactobacillus* spp. [Santiago *et al.*, 2012; Ravel, 2013].

Longitudinal studies have revealed factors influencing the stability of the vaginal microbiota. One of the major influencing factors is the menstrual cycle. Over the course of the menstrual cycle, hormonal and glycogen levels in the vagina fluctuate, and menstrual blood increases the vaginal pH and offers a new niche for bacteria. Menses have been shown to have a negative effect on the stability of the vaginal microbiota [Santiago *et al.*, 2012], with a prominent increase of *L. iners*, *A. vaginae* and *G. vaginalis* and a decrease of *L. crispatus.*

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A multi-country cross-sectional study of vaginal carriage of group B streptococci (GBS) and *Escherichia coli* in resource-poor settings: prevalences and risk factors

— CHAPTER 7

7.1 Abstract

Background

One million neonates die each year in low- and middle-income countries because of neonatal sepsis; group B *Streptococcus* (GBS) and *Escherichia coli* are the leading causes. In sub-Saharan Africa, epidemiological data on vaginal GBS and *E. coli* carriage, a prerequisite for GBS and *E. coli* neonatal sepsis, respectively, are scarce but necessary to design and implement prevention strategies. Therefore, we assessed vaginal GBS and *E. coli* carriage rates and risk factors and the GBS serotype distribution in three sub-Saharan countries.

Methods

A total of 430 women from Kenya, Rwanda and South Africa were studied cross-sectionally. Vaginal carriage of GBS and *E. coli*, and GBS serotype were assessed using molecular techniques. Risk factors for carriage were identified using multivariable logistic regression analysis.

Results

Vaginal carriage rates in reference groups from Kenya and South Africa were 20.2% (95% CI, 13.7–28.7%) and 23.1% (95% CI, 16.2–31.9%), respectively for GBS; and 25.0% (95% CI, 17.8–33.9%) and 27.1% (95% CI, 19.6–36.2%), respectively for *E. coli*. GBS serotypes Ia (36.8%), V (26.3%) and III (14.0%) were most prevalent. Factors independently associated with GBS and *E. coli*

carriage were *Candida albicans*, an intermediate vaginal microbiome, bacterial vaginosis, recent vaginal intercourse, vaginal washing, cervical ectopy and working as a sex worker. GBS and *E. coli* carriage were positively associated.

Conclusions

Reduced vaginal GBS carriage rates might be accomplished by advocating behavioral changes such as abstinence from sexual intercourse and by avoidance of vaginal washing during late pregnancy. It might be advisable to explore the inclusion of vaginal carriage of *C. albicans*, GBS, *E. coli* and of the presence of cervical ectopy in a risk- and/or screening-based administration of antibiotic prophylaxis. Current phase II GBS vaccines (a trivalent vaccine targeting serotypes Ia, Ib, and III, and a conjugate vaccine targeting serotype III) would not protect the majority of women against carriage in our study population.

7.2 Introduction

One million children die each year in low- and middle-income countries in the first 4 weeks of life because of neonatal sepsis [Lawn *et al.*, 2005]. Early-onset neonatal sepsis (EOS), occurring in the first week of life, accounts for approximately 80% of cases, and is caused by bacteria that are transmitted vertically from the genital tract of the mother to infant before or during delivery [Hornik *et al.*, 2012]. Late-onset neonatal sepsis (LOS) occurs between week 1 and month 2 to 3 of life and may be caused by bacteria acquired vertically or horizontally [Schuchat 1998]. Because the transfer of a single species from the maternal genitourinary tract to the neonate before or during delivery is a prerequisite for EOS [Cutland *et al.*, 2009], there are unique opportunities for prevention of EOS.

At present, *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) and *Escherichia coli* are the leading causes of EOS worldwide [Simonsen *et al.*, 2014]. Furthermore, GBS and *E. coli* are associated with preterm birth, very-low-birth-weight delivery and puerperal sepsis [Krohn *et al.*, 1997; Acosta *et al.*, 2014], which cause substantial morbidity and mortality in sub-Saharan Africa (SSA) [Hornik *et al.*, 2012; Beck *et al.*, 2010; Khan *et al.*, 2006].

To prevent EOS, efforts have been focusing mainly on GBS and high-income countries, based on two strategies, namely the screening- or risk-based administration of intrapartum antibiotic prophylaxis (IAP) and the development of vaccines [Melin and Efstratiou 2013].

IAP has been shown to reduce the incidence of GBS EOS from 1.7/1000 to 0.6/1000 in the US [Schrag *et al.*, 2000], but is not effective against *E. coli* EOS, LOS, and adverse perinatal outcomes related to GBS [Regan *et al.*, 1996; Ohlsson *et al.*, 2014]. Furthermore, according to the current universal guidelines (Centers for Disease Control and Prevention, CDC), IAP should be administered to women found positive for GBS at 35–37 weeks of gestation [Verani *et al.*, 2010]. However, these guidelines are not followed in most health-care facilities in low-income countries. The use of intravaginal washes with chlorhexidine (a wide-spectrum microbicide) during labour and neonatal wipes with chlorhexidine, has been explored in low- and middle-income countries, but is unlikely to prevent vertically acquired neonatal infections in any setting or population [Cutland *et al.*, 2009].

Most GBS vaccines under development aim at eliciting protective antibodies against capsular polysaccharides (CPS), the most important GBS virulence factor of which ten antigenically distinct CPS are known [Melin and Efstratiou 2013], and are attractive as some of the IAP-related problems may be circumvented [Melin and Efstratiou 2013]. However, these vaccines might not be effective in low-income countries because of different serotype distribution [Johri *et al.*, 2006].

Although SSA has the highest rates of neonatal sepsis mortality worldwide, epidemiological data on vaginal GBS and *E. coli* carriage are very limited but necessary to develop and implement prevention strategies [Capan *et al.*, 2012; Stoll *et al.*, 1998]. Therefore, in this multi-country cross-sectional study, we assessed the vaginal GBS and *E. coli* carriage prevalence, risk factors for GBS and *E. coli* carriage, and GBS serotype distribution in populations from three countries: Kenya, Rwanda and South Africa.

7.3 Patients and methods

Study design and population

In 2010–2011, we conducted a multi-country follow-up study entitled ''Characterisation of novel microbicide safety biomarkers in East and South Africa''. The main aim of that project was to characterise the vaginal microbiome and the cervicovaginal mucosal immune system in African women and to assess changes of these over time [Gautam *et al.*, 2015; Jespers *et al.*, 2014; Jespers *et al.*, 2015; Kyongo *et al.*, 2015]. In that study, 430 women were recruited at three study sites, i.e. the International Centre for Reproductive Health Kenya

(ICRHK) in Mombasa, Kenya (170 women); the non-governmental organisation Rinda Ubuzima (RU) in Kigali, Rwanda (60 women), and the Wits Reproductive Health and HIV Institute (Wits RHI) in Johannesburg, South Africa (SA) (200 women). The women were recruited into 6 predefined study groups: a reference group of 219 women (adult, non-pregnant, HIV-negative women at average risk of HIV), 60 pregnant women (up to 14 weeks of gestational age as determined by abdominal ultrasound at recruitment), 60 adolescent girls (16–17 years), 31 HIV-negative women engaging in vaginal practices (usage of cloth, lemon juice, or detergents to clean, dry or tighten the vagina on a regular basis), 30 self-acknowledged female sex workers (FSW), and 30 HIV-positive women (on antiretroviral treatment for at least 6 months, asymptomatic and with a CD4 count of more than 350 cells/µl) (Table 2). Participants were eligible for inclusion if they were in good physical and mental health, able and willing to participate in the study as required by the protocol, able and willing to give written informed consent (including written parental or guardian consent for adolescents). Women were excluded if they had never had penetrative vaginal intercourse, if they had a history of hysterectomy or other genital tract surgery in the three months prior to the screening visit, if external and/or internal genital warts were found, if they were enrolled in HIV prevention trials involving investigational products, if they were less than 6 months post-partum at the time of enrolment, if they were HIV-positive (unless for inclusion in the HIV-positive women group), or if they were pregnant (unless for inclusion in the pregnant women group). The study population, followed up for approximately eight months per person over 8 visits, is described in detail by Jespers and coworkers [Jespers *et al.*, 2014].

TABLE 2 Study population and vaginal GBS and *E. coli* carriage rates.

City, Country	Group	n	GBS prevalence % (95% CI)	*E. coli* prevalence % (95% CI)
Mombasa, Kenya	Reference group	110	20.2 (13.7–28.7)	25.0 (17.8–33.9)
Mombasa, Kenya	Pregnant women	30	14.3 (5.7–31.5)	14.3 (5.7–31.5)
Mombasa, Kenya	Adolescents	30	3.6 (0.6–17.7)	28.6 (15.3–47.1)
Kigali, Rwanda	FSW	30	20.0 (9.5–37.3)	70.0 (52.1–83.3)
Kigali, Rwanda	HIV+ women	30	0.0 (0.0–11.4)	20.0 (9.5–37.3)
Johannesburg, SA	Reference group	109	23.2 (16.2–31.9)	27.1 (19.6–36.2)
Johannesburg, SA	Pregnant women	30	10.0 (3.5–25.6)	33.3 (19.2–51.2)
Johannesburg, SA	Adolescents	30	0.0 (0.0–11.4)	13.3 (5.3–29.7)
Johannesburg, SA	Vaginal practices	31	25.8 (13.7–43.2)	30.0 (16.7–47.9)

The current study presents one of the tertiary objectives of the above-mentioned study, namely to document the vaginal carriage rates of the main pathogens associated with EOS (GBS and *E. coli*) and the risk factors for their carriage. These analyses are based on the screening visit and the first visit (scheduled soon after the last day (day 9 ± 2 days) of the menstrual period) from the follow-up study.

Study procedures

At the screening visit, blood, vaginal, endocervical and urine samples were taken for diagnostic testing of HIV, HSV-2, syphilis, *Neisseria gonorrheae* (NG), *Chlamydia trachomatis* (CT), *Trichomonas vaginalis* (TV), urinary tract infection (UTI), pregnancy, cervical dysplasia (by Pap smear), bacterial vaginosis (BV) (Amsel criteria), and vaginal candidiasis. Treatment was provided according to national guidelines, voluntary HIV counseling was offered, and condoms were provided free-of-charge.

At visit 1, two sterile Copan flocked® vaginal swabs (Copan Diagnostics, Inc., Murrieta, CA), to be used for the molecular detection of GBS and *E. coli*, were brought into the vaginal vault by the study clinician, rotated against the vaginal wall at the midportion of the vault, gently dipped in the posterior fornix and carefully removed to prevent contamination with the microbiome of the vulva and introitus. The swab heads were collected into two 1.5 ml cryovials, labelled and immediately frozen at -80 °C until shipment to the central laboratory at the Institute of Tropical Medicine (ITM, Antwerp, Belgium) using temperature-monitored dry shippers filled with liquid nitrogen. One Amies swab (Copan Diagnostics, Inc.) for culturing was taken in a likewise manner, placed in the Amies tube, and transported at 4 °C in a temperature-monitored cooler to the local laboratory, where it was processed immediately. At both visits, women were interviewed during face-to-face interviews about their general and sexual health, vaginal habits and sociodemographic characteristics. A physical examination including speculum and bimanual pelvic examination was carried out by a clinician. At each visit, participants were compensated for their time and transportation.

Diagnosis of genital infections

At the local laboratories, tests for HIV, HSV-2, syphilis, CT and NG were performed. For immediate detection of *Candida* cells and hyphae, TV, and clue cells,

wet mount microscopy was used. For the purpose of this study, a commercially available TV InPouch™ system (BioMed Diagnostics, White City, Oregon) was used. For this, a vaginal swab was inoculated according to the manufacturer's instructions and InPouch cultures were monitored on a daily basis. InPouch bags with no growth at the end of five days were considered negative. BV diagnosed according to the Amsel criteria was used for immediate treatment. For research, vaginal smears were made and sent to ITM for Gram staining and Nugent scoring, a scoring system to diagnose BV. Briefly, smears from vaginal swabs were prepared by rolling the swab onto a glass slide. Slides were air-dried and fixed using 70% ethanol. For the Gram-staining at ITM, the fixed smear was covered with crystal violet for 1 minute, washed with water, flooded with Lugol's iodine for 1 minute, washed with water, and then decolorized with acetone-alcohol for 2–3 seconds. The smears were rinsed quickly under running water to stop the decolorisation and then counterstained with safranin for 1 minute. All reagents were from Becton Dickinson (BD). All smears were examined microscopically with the 40x objective to check the staining and the distribution of the material, and then assessed under oil immersion objective (1000x magnification) using the grading system described by Nugent and co-workers [Nugent *et al.*, 1991]. The Nugent score is calculated by assessing for the presence of *Lactobacillus* cell types, small Gram-variable coccobacilli, and curved Gram-variable rods. A score of 0–3 is considered as normal (BV-negative); a score of 4–6 as an intermediate vaginal microbiome; and a score of 7–10 as BV-positive.

DNA extraction

For the molecular detection of GBS and *E. coli*, DNA extraction from the two Copan swabs of each subject was carried out at ITM by thawing the swabs at room temperature for 30 minutes. After adding 1200 µL of diluted PBS, each swab was gently vortexed for 15 seconds, and 1 mL of each swab suspension was pooled into a final volume of 2 mL. An aliquot of 250 µL was extracted using the Abbott m24sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacturer's instructions, and 200 µl of eluted DNA – to be used in the quantitative PCR (qPCR) assays – was stored at -80 °C.

For the construction of qPCR standard curves, DNA was extracted from overnight cultures of *S. agalactiae* LMG 14694T on TSA + 5% sheep blood, *E. coli* ATCC 25922 grown on TSA + 5% sheep blood, and *C. albicans* ATCC 90028 grown on Sabouraud agar (all BD). All growth was harvested from the plate and

resuspended in 1 ml of saline. DNA of this suspension was extracted using the High Pure PCR Template Preparation Kit (Roche Applied Science, Basel, Switzerland) according to the manufacturer's instructions.

For capsular genotyping of GBS, 1 ml of inoculated Lim Broth medium (see Microbiological culturing) was used for DNA extraction using the High Pure PCR Template Preparation Kit (Roche), according to the the manufacturer's instructions.

Streptococcus agalactiae qPCR

To detect *S. agalactiae* in vaginal DNA extracts, a *S. agalactiae* specific qPCR was carried out, using primers previously described [Bergh *et al.*, 2004]. The qPCR reactions for *S. agalactiae* were performed in a final volume of 10 µl, containing 5 µl of LightCycler 480® SYBR Green I Master (Roche), 0.5 µM of both forward primer Sip1 (5'-ATCCTGAGACAACACTGACA-3') and reverse primer Sip2 (5'-TTGCTGGTGTTTCTATTTTCA-3'), 0.3 µM of probe (5'- 6-FAM–ATCAGAAGAGTCATACTGCCACTTC–TAMRA-3') (Eurogentec, Liège, Belgium) and 2 µl of DNA extract or 2 µl of HPLC water (as negative template control). Cycling conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. For the standard series, DNA concentration of the extract of *S. agalactiae* LMG 14694T was determined using the Qubit® Fluorometer (Invitrogen, Auckland, New Zealand) and the genomic concentration was calculated based on the GC% content and genome size of the type strain. A tenfold dilution standard series of *S. agalactiae* LMG 14694T DNA was prepared by dilution of the DNA stock in HPLC grade water. All standard tenfold dilution series and samples were run in duplicate. Amplification, detection and quantification were carried out using the LightCycler480® platform and the LightCycler® 480 Software Version 1.5 (Roche).

Escherichia coli qPCR

To detect *E. coli* in vaginal DNA extracts, an *E. coli* specific qPCR was carried out, using primers targeting the β-glucuronidase encoding gene uidA, previously described [Chern *et al.*, 2011]. The qPCR reactions were performed in a final volume of 10 µl, containing 5 µl of LightCycler 480® SYBR Green I Master (Roche), 0.3 µM of both forward primer EcoliFW (5'-CAACGAACTGAACTGGCAGA-3') and reverse primer EcoliRV (5'- CATTACGCTGCGATGGAT -3') (Eurogentec) and 2 µl of

DNA extract or 2 µl of HPLC water (as negative template control). Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard series (using *E. coli* ATCC 25922 grown on TSA + 5% sheep blood (BD)), was constructed as described for *S. agalactiae.*

Candida albicans qPCR

To detect *C. albicans* in vaginal DNA extracts, a *C. albicans* specific qPCR was carried out, using primers targeting the ITS-1 gene (adapted from [25]). The qPCR reactions were performed in a final volume of 10 µl, containing 5 µl of LightCycler 480® SYBR Green I Master (Roche), 0.3 µM of both forward primer CA_FW (5'-CAACGAACTGAACTGGCAGA-3') and reverse primer CA_RV (5'- CATTACGCTGCGATGGAT -3') (Eurogentec) and 2 µl of DNA extract or 2 µl of HPLC water (as negative template control). Cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min; 40 cycles of 95 °C for 15 s and 60 °C for 1 min. A standard series (using *C. albicans* ATCC 90028 grown on Sabouraud agar (BD)), was constructed as described for *S. agalactiae.*

Microbiological culturing

At the local laboratories, the Amies swab was inoculated on in-house TMB^{plus} plates (a medium supporting growth of anaerobes and allowing assessment of hydrogen peroxide production of strains) [26], after which the plates were incubated anaerobically as described previously [Verhelst *et al.*, 2004]. After 48–72 h, depending on the growth, all biological material of the culture plate was harvested using sterile cotton swabs and stored in cryovials with 1 ml of tryptic soy broth + 5% glycerol at -80 °C until shipment. After shipment to the ITM, bacteria from the cryovial were inoculated in commercial Lim Broth medium (BD) – a selective enrichment medium for GBS – according to the manufacturer's instructions (5% CO_2 at 35°C for 24 hours). The latter procedure was performed only for women found to be positive for vaginal GBS carriage by means of qPCR. DNA extracts of inoculated Lim Broth medium was used for direct molecular capsular typing of GBS.

S. agalactiae molecular capsular typing

To determine the GBS serotype, we used a flowchart described by [Yao *et al.*, 2013], based on the multiplex PCRs with primers as described by Poyart and co-workers and Imperi and co-workers [Poyaert *et al.*, 2007; Imperi *et al.*, 2010]. The multiplex PCRs were performed directly on DNA extracted from the inoculated Lim Broth medium. The reactions were performed in a final reaction mixture of 20 µl, containing 10 µl of FastStart PCR Master Mix (Roche), 0.2 µM of each primer, and 2 µl of DNA template. Using a Veriti 96-well thermal cycler (Applied Biosystems, Foster City, CA), the following PCR program was run: 94 °C for 5 min, 3 cycles of 45 s at 94 °C, 2 min at 50 °C, 1 min at 72 °C, and 30 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min at 72 °C, with a final extension at 72 °C for 7 min. PCR amplification products were visualised under UV light after electrophoresis on 1% agarose gels (30 minutes at 10 V/cm) and staining with ethidiumbromide. Twenty-five control strains (covering all GBS serotypes and provided by the Belgian *Streptococcus agalactiae* reference center (Dr. Pierette Melin, University of Liège, Belgium)) were used as a positive control.

Physiological parameters

Vaginal pH was measured during the speculum examination by pressing commercial pH strips (pH Fix 3.6–6.1, Machery-Nagel) against the vaginal wall. Detection of prostate-specific antigen (PSA), a marker for sexual intercourse within the past 24 hours [Macaluso *et al.*, 1999] in vaginal swab fluid was performed using a chromatographic immune assay (the Seratec® PSA SemiQuant Cassette Test, Seratec, Gottingen, Germany) according to the manufacturer's instructions. Pregnancy was assessed by testing urine with a rapid hCG test (QuickVue One-Step hCG Test (Kigali, Johannesburg) or Unimed First Sign hCG test (Mombasa)). Leucocytes and erythrocytes in urine were detected using dipsticks according to the manufacturer's instructions (Siemens Multistix 10 sg in Kigali, Mission® urinalysis strips in Mombasa, and Neotest 4 Urine Dipstick in Johannesburg).

Statistical analysis

Data were analyzed with SPSS software version 22 (SPSS Inc.). Prevalences were reported with their 95% confidence interval. Outcomes for this analysis were vaginal GBS carriage and vaginal *E. coli* carriage, as determined by a positive qPCR.

Independent variables considered were study site, sociodemographic characteristics, reproductive health characteristics, sexual behavioural factors, vaginal practices characteristics, cervicovaginal signs and symptoms and microbiological characteristics. Variables were analyzed using logistic regression in univariable and multivariable ways, with p-values < 0.05 indicating significance. In order not to overfit our multivariable models, variables were restricted in proportion to the number of cases positive for GBS and *E. coli*, i.e. maximum one degree of freedom per 10 cases [Babyak *et al.*, 2004]. Variables included in the models were selected as follows [Wakkee *et al.*, 2014]: firstly, only variables found to be significantly associated with GBS or *E. coli* carriage in univariable analysis were considered for inclusion the multivariate GBS or *E. coli* model, respectively. Subsequently, of correlated variables (e.g. 'having had recent vaginal intercourse' and a positive PSA test), only one was kept for further consideration to avoid collinearity. The final selection of variables was based on literature and clinical expertise/relevance. The multivariable models were controlled for possible confounding variables and were validated with bootstrap analysis.

Ethics statement

Written information and consent forms in the local language were provided to the women or to the Legally Authorized Representatives for their review. After the interview, the participants were asked to express their willingness to participate in the study by signing (or thumb-printing in case they were illiterate) the consent form. In case they were of minor age (age below 18 in Kenya and SA, and below 21 in Rwanda), also the parents or guardians were asked to give consent. The study was approved by the Kenyatta National Hospital Ethical Review Committee, Kenya; the Human Research Ethics Committee (Medical), University of the Witwatersrand, SA; the Rwanda National Ethics Committee, Rwanda; the Institutional Review Boards of the Institute of Tropical Medicine in Antwerp, of Ghent University, and of the University Teaching Hospital in Antwerp, Belgium. In addition, the study was approved by the National Council on Science and Technology in Kenya, and the National AIDS Control Commission in Rwanda. The study is registered at the Trial Registration at the National Health Research Ethics Council South Africa (DOH2709103223) [Jespers *et al.*, 2014].

7.4 Results

Vaginal GBS and *E. coli* carriage and GBS serotype distribution

Of the 430 women enrolled in the study, 424 and 421 vaginal swab DNA extracts were analysed for the presence of GBS and *E. coli*, respectively. The vaginal GBS and *E. coli* carriage rates in the different study groups are presented in Table 1.

The GBS serotype distribution is presented in Table 3 and Fig 7. For 12 GBS carriers, the serotype could not be determined because samples were no longer available. Serotype distribution was largely comparable between sites. The most prevalent serotypes were Ia (27.3%), V (27.3%), and III (22.7%) in Kenya; Ia (34.5%), V (31.0%), and IV (13.8%) in SA; and Ia (83.3%) and II (16.7%) in Rwanda.

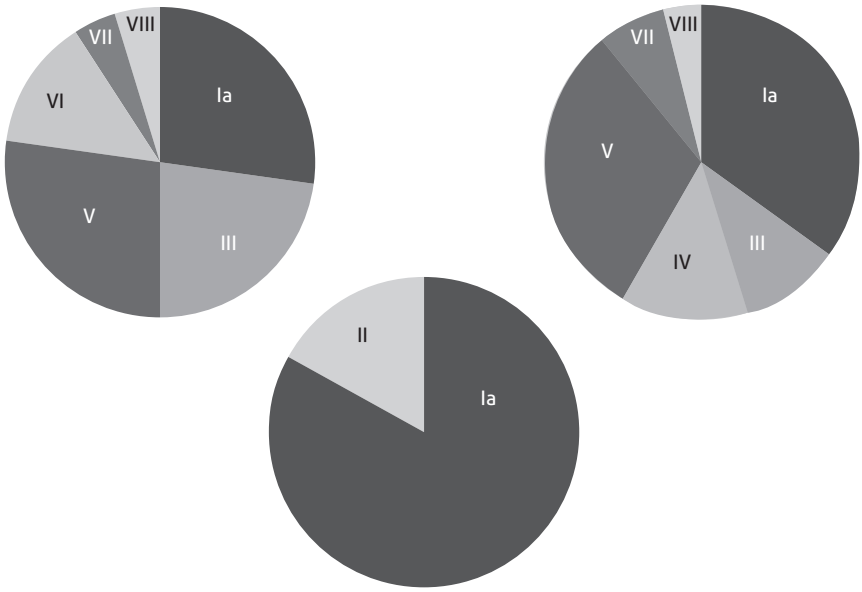

FIGURE 7 Distribution of GBS capsular serotypes.
Left, Kenya (n=22);
Middle, Rwanda (n=6);
Right, South Africa (n=29).

TABLE 3 Studies reporting GBS serotype distribution of (recto)vaginal isolates in SSA.

Country	Year	Population	Ia	Ib	II	III	IV
The Gambia	1994	P	19		28	6	3
Malawi	2011	P, HIV+, HIV-	18.2	6.2	10.3	39.0	0.3
SA	2011	P	30.1	6.7	11.3	37.3	3.7
SA	2014	P	36.2–41.4	3.5–4.6	7.2–7.5	31.3–34.9	2.0–4.0
Kenya	**2015**	**P, NP**	**27.3**			**22.7**	
Rwanda	**2015**	**NP**	**83.3**		**16.7**		
SA	**2015**	**P, NP**	**34.5**			**10.3**	**13.8**
Europe	2010	N/A	18.2	12.4	14.4	28.1	3.7
US	2010	N/A	26.8	8.1	10.9	24.8	1.0

P, pregnant;
NP, non-pregnant;
[$]determined serotypes I-VI (no differentiation between Ia and Ib);
N/A, not applicable (review);
[£]data from meta-analysis but excluding isolates from non-sterile sites and from neonates were excluded.

V	VI	VII	VIII	IX	NT	Reference
38						[Suara *et al.*, 1994][S]
23.9	0.8		0.8		1.5	[Gray *et al.*, 2011]
10.2						[Madzivhandila *et al.*, 2011]
10.3–15.6				0.0–3.3		[Kwatra *et al.*, 2014]
27.3	**13.6**	**4.5**	**4.5**			**This study**
						This study
31.0		**6.9**	**3.4**			**This study**
14.9	0.6	0.6	0.6			[Ippolito *et al.*, 2010][E]
15.0	0.3	0.0	0.2			[Ippolito *et al.*, 2010][E]

Univariable and multivariable analyses

Tables 4 and 5 present the univariable associations of the sociodemographics, sexual behavior, vaginal practices, cervicovaginal signs and symptoms, and microbiological characteristics with vaginal GBS and *E. coli* carriage, respectively. Because of the low prevalence, CT, NG, TV, and syphilis were not considered for further analysis.

TABLE 4 Sociodemographic characteristics, reproductive health, sexual behavior, vaginal practices, vaginal signs & symptoms, and microbiological associations with vaginal GBS carriage (univariable analysis).

	n	GBS+ n (%)	Crude OR (95% CI)	p-value*
	424	69 (16.3)		
Sociodemographic characteristics				
City (Country)				
Mombasa (Kenya)	165	27 (16.4)	0.89 (0.51–1.53)	0.665
Kigali (Rwanda)	60	6 (10.0)	0.50 (0.20–1.26)	0.142
Johannesburg (SA)	199	36 (18.1)	1	-
Age (years)				
<18	58	1 (1.7)	0.07 (0.01–0.50)	**0.008**
18–24	148	23 (15.5)	0.71 (0.41–1.23)	0.219
>24	218	45 (20.6)	1	-
Educational level				
Higher educational level[£]	189	39 (20.6)	1	-
Lower educational level[££]	235	30 (12.8)	0.56 (0.33–0.95)	**0.030**
Marital status				
Never married	242	34 (14.0)	1	-
Married	148	30 (20.2)	1.55 (0.91–2.67)	0.109
Separated/divorced/widowed	34	5 (14.7)	1.06 (0.38–2.91)	0.918
Socio-economic status[#]				
Low	106	17 (13.0)	1	-
Medium	163	31 (19.0)	1.23 (0.64–2.36)	0.533
High	155	21 (13.5)	0.82 (0.41–1.64)	0.576

Reproductive health				
Pregnant				
No	366	62 (16.9)	1	-
Yes	58	7 (12.1)	0.67 (0.29–1.55)	0.353
Parity				
0	149	18 (12.1)	1	-
1–2	211	42 (19.9)	1.81 (1.00–3.29)	0.052
>2	64	9 (14.1)	1.19 (0.50–2.81)	0.690
Gravity				
0	118	14 (11.9)	1	-
1–2	210	38 (18.1)	1.64 (0.85–3.17)	0.141
>2	96	17 (17.7)	1.60 (0.74–3.44)	0.230
Regular cycle				
Yes	256	42 (16.4)	1	-
No/unknown	168	27 (16.1)	0.98 (0.58–1.66)	0.927
Menstrual cycle				
No cycle	194	33 (17.0)	1	-
With cycle	230	36 (15.7)	0.91 (0.54–1.51)	0.706
Contraceptive				
None	73	15 (20.5)	1	-
Condom only	108	10 (9.3)	0.40 (0.17–0.94)	**0.035**
Others (hormones/IUD/ sterilisation/pregnant)	243	44 (18.1)	0.86 (0.44–1.65)	0.639
Currently breastfeeding				
No	390	64 (16.4)	1	-
Yes	34	5 (14.7)	0.88 (0.33–2.36)	0.796
Sexual behaviour				
Age at first sexual encounter (years)				
<16	80	8 (10.0)	1	-
16–18	197	36 (18.3)	2.01 (0.89–4.5)	0.093
19–21	104	16 (15.4)	1.64 (0.66–4.04)	0.286
>21	43	9 (20.9)	2.38 (0.85–6.71)	0.101

Sexually active (last 3 months)				
No	55	4 (7.3)	1	-
Yes	369	65 (17.6)	2.73 (0.95–7.81)	0.062
Condom use (at last sexual encounter)[@]				
No	229	47 (20,5)	1.75 (0.94–3.16)	0.050
Yes	140	18 (12.9)	1	-
Lifetime n° of sex partners				
1	112	13 (11.6)	1	-
2–3	186	32 (17.2)	1.58 (0.79–3.16)	0,194
> 3	126	24 (19.0)	1.79 (0.86–3.71)	0,117
N° of sex partners in the last 3 months				
0	25	0 (0.0)	1	-
>=1	399	69 (17.3)	N/A	**0.010**
Recent vaginal sex[%]				
No	343	49 (14.3)	1	-
Yes	81	20 (24.7)	2.73 (0.95–7.81)	**0.024**
Sexual risk taking[€]				
Low	167	25 (15.0)	1	-
Medium	155	30 (19.4)	1.36 (0.76–2.44)	0.297
High	102	14 (13.7)	0.90 (0.45–1.83)	0.779
Estimated frequency of sexual encounters in last 3 months[@, &]				
0	55	4 (7.3)	1	-
< 10 times	137	18 (13.1)	1.93 (0.62–5.98)	0.255
11–30 times	129	24 (18.6)	2.91 (0.96–8.84)	0.059
> 30 times	98	23 (23.5)	3.91 (1.27–11.98)	**0.017**
HIV status partner[@]				
HIV positive	38	3 (7.9)	1	-
HIV negative	250	52 (20.8)	3.06 (0.91–10.36)	0.072
Unknown	79	10 (12.6)	1.69 (0.44–6.54)	0.447

Estimated frequency of unprotected sex in last 3 months				
No sexual contacts	55	4 (7.3)	1	-
Never unprotected	104	12 (11.5)	1.66 (0.51–5.42)	0.399
< 10 times	88	14 (15.9)	2.41 (0.75–7.75)	0.139
>= 10 times	177	39 (22.0)	3.60 (1.23–10.59)	**0.020**
New partner (within 3 months)				
No	378	60 (15.9)	1	-
Yes	46	9 (19.6)	1.29 (0.59–2.81)	0.523
Circumcision status partner[@]				
Circumcised	240	38 (15.8)	1	-
No/don't know Not circumcised/ don't know	129	27 (20.9)	1.41 (0.81–2.43)	0.222
Female sex worker				
Yes	30	6 (20.0)	1	-
No	394	64 (16.2)	1.03 (0.38–2.97)	0.952
Vaginal practices				
Washing inside the vagina when bathing				
No	176	15 (8.5)	1	-
Yes	248	54 (21.8)	2.99 (1.63–5.49)	**<0.001**
Drying the vagina before sex				
Yes	10	4 (40.0)	1	-
No	414	65 (15.7)	3.58 (0.98–13.04)	0.053
Washed inside the vagina recently (morning or evening before study visit)				
No	231	28 (12.1)	1	-
Yes	193	41 (21.2)	1.96 (1.16–3.30)	**0.012**
Products to wash/clean/dry/tighten the vagina				
None	153	13 (8.5)	1	-
Water/fingers only or water/soap	211	44 (20.9)	2.84 (1.47–5.48)	**0.002**
Cloth	48	9 (18.8)	2.49 (0.99–6.24)	0.053
Lemon juice/detergents	12	3 (25.0)	3.59 (0.86–14.92)	0.079

Cleaning the vagina after sexual intercourse				
No	227	28 (12.3)	1	-
Yes	197	41 (20.8)	1.87 (1.11–3.16)	**0.019**
Cervicovaginal signs and symptoms				
Ectopy[¶]				
No	226	34 (15.0)	1	-
Yes	197	34 (17.3)	1.18 (0.70–1.98)	0.536
Degree of ectopy[¶]				
Absent	226	34 (15.0)	1	-
Small	53	8 (15.1)	1.00 (0.44–2.32)	0.993
Moderate	139	24 (17.3)	1.18 (0.67–2.09)	0.573
Large	5	2 (40.0)	3.77 (0.61–23.4)	0.155
Colposcopic findings[§,¶]				
No	380	59 (15.5)	1	-
Yes	43	10 (23.3)	1.65 (0.77–3.53)	0.197
Cervical mucus				
No	270	39 (14.4)	1	-
Mild to moderate	140	28 (20.0)	1.48 (0.87–2.53)	0.151
Abundant	14	2 (14.3)	0.99 (0.21–4.58)	0.987
Reported abnormal discharge				
No	399	64 (16.0%)	1	-
Yes	25	5 (20%)	1.31 (0.47–3.61)	0.604
Vaginal discharge on speculum				
No	332	52 (15.7)	1	-
Yes	92	17 (18.5)	0.82 (0.45–1.50)	0.518
Vaginal epithelial abnormalities				
No	419	67 (16.0)	1	-
Yes	5	2 (40.0)	3.50 (0.57–21.36)	0.174
Cervical epithelial abnormalities				
No	379	64 (16.9)	1	-
Yes	45	5 (11.1)	0.62 (0.23–1.62)	0.325

Red blood cells in urine				
No	358	58 (16.2)	1	-
Yes	66	11 (16.7)	1.03 (0.51–2.10)	0.925
White blood cells in urine				
No	328	56 (17.1)	1	-
Yes	96	13 (13.5)	0.76 (0.40–1.46)	0.411
Microbiological factors				
BV visit 1 (Amsel criteria)				
No BV	346	61 (17.6)	1	-
BV	78	8 (10.3)	0.53 (0.24–1.17)	0.116
BV (Nugent) [1]				
No BV (Nugent 0–3)	217	37 (17.1)	1	-
Intermediate (Nugent 4–6)	29	6 (20.7)	1.30 (0.60–2.83)	0.507
BV (Nugent 7–10)	137	13 (9.5)	0.29 (0.12–0.70)	**0.006**
Reproductive tract infection (RTI)				
No RTI	352	60 (17.0)	1	-
1 or more RTI	60	8 (13.3)	0.75 (0.34–1.66)	0.475
>1 RTI	12	1 (8.3)	0.44 (0.06–3.50)	0.439
Syphilis				
No	415	69 (16.6)	1	-
Yes	9	0 (0.0)	N/A	**0.001**
Chlamydia trachomatis				
No	382	62	1	-
Yes	42	7	1.03 (0.44–2.43)	0.942
Neisseria gonorrhoeae				
No	415	69 (16.6)	1	-
Yes	9	0 (0.0)	N/A	**0.001**
Trichomonas vaginalis				
No	390	66 (16.9)	1	-
Yes	26	3 (11.5)	0.64 (0.19–2.20)	0.478

***Candida albicans* (qPCR)**				
No	375	50 (13.3)	1	-
Yes	46	17 (37.0)	3.81 (1.95–7.44)	**<0.001**
***Escherichia coli* (qPCR)**				
No	354	89 (25.1)	1	-
Yes	67	29 (43.3)	2.27 (1.33–3.90)	**0.003**
HSV-2 serology				
No	276	42 (15.2%)	1	-
Yes	147	27 (18.4%)	1.25 (0.74–2.13)	0.404
Vaginal pH				
< 4.4	126	15 (11.9)	1	-
4.4 – 5.3	240	42 (17.5)	1.57 (0.83–2.96)	0.163
5.4 and more	58	12 (20.7)	1.93 (0.84–4.44)	0.122
PSA present				
No	233	31 (13.3)	1	-
Yes	181	38 (21.0)	1.73 (1.03–2.91)	**0.039**
Systemic antibiotics visit 1				
No	341	62 (18.1)	1	-
Yes	83	7 (8.4)	0.41 (0.18–0.94)	**0.036**
Systemic antibiotics screening visit				
No	391	68 (17.4)	1	-
Yes	33	1 (3.0)	0.15 (0.02–1.11)	0.063

*bold: significant at the 5% level; [£]completed secondary school, or post-secondary school; [ff]Primary school (completed or not), secondary school but not completed; [#]Socio-economic-status was constructed from total income, type of housing, type of toilet; [@]with partners within three months prior to enrolment; [&]missing data for 5; [%]sex morning or evening before visit; [&,€]low risk: 1 or no partners in last year and did not have any partner (in the last 3 months) with multiple partners and age first sex at least 15 years; medium risk: 2 partners last year or had at least one sexual partner (in the last 3 months) who had multiple partners; high risk: sex worker or at least 3 partners last year or at had at least one sexual partner with HIV in the last 3 months or age first sex less than 15 years; N/A, no odds ratio due to no cases in one category;

¶data missing for 1 (ectopy, colposcopic findings, HSV-2 serology), 3 (*C. albicans*, *E. coli*), 8 (*T. vaginalis*), 41 (BV Nugent, unreadable slides); §petechiae (6 GBS cases/20), abrasion (2 GBS cases/5), erythema (1 GBS case/10), laceration (1 GBS case/4); BV, bacterial vaginosis; IUD, intrauterine device.

TABLE 5 **Sociodemographic characteristics, reproductive health, sexual behavior, vaginal practices, vaginal signs & symptoms, and microbiological associations with vaginal *E. coli* carriage (univariable analysis).**

	n	*E. coli* + n (%)	Crude OR (95% CI)	p-value*
	421	118 (28.0)		
Sociodemographic characteristics				
City (Country)				
Mombasa (Kenya)	164	39 (23.6)	0.87 (0.54–1.41)	0.569
Kigali (Rwanda)	60	27 (45.0)	2.28 (1.25–4.15)	**0.007**
Johannesburg (SA)	197	52 (26.5)	1	-
Age (years)				
<18	58	12 (20.7)	0.73 (0.36–1.47)	0.376
18–24	147	49 (33.3)	1.40 (0.88–2.20)	0.154
>24	216	57 (26.4)	1	-
Educational level				
Higher educational level£	187	56 (29.9)	1	-
Lower educational level££	234	62 (26.5)	0.84 (0.55–1.29)	0.434
Marital status				
Never married	240	71 (29.6)	1	-
Married	147	37 (25.2)	0.80 (0.50–1.27)	0.348
Separated/divorced/widowed	34	10 (29.4)	0.99 (0.45–2.18)	0.984
Socio-economic status#				
Low	106	27 (25.5)	1	-
Medium	163	46 (28.2)	1.15 (0.66–2.00)	0.620
High	152	45 (29.6)	1.23 (0.70–2.15)	0.467

Reproductive health				
Pregnant				
No	363	104 (28.7)	1	-
Yes	58	14 (24.1)	0.79 (0.42–1.51)	0.478
Parity				
0	149	35 (23.5)	1	-
1–2	209	62 (29.7)	1.37 (0.85–2.22)	0.196
>2	63	21 (33.3)	1.63 (0.85–3.11)	0.139
Gravity				
0	118	25 (21.2)	1	-
1–2	208	63 (30.3)	1.62 (0.95–2.75)	0.077
>2	95	30 (31.6)	1.72 (0.93–3.19)	0.087
Regular cycle				
Yes	254	65 (25.6)	1	-
No/unknown	167	54 (33.3)	1.42 (0.92–2.18)	0.111
Menstrual cycle				
No cycle	194	58 (29.9)	1	-
With cycle	227	60 (26.4)	0.84 (0.55–1.29)	0.430
Contraceptive				
None	72	20 (27.8)	1	-
Condom only	106	28 (26.4)	0.93 (0.48–1.83)	0.841
Others (hormones/IUD/ sterilisation/pregnant)	243	70 (28.8)	1.05 (0.59–1.89)	0.865
Currently breastfeeding				
No	387	106 (27.4)	1	-
Yes	34	12 (35.3)	1.45 (0.69–3.03)	0.327
Sexual behaviour				
Age at first sexual encounter (years)				
<16	79	29 (36.7)	1	-
16–18	197	52 (26.4)	0.62 (0.35–1.08)	0.090
19–21	103	26 (25.2)	0.58 (0.31–1.10)	0.096
>21	42	11 (26.2)	0.61 (0.27–1.40)	0.224

Sexually active (last 3 months)				
No	55	16 (29.1)	1	-
Yes	366	102 (27.9)	0.94 (0.50–1.76)	0.851
Condom use (at last sexual encounter)				
No	283	68 (24.0)	1.80 (1.16–2.79)	**0.009**
Yes	138	50 (36.2)	1	-
Lifetime n° of sex partners				
1	110	28 (25.5)	1	-
2–3	185	42 (22.7)	0.86 (0.50–1.49)	0.591
> 3	126	48 (38.1)	1.80 (1.03–3.15)	**0.039**
N° of sex partners in the last 3 months				
0	25	6 (24.0)	1	-
>=1	396	112 (28.3)	1.25 (0.49–3.21)	0.644
Recent vaginal sex[%]				
No	340	91 (26.8)	1	-
Yes	81	27 (33.3)	1.37 (0.81–2.30)	0.238
Sexual risk taking[€]				
Low	167	44 (26.3)	1	-
Medium	152	37 (22.4)	0.90 (0.54–1.49)	0.681
High	102	37 (36.3)	1.59 (0.94–2.71)	0.086
Estimated frequency of sexual encounters in last 3 months[@,&]				
0	55	16 (29.1)	1	-
< 10 times	135	31 (23.0)	0.73 (0.36–1.47)	0.376
11–30 times	128	43 (33.6)	1.23 (0.62–2.45)	0.550
> 30 times	98	27 (27.6)	0.93 (0.45–1.93)	0.839
HIV status partner[@]				
HIV positive	38	11 (28.9)	1	-
HIV negative	247	63 (25.5)	0.84 (0.39–1.79)	0.653
Unknown	79	28 (35.4)	1.35 (0.58–3.12)	0.486

Estimated frequency of unprotected sex in last 3 months				
No sexual contacts	55	16 (29.1)	1	-
Never unprotected	102	34 (33.3)	1.22 (0.60–2.49)	0.586
< 10 times	87	19 (21.8)	0.68 (0.31–1.48)	0.330
>= 10 times	177	49 (27.7)	0.93 (0.48–1.82)	0.839
New partner (within 3 months)				
No	375	96 (25.6)	1	-
Yes	46	22 (47.8)	2,66 (1.43–4.97)	**0.002**
Circumcision status partner@				
Circumcised	238	62 (26.1)	1	-
Not circumcised/don't know	128	40 (31.3)	1.29 (0.80–2.07)	0.291
Female sex worker				
No	391	97 (24.8)	1	-
Yes	30	21 (70.0)	7.07 (3.13–15.96)	**<0.001**
Vaginal practices				
Washing inside the vagina when bathing				
No	175	44 (25.1)	1	-
Yes	246	74 (30.1)	1.28 (0.83–1.98)	0.267
Drying the vagina before sex				
No	411	114 (27.3)	1	-
Yes	10	4 (40.0)	1.74 (0.48–6.27)	0.399
Washed inside the vagina recently (morning or evening before study visit)				
No	229	64 (27.9)	1	-
Yes	192	54 (28.1)	1.01 (0.66–1.55)	0.968
Products to wash/clean/dry/tighten the vagina				
None	153	40 (26.1)	1	-
Water/fingers only or water/ soap	210	59 (28.1)	1.10 (0.69–1.77)	0.680
Cloth	46	16 (34.8)	1.51 (0.74–3.05)	0.255
Lemon juice/detergents	12	3 (25.0)	0.94 (0.24–3.65)	0.931
Cleaning the vagina after sexual intercourse				
No	226	61 (27.0)	1	-
Yes	195	57 (29.2)	1.12 (0.73–1.71)	0.610

Cervicovaginal signs and symptoms				
Ectopy[¶]				
No	224	49 (21.9)	1	-
Yes	196	69 (35.2)	1.94 (1.26–2.99)	**0.003**
Degree of ectopy[¶]				
Absent	224	49 (21.9)	1	-
Small	52	22 (42.3)	2.62 (1.39–4.94)	**0.003**
Moderate	139	46 (33.1)	1.77 (1.10–2.84)	**0.019**
Large	5	1 (20.0)	0.89 (0.10–8.17)	0.920
Colposcopic findings[§,¶]				
No	377	102 (27.1)	1	-
Yes	43	16 (37.2)	1.60 (0.83–3.09)	0.163
Cervical mucus				
No	269	67 (24.9)	1	-
Mild to moderate	138	47 (34.1)	1.56 (1.00–2.44)	0.052
Abundant	14	4 (28.6)	1.21 (0.37–3.97)	0.758
Reported abnormal discharge				
No	396	112 (28.3)	1	-
Yes	25	6 (24.0)	0.80 (0.31–2.06)	0.644
Vaginal discharge on speculum				
No	331	85 (25.7)	1	-
Yes	90	33 (36.7)	1.68 (1.02–2.75)	**0.041**
Vaginal epithelial abnormalities				
No	416	116 (27.9)	1	-
Yes	5	2 (40.0)	1.72 (0.28–10.45)	0.554
Cervical epithelial abnormalities				
No	377	105 (27.9)	1	-
Yes	44	13 (29.5)	1.09 (0.55–2.16)	0.813
Red blood cells in urine				
No	355	101 (28.5)	1	-
Yes	66	17 (25.8)	0.87 (0.48–1.59)	0.655

White blood cells in urine				
No	325	81 (24.9)	1	-
Yes	96	37 (38.5)	1.89 (1.17–3.06)	**0.010**
Microbiological factors				
BV visit 1 (Amsel criteria)				
No BV	344	99 (28.8)	1	-
BV	77	19 (24.7)	0.81 (0.46–1.43)	0.469
BV visit 1 (Nugent) [¶]				
No BV (Nugent 0–3)	217	60 (27.6)	1	-
Intermediate (Nugent 4–6)	29	15 (51.7)	2.80 (1.28–6.16)	**0.010**
BV (Nugent 7–10)	137	33 (24.1)	0.83 (0.51–1.36)	0.459
GBS				
No	354	89 (25.1)	1	-
Yes	67	29 (43.3)	2.27 (1.33–3.90)	**0.003**
Reproductive tract infection (RTI)				
No RTI	349	96 (27.5)	1	-
1 or more RTI	60	19 (31.7)	1.22 (0.68–2.21)	0.508
>1 RTI	12	3 (25.0)	0.88 (0.23–3.31)	0.848
Syphilis				
No	412	117 (28.4)	1	-
Yes	9	1 (11.1)	0.32 (0.04–2.55)	0.279
Chlamydia trachomatis				
No	379	107 (28.2)	1	-
Yes	42	11 (26.2)	0.90 (0.44–1.86)	0.780
Neisseria gonorrhoeae				
No	412	114 (27.7)	1	-
Yes	9	4 (44.4)	2.09 (0.55–7.93)	0.278
Trichomonas vaginalis[¶]				
No	387	105 (27.1)	1	-
Yes	26	11 (42.3)	1.97 (0.88–4.43)	0.101

***Candida albicans* (qPCR)**				
No	375	102 (27.2)	1	-
Yes	46	16 (34.8)	1.43 (0.75–2.73)	0.282
HSV-2 serology[¶]				
No	274	80 (29.2)	1	-
Yes	146	38 (26.0)	0.85 (0.54–1.34)	0.492
Vaginal pH				
< 4.4	125	34 (27.2)	1	-
4.4 – 5.3	237	65 (27.4)	1.01 (0.62–1.65)	0.963
5.4 and more	58	19 (32.8)	1.30 (0.66–2.56)	0.441
PSA present				
No	231	69 (29.9)	1	-
Yes	180	48 (26.7)	0.85 (0.55–1.32)	0.475
Systemic antibiotics visit 1				
No	338	98 (29.0)	1	-
Yes	83	20 (24.0)	0.78 (0.45–2.36)	0.374
Systemic antibiotics screening visit				
No	388	111 (28.6)	1	-
Yes	33	7 (21.2)	0.67 (0.28–1.59)	0.366

*Bold: significant at the 5% level; [£]completed secondary school, or post-secondary school;
[££]Primary school (completed or not), secondary school but not completed;
[#]Socio-economic-status was constructed from total income, type of housing, type of toilet;
[@]with partners within three months prior to enrolment;
[ß]missing data for 5; [%]sex morning or evening before visit;
[€]low risk: 1 or no partners in last year and did not have any partner (in the last 3 months) with multiple partners and age first sex at least 15 years; medium risk: 2 partners last year or had at least one sexual partner (in the last 3 months) who had multiple partners; high risk: sex worker or at least 3 partners last year or at had at least one sexual partner with HIV in the last 3 months or age first sex less than 15 years; N/A, no odds ratio due to no cases in one category;
[¶]data missing for 1 (ectopy, colposcopic findings, HSV-2 serology), 8 (*T. vaginalis*), 38 (BV Nugent, unreadable slides); §petechiae (6 *E. coli* cases/20), abrasion (2 *E. coli* cases/5), erythema (3 *E. coli* cases/10), laceration (2 *E. coli* cases/4), ulcer (2 E. coli cases/6), ecchymosis (2 *E. coli* cases/6); BV, bacterial vaginosis.

In our final multivariable GBS model (Table 6), BV by Nugent score remained significantly negatively associated with GBS carriage (AOR, 0.43; 95% CI, 0.21–0.88; p=0.022), and a positive association was observed for vaginal *Candida albicans* carriage (AOR, 3.25; 95% CI, 1.50–7.06; p=0.003), vaginal *E. coli* carriage (AOR, 2.01; 95% CI, 1.10–3.80; p=0.023), recent vaginal intercourse (AOR, 2.63; 95% CI, 1.35–5.15; p=0.005), and currently washing the vagina (AOR, 2.26; 95% CI, 1.16–4.37; p=0.016).

TABLE 6 Multivariable associations with vaginal GBS carriage.

	n	GBS+ (%)	adjusted OR (95% CI)	p-value[$]
	424	69 (16.3)		
Recent vaginal sex[%]				
No	343	49 (14.3)	1	-
Yes	81	20 (24.7)	2.63 (1.35–5.15)	**0.005**
Washing inside the vagina[#]				
No	176	15 (8.5)	1	-
Yes	248	54 (21.8)	2.26 (1.16–4.37)	**0.016**
BV (Nugent)[¶]				
No BV (Nugent 0–3)	217	37 (17.1)	1	-
Intermediate (Nugent 4–6)	29	6 (20.7)	0.93 (0.33–2.64)	0.898
BV (Nugent 7–10)	137	13 (9.5)	0.43 (0.21–0.88)	**0.022**
***Candida albicans* (qPCR)[¶]**				
No	375	50 (13.3)	1	-
Yes	46	17 (37.0)	3.25 (1.50–7.06)	**0.003**
***Escherichia coli*[¶]**				
No	303	38 (12.5)	1	-
Yes	118	29 (24.6)	2.01 (1.10–3.80)	**0.023**

[$]bold, significant at the 5% level;

[#]when having shower or bath;

[%]morning or evening before study visit;

[¶]data missing for 3 (*C. albicans*, *E. coli*), 41 (BV, unreadable slides).

In our multivariable *E. coli* model, an intermediate Nugent score remained significantly negatively associated with vaginal *E. coli* carriage (AOR, 2.61; 95% CI, 1.15–5.94; p=0.023), and a positive association was observed with working as a FSW (AOR, 7.83; 95% CI, 2.88–21.30; p<0.001), vaginal GBS carriage (AOR, 2.05; 95% CI, 1.09–3.83; p=0.025), and cervical ectopy (AOR, 1.64; 95% CI, 1.01–2.68; p=0.046) (Table 7).

TABLE 7 Multivariable associations with vaginal *E. coli* carriage.

	n	*E. coli* + (%)	adjusted OR (95% CI)	p-value[$]
	421	118 (25.2)		
Condom use (at last sexual encounter)				
No	283	68 (24.0)	1.53 (0.92–2.56)	0.104
Yes	138	50 (36.2)	1.0	-
Female sex worker				
No	391	97 (24.8)	1	-
Yes	30	21 (70.0)	7.83 (2.88–21.30)	**<0.001**
Ectopy[¶]				
No	224	49 (21.9)	1	-
Yes	196	69 (35.2)	1.64 (1.01–2.68)	**0.046**
Vaginal discharge on speculum				
No	331	85 (25.7)	1	-
Yes	90	33 (36.7)	1.63 (0.92–2.88)	0.095
BV (Nugent)[¶]				
No BV (Nugent 0–3)	217	60 (27.6)	1	-
Intermediate (Nugent 4–6)	29	15 (51.7)	2.61 (1.15–5.94)	**0.023**
BV (Nugent 7–10)	137	33 (24.1)	0.66 (0.38–1.15)	0.140
GBS				
No	354	89 (25.1)	1	-
Yes	67	29 (43.3)	2.05 (1.09–3.83)	**0.025**

[$]bold, significant at the 5% level;

[¶]data missing for 1 (ectopy), 38 (BV, unreadable slides).

7.5 Discussion

Group B streptococci (GBS) and *E. coli* account for the majority of EOS cases worldwide. Vaginal carriage of GBS and *E. coli* is considered a prerequisite for GBS or *E. coli* transmission to the neonate in GBS EOS and *E. coli* EOS, respectively. However, epidemiological data of vaginal GBS and *E. coli* carriage, which are essential for the development and implementation of prevention strategies, are very limited in sub-Saharan Africa (SSA) [Capan *et al.*, 2012; Stoll and Schuchat 1998]. In this study, we aimed to present vaginal GBS and *E. coli* carriage rates, GBS serotype distribution and define risk factors for carriage in populations from three SSA countries.

Vaginal GBS and *E. coli* carriage rates

We found a vaginal GBS carriage rate of 20.2% and 23.2% in the Kenyan and South African reference groups (adult, non-pregant, HIV-negative women at average risk of HIV), respectively. Compared to these reference groups, adolescents in our study were found to have lower GBS carriage rates: 3.6% of the Kenyan and 0% of the SA adolescents carried GBS vaginally. Other studies report conflicting associations between age and vaginal GBS carriage [Kim *et al.*, 2011; Regan *et al.*, 1991; Hastings *et al.*, 1986]. All of these studies (except for [Valkenburg-van den Berg *et al.*, 2006]) report on pregnant women. Interestingly, when we compare different age groups (< 18 years, 18–24 years, > 24 years) in our Kenyan and SA population, we see no age-group dependent GBS colonization in the pregnant women. However, we do see a statistically significant age-group dependent GBS association in the non-pregnant women, with the lowest and the highest GBS carrier rates in the youngest and the oldest age groups, respectively (Pearson Chi-Square test, data not shown).

The pregnant women in our study population had vaginal GBS carriage rates of 14.3% and 10.0% in Kenya and SA, respectively. This is lower than most other studies reporting (recto)vaginal GBS carriage rates in SSA (see Table 8). Although the CDC recommends rectovaginal sampling for detection of GBS in pregnant women, we only swabbed vaginally, to be able to study the interaction of GBS with the vaginal immune system and vaginal microbiome (to be published). This vaginal sampling may (partly) explain the lower GBS carriage rates found by us, as rectovaginal sampling has been shown to yield higher GBS recovery rates compared to vaginal sampling alone [Gupta *et al.*, 2004; El Aila *et*

al., 2010]. Furthermore, in contrast to other studies (listed in Table 8) using culturing techniques, we used qPCR without prior enrichment step to detect GBS. Although the CDC allows PCR for the detection of GBS (albeit recommending an enrichment step), this difference with other studies probably does not account (or to a lesser extent) for the lower rates found by us, as PCR (even without an enrichment step) has been shown to be more sensitive than culture [Verani *et al.*, 2010; El Aila *et al.*, 2011; Davies *et al.*, 2004; Convert *et al.*, 2005; Rallu *et al.*, 2006]. A further difference with other studies regards the fact that the pregnant women in our study were up to 14 weeks of gestation, while the other studies listed in Table 7 sampled pregnant women at 35–37 weeks of gestation, which also may account for differences, as some authors have reported on varying GBS rates during pregnancy [Baker *et al.*, 1975].

In the group of HIV positive women, we did not observe any GBS carriers, which is probably explained by the fact that most of the HIV positive women (26/30) received prophylactic cotrimoxazole, which is largely effective against GBS [Joachim *et al.*, 2009].

TABLE 8 Studies reporting (recto)vaginal GBS carriage rates in SSA.

Country	Year	n	Population	% GBS	Sample	Detection	Reference
Nigeria	1980	588	P, L	19	V	SB+C	[Onile 1980]
Nigeria	1983	225	P	20	V	SB+C	[Dawodu *et al.*, 1983]
Zimbabwe	1990	89	P	31	V	SB+C	[Nathoo *et al.*, 1990]
Togo	1991	106	P	4	V, R	SB+C	[David-Prince *et al.*, 1991]
Gambia	1994	136	P	22	V, R	SB+C	[Suara *et al.*, 1994]
Malawi	2005	97	P	16.5	V, R	SA	[Dzowela *et al.*, 2005]
Mozambique	2008	113	P	1.8	V, R	SB+C	[de Steenwinkel *et al.*, 2008]
Tanzania	2009	300	P	23.0	V, R	SB+C	[Joachim *et al.*, 2009]

Zimbabwe	2010	780	P	47, 24, 21[#]	V, R	SB+C	[Mavenyengwa *et al.*, 2010]
Malawi	2011	1840	P, HIV+ and HIV-	21.2	V, R	SB+C	[Gray *et al.*, 2011]
South Africa	2014	661, 621, 595, 521$	P	33.0, 32.7, 28.7, 28.4[$]	V, R	SA	[Kwatra *et al.*, 2014]
DR Congo	2015	509	P	20.2	V	SA	[Mitima *et al.*, 2014]

L, women in labor; NP, non-pregnant women; P, pregnant women; V, vaginal swab; R, rectal swab; SB+C, selective broth and culturing; SA, selective agar; [#]week 20, 26, and delivery, respectively; [$]week 20–25, week 26–30, week 31–35, and week 37+, respectively.

Our reference groups from Kenya and SA had vaginal *E. coli* carriage rates of 25.0% and 27.1%, respectively. Compared to other studies from SSA reporting vaginal carriage of *E. coli*, these prevalences are higher than the ones reported by Karou and coworkers (2012) and Ekwempu and coworkers (1981), lower than the ones reported by Schellenberg and coworkers (2011) and Cutland and coworkers (2012), and comparable with the prevalence reported by Sagna and coworkers (2010) (See Table 9) [Karou *et al.*, 2012; Ekwempu *et al.*, 1981; Schellenberg *et al.*, 2011; Cutland *et al.*, 2012; Sagna *et al.*, 2010]. Vaginal *E. coli* carriage rates in Asia, Europe, North and South America appear lower. Different study populations, sampling and detection techniques might account for these differences.

Compared to the reference group in Kenya, pregnant women had a lower prevalence of vaginal *E. coli* carriage; compared to the reference group in SA, adolescent women had a lower prevalence of *E. coli* carriage. The FSW study group in Kigali had a very high prevalence of *E. coli* carriage, i.e. 70%, and will be discussed below (risk factors).

TABLE 9 **Studies reporting (recto)vaginal *E. coli* carriage rates**.

Country	Year	n	Population	% E. coli	Sample	Detection	Reference
Africa **(pooled prevalence 36.0% (2846/7912); range 9.1–46.5%)**							
Burkina Faso	2010	156	HIV+	28.4	V	C	[Sagna *et al.*, 2010]
Burkina Faso	2012	2000	S	16.7	V	C	[Karou *et al.*, 2012]
Kenya	2011	44	HIV+, HIV-, HESN	40.1	V	cpn60	[Schellenberg *et al.*, 2011]
Nigeria	1981	187	L	9.1	C	C	[Ekwempu *et al.*, 1981]
SA	2012	1347	P, HIV+	42.3	V	C	[Cutland *et al.*, 2012]
SA	2012	3752	P, HIV-	46.5	V	C	[Cutland *et al.*, 2012]
Asia **(pooled prevalence 5.3% (163/3072); range 0–25.8%)**							
Iraq	2011	90	S, NP	16.2	V	C	[Razzak *et al.*, 2011]
Iraq	2011	20	S, P	25.8	V	C	[Razzak *et al.*, 2011]
Iran	2014	85	S, P	18.0	V	C	[Mobasheri *et al.*, 2014]
Japan	2002	2575	NP, P	3.4	V	C	[Obata-Yasuoka *et al.*, 2002]
Pakistan	2012	100	HC	28	V	C	[Kazi *et al.*, 2012]
Pakistan	2012	100	H	6	V	C	[Kazi *et al.*, 2012]
Turkey	2007	34	IUD	14.7	V	C	[Ocak *et al.*, 2007]
Turkey	2007	34	HC	2.9	V	C	[Ocak *et al.*, 2007]
Turkey	2007	34	H	0.0	V	C	[Ocak *et al.*, 2007]

Europe **(pooled prevalence 13.4% (670/4980); range 3.1–51.2%)**							
Croatia	2011	114	IUD	25.5	V	C	[Kaliterna *et al.*, 2011]
Croatia	2011	122	H	8.2	V	C	[Kaliterna *et al.*, 2011]
Denmark	2014	668	P	11.7	V	C	[Stokholm *et al.*, 2014]
Germany	2007	166	H	16.3, 51.2, 25.9*	V	C	[Chase *et al.*, 2007]
Greece	2008	1632	S	3.1	V	C	[Iavazzo *et al.*, 2008]
Lithuania	2012	970	P	19.9	V, R	C	[Tameliene *et al.*, 2012]
Spain	2002	623	P	27.0	V	C	[Bayo *et al.*, 2002]
Spain	2011	321	P	15	E, V	C	[Guiral *et al.*, 2011]
Spain	2011	327	NP	12	E, V	C	[Guiral *et al.*, 2011]
Sweden	2008	37	H	5.4	V	C	[Nikolaitchouk *et al.*, 2008]
North America **(pooled prevalence 12.7% (430/3373); range 0–29.5%)**							
Canada	1983	495	H	12.3	V	C	[Percival-Smith *et al.*, 1983]
US	1997	2646	P	13.0	V	C	[Krohn *et al.*, 1997]
US	2001	44	H	18.2, 9.1, 29.5, 6.8, 6.8 #	V	C	[Eschenbach *et al.*, 2001]
US	2005	20	H	0	V	16S	[Hyman *et al.*, 2005]
US	2012	70	P	10	V	C	[Ghartey *et al.*, 2012]
US	2012	35	NP	23	V	C	[Ghartey *et al.*, 2012]

US	2013	47	P	2.1, 2.2, 5.6, 8.3[£]	V	C	[Anderson *et al.*, 2013]
US	2013	16	NP	6.3, 0, 12.5, 20	V	C	[Anderson *et al.*, 2013]
South America **(pooled prevalence 19.7% (135/684); range 14.3–23.0%)**							
Argentina	2013	259	P	14.3	V	C	[Villar *et al.*, 2013]
Chile	2009	425	S	23.0	V	C	[Lobos *et al.*, 2009]

The electronic bibliographic database PubMed was searched for articles using the search terms '(Escherichia) AND (coli) AND (vaginal)' with no date or language restriction. Studies were included if the number of vaginal *E. coli* carriers and the total number of individuals tested were reported; studies were excluded if women were not of childbearing age.
16S, deep sequencing of the 16S rRNA gene; cpn60, deep sequencing of the cpn60 gene;
C, conventional culturing and identification; E, endocervial swab;
FSW, female sex workers; H, healthy women; HC, women using hormonal contraception;
HESN, HIV exposed seronegative women; IUD, women using an intrauterine device as contraception; L, women in labor; NP, non-pregnant women; P, pregnant women; Q, qPCR;
R, rectal swab; S, women with vaginal symptoms or clinical diagnosis of infection;
V, vaginal swab; *pre, mid, post cycle, respectively;
visit 1at 1 month before visit 2 and 19–24 days after cycle, 1–2 days before intercourse, 8–12h after intercourse, 3–4 days after intercourse, 5–6 days after intercourse;
£ <14weeks, between 14–28 weeks, >28weeks, postpartum.

To our knowledge, this is the first study to determine simultaneously the vaginal GBS and *E. coli* carriage rates in SSA populations using qPCR, known to be more sensitive than culture-based techniques. Moreover, vaginal carriage rates of GBS in Kenya and Rwanda and *E. coli* in Rwanda have not yet been described.

Risk factors for vaginal *E. coli* and GBS carriage

The presence of vaginal *C. albicans*, recent vaginal intercourse, working as a FSW, an intermediate vaginal microbiome, BV, washing the vagina and cervical ectopy were independent risk factors for vaginal GBS or *E. coli* colonization.

Women carrying *C. albicans* were 3.6 times more likely also to carry GBS. Three US based studies have shown this same significantly positive association between GBS and *Candida* or yeast [Regan *et al.*, 1991; Cotch *et al.*, 1998; Beigi *et al.*, 2004].

Intravaginal practices, like e.g. cleaning inside the vagina beyond the introitus or insertion of substances into the vagina to dry or tighten the vagina, are common in Africa and are associated with adverse outcomes including increased risk for BV and for sexually transmitted infections [Low *et al.*, 2011]. Our model showed that washing inside the vagina was an independent risk factor for vaginal GBS carriage: women were twice as likely to be colonized with GBS compared to women not washing inside the vagina. A study by van de Wijgert and coworkers [van de Wijgert *et al.*, 2000] showed that women using substances other than plain water to finger-clean or wipe inside the vagina had a GBS prevalence of 26.3% (n=99), whereas women not engaging in these practices had a GBS prevalence of 14.7% (n=70). However, their findings did not reach significance, most probably because of the smaller sample size (169 women compared to 424 women in our study).

Women who had recent vaginal sex (the morning or evening before the study visit) were more than twice as likely to carry GBS vaginally than women who did not. Accordingly, a positive PSA test was also significantly correlated with GBS carriage. GBS is generally not considered an STI, and the influence of sexual behavior on vaginal GBS carriage or acquisition is a matter of debate [Foxman *et al.*, 2008; Foxman *et al.*, 2007; Honig *et al.*, 2002]. Based on published literature and our own data, we hypothesize that sexual activity might lead to a brief temporal GBS colonization of the vagina. This hypothesis is strengthened by a recent longitudinal deep-sequencing study of the vaginal microbiome, where 25 women were sampled on a daily basis over a 10 week period, revealing an average of 0.39 GBS episodes per week and an average GBS episode of 2.8 days (Fig 1 and additional file 4 in [Ravel *et al.*, 2013]), contrasting with earlier studies – where sampling occurred every 3 weeks – that report average GBS episodes of 13.7 weeks [Foxman *et al.*, 2006]. The brief colonization might explain why we and other authors find parameters such as 'age of first sexual intercourse' not to be associated with GBS carriage (they do not cover the recent aspect), while parameters such as 'high frequency of intercourse during last month' (as a consequence, a higher chance of also having had recent intercourse) do correlate. Taken together, GBS should be considered as a potentially pathogenic micro-organism that can be sexually transmitted and whose vaginal presence can be enhanced by sexual activity.

Cervical ectopy was an independent risk factor for vaginal *E. coli* carriage, 21.9% of women without cervical ectopy were *E. coli* carriers as opposed to 35.2% of women with cervical ectopy. Cervical ectopy has been associated with CT [Lee *et al.*, 2006], HPV [Rocha-Zavaleta *et al.*, 2004] and an increased

susceptibility to HIV infection [Venkatesh *et al.*, 2013]. Although we could not determine the cause-effect relation of this association, it seems biologically plausible that a niche is created by the glandular columnar epithelium of women with cervical ectopy that somehow – directly or indirectly – favors the colonization by *E. coli*. Some studies have e.g. related cervical ectopy with a reduced cell-mediated or changed humoral immunity [Kyongo *et al.*, 2015; De Luca Brunori *et al.*, 1994].

Working as a FSW was an independent risk factor for vaginal *E. coli* carriage. We could not explain this by any of the sexual behavioural or other parameters presented in Table 5. In our study, none of the participants, including the female sex workers, reported having had anal intercourse during the last 3 months. These percentages probably are underestimates, since stigma associated with anal intercourse often leads to reduced reporting [Veldhuijzen *et al.*, 2011]. Furthermore, Ghanem and coworkers [Ghanem *et al.*, 2005] showed that regarding anal intercourse, significantly more women reported to engage in these practices when asked by means of computer assisted self-interviews compared to face-to-face interviews, which was used in our study. It is not unlikely that FSW engage more in anal intercourse, compared to the general population, and that in our FSW population, vaginal contamination with *E. coli* is higher by transfer of (peri)anal bacteria during anal/vaginal intercourse. Other studies from East-Africa report anal intercourse prevalences in FSW of up to 40.8% [Schwandt *et al.*, 2006]. Interestingly, anal intercourse during pregnancy has been reported as a significant risk factor for neonatal *E. coli* colonization [Tameliene *et al.*, 2012].

Besides above-mentioned risk factors for GBS or *E. coli* carriage, we show for the first time that colonization with GBS and *E. coli*, the leading causes of EOS, are positively associated.

Above-mentioned risk factors can be translated and implemented into strategies that aim to reduce the maternal carriage of GBS and *E. coli*. First, behavioral change by advocating abstinence from sexual intercourse and vaginal washing during late pregnancy, e.g. via counseling in family planning facilities, could help to reduce the risk of maternal GBS and *E. coli* colonization in resource-poor settings. Second, the extension of GBS screening with screening for *C. albicans* and *E. coli* – risk factors for vaginal GBS carriage – should be further investigated. Furthermore, the screening for *E. coli* itself also merits further investigation because of its role as a major EOS causative agent for which currently no prevention measures are taken, nor in low-income, nor in high-income countries [Stoll *et al.*, 2011]. In this context, the presence of cervical ectopy – a risk factor for vaginal *E. coli* carriage – should be further investigated.

GBS serotype distribution

As IAP is not effective against LOS and culture-based screening and administration of costly intravenous antibiotics might not be feasible in most low-income countries, an alternative and long-term solution lies in the development of effective GBS vaccines, that however would not cover for other micro-organisms causing EOS. As most GBS vaccines under development aim at eliciting protective antibodies against capsular polysaccharides, the principal difficulty in developing globally effective GBS vaccines is the existence of several serotypes with different geographical distributions. In our study, the most prevalent GBS serotypes were Ia (27.3%), V (27.3%) and III (22.7%) in Kenya; Ia (34.5%), V (31.0%), and IV (13.8%) in SA; and Ia (83.3%) and II (16.7%) in Rwanda. Only few other studies have documented vaginal GBS serotype distribution in Africa and these are largely in line with our findings (Table 3 and Fig 7).

Interestingly, compared to the low prevalences in Europe and the US, we found relatively high prevalences of serotypes IV, VI, VII – shown for the first time in sub-Saharan Africa – and VIII in the Kenyan and South African population (VI, 13.6%; VII, 4.5%; VIII 4.5% in Kenya, and IV, 13.8%; VI, 6.9%; VIII, 3.4% in SA) (Table 4). In contrast, we did not detect any serotype Ib, which has, according to a recent meta-analysis, a prevalence of 8.1% in the US and 12.4% in Europe [Ippolito *et al.*, 2010].

Differences in serotype distribution between our study and other studies (Table 3) might be explained by differences in study populations, most studies listed in Table 3 typed strains isolated from pregnant women whereas most of our GBS strains were isolated from non-pregnant women. Methodological differences might also contribute. Many GBS capsular polysaccharide typing methods have been described, with the most commonly used method being a serological test, used by all studies listed in Table 3, except our study. We used a molecular capsular typing method, developed and applied by European (reference) laboratories [Joubrel *et al.*, 2015; Afshar *et al.*, 2011]. Brigtsen and coworkers (2015) compared capsular typing of 426 GBS strains by a conventional latex agglutination test with PCR, and found that a substantial proportion of the strains were non-typeable by serotyping, but typeable by genotyping, and that an agreement between serotyping and genotyping was shown in 71.1% (of the isolates that were typeable by both methods) [Brigtsen *et al.*, 2015]. Moreover, we used a molecular technique directly on DNA extracts from vaginal swabs (and not on DNA extracts from isolates), which could lead to the detection of certain serotypes that would not have been isolated by culture.

Currently, there are two candidate vaccines in phase II clinical trials, i.e. a trivalent vaccine targeting serotypes Ia, Ib, and III, and a conjugate vaccine targeting serotype III (www.clinicaltrials.gov). In theory, the first vaccine could cover for 50.0%, 83.3% and 44.8% of vaginal GBS cases in our Mombasa, Kigali and Johannesburg population, respectively, but only a minority of women would be protected by the conjugate vaccine (22.7%, 0% and 10.3%, respectively).

GBS, *E. coli* and the vaginal microbiome

Our results show that vaginal GBS and *E. coli* carriage were significantly associated with disturbances of the vaginal microbiome: compared to women with a normal vaginal microbiome, women with an intermediate vaginal microbiome were 2.61 times more likely to carry *E. coli*, whereas women with BV were 2.33 less likely to carry GBS. The latter finding is in accordance a study of Hillier and coworkers [Hillier *et al.*, 1992], reporting a significant negative association between GBS carriage and BV by Nugent scoring, studying 7,918 pregnant women. Two other studies did not confirm these findings [Honig *et al.*, 2002; Rocchetti *et al.*, 2011]. In depth analysis of above-mentioned interdependencies of GBS, *E. coli*, *C. albicans*, BV and the vaginal microbiome will be published elsewhere.

Our study was limited by the fact that we used vaginal sampling instead of rectovaginal sampling for the detection of GBS (as recommended by the CDC), which has been shown to have higher recovery rates for GBS. Furthermore, we did not use a selective broth enrichment prior to PCR, as recommended by the CDC. In our study, pregnant women were up to 14 weeks of gestation, and were not sampled at 35–37 weeks' gestation as recommended by the CDC, which could have biased our results. Our study was further limited by the rather small sample size of our Rwanda study population.

In conclusion, vaginal GBS carriage rate and serotype distribution were similar to high-income countries, except for the higher prevalence of serotypes VI, VII and VIII. *E. coli* carriage rate was higher compared to high-income countries. We identified risk factors for GBS or *E. coli* carriage, ie. recent sexual intercourse, vaginal washing, *C. albicans* colonization and presence of cervical ectopy, that can be implemented in strategies to reduce maternal colonization. Immunoprophylaxis with current phase II candidate GBS vaccines would not protect the majority of women against vaginal GBS carriage in our study population. The most important causative agents of EOS, GBS and *E. coli*, both associated with disturbances of the vaginal microbiome, are positively associated.

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Role of HIV exposure and infection in relation to neonatal GBS disease and rectovaginal GBS carriage: a systematic review and meta-analysis

— CHAPTER 8

8.1 Introduction

One million children die each year in the first 4 weeks of life because of neonatal disease [Lawn *et al.*, 2005]. Early-onset neonatal disease (EOD), which occurs in the first week of life and most often within 24 hours after birth, is caused by bacteria that are transmitted from the genital tract of the mother before or during delivery [Hornik *et al.*, 2012]. Late-onset neonatal disease (LOD) occurs between the first week and the third month of life and may be caused by bacteria acquired vertically or horizontally [Schuchat 1998].

Streptococcus agalactiae (Group B *Streptococcus* (GBS)), present in the vagina and/or gastro-intestinal tract of 20 to 30 % of pregnant women, is the most common infectious agent identified in case of EOD [Stoll *et al.*, 2011] and an important pathogen in LOD [Dillon, Khare, and Gray 1987]. Although vaginal carriage of GBS usually remains asymptomatic, it has been associated with chorioamnionitis [Yancey *et al.*, 1994], spontaneous abortion [Temmerman *et al.*, 1992; McDonald and Chambers 2000], premature labor [Allen *et al.*, 1999], premature delivery [Regan *et al.*, 1996], premature rupture of membranes [Matorras *et al.*, 1989] and puerperal sepsis [Acosta *et al.*, 2014; Koenig and Keenan 2009], causing substantial neonatal and maternal morbidity and mortality [Beck *et al.*, 2010; Hornik *et al.*, 2012; Khan *et al.*, 2006; Lawn *et al.*, 2005].

Infants born to HIV-infected mothers have increased rates of infectious morbidity and mortality compared to non-exposed infants, even if they remain HIV-uninfected [Madhi *et al.*, 2011; Epalza *et al.*, 2010]. Since the first study reporting an increased risk of GBS neonatal disease in HIV-exposed neonates [Epalza *et al.*, 2010], evidence for this association has been accumulating.

A possible explanation for these observations might be higher rectovaginal GBS carriage rates in HIV-infected women, as rectovaginal GBS carriage is a major risk factor for GBS neonatal sepsis.

Indeed, HIV might influence the microbial composition of the vagina, rectum and colon [Spear *et al.*, 2008; Schellenberg *et al.*, 2011; Jespers *et al.*, 2015; McHardy *et al.*, 2013; Dillon *et al.*, 2014] and HIV infection in women has been associated with increased prevalence of vaginal candidiasis and sexually transmitted infections [Weiss *et al.*, 2001; Vandepitte *et al.*, 2012]. However, conflicting results have been reported for the association between HIV infection and rectovaginal carriage of GBS [Mitima *et al.*, 2014; El Beitune *et al.*, 2006].

The objectives of this systematic review and meta-analysis were to assess to which extent HIV exposure of neonates is associated with GBS neonatal disease and to clarify to which extent HIV infection of women is associated with rectovaginal GBS carriage, a major risk factor for GBS neonatal disease.

RESEARCH IN CONTEXT

Evidence before this study Mother-to-child transmission of HIV is one of the great tragedies of the HIV pandemic. Although the number of HIV-infected infants is declining due to interventions for the elimination of pediatric HIV infection, the number of newborns that are HIV-uninfected but HIV-exposed through their HIV-infected mothers is on the rise. Interest in the health outcomes of these HIV-exposed infants has grown in the past decade, and several studies suggest increased mortality rates and increased infectious morbidity.

Neonatal sepsis causes one million neonatal deaths yearly, and Group B *Streptococcus* (GBS) is the leading cause. Since 2010, evidence has also been accumulating for an association between HIV-uninfected neonates born to HIV-infected mothers and neonatal sepsis caused by GBS. Regarding the association between maternal vaginal GBS carriage – the single most important risk factor, if not, a prerequisite, for GBS neonatal sepsis – and HIV-infection in mothers, conflicting results have been reported.

Added value of this study This systematic review and meta-analysis is the first to examine the association between HIV-exposure of neonates and the risk for neonatal disease caused by GBS. We performed subgroup analysis to

further clarify the risk for the early-onset form of GBS neonatal sepsis, and the late-onset form. Furthermore, we performed a meta-analysis to assess the risk for maternal vaginal GBS carriage in HIV-infected compared to uninfected women. We present a framework the associations between neonatal HIV-exposure and early-and late-onset neonatal sepsis, offering opportunities for the clinical practice and global health policy to reduce HIV-related neonatal morbidity and mortality rates.

Implications of all the available evidence. Neonates born to HIV-infected mothers, but themselves uninfected, are at increased risk for GBS neonatal sepsis. Subgroup analysis showed that these neonates were four time more likely to have the GBS late-onset form, but were not at increased risk for the early-onset form. We found no association between maternal vaginal GBS carriage and HIV-infection, but these studies suffered from major limitations.

The health needs of HIV-exposed, uninfected infants should be prioritized further. Particularly in sub-Saharan Africa, were 90% of new HIV infections occurs and the burden of neonatal mortality is the highest, public health interventions reducing neonatal mortality rates are urgently needed, and could be found in improved nutrition strategies.

8.2 Methods

Protocol and registration We followed the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines [Moher *et al.*, 2009] for carrying out and reporting this systematic review and meta-analysis, using an *a priori* defined protocol. This systematic review and meta-analysis has been registered in PROSPERO under registration number CRD42016033414.

Eligibility criteria We included studies that assessed the association between neonatal GBS disease and HIV-status of the mother and studies that assessed the association between rectovaginal GBS colonization and HIV status in adult women. We only included studies that reported a sample size and a measure of effect for the associations assessed or provided the data to calculate the latter. There were no restrictions in terms of study design, manuscript language, or date of publication. We included papers that were published up to 1 November 2015.

Reviews, conference abstracts, comments, guidelines, case reports or case series, unpublished articles, multiple reports of the same data and *in vitro* and animal studies were not considered for inclusion. We excluded studies that had no clear description of the assessment of the HIV status of the women, of the HIV exposure of the neonates, or of the laboratory methodology to assess rectovaginal GBS carriage. GBS neonatal disease was defined as the presence of GBS as assessed by molecular methods, culture from blood, cerebrospinal fluid or another normally sterile site, or by latex agglutination in cerebrospinal fluid, in neonates below 90 days of age.

Search strategy We searched the MEDLINE (through PubMed), Web of Science, and EMBASE databases for bibliographic references. We used the combination of all of the following search terms (*Streptococcus agalactiae*, *S. agalactiae*, GBS, Group B *Streptococcus*, Group B streptococci, early-onset disease, early-onset sepsis, late-onset disease, late-onset sepsis, neonatal sepsis, neonatal septicemia, neonatal pneumonia or neonatal meningitis) with all of the following search terms (human immunodeficiency virus, HIV, HIV-1, or AIDS).

The bibliographic references of the studies that were considered eligible were also hand searched.

Study selection Studies were selected in a two-stage process (Figure 8). First, all bibliographic references were screened to identify studies for full text evaluation by one reviewer (PC), on the basis of only the information present in the title and abstract. Subsequently, the full texts of those articles not excluded in the screening process were assessed for eligibility, using the aforementioned criteria, by two authors independently (PC and MV). Reasons for inclusion or exclusion were recorded and categorized, and disagreements were resolved by consensus.

Selected studies were divided into two categories, i.e. studies reporting an association between HIV-exposure and GBS invasive neonatal disease in infants (or providing data to calculate this), and studies reporting an association between HIV infection and rectovaginal GBS carriage in adult women (or providing data to calculate this).

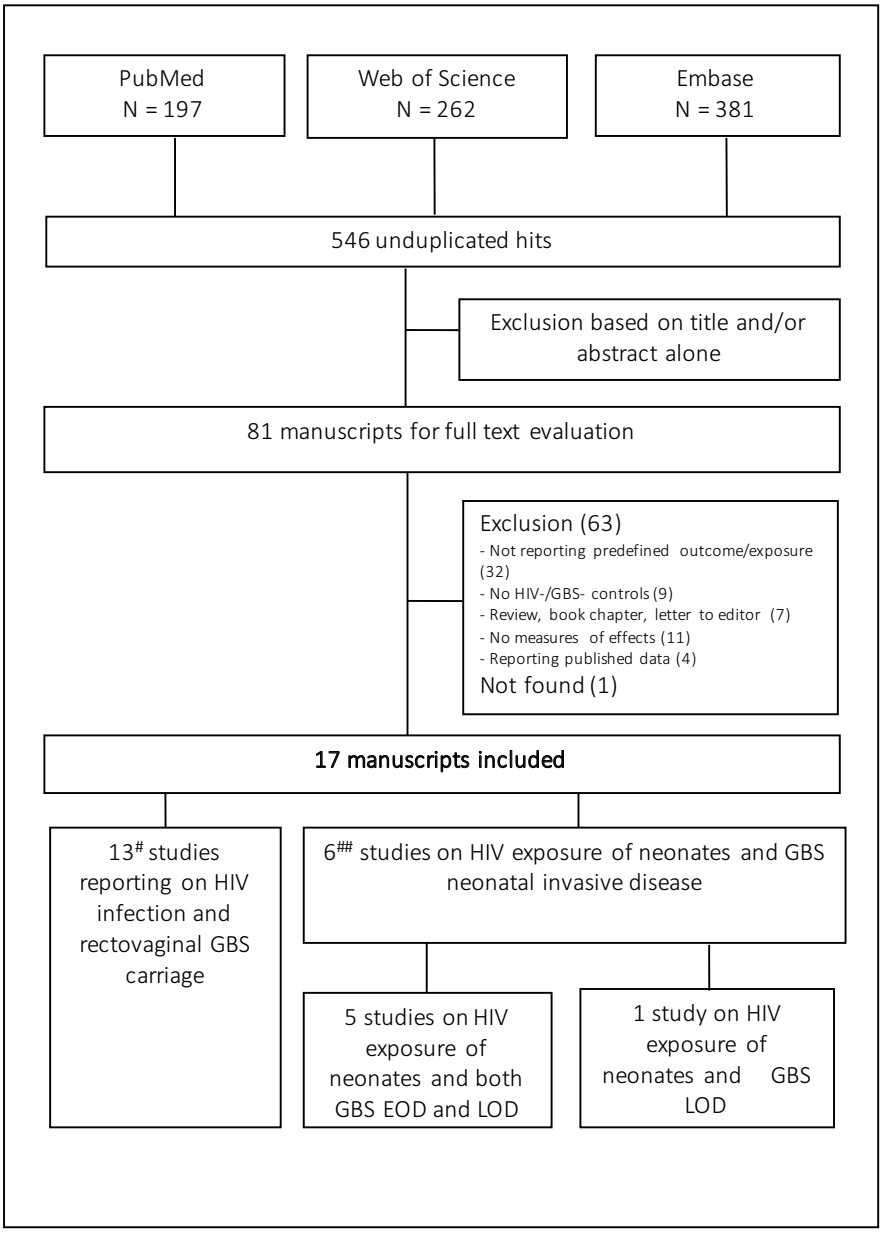

FIGURE 8 Identification of studies included in the meta-analysis.

#, including two studies reporting on neonatal GBS disease;

##, including two studies reporting on rectovaginal carriage.

Data collection process A predefined data extraction form was pilot-tested and used for data extraction. From each individual study, information was collected on authors, year of publication, journal, country where the study had been carried out, study design, sample collection period, study population, age of the participants, total number of study participants, total number of samples included in the analysis, HIV assessment and prevalence, GBS neonatal disease assessment and incidence, rectovaginal GBS carriage assessment and prevalence, relevant measures of effects, statistics, correction for confounding, and epidemiological strengths and weaknesses.

This procedure was performed for each article independently by two authors (PC, and TC, VJ, ES, JVDW or MV) and disagreements were resolved by discussion between both authors. Further information or clarification from manuscript authors was sought by PC on seven occasions.

Risk of bias in individual studies To assess the quality and risk of bias of the included studies, we developed a quality appraisal tool (Supplementary Table 1), based on the Newcastle-Ottawa Scale [GA Wells 2013]. This scale is a bias assessment tool for observational studies, recommended by the Cochrane Collaboration [Higgins *et al.*, 2011]. We adapted this tool to the content of the meta-analysis. For example, consideration was given to the methodology used to assess rectovaginal GBS carriage.

Stars were assigned by PC for three broad criteria (representativeness of the study groups, comparability of the study groups, and quality of the outcome assessment) in order to provide a measure of their quality. In this context, good representativeness is defined as those exposed/unexposed being similar to others in the community they come from, and good comparability is defined as the exposed and unexposed being similar in all respects other than their HIV or HIV exposure status.

Studies that scored none or one, two or three, or four stars for representativeness were classified as having respectively a high, medium or low risk of bias for this criterion. Studies assigned none, one, or two stars for comparability or outcome were considered to have respectively a high, medium or low risk of bias for comparability and outcome.

Data analysis First, an odds ratio (OR) was calculated for studies that did not present them. We compared the odds of neonatal GBS disease in neonates born to HIV-infected mothers versus infants born to HIV-uninfected mothers, and the odds of rectovaginal GBS carriage in HIV-positive versus HIV-negative women,

using unadjusted counts. All neonates born from HIV-positive women were considered as HIV-exposed, regardless of their infection status. Rectovaginal GBS carriage was defined as GBS detected in the vagina and/or rectum and/or (peri)anal region by culture. While our protocol also included detection of rectovaginal and neonatal GBS by molecular testing, none of the eligible studies used molecular testing, and the results reported in this manuscript are based on culture and latex agglutination results only.

We reported the results of the meta-analyses obtained after pooling individual study estimates with random effects model as ORs with 95% confidence interval (CI). The degree of heterogeneity between the studies was assessed using the I^2 index, with percentages of 30–50%, 50–75% and 75–100% being indicative of moderate, substantial and considerable heterogeneity, respectively [Higgins 2011].

A priori planned subanalyses were performed for GBS neonatal disease by considering early-onset and late-onset neonatal GBS disease separately, relying on the authors' own classification into these two groups.

To assess publication bias, funnel plots were created by plotting the natural logarithm of the ORs against the inverse of the standard error. The asymmetry of the funnel plots was visually inspected and statistically checked using Egger's regression test and Begg and Mazumdar's rank correlation test [Begg and Mazumdar 1994; Egger *et al.*, 1997]. All statistical analyses were carried out using the Comprehensive Meta-Analysis software package v3 (Biostat Inc., Englewood, NJ).

8.3 Results

Manuscript and study selection A total of 546 unduplicated citations were identified, we selected 81 abstracts for full text evaluation (Figure 8). Of these, 32 manuscripts did not report results on GBS disease or carriage or HIV exposure, seven were reviews, book chapters or letter to editors, nine did not include an HIV-negative or GBS-negative group, 11 did not present measures of effect or the data to calculate these, four reported already published data, and one paper could not be found.

Seventeen manuscripts reporting on 19 studies met the inclusion criteria. These included four studies reporting on neonatal GBS disease only, 11 studies reporting on rectovaginal carriage only, and two studies reporting both (Figure 8). A list of these studies and a digest of the extracted information is presented in the Supplementary Tables 2 and 3.

Description of selected studies Six studies reported cases of infants with GBS neonatal disease born to HIV-infected mothers compared to infants with GBS neonatal disease born to HIV-uninfected mothers [Dangor, Lala, *et al.*, 2015; Dangor, Kwatra, *et al.*, 2015; Cutland *et al.*, 2012; Cutland *et al.*, 2015; Epalza *et al.*, 2010; Frigati *et al.*, 2014]. Five of these studies were conducted in South Africa [Dangor, Lala, *et al.*, 2015; Dangor, Kwatra, *et al.*, 2015; Cutland *et al.*, 2012; Cutland *et al.*, 2015; Frigati *et al.*, 2014] and one was performed in Belgium [Epalza *et al.*, 2010]. All infants included were born in a hospital. For the laboratory diagnosis of GBS neonatal disease, all studies used the BacT/ALERT microbial detection system to detect bacteria in blood and/or cerebrospinal fluid samples. Positive samples were subcultured on blood agar plates and identification was subsequently performed using standard identification methods.

For the second category, thirteen studies, representing a total of 10.105 women, provided rectovaginal GBS carriage ratios of HIV-infected versus HIV-uninfected women [Cutland *et al.*, 2012; Dangor, Kwatra, *et al.*, 2015; Djigma *et al.*, 2011; El Beitune *et al.*, 2006; Ernest *et al.*, 2015; Gray *et al.*, 2011; Joachim *et al.*, 2009; Mavenyengwa, Moyo, and Nordbø 2010; Mitima *et al.*, 2014; Sebitloane, Moodley, and Esterhuizen 2011; Shah *et al.*, 2011; Temmerman *et al.*, 1992; Ulla *et al.*, 1993]. Eleven of these studies were conducted in low-or-middle income countries, i.e. three studies in South Africa [Cutland *et al.*, 2012; Dangor, Kwatra, *et al.*, 2015; Sebitloane, Moodley, and Esterhuizen 2011], two studies in Tanzania [Joachim *et al.*, 2009; Ernest *et al.*, 2015], and one study in Burkina Faso, Brazil, Democratic Republic Congo, Kenya, Malawi, and Zimbabwe [Djigma *et al.*, 2011; El Beitune *et al.*, 2006; Gray *et al.*, 2011; Mavenyengwa, Moyo, and Nordbø 2010; Mitima *et al.*, 2014; Temmerman *et al.*, 1992] and two in a high-income country namely Spain and the US [Shah *et al.*, 2011; Ulla *et al.*, 1993]. All these studies recruited pregnant women in a hospital setting, except for the study of Djigma and coworkers in Burkina Faso (2011), who recruited non-pregnant women, and the study of Ulla and coworkers in Spain (1993), who recruited female sex workers attending a family planning center.

Rectovaginal GBS carriage was assessed by culture in all studies: five studies performed rectovaginal sampling and culturing with the use of an enrichment broth, as recommended by the Centers for Disease Control and Prevention (CDC) guidelines [El Beitune *et al.*, 2006; Gray *et al.*, 2011; Joachim *et al.*, 2009; Mavenyengwa, Moyo, and Nordbø 2010; Shah *et al.*, 2011], two studies only sampled vaginally with cultures performed according to the CDC guidelines [Cutland *et al.*, 2012; Dangor, Kwatra, *et al.*, 2015] and the remaining six

studies did not culture according to CDC guidelines or did not report the exact culture methods used [Djigma *et al.*, 2011; Ernest *et al.*, 2015; Mitima *et al.*, 2014; Sebitloane, Moodley, and Esterhuizen 2011; Temmerman *et al.*, 1992; Ulla *et al.*, 1993].

Of the 19 studies described of the 17 manuscripts, three were retrospective [Epalza *et al.*, 2010; Shah *et al.*, 2011; Frigati *et al.*, 2014], fourteen were cross-sectional [Dangor, Lala, *et al.*, 2015; Dangor, Kwatra, *et al.*, 2015; Cutland *et al.*, 2012; Cutland *et al.*, 2015; El Beitune *et al.*, 2006; Ernest *et al.*, 2015; Gray *et al.*, 2011; Joachim *et al.*, 2009; Mavenyengwa, Moyo, and Nordbø 2010; Mitima *et al.*, 2014; Sebitloane, Moodley, and Esterhuizen 2011; Temmerman *et al.*, 1992] and none were prospective.

One study reported two different analyses for rectovaginal GBS carriage in HIV-infected versus uninfected women, i.e. using the total cohort versus matched subsets [Cutland *et al.*, 2012]. The analysis least prone to bias (i.e. the matched subsets) was used for this meta-analysis. The same study analyzed matched subsets for the association of HIV exposure of neonates and GBS neonatal disease and early-onset GBS neonatal disease, but not for late-onset GBS neonatal disease. Therefore, we used the total cohort analysis for these associations.

Risk of bias within studies The results of the critical appraisals of the included studies are shown in Figure 9 and Supplementary Table 4. All six studies addressing the relationship between HIV exposure of neonates and GBS neonatal disease were assessed as having a low risk of bias for representativeness [Dangor, Lala, *et al.*, 2015; Dangor, Kwatra, *et al.*, 2015; Cutland *et al.*, 2012; Cutland *et al.*, 2015; Frigati *et al.*, 2014; Epalza *et al.*, 2010]. Two out of the six studies had a low risk of bias for comparability [Cutland *et al.*, 2012; Dangor, Lala, *et al.*, 2015], two had a medium risk [Cutland *et al.*, 2015; Dangor, Kwatra, *et al.*, 2015] and two had a high risk [Epalza *et al.*, 2010; Frigati *et al.*, 2014]. For the GBS disease outcome assessment, four studies were assessed as having a low risk of bias [Cutland *et al.*, 2012; Cutland *et al.*, 2015; Dangor, Lala, *et al.*, 2015; Frigati *et al.*, 2014] and two had a medium risk [Dangor, Kwatra, *et al.*, 2015; Epalza *et al.*, 2010].

Of the studies on HIV infection and maternal GBS carriage only two out of the thirteen studies were assessed as having a low risk of bias for representativeness [Cutland *et al.*, 2012; Gray *et al.*, 2011], nine studies a medium risk [Dangor, Kwatra, *et al.*, 2015; El Beitune *et al.*, 2006; Ernest *et al.*, 2015; Joachim *et al.*,

2009; Mavenyengwa, Moyo, and Nordbø 2010; Mitima *et al.*, 2014; Sebitloane, Moodley, and Esterhuizen 2011; Temmerman *et al.*, 1992; Ulla *et al.*, 1993] and two studies a high risk [Djigma *et al.*, 2011; Shah *et al.*, 2011].

Three out of the thirteen studies were assessed as having a low risk of bias for comparability [Cutland *et al.*, 2012; Gray *et al.*, 2011; Shah *et al.*, 2011], four a medium risk [Dangor, Kwatra, *et al.*, 2015; Ernest *et al.*, 2015; Joachim *et al.*, 2009; Sebitloane, Moodley, and Esterhuizen 2011], and six a high risk [Djigma *et al.*, 2011; El Beitune *et al.*, 2006; Mavenyengwa, Moyo, and Nordbø 2010; Mitima *et al.*, 2014; Temmerman *et al.*, 1992; Ulla *et al.*, 1993]. The risk of bias for outcome assessment was low in five out of the thirteen studies [El Beitune *et al.*, 2006; Gray *et al.*, 2011; Joachim *et al.*, 2009; Mavenyengwa, Moyo, and Nordbø 2010; Shah *et al.*, 2011]; medium in two studies [Cutland *et al.*, 2012; Dangor, Kwatra, *et al.*, 2015] and high in six studies [Djigma *et al.*, 2011; Ernest *et al.*, 2015; Mitima *et al.*, 2014; Sebitloane, Moodley, and Esterhuizen 2011; Temmerman *et al.*, 1992; Ulla *et al.*, 1993].

Association of maternal HIV status with GBS neonatal disease The meta-analysis of the association between HIV exposure of neonates and GBS neonatal disease showed that HIV-exposed neonates were more than twice as likely to have neonatal GBS disease compared to unexposed neonates (OR, 2.39; CI, 1.31–4.38; p=0.005) (Figure 10). The crude OR in the individual studies ranged from 1.72 to 19.86, with three of the six individual studies showing a statistically significant association. The subanalyses showed that HIV-exposed neonates were not at increased risk for early-onset neonatal disease (OR, 1.31; 95% CI, 0.84–2.04; p=0.240), but were 4.43 times more likely to have late-onset neonatal disease (95% CI, 1.81–10.85; p=0.001).

As recommended by Sterne and co-workers [2011] to only create funnel plots when the number of studies is ten or more, we did not create a funnel plot for this meta-analysis.

Association of HIV infection and rectovaginal GBS carriage In our meta-analysis of the association between HIV infection and rectovaginal GBS carriage, we found no significant association between HIV infection status and rectovaginal GBS carriage (OR 1.09; 95% CI 0.82–1.44; p=0.55) (Figure 11). The crude ORs in the individual studies ranged from 0.26 to 4.22, with only three studies reporting a statistically significant association [Temmerman *et al.*, 1992; Mitima *et al.*, 2014; Cutland *et al.*, 2012]. Two of these studies found a significantly

higher rectovaginal carriage rate in HIV-positive women [Temmerman *et al.*, 1992; Mitima *et al.*, 2014], whereas one study found a significantly lower rate [Cutland *et al.*, 2012].

The heterogeneity among these studies is substantial (I^2 = 68.7%). Visual inspection of the funnel plot shows an asymmetrical distribution of studies, suggesting publication bias (Figure 6), which is marginally supported by Egger's regression intercept (0.94; 95% CI -0.48 -2.37; p=0.09), but not by the Begg and Mazumdar's rank correlation test (Kendall's tau = -0.10, one-tailed p-value = 0.313). Two studies [Mitima *et al.*, 2014; Temmerman *et al.*, 1992] fall outside the pseudo 95% confidence interval, providing further evidence of heterogeneity and bias.

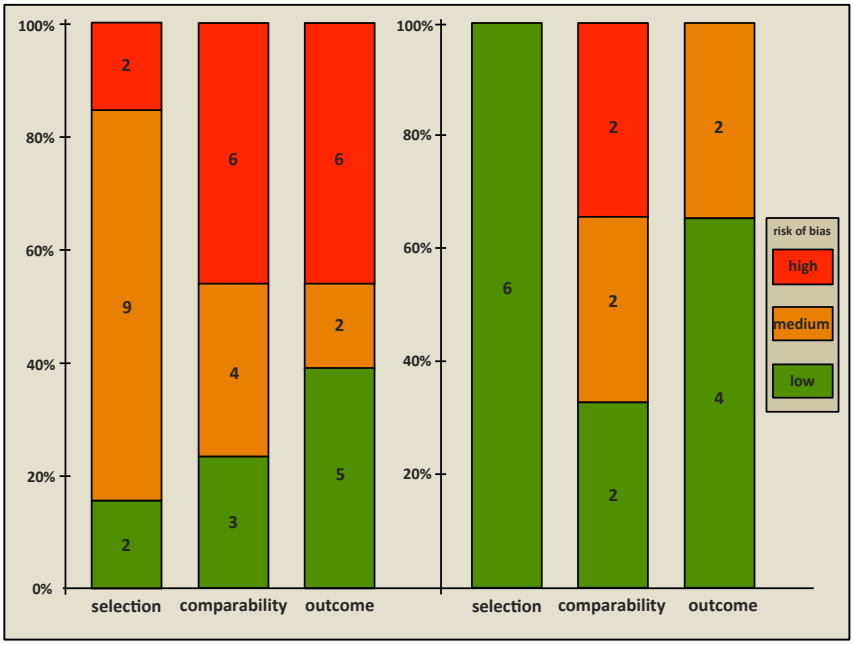

FIGURE 9 **Quality assessment using an adapted Newcastle-Ottawa Scale for risk of bias of studies included in the systematic review.** Left, studies (n = 13) reporting an association between HIV infection and rectovaginal GBS carriage. Right, studies (n = 6) reporting an association between neonatal HIV exposure and neonatal GBS disease. The absolute numbers of studies are shown in the boxes.

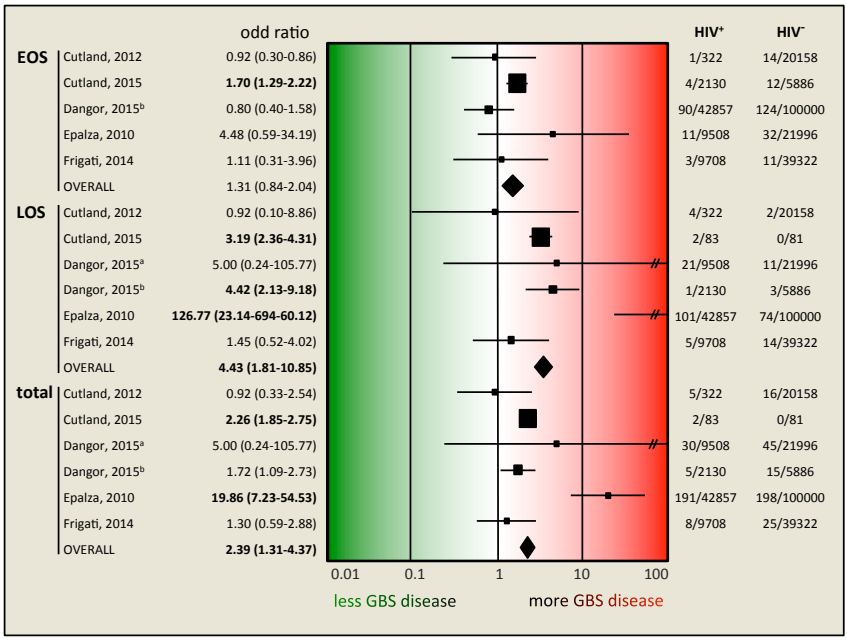

FIGURE 10 Forest plot of estimates of association between HIV exposure and GBS neonatal disease. Studies are plotted alphabetically according to the last name of the first author and followed by the publication year. Each study is represented by a black square and a horizontal line, which corresponds to the OR and 95% CI, respectively. The areas of the black squares reflect the weight of the study (determined by random effects analysis) in the meta-analysis. The vertical line in the middle corresponds to an OR of 1.0. The diamonds represent the overall summary estimate for EOD, LOD and total GBS disease, respectively, with the 95% CIs given by the width. In the columns on the right, the number of cases on the total number of HIV-positive and of HIV-negative mothers is given, respectively, for each study. [a], Dangor, 2015, JID; [b], Dangor, 2015, PONE; CI, confidence interval; OR, odds ratio; //, 95% CI line exceeding OR 100.

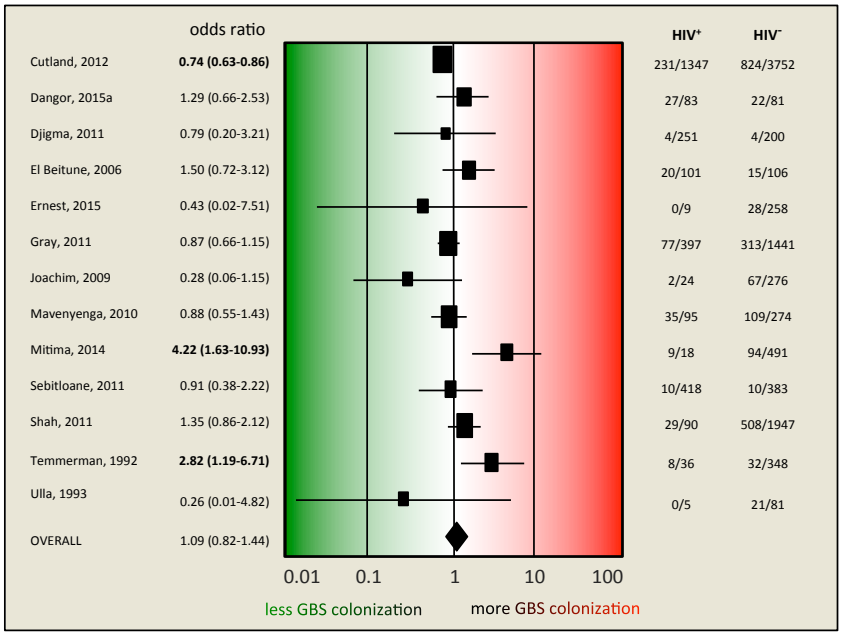

FIGURE 11 **Forest plot of estimates of association between HIV infection and rectovaginal GBS carriage.** Studies are plotted alphabetically according to the last name of the first author and followed by the publication year. Each study is represented by a square and a horizontal line, which corresponds to the OR and the 95% CI, respectively. The magnitude of the squares reflects the weight of the study (determined by random effects analysis) in the meta-analysis. The vertical line in the middle indicates the OR 1.0. The diamond at the bottom represents the summary estimate, with its width indicating the 95% CI. The two columns on the right document the number of GBS cases on the total of HIV-positive and of HIV-negative women, respectively, for each study. [a], Dangor, 2015, JID; CI, confidence interval; OR, odds ratio; bold, $p<0.05$.

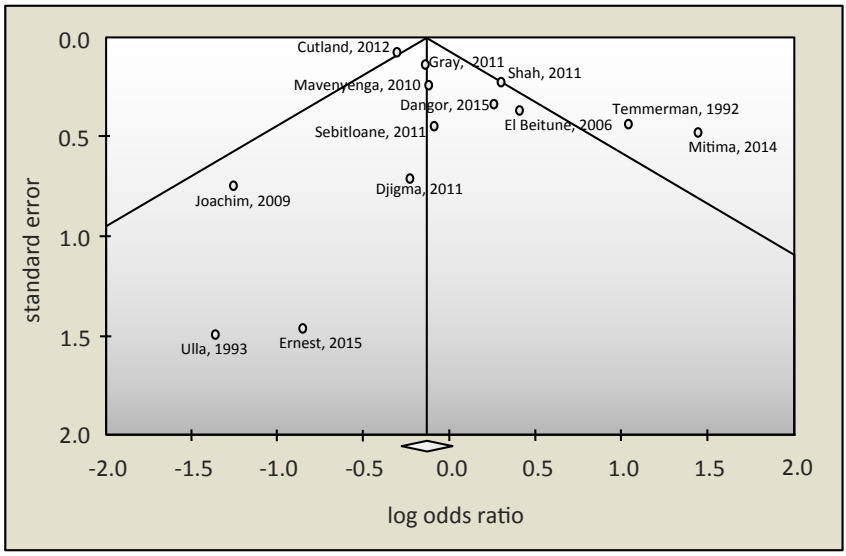

FIGURE 12 Funnel plot to assess publication bias among studies evaluating the association between HIV infection and rectovaginal GBS carriage. The circles represent the estimates of the 11 included studies of association between HIV infection and rectovaginal GBS carriage. The log of the odds ratio is plotted on the horizontal axis, against the inverse of the standard error of the odds ratio on the vertical axis. The vertical line in the funnel plot indicates the fixed-effects summary estimate and the sloping lines indicate the expected 95% confidence intervals for a given standard error.

8.4 Discussion

Group B streptococci (GBS) are a major cause of neonatal sepsis, which results in one million neonatal deaths every year [Lawn *et al.*, 2005]. Also, studies have repeatedly shown that HIV-negative neonates that had been exposed to HIV during pregnancy are at increased risk for infectious morbidity and mortality (reviewed in [Afran *et al.*, 2014]). In this systematic review and meta-analysis, we assessed the risk for GBS neonatal disease in HIV-exposed neonates. We also assessed the risk for rectovaginal GBS carriage in HIV-infected women, as rectovaginal carriage is a major risk factor for neonatal GBS disease.

We found convincing evidence that HIV-exposed neonates were at higher risk for GBS neonatal disease. When analyzing cases of early-onset GBS and late-onset GBS disease separately, we found that this increased risk pertains to late-onset disease only.

There are several factors associated with HIV infection in pregnant women or mothers that might explain why infants born to HIV-infected mothers are at increased risk for late-onset, but not early-onset GBS disease. In the case of early-onset GBS disease, the factors that increase or decrease the risk for early-onset GBS disease might be well-balanced, while in the case of late-onset GBS disease, the net result may be an enhanced risk (Figure 13).

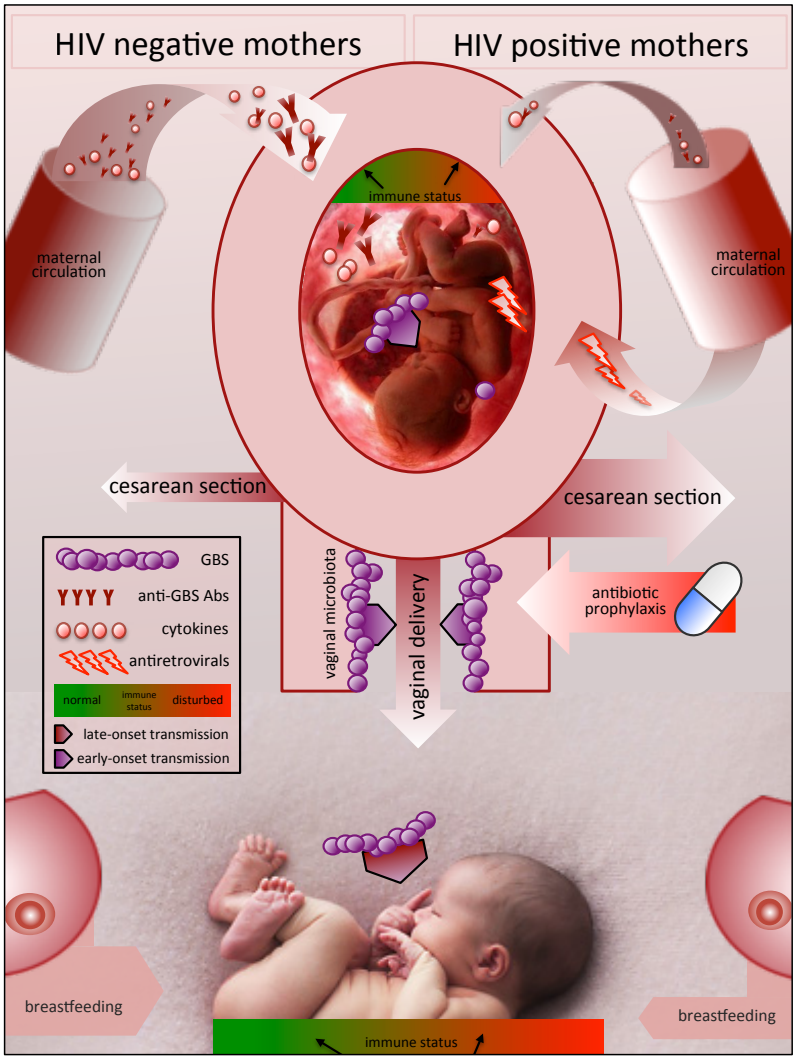

FIGURE 13 **Integrated model of factors influencing rectovaginal GBS carriage, EOD and LOD.** Overview of the different factors and their influence on early- and late-onset sepsis in HIV- and HIV+ mothers.

Factors that might decrease early-onset GBS disease rates in HIV-exposed neonates

First, HIV-infected pregnant women widely use antibiotic prophylaxis (co-trimoxazole), as recommended by the World Health Organization (WHO) [WHO 2006]. Co-trimoxazole, largely effective against GBS [Joachim *et al.*, 2009], is likely to be responsible for lower GBS rectovaginal carriage rates in HIV-infected pregnant women. For instance, we previously showed, using GBS-specific quantitative PCR (qPCR), that none of 30 Rwandan HIV-infected women, of which 93.3% were on antibiotic prohylaxis, carried GBS [Cools *et al.*, 2016]. As vaginal GBS carriage is a prerequisite for GBS early-onset disease, reduction of carriage rates might reduce the risk for the early-onset form, but not for the late-onset form, where GBS is considered to be more frequently community- or hospital-acquired.

Second, the prevalence of EOD might be reduced by the practice of elective caesarean section. Elective caesarean section, i.e. caesarean delivery before labor and before rupture of membranes, has been shown to reduce the risk of mother-to-child transmission of HIV by 80% (60% to 70% of mother-to-child transmissions occur during labor and vaginal delivery [Chama and Morrupa 2008; Jamieson *et al.*, 2007]. This has led to dramatically increased ECS rates in HIV-infected women in some countries [Jamieson *et al.*, 2007]. Because in EOD, GBS is transmitted to the neonate when passing the birth channel or, more commonly *in utero* after rupture of the membranes, ECS might reduce the risk for EOD. Interestingly, Hook and colleagues [Hook *et al.*, 1997] compared pregnancy outcomes among 497 women undergoing elective repeat cesarean delivery to 492 who attempted trial of labor and 989 women with routine vaginal delivery. They found that rates of suspected and confirmed neonatal sepsis were significantly lower in the elective repeat cesarean delivery group, although absolute numbers of cases were very small (9, 26 and 26 cases of suspected sepsis in elective repeat cesarean delivery, trial of labor and vaginal delivery, respectively; and 0, 4 and 1 cases of confirmed sepsis in elective repeat cesarean delivery, trial of labor and vaginal delivery, respectively).

Factors that might increase late-onset GBS disease rates in HIV-exposed neonates

Since 2009, the WHO recommends HIV-positive mothers to practice exclusive breastfeeding for their infants in combination with antiretroviral therapy (ART)

for mother or infant. However, compared to HIV-negative women, HIV-positive women have been shown to preferentially choose exclusive replacement feeding or mixed feeding, or stop breastfeeding early, because of fear of HIV transmission through breast milk. This is a consequence of previous WHO guidelines recommending total avoidance of breastfeeding and/or because many advocates of programs for prevention of mother-to-child transmission recommend early weaning [Aishat UO 2015; Lunney *et al.*, 2008; Kuhn *et al.*, 2010; Slater, Stringer, and Stringer 2010; Haile 2015; Sadoh WE 2008; Adejuyigbe *et al.*, 2008]. Lack of breastfeeding might hamper passive protection of the neonate against GBS neonatal disease as GBS specific IgA, present in breast milk, could offer GBS-specific protection [Weisman and Dobson 1991]. Furthermore, HIV-infected mothers might have reduced IgA in breast milk, as has been shown to be the case for saliva [Challacombe and Sweet 1997]. These altered infant feeding practices are likely to have more effect on the incidence of LOD, considering the fact that most infants with EOD are already septic at birth or become septic shortly after birth. Concordantly, clinical studies have shown that lack of breastfeeding is a risk factor for neonatal LOD and meningitis [Schanler, Shulman, and Lau 1999; Hylander, Strobino, and Dhanireddy 1998].

Factors that might increase EOD and LOD GBS disease rates in HIV-exposed neonates

Low maternal GBS-specific antibody concentrations have been shown to be correlated with susceptibility of neonates for GBS EOD and LOD [Baker and Kasper 1976; Baker, Edwards, and Kasper 1981; Baker *et al.*, 2014]. Some important observations show that these protective antibodies are reduced in HIV-exposed neonates and HIV-infected mothers. Significantly lower GBS-specific antibody concentrations were measured in maternal/cord blood from HIV-infected mothers and neonates born from HIV-infected mothers [Dangor, Kwatra, *et al.*, 2015; Le Doare *et al.*, 2015]. Furthermore, a reduced transplacental transfer of GBS-specific antibodies has been shown in HIV-infected mothers [Dangor, Kwatra, *et al.*, 2015; Le Doare *et al.*, 2015]. In addition, the neonatal anti-GBS antibodies remained significantly lower at 16 weeks [Le Doare *et al.*, 2015]. Le Doare and co-workers demonstrated a significantly lower antibody-mediated C3b/iC3b deposition on all investigated GBS serotypes (Ia, Ib, II, III and V) in HIV-infected women and neonates born to HIV-infected women, suggesting a reduced opsonophagocytotic immune effector function [Le Doare *et al.*, 2015]. A recent phase 2 GBS vaccination trial (with a trivalent vaccine consisting of

conjugated capsular polysaccharides of GBS serotypes Ia, Ib and III) in African HIV-positive and HIV-negative pregnant women showed that the vaccine was less immunogenic in HIV-infected women, irrespective of their CD4 count [Heyderman *et al.*, 2016].

Other perturbations of the immune system of HIV-infected women (even in the absence of overt immune dysfunction) might also play an important role. HIV-infected women were shown to have reduced transplacental transfer of hematopoietic cytokines. This may result in lower thymic output of CD4+ cells in their infants and a delay in immune cell maturation [Epalza *et al.*, 2010]. Furthermore, intrauterine exposure to HIV or its soluble products appears to cause a disturbed thymic function in HIV-uninfected newborns [Clerici *et al.*, 2000].

ART, used to prevent mother-to-child transmission of HIV, might play a role as well. Nucleoside reverse transcriptase inhibitors (NRTIs) crossing the placenta could cause mitochondrial dysfunction in HIV-exposed uninfected infants, explaining a decrease in neutrophils, total lymphocytes, CD4+ and CD8+ cells counts [Epalza *et al.*, 2010].

Factors that might increase or decrease early-onset GBS disease rates in HIV-exposed neonates: meta-analysis of the risk for rectovaginal GBS carriage in HIV-infected women.

Because rectovaginal GBS carriage is a major risk factor for early-onset neonatal GBS disease, we also assessed the risk for rectovaginal GBS carriage in HIV-infected women.

Our meta-analysis, based on 13 studies, did not show an association between HIV infection and rectovaginal GBS carriage, but most of the studies suffered from major limitations and we consider the currently available evidence weak and call for further study.

Few studies had adequate statistical power and few were designed to assess the association between HIV infection and rectovaginal GBS carriage. Most studies were at medium or high risk of bias related to representativeness of study groups, comparability of study groups, and/or outcome assessment.

Heterogeneity was considerable. Technical biases, such as the specimen collection and culture methods are likely to contribute substantially to this heterogeneity. For example, rectovaginal sampling has been shown to yield significantly higher recovery rates compared to vaginal sampling alone [El Aila *et al.*, 2010; Dillon *et al.*, 1982]. Furthermore, the use of a selective enrichment broth

has been shown to improve GBS detection substantially [Baker *et al.*, 1977; El Aila *et al.*, 2010]. However, rectovaginal sampling and the use of a selective enrichment broth, both recommended by the CDC, were used by only five studies. Also, the different study populations and eligibility criteria are likely to contribute to the observed heterogeneity.

Some important possible confounding factors were not taken into account. For instance, none of the studies corrected for bacterial vaginosis (BV), a disturbance of the vaginal microbiome characterised by the deprivation of lactobacilli and the presence of anaerobes such as *Gardnerella vaginalis* and *Atopobium vaginae*. BV has been shown to be a risk factor for the acquisition of HIV [Atashili *et al.*, 2008] but also to be negatively associated with vaginal GBS carriage [Cools *et al.*, 2016; Hillier *et al.*, 1992], possibly masking an association between HIV and GBS.

Furthermore, as described above, the use of antibiotic prophylaxis in HIV positive women, recommended by the WHO [WHO 2006], might be responsible for lower GBS rectovaginal carriage rates in HIV-infected pregnant women but was only taken into account in few studies. Also, risk factors for early-onset GBS disease such as urinary tract infections and intrapartum fever have been reported to be more prevalent in HIV-infected women but were considered only by Cutland and coworkers [2012].

As the risk for EOD increases with heavier vaginal GBS colonization at the onset of labor or rupture of membranes [Verani and Schrag 2010], future studies should also include quantitative molecular tests such as GBS-specific qPCR to determine the vaginal GBS load in relation to the HIV status. Furthermore, including the assessment of GBS serotype/genotype distribution in HIV-positive and –negative women will document whether more virulent GBS strains are present in HIV-positive women.

In conclusion, the evidence that HIV-exposed infants are at increased risk for late-onset GBS neonatal disease is accumulating and public health intervention is urgently needed. A compromised immune system, a lesser tendency to breastfeed but higher tendency for cesarean section and the use of prophylactic antibiotics in HIV-positive women might partly explain these findings. While we did not find evidence for an increased risk of rectovaginal GBS colonization in HIV-infected women, most studies conducted to date suffered major limitations. Further well-designed studies incorporating sensitive, quantitative (and serotype specific) GBS detection methods may further clarify the relation between HIV-positivity and GBS carriage.

Supplementary Tables

Selection	
1 Representativeness of the exposed group	
a Truly representative of the average women *(neonates)* in the community	*
b Somewhat representative of the average women *(neonates)* in the community	*
c Selected group of women *(neonates)*	
d No description of the sampling strategy	
2 Representativeness of the non-exposed group	
a Drawn from the same community as the exposed group	*
b Drawn from a different source	
c No description of the derivation of the non-exposed group	
3 Sample size (total number of cases analyzed)	
a Justified and satisfactory	*
b Not justified or not satisfactory	
4 Ascertainment of the HIV infection (exposure)	
a Validated assay performed for the study or through medical records	*
b Self-reported without further documentation	
Comparability	
The subjects in different comparison groups are comparable, based on the study design or analysis	
a Controlling for confounding factors (logistic regression or propensity scoring)	**
b Subjects in different groups are comparable	*
c No reporting on comparability between comparison groups and no control for confounding	
Outcome	
Assessment of the outcome (rectovaginal GBS carriage)	
a Sampling, culturing, and identification according to CDC# guidelines	**
b Culturing and identification according to CDC guidelines	*
c Sampling, culturing, and identification not adequately performed/described	
d No description	

Assessment of the outcome (GBS neonatal disease):	
a Blood samples, cerebrospinal fluid (CSF), or other normal sterile site; BactAlert and/or GBS specific latex agglutination on CSF	**
b Culture methods not described	
Risk of bias	
Selection	4 stars, low risk of bias; 2 or 3 stars, medium risk of bias; 0 or 1 star(s), high risk of bias
Comparability	2 stars, low risk of bias; 1 star, medium risk of bias; 0 stars, high risk of bias
Outcome	2 stars, low risk of bias; 1 star, medium risk of bias; 0 stars, high risk of bias

SUPPLEMENTARY TABLE 1 **Quality appraisal tool: adjusted Newcastle-Ottawa Quality Assessment Scale.**
#CDC, Centers for Disease Control and Prevention, Bethesda, Maryland.

SUPPLEMENTARY TABLE 2 (see next page)

Digest of included studies reporting HIV exposure of neonates and GBS neonatal disease.

- Author: [a], Dangor, 2015, JID; [b], Dangor, 2015, Plos ONE
- Country: B, Belgium; SA, South-Africa
- Study design: CC, case-control; R, retrospective; XS, cross-sectional
- Study setting and population: EOD, early-onset disease; I, inclusion criteria; LOD, late-onset disease; SSA, sub-Saharan Africa
- Exposure assessment: CON, confirmatory test; RT, rapid test; S, serology; WB, Western blotting; PC, personal communication
- Outcome assessment: EOD, early-onset disease; LOD, late-onset disease; d, days; PC, personal communication
  1. *Sampling and transport medium*: B, blood sample; CSF, cerebrospinal fluid
  2. *Culture conditions*: AER, aerobic conditions; BA, blood agar; BHIB, brain heart infusion broth; CA, chocolate agar
  3. *Identification:* BEA, growth on bile esculin agar; CAMP, Christie, Atkins, and Munch-Petersen test; CAT, catalase test; ESC, esculine hydrolase test; GS, Gram stain; LA, GBS specific latex agglutination
- Correction confounding: PSM, propensity score matching

Author, year	Country	Study design	Study period	Study setting and population
Cutland, 2012	SA	XS	2004–2007	Infants born at a public secondary-tertiary level of care hospital
Cutland, 2015	SA	R	2004–2008	Infants born at a public secondary-tertiary level of care hospital
Dangor, 2015[a]	SA	XS	2013	Tertiary-level care hospital serving black-African community of Soweto and surrounding areas. I, >= 2500 g.
Dangor, 2015[b]	SA	CC	2012–2014	Infants with GBS disease recruited from three secondary-tertiary care public hospitals; EOD controls from one public hospital; LOD controls identified through the birth registries
Epalza, 2010	B	R	2001–2008	Hospital with tertiary neonatal center and HIV referral center in an urban setting. Most infants born from mothers of SSA origin living in poor socioeconomic conditions
Frigati, 2014	SA	R	2010–2011	Infants born at a primary and a tertiary level of care hospital

SUPPLEMENTARY TABLE 2

	HIV exposure assesment	Outcome assessment and EOS/LOS definition	Correction confounding
	Medical records, active voluntary HIV testing programme for pregnant women (in the community, >96% of pregnant women are tested (PC), in parent study, 99% were tested)	EOS, 0–3 d; LOS, 4–28 d. Active surveillance of neonatal/pediatric wards to ID study neonates, abstraction information related to neonatal sepsis from medical records. **1.** B, samples other sterile sites **2.** B: BacT/Alert, other samples: routine methods **3.** Routine methods	PSM (EOS only)
	Medical records, HIV ELISA results from maternal blood tests (>96% of pregnant women accept testing (PC). Sensitivity analysis of incidence accounted for GBS case-patients with unknown maternal HIV status	GBS disease, 0–90 d. Cases identified through screening of ward admissions and microbiological records within 24h of GBS ID. **1.** B, CSF. **2.** BacT/Alert	None
	Medical records, pregnant women HIV tested by RT, CON ELISA	GBS disease, <90 d; EOS, < 7 d; LOS, 7–89 d. GBS cultured from blood, CSF or normally sterile sites, or GBS in CSF by LA. **1.** B, CSF **2.** B positive on BacT/Alert and CSF: BA or CA, BHIB (CSF), AER, CO_2, 35°C, 72h. **3.** direct on CSF: LA, GS. Colonies ID: CAT, BEA, ESC, CAMP, GBALA (all PC)	None
	HIV testing of pregnant women routinely performed and confirmed using two independent RT	GBS disease, <90 d; EOS, < 7 d; LOS, 7–89 d. GBS cultured from blood, CSF or normally sterile sites, or GBS in CSF by LA. **1.** B, CSF **2.** B positive on BacT/Alert and CSF: BA or CA, BHIB (CSF), AER, CO_2, 35°C, 72h. **3.** direct on CSF: LA, GS. Colonies ID: CAT, BEA, ESC, CAMP, GBALA.	None
	Review of medical records, all pregnant women are tested for HIV (ELISA, CON WB)(PC)	EOS, 0–6 d; LOS, 7–90 d. **1.** B, CSF (PC) **2.** BacT/Alert (PC)	None
	Medical records	GBS disease, 0–90 d; EOS, 0–6 d; LOS, 7–90 d. Cases identified throught the hospital database. **1.** B, CSF **2.** BacT/Alert	None

SUPPLEMENTARY TABLE 3

Digest of studies reporting HIV infection of women and rectovaginal GBS carriage.

- » Author: a, Dangor, 2015, JID
- » Country: Br, Brazil; BF, Burkina Faso; DRC, Democratic Republic Congo; K, Kenya; M, Malawi; S, Spain; SA, South-Africa; T, Tanzania; US, United States; Z, Zimbabwe;
- » Study design: R, retrospective; XS, cross-sectional
- » Age: M, mean; Mdn, median; R, range
- » Study setting and population: E, exclusion criteria; GA, gestation age; I, inclusion criteria; w, weeks
- » Exposure assessment: HEU, HIV-exposed uninfected; 2X, positive samples repeated with same method; CON, confirmatory test; RT, rapid test; S, serology; WB, Western blotting; PC, personal communication

Author, year	Country	Study design	Study period	Study setting and population
Cutland, 2012	SA	XS	2004–2007	Pregnant women presenting in labor at a secondary-tertiary level of care hospital
Dangor, 2015a	SA	XS	2013	Pregnant women delivering at a tertiary-level care hospital serving black-African community of Soweto and surrounding areas.
Djigma, 2011	BF	XS	2009	Non-pregnant women seeking gynaecological assistance
Ernest, 2015	T	XS	2011–2012	Pregnant women (GA, 28–42 w) attending consultant and teaching hospital, E,: ab within 2 w before recruitment

SUPPLEMENTARY TABLE 3 / part 1 of 3

» Outcome assessment:

1. **Sampling and transport medium:** A, anal swab; B, blood sample; CSF, cerebrospinal fluid; HV, high vaginal swab; LV, low vaginal swab; PA, perianal swab; R, rectal swab; RV, rectovaginal swabs; V, vaginal swab;
2. **Culture conditions:** A, Amies transport medium; AER, aerobic conditions; BA, blood agar; BAC, bacitracin; BHIB, brain heart infusion broth; C, colistin; CA, chocolate agar; CA+SB, Columbia agar + 5% sheep blood; CAN, colistin nalidixic acid agar; CHA, CHROMagar StrepB; CEF CLED, cefoxitine CLED plates; G, gentamycin; HBA, 5% horse blood agar; NA, nalidixic acid; S, Stuart's transport medium; SB, sheep blood; SBA, 5% sheep blood agar; SC, subcultures; TH, Todd Hewitt broth; TSA-HB, tryptic soy agar + 5% human blood; (-48h), if no growth after 24h, another day was incubated
3. **Identification:** BEA, growth on bile esculin agar; CAMP, Christie, Atkins, and Munch-Petersen test; CAT, catalase test; ESC, esculine test; GS, Gram stain; (GP) BHC, (Gram-positive) β-haemolytic colonies; HAEM, haemolytic activity; HIP, hippurate test; LA, latex agglutination; PC, personal communication

Age (years)	HIV exposure assesment	Outcome assesment	Correction confounding
Mdn, 26; R, 12–51	Medical records, active voluntary HIV testing programme for pregnant women (in the community, >96% accept testing (PC))	**1.** LV **2.** A; HBA ± C (10 µg/mL) and NA (15 µg/mL); TH+G (8 µg/mL) and NA (15 µg/mL), HBA. **3.** GPBHC: CAMP, ESC, LA.	PSM
R, 18.2–42.7	Medical records, pregnant women HIV tested by RT, CON ELISA	**1.** LV, R. **2.** A w/o charcoal; CHA; 37 °C; 18–24-(48)h; AER. **3.** GBS-like colonies: CAMP; ESC, CAT, LA	None
HIV+: Mdn, 33.0; R, 28–44; HIV-: Mdn, 35.0; R, 27–45	Questionnaire	**1.** V. **2.** CA; 37 °C; 24h **3.** mini-galleries	None
M 25.6 ± 0.31; R, 14–39	Questionnaire	**1.** LV, PA **2.** SBA, 37 °C, CO_2, 24 h. GPBHC, CAT negative, SC SBA + SXT and BAC discs. **3.** Confirmation CAMP, LA	None

El Beitune, 2006	Br	XS	2002–2004	Pregnant women (GA, 35–37 w), singleton gestation; E, hepatitis B, hepatitis C, syphilis, previous history of a newborn with GBS EOD.
Gray, 2011	M	XS	2008–2010	Pregnant women (GA, 3th trimester) recruited from the labor ward of hospital
Joachim, 2011	T	XS	2008–2009	Pregnant women (GA, >= 37 w) attending antenatal clinics of a tertiary facility which handles referrals from peripheral and upcountry hospitals for routine antenatal visits; E, ab within 2 weeks prior to recruitment
Mavenyengwa, 2010	Z	XS	2003–2005	Pregnant women (GA, ± 20 w) attending antenatal clinic and recruited consecutively at three centres (rural, an urban, and a mixed rural-urban).
Mitima, 2014	DRC	XS	2012–2013	Pregnant women, (GA, 3th trimester), attending prenatal care in 12 healthcare centers. Women mostly of low socio-economic status, urban population. E, women with fistulae, women currently on ab.
Sebitloane, 2011	SA	XS	2 years	Pregnant women (GA, >= 36 w) in whom vaginal delivery was anticipated

SUPPLEMENTARY TABLE 3 / part 2 of 3

	R, 16–43	2 ELISAs, CON WB	**1.** LV, A **2.** TH + 8 mg/ml G and 15 mg/ml NA (transport), +250 ul SB, 35 °C, 18 h; BA + G and NA (idem conc), 35 °C, 48h. **3**. (GP) BHC, LA	None
	>= 16; M, 25.2 ± 5.9	2 RT (Detemine™, Uni-Gold™), discordant results: 3rd RT (SD-Bioline)	**1.** LV, R **2.** TH+ 15 ug/ml NA and 10 ug/ml C; 18–24 h. **3.** Phenotypic characteristics, CAMP, LA	LR
	M, 26.6 ± 5.1; R, 16–44	RT (SD-Bioline HIV 1/2), if negative: HIV status negative, if reactive: CON 2nd RT (Determine™). HIV status positive if both tests positive	**1.** HV, R **2.** TH (transport), TH + NA (15 mg/L) and G (8 mg/L), 37°C, 5% CO_2, 24 h-(48h), SC on SBA. **3.** Presumptive ID: GS, CAT, HAEM, HIP, CAMP, LA; Confirmation, LA.	None
	R, 16–45	RT (HIV Ag/Ab Combo). CON WB. Interpretation according to American Red Cross guidelines.	**1.** LV, R **2.** S, CAN agar base with SB, and in TH+C (10 mg/L) and NA 15 mg/L, 37°C, 24 h-(48), looking for b-haemolytic or nonhemolytic streptococcal colonies typical of GBS. TH was sub-cultured onto CAN blood agar plates before 24 h incubation at 37 °C.	None
	10%, <18; 66%, R, 18–35; 24% >35	Medical records; RT (Determine), CON 2nd RT (Unigoldor Double Check Gold). Positive if 2 RT positive.	**1.** V **2.** A, BA + NA (15 mg/mL) and C (10 µg/mL); 37°C, CO_2, 48 h **3.** BHC: GS, CAT, HIP, CAMP.	None
	>= 18	RT (DetermineTM HIV1/2), CON 2nd RT (SmartCheck).	**1.** V **2.** A, CEF CLED, BAC CA, and laked blood CEF	None

Shah, 2011	US	R	1997–2007	Ethically diverse pregnant women attending prenatal care in an urban public hospital
Temmerman, 1992	K	XS	1989–1990	Pregnant women with clinical signs and symptoms of spontaneous abortion (GA, < 20 w) and pregnant women (GA, >20 w) visiting a non-private referral university hospital. E, women on ab 24h before visit, women with induced or clinically septic abortion or with axillary temp >38°C, severe anemia, shock.
Ulla, 1993	S	XS	1986–1991	Female sex workers recruited at a family planning center

SUPPLEMENTARY TABLE 3 / part 3 of 3

	HIV+: M, 29.5 ± 5.8; HIV-: M, 28.0 ± 6.3	HIV status abstracted from medical records, HIV test not mentioned.	Medical records.: defined as having a positive rectovaginal swab recovered within 5 weeks of delivery or GBS bacteriuria at any point during index pregnancy, as per the 2002 guidelines CDC	LR
	M, 25.9	EIA (2X), CON WB	**1.** HV **2.** CAN CA+SB	None
	M, 25; R, 18–50	ELISA, CON WB	**1.** V **2.** TSA-HB	None

Study	Selection		
	Representativeness exposed group	Representativeness non-exposed group	Total number cases analyzed
HIV infection in women and rectovaginal GBS carriage			
Cutland, 2012	*	*	*
Dangor, 2015[a]		*	
Djigma, 2011		*	
Ernest, 2015	*	*	
El Beitune, 2006			*
Gray, 2011	*	*	*
Joachim, 2011	*	*	
Mavenyengwa, 2010		*	
Mitima, 2014	*	*	
Sebitloane, 2011	*	*	
Shah, 2011			
Temmerman, 1992		*	
Ulla, 1993		*	
HIV exposure neonates and GBS neonatal disease			
Cutland, 2012	*	*	*
Cutland, 2015	*	*	*
Dangor, 2015[a]	*	*	*
Dangor, 2015[b]	*	*	*
Epalza, 2010	*	*	*
Frigati, 2014	*	*	*

SUPPLEMENTARY TABLE 4 **Critical appraisal of the included studies.**

[a], Dangor, 2015, JID; [b], Dangor, 2015 PONE

	Ascertainment HIV infection/ exposure	Comparability	Outcome
	⭐	⭐⭐	⭐
	⭐	⭐	⭐
		⭐	
	⭐		⭐⭐
	⭐	⭐⭐	⭐⭐
	⭐	⭐	⭐⭐
	⭐		⭐⭐
	⭐		
	⭐	⭐	
	⭐	⭐⭐	⭐⭐
	⭐		
	⭐		
	⭐	⭐⭐	⭐⭐
	⭐	⭐	⭐⭐
	⭐	⭐	⭐
	⭐	⭐⭐	⭐⭐
	⭐		⭐
	⭐		⭐⭐

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Mucosal immune correlates of vaginal *Streptococcus agalactiae* colonization in women from sub-Saharan Africa

— CHAPTER 9

9.1 Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a commensal bacterium with an unique ability to colonize the vagina of adult women asymptomatically but causing acute disease in neonates. In this population, it is worldwide the leading cause of sepsis and meningitis, commonly named neonatal disease [Melin, 2011]. Neonatal disease causes one million neonatal deaths each year, and over 99% occur in low- and middle-income countries [Lawn *et al.*, 2005].

Early-onset neonatal disease (EOD), occurs in the first week of life, but in over 90% of cases, signs are evident at birth or within 12 hours after birth and presentation is typically with pneumonia/sepsis [Heath *et al.*, 2016]. In EOD, vertical transmission of GBS takes place *in utero* when GBS ascends from the vagina and causes an intra-amniotic infection after rupture of the membranes, or intrapartum when the neonate passes the birth canal [Simonsen *et al.*, 2014; Hornik *et al.*, 2012]. Late-onset disease (LOD), mostly presenting as meningitis, occurs between week 1 and month 3 of life, and transmission is less well-understood but thought to be caused by GBS acquired vertically or horizontally [Schuchat *et al.*, 1998].

To prevent GBS disease in infants, huge efforts have been made since the late 1980s based on two strategies, *i.e.* intrapartum antibiotic prophylaxis (IAP) and vaccination [Melin *et al.*, 2013]. Women at target for IAP are identified on a risk-based screening or by a late-gestation culture-based screening [Verani *et al.*, 2010]. Although IAP has reduced EOD in high-income countries [Schrag *et al.*, 2000], it has no effect on LOD or adverse perinatal outcomes related to GBS such as chorioamnionitis [Galask *et al.*, 1984; Yancey *et al.*, 1994], spontaneous abortion [Temmerman *et al.*, 1992; McDonald *et al.*, 2000], stillbirth [Nan *et al.*,

2015], premature labour [Allen *et al.*, 1999], premature rupture of membranes [Matorras *et al.*, 1989], premature delivery [Regan *et al.*, 1996], and puerperal sepsis [Acosta *et al.*, 2014; Koenig *et al.*, 2009].

Vaccination of pregnant women represents an attractive alternative to protect neonates against EOD, LOD and GBS-related adverse perinatal outcomes [Heath *et al.*, 2016; Melin *et al.*, 2013]. Most GBS vaccines under development aim at eliciting maternal serum immunoglobuline G (IgG) against capsular polysaccharides (CPS) that protect the fetus or neonate after crossing the placenta [Kasper *et al.*, 1996; Heath *et al.*, 2016]. However, as transplacental IgG is only sufficiently from week 32 of gestation onwards [Boyer *et al.*, 1984], premature neonates – at high risk for EOD – might not be protected by a systemic vaccine [Hordnes *et al.*, 1996]. Vaginal mucosal immunity to GBS is an important first-line defense against EOD and some GBS-related adverse perinatal outcomes, as colonization of the vaginal epithelium by GBS is the first step in pathogenesis [Simonsen *et al.*, 2014]. Therefore, an optimal vaccine should also induce mucosal immunity [Shen *et al.*, 2000].

However, the human vaginal mucosal immune response to GBS colonization of the vaginal tract remains to be elucidated. Gaining insight into this is essential for our understanding of the biological factors favoring colonization and persistence of GBS in the complex environment of the vaginal microbiome. Furthermore, knowledge of protective vaginal mucosal cytokines in response to GBS could provide a template for the design or refinement of more appropriate and effective vaccines [Morel *et al.*, 2010].

To our knowledge, the cytokine responses to GBS have only been characterized using *in vitro* cell-culture studies and murine models.

In this study, we describe mucosal cytokine patterns associated with human vaginal GBS colonization in populations at the highest need for prevention measures to counter the extremely high burden of GBS EOD, LOD and GBS-related adverse perinatal outcomes.

9.2 Materials and methods

Study design and population A total of 430 women were recruited during 2010–2011 at three study sites, i.e. the International Centre for Reproductive Health Kenya (ICRHK), Mombasa, Kenya (170 women); the Wits Reproductive Health and HIV Institute (WrHI), Johannesburg, Republic of South Africa (RSA) (200

women); and the non-governmental organisation Rinda Ubuzima (RU), Kigali, Rwanda (60 women) (Table 10). The women were recruited into 6 predefined study groups: a reference group of 219 women (adult, non-pregnant, HIV-negative women at average risk of HIV), 60 pregnant women (up to 14 weeks of gestational age as determined by abdominal ultrasound at recruitment), 60 adolescent girls (16–17 years), 31 HIV-negative women engaging in vaginal practices (usage of cloth, lemon juice, detergents to clean, dry or tighten the vagina on a regular basis), 30 female sex workers (FSW), and 30 HIV-positive women (on antiretroviral treatment for at least 6 months, asymptomatic and with a CD4 count of more than 350 cells/µl). Women in the reference group and pregnant women were recruited from family planning clinics, 'women's groups' and antenatal clinics in Mombasa County and Johannesburg. Adolescents were recruited at youth centres and youth-friendly family planning services in Mombasa County and Johannesburg. Women engaging in vaginal practices were recruited in Johannesburg only. FSW and HIV-positive women were recruited in Kigali from the sex worker community, using community mobilizers and from public HIV treatment clinics, respectively.

This study reports on a cross-sectional analysis of the first study visit of a longitudinal parent study presented in detail previously [Jespers *et al.*, 2014]. After screening and consenting, this first study visit was scheduled soon after the last day (day 9 ± 2 days) of the menstrual period.

Study site	Study group	n	Prevalence of vaginal GBS (95% CI)
Mombasa, Kenya	Reference group	110	20.2 (13.7–28.7)
Mombasa, Kenya	Pregnant women	30	14.3 (5.7–31.5)
Mombasa, Kenya	Adolescents	30	3.6 (0.6–17.7)
Kigali, Rwanda	FSW	30	20.0 (9.5–37.3)
Kigali, Rwanda	HIV+ women	30	0.0 (0.0–11.4)
Johannesburg, SA	Reference group	109	23.2 (16.2–31.9)
Johannesburg, SA	Pregnant women	30	10.0 (3.5–25.6)
Johannesburg, SA	Adolescents	30	0.0 (0.0–11.4)
Johannesburg, SA	Vaginal practices	31	25.8 (13.7–43.2)

TABLE 10 **Study population and vaginal GBS carriage rates.**

FSW, female sex workers

Ethical committee Written information and consent forms in the local language were provided to the women or Legally Authorized Representatives for their review. After the interview, the participants and, in case they were of minor age (age below 18 in Kenya and South Africa and below 21 in Rwanda), the parents or guardians were asked to confirm their willingness to participate in the study by signing (or thumb-printing in case they were illiterate) the consent form. The protocol was approved by the Kenyatta National Hospital Ethical Review Committee, Kenya; the Human Research Ethics Committee (Medical), University of the Witwatersrand, RSA; the Rwanda National Ethics Committee, Rwanda; the Institutional Review Boards of the Institute of Tropical Medicine in Antwerp, of Ghent University, and of the University Teaching Hospital in Antwerp, Belgium. In addition, the study was approved by the National Council on Science and Technology in Kenya; the RSA Department of Health; and the National AIDS Control Commission in Rwanda. The study is registered at the Trial Registration at the National Health Research Ethics Council South Africa with the number DOH2709103223.

Collection of samples Two sterile Copan flocked® swabs (CopanDiagnostics, Inc., Murrieta, CA) were brought into the vaginal vault by the study clinician, rotated against the vaginal wall at the midportion of the vault, gently dipped in the posterior fornix and carefully removed to prevent contamination with the microbes of the vulva and introitus. The swab heads were collected into two 1.5 ml cryovials, labeled and immediately frozen at -80 °C until shipment.

For cervicovaginal lavage (CVL) samples, 10 ml normal saline at room temperature was flushed using a sterile pipette over the cervix and the lateral vaginal walls. This fluid was aspirated from the posterior fornix using the same pipette and collected in a 15 ml Falcon tube and immediately transported in a cooled box (2–8°C) to the laboratory for processing. At the laboratory, CVL processing was started within one hour after sample collection. CVLs were centrifuged at 1,000g for 10 minutes at 4°C and the supernatant stored at -80°C until shipment.

Shipment of samples Cryovials with the vaginal swab heads and CVLs were placed in a dry shipper, which was filled with liquid nitrogen according to the manufacturer's instructions. The dry shippers were sent to the central laboratory at the Institute of Tropical Medicine (ITM) in Antwerp, Belgium, using a temperature-monitor to guarantee maintenance of the cold chain.

Extraction and purification of DNA For the quantitative PCR (qPCR) assays, DNA extraction from the two Copan swabs of each subject was performed by thawing the swabs at room temperature for 30 minutes. After adding 1200 µL of diluted PBS, each swab was gently vortexed for 15 seconds, and 1 mL of each swab suspension was pooled into a final volume of 2 mL. An aliquot of 250 µL was extracted using the Abbott m24sp automated extraction platform (Abbott, Maidenhead, UK), according to the manufacterer's instructions, and 200 µl of eluted DNA was stored at -80 °C.

GBS-specific qPCR To detect and quantify GBS in vaginal swab DNA extracts, a GBS-specific qPCR was carried out as described previously [Cools *et al.*, 2016]. Briefly, the qPCR reactions were performed in a final volume of 10 µl, containing 5 µl of LightCycler 480® SYBR Green I Master (Roche Applied Science, Basel, Switzerland), 0.5 µM of both forward Sip1 (5'-ATCCTGAGACAACACTGACA-3') and reverse primer Sip2 (5'-TTGCTGGTGTTTCTATTTTCA-3'), 0.3 µM of probe (5'- 6FAM-ATCAGAAGAGTCATACTGCCACTTC–TAMRA-3') (Eurogentec, Liège, Belgium) and 2 µl of DNA extract or 2 µl of HPLC water (as negative template control). Cycling conditions were as follows: 95 °C for 5 min; 40 cycles of 95 °C for 10 s, 58 °C for 15 s and 72 °C for 20 s. Amplification, detection and quantification were carried out using the LightCycler480 platform and the LightCycler® 480 Software Version 1.5 (Roche, Basel, Switzerland). Concentrations were expressed as bacteria/ml and log transformed.

Quantification of cytokines and elafin Quantification of the concentrations of the cytokines IL-1α, IL-1β, IL-6, IL-8 and IL-12, MIP-1β, interferon (IFN)-γ–induced protein (IP-10), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) in CVL samples was done using the Bio-Plex™ human cytokine assay kit (Bio-Rad Laboratories NV-SA, Nazareth, Belgium), elafin was measured by ELISA kits from R&D Systems (Minneapolis, MN), and IL-1RA was measured using the Meso Scale Discovery (MSD) multiplex platform and Sector Imager 2400 (MSD, Gaithersburg, MD). All measurements were performed according to the manufacturer's instructions as described previously [Kyongo *et al.*, 2015]. Concentrations of immune markers were expressed as pg/mL and log transformed.

Data analysis Women with a positive qPCR result for GBS were considered GBS carriers. Correlations of the concentrations of GBS and the different

cytokines/elafin were investigated by calculating the Spearman correlation coefficients, with p-values < 0.05 indicating significance.

A multivariate analysis of variance (MANOVA) was performed to simultaneously compare the means of all cytokines and elafin between the GBS carriers and the non-carriers. Tests for mean differences between these two groups were based on Hotelling's Trace statistic. If a statistically significant difference was indicated, analysis of variance (ANOVA) was carried out for cytokines or elafin separately. All analyses were performed using SPSS software version 22 (SPSS Inc.).

9.3 Results

A total of 595 women were screened and 430 were enrolled in the study. Details on screening, screening failure and enrolment have been reported previously [Jespers *et al.*, 2014]. Of the 430 women enrolled, 424 women were analysed by qPCR for vaginal GBS colonization. Overall, 69 of the 424 women (16.3%) were found positive for vaginal GBS carriage. The prevalences in the different study groups are presented in Table 10. Details on associations of GBS carriage and sociodemographic characteristics, reproductive health, sexual behaviour, vaginal practices, cervicovaginal signs and symptoms, and genital tract infections have been presented previously [Cools *et al.*, 2016].

MANOVA analysis showed that there were statistically significant differences in the immune parameters in the CVLs of the women carrying GBS versus the women not carrying GBS vaginally ($F(11,412) = 2{,}240$, $p<0.05$). The results of the different ANOVAs are presented in Table 11. Women colonized with GBS had higher cervicovaginal concentrations of IL-8 ($\log_{10}$ concentration 2.41 pg/mL versus 2.14 pg/mL), IL-12 ($\log_{10}$ concentration 0.26 pg/mL versus 0.13 pg/mL) and IP-10 ($\log_{10}$ concentration 2.81 pg/mL versus 2.53 pg/mL) compared to non-colonized women.

The Spearman correlations – only providing a measure of linear associations and not suited to show logaritmic or other non-linear assocations – between the concentrations of the different cytokines/elafin and GBS are shown in Table 12. GBS loads were significantly positively correlated with concentrations of IL-8 ($r_s = 0.161$), IP-10 ($r_s = 0.134$), IL-12 ($r_s = 0.130$), IL-1 ($r_s = 0.101$) and IL-6 ($r_s = 0.097$).

	GBS positive (n=69)		GBS negative (n=355)		ANOVA	
	mean	SD	mean	SD	F	p
IL-1α	1.41	0.500	1.39	0.641	0.064	0.801
IL-1β	1.19	0.788	0.99	0.753	3.726	0.054
IL-1Ra	4.73	0.472	4.71	0.542	0.065	0.799
IL-6	1.05	0.658	0.91	0.613	2.733	0.099
IL-8	2.41	0.658	2.14	0.547	12.967	**<0.001**
IL-12	0.26	0.303	0.13	0.371	8.213	**0.004**
Elafin	5.22	0.437	5.10	0.535	3.126	0.078
G-CSF	2.07	0.621	1.98	0.641	1.237	0.267
GM-CSF	0.36	0.400	0.30	0.410	1.514	0.219
IP-10	2.81	0.606	2.53	0.727	8.882	**0.003**
MIP-1β	1.01	0.667	0.87	0.579	3.423	0.065

TABLE 11 **Mean log_{10} concentrations (pg/mL) of the different cytokines and elafin in the GBS carrier group versus the non-carrier group.**
ANOVAs are presented with their F value and p value.
Bold: significant at the 0.05 level.

	GBS	IL-1β	GM-CSF	IL-1α	IL-6	IL-8	IL-12	G-CSF	IP-10	MIP-1β	Elafin
IL-1β	**0.101**										
GM-CSF	0.083	**0.197**									
IL-1α	0.014	**0.532**	**0.196**								
IL-6	**0.097**	**0.701**	**0.202**	**0.209**							
IL-8	**0.161**	**0.743**	**0.257**	**0.434**	**0.602**						
IL-12	**0.130**	**0.569**	**0.238**	**0.310**	**0.514**	**0.561**					
G-CSF	0.064	**0.646**	**0.189**	**0.224**	**0.799**	**0.560**	**0.476**				
IP-10	**0.134**	**0.150**	**0.247**	**0.184**	**0.221**	**0.365**	**0.277**	**0.300**			
MIP-1β	0.076	**0.558**	**0.159**	0.049	**0.668**	**0.546**	**0.550**	**0.568**	**0.248**		
Elafin	0.069	0.073	**0.102**	**0.219**	-0.008	**0.187**	**0.102**	**0.174**	**0.391**	-0.037	
IL-1RA	0.024	**0.331**	**0.172**	**0.402**	0.088	**0.361**	**0.380**	0.152	**0.205**	0.080	**0.295**

TABLE 12 **Spearman correlation of the cytokines and GBS.**
Bold, p<0.05; grey; GBS, Group B *Streptococcus*; IL-1β, Interleukin-1β; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL-1α, Interleukin-1α; IL-6, Interleukin-6; IL-8, Interleukin-8; IL-12, Interleukin-12; G-CSF, Granulocyte colony-stimulating factor; MIP-1β, Macrophage inflammatory protein-1β; elafin; IL-1RA, Interleukin-1 receptor antagonist.

9.4 Discussion

We assessed the immune correlates of vaginal GBS colonization and found that concentrations of IL-8, IL-12 and IP-10 in CVLs of women who were vaginally colonized with GBS were significantly elevated compared to non-colonized women, and these concentrations correlated with vaginal GBS concentrations as determined by qPCR.

IL-8 was elevated in GBS carriers and correlated with GBS loads

IL-8 is a potent chemotactic and activating factor for neutrophils, which respond to pathogens through phagocytosis, production and release of reactive oxygen species and antimicrobial peptides. Epithelial cells of the female reproductive tract (FRT), in addition to providing a physical barrier for protection, are immunologically active cells and act as key initiators of innate and adaptive immunity by secreting cytokines such as IL-8 [Agrawal *et al.*, 2009]. There is general consensus that the number of neutrophils in the different FRT tissues is dependent on IL-8 [Weissenbacher *et al.*, 2014]. Although IL-8 has been shown to be the major chemoattractant secreted by epithelial cells of the FRT, GM-CSF has been shown to work synergetically with IL-8 in the chemoattraction of neutrophils [Shen *et al.*, 2004].

Numerous *in vitro* studies have shown that GBS induced IL-8 transcription or IL-8 expression after stimulation of human vaginal epithelial cells, lung epithelial cells, cord/neonatal/adult blood mononuclear cells, neutrophils, monocytes, macrophages, placental trophoblasts, decidual cells and brain endothelial cells [Patras *et al.*, 2013; Doran *et al.*, 2002; Berner *et al.*, 2002; Griesinger *et al.*, 2001; Dudley *et al.*, 1997; Kwak *et al.*, 2000; Doran *et al.*, 2003; Mikamo *et al.*, 2004; Albanyan *et al.*, 2000; De Francesco *et al.*, 2008; Berner *et al.*, 2002; Sagar *et al.*, 2013]. IL-8 production has been shown to differ significantly after stimulation with different GBS serotypes and genotyes and to elicit significantly more IL-8 from adult monocytes compared to cord monocytes [Mikamo *et al.*, 2004; De Francesco *et al.*, 2008; Rowen *et al.*, 1995].

Functionally, GBS-induced IL-8 has been shown to induce neutrophil migration across human brain microvascular endothelial cells [Doran *et al.*, 2003]. GBS serotype III-induced secretion of IL-8 by neutrophils did not result in ingestion of GBS nor did it trigger superoxide production [Albanyan *et al.*, 2000].

Despite these *in vitro* findings, the only clinical study carried out thus far documented that IL-8 was not elevated in the maternal and fetal plasma of

women (recto)vaginally colonized with GBS compared to non-carriers [Mitchell *et al.*, 2013].

Our study confirms the above-mentioned *in vitro* findings. Although the cross-sectional study design of our study does not allow mechanistic inferences regarding GBS colonization and the observed cytokine patterns, the fact that IL-8 is readily released by many cell types upon stimulation by GBS, not in the least by vaginal epithelial cells [Patras *et al.*, 2013], justifies us to hypothesize/speculate that the presence of GBS in the cervicovaginal mucosa of these women induced IL-8, rather than that GBS was present because of elevated levels of IL-8.

In summary, although our finding, i.e. that GBS colonization is associated with elevated IL-8 transcription, seems to contradict the findings of the only other cross-sectional clinical study [Mitchell *et al.*, 2013] carried out thus far, they are in agreement with the general notion that the epithelial cells of the FRT act as key initiators of innate and adaptive immunity by secreting cytokines such as IL-8 [Agrawal *et al.*, 2009], and with numerous *in vitro* studies indicating IL-8 transcription and expression by different cell types upon challenge with GBS (see above for relevant references).

In our study, following elevated IL-8 concentrations in the CVLs of the GBS carriers, increased neutrophil levels could be expected. However, GBS carriage was asymptomatically and not associated with the presence of neutrophils [Cools *et al.*, 2016]. Possible, although significantly elevated, IL-8 concentrations in GBS carriers were not high enough to chemoattract neutrophils, as it has been shown that chemotaxis is strongly influenced by the concentration of IL-8 [Lin *et al.*, 2004].

IL-12 and IP-10 were elevated in GBS carriers and both correlated with GBS loads

Women vaginally colonized with GBS had significantly higher cervicovaginal concentrations of IL-12 compared to non-colonized women. Furthermore, in GBS carriers, IL-12 concentrations were modestly but significantly correlated with the GBS concentrations.

IL-12 is a pro-inflammatory cytokine that induces the production of IFN-γ and favours the differentiation of T helper 1 (T_H1) cells [Trinchieri *et al.*, 2003].

In vitro studies have shown that GBS induced IL-12 production in murine macrophages, human neonatal lymphocytes, T-cells and NK cells [Peoples *et al.*, 2009; Kwak *et al.*, 2000; Derrico *et al.*, 1996]. In human epithelial cells, this

production differed significantly upon stimulation with different serotypes [Mikamo *et al.*, 2004]. Interestingly, GBS-induced IL-12 and IFN-γ production by mixed mononuclear cells from cord blood was found to be signifantly less compared to production by mixed mononuclear cells from adults [La Pine *et al.*, 2003; Martins *et al.*, 2002]. This T_H1 type cytokine deficiency was suggested to contribute to the enhanced susceptibility of neonates to GBS [La Pine *et al.*, 2003].

Different animal models have documented the importance of IL-12 in protection of the neonate against GBS infections. In a murine model of neonatal GBS sepsis, IL-12 was found to be elevated in the plasma of GBS-infected pups, and pretreatment with antibodies that neutralized IL-12 resulted in significantly greater mortality and GBS bacteremia [Mancuso *et al.*, 1997]. In contrast, prophylactic or therapeutical administration of recombinant IL-12 was significantly associated with a lower mortality and a decreased bloodstream replication of GBS. These beneficial effects of IL-12 were at least partially explained by IL-12 mediated induction of IFN-γ.

Likewise, in a murine GBS-induced arthritis model, prophylactic administration of IL-12 to infected mice was shown to have beneficial effects, mostly attributable to the IL-12-induced IFN-γ [Puliti *et al.*, 2002]. In a murine model, where GBS was infected peritoneally, the mRNA of IL-12 and IFN-γ, but not of T_H2 associated cytokines IL-4 and IL-5, was upregulated in spleen cells [Rosati *et al.*, 1998].

In our study, the mean $\log_{10}$ concentration of IP-10 in the CVLs of GBS colonized women (2.81 pg/mL) was significantly higher compared to uncolonized women (2.53 pg/mL) and these concentrations correlated in a significant manner with the GBS concentrations.

IP-10 is a pleiotropic cytokine induced by IFN-γ, the T_H1 cytokine signature, and is capable of exerting potent biological functions in infectious diseases [Liu *et al.*, 2011; Holdren *et al.*, 2014]. IP-10 chemoattracts monocytes/macrophages, dendritic cells, NK cells, cytotoxic T cells and T_H1 cells towards inflamed and infected sites [Liu *et al.*, 2011; Holdren *et al.*, 2014; Bonecchi *et al.*, 1998; Lebre *et al.*, 2005].

Only few studies investigated IP-10 in the context of GBS infection. Murine macrophages did not produce IP-10 after stimulation with heat-killed GBS [Fan *et al.*, 2007]. Interestingly, IP-10 has been shown to be a sensitive early marker of late-onset bacterial sepsis and a good predictor of serious bacterial infection in general in infants < 4 months of age [Ng *et al.*, 2007; Chen *et al.*, 2011].

Vaginal GBS colonization is associated with a T_H1-associated cytokine environment

Our observations of elevated concentrations of IL-12 and IP-10 in CVLs of GBS colonized women point to a cytokine environment associated with a T_H1 response, as IL-12 is the main inducer of such a response, and IP-10 is induced by IFN-γ, the main effector cytokine of T_H1 cells and itself induced by IL-12 [Abbas *et al.*, 1996].

Our cross-sectional study design does not allow to infere whether this T_H1-associated cytokine pattern preceeds or follows vaginal GBS colonization. However, there are numerous indications suggesting that a GBS infection induces a T_H1 response. First, all above-mentioned cell-culture and mouse models do conclude that IL-12 (and IFN-γ) followed GBS infection. Second, other studies demonstrated a T_H1 response following GBS infection. Total splenocytes from mice infected with GBS produced a type 1 proinflammatory response inducing $CD4^+$ T cells to differentiate into T_H1 cells, which produced large amounts of IFN-γ [Clarke *et al.*, 2016]. Similarly, in a mouse model of GBS neonatal sepsis, IFN-γ was produced by splenocytes after GBS challenge, and administration of recombinant IFN-γ partially restored impaired host defense [Cusumano *et al.*, 1996]. Concordantly, *in vitro* and clinical studies reported T_H1 (and T_H17) responses against pneumococcal and group A streptococcal infections [Olliver *et al.*, 2011; Mortensen *et al.*, 2015]. Interestingly, IFN-γ was found to inhibit the intracellular survival of GBS in human umbilical vein endothelial cells, although no cause/effect relationship could be assessed [Lione *et al.*, 2014].

Taken together, a vast literature of *in vitro* and animal studies, suggests that GBS induces a T_H1 reponse that confers protection and the asymptomatic vaginal GBS colonization of the women in our study was associated with a mucosal T_H1-type cytokine pattern, probably protective and following colonization.

The cervicovaginal mucosal T_H1-cytokine environment during vaginal GBS colonization might profoundly influence the isotype of naturally acquired antibodies by isotype switching. Indeed, rectovaginal colonization with GBS has been associated with significantly elevated levels of cervicovaginal and systemic IgA and IgG [Hordnes *et al.*, 1996], and human naïve B cells have the potential to switch to any Ig isotype production under influence of cytokines. For instance, T_H1-related cytokines are suggested to induce IgG1, IgG2 and IgG3 isotypes [Abbas *et al.*, 1996; Sousa *et al.*, 1998; Mortensen *et al.*, 2015], which are the only isotypes (together with IgG4) that can cross the placenta [Guttormsen *et*

al., 2008; Palmeira *et al.*, 2012; Vidarsson *et al.*, 2014], and maternal/cord serum IgG have been linked with neonatal protection against GBS disease [Baker *et al.*, 1976]. Furthermore, IgG1, IgG2, and IgG3 (but not IgG4) are opsonizing antibodies, important for protection against invasive GBS infections. Also, Ig switching towards the production of IgA antibodies, which might be important for GBS immunity at mucosal sites, is induced by TGF-β but inhibited by T_H1 cytokine IFN-γ [Macpherson *et al.*, 2008; Trinchieri *et al.*, 2003].

The development of vaccins is one of the main strategies to prevent GBS disease [Melin *et al.*, 2013]. One of the most important strategies for the development of effective new vaccines is the selection and usage of a suitable adjuvant. Immunologic adjuvants are essential for enhancing vaccine potency by improvement of the humoral and/or cell-mediated immune response to vaccine antigens [Lee S and Nguyen 2015]. Most GBS vaccine studies so far have used conjugate vaccins without an adjuvant [Heath *et al.*, 2011]. One study demonstrated that aluminium hydroxide as an adjuvant did not enhance the immune response to a GBS serotype III conjugated vaccine [Paoletti *et al.*, 2001].

Because IL-12 directs the differentiation of T_H cells to the T_H1 phenotype, its use as a vaccine adjuvant to promote T_H1 responses is of great interest [Romani *et al.*, 1997]. IL-12 has been shown to enhance the efficacy of systemic vaccines against bacterial pathogens such as *Bordetella pertussis* and *Listeria monocytogenes* [Miller *et al.*, 1997; Mahon *et al.*, 1996]. Interestingly, administration of IL-12 as adjuvant together with tetanus toxoid (TT) at the nasal mucosa not only induced sharp increases in TT-specific serum IgG and IgA, but was also shown to enhance mucosal IgA [Boyaka *et al.*, 1999], pointing to IL-12 as a promising mucosal vaccine adjuvant [Stevceva *et al.*, 2006]. As our results seem to point to a protecting effect of IL-12 in controlling mucosal colonization of GBS, IL-12 might be considered as an adjuvans in GBS vaccination.

Our findings are limited by the cross-sectional design of our study, but has advantages compared to animal studies. Animal models have undoubtedly improved our understanding of the immune response against GBS, but only a few murine models studied the vaginal mucosal immune response against GBS [Patras *et al.*, 2015; Patras *et al.*, 2013; Carey *et al.*, 2014]. However, like many other pathogens, strains of *S. agalactiae* isolated from human infections appear to be adapted to their human host [Lindahl *et al.*, 2005], reducing the value of animal models. For instance, most human GBS isolates have important virulence factors such as surface proteins ScpB, Lmb, and IgA-binding beta protein, that are not present in strains isolated from bovine mastitis. Moreover, the

C5a peptidase of GBS may degrade human C5a but not mouse C5a [Lindahl *et al.*, 2005]. Importantly, the composition of murine vaginal microbial community differs substantially from that of the human vagina [Barfod *et al.*, 2013], normally dominated by lactobacilli. Furthermore, the murine vagina has a neutral pH and not the characteristic low pH of the human vagina [Yano *et al.*, 2011], shown to be important in GBS adherence to epithelial cells [Tamura *et al.*, 1994].

In conclusion, in populations at the highest need for better prevention measures to control rectovaginal GBS colonization and GBS neonatal disease, asymptomatic vaginal colonization was associated with elevated CVL levels of IL-8 and Th1-associated cytokines IL-12 and IP-10. This might have important consequences in our understanding of the mucosal immune reactions against GBS and for current and future vaccination efforts.

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Summary and future perspectives

— CHAPTER 10

10.1 Objective 1

To assess the prevalence of vaginal GBS and *E. coli* carriage in different populations from sub-Saharan Africa

Background

Sub-Saharan Africa (SSA) has the highest rates of neonatal sepsis mortality worldwide. However, epidemiological data on GBS and *E. coli* vaginal carriage – the main risk factors for early-onset sepsis (EOS) – are very limited, although they are necessary to design and implement EOS prevention strategies [Capan, 2012; Stoll and Schuchat 1998]. Therefore, the first objective of this thesis was to assess the prevalence of vaginal GBS and *E. coli* in different populations from SSA.

How did we study this?

A total of 430 women from Mombasa (Kenya), Kigali (Rwanda) and Johannesburg (South Africa (SA)) were recruited during 2010–2011 (Table 13). To capture different relevant populations groups, these women were recruited into different predefined study groups:

- **A group of reference women** (adult, non-pregnant, HIV-negative women at average risk of HIV)
- **Pregnant women** (up to 14 weeks of gestational age as determined by abdominal ultrasound at recruitment)
- **Adolescents** (16–17 years)
- **Female sex workers** (self-acknowledged)

» **HIV-positive women** (on antiretroviral treatment for at least 6 months, asymptomatic and with a CD4 count of more than 350 cells/µl)
» **Women engaging in vaginal practices** (HIV-negative women who use cloth, lemon juice, or detergents to clean, dry or tighten the vagina on a regular basis)

Vaginal carriage of GBS and *E. coli* was assessed by collecting vaginal swabs and detecting GBS and *E. coli* using quantitative polymerase-chain reaction (qPCR) in vaginal swab DNA extracts.

TABLE 13 Vaginal GBS and *E. coli* carriage rates.

Group	City, Country	n	GBS prevalence % (95% CI)	E. coli prevalence % (95% CI)
Reference group	Mombasa, Kenya	110	20.2 (13.7–28.7)	25.0 (17.8–33.9)
	Johannesburg, SA	109	23.2 (16.2–31.9)	27.1 (19.6–36.2)
Pregnant women	Mombasa, Kenya	30	14.3 (5.7–31.5)	14.3 (5.7–31.5)
	Johannesburg, SA	30	10.0 (3.5–25.6)	33.3 (19.2–51.2)
Adolescents	Mombasa, Kenya	30	3.6 (0.6–17.7)	28.6 (15.3–47.1)
	Johannesburg, SA	30	0.0 (0.0–11.4)	13.3 (5.3–29.7)
FSW	Kigali, Rwanda	30	20.0 (9.5–37.3)	70.0 (52.1–83.3)
HIV+ women	Kigali, Rwanda	30	0.0 (0.0–11.4)	20.0 (9.5–37.3)
Vaginal practices	Johannesburg, SA	31	25.8 (13.7–43.2)	30.0 (16.7–47.9)

What were our major findings?

Vaginal GBS carriage rates

» Vaginal carriage rates in reference groups from Kenya and SA were 20.2% (95% CI, 13.7–28.7%) and 23.1% (95% CI, 16.2–31.9%).
» The pregnant women in our study population had vaginal GBS carriage rates of 14.3% and 10.0% in Kenya and SA, respectively. In chapter 7, we discuss the observed lower prevalences compared to most other studies in SSA (see Table 14).

TABLE 14 Studies reporting (recto)vaginal GBS carriage rates in SSA.

Country	Year	n	Population
Nigeria	1980	588	P, L
Nigeria	1983	225	P
Zimbabwe	1990	89	P
Togo	1991	106	P
Gambia	1994	136	P
Malawi	2005	97	P
Mozambique	2008	113	P
Tanzania	2009	300	P
Zimbabwe	2010	780	P
Malawi	2011	1840	P, HIV+ and HIV-
South Africa	2014	661, 621, 595, 521[$]	P
DR Congo	2015	509	P

L, women in labor; NP, non-pregnant women; P, pregnant women; V, vaginal swab; R, rectal swab; SB+C, selective broth and culturing; SA, selective agar; [#]week 20, 26, and delivery, respectively; [$]week 20–25, week 26–30, week 31–35, and week 37+, respectively.

% GBS	Sample	Detection	Reference
19	V	SB+C	[Onile, 1980]
20	V	SB+C	[Dawodu, 1983]
31	V	SB+C	[Nathoo, 1990]
4	V, R	SB+C	[David-Prince, 1991]
22	V, R	SB+C	[Suara, 1994]
16.5	V, R	SA	[Dzowela, 2005]
1.8	V, R	SB+C	[de Steenwinkel, 2008]
23.0	V, R	SB+C	[Joachim, 2009]
47, 24, 21[#]	V, R	SB+C	[Mavenyengwa, 2010]
21.2	V, R	SB+C	[Gray, 2011]
33.0, 32.7, 28.7, 28.4[$]	V, R	SA	[Kwatra, 2014]
20.2	V	SA	[Mitima, 2014]

» Adolescents in our study were found to have low GBS carriage rates: 3.6% of the Kenyan and 0% of the SA adolescents carried GBS vaginally. Other studies report conflicting associations between age and vaginal GBS carriage and are discussed in chapter 7.
» Women engaging in vaginal practices had higher prevalences of vaginal GBS carriage and will be discussed in objective 3.
» In the group of HIV positive women, we did not observe any GBS carriers. This group will be discussed in objective 4.

Vaginal E. coli carriage rates

» The group of reference women from Kenya and SA had vaginal *E. coli* carriage rates of 25.0% and 27.1%, respectively. In chapter 7, we performed a systematic review on global epidemiology of rectovaginal *E. coli* carriage. Vaginal *E. coli* carriage rates in our references groups were somewhat lower than pooled prevalences from Africa (36.0%), but higher than pooled prevalences in Asia (5.3%), Europe (13.4%), North America (12.7%) and South America (19.7%).
» Compared to their reference groups, pregnant women in our Kenyan and SA population had lower (14.3%) and higher (33.3%) prevalences of vaginal *E. coli* carriage, respectively.
» The FSW study group in Kigali had a very high prevalence of *E. coli* carriage, i.e. 70%, and will be discussed further (objective 3).

What do our findings mean?

» Vaginal carriage rates of GBS are prevalent among pregnant women from Kenya and SA but lower compared to populations from high-income countries. Considering the lack of implementation of IAP in these countries, other prevention measures to reduce vaginal GBS carriage in pregnant women are urgently needed.
» *E. coli* is the leading pathogen in EOS among preterm neonates [Stoll, 2011] and SSA has the highest rates of preterm births worldwide, with rates as high as 14.3% and 17.5% in Eastern and Southern Africa, respectively [Beck *et al.*, 2010]. Therefore, the high vaginal *E. coli* carriage prevalences in our pregnant populations urgently warrant for prevention measures to reduce these carriage rates during pregnancy to reduce transmission hence *E. coli* EOS.

10.2 Objective 2

To assess the GBS serotype distribution in different population of SSA

Background

Prevention measure to reduce neonatal GBS disease are based on two main strategies: intrapartum antibiotic prophylaxis and vaccination, discussed in chapter 3. Most GBS vaccines under development aim at eliciting protective antibodies against capsular polysaccharides (CPS), of which ten antigenically distinct CPS are known [Melin, 2013]. Vaccines under development are serotype-specific but might not be effective in SSA populations because of different serotype distribution [Johri *et al.*, 2006]. Only a few studies have documented on GBS serotype distributions of vaginal isolates in SSA. Therefore, the second objective of this thesis was to assess the GBS serotype distribution in different population of SSA.

How did we study this?

Of all women in our study, we collected vaginal swabs and inoculated these on TMB^{plus} culture plates, after 24–72h of anaerobic incubation, all biological material was harvested, frozen at – 80 °C and shipped to the Institute of Tropical Medicine, Antwerp, Belgium. Of all women found positive for vaginal GBS carriage as assessed by qPCR (see objective 1), we inoculated Limbroth culture bottles with the harvested and frozen biological material. We performed multiplex PCR directly on DNA extracts of these Limbroth cultures in order to determine to capsular serotype.

What were our major findings?

- In Kenya and SA, the most prevalent serotypes were Ia (27.3% and 34.5%, respectively), V (27.3% and 34.5%, respectively), and III (22.7% and 13.8%, respectively) (Table 15).
- In Rwanda, we found Ia (83.3%) and II (16.7%) to be most prevalent, but absolute numbers were too small to make significant conclusions.
- Overal GBS serotypes Ia, II, III, IV, V, VI, VII and VIII were prevalent.
- GBS serotypes Ib and IX were not prevelant.

In chapter 7, we discuss different factors, such as the use of molecular techniques, that might account for observed differences in our population and other populations from SSA.

TABLE 15 Studies reporting GBS serotype distribution of (recto)vaginal isolates in SSA.

Country	Year	Population	Ia	Ib	II	III
The Gambia	1994	P	19		28	6
Malawi	2011	P, HIV+, HIV-	18.2	6.2	10.3	39.0
SA	2011	P	30.1	6.7	11.3	37.3
SA	2014	P	36.2–41.4	3.5–4.6	7.2–7.5	31.3–34.9
Kenya	**2015**	**P, NP**	**27.3**			**22.7**
Rwanda	**2015**	**NP**	**83.3**		**16.7**	
SA	**2015**	**P, NP**	**34.5**			**10.3**

P, pregnant; NP, non-pregnant; [$]determined serotypes I-VI
(no differentiation between Ia and Ib); N/A, not applicable (review)

TABLE 16 GBS serotype distribution of (recto)vaginal isolates in Europa and the US.

Region	Year	Ia	Ib	II	III
Europe	2010	18.2	12.4	14.4	28.1
US	2010	26.8	8.1	10.9	24.8

Data from a systematic review

	IV	V	VI	VII	VIII	IX	NT	Reference
	3	38						[Suara, 1994][5]
	0.3	23.9	0.8		0.8		1.5	[Gray, 2011]
	3.7	10.2						[Madzivhandila, 2011]
	2.0–4.0	10.3–15.6				0.0–3.3		[Kwatra, 2014]
		27.3	**13.6**	**4.5**	**4.5**			[Cools, 2016]
								[Cools, 2016]
	13.8	**31.0**		**6.9**	**3.4**			[Cools, 2016]

	IV	V	VI	VII	VIII	IX	NT	Reference
	3.7	14.9	0.6	0.6	0.6			[Ippolito, 2010]
	1.0	15.0	0.3	0.0	0.2			[Ippolito, 2010]

What do our findings mean?

» Currently, vaccines in clinical trials phase II target serotypes Ia, Ib, and III (trivalent vaccine) or serotype III (monovalent vaccine). Assuming equal transmission rates of GBS serotypes from mother to fetus/neonate and 100% efficacy of vaccines, in our Kenyan population, immunoprophylaxis with the trivalent and monovalent vaccine would not be protective in 50.0% and 77.3% of cases, respectively. These percentages are even higher in our SA study population, i.e. 55.2% and 89.7%.

» Compared populations from Europe and the US (Table 16), we found relatively high prevalences of serotypes IV, VI, VII (shown for this first time in SSA) and VIII in the Kenyan and SA population (VI, 13.6%; VII, 4.5%; VIII 4.5% in Kenya, and IV, 13.8%; VI, 6.9%; VIII, 3.4% in SA), but did not detect any serotype Ib, with prevalences of 8.1% and 12.4% in the US and Europe, respectively [Ippolito *et al.*, 2010]. This might warrant development of protein based vaccines that are not serotype specific and cover all GBS strains.

10.3 Objective 3
To determine risk factors for vaginal GBS and *E. coli* carriage in populations from SSA

Background

Efforts to prevent EOS, i.e. IAP and development of vaccines, have been focusing mainly on GBS and high-income countries [Melin, 2013]. Vaccines are still under development and both IAP and vaccination strategies have their drawbacks, discussed in chapter 3. IAP is not effective against *E. coli* EOS, LOS, and other adverse perinatal outcomes related to GBS [Regan *et al.*, 1996; Ohlsson *et al.*, 2014]. Furthermore, to our knowledge, national guidelines for IAP are lacking in SSA countries, and might be difficult to implement. In a large randomized controlled trial, the use of the wide-spectrum microbicide chlorhexidine as an alternative prevention measure has been shown to have no effect on the vertically transmission of GBS (55% and 54% transmission rate in the chlorhexidine and control group, respectively) [Cutland *et al.*, 2009].

Therefore, the third objective of this thesis was to identify risk factors for vaginal GBS and *E. coli* carriage that can be implemented in public health recommendations or interventions to reduce neonatal sepsis.

How did we study this?

Risk factors for GBS and *E. coli* carriage were identified using logistic regression analysis. The variables investigated were sociodemographic factors, sexual behavior, reproductive history, vaginal practices, vaginal signs and symptoms, and microbial characteristics other than GBS and *E. coli* (see Tables 4 and 5 in Chaper 7).

What were our major findings?

Independent risk factors for vaginal GBS carriage

- **Recent vaginal intercourse** (AOR, 2.63; 95% CI, 1.35–5.15; p=0.005) Women who had recent vaginal sex, defined as the morning or evening before the study visit, were more than twice as likely to carry GBS vaginally than women who did not. Other papers reporting conflicting results on the relationship between vaginal GBS carriage and sexual behavior, and the argumentation why we think that vaginal intercourse only induces a brief temporal colonization of the vagina are discussed in chapter 7.
- **Vaginal washing** (AOR, 2.26; 95% CI, 1.16–4.37; p=0.016) Women engaging in the practice of washing inside the vagina were more than twice as likely to be colonized with GBS compared to women not washing inside the vagina.

Independent risk factors vaginal E. coli carriage

- **working as a FSW** (AOR, 7.83; 95% CI, 2.88–21.30; p<0.001) Women who were self-acknowledged sex workers were nearly eight times as likely to carry *E. coli* vaginally. We could not explain this high prevalence in this study group by any of the sexual behavioral or other parameters assessed. In chapter 7, we discuss why we hypothesize that unreported anal intercourse is not unlikely to, at least partly, explain these observations.
- **cervical ectopy** (AOR, 1.64; 95% CI, 1.01–2.68; p=0.046) Twenty two percent of women without cervical ectopy were *E. coli* carriers as opposed to 35.2% of women with cervical ectopy.

Vaginal carriage of GBS and E. coli were positively associated

Importantly, we found that the two major causes of EOS, i.e. GBS and *E. coli*, were independent risk factors for one another (AOR, 2.05; 95% CI, 1.09–3.83; p=0.025).

Vaginal carriage of GBS and/or E. coli were independently associated with disturbances of the vaginal microbiome and C. albicans. Vaginal pH was not associated with vaginal presence of GBS

» GBS was negatively associated with bacterial vaginosis as assessed by Nugent score (AOR, 0.43; 95% CI, 0.21–0.88; p=0.022).
» *E. coli* was positively associated with an intermediate Nugent score (AOR, 2.61; 95% CI, 1.15–5.94; p=0.023)
» GBS was independently and positively associated with the vaginal *Candida albicans* as assessed by qPCR (AOR, 3.25; 95% CI, 1.50–7.06; p=0.003).
» GBS was not associated with vaginal pH.

Studies confirming or not confirming these findings are discussed in chapter 7.

What do these findings mean?

» In populations suffering from the highest burden of neonatal sepsis and intrapartum maternal sepsis, prevention of maternal GBS and *E. coli* carriage might be accomplished by advocating behavioral changes such as abstinence of sexual intercourse and vaginal washing during late pregnancy, e.g. via counseling in family planning facilities.
» Inclusion of vaginal carriage of *C. albicans*, *E. coli* and GBS and of the presence of cervical ectopy in a risk- and/or screening-based administration of IAP should be explored.
» The screening for *E. coli* itself also merits further investigation because of its role as a major EOS causative agent for which currently no prevention measures are taken, nor in low-income, nor in high-income countries [Stoll *et al.*, 2011]. In this context, the presence of cervical ectopy – a risk factor for vaginal *E. coli* carriage – should be further investigated.
» We found a higher vaginal pH and absence of lactobacilli not related to higher carriage rates of GBS, which contradicts other report [Leclair *et al.*, 2010; Le Doare and Heath 2013; Kubota *et al.*, 2002].

10.4 Objective 4

To determine to which extent HIV infection in women is associated with rectovaginal GBS carriage by means of systematic review and meta-analysis

Background

SSA remains the most heavily HIV affected region with 67% of the global burden [Kilmarx, 2009]. HIV infection in women has been associated with increased rates of genital tract infections [Vandepitte *et al.*, 2012; Umeh *et al.*, 2010; Weiss *et al.*, 2001]. Therefore, we wanted to assess to which extent HIV women in our study population were at risk for vaginal GBS colonization.

In our study population of HIV positive women, we found that none of these women was vaginally colonized with GBS. This was a significant finding in univariate analysis, but not after correcting for antibiotic use. Indeed, most of the HIV positive women (26/30) received prophylactic cotrimoxazole - largely effective against GBS [Joachim *et al.*, 2009]- and which is a probable explanation for our observations of the lack of GBS positive women in our HIV-positive group but leaving us with unresolved issues. Therefore, our fourth objective was to clarify to which extent HIV infection in women is associated with rectovaginal GBS carriage by means of systematic review and meta-analysis.

How did we perform this systematic review and meta-analysis?

We searched the MEDLINE, Web of Science, and EMBASE databases for bibliographic references using all combinations of search terms listed in columns A and B below.

A	B
Streptococcus agalactiae, *S. agalactiae*, Group B *Streptococcus*, GBS, Group B streptococci, early-onset disease, early-onset sepsis, late-onset disease, late-onset sepsis, neonatal sepsis, neonatal septicemia, neonatal pneumonia, neonatal meningitis	Human immunodeficiency virus, HIV, HIV-1, AIDS

We included studies that assessed the association between rectovaginal GBS colonization and HIV-status in adult women. We only included studies that reported a sample size and a measure of effect for the associations assessed, or did provide the data to calculate the latter.

Studies were scored for quality and risk of bias using a study quality appraisal tool, based on the Newcastle-Ottawa Scale [Wells *et al.*, 2013]. We compared the odds of rectovaginal GBS carriage in HIV-positive to HIV-negative women, using unadjusted counts.

What were our major findings?

- Based on thirteen studies, representing a total of 10,105 women, we found no significant association between HIV infection status and rectovaginal GBS carriage (OR 1.09; 95% CI 0.82–1.44; p=0.55).
- Most studies had a medium or high risk of bias for study group selection, showed lack of adequate statistical power, and did not sample and culture according to CDC guidelines for GBS detection.
- Heterogeneity among the studies was substantial (I^2 = 68.7%), publication bias was not unlikely and important possible confounding factors, such as antibiotic prophylaxis and bacterial vaginosis, were not taking into account in most studies.

What do these findings mean?

- Most of the studies suffered from major limitations and we consider the currently available evidence weak and call for further study.
- Well-designed studies are urgently needed to clarify the relation between HIV-positivity and GBS carriership, the most important risk factor for GBS EOS.
- These studies should incorporate quantitative and serotype-specific (molecular) GBS detection methods to clarify if HIV-status is related to vaginal GBS load (the risk for EOS increases with heavier vaginal GBS colonization [Verani *et al.*, 2010]) and to more virulent serotypes.

10.5 Objective 5

To determine if neonates born from HIV-infected women are at increased risk for GBS EOS and LOS by means of systematic review and meta-analysis

Background

Infants born to HIV-infected mothers have increased rates of infectious morbidity and mortality compared to non-exposed infants, even if they remain HIV-uninfected [Madhi *et al.*, 2011; Epalza *et al.*, 2010]. Since the first study reporting an increased risk of GBS neonatal sepsis in HIV-exposed neonates [Epalza *et al.*, 2010], evidence for this association has been accumulating. Therefore, the fifth objective of this thesis was to assess to which extent neonates born to HIV-infected mothers were at increased risk for GBS neonatal disease by means of systematic review and meta-analysis.

How did we perform this systematic review and meta-analysis?

We followed the same strategy as summarized in objective 4, exept that for this meta-analyses, we compared the odds of GBS neonatal sepsis, GBS EOS and GBS LOS in HIV-exposed neonates compared to HIV-unexposed neonates. Neonatal HIV-exposure was defined as neonates who were born to HIV-infected mothers, regardless of the neonatal HIV-status at birth.

What were our major findings?

» Based on outcomes from 64608 births from HIV-infected mothers and 187443 births from HIV-uninfected mothers, HIV-exposed neonates were more likely to have neonatal GBS disease compared to unexposed neonates (OR, 2.39; CI, 1.31–4.38; p=0.005).
» When analyzing cases of GBS EOS and GBS LOS separately, this increased risk pertained to LOS only. (EOS; OR, 1.31; 95% CI, 0.84–2.04; p=0.240; LOS; OR, 4.43; 95% CI, 1.81–10.85; p=0.001).

We integrated several factors associated with HIV infection in pregnant women or mothers into a model that might explain why infants born to HIV-infected mothers were at increased risk for GBS LOS but not GBS EOS. In the case of

GBS EOS, the factors that might increase or decrease the risk for GBS EOS might balance out, while in the case of GBS LOS, the net result may be an enhanced risk. The model is explained and argued in detail in Chapter 8, and takes into account the following parameters, summarized below, that might decrease/increase EOS/LOS (Figure 11):

Factors that might decrease EOS GBS rates in HIV-exposed neonates

» **antibiotic prophylaxis (co-trimoxazole)** is widely used in HIV-positive pregnant women as recommended by the WHO [WHO, 2006], and is largely effective against GBS [Joachim *et al.*, 2009], consequently likely to be responsible for lower GBS rectovaginal carriage rates in HIV-infected pregnant women, and consequently might reduce the risk for the GBS EOS but not for the GBS LOS
» **elective caesarean section** defined as a caesarean delivery before labor and before rupture of membranes, is increasingly used in HIV-infected women because of proven reduced risk of mother-to-child transmission of HIV [Jamieson *et al.*, 2007], and might reduce the risk for EOS as GBS is thought to be mainly transmitted *in utero* after rupture of the membranes.

Factors that might increase LOS GBS disease in HIV-exposed neonates

» **breastfeeding** might not be the preferred choice of infant feeding or might be stopped early by HIV-positive mothers, because of fear of HIV transmission through breast milk, which might lead to a hampered passive antibody-mediated protection, likely to have more effect on the incidence of LOS, considering the fact that most EOS cases are already septic at birth or become septic shortly after birth.

Factors that might increase EOS and LOS GBS rates in HIV-exposed neonates

» **immune dysfunction and perturbations** contributed to HIV infection has been shown in HIV-exposed neonates and HIV-infected mothers. Protective antibodies are reduced in both mother and neonates (even at 16 weeks) [Dangor *et al.*, 2015; Le Doare *et al.*, 2015], and transplacental transfer of protective antibodies and hematopoietic cytokines is reduced in HIV-infected mothers [Epalza *et al.*, 2010; Dangor *et al.*, 2015; Le Doare *et al.*, 2015], lower antibody-mediated complement fixation in HIV-infected mothers [Le Doare *et al.*, 2015].

» **antiretroviral therapy** is used to prevent mother-to-child transmission of HIV, but nucleoside reverse transcriptase inhibitors crossing the placenta might be responsible for decreased immune cell counts in HIV-exposed uninfected infected infants [Epalza *et al.*, 2010].

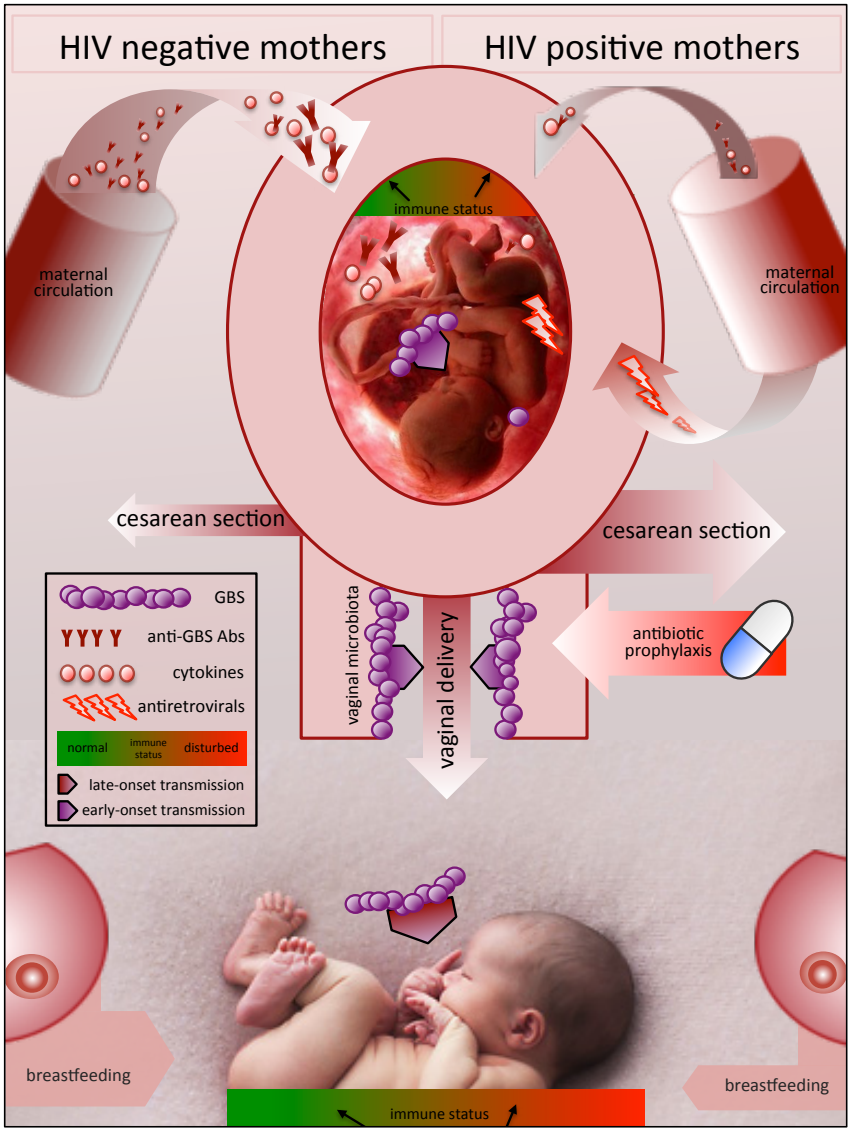

FIGURE 13

Factors that might increase or decrease GBS EOS rates in HIV-exposed neonates

» **rectovaginal GBS carriage** is the single most important risk factor for GBS EOS, if not, a prerequisite. The extent to which HIV infection is associated with rectovaginal GBS carriage, is discussed in detail in chapter 8 and summarized in objective 4.

What do these findings mean?

» The evidence that HIV-exposed infants are at increased risk for GBS LOS is convincing and accumulating.
» Public health interventions, especially in SSA, where the burden of HIV and neonatal sepsis is the highest, are urgently needed.

10.6 Objective 6
To characterize the vaginal mucosal cytokine pattern associated with asymptomatic vaginal GBS carriage

Background

Most current GBS vaccines aim to protect the fetus or neonate against neonatal GBS disease by immunoglobuline G (IgG) transferred transplacentally from maternal serum [Kasper *et al.*, 1996; Heath *et al.*, 2016]. However, fetal IgG concentrations increase with gestational age [Boyer *et al.*, 1984] and might not be high enough to protect premature newborns, at high risk for EOS [Hordnes *et al.*, 1996].

Vaginal mucosal immunity to GBS is an important first-line defense against GBS EOS and some GBS-related adverse perinatal outcomes, as vaginal colonization is the first step in pathogenesis [Simonsen *et al.*, 2014]. Therefore, an optimal vaccine should also induce vaginal mucosal immunity [Shen *et al.*, 2000].

However, the human vaginal mucosal immune response to GBS colonization of the vaginal tract remains to be elucidated. Cytokine responses to GBS have only been characterized using *in vitro* cell-culture studies and murine models.

Therefore, or sixth objective was to characterize the vaginal mucosal cytokine pattern associated with vaginal GBS colonization in populations at the highest need for better prevention measures against neonatal GBS disease.

How did we study this?

Cervicovaginal lavage (CVL) samples were obtained from the women in our study. In these CVLs, the concentrations of different cytokines (interleukin (IL)-1α, IL-1RA, IL-1β, IL-6, IL-8 and IL-12, MIP-1β, interferon (IFN)-γ–induced protein (IP-10), granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF)) and elafin were measured by means of ELISA. Differences in cytokine patterns were analyzed by means of MANOVA and ANOVA, and linear correlations between concentrations of GBS in carriers and the different immune parameters were assessed by calculating the Spearman correlations.

What were our major findings?

» **IL-8 was elevated in GBS carriers and correlated with GBS loads**

IL-8 is a potent chemotactic and activating factor for neutrophils, essential in the responce to pathogens. Epithelial cells of the female reproductive tract act as key initiators of innate and adaptive immunity by secreting cytokines such as IL-8 [Agrawal *et al.*, 2009] and there is general consensus that IL-8 is the major chemoattractant for neutrophils in the different FRT tissues [Weissenbacher *et al.*, 2014]. Numerous *in vitro* studies have shown that GBS induced IL-8 transcription or IL-8 expression after stimulation of different human cell types. These studies are discussed in chapter 9.

» **IL-12 and IP-10 were elevated in GBS carriers and both correlated with GBS loads**

IL-12 is a pro-inflammatory cytokine that induces the production of IFN-γ and favours the differentiation of T helper 1 (T_H1) cells [Trinchieri *et al.*, 2003]. IP-10 is a pleiotropic cytokine induced by IFN-γ, the T_H1 response cytokine signature, and chemoattracts monocytes/macrophages, dendritic cells, NK cells, cytotoxic T cells and T_H1 cells towards inflamed and infected sites [Liu *et al.*, 2011].

In vitro studies have shown that GBS induced IL-12 production in different human cell types and animal models have documented the importance of IL-12 in the protection of the neonate against GBS infections. These studies are discussed in chapter 9.

What do these findings mean?

» This first clinical study characterizing the vaginal mucosal cytokine patterns associated with GBS colonization, confirms findings of *in vitro* cell models and murine models that document elevated levels of IL-8 and IL-12 after GBS challenge.
» Our observations of elevated concentrations of IL-12 and IP-10 point to a local T_H1-associated cytokine environment in asymptomatically vaginally colonized with GBS.
» This T_H1-cytokine environment during vaginal GBS colonization might profoundly influence the isotype distribution of naturally acquired antibodies by isotype switching. T_H1-related cytokines are important to induce IgG1, IgG2 and IgG3 isotypes that can cross the placenta, but inhibit IgA antibodies important for mucosal immunity. These findings may be important for the development of vaccination strategies.
» IL-12 might be considered as an adjuvans in GBS vaccines, as our results seem to point to a protecting effect of IL-12 in controlling mucosal colonization of GBS and the use of IL-12 as a vaccine adjuvant to promote T_H1 responses is of great interest [Romani *et al.*, 1997].

10.7 Future perspectives

One million neonates die each year because of neonatal sepsis, and Sub-Saharan Africa (SSA) suffers from the highest rates worldwide [Capan *et al.*, 2012; Stoll *et al.* 1998; Lawn *et al.*, 2005]. Early-onset sepsis (EOS) occurs in the first week of life while late-onset sepsis (LOS) occurs between week 1 and month 3 of life [Schuchat *et al.*, 1998].

EOS poses unique opportunities for prevention because transfer of a single species from the maternal genitourinary tract to the neonate before/during delivery is a prerequisite.

In high-income countries, the leading causes of EOS are Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) and *Escherichia coli*.

Efforts to prevent EOS in high-income countries have been focusing on GBS only, based on two strategies: the culture-based or risk-based intrapartum antibiotic prophylaxis (IAP) and the development of GBS vaccines.

Although the highest rates of neonatal sepsis occur in SSA, there is very limited epidemiological data on (recto)vaginal carriage rates of GBS and *E. coli* in SSA. These data are necessary to design and implement EOS prevention

strategies. In our study population, pregnant women from Kenya and South-Africa had vaginal GBS carriage rates of 14.3% and 10.0%, respectively; and vaginal *E. coli* carriage rates of 14.3% and 33.3%, respectively.

Further research should identify not only document further carriage rates for GBS and *E. coli* in different populations of SSA, but also identify pathogens responsible for EOS and LOS. Indeed, in contrast to high-income countries where it is well documented that GBS is the most common cause of EOS, while *E. coli* is the most common cause of mortality related to EOS; in SSA, - although GBS and *E. coli* are regarded as major pathogens causing EOS -, there is a lack on data on etiological agents. A limited number of studies report that GBS is a major cause of EOS but is preceded by *E. coli*, *Klebsiella pneumoniae* and *Staphylococcus areus* [Newton and English 2007; Zaidi *et al.*, 2009]. Future research regarding (recto)vaginal organisms and EOS should include these organisms in the study protocols.

Furthermore, the limited number of studies carried out in SSA are hospital-based. According to UNICEF, most of deliveries in SSA take place at home and only half of the pregnant women in SSA in 2015 had skilled attendance at birth. Therefore, the target population for future studies on neonatal sepsis in SSA should also include pregnant women delivering at home.

IAP has reduced GBS EOS in high-income countries [Schrag, 2000], but it has had no effect on EOS caused by other pathogens, GBS LOS, and other adverse perinatal outcomes related to GBS.

In SSA settings, an IAP strategy could be considered, although in modified form compared to the IAP implemented in many high-income countries. Exploring the possibilities for including not only GBS, but also or only *E. coli, K. pneumoniae* and/or *S. aureus* should be considered, as the latter pathogens may be more important than GBS.

Studies should document transmission (rates) of *E. coli, K. pneumoniae* and/or *S. aureus* from the maternal vagina and/or rectum to the neonate. These studies should use typing techniques, and transmission should not be merely based on the detection of the same pathogen in mother and neonate. Longitudinal studies should clarify if a late-gestational (i.e., the time point in pregnancy of screening for GBS in high-income countries) positive screening of a *E. coli, K. pneumoniae* or *S. aureus* is predictive for (recto)vaginal carriage of the same pathogen at birth. Furthermore, vaginal sampling, rectal sampling and rectovaginal sampling, should be compared to identify optimal sampling technique – if any – for these different pathogens.

We have shown that vaginal carriage of *E. coli*, GBS and *Candida* are positively associated, which might offer unique opportunities by screening for the three organisms, as a culture-based and risk-based screening would be intertwined. For example, a screening positive for *E. coli* and *Candida* but negative for GBS, would still imply increased odds for GBS carriage.

For IAP strategies to be feasible in resource-limited settings, administration of antibiotics other than the intravenous route should be considered.

Vaccination of pregnant women represents an attractive alternative to protect neonates against GBS EOS, LOS and GBS-related adverse perinatal outcomes [Heath, 2016; Melin, 2013]. Most GBS vaccines under development aim at eliciting protective antibodies against capsular polysaccharides (CPS), of which ten antigenically distinct CPS are known and offer only a serotype specific protection. Hence, a vaccine suitable for American or European populations may not be suitable for populations in SSA. For this reason, data on GBS serotype distributions in different populations in SSA are urgently needed. We found that GBS serotypes Ia, V and III were most prevalent, as in most high-income countries, whereas serotypes VI, VII and VIII were more prevalent in our study population and we did not detect serotype Ib, prevalent in high-income countries. Immunoprophylaxis with phase II GBS vaccines would not protect the majority of women against carriage in our study population.

All GBS vaccines under current development aim at eliciting maternal serum antibodies that protect the fetus or neonate after crossing the placenta [Kasper, 1996; Heath, 2016]. However, an optimal vaccine should also induce mucosal immunity [Shen, 2000], as transplacental IgG is insufficiently early in gestatation [Hordnes, 1996].

We aimed at elucidating the human vaginal mucosal immune response to vaginal GBS colonization because knowledge of protective vaginal mucosal cytokines in response to GBS could provide a template for the design or refinement of more appropriate and effective vaccines [Morel and Turner 2010].

Our study was the first clinical study characterizing the vaginal mucosal cytokine patterns associated with GBS colonization, and we confirmed findings of *in vitro* cell models and murine models that document elevated levels of IL-8 and IL-12 after GBS challenge. Our observations of elevated concentrations of IL-12 and IP-10 point to a local TH1-associated cytokine environment in women asymptomatically vaginally colonized with GBS. As our results point to a protecting effect of IL-12 in controlling mucosal colonization of GBS, IL-12 might be considered as an adjuvans in GBS vaccination.

In future GBS vaccination trials in SSA, two infections might profoundly influence vaccine efficacy and immunogenicity. Helminths, multicellular parasitic worms that are highly endemic in SSA, have been shown to confound vaccine efficacy [Urban *et al.*, 2007]. Helminths are potent inducers of a TH2 response and it has been shown that worm antigens induce amplification of the TH2 response to bystander antigens [Elias *et al.*, 2007]. Future trials assessing GBS vaccine efficacy in countries endemic for these helminthes should take this into consideration and might consider to overcome the possible effects of immunomodulation by helminthes by the use of adjuvants [Elias *et al.*, 2007]. Another infection highly affecting SSA is HIV. Recently, it has been shown in phase II trails that GBS vaccines were less immunogenic in HIV-infected women compared to HIV-uninfected women.

Vaccination trials so far assessed the serum concentration of CPS specific antibodies. However, knowledge on the effect of these vaccines on the concentration of antibodies and GBS at the vaginal mucosa would allow us to further understand the mucosal immune response induced by current vaccines.

Furthermore, vaccination at rectal, vaginal or oral mucosa might be explored given the possible protective benefits early in gestation, as GBS has also been associated with spontaneous abortion and stillbirth.

In settings where future implementation of IAP and/or vaccination for the prevention of GBS EOS is unlikely and were prevention measure for other EOS related pathogens such as *E. coli* are more urgently needed, risk factors for (recto) vaginal GBS and/or *E. coli* carriage that are easily modifiable might be considered in prevention strategies that aim to change behavior. Such risk factors identified in our study include practices of intravaginal washing/douching/drying, recent sexual intercourse and possibly anal intercourse. Further research should explore of behavioral change, e.g. through counselling in family planning clinics, youth/adolescent centers or health care centers, - if found feasible - could reduce the (recto)vaginal carriage rate of GBS and/or *E. coli* in late pregnancy.

We could not assess the role of HIV as a possible risk factor for (recto)vaginal carriage of GBS and/or *E. coli* in our clinical study because nearly all HIV-infected women were on antibiotic prophylaxis. We therefore conducted a systematic review and meta-analysis on the association between HIV-infected women and (recto)vaginal GBS carriage; and on the association between neonatal HIV-exposure and GBS EOS / LOS.

We did not found an association between HIV infection and rectovaginal carriage of GBS, but studies included were poor. Well-designed studies that asses the association between HIV-infection in women and (recto)vaginal carriage of not only GBS, but also other major pathogens related to neonatal sepsis should be performed, and preferable document (recto)vaginal loads.

We found convincing evidence that HIV exposure of neonates is associated with higher odds of GBS LOS but not GBS EOS. Health workers in the clinical practice should be aware of these increased risks and health policy makers should advocate for a better follow-up of neonates born to HIV-infected mothers.

The model explaining our findings on the influence of neonatal HIV exposure and GBS neonatal sepsis integrates breastfeeding, mode of delivery, antibiotic prophylaxis and reduced immune status; and should be further explored to identify opportunities to reduce the risk for GBS LOS. Furthermore, given our findings for GBS, the relation between HIV-exposure of neonates of LOS due to other pathogens than GBS, should be further investigated.

Most studies that investigate GBS and/or *E. coli* in relation to adverse pregnancy outcomes use culture-based techniques for the detection of GBS and/or *E. coli* in (recto)vaginal samples, as do most hospitals were screening-based IAP is implemented to prevent GBS EOS. However, in general, molecular techniques such as qPCR and deep-sequencing are increasingly used in a clinical or research setting to detected infections or to study (vaginal) microbiota. Regarding research on the vaginal microbiome, deep-sequencing studies are increasingly published. These deep-sequencing studies are usually performed to describe the vaginal microbiome in different populations, but caution is warranted as these often lack the resolution to detect *E. coli* and GBS.

For research, qPCR, possibly in a multiplex format including GBS, *E. coli*, *S. aureus*, and/or *K. pneumoniae* might be the most cost-effective technique for studying these species in relation to adverse pregnancy outcomes.

However, for obtaining data on antibiotic sensitivities of the different pathogens, and for typing studies that document transmission, isolated strains are still needed, necessitating culturing. Furthermore, typing of isolates will allow to document if certain 'pathotypes' of *E. coli* (and other pathogens) associated with the cervicovaginal mucosa and/or downstream pathological anatomical locations (such as chorion, amnion, amniotic fluid) exist, as is the case for e.g. *E. coli* related to urinary tract infections.

In resource-limited settings, the use of Gram-stained vaginal smears for the detection of Gram-positive streptococci (including GBS), Gram-positive staphylococci (including *S. aureus*), Gram-negative rods (include *E. coli* and *K. pneumoniae*) and *Candida* could offer possibilities to be used in research and/or clinical setting.

Regarding adverse pregnancy outcomes, research has mostly focused on EOS. However, stillbirth is one of the most common causes of pregnancy loss worldwide, and in spite of advances in perinatal medicine, an estimated 3.2 million cases occur each year [McClure and Goldenberg 2009]. Few studies have shown GBS and *E. coli* as causative organisms in stillbirth. Future longitudinal studies should include investigation on the role of infections in stillbirths.

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