**Molecular Characterization of *Streptococcus agalactiae* Isolates from Human Cases of Invasive Disease Collected in Alberta, Canada: 2003-2013**

by

Areej Alhhazmi

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Laboratory Medicine and Pathology

University of Alberta

© Areej Alhhazmi, 2018

**ABSTRACT**

Capsular polysaccharide (CPS) surrounding the Group B streptococcus (GBS) is a major virulence factor for GBS. There are 10 recognized CPS types (Ia, Ib and II-IX). A GBS isolate is non-typeable (NT) when CPS cannot be identified as one of the 10 CPS types. All genes required for CPS synthesis are found on the GBS *cps* operon, which contains a highly variable CPS-determining region (*cpsG-cpsK*). Sialic acid (sia) is located at the terminal end of the side chain of all known GBS CPS types. In GBS, CovRS is an important regulatory system that controls GBS virulence factors, including the capsule.

Invasive GBS (iGBS) from Alberta, Canada between 2003-2013 were analyzed to determine prevalence rates of GBS disease, CPS type distribution, and antimicrobial susceptibility patterns. Over the 11 years, a noticeable increase in the rate of neonatal GBS disease as well as adult infections was documented including CPS Ia, Ib, III, and IV. The increased rates of iGBS disease have been accompanied by elevated rates of erythromycin and clindamycin resistance.

A comprehensive CPS typing system was developed to detect sialic acid on the GBS cell surface followed by a genotypic PCR CPS typing assay. Sialic acid can be bound to commercially available lectins such as slug *Limax flavus* (LFA) lectin. Biotinylated LFA-streptavidin-peroxidase complex was used in EIA and dot blot assays to detect sialic acid. That was followed by a PCR typing scheme targeting the serotype-determining region of the *cps* locus for Ia, Ib, and II-IX. The combination of sialo-CPS lectin binding and duplex real-time PCR typing assays provided a simple and reliable tool for CPS expression confirmation and CPS genotype. This system

enabled the characterization of GBS CPS Ia, Ib, II-IX, thereby reducing the rate of detection of NT isolates.

Two groups of GBS NT isolates were studied, isolates without surface sialic acid sia (-) and isolates with surface sialic acid sia (+). NT sia (-) isolates were characterized by assaying *in vitro* virulence changes and identifying *covR/S* mutations potentially responsible for altered virulence phenotypes. NT sia (+) isolates were investigated to identify genetic changes in the *cps* operon that failed to identify as one of the 10 CPS types yet expressed capsule. A subset of non-capsule producing and sia (-) isolates identified a series of *covR/S* mutations not only affecting CPS production but also an array of other phenotypic properties. Of the NT CPS expressing strains and sia (+), two isolates were identified as a subtype CPSIIa encoded by CPSIIa gene operon. One isolate was found to encode a novel CPS type, CPSIIa/V hybrid.

Whole genome sequence (WGS) technologies elucidated the complete genome of a ST 1/serotype V PLGBS13 strain recovered from an adult case in Alberta, Canada in 1997. Polymorphism and recombinational studies from four iGBS ST1 isolates revealed that the genetic diversity among ST 1/serotype V GBS isolates is mainly driven by small genetic changes such as insertions, deletions or mutations. However, the genetic diversity of ST 1 GBS isolates that have serotypes other than serotype V (CPS type IIa and cpsIIa and V hybrid) is mainly explained by recombination in the *cps* gene cluster and few genes.

In conclusion, the research presented in this thesis revealed that Alberta has experienced an increase in the incidence of GBS disease. A dual GBS typing system involving phenotypic and genotypic assays was developed. These assays were reliable, sensitive and specific and enable the characterization of GBS CPS Ia, Ib, II-IX, thereby reducing the rate of

detection of NT isolates. A series of *covRS* mutations among NT sia (-) isolates was identified. Of the NT CPS expressing strains, two subtypes of CPSII do exist, CPSIIa and CPSIIb each encoded by *cpsIIa* and *cpsIIb* gene operons, respectively. Sia (+) detection assay allows for the discovery of novel CPS producing strains such as the CPSIIa/V hybrid. This work also contributed to the understanding of the genetic diversity among ST1 isolates commonly found among adult population.

## PREFACE

The experiment design referred to in chapters 2, 3, 4 and 5 were designed by myself. The data analysis and concluding analysis in chapters 2, 3, 4 and 5 are my original work. Chapter 2 of this thesis has been published as A. Alhhazmi, D. Hurteau, and G. J. Tyrrell, 2016, "Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013," Journal of Clinical Microbiology, vol. 54, issue 7, pages 1774-1781 and A. Alhhazmi, D. Hurteau, and G. J. Tyrrell, 2017, " correction for Alhhazmi etal., Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013," Journal of Clinical Microbiology, vol. 55, issue 1, pages 342-343. I was responsible for the data collection, performing experiments and analyses as well as the manuscript composition. D. Hurteau assisted with the data collection and GBS typing. G.J. Tyrrell was the supervisory author and was involved with concept formation and manuscript composition, assisted with the data collection, and contributed to manuscript edits. Chapter 3 of this thesis has been published as A. Alhhazmi, A. Pandey and G. J. Tyrrell, 2017, "Identification of Group B Streptococcus Capsule Type by Use of a Dual Phenotypic/Genotypic Assay," Journal of Clinical Microbiology, vol. 55, issue 11, pages 00300-17. I was responsible for the experiment design, the data collection, performing experiments and the analysis as well as the manuscript composition. A. Pandey assisted with performing experiments. G.J. Tyrrell was the supervisory author and was involved with concept formation and manuscript composition and edits. Chapter 4 of this thesis has been accepted for publication as A. Alhhazmi and G. J. Tyrrell, "Phenotypic and molecular analysis of nontypeable Group B streptococci: identification of cpsIIa and hybrid cpsIIa/cpsV Group B streptococcal capsule gene clusters," Journal of

Emerging Microbes & Infections, in press. I was responsible for the experiment design, the data collection, performing experiments and the analysis as well as the manuscript composition. G.J. Tyrrell was the supervisory author and was involved with concept formation and manuscript composition and edits. Chapters 1, 5 and 6 of this thesis are original works by A. Alhhazmi.

**ACKNOWLEDGMENTS**

The completion of this project would not have been possible without the kindness, knowledge, generosity, resources, and support of a great number of people. I would like to thank first and foremost, my supervisor, Dr. Gregory Tyrrell, for his guidance and support. His experience, knowledge, and willingness to help have been a greatly appreciated. I am also extremely grateful to the members of my committee, Dr. Linda Chui and Dr. Bart Hazes for their input and interest in this research. I would also like to say thank you to whom I worked with on a regular basis. I would like to express my gratitude to LeeAnn Turnbull for her technical expertise in the laboratory This project would not have been possible without her assistance. I also would like to thank the members of the Tyrrell Laboratory who were involved in one way or another in my project: Amy Delorme, Faisal Hirji, and Khalida Siddiqua I would like to thank the members of the Dr. Xiao-Li Pang laboratory for their assistance in my project Min Cao, Dr. Ran Zhuo, Dr. Maria Hasing Rodriguez, Dr. Yuanyuan Qiu and Chen Liu. I would like to thank my parents, husband, my sons, sisters, and brothers, for their unconditional love, encouragement and support of my educational pursuits. Thanks for being there for me, even during some of the more stressful times! I would like to thank "Taibh University" for providing financial support. Lastly, I would like to thank Sarah Fatani and Deema Alfareeh for being my best friends. I could not have done it without all of you!

# Table of Contents

## LIST OF TABLES

**Chapter 2**



**Chapter 3**

**Chapter 4**



**Chapter 5**

## LIST OF FIGURES

**Chapter 1**



**Chapter 2**



**Chapter 3**

**Chapter 6**

## LIST OF ABBREVIATIONS

| | |
|---|---|
| *aad* | streptomycin aminoglycoside 6-adenyltransferase gene |
| *adhP* | alcohol dehydrogenase gene |
| Alp (*alp*) | alpha-like protein (gene) |
| α | alpha |
| *aph* | aminoglycoside 3'-phosphotransferase gene |
| AR | antimicrobial resistance |
| *atr* | aminoacid transporter gene |
| β | beta |
| beta-h/c (*cyl*) | beta-hemolysin/cytolysin (gene) |
| BibA (*bibA*) | GBS immunogenic bacterial adhesin protein (gene) |
| BLAST | basic local alignment search tool |
| bp | base pair |
| BSA | bovine serum albumin |
| °C | degrees Celsius |
| C5a | human complement factor C5a |
| CAMP (*cfb*) | Christie–Atkins–Munch-Petersen factor (gene) |
| CC | clonal complex |
| CFU | colony forming unit |
| cMLSB | constitutive macrolide, lincosamide, and streptogramin B |

| | |
|---|---|
| Cov (*cov*) | control of virulence (gene) |
| CPS (*cps*) | capsular polysaccharide serotype (gene) |
| CRM | cross-reacting material |
| Csr | capsule synthesis regulator |
| C-terminal | carboxy terminal |
| CT | cycle threshold |
| CMP-sialic acid | cytidine-5'-monophosphate N-acetylneuraminic acid |
| DLVs | double-locus variants |
| DNA | deoxyribonucleic acid |
| EIA | enzyme immunosorbent assay |
| EOD | early onset disease |
| *erm* | erythromycin ribsome methylase gene |
| Fbs (*fbs)* | fibrinogen-binding protein (gene) |
| GAS | group A streptococci |
| GBS | group B streptococci |
| *glcK* | glucokinase gene |
| *glnA* | glutamine synthetase gene |
| HGT | horizontal gene transfer |
| HRP | horseradish peroxidase |
| hrs | hours |
| Hvg *(hvg)* | hypervirulent adhesin (gene) |
| IAP | intrapartum antibiotic prophylaxis |

| | |
|---|---|
| iGBS | invasive GBS |
| IgG | immunoglobulin |
| iMLSB | inducible macrolide, lincosamide, and streptogramin B |
| IS | insertion sequence |
| LB | Luria-Bertani |
| LFA | *Limax flavus* lectin |
| *linB* | lincosamide nucleotidyltransferase gene |
| Lmp | laminin binding protein |
| LOD | late onset disease |
| LSA | lincosamides, streptogramin A |
| LPXTG | leucine-proline-any amino acid-threonine-glycine |
| M phenotype | macrolides and streptogramin B resistance and lincosamide susceptibility |
| *mef* | macrolide efflux gene |
| mg | milligram |
| µg | microgram |
| MGE | mobile genetic lement |
| MIC | minimum inhibitory concentration |
| min | minute |
| ml | milliliter |
| µl | microliter |
| MLEE | multi-locus enzyme electrophoresis |

| | |
|---|---|
| MLSB | macrolides, lincosamides and streptogramin B |
| MLST | multi-locus sequence typing |
| MLVA | multi-locus variable number tandem repeat analysis |
| mM | millimolar or m moles per liter |
| µm | micrometer |
| *mreA* | macrolides resistance gene |
| mRNA | messenger Ribonucleic acid |
| MUSCLE | multiple sequence comparison by log-expectation |
| NaCl | sodium chloride |
| $NaH_2PO_4$ | sodium dihydrogen phosphate |
| NaOH | sodium hydroxide |
| NCCLS | National Committee for Clinical Laboratory Standared |
| Neu5Ac | N-Acetylneuraminic acid |
| nm | nanometers |
| NT | nontypeable |
| N-terminal | amino terminal |
| *neuA* | N-acylneuraminate cytidylyltransferase gene |
| OD | optical density |
| PBS | phosphate buffered saline |
| PBP | penicillin-binding protein |
| PCR | polymerase chain reaction |
| PFGE | pulsed field gel electrophoresis |

| | |
|---|---|
| *pheS* | phenylalanyl tRNA synthetase gene |
| PBSAT | PBS with 1% BSA and 0.05% Tween 20 |
| PI | pillus island |
| PLGBS | plasminogen-binding GBS |
| PPHL | Provincial Public Health Laboratory |
| PTS | phosphotransferase system |
| RAST | Rapid Annatoation using Subsystem Technology |
| RFLP | Restriction Fragment Length Polymorphism |
| *rps* | receptor polysacchrides locus |
| RT | room temprature |
| ScpB (*scpB)* | streptococcal C5a peptidase protein (gene) |
| SD | standard deviation |
| *sdhA* | L-serine dehydratase gene |
| sia | sialic acid |
| Sip (*sip)* | surface immunogenic protein (gene) |
| SLVs | single-locus variants |
| SNPs | single nucleotide polymorphisms |
| Srr | serine rich repeat |
| ST | sequence type |
| Stk (*stk*) | serine/threonine kinase protein (gene) |
| TBS | Tris Buffered Saline |
| TBST | Tris Buffered Saline with Tween |

| | |
|---|---|
| TCS | two component system |
| tet *(tet)* | tetracycline resistance protein (gene) |
| THB | Todd Hewitt Broth |
| *tkt* | transkelotase gene |
| Tn | transposon |
| tRNA | transfer Ribonucleic acid |
| yr | year |
| WGS | whole genome sequence |

# Chapter 1: Literature Review

## 1.1 Introduction

*Streptococcus agalactiae* also known as Group B Streptococci (GBS) is a commensal organism in humans but can cause life threatening infections in susceptible hosts such as neonates, pregnant women and nonpregnant adults with underlying medical conditions (1-7). Understanding the epidemiology of GBS invasive disease in a large population provides important information regarding disease rates, polysaccharide capsule changes, sequence type variations and antimicrobial susceptibility trends. With more attention being focused recently on GBS vaccine development especially for protection against invasive GBS (iGBS) neonatal disease, this epidemiological information is important. It also provides data that can direct clinicians in treating patients with iGBS and for public health officials with respect to understanding changes in iGBS disease trends.

One goal of this work was to describe the epidemiology of iGBS disease in the Alberta population with respect to rates, capsular polysaccharide type distribution and GBS antimicrobial susceptibility from 2003 to 2013. A detailed description of the epidemiology of GBS isolates recovered from invasive infections in newborns and adults in Alberta was reported in this work. A noticeable increase in the rate of neonatal GBS disease in Alberta between 2003-2013 was documented, similarly to the increase reported in North America in the past 15 years (11). In addition, infection in adults rose in Alberta in the 11 years surveyed as reported globally (1-7, 12). Edwards and Baker documented an increased rate of adult cases in 2005 in the United States for which two thirds of all iGBS infections were in older adults (13).

Typing GBS based on the different capsular polysaccharides has uncovered a high prevalence of serotype III in neonatal disease and the emergence of serotype Ia and V in the

adult population as reported previously (14-25). The emergence of CPS IV, a previously uncommon serotype, was also documented in Alberta as an important cause of both neonatal and adult infections. Recent studies have also reported this increase (16, 21, 26-30). Type IV isolates recovered from invasive infections in neonates and adults were studied, with the aim of evaluating their GBS clonal structure. Multi-locus sequence typing (MLST) (31) was used in this study to determine the clonal structure of CPS type IV in Alberta. This typing system revealed the predominance of sequence type 459 (ST459) belonging to clonal complex (CC) CC 1 among CPS type IV isolates in 2013. All the isolates that were found to belong to ST459/CPS type IV were resistant to macrolide elements and tetracycline, explaining the expansion of ST459/CPS type IV clones in Alberta and globally (32, 33).

Typing of GBS is performed to gain insight into the epidemiology. Multiple typing techniques have been developed and used over the years. Typing of GBS generally begins with the determination of the capsular polysaccharide serotypes (CPS). There are currently ten recognized GBS CPS's designated Ia, Ib, and II through IX (34-37). A GBS isolate without identifiable capsule type is known as a nontypeable (NT) isolate. However, the most used CPS typing methods, serological assays, are limited in their ability to type GBS capsule resulting in a significant number of ambiguous results and NT isolates (31, 37, 38). Molecular based PCR-assays targeting serotype specific capsule genes have been previously developed to assign capsule types (39-42). These typing methods have several advantages over serological typing methods, among them the generation of more unambiguous results. However, these assays were associated with a number of limitations (38, 43). These methods are expensive and time-consuming as all the developed molecular typing methods of GBS isolates require two different

techniques, the first one is a PCR and the second is a detection method, such as sequencing (40), blot hybridization (38, 43), enzymatic restriction (44), or agarose gel electrophoresis (39, 41). Generally, molecular typing assays target capsule genes rather than expression; therefore, the identified cps gene(s) by the PCR-based assays does not confirm if the capsule polysaccharide is expressed or not. Previous work has shown the conservation of sialic acid among all known GBS capsular types (9, 39, 45). Due to the essential role of this structure to GBS capsular polysaccharide biosynthesis and expression, sialic acid on the surface of GBS capsule was proposed in this work to be used as a universal phenotypic assay to ascertain capsule expression on the surface followed by a CPS specific real-time-PCR assay.

One of the goals of this work was to develop a dual GBS typing system involving phenotypic and genotypic assays that are reliable, sensitive and specific and enable the characterization of GBS CPSs Ia, Ib, II-IX, thereby reducing the rate of detection of NT isolates. The first step is sialic acid detection on GBS cell surface. To identify sialic acid on the different GBS CPS types, lectin from *Limax flavus* agglutinin (LFA) was chosen because LFA binds to sialic acid regardless of its linkage and non-specific binding has not been identified for LFA (46). This method can confirm capsule expression of the surface and bypass the requirement of CPS-specific antisera to identify each capsule type. A genotypic assay is a duplex real-time PCR/DNA assay targeting CPS-determining genes (*cpsG*-*cpsK*) in *cps* gene cluster. This assay succeeded in assigning a CPS type to known GBS type isolates and to isolates failed to be typed by serological assays. The developed typing system in this work could be well adapted particularly for GBS CPS typing in large-scale epidemiological studies

GBS isolates that failed to be assigned a capsule type are known as NT, but fewer isolates were identified as NT in molecular assays. The NT phenotype and genotype could be a result of technical issues, the expression of undetectable amount of capsular polysaccharide by serological methods, no capsule expression, or production of uncharacterized capsular polysaccharide for which antibody is not yet available (47). The work in this thesis described the characterization of GBS NT isolates with an aim of identifying new CPS type(s) and/or other underlying causes of capsule expression loss. The combination of sialic acid detection and real-time PCR typing assays described in this work enabled the characterization of GBS NT isolates into two groups: sia (+) with unidentifiable CPS types, suggesting these isolates may represent new CPS type(s) and sia (-), indicating capsule loss on the surface. A whole genome sequencing (WGS) method has been utilized in this work to investigate cps gene clusters and identify new cps genes among GBS with a capsule, but unidentifiable CPS type. One GBS isolate was found to be a CPS hybrid of IIa and V. This isolate may represent a potential new CPS type.

Previous work has shown that a CovRS (a global regulatory system) controls GBS virulence genes, including GBS capsule (39, 86). In this study, we identified isolates with inactivated *covR* or *covS* genes among GBS isolates that lacked sialic acid. These findings suggest that mutations in *covR*/S genes occurred among GBS NT isolates. These mutations could affect capsule expression and explain the NT phenotypes of these isolates.

MLST is a valuable typing method that has the ability to differentiate strain collections into many types. However, it has some drawbacks, in comparison to whole genome sequencing, MLST has limited resolution in identifying and characterizing intraspecies genetic variability among bacterial clones (33, 48-51). Hence, in recent years WGS has been proposed

as the method for investigating genetic variations in highly related bacterial population. This method enhances the understanding of bacterial population diversity and changing of bacterial adaptive genetic traits, such as elements encoding antibiotic resistance and virulence determinants (52, 53). In this work, both methods have been used to understand the genomic makeup, antimicrobial drug resistance profiles and the molecular mechanisms underlying the emergence of GBS ST1 isolates. ST1/CPS type V isolates were associated with the majority of GBS infections among nonpregnant adults in North America (49). In this thesis, four ST1 GBS isolates that identified as i) CPS type IIa (1 isolate), ii) CPS hybrid of CPS IIa and V (1 isolate) and iii) CPS type V (2 isolates) were compared to a complete genome of ST1/CPS type V (PLGBS13 strain). Elucidation of the complete genome of a ST1/serotype V PLGBS 13 strain, isolated from invasive adult case from Alberta, Canada in 1997, was reported in this study. This work provided evidence for the acquisition of capsule-specific genes between GBS CPS types by recombinational events and capsular switching of the entire capsular locus. In addition, recombination has been found to play a role for GBS genetic diversity among ST1 genotype with serotypes other than serotype V, but the diversity among ST1 asscoiated with serotype V is mainly driven by small genetic changes rather than extensive recombination.

The purpose of the work presented in this thesis was to obtain insights on different aspects of the molecular epidemiology of GBS with a particular emphasis on invasive human infections. This thesis also focuses on the development of an accuate capsular typing system for GBS isolates and the characterization of GBS isolates without an identifiable CPS type. The intent of the introductory chapter is to orient the reader and address the problems in context, by briefly reviewing essential aspects of GBS disease and colonization, as well as of the impact

of preventive strategies. The contribution of virulence factors and antimicrobial resistance on GBS epidemiology, the importance of surveillance and characterization of the isolates, as well as the major findings in recent years on the structure of the GBS population are also reviewed. This chapter also describes the most commonly used epidemiological typing methods. It also intended to introduce the reader to the nontypeable phenotype of GBS isolates and recent findings to explain this phenotype.

The work described int this thesis documents the ever-changing epidemiology of GBS invasive disease in a large population such as Alberta. This provides important information regarding disease rates, polysaccharide capsule changes, sequence type variations, antimicrobial susceptibility trends. It also provides data that allows clinicians to make more informed decisions regarding the treatment of patients with iGBS and for public health officials with respect to understanding changes in iGBS disease trends. Moreover, this information is critical for GBS vaccine development especially for protection against neonatal iGBS disease. In addition, my work has focused on describing the epidemiology of iGBS disease recovered from invasive infections in newborns and adults in the Alberta population with respect to CPS distribution and GBS antimicrobial susceptibility from 2003 to 2013. The work described within focuses on developing a more comprehensive capsular polysaccharide typing system for GBS, as well as developing a dual GBS typing system that provides information regarding capsule expression and identification of capsule type. I also describe the use of whole genome sequencing and genome comparative analysis to understand the genomic makeup, antimicrobial drug resistance profiles and the molecular mechanisms underlying the emergence of GBS ST1 isolates.

## 1.2 History of *Streptococcus agalactiae*

Streptococcal species of clinical importance are divided into six groups based on pathogenic and clinical features (54-56). *Streptococcus agalactiae* is a species in the genus of Streptococci and a member of the pyogenic group. The pyogenic streptococci are identified on 5% sheep blood agar plates by the typical zone of β-hemolysis surrounding bacterial colonies. *S. agalactiae* is serologically classified as Lancefield Group B; hence it is commonly recognized as Group B Streptococcus (GBS) (54-56). This classification was described by Rebecca Lancefield in 1933, when she published her extensive studies to distinguish among hemolytic streptococci. In this classification, *S. agalactiae* is the only species belonging to the group B (54). GBS was historically associated with bovine mastitis and dairy sources (55). The recognition of GBS as an etiological agent of severe human infections among neonates was first described in the 1930s and by the 1970s, GBS was already established as a leading cause of infections in the newborns (57). In the 1990s, GBS has been increasingly associated with invasive infections in nonpregnant adults (58). GBS is also a significant veterinary problem, since it is considered a pathogen causing infections in chickens, cattle, camels, dogs, bottlenose dolphins, horses, cats, fish, frogs, hamsters, mice, and monkeys (59-66).

## 1.3 Bacteriology of GBS

GBS isolates are Gram-positive cocci, facultative anaerobic and catalase-negative. GBS isolates divide in one plane and therefore occur as pairs or chains composed of 4 cells or more. The size of overnight GBS culture colonies on blood agar media tends to be >0.5 mm in diameter. GBS colonies appear flat, slightly mucoidal, grayish-white but some strains can be

pigmented from yellowish to orange, which is unique in the genus, when grown on blood agar plates (67, 68).

GBS are heterotrophic bacteria that require a carbon source for energy and substrates. GBS are able to ferment a variety of carbon sources resulting in multiple by-products, such as lactate, acetate, ethanol, formate and acetoin. Cattle GBS isolates are able to ferment lactose, due to possessing lac.2 operon in their genome (69). In contrast, the majority of human GBS isolates lacked this ability (51). This organism is auxotrophic for multiple amino acids that it must obtain as nutrients from its host, supported by the absence of multiple biosynthetic ways and the presence of a wide array of transporters suggesting a broad catabolic capacity (70, 71).

### 1.4 Identification of GBS

To detect GBS in samples from the vagina and/or rectum, enrichment broths such as Todd-Hewitt broth, with antimicrobial agents inhibiting Gram negative flora is recommended for detection of colonization. Then, subculture of the enriched cultured on blood agar plates is used for further processing (72, 73). The Christie Atkinson Munch-Petersen (CAMP) test is one of the standard tests for identification of GBS. It involves the hemolytic activity of staphylococcal sphingomyelinase by some *Staphylococcus aureus* strains on red blood cells which is enhanced by the CAMP factor produced only by GBS strains (74, 75). Currently, verification of the suspected GBS colonies is carried out using latex agglutination tests reacting with the group B antigen (76, 77) or other specific methods such as genetic probes and fluorescent antibodies (55, 56, 78-80). Recently, chromogenic media have been introduced to improve the detection of GBS isolates. It is based on color change in the presence of GBS colonies (81). Molecular assays based on PCR or probes have been already developed; several

GBS genes have been used as targets such as the *cfb* gene coding for the CAMP factor (82), the *sip* gene (83, 84) or the *pts1* gene (85). Real-time PCR assays permit the rapid detection of GBS with high sensitivity. The use of traditional enrichment broth and subsequent culture on agar media consume time (turnaround time is two days). Molecular tests without enrichment are time saving assays (turnaround time is two hours). These allow screening of pregnant women in labor and give a more correct diagnosis of colonization than screening in week 35-37. These rapid tests can detect GBS colonization status at delivery and thereby replace screening in weeks 35-37 by intrapartum screening. Generally, they also have high sensitivity and specificity (86). However, these molecular tests are regarded as supplementary to screening by culture and risk based approaches at delivery (86).

## 1.5 GBS epidemiology

### 1.5.1 Colonization and transmission

GBS is part of the normal human microbiota and approximately 10-30% of pregnant women are colonized with GBS in the vagina or rectum (87). The gastrointestinal tract serves as the natural reservoir for GBS and is likely to be the source of vaginal and rectal colonization (88). GBS has been isolated from the rectum, perianal area, vagina, cervix and urethra (89). Colonization is a prerequisite to proceed to invasive neonatal diseases. GBS colonization can be transient, chronic or intermittent (88). Prevalence studies looking at maternal GBS colonization have been carried out and from these studies a number of risk factors affecting the prevalence rates have been identified. The prevalence of maternal GBS colonization varies based on socio-economic status, race, maternal age, presence of sexually transmitted disease and sexual behavior (87, 90-94).

The prevalence of GBS vaginal colonization among pregnant women also varies regionally. This may be due to the absence of standardization methodology for sampling and processing of samples. Specifically, differences in culturing techniques, culture media and sites of sampling may contribute partially to the variability in the detection of GBS colonization between studies. Recto-vaginal swabbing is the recommended method to improve the sensitivity of detecting GBS carriers. Both rectal and vaginal swabs had higher sensitivity 18.5-51.0% than vaginal swabs alone in past studies (95-97). The variability in maternal GBS prevalence ranging from 6.6-36% was documented in different countries worldwide. The highest prevalence was reported in Denmark (36%) (88), followed by the United States (Florida) (33.5%) (98), New Zealand (22%) (99), Mexico (13.3%) (100), Australia (12.9%) (101), France (8%) (102) and Greece (6.6%) (103). The low prevalence of GBS colonization may be due to difference in sampling sites or culturing techniques. In France and Australia, GBS was detected from the vagina alone (102) whereas in Greece nonselective culturing media was used (Chocolate and blood agar instead of colistin nalidixic acid) (103) which could have decreased isolation rates.

Studies from Africa that assessed the prevalence of GBS maternal colonization showed a similar prevalence rate for various countries, South Africa (21.6%) (104), Gambia (22.1%) (105) and Tanzania (23.0%) (106). These studies used different criteria in terms of gestational age at sampling, the site of sampling and culturing media. A low maternal GBS prevalence has been reported in Mozambique ranging from 0.9% to 1.8% in two different studies (107, 108). The low prevalence rate reported in both studies may be due to culturing the bacteria without the use of selective media which may reduce the yield of GBS recovery by 50%. (73).

The prevalence of GBS colonization among pregnant women in Asia was consistently low, specifically, 1.3% in India (109), 8.5% in Pakistan, 7.5% in Philippines and 7.1% in Myanmar (110), irrespective if the sampling site was vaginal swabbing or recto-vaginal swabbing. In contrast, a high prevalence rate was observed in Saudi Arabia 31.6% (111). Little is known regarding the low prevalence of GBS colonization in Asian countries and geographical variation in general remains unknown.

During labor, heavy colonization with GBS is a great risk factor for vertical transmission (112). A study from Italy reported an increase rate of neonatal colonization for women with high density of colonization (50%) compared to those with a lower density of colonization (30.4%) (112). Prolonged rupture of the amniotic sac membrane is another risk factor that can determine the acquisition of GBS in infants. Specifically, vertical transmission has been reported as 73.3% in membrane rupture of more than 12 hours before delivery compared to 38.4% in less than 12 hours before delivery (91). Other studies have reported neonatal colonization in neonates born from noncolonized mothers, specifically, 2.0-5.6% of neonates born to noncolonized mothers are colonized by GBS during birth (91, 104). These results indicate poor sensitivity of culturing methods in these studies or acquisition of GBS from other sources than the mothers.

### 1.5.2 GBS diseases

GBS is commonly found in humans as a colonizing organism without causing disease symptoms, but it is also an opportunistic pathogen with the potential to cause human infections. Three patient groups can be classified as targets of iGBS disease. These are pregnant women, newborns, and nonpregnant adults.

#### 1.5.2.1 Infection in pregnancy

Female genital tract colonization with GBS in pregnant women can cause maternal infection during pregnancy or labour (113, 114). Past studies have shown that GBS was linked to approximately 12-25% of puerperal fever. The clinical presentations of GBS disease among pregnant women are varied, and include urinary tract infection (usually asymptomatic bacteriuria), endometritis, chorioamnionitis (intraamniotic infection), wound infections associated with caesarean delivery or episiotomy, less commonly, puerperal sepsis and meningitis (113-115). Women colonised with GBS during pregnancy are at an increased risk of stillbirths and premature delivery (89, 113, 114). Based on an active, laboratory-based surveillance study for GBS infections in the United States, invasive GBS infections among pregnant women accounted for 6.3% of all adult cases (116).

#### 1.5.2.2 Neonatal infection

The neonatal patients are subclassified into two subgroups: early onset disease (EOD), in which presentation occurs within the first week of life, and late on set disease (LOD) for cases presenting between one week and up to three months of life (90 days). Neonates with invasive GBS disease may present with septicemia, meningitis and pneumonia, with septicemia being the most common (117, 118).

##### 1.5.2.2.1 Early onset disease

EOD disease is preceded by the asymptomatic colonization of the female genital tract during pregnancy. With a maternal colonization rate of 30%, a neonate colonization rate was found to be 50%. However, only few cases (1-3%) of neonates develop EOD disease (89, 119, 120). The clinical presentations of EOD are septicemia, pneumonia, or meningitis. GBS was

found to be the cause of 86.1% of bacterial meningitis within the first two months of age in the United States between 1998 and 2007 (121). EOD patients have the highest fatal cases with 20% fatality rate in neonates before 33 weeks of gestation and 2-3% in full term neonates (122). Approximately, 85% of EOD cases occur within 24 hours of birth. Most premature newborns tend to be in this group while babies with onset after 24 hours tend to be born at term

Vertical transmission of GBS from colonized mothers to their newborns is the route of infection in EOD (123). Transmission could occur by the ascending route into the uterus. GBS may travel through intact or ruptured amniotic membranes into the amniotic fluid, where it is able to replicate and be aspirated. The fetus could also aspirate contaminated vaginal contents during passage through the birth canal. Following aspiration by the fetus, the organism can remain localised in the lungs, causing pneumonia, or progress to sepsis (72%) and meningitis (2.5%) (34, 124) (Fig 1.1).

A number of risk factors associated with EOD development have been identified, such as i) mothers heavily colonized with GBS (87, 88, 102, 125), ii) Premature birth or low birth weight, iii) Prolonged rupture of amniotic membranes, iv) Maternal fever during labour or chorioamnionitis, v) GBS isolated from urine samples during pregnancy and vi) A previous baby being born with GBS disease (89, 124). An estimation of the global burden of EOD in different countries has been reported in a systemic review (6). The overall incidence rate was 0.43 cases per 1000 live birth, with the highest rate documented in Africa (0.53 cases) and followed by Americas (0.5 cases) and Europe (0.45 cases). The lowest incidence rate was reported in South-East Asia, 0.11 cases per 1000 live births (6).

#### 1.5.2.2.2 Late onset disease

Approximately of half the reported cases of neonatal GBS disease are classified as LOD patients (126). A study in the United States between 1999-2005 reported a LOD proportion to be around 50% of neonatal iGBS diseases (15). LOD commonly presents as meningitis, septicemia, or bacteraemia. 65% of LOD patients develop septicemia without identified focus (123). Meningitis is a frequent presentation and found in 25% of cases (127). Meningitis develops when the entry of bacteria into the bloodstream is followed by the invasion of the cerebrospinal fluid. Severe long-term sequelae such as blindness, deafness and global developmental delay occur in 50% of neonatal meningitis survivors (127).

Little is known regarding the pathogenesis of LOD, although some cases also suggest a maternal source, probably reflecting acquisition of the microorganism during passage through the birth canal (128). GBS may then gain accesses to the blood stream by adhering to and translocation of the gut epithelium (129). 50% of mothers of infants with LOD were found to carry the same GBS serotype as that causing infection in their infants (128). It has also been shown that breast feeding may be a source of LOD infections (130). Another possible source of LOD infection is nosocomially and community acquired infection (131, 132). An estimated mortality rate of LOD patients was found to be lower than EOD at 2-6% (1, 133), but deaths caused by GBS meningitis were higher at 14.5% of all LOD fatalities, compared to 2.5% in EOD (124, 134).

The overall LOD incidence rate reported globally was 0.24 cases per 1000 live births with the highest rate in Africa (0.71), followed by the Americas (0.31) (6). In the United States and Europe, the rate for LOD was 0.47 and cases 0.36 per 1000 live births, respectively (126).

Studies from South Africa, Malawi, and Kenya have reported LOD rate to be 1.0, 0.66 and 0.89 cases per 1000 live births, respectively (135-137). The burden of LOD in Asian countries is poorly documented. More studies are needed to accurately estimate the burden of LOD due to GBS. (6).

**1.5.2.3 Adult disease**

GBS has recently emerged as an important pathogen among non-pregnant adults. In the United States, almost two-thirds of all iGBS cases in 2001 were encountered among non-pregnant adults and the frequency of invasive diseases in this patient group seems to increase further (138). The majority of GBS infection among non-pregnant adults (75%) occur in people with underlying diseases such as diabetes, liver disease, cardiovascular disease or malignancies (13, 139). Increasing age (>65 years) is also an important risk factor for this group (13). GBS infection in non-pregnant adults occurs more frequently among nursing facility residents than in the community, and is considered responsible for significant morbidity and mortality, with case-fatality rates of nearly 25% (139, 140). The most common clinical presentations widely associated with non-pregnant adults are septicemia without identifiable focus, skin and/or soft tissue infection, urinary tract infection, pneumonia and septic arthritis (12, 141). Cases of necrotizing fasciitis and toxic shock are observed infrequently (142, 143). Relapsing infection has been seen in adults, especially presenting as cellulites, this could be due to a persistent carriage or poor clinical management of primary infection (144). It is possible that the organism inhabits a privileged niche such as within host cells, preventing clearance (93) Nosocomial disease is also raising concerns as more than 20% of patients with GBS invasive infection are thought to have acquired the bacteria from hospital settings (145).

Importantly, rates of iGBS disease have been increasing during the past 25 years in non-pregnant adults (11, 16, 17, 19, 24). In the early 1990s, the incidence of invasive GBS disease in adults has been reported as 2.4-4.4 cases per 100 000; this rate is rising, which could be a reflection of the increasing population at risk (12). In a more recent population based study, the rate of iGBS infections was found to be 7.3 cases per 100 000 population (12).

### 1.6 Preventive measurements

Strategies for prevention of perinatal GBS disease have been implemented in the United States and Canada. The United States (1996) and Canadian (1997) guidelines recommend two preventive measures. These strategies involve a universal culture based screening at 35-37 weeks of gestation with the administration of intrapartum prophylaxis antibiotics (IAP), usually benzylpenicillin or ampicillin, to women who are colonised with GBS and with an identified risk factor (146-149). To identify maternal colonization status, the United States and Canadian guidelines recommend to culture swabs collected from the lower vagina and rectum. The use of selective enrichment broth is recommended and improves the chance of laboratory detection of GBS substantially (122, 146). On the other hand, the UK, New Zealand and Australian guidelines recommended only a risk based approach (150, 151) This approach recommends that all women with one or more of the aforementioned risk factors to be offered intravenous intra-partum antibiotic prophylaxis. The use of IAP resulted in a decrease in EOD cases, from 3.2 cases per 1000 live births in 1987-1989 to 2.0 cases per 1000 live births in 1990-1992 and even further to 0.37 cases per 1000 live births between 1997-2003 in the United States (152). The incidence rate of neonatal GBS disease in Canada and the United States has also decreased from 1-3 cases per 1000 live births in the early 1990s to 0.35-0.5 cases per 1000 live births with

the application of intra-partum chemoprophylaxis prevention measures (15, 86, 153, 154). The mortality rate for EOD has decreased from 50% to 4-20%, coinciding with the introduction of neonate GBS preventive measures. Although the increased use of the IAP in most European countries during the 1990s had led to significant decrease in EOD patients, a high residual incidence of EOD disease has been reported in countries such as Finland (0.6-0.7 cases per 1000 live births) (155), Spain (0.89 cases per 1000 live births) (156) and Czech Republic (0.7-1.0 cases per 1000 live births) (157). In Africa and Asia screening for GBS colonization during pregnancy has not be implemented. Two separate studies have been carried out in Soweto and South Africa. The first study was between 1997 and 1999, and reported an incidence of 2.06 cases per 1000 live births for EOD (158). The second study carried out between 2005-2007 documented a similar rate (2.0 cases per 1000 live births) (159). In Malawi, a high incidence of EOD (0.92) has also been reported (135). In some Asian countries, such as Taiwan and Japan, high incidence rates were reported of 0.7-1.83 cases per 1000 live births (160, 161). This may be due to the lack of practice of neither universal GBS screening during pregnancy nor risk based IAP protocols. In contrast, some of the Asian and African countries documented low EOD rates, such as India (0.17 cases per 1000 live births) (162) and Nigeria (0.24 cases per 1000 live births) (163). This may be attributed to inadequate laboratory facilities in rural regions. In addition to the high number of cases occurring among home-births which may be missed (164). The implementation of GBS preventive measurements has decreased the burden of GBS invasive neonatal diseases, but it has not completely prevented EOD. Also, they have not prevented LOD, nor have they addressed GBS disease in non-pregnant adults (147). A new strategy, such as the development of vaccines against GBS, is needed to prevent GBS disease.

## 1.7 Treatment and antibiotic resistance

### 1.7.1 Penicillin and β lactam

Penicillin and other β-lactam antibiotics, such as ampicillin are the agents of choice for IAP as well as treatments of GBS infections in all patients. These agents have a narrow-spectrum, effective transplacental crossing and low cost (165). Penicillin and other β lactam antibiotics covalently bind to and inactivate the bacterial proteins (penicillin-binding proteins, PBPs) responsible for the formation of peptidoglycan by crosslinking its subunits in the bacterial cell wall. GBS was thought to be susceptible to these agents; however, recent reports from Japan (166) and the United States (167) reported reduced penicillin susceptibility in noninvasive and iGBS isolates, respectively. These isolates were found to have mutations in a PBP-2x resulting in an increased minimum inhibitory concentration (MIC) to penicillin (166, 167). These findings raise a concern related to the possibility of the emergence of penicillin resistance in GBS isolates, but the clinical impact needs to be identified.

### 1.7.2 Macrolides and related agents

Clindamycin and erythromycin are alternative agents for patients who are allergic to β-lactam agents (168-170). Macrolides are protein synthesis inhibitors that act by binding to a specific location of the 50S subunit of the bacterial ribosome and preventing its association to tRNA. The wide use of these antibiotics is a major concern because of the increase in GBS clindamycin and erythromycin resistant strains as reported in North America and Europe (171-175).

Resistance to macrolides in GBS is divided in to two groups; i) resistance to erythromycin alone or ii) resistance to both erythromycin and clindamycin (176). Resistance can

occur by three mechanisms, one by target site modification, a second by an active efflux pump and a third by drug inactivation (176). Target-site modification by a methylase is encoded by an erythromycin ribosome methylase (erm) gene. The methylation of 23S rRNA at the erythromycin-binding nucleotide target changes the binding site for antibiotics on the ribosomes, conferring resistance to macrolides, lincosamides and streptogramin B (MLSB phenotype). Phenotypic expression of MLSB is either constitutive (cMLSB) or inducible (iMLSB). Constitutive expression occurs in the presence or absence of an inducer antibiotic, such as erythromycin, whereas inducible resistance takes place only in the presence of the inducer antibiotic (176-178). In the second mechanism, an active drug efflux system that functions via a membrane bound pump encoded by the *mefA* or *mefE* gene is responsible for macrolide resistance only (176-178). GBS isolates encoding either *mefA* or *mefE* remain susceptibility to lincosamides and streptogramin B (M phenotype) (176).

In GBS isolates, the wide spread *erm* genes belonging to the *ermB* class (179-185) and, infrequently, to the *ermTR* subset of the *ermA* class (186) are responsible for the bulk of macrolide resistance. The *ermB* gene is linked to the cMLSB phenotype whereas the ermTR gene is associated with iMLSB phenotype (180, 187). A recent report identified the *ermT* gene among iMLSB GBS strains, a gene that had only been described previously in *Streptococcus bovis* (188). Two genes, the *mefA* and *mefE*, were found to be responsible for the M phenotype macrolide resistance among GBS isolates (180, 189). A study from Canada identified one isolate that was susceptible to erythromycin but resistant to clindamycin. This isolate harbored *linB*, responsible for lincosamide nucleotidylation and previously identified only in *Enterococcus faecium,* and conferred resistance to lincosamides only (186). This resistance element was also

described in Korea (185). *linB* can be colocalized in the same mobile genetic element as the *ermB* gene (179, 185). A new resistance phenotype in GBS known as lincosamide and streptogramin A (LSA) has been also identified in New Zealand (190). This study found that some GBS isolates are simultaneously resistant to lincosamide and streptogramin A (190). This could be a result of acquisition of resistance elements through horizontal gene transfer (HGT). This phenotype has also been detected in Taiwan (191) and Korea (192).

### 1.7.3 Tetracycline

Tetracycline is a protein synthesis inhibitor that acts by preventing the association of tRNA with the bacterial ribosome (193). Among GBS isolates, resistance to tetracycline is almost ubiquitous and is most often caused by a protein encoded by *tetM* or *tetO*. They protect the ribosome from the tetracycline. Other genes less frequent in prevalence are *tetK* and *tetL* genes encoding a tetracycline efflux pump (193). *tetM* has been identified as the most common tetracycline resistance determinant among GBS isolates from human infections whereas *tetO* is the most frequent among those bacteria recovered from bovines (194, 195). Tetracycline is not the drug of choice to treat streptococcal infections. The genetic elements carrying tet genes are commonly the same that carry genes encoding resistance to macrolides, lincosamides and chloramphenicol, thereby, it is recommended to maintain surveillance of tetracycline resistance trend (193). Two mechanisms regarding the dissemination of tet genes and *ermB* have been proposed. The first theory supports the dependent dissemination of tetracycline and erythromycin resistance determining genes. In GBS isolates, the macrolide resistance gene *ermB* and *tetM* in human or *tetO* in bovine were found to be carried by the same transposons, supporting the importance of HGT for antimicrobial resistance evolution and spread in GBS

(183, 195-197). In contrast, the second theory supports the independent dissemination of tetracycline resistance determining genes and erythromycin resistant elements (198).

### 1.8 Potential GBS vaccines

Development of a universal vaccine is an attractive preventive measure of GBS infections which has led to investigation into potential vaccine targets. The preventive measures of GBS infections, specifically IAP, in use has only controlled EOD infections (199). It has been proposed that vaccination could prevent GBS infection in LOD, pregnant women and in nonpregnant adults. Moreover, vaccination of pregnant women against GBS would be more effective at preventing neonatal GBS disease than the current antibiotic prophylaxis (200). A GBS vaccine could be administered to adolescent females or late in pregnancy. Also, adults at risk (>65 years, underlying medical conditions) could be selected for targeted immunization. Currently, there is no vaccine available for GBS infections, but multiple antigens of GBS have been proposed as candidates for a vaccine. Initially, GBS vaccine candidates included polysaccharide capsule. It is known that high levels of capsular type specific antibodies are protective against invasive neonatal disease (201). Candidate vaccines using CPS alone have shown poor immunogenicity (202). When capsule based vaccine ability to provide protection against neonatal GBS diseases was assayed, the vaccine was found to elicit a protective immune response in 63% of vaccinated mothers (202). The current efforts have focused on developing GBS polysaccharide protein conjugate, in which polysaccharide epitopes of specific serotypes are conjugated to an immunogenic carrier protein (203, 204). GBS serotype specific polysaccharide-protein conjugate vaccines containing serotypes Ia, Ib and III, have been assayed for safety and immunogenicity (203, 204). Conjugation of serotype specific

polysaccharide to tetanus toxin improved the protective immune response to 80-93% (205), raising the possibility of the prevention of perinatal GBS disease through maternal immunization. It has also been found that antibodies raised against the capsule are function against homologous serotypes only; therefore, a successful vaccine would need to be multivalent (205). The trivalent vaccine, including serotypes Ia, Ib and III, conjugated to tetanus toxoid or CRM conjugates are currently in human clinical trials (199).

The disadvantage of serotype specific capsular polysaccharide conjugate vaccine is notably the serotype specificity. A capsular polysaccharide based vaccine against serotypes Ia, Ib, II, III, and V could potentially offer protection to 80-95% of the population, including neonates, pregnant women and nonpregnant adults in North American and most of the European countries. However, GBS serotypes that are more prevalent in other parts of the world are not covered. Moreover, the distribution of GBS isolates without capsule among maternal colonization and invasive isolates in non-pregnant adults would not be covered by a polysaccharide based vaccine formulation. Another limitation associated with the introduction of such a vaccine is serotype replacement and expansion of less common serotypes that are not included in the vaccine formulation along with the decrease in the infections caused by vaccine serotypes as observed previously in *S. pneumoniae* (206). Recent reports have described the emergence and circulation of CPS type IV as cause of neonate and adult infections (16, 21, 26-30) including Canada (30, 207). Furthermore, GBS vaccination could provide selective pressure for virulent genotypes to switch capsules and escape vaccine induced immunity (32, 207).

To overcome limitations associated with the multivalent serotype-specific vaccine, GBS vaccine should contain multivalent serotype-specific vaccine conjugated with a conserved surface protein. This may be an effective approach to provide protection against all GBS serotypes. Five GBS surface proteins that have been found to be present on virtually all GBS serotype isolates recovered from humans are ScpB and Lmb (195, 208), pilus proteins (209, 210), C5a (211) and Sip (209). A vaccine that has one of GBS surface proteins, such as Lmb, pilus proteins, C5a and Sip has been found to provide protection against GBS infections using mouse models (209-213). Hence, a vaccine formulation that includes one of the GBS surface proteins ScpB, Lmb, Sip and the pilus along with the multivalent serotype-specific antigens could provide a broad protection against GBS infections.

Introduction of a GBS vaccine, generally, imposes some difficulties as well. One of the target populations for GBS vaccine immunization is pregnant women. Introduction of vaccines into the pregnant population is risky. Moreover, to assess the efficacy of GBS vaccine to prevent EOD, the current preventive use of antibiotic prophylaxis needs to end in a clinical trial which is not feasible (212, 214). However, the vaccine could be evaluated in different patient group, such as elderly population (215). When the vaccine has been proved to be protective and safe in the elderly population, it would be easier to introduce into the pregnant population to prevent GBS diseases.

### 1.9 GBS virulence factors

GBS usually resides as a commensal microorganism in genital and gastrointestinal tracts. It colonizes the mucus membranes lining the gastrointestinal and genitourinary tract of humans (1-7, 87). Once GBS breaches these barriers, it can gain access to soft tissues and the

bloodstream leading to serious diseases. To cause an infection, GBS requires establishing a successful colonization and efficient capacity to penetrate host physical barriers. This requires an appropriate expression and regulation of surface-associated and secreted virulence factors that mediate host-cell interactions, including adherence to host epithelial surfaces, invasion across epithelial and endothelial barriers, and interference with innate immune clearance mechanisms (216). GBS has a survival advantage in that it can adapt to different niches (217). Multiplication of GBS in the bloodstream can lead to bacteraemia and sepsis. GBS also can access several other niches such as the intrauterine compartment and multiple organs. For example, GBS can adhere to endothelial cells and penetrate the blood brain barrier leading to the development of meningitis (129, 218-221) .

### 1.9.1 Polysaccharide Capsule

The capsule is a major virulence determinant of GBS, of which there are ten recognized antigenic variants (Ia, Ib, II-IX) differing in their chemical composition, structure, and serological properties (34-37). The capsular polysaccharide is predominantly composed of repeating units containing four elements: glucose, galactose, N-acetylglucosamine and sialic acid, the terminal sugar on the side chain of all serotypes (Fig 1.2). Nevertheless, serotypes VI and VIII capsule composition lack the N-acetylglucosamine and serotype VIII has an additional rhamnose residue (9).

The polysaccharide capsule biosynthetic genes are encoded in a gene cluster known as the cps operon. This operon encodes 16-18 genes consisting of a central group of genes that encode serotype-specific glycosyltransferases and polymerases, which are flanked by genes conserved across all serotypes. The genes upstream of the serotype-specific region encode enzymes that

direct the synthesize and activation of sialic acid, and the genes downstream are hypothesized to function in regulation and export of the polysaccharide capsule (222). The capsule gene operons of all ten GBS serotypes are illustrated in Fig 1.3. cps genes have shown to be both structurally and genetically closely related (9). In contrast, the amino acid sequences of proteins having similar functions in different capsular types were found to exhibit significant heterogeneity (9).

The diversity in the existing GBS 10 CPS types has arisen through the introduction of novel DNA sequences that may have occurred by intra-and interspecies recombination events (8, 9). Moreover, it has been proposed that the diversity at the capsular locus could to be driven by the equilibrium between the selective pressures imposed by host immunity and the conservation of important structures (8, 9). This could lead to capsular variation and the conservation of structural elements of a particular capsular polysaccharide that might be required for pathogenicity, such as sialic acid (9, 223).

GBS capsular polysaccharide plays a role in resistance to opsonophagocytic killing and phagocytosis, as well as for the inhibition of complement system clearance (10). This is achieved by preventing complement factor C3b deposition on the surface of GBS reducing the production of the chemokine C5a (216, 224). It has been found that a non-capsulated mutant of GBS strain exhibited significantly reduced virulence as compared to the encapsulated strain when the virulence role of the GBS capsule was evaluated in a rat model of neonatal infection (225). Capsular sialic acid was found to play an important role in bacterial evasion of host mechanisms (226). Loss of GBS capsule sialic acid was associated with loss of virulence in a neonatal rat model of lethal GBS infection (226). Furthermore, GBS capsular polysaccharide is

required for the process of biofilm formation in the presence of human plasma (227), contributing to GBS persistence and pathogenicity. Inactivation of capsule biosynthesis gene(s), accordingly, was associated with the loss of GBS capsule functions regarding resistance toward phagocytic killing (29) and biofilm formation (227).

#### 1.9.1.1 Sialylated capsular polysaccharide

GBS is unusual among bacteria in that all serotypes display a terminal sialic acid (N-acetylneuramininc acid) on the capsular polysaccharide. *Streptococcus suis* is the only other Gram positive organism where sialic acid has been detected (228). Sialic acid holds the terminal position of the side chain and is a pathogenicity factor in itself because it inhibits the activation of the alternative complement pathway (224, 229-231). Sialic acid is also present on the glycan of vertebrate cells (226). GBS is decorated with sugars that mimic the host cell, a form of molecular mimicry that has been proposed to evade the host immune system (226). The sialyated capsule enhances GBS affinity for factor H, an inhibitor of complement activation, therefore preventing direct complement mediated killing (232). Furthermore, sialyation of GBS capsule was found to be an essential process for full GBS capsule polysaccharide biosynthesis and expression (45). Sialylation of GBS capsule biosynthesis pathway has been previously described (233). It starts with the synthesis of free intracellular sialic acid (endogenous) from N-acetyl-mannosamine and phosphoenolpyruvate by sialic acid synthase. An essential step for the sialylation process is the activation of endogenous sialic acid to a nucleotide sugar precursor (CMP-sialic acid) (233). Sialic acid polymerization with capsule repeating units and exportation are the final steps. Wessels et al., (233) created a mutant that was unable to activate sialic acid. This mutant had an asialo phenotype, but an increased amount of endogenous sialic acid (233).

It has been demonstrated that the sialylation process of GBS capsular polysaccharide is required for full synthesis of CPS by GBS (45). These investigators found a 80% reduction in surface associated CPS produced by asialo mutant strains that had a deletion in the *cpsK* gene encoding sialyltransferase (45) and *neuA* encoding CMP-sialic acid synthase (233) compared to the parental strain.

### 1.9.2 Surface proteins

Surface proteins of GBS play important roles during different infections stages, such as adherence to epithelial cells, interactions with human extracellular matrix or plasma proteins and escape from the host immunity response (234). Some of the surface proteins are conserved among all GBS isolates, others are found to be expressed in some but not all strains and have been used for subtyping purposes (234). GBS isolates encode a number of surface proteins, such as C proteins (α-protein and β protein) (235), the Alp like protein family (α, Alp1, Alp2, Alp3, Rib, and R4) (236), FbsA protein (237), Sip (238), (Srr1) (218, 239) and Srr2 (240), Laminin-binding protein (Lmb) (241) and C5a peptidase (217).

Another surface protein is BibA, encoded by *bibA* gene and found in all GBS strains. This protein consists of a helix-rich N-terminal domain, a proline-rich region and a canonical LPXTG cell wall-anchoring domain (242, 243). BibA, specifically the surface portion, was associated with protection in mice immunized with BibA. It also confers resistance to phagocytic killing and confers adhesion to host cells (242, 243). *bibA* has allelic variants which are associated with specific serotypes and clonal complexes (CCs) assigned by MLST assay (242, 244) One of the four allelic variants known as *hvgA* was thought to be strongly associated with clonal complex 17 (CC17) (244).

Pili are cell-surface appendages (245, 246). The genes encoding pili in GBS are located within two distinct loci in different regions of the genome, designated pilus-islands 1 and 2 (PI-1 and PI-2), the latter presenting two distinct variants, PI-2a and PI-2b (247). Pili consist of three structures: a backbone protein, the bona fide pilin, and two ancillary proteins, a pilus associated adhesin and a component that anchors the pili to the cell wall. Both the polymerization and attachment of the pili to the peptidoglycan cell wall occur by sortase-dependent mechanisms (248).

PI-2 is expressed in virtually all GBS strains, but PI-1 is only found in some GBS strains and they often appear in combinations (210). Certain pilus or combinations seem to be associated with particular serotypes (210). They have evoked protective immunity in mice and are hence recognized as a new candidate for a GBS vaccine (210). The three pilus-islands have well conserved sequences, except PI-2a that appears to show some extent of variability (210). A pilus structure has been recognized to play a role in adherence, invasion and translocation of epithelial cells (249-251) and biofilm formation (252, 253).

### 1.9.3 Other virulence factors

A number of secreted virulence factors have been described in GBS. CAMP factor has been routinely used to identify GBS isolates. GBS secretes a CAMP factor that lyses sheep red blood cells previously sensitized with a sphingomyelinase produced by some *Staphylococcus aureus* strains (74, 75). It has been shown that a CAMP factor is able to produce pores in target cells (254). However, its role in GBS pathogenicity needs to be confirmed.

GBS typically produces a narrow zone of β-hemolysis on 5% sheep blood agar (255). β-hemolysin/cytolysin is encoded by the cyl operon (255). β-hemolysin/cytolysin (β-h/c) is a pore-

forming membrane-associated toxin that promotes injury of a broad range of eukaryotic cell types (255).

### 1.10 Regulation of GBS virulence factors

GBS has a number of regulatory systems permitting the bacteria to respond and adapt to different environments encountered in the host. Generally, regulation is commonly controlled through two component systems (TCS), which senses environmental signals such as changes in $Mg^{2+}$ concentration, chemical gradients, osmolarity and the presence of auto-inducing or antimicrobial peptides (256). A two component system generally contains of a sensor histidine kinase on the bacterial membrane which becomes autophosphorylated and then phosphorylates a cognate DNA binding protein. In GBS, there are 17-20 identified two component systems (70, 257, 258) and six standalone transcriptional regulators (70). In contrast, other streptococci have fewer numbers of TCS, *S. pneumoniae* has 14, *S. pyogenes* contains 13 and *Lactobacillus lactis* encodes 8 TCSs (70). This likely reflects the lifestyle of GBS which needs the bacteria to adapt and thrive in different host environments.

The CovR/CovS (Control of virulence) is a TCS that allows the bacteria to adapt to a variety of environmental conditions during the infection process. TCS can regulate multiple virulence factors required for host adaptation and intracellular survival. For GBS, CovR/S is an orthologue of the CovR/S system (Capsule synthesis regulator, CsrS/CsrR) known to play a role in the virulence of *Streptococcus pyogenes* (group A streptococci, GAS). It is a major global regulatory system that is responsible for modulating the transcription of up to 7% of total GBS genomic genes including cell envelope, cellular process, metabolism and host-pathogen interaction

genes (259). In addition, a serine/threonine kinase regulator (Stk1) has been identified in GBS, which can alternatively phosphorylate the DNA binding protein CovR, deleting the repression effect of CovR. In GAS, CovR acts only as a repressor. Conversely, the CovR in GBS acts both as a repressor and an activator. In GAS and *S. suis*, mutations in c*ovR* or *covS* lead to increased intracellular survival and virulence in animal models (260, 261). In GBS, deletion of *covR* or *covS* has been found to display reduced virulence in mice and rat models (262, 263). In contrast, deletion of *covR* has shown to have an increased sepsis and blood brain barrier penetration in animal models, potentially explained by the lack of repression of β-hemolysin/cytolysin in these mutants (264).

In response to different environmental stimuli, the GBS transcriptome undergoes changing and remodelling (265, 266). Specifically, upon GBS growth in amniotic fluid, the organism up regulates genes responsible for aminoacid transport and down regulates stress genes (266). However, after 90 minutes incubation in blood in which GBS cannot grow, stress response genes and cell surface factors that bind or activate fibrinogen are highly upregulated (265, 267). In the amniotic fluid and during high temperature, the *cylE* (a gene encoded by cyl operon responsible for β-h/c activity) is upregulated, reflecting the destructive and immunomodulatory properties of this toxin during infection (265, 266). In low pH, CovR/CovS was found to remodel gene expression, specifically, down regulation of the virulence factors such as the β-hemolysin/cytolysin, BibA, and C5a peptidase. It also changed the expression of genes responsible for metabolism and transport proteins. (268).

Inactivation of *covR/covS* has been shown to display pleiotropic effect on GBS phenotypes, such as i) increased hemolytic activity, ii) reduced CAMP activity, iii) increased

adherence to epithelial cells, iv) increased expression of surface fibrous extracellular matrix, v) inability to initiate growth in minimal media and vi) reduction in capsular and sialic acid content (39). At the transcriptional level, mutations in *covR* were found to derepress *cylE* (β-hemolysin), *scpB* (encoding C5a peptidase), and *fbsA* (encoding a surface protein FbsA) whereas repress *cfb* (encoding CAMP factor) (262) and genes essential for capsule synthesis such as *cpsE* (262) and *cpsG* (269).

### 1.11 Characterization of GBS isolates

Bacterial epidemiologists use a combination of phenotypic and molecular typing methods to study the dissemination and population dynamics of human bacterial pathogens in clinical and environmental settings (270). Typing methods also permit the study of bacterial clonal lineages associated with colonization or invasive disease (271, 272). Furthermore, typing methods can also be used to address whether two or several isolates are epidemiologically connected, such as in nosocomial outbreaks. Epidemiological surveillance of an infectious disease over time using typing methods allows identification of disease trends and possible ways to control infection.

Multiple typing methods have been developed which vary in their discriminatory power. The discriminatory power is defined as the ability of a typing method to assign a different type to two unrelated strains sampled randomly from the population of a given species (270, 273). Based on the discriminatory power of each typing tool, the bacterial isolates could be clustered in specific groups. There is no optimal typing system for all epidemiological investigation purposes. Each typing system can answer a specific epidemiological question, such as identifying epidemiological linkage in outbreak, describing the distribution of bacterial types, or

generating hypotheses regarding epidemiological relationships between bacterial isolates (270, 273).

Typing systems identify variations in bacterial genomes. Two mechanisms are the main drivers of genetic variations in bacterial genomes, recombination and mutation (274). Recombination is thought to be responsible for the vast majority of genetic changes in a number of streptococci, including GBS (274-276). Recombination events alter the bulk of genome sequences whereas a point mutation changes only one nucleotide. Throughout the bacterial genome, recombination events and point mutations occur, but with different frequencies. Housekeeping and essential genes are generally conserved among all bacterial isolates for a given species. These genes have a very low mutation rate for which such mutations in these genes could be lethal or disadvantageous and may result in negative selection (277). The genome of bacterial isolates of the same species may vary in genetic islands. Identification of genetic islands is generally based on the differences in G+C content than the core genome (278). Genetic islands are acquired from another genome thorough horizontal gene transfer (HGT). Upon the loss of all movable genetic elements encoded in genetic islands, they become part of the core genome of a species which could increase the fitness of the host organism (279). However, some genetic islands carry some features of mobility, such as transposons, integrases and genomic materials from bacteriophages. Such islands are thought to be “hotspots” for variability for which mutations in these regions could help bacteria to adapt to different environments or change gene expression (270, 273, 280). Bacterial conserved and variable genome varies in their mutation rate. There are different genetic events that could affect the mutation rate, such as negative selection, HGT and homologous recombination or slipped stream mispairing in repeated sequences (270, 273,

280). To select the optimal genes for a molecular typing system, the purpose of the epidemiological investigation needs to be defined. Genes with low mutation rates are favorable for phylogenetic relationships and population dynamics studies (270, 273, 280). On the other hand, genes with high mutation rates are beter suited for outbreak investigations (270, 273, 280).

### 1.11.1 Typing methods of GBS isolates

Methods have been developed to permit the subdivision of bacterial strains within a species. A number of simple phenotypic methods such as colony appearance, biochemical differences, bacteriocin typing (susceptibility to a specific group of bacteriocins) and antibiotic susceptibility profiles have been used as initial typing techniques with low resolution (281). Immunological methods, phage typing or multi-locus enzyme electrophoresis (MLEE) (282) have been used for typing and have high discriminatory power. For GBS, typing of the capsular polysaccharides was originally performed by immunological methods (77-80, 283, 284), but recently, there is high propensity to use gene based methods (38-41, 43, 44). Typing methods based on the bacterial genome are more common because they have high resolution and discriminatory power and high reproducibility. However, most of these methods require a descend technology which is not available in developing countries. Some examples of these methods are DNA hybridization, restriction fragment length polymorphism (RFLP) (285), random amplification of polymorphic DNA-analysis (286), multilocus variable number of tandem repeat analysis (MLVA) assays, MLST (31) and pulsed field gel electrophoresis method (PFGE) (270, 287). Recently, the availability of complete genome sequences and comparative analysis methods of these

sequences offers the possibility to assess genetic differences within a bacterial species, providing insights on how genetic variability drives the evolution of virulence mechanisms.

#### 1.11.1.1 Capsular polysaccharide typing

GBS capsule designations are assigned by phenotypic or genotypic assays. Serological CPS typing is the most common phenotypic method for GBS CPS assignment. It is based on the expression of distinct polysaccharide capsules at the bacterial surface that react with serotype-specific antibodies raised in rabbits or mice. There are ten different serotypes recognized: Ia, Ib and II-IX (37, 128). A number of assays have been developed and used for serological CPS typing such as capillary precipitin (80), immunodiffusion (80, 283), latex agglutination (77, 284), coaggulation (79), or enzyme immunoassay (78). These assays have been found to be invaluable to identifying CPS types, but they have also resulted in a number of incorrect typing assignments due to poor capsule expression, capsule operon mutations, rearrangements or limited accuracy (230). GBS strains that tend to be difficult to type with serological methods are known as NT.

The sequences of the GBS CPS-determining regions have been published within the last ten years (9, 37, 40). This information has allowed the development of molecular typing based on PCR-assays that target *cps*-determining genes in the *cps* operon (39-42). These typing assays have been successful in typing a great number of GBS isolates that were found nontypeable by serological methods because of their high discriminatory power for identifying CPS types and the fact that they are genotypic, not phenotypic (39-42). However, these assays target capsule gene detection rather than capsule expression. Thus, the identified *cps* gene by the molecular assays does not confirm if the capsule polysaccharide is expressed or not. Moreover, such

assays tend to involve a combination of two different techniques, e.g., PCR plus sequencing (40), PCR plus blot hybridization (38, 43), PCR plus enzymatic restriction (44), or multiplex PCR plus agarose gel electrophoresis (39, 41).

#### 1.11.1.1.1 The nontypeable GBS phenotype

The proportion of NT isolates has increased over time (31, 37, 38), which could lead to misrepresentation of some of the serotypes in a study population worldwide, 7 to 32% of GBS isolates are believed to be NT (288) and the proportions are similar between invasive (37, 289-292) and noninvasive isolates (293-295). In contrast, GBS isolated from animals, such as bovine, were found to have a much higher proportion of NT (296). This might be due to the antisera used in the serological typing assays which was initially developed for human isolates (289, 296). In comparison to human NT isolates, bovine isolates had a higher incidence of mutations in the c*psE*, *cpsF* and *cpsG* region (296).

The NT phenotype of human GBS isolates may also be due to the expression of an undetectable amount of capsular polysaccharide by the serological methods, complete lack of capsule expression, or production of uncharacterized capsular polysaccharide for which antibody is not yet available (47). Technical reasons may also have contributed to the nontypeable phenotype of some GBS isolates. A number of factors could affect the sensitivity of the serological assays to detect GBS capsule polysaccharide. These methods depend on different capsule extraction procedures, the quality of the antibodies, the technical experience of the operator, and expression of detectable amount of capsule (47). A study by Kilian *et al.,* described drawbacks associated with GBS latex agglutination serotyping (297). In comparison to the flow cytometric assay, latex agglutination serotyping was less sensitive. Approximately one-

half of the strains assigned as NT in the agglutination test were found to express serotype-specific polysaccharides by the flow cytometric method (297).

A number of genetic alterations in *cps* genes also could affect capsule expression, such as insertion or deletion of DNA fragments in the *cps* operon, or mutation(s) in *cps* biosynthetic genes (38, 75, 298). It has been shown that an insertion sequence element designated IS1381 in the C terminus of *cpsE* could explain the nontypeable phenotype of GBS isolates since *cpsE* encodes a glycosyltransferase and is thought to have a role as the initiating enzyme in the biosynthesis of polysaccharide repeating units (299). The IS1381 insertion was initially identified in *S. pneumoniae* R6 strain (300) and has been found in GBS isolates of both human and bovine origin (301-303). A knockout mutant of *cpsE* resulted in a reduction of capsule production in both cell wall and protoplast fractions when compared with the wild-type parent strain (45). In addition, a previous study identified two NT isolates with two different insertional sequences within *cpsD*, one isolate encoded IS1548, and the other encoded IS861 (304). The insertion sequence IS1548 has been previously reported in the *hylB* gene of GBS and led to loss of the hemolysin function (304). Another genetic mechanism that could explain the nontypeable phenotype of GBS isolates is combination of mutations in *cps* genes, such as *cpsH*, *cpsJ* and *cpsF* or *cpsG* and *cpsA* as described previously (75). A further explanation for the inability of GBS to produce GBS capsular polysaccharide is the loss of the entire capsule operon by a recombination event. A study by Creti *et al*., identified one GBS isolate from human that is nontypeable because of the natural loss of the entire capsular operon (305). PCR-based typing methods have shown that some GBS NT isolates by serological assays still encode the genetic information for CPS formation (40, 77). This suggests that regulatory mechanisms of capsular

polysaccharide could be involved in the nontypeable phenotype and should be further considered and studied.

#### 1.11.1.1.2 Serotype distribution among colonized and iGBS diseases

Serotyping based on GBS capsular polysaccharide is an important methodology in epidemiological studies to evaluate fluctuations of different GBS clones in the human population (91, 166, 306). Distribution of GBS serotypes and the proportion of individual serotypes may vary geographically and over time, which may have a serious impact on a future implementation for CPS based vaccines. The prevalence of GBS serotypes also may vary among different age groups and colonizing and invasive isolates. Five serotypes (Ia, Ib, II, III and V) are responsible for 85% of GBS disease in North America and European countries (14-25) whereas serotypes VI and VIII are more commonly associated with colonization and iGBS disease in Japan (307). Previous studies from the United States and Canada described serotype Ia, III, and V as the most frequent serotypes among colonizing isolates (308). Serotype III is the most frequent among European countries (308) except Greece and Germany where serotype Ib is the most common (309). In Africa, the vast majority of GBS isolates from the few studies done belong to serotype III and V (310). The most common serotype reported in Asian countries are serotype III, followed by serotype Ib and V (310). In the Middle-east, serotypes Ia, II, III and V are predominant among pregnant women (310). Interestingly, recent studies have reported the emergence and circulation of CPS IV, a previously uncommon serotype, as an important cause of both neonatal and adult infections (16, 21, 26-30). Also, serotype IV has been found to be a frequent colonizer as well. In the United Arab Emirates, serotype IV is the most frequent colonizing isolate (311). Serotype IV is also common among colonizing isolates from Brazil

13.1% (312). The emergence of serotype IV has been linked to the capsular switch from serotype III to serotype IV or V, probably due to the selective immune pressure (16, 32). It has also been suggested that the acquisition of resistance to antibiotic classes along with other genetic determines could be the main driver for the emergence and expansion of some of GBS clones (49, 313).

GBS acquisition by newborns from maternal recto-vaginal colonization was assumed to reflect serotype prevalence among colonizing pregnant women and their neonates. In contrast, there are differences in the relative contribution of individual serotypes that cause invasive disease in neonates compared to their relative prevalence in maternal colonization (314, 315). These differences could reflect an increased invasive disease potential of certain GBS serotypes, rather than an increased risk of transmission of these serotypes from mothers to neonates.

In the past two decades, GBS has been increasingly associated with invasive disease in non-pregnant adults, particularly the elderly. Serotype V was rarely isolated from human before the mid-1980s (19), but it now accounts for the bulk of invasive GBS diseases in non-pregnant adult in North America and European countries (11, 16, 17, 19, 24, 316). A recent report described both serotypes V and III as equally frequent in Korea (185). Although serotypes VI and VIII are commonly found among maternal colonization, they are infrequent causes of invasive disease in Japan (307), serotype Ib is the most common among invasive diseases in non-pregnant adults (317).

A systematic review on serotype distribution in infant patients under three months reported that five serotypes (Ia, Ib, II, III, and V) accounted for more than 85.0% of serotypes in all regions, including 98.0% in Africa, 96.0% in the Americas, 93.0% in Europe, 89.0% in the

Western Pacific and 88.0% in the Eastern Mediterranean (6). When serotype distribution was stratified based on the onset of invasive disease, 37.0% of EOD was due to serotype III compared to 53.0% in LOD. Conversely, 40.0% of EOD was caused by serotype Ia compared with 30.0% of LOD(6).

There was limited data from Africa and the Eastern Mediterranean and none from South East Asia. In Africa, serotype Ia and III were consistently found to be the dominant serotypes, collectively associated with 80% and 77% of EOD in South Africa and Malawi, respectively. Similarly both serotypes accounted for 100% and 92% of LOD in South Africa and Malawi, respectively (135, 136). Serotype III was more likely to be isolated from LOD (72.0-75.7%) than in EOD (49.2-51.8%), and conversely serotype Ia was more common in EOD (26.0-31.0%) compared to LOD (20.0%-24.3%). This breakdown was consistent with invasive neonatal serotype epidemiology from the United States and European countries. All of all, GBS CPS distribution is dynamic in nature with some CPS's having a higher tropism for specific populations and emergence and circulating uncommon CPS types. This has direct implications for the design of serotype-specific polysaccharide vaccines for prevention of GBS patients; hence CPS type distribution studies provide crucial information for capsule-based vaccine formulation.

**1.11.1.2 Multilocus sequence typing**

A MLST scheme is generally used to assign GBS strains in to more than 700 sequence types (STs), which are grouped in a few clonal complexes (CCs) (31). MLST is based on the DNA sequencing of seven GBS housekeeping genes, which include alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*PheS*), amino acid transporter (*atr*), glutamine synthetase (*glnA*),

serine dehydratase (*sdhA*), glucose kinase (*glcK*) and transkelotase (*tkt*) (31). The sequence polymorphisms at each locus are classified and each allele is given a number. The combination of alleles found in each isolate defines the sequence type, and sequence types having allelic profiles that share at least five identical alleles are grouped within a particular clonal complex (CC) (318, 319).

MLST has been used to infer levels of relatedness between bacterial isolates and to reconstruct evolutionary events (318). It also has been increasingly used for the characterization of bacterial populations (318). An algorithm, eBURST, was developed to divide an MLST data set into groups of related isolates by implementing a simple model of clonal expansion and diversification (318). This model predicts that the emergence of clonal complexes (CCs) is due to an increase in the frequency of the founding genotype in a population, as a consequence of either a fitness advantage or of random genetic drift. This genotype increases in numbers and by gradual diversification (point mutation or recombination) that allows the emergence of a CC (318). In MLST, the descendants of the founder allelic profile will initially remain unchanged, but over time, variants in one of the seven alleles will occur (318). The genotypes, which have allelic profiles that differ from that of the founder at only one of the seven MLST loci, are called single-locus variants (SLVs). SLVs will finally diversify further, to produce variants that differ at two of the seven loci, called double-locus variants (DLVs) (318).

A wide number of advantages have been recognized for MLST. MLST uses standardized protocols, and provides specific data on single nucleotide changes. Also, the MLST data from different laboratories can be stored and compared on an open access online database

(http://pubmlst.org/agalactiae/). Therefore, MLST has become the most used method to compare the genetic relatedness of GBS isolates (320). In contrast, MLST has some drawbacks, such as high costs, long sample processing time of 3-4 work days and the need for a technical experience. MLST also does not provide information regarding the variability in genes other the seven housekeeping genes used in the MLST assay among bacteria within the same species (320).

#### 1.11.1.2.1 Distribution of clonal complex among colonized and iGBS isolates

MLST has been successfully used to characterize the clonal diversity of a number of human bacterial pathogens such as *Neisseria meningitides* and *S. pneumoniae* (321, 322). Based on the MLST data, identification the clonal lineages among colonized and iGBS isolates has been described in a wide range of studies from Europe and the United States (14, 316, 323-329). Regardless of geographical and epidemiological distinction, characterization of GBS colonizing isolates in maternal and neonatal hosts using MLST typing has identified a limited number of sequence types, ST1, ST19, ST17 and ST23 (330, 331). ST1 and ST19 were found to be responsible for more than 80% of sequence types in colonizing isolates (31). The four major sequence types belong to clonal complexes, CC1, CC19, CC17 and CC23, respectively. Among colonizing isolates, these clonal complexes accounted for 63.7% in Europe, 74.4% in the United States, 65.8% in Africa, 69.0% in Canada and 72.2% in the Middle East (31, 197, 325, 328, 330-333). In comparison with other pathogens in terms of clonal diversity based on MLST analysis, especially *S. pneumoniae* and *S. pyogenes*, GBS are less diverse (321, 334).

Manning *et al* have reported two different groups of GBS clones among different stages of pregnancy (324). Specifically, CC10 was significantly associated with GBS clearance whereas

CC17 and CC9 were associated with persistent colonization after pregnancy (324). These findings suggested that particular GBS clones are more likely to persistently colonize, after acquisition during pregnancy which permits their transmission to newborns during birth. The association between serotype III and CC17 has been widely documented (244, 335, 336). A study from Canada reported the prevalence of CC17 among colonized mothers and iGBS diseases in neonates (333).

CC17 and CC23 have been reported to be responsible for the majority of EOD and LOD (327). In EOD isolates, CC17 was found in 17.0% of EoD isolates in Canada (325) whereas CC19 accounted for 57.1% in Canada (333). The prevalence of CC19 in EOD supports the theory that maternal colonization is a major risk factor for EOD development. A higher proportion of LOD is associated with CC17 with prevalence ranging from 82.6% in France (129) to 81.6% in Italy (337). CC17 has consistently been found to be associated with neonatal iGBS disease; indicating intrinsic genetic properties of CC17 isolates to cause neonatal invasive disease (31, 129, 338). Among CC17 causing EOD and LOD, ST17 is the most predominant (129). The enhanced invasiveness abilities of ST17 clone in neonates has been attributed to a genomic locus (gbs2018C) that encodes a cell wall anchored protein HvgA (244). *hvgA* has been exclusively found in ST17 isolates and this gene was used as a rapid detection for this highly virulent GBS clone ST17 (244). *hvgA* was present in (25.6%) of GBS isolates, and all *hvgA* positive isolates were confirmed as ST17 by MLST (244). The overall prevalence of CC19 in LOD ranged between 5.3% in Italy and 20.0% in Canada isolates (325, 337). CC19 is predominant by a ST19, accounting for over 72.0% of all of CC19 sequence types (31, 330).

#### 1.11.1.2.2 Distribution of clonal complex among colonizing and iGBS capsular serotypes

Distribution of clonal complexes among colonizing and invasive capsular serotype has been investigated in many studies. CCs (CC17, CC19 and CC23) for both colonization and invasive disease are associated with particular capsular polysaccharides (316, 325, 330, 339). CC17 and CC19 (more than 90%) were associated with serotype III isolates whereas CC23 (90%) belongs to serotype Ia isolates (327, 330, 337). It has been thought that CC17 is a genetically homogenous group (32, 244). All described CC17 strains have been found to be serotype III (32), but capsular switching from type III to IV within CC17 background genome has been recently reported due to capsular switch of the entire cps operon (32, 207).

Between CC17 and CC19, there is a degree of variability between the proportion of colonizing and invasive disease isolates of serotypes III which are grouped either in CC17 or CC19. The bulk of serotype III invasive GBS isolates (56.4-73.1%) belong to CC17 whereas 18.8-21.8% of serotype III colonizing isolates are clustered in CC19 (31, 329, 336). Isolates representing serotype Ia regardless of colonizing (68.7-96.4%) or invasive isolates (66.6-84.0%) belong to CC23 (31, 329, 332, 337). In contrast, some CCs are associated with different GBS serotypes. ST1/CC1 encompasses of isolates representing mostly serotypes Ib and V. This interesting observation implies that serotypes Ib and V grouped with the same clonal complex, CC1, although each serotype has different invasiveness potential and prevalence (327, 330). However, serotype V was largely associated with ST1/CC1 in Europe and the United States (327, 330). This manifestation of GBS ST1/CC1 with serotype V isolates needs to be addressed to study the genomic, resistance and virulence gene make up of their genomes.

#### 1.11.1.3 Whole-genome sequences comparisons

Although MLST has been used to describe population structure in many studies, It has limited capability to identify and characterize genetic variations associated with extensive HGT and recombination among the different and the same bacterial clone (33, 48-51). Whole genome sequencing (WGS) has become accessible and affordable for more and more laboratories. Also, development of novel bioinformatics analyses and powerful computational tools has enabled studying the genetic variability among GBS isolates. WGS permits the interpretation and comparison with other genomes (30, 48, 49). The comparison of the complete sequences of pathogens within the species or other species enriches our understanding of the evolution of virulence mechanisms.

The first complete GBS genome sequences NEM316 strain (serotype III) and 2603V/R (serotype V) were made available in 2002 (70, 258)(65, 172). The size of the GBS genome is almost 2.2 Mbp long and has over 2100 predicted coding regions (65, 172). Both sequences revealed substantial similarity with the genomes of the related human pathogens *S. pyogenes* and *S. pneumoniae*, sharing a conserved backbone between streptococcal species (65, 172). In contrast, the GBS genome varied from other streptococci in regions encoding virulence genes, such as genes encoding surface proteins and genes related to mobile elements (65, 172). These regions are more likely part of pathogenicity islands (70). Genes encoded in pathogencity islands were found to be involved in GBS adaptation to distinct niches in GBS human and animal hosts and were expected to play a role in colonization or disease. The abundance of genes associated with mobile elements found in the variable regions of the genome, suggested the acquisition of the majority of strain specific traits depends on HGT (257). Among GBS isolates,

even of the same serotype, heterogeneity has been identified, including genes encoding surface and secreted proteins and capsule biosynthetic genes. The evolution of these genes is mainly driven through recombination events leading to gene acquisition, duplication, and reassortment. This could result in to the replacement of a region of several genes or to the allelic exchange of the internal part of a gene. These events likely explain the ability of a group of GBS isolates to express various combinations of virulence factors, which are likely to serve as means of adapting to host immunity and different environmental settings (223, 257). Integrative conjugative elements are also involved in the evolution of the GBS genome through their plasticity and potential for gene acquisition and mobilization, as well as exchange with other species (340). Large recombinational exchange (up to 334 kb) through mobilization by conjugative elements is another mechanism of GBS genome evolution. The GBS chromosome has been identified as a mosaic of large chromosomal fragments from different ancestors (257). All these evolution mechanisms could serve to enhance fitness and increase GBS ability to cause disease, escape human immune system or colonize their hosts.

### 1.12 Objectives and hypotheses

**Hypothesis Chapter 2:** Invasive GBS disease has decreased in neonates and increased in the older population in Alberta. This has led to change in GBS capsule distribution and increase in antimicrobial resistance.

**Objective Chapter 2:** to describe the epidemiology of iGBS disease in the Alberta population with respect to rate, CPS distribution and antimicrobial susceptibility from invasive GBS isolates collected from years 2003 to 2013.

**Hypothesis Chapter 3.1:** Sialic acid is as recognition marker for GBS surface capsule expression.

**Hypothesis Chapter 3.2:** GBS serotype-*cps* specific genes (*cpsG*-*cpsK*) assist in the identification of the 10 GBS type.

**Objective Chapter 3.1:** Develop a sialic acid detection assays to identify iGBS expressing surface sialic acid.

**Objective Chapter 3.2:** To develop a real-time PCR assay that can identify the GBS capsule type based on a CPS-specific gene scheme.

**Hypothesis Chapter 4.1:** *covR* and *covS* mutations result in the loss of the expression of a number of virulence attributes.

**Hypothesis Chapter 4.2:** iGBS isolates that fail to assign a CPS type by serological and molecular assays, express surface capsule that encode novel *cps* genes.

**Objective Chapter 4.1:** To identify *covRS* mutants among sialic acid negative iGBS isolates and characterize these mutants with respect to a variety of virulence characteristics.

**Objective Chapter 4.2:** To identify novel CPS type(s) among iGBS isolates that express capsule but fail to be typed by a serological assay and a molecular assay.

**Objective Chapter 5.1:** To elucidate the genome sequence of the adult serotype V/ST1 PLGBS 13 strain and gain a better understanding of the strain virulence attributes and antimicrobial resistance profile

**Objective Chapter 5.2:** To compare the complete genome of the PLGBS 13 strain to other GBS genomes available in the GenBank database and to the genomes of four adult ST1 GBS isolates collected from Alberta.

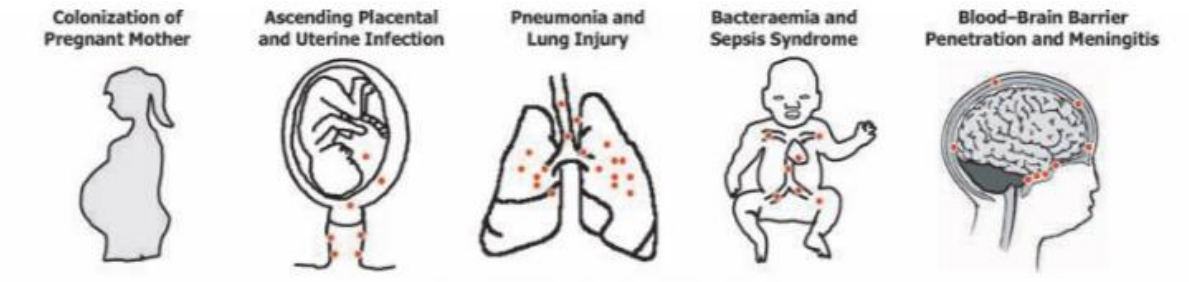
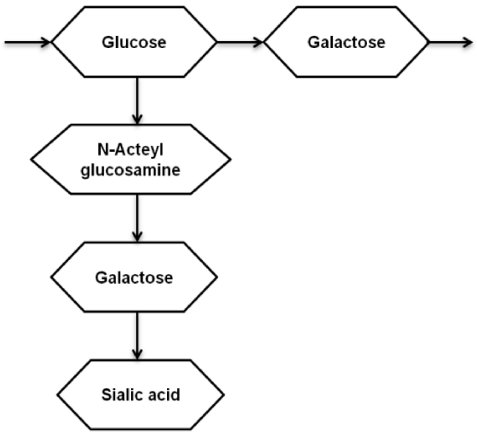



Figure 1.1. Stages in the molecular and cellular pathogenesis of neonatal Group B Streptococcal (GBS) infection [adapted from Doran and Nizet 2004, (10)

CPS type III




CPS type V

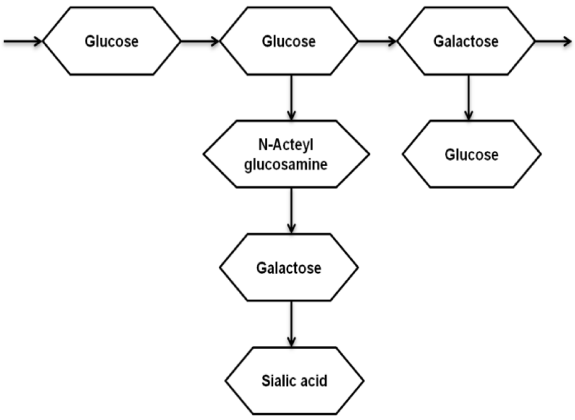



**Figure 2.2. Chemical structure of the oligosaccharide subunits of two of the most common GBS capsular polysaccharide types, CPS type III and CPS type V.**
The subunits consist of a backbone with side chains. The subunits are usually repeated 100 times and more. Type III and type V are illustrated with a subunit of five monosaccharides and seven monosaccharides, respectively.

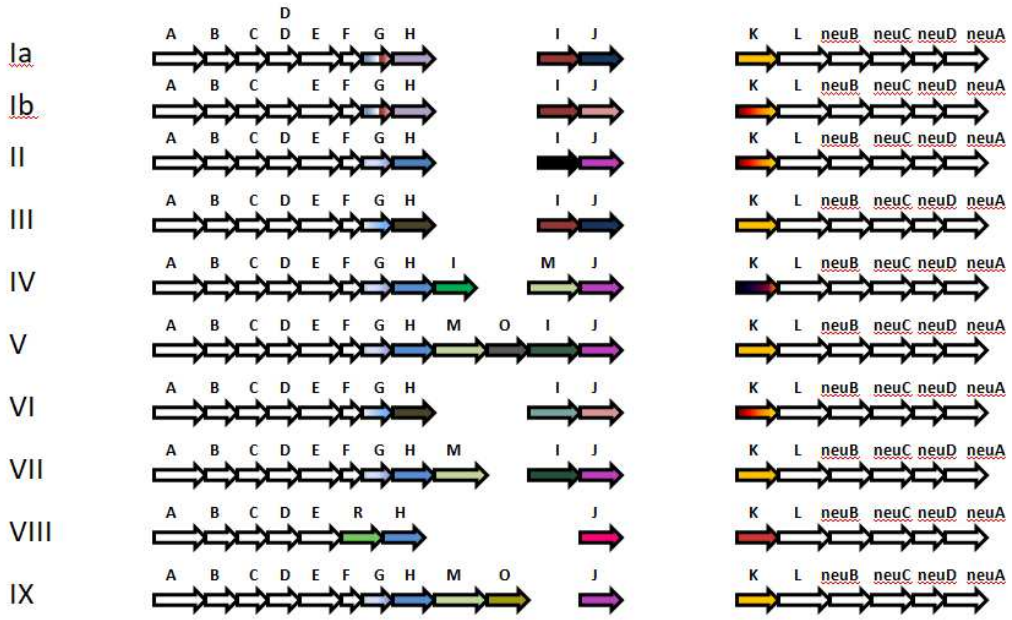



Figure 1.3. Capsule gene clusters of the ten GBS serotypes.

The colours inside each arrow of GBS cps genes indicate the degree of similarity to other cps genes at the aminoacid sequence level. Arrows with the same color indicate 100% identical aminoacid sequences whereas arrows with different shade of the same color indicate related amino acid sequences. White arrows indicate conserved aminoacid sequences of cps genes among all the ten capsule types. A gap was introduced between genes *cpsH-cpsI* and *cpsJ-cpsK* to permit an alignment of corresponding cps genes [Adapted from Berti *et al*. 2014 and Cieslewicz *et al*. 2005, (8, 9)].

## 1.13 References


1. **Schuchat A.** 1999. Group B streptococcus. Lancet **353:**51-56.
2. **Baker CJ, Barrett FF.** 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr **83:**919-925.
3. **Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.
4. **Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.
5. **Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol **96:**498-503.
6. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.
7. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.
8. **Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, Romano MR, Pinto V, Brogioni B, Torricelli G, Janulczyk R, Grandi G, Margarit I.** 2014. Structure of the type IX

group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem **289:**23437-23448.

9. **Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

10. **Doran KS, Nizet V.** 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Molecular microbiology **54:**23-31.

11. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ, Network ABCsEIP.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299:**2056-2065.

12. **Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER, Jackson D, Thompson T, Schrag SJ.** 2009. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis **49:**85-92.

13. **Edwards MS, Baker CJ.** 2005. Group B streptococcal infections in elderly adults. Clin Infect Dis **41:**839-847.

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

15. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A,**

**Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

16. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol doi:10.1128/JCM.03554-13.

17. **Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol doi:10.1128/JCM.00355-16.

18. **Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand **87:**260-271.

19. **Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis **173:**365-373.

20. **Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microb Drug Resist **10:**31-36.

21. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus

Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

22. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.

23. **Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A.** 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis **177:**998-1002.

24. **Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.

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

26. **Ferrieri P, Lynfield R, Creti R, Flores AE.** 2013. Serotype IV and invasive group B Streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis **19:**551-558.

27. **Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B.** 2011. Emergence of group B Streptococcus serotype IV in women of child-bearing age in Ireland. Epidemiol Infect **139:**236-238.

28. **Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P.** 2010. Clonal analysis of colonizing group B Streptococcus, serotype IV, an emerging pathogen in the United States. J Clin Microbiol **48:**3100-3104.

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

30. **Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

31. **Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

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

33. **Campisi E, Rinaudo CD, Donati C, Barucco M, Torricelli G, Edwards MS, Baker CJ, Margarit I, Rosini R.** 2016. Serotype IV Streptococcus agalactiae ST-452 has arisen from

large genomic recombination events between CC23 and the hypervirulent CC17 lineages. Sci Rep **6:**29799.

34. **Doran KS, Nizet V.** 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol **54:**23-31.

35. **Herbert MA, Beveridge CJ, Saunders NJ.** 2004. Bacterial virulence factors in neonatal sepsis: group B streptococcus. Curr Opin Infect Dis **17:**225-229.

36. **Rubens CE, Wessels MR, Kuypers JM, Kasper DL, Weiser JN.** 1990. Molecular analysis of two group B streptococcal virulence factors. Semin Perinatol **14:**22-29.

37. **Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL.** 2007. Serotype IX, a Proposed New Streptococcus agalactiae Serotype. J Clin Microbiol **45:**2929-2936.

38. **Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL.** 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol **46:**2745-2750.

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

40. **Kong F, Gowan S, Martin D, James G, Gilbert GL.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol **40:**216-226.

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

42. **Wen L, Wang Q, Li Y, Kong F, Gilbert GL, Cao B, Wang L, Feng L.** 2006. Use of a serotype-specific DNA microarray for identification of group B Streptococcus (Streptococcus agalactiae). J Clin Microbiol **44:**1447-1452.

43. **Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae using multiplex PCR and reverse line blot hybridization. J Med Microbiol **54:**1133-1138.

44. **Sellin M, Olofsson C, Håkansson S, Norgren M.** 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol **38:**3420-3428.

45. **Chaffin DO, Mentele LM, Rubens CE.** 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol **187:**4615-4626.

46. **Geisler C, Jarvis DL.** 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology **21:**988-993.

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

48. **Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D,**

**Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P, Consortium D.** 2014. Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **5:**4544.

49. **Flores AR, Galloway-Peña J, Sahasrabhojane P, Saldaña M, Yao H, Su X, Ajami NJ, Holder ME, Petrosino JF, Thompson E, Margarit Y Ros I, Rosini R, Grandi G, Horstmann N, Teatero S, McGeer A, Fittipaldi N, Rappuoli R, Baker CJ, Shelburne SA.** 2015. Sequence type 1 group B Streptococcus, an emerging cause of invasive disease in adults, evolves by small genetic changes. Proc Natl Acad Sci U S A **112:**6431-6436.

50. **Neemuchwala A, Teatero S, Athey TB, McGeer A, Fittipaldi N.** 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B Streptococcus. Emerg Infect Dis **22:**1941-1944.

51. **Sørensen UB, Poulsen K, Ghezzo C, Margarit I, Kilian M.** 2010. Emergence and global dissemination of host-specific Streptococcus agalactiae clones. MBio **1**.

52. **Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, Corander J, Bryant J, Parkhill J, Nejentsev S, Horstmann RD, Brown T, Drobniewski F.** 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet **46:**279-286.

53. **Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, Pessia A, Aanensen DM, Mather AE, Page AJ, Salter SJ, Harris D, Nosten F, Goldblatt D, Corander J, Parkhill J, Turner P, Bentley SD.** 2014. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet **46:**305-309.

54. **Lancefield RC.** 1933. A serological differention of human and other groups of hemolytic streptococci. J Exp Med **57:**571-595.

55. **Lancefield RC.** 1934. A serological differention of specific types of bovine hemolytic streptococci (Group B streptococci). J Exp Med **59:**441-458.

56. **Lancefield RC, Hare R.** 1935. The serological differention of pathogenic and non-pathogenic strains of hemolytic from parturent women. J Exp Med **61:**335-349.

57. **Harper IA.** 1971. The importance of group B streptococci as human pathogens in the British Isles. J Clin Pathol **24:**438-441.

58. **Farley MM, Harvey RC, Stull T, Smith JD, Schuchat A, Wenger JD, Stephens DS.** 1993. A population-based assessment of invasive disease due to group B Streptococcus in nonpregnant adults. N Engl J Med **328:**1807-1811.

59. **Elliott JA, Facklam RR, Richter CB.** 1990. Whole-cell protein patterns of nonhemolytic group B, type Ib, streptococci isolated from humans, mice, cattle, frogs, and fish. J Clin Microbiol **28:**628-630.

60. **Evans JJ, Klesius PH, Pasnik DJ, Bohnsack JF.** 2009. Human Streptococcus agalactiae isolate in Nile tilapia (Oreochromis niloticus). Emerg Infect Dis **15:**774-776.

61. **Evans JJ, Pasnik DJ, Klesius PH, Al-Ablani S.** 2006. First report of Streptococcus agalactiae and Lactococcus garvieae from a wild bottlenose dolphin (Tursiops truncatus). J Wildl Dis **42:**561-569.

62. **Geng Y, Wang KY, Huang XL, Chen DF, Li CW, Ren SY, Liao YT, Zhou ZY, Liu QF, Du ZJ, Lai WM.** 2012. Streptococcus agalactiae, an emerging pathogen for cultured ya-fish, Schizothorax prenanti, in China. Transbound Emerg Dis **59:**369-375.

63. **Wagner M, Kaatz M.** 1997. Virulence of strains of new types and type candidates of group B streptococci (Streptococcus agalactiae). Comparative evaluation using mice and a chicken embryo model. Adv Exp Med Biol **418:**819-821.

64. **Wilkinson HW, Thacker LG, Facklam RR.** 1973. Nonhemolytic group B streptococci of human, bovine, and ichthyic origin. Infect Immun **7:**496-498.

65. **Yildirim AO, Lämmler C, Weiss R.** 2002. Identification and characterization of Streptococcus agalactiae isolated from horses. Vet Microbiol **85:**31-35.

66. **Zappulli V, Mazzariol S, Cavicchioli L, Petterino C, Bargelloni L, Castagnaro M.** 2005. Fatal necrotizing fasciitis and myositis in a captive common bottlenose dolphin (Tursiops truncatus) associated with Streptococcus agalactiae. J Vet Diagn Invest **17:**617-622.

67. **Liddy H, Holliman R.** 2002. Group B Streptococcus highly resistant to gentamicin. J Antimicrob Chemother **50:**142-143.

68. **Wheeler SM, Foley GE.** 1943. A Note on Non-Group-A Streptococci Associated with Human Infection. J Bacteriol **46:**391-392.

69. **Richards VP, Choi SC, Pavinski Bitar PD, Gurjar AA, Stanhope MJ.** 2013. Transcriptomic and genomic evidence for Streptococcus agalactiae adaptation to the bovine environment. BMC Genomics **14:**920.

70. **Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, Msadek T, Zouine M, Couvé E, Lalioui L, Poyart C, Trieu-Cuot P, Kunst F.** 2002. Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease. Mol Microbiol **45:**1499-1513.

71. **Jones AL, Knoll KM, Rubens CE.** 2000. Identification of Streptococcus agalactiae virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. Mol Microbiol **37:**1444-1455.

72. **Baker CJ, Byington CL, Polin RA.** 2011. Policy statement-Recommendations for the prevention of perinatal group B streptococcal (GBS) disease. Pediatrics **128:**611-616.

73. **Schrag S, Gorwitz R, Fultz-Butts K, Schuchat A.** 2002. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recomm Rep **51:**1-22.

74. **Tapsall JW, Phillips EA.** 1987. Presumptive identification of group B streptococci by rapid detection of CAMP factor and pigment production. Diagn Microbiol Infect Dis **7:**225-228.

75. **Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC.** 2006. Identification of novel cps locus polymorphisms in nontypable group B Streptococcus. J Med Microbiol **55:**775-783.

76. **Daly JA, Seskin KC.** 1988. Evaluation of rapid, commercial latex techniques for serogrouping beta-hemolytic streptococci. J Clin Microbiol **26:**2429-2431.

77. **Slotved HC, Elliott J, Thompson T, Konradsen HB.** 2003. Latex assay for serotyping of group B Streptococcus isolates. J Clin Microbiol **41:**4445-4447.

78. **Arakere G, Flores AE, Ferrieri P, Frasch CE.** 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. J Clin Microbiol **37:**2564-2567.

79. **Håkansson S, Burman LG, Henrichsen J, Holm SE.** 1992. Novel coagglutination method for serotyping group B streptococci. J Clin Microbiol **30:**3268-3269.

80. **Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

81. **Verhoeven PO, Noyel P, Bonneau J, Carricajo A, Fonsale N, Ros A, Pozzetto B, Grattard F.** 2014. Evaluation of the new brilliance GBS chromogenic medium for screening of Streptococcus agalactiae vaginal colonization in pregnant women. J Clin Microbiol **52:**991-993.

82. **Ke D, Ménard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, Bergeron MG.** 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem **46:**324-331.

83. **Bergh K, Stoelhaug A, Loeseth K, Bevanger L.** 2004. Detection of group B streptococci (GBS) in vaginal swabs using real-time PCR with TaqMan probe hybridization. Indian J Med Res **119 Suppl:**221-223.

84. **Bergseng H, Bevanger L, Rygg M, Bergh K.** 2007. Real-time PCR targeting the sip gene for detection of group B Streptococcus colonization in pregnant women at delivery. J Med Microbiol **56:**223-228.

85. **Uhl JR, Vetter EA, Boldt KL, Johnston BW, Ramin KD, Adams MJ, Ferrieri P, Reischl U, Cockerill FR.** 2005. Use of the Roche LightCycler Strep B assay for detection of group B Streptococcus from vaginal and rectal swabs. J Clin Microbiol **43:**4046-4051.

86. **Prevention CfDCa.** 2010. Prevention of Perinatal Group B Streptococcus Diseases Revised Guidlines from CDC

87. **Regan JA, Klebanoff MA, Nugent RP.** 1991. The epidemiology of group B streptococcal colonization in pregnancy. Vaginal Infections and Prematurity Study Group. Obstet Gynecol **77:**604-610.

88. **Hansen SM, Uldbjerg N, Kilian M, Sørensen UB.** 2004. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. J Clin Microbiol **42:**83-89.

89. **Melin P.** 2011. Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. Clin Microbiol Infect **17:**1294-1303.

90. **Foxman B, Gillespie BW, Manning SD, Marrs CF.** 2007. Risk factors for group B streptococcal colonization: potential for different transmission systems by capsular type. Ann Epidemiol **17:**854-862.

91. **Hickman ME, Rench MA, Ferrieri P, Baker CJ.** 1999. Changing epidemiology of group B streptococcal colonization. Pediatrics **104:**203-209.

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

93. **Sendi P, Johansson L, Norrby-Teglund A.** 2008. Invasive group B Streptococcal disease in non-pregnant adults : a review with emphasis on skin and soft-tissue infections. Infection **36:**100-111.

94. **Rocchetti TT, Marconi C, Rall VL, Borges VT, Corrente JE, da Silva MG.** 2011. Group B streptococci colonization in pregnant women: risk factors and evaluation of the vaginal flora. Arch Gynecol Obstet **283:**717-721.

95. **Dillon HC, Gray E, Pass MA, Gray BM.** 1982. Anorectal and vaginal carriage of group B streptococci during pregnancy. J Infect Dis **145:**794-799.

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

97. **Quinlan JD, Hill DA, Maxwell BD, Boone S, Hoover F, Lense JJ.** 2000. The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. J Fam Pract **49:**447-448.

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

99. **Grimwood K, Stone PR, Gosling IA, Green R, Darlow BA, Lennon DR, Martin DR.** 2002. Late antenatal carriage of group B Streptococcus by New Zealand women. Aust N Z J Obstet Gynaecol **42:**182-186.

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

101. **Garland SM, Kelly N, Ugoni AM.** 2000. Is antenatal group B streptococcal carriage a predictor of adverse obstetric outcome? Infect Dis Obstet Gynecol **8:**138-142.

102. **van der Mee-Marquet N, Jouannet C, Domelier AS, Arnault L, Lartigue MF, Quentin R.** 2009. Genetic diversity of Streptococcus agalactiae strains and density of vaginal carriage. J Med Microbiol **58:**169-173.

103. **Maniatis AN, Palermos J, Kantzanou M, Maniatis NA, Christodoulou C, Legakis NJ.** 1996. Streptococcus agalactiae: a vaginal pathogen? J Med Microbiol **44:**199-202.

104. **Cutland CL, Madhi SA, Zell ER, Kuwanda L, Laque M, Groome M, Gorwitz R, Thigpen MC, Patel R, Velaphi SC, Adrian P, Klugman K, Schuchat A, Schrag SJ, Team PT.** 2009. Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised, controlled trial. Lancet **374:**1909-1916.

105. **Suara RO, Adegbola RA, Baker CJ, Secka O, Mulholland EK, Greenwood BM.** 1994. Carriage of group B Streptococci in pregnant Gambian mothers and their infants. J Infect Dis **170:**1316-1319.

106. **Joachim A, Matee MI, Massawe FA, Lyamuya EF.** 2009. Maternal and neonatal colonisation of group B streptococcus at Muhimbili National Hospital in Dar es Salaam, Tanzania: prevalence, risk factors and antimicrobial resistance. BMC Public Health **9:**437.

107. **de Steenwinkel FD, Tak HV, Muller AE, Nouwen JL, Oostvogel PM, Mocumbi SM.** 2008. Low carriage rate of group B streptococcus in pregnant women in Maputo, Mozambique. Trop Med Int Health **13:**427-429.

108. **Osman NB, Folgosa E, Bergström S.** 1995. An incident case-referent study of threatening preterm birth and genital infection. J Trop Pediatr **41:**267-272.

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

110. **Whitney CG, Daly S, Limpongsanurak S, Festin MR, Thinn KK, Chipato T, Lumbiganon P, Sauvarin J, Andrews W, Tolosa JE, Health GNFPAR.** 2004. The international infections in pregnancy study: group B streptococcal colonization in pregnant women. J Matern Fetal Neonatal Med **15:**267-274.

111. **Zamzami TY, Marzouki AM, Nasrat HA.** 2011. Prevalence rate of group B streptococcal colonization among women in labor at King Abdul-Aziz University Hospital. Arch Gynecol Obstet **284:**677-679.

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

113. **Krohn MA, Hillier SL, Baker CJ.** 1999. Maternal peripartum complications associated with vaginal group B streptococci colonization. J Infect Dis **179:**1410-1415.

114. **Yancey MK, Duff P, Clark P, Kurtzer T, Frentzen BH, Kubilis P.** 1994. Peripartum infection associated with vaginal group B streptococcal colonization. Obstet Gynecol **84:**816-819.

115. **Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P, Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M.** 2012. Prevention of group B

streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis **31:**2097-2104.

116. **Schrag SJ, Zywicki S, Farley MM, Reingold AL, Harrison LH, Lefkowitz LB, Hadler JL, Danila R, Cieslak PR, Schuchat A.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. N Engl J Med **342:**15-20.

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

118. **Puopolo KM, Madoff LC, Eichenwald EC.** 2005. Early-onset group B streptococcal disease in the era of maternal screening. Pediatrics **115:**1240-1246.

119. **Daniels JP, Gray J, Pattison HM, Gray R, Hills RK, Khan KS, Group GC.** 2011. Intrapartum tests for group B streptococcus: accuracy and acceptability of screening. BJOG **118:**257-265.

120. **Heath PT, Schuchat A.** 2007. Perinatal group B streptococcal disease. Best Pract Res Clin Obstet Gynaecol **21:**411-424.

121. **Thigpen MC, Whitney CG, Messonnier NE, Zell ER, Lynfield R, Hadler JL, Harrison LH, Farley MM, Reingold A, Bennett NM, Craig AS, Schaffner W, Thomas A, Lewis MM, Scallan E, Schuchat A, Network EIP.** 2011. Bacterial meningitis in the United States, 1998-2007. N Engl J Med **364:**2016-2025.

122. **Verani JR, McGee L, Schrag SJ, Division of Bacterial Diseases NCfIaRD, C.nters for Disease Control and Prevention (CDC).** 2010. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep **59:**1-36.

123. **Edwards MS.** 1990. Group B streptococcal infections. The Pediatric Infectious Disease Journal **9:**779-780.

124. **Poyart C, Réglier-Poupet H, Tazi A, Billoët A, Dmytruk N, Bidet P, Bingen E, Raymond J, Trieu-Cuot P.** 2008. Invasive group B streptococcal infections in infants, France. Emerg Infect Dis **14:**1647-1649.

125. **Ferrieri P, Blair LL.** 1977. Pharyngeal carriage of group B streptococci: detection by three methods. J Clin Microbiol **6:**136-139.

126. **CDC.** 2005. Early-onset and late-onset neonatal group B streptococcal disease--United States, 1996-2004. MMWR Morb Mortal Wkly Rep **54:**1205-1208.

127. **Bedford H, de Louvois J, Halket S, Peckham C, Hurley R, Harvey D.** 2001. Meningitis in infancy in England and Wales: follow up at age 5 years. BMJ **323:**533-536.

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

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

130. **Kotiw M, Zhang GW, Daggard G, Reiss-Levy E, Tapsall JW, Numa A.** 2003. Late-onset and Recurrent Neonatal Group B Streptococcal Disease Associated with Breast-milk Transmission. Pediatric and Developmental Pathology **6:**251-256.

131. **Jordan HT, Farley MM, Craig A, Mohle-Boetani J, Harrison LH, Petit S, Lynfield R, Thomas A, Zansky S, Gershman K, Albanese BA, Schaffner W, Schrag SJ, Active**

**Bacterial Core Surveillance (ABCs)/Emerging Infections Program Network CDC.** 2008. Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. Pediatr Infect Dis J **27:**1057-1064.

132. **Green PA, Singh KV, Murray BE, Baker CJ.** 1994. Recurrent group B streptococcal infections in infants: clinical and microbiologic aspects. J Pediatr **125:**931-938.

133. **Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nat Rev Micro **4:**932-942.

134. **Tazi A, Bellais S, Tardieux I, Dramsi S, Trieu-Cuot P, Poyart C.** 2012. Group B Streptococcus surface proteins as major determinants for meningeal tropism. Curr Opin Microbiol **15:**44-49.

135. **Gray KJ, Bennett SL, French N, Phiri AJ, Graham SM.** 2007. Invasive group B streptococcal infection in infants, Malawi. Emerg Infect Dis **13:**223-229.

136. **Madhi SA, Radebe K, Crewe-Brown H, Frasch CE, Arakere G, Mokhachane M, Kimura A.** 2003. High burden of invasive Streptococcus agalactiae disease in South African infants. Ann Trop Paediatr **23:**15-23.

137. **Berkley JA, Lowe BS, Mwangi I, Williams T, Bauni E, Mwarumba S, Ngetsa C, Slack MP, Njenga S, Hart CA, Maitland K, English M, Marsh K, Scott JA.** 2005. Bacteremia among children admitted to a rural hospital in Kenya. N Engl J Med **352:**39-47.

138. **Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER, Jackson D, Thompson T, Schrag SJ.** 2009. Increasing Burden of Invasive Group B Streptococcal Disease in Nonpregnant Adults, 1990–2007. Clinical Infectious Diseases **49:**85-92.

139. **Farley MM.** 2001. Group B streptococcal disease in nonpregnant adults. Clin Infect Dis **33:**556-561.

140. **Henning KJ, Hall EL, Dwyer DM, Billmann L, Schuchat A, Johnson JA, Harrison LH, Program MEI.** 2001. Invasive group B streptococcal disease in Maryland nursing home residents. J Infect Dis **183:**1138-1142.

141. **Schuchat A, Balter S.** 2006. Epidemiology of Group B Streptococcal Infections, Gram-Positive Pathogens, Second Edition doi:doi:10.1128/9781555816513.ch15. American Society of Microbiology.

142. **Sendi P, Johansson L, Norrby-Teglund A.** 2008. Invasive Group B Streptococcal Disease in Non-pregnant Adults. Infection **36:**100-111.

143. **Sendi P, Johansson L, Dahesh S, Van-Sorge NM, Darenberg J, Norgren M, Sjölin J, Nizet V, Norrby-Teglund A.** 2009. Bacterial phenotype variants in group B streptococcal toxic shock syndrome. Emerging infectious diseases **15:**223-232.

144. **Harrison LH, Ali A, Dwyer DM, Libonati JP, Reeves MW, Elliott JA, Billmann L, Lashkerwala T, Johnson JA.** 1995. Relapsing invasive group B streptococcal infection in adults. Annals of internal medicine **123:**421-427.

145. **Jackson LA, Hilsdon R, Farley MM, Harrison LH, Reingold AL, Plikaytis BD, Wenger JD, Schuchat A.** 1995. Risk factors for group B streptococcal disease in adults. Annals of internal medicine **123:**415-420.

146. **Money D, Allen VM.** 2013. The prevention of early-onset neonatal group B streptococcal disease. J Obstet Gynaecol Can **35:**939-951.

147. **Schrag S, Gorwitz R, Fultz-Butts K, Schuchat A.** 2002. Prevention of perinatal group B streptococcal disease. MMWR Recomm Rep **51:**1-22.

148. **CDC.** 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. Pediatrics **99:**489-496.

149. **Pearlman M.** 2003. Prevention of Early-Onset Group B Streptococcal Disease in Newborns. Obstetrics & Gynecology **102:**414-415.

150. **Boyer KM, Gotoff SP.** 1986. Prevention of early-onset neonatal group B streptococcal disease with selective intrapartum chemoprophylaxis. New England Journal of Medicine **314:**1665-1669.

151. **Darlow B, Campbell N, Austin N, Chin A, Grigg C, Skidmore C, Voss L, Walls T, Wise M, Werno A.** 2015. The prevention of early-onset neonatal group B streptococcus infection: New Zealand Consensus Guidelines 2014.

152. **Chen KT, Puopolo KM, Eichenwald EC, Onderdonk AB, Lieberman E.** 2005. No increase in rates of early-onset neonatal sepsis by antibiotic-resistant group B Streptococcus in the era of intrapartum antibiotic prophylaxis. American journal of obstetrics and gynecology **192:**1167-1171.

153. **Davies HD, Leblanc J, Bortolussi R, McGeer A, PICNIC.** 1999. The Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) study of neonatal group B streptococcal infections in Canada. Paediatr Child Health **4:**257-263.

154. **Davies HD, Adair CE, Schuchat A, Low DE, Sauve RS, McGeer A, Network ANGBSNatTIBD.** 2001. Physicians' prevention practices and incidence of neonatal group B streptococcal disease in 2 Canadian regions. CMAJ **164:**479-485.

155. **Lyytikäinen O, Nuorti JP, Halmesmäki E, Carlson P, Uotila J, Vuento R, Ranta T, Sarkkinen H, Ämmälä M, Kostiala A.** 2003. Invasive group B streptococcal infections in Finland: a population-based study. Emerging infectious diseases **9:**470.

156. **López Sastre JB, Fernández Colomer B, Coto Cotallo GD, Ramos Aparicio A.** 2005. Trends in the epidemiology of neonatal sepsis of vertical transmission in the era of group B streptococcal prevention. Acta Paediatrica **94:**451-457.

157. **Strakova L, Motlova J.** 2004. Active surveillance of early onset disease due to group B streptococci in newborns. Indian Journal of Medical Research **119:**205.

158. **Madhi SA, Kuwanda L, Cutland C, Klugman KP.** 2005. The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and-uninfected children. Clinical Infectious Diseases **40:**1511-1518.

159. **Cutland CL, Madhi SA, Zell ER, Kuwanda L, Laque M, Groome M, Gorwitz R, Thigpen MC, Patel R, Velaphi SC.** 2009. Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised, controlled trial. The Lancet **374:**1909-1916.

160. **Matsubara K, Katayama K, Baba K, Nigami H, Harigaya H, Sugiyama M.** 2002. Seroepidemiologic studies of serotype VIII group B Streptococcus in Japan. Journal of Infectious Diseases **186:**855-858.

161. **Yu H-W, Lin H-C, Yang P-H, Hsu C-H, Hsieh W-S, Tsao L-Y, Chen C-H, Lin H-C, Tseng Y-C.** 2011. Group B streptococcal infection in Taiwan: maternal colonization and neonatal infection. Pediatrics & Neonatology **52:**190-195.

162. **Kuruvilla K, Thomas N, Jesudasan M, Jana A.** 1999. Neonatal group B streptococcal bacteraemia in India: ten years' experience. Acta Paediatrica **88:**1031-1032.

163. **Ojukwu JU, Abonyi LE, Ugwu J, Orji IK.** 2006. Neonatal septicemia in high risk babies in South-Eastern Nigeria. Journal of perinatal medicine **34:**166-172.

164. **Shet A, Ferrieri P.** 2004. Neonatal & maternal group B streptococcal infections: a comprehensive review. Indian Journal of Medical Research **120:**141.

165. **Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta obstetricia et gynecologica Scandinavica **87:**260-271.

166. **Kimura K, Suzuki S, Wachino J-i, Kurokawa H, Yamane K, Shibata N, Nagano N, Kato H, Shibayama K, Arakawa Y.** 2008. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob Agents Chemother **52:**2890-2897.

167. **Dahesh S, Hensler ME, Van Sorge NM, Gertz RE, Schrag S, Nizet V, Beall BW.** 2008. Point mutation in the group B streptococcal pbp2x gene conferring decreased susceptibility to β-lactam antibiotics. Antimicrob Agents Chemother **52:**2915-2918.

168. **Verani JR, McGee L, Schrag SJ; Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC).** 2010. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. **19:**1-36.

169. **Schoening T, Wagner J, Arvand M.** 2005. Prevalence of erythromycin and clindamycin resistance among Streptococcus agalactiae isolates in Germany. Clinical Microbiology and Infection **11:**579-582.

170. **Pinheiro S, Radhouani H, Coelho C, Gonçalves A, Carvalho E, Carvalho JA, Ruiz-Larrea F, Torres C, Igrejas G, Poeta P.** 2009. Prevalence and mechanisms of erythromycin resistance in Streptococcus agalactiae from healthy pregnant women. Microbial Drug Resistance **15:**121-124.

171. **Borchardt SM, DeBusscher JH, Tallman PA, Manning SD, Marrs CF, Kurzynski TA, Foxman B.** 2006. Frequency of antimicrobial resistance among invasive and colonizing Group B streptococcal isolates. BMC Infect Dis **6:**57.

172. **Castor ML, Whitney CG, Como-Sabetti K, Facklam RR, Ferrieri P, Bartkus JM, Juni BA, Cieslak PR, Farley MM, Dumas NB, Schrag SJ, Lynfield R.** 2008. Antibiotic resistance patterns in invasive group B streptococcal isolates. Infect Dis Obstet Gynecol **2008:**727505.

173. **Domelier AS, van der Mee-Marquet N, Arnault L, Mereghetti L, Lanotte P, Rosenau A, Lartigue MF, Quentin R.** 2008. Molecular characterization of erythromycin-resistant Streptococcus agalactiae. J Antimicrob Chemother **62:**1227-1233.

174. **Betriu C, Culebras E, Gómez M, Rodríguez-Avial I, Sánchez BA, Agreda MC, Picazo JJ.** 2003. Erythromycin and clindamycin resistance and telithromycin susceptibility in Streptococcus agalactiae. Antimicrob Agents Chemother **47:**1112-1114.

175. **Heelan JS, Hasenbein ME, McAdam AJ.** 2004. Resistance of group B streptococcus to selected antibiotics, including erythromycin and clindamycin. J Clin Microbiol **42:**1263-1264.

176. **Leclercq R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clinical Infectious Diseases **34:**482-492.

177. **Gygax SE, Schuyler JA, Kimmel LE, Trama JP, Mordechai E, Adelson ME.** 2006. Erythromycin and clindamycin resistance in group B streptococcal clinical isolates. Antimicrob Agents Chemother **50:**1875-1877.

178. **Varaldo PE, Montanari MP, Giovanetti E.** 2009. Genetic elements responsible for erythromycin resistance in streptococci. Antimicrob Agents Chemother **53:**343-353.

179. **Brzychczy-Włoch M, Gosiewski T, Bodaszewska M, Pabian W, Bulanda M, Kochan P, Strus M, Heczko PB.** 2010. Genetic characterization and diversity of Streptococcus agalactiae isolates with macrolide resistance. J Med Microbiol **59:**780-786.

180. **Castor ML, Whitney CG, Como-Sabetti K, Facklam RR, Ferrieri P, Bartkus JM, Juni BA, Cieslak PR, Farley MM, Dumas NB.** 2009. Antibiotic resistance patterns in invasive group B streptococcal isolates. Infect Dis Obstet Gynecol. **2008**:727505.

181. **Figueira-Coelho J, Ramirez M, Salgado M, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microbial Drug Resistance **10:**31-36.

182. **Fluegge K, Supper S, Siedler A, Berner R.** 2004. Antibiotic susceptibility in neonatal invasive isolates of Streptococcus agalactiae in a 2-year nationwide surveillance study in Germany. Antimicrob Agents Chemother **48:**4444-4446.

183. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.

184. **Janapatla RP, Ho Y-R, Yan J-J, Wu H-M, Wu J-J.** 2008. The prevalence of erythromycin resistance in group B streptococcal isolates at a University Hospital in Taiwan. Microbial Drug Resistance **14:**293-297.

185. **Seo YS, Srinivasan U, Oh K-Y, Shin J-H, Chae JD, Kim MY, Yang JH, Yoon H-R, Miller B, DeBusscher J.** 2010. Changing molecular epidemiology of group B streptococcus in Korea. Journal of Korean medical science **25:**817-823.

186. **de Azavedo JC, McGavin M, Duncan C, Low DE, McGeer A.** 2001. Prevalence and mechanisms of macrolide resistance in invasive and noninvasive group B streptococcus isolates from Ontario, Canada. Antimicrob Agents Chemother **45:**3504-3508.

187. **Sadowy E, Matynia B, Hryniewicz W.** 2010. Population structure, virulence factors and resistance determinants of invasive, non-invasive and colonizing Streptococcus agalactiae in Poland. Journal of antimicrobial chemotherapy **65:**1907-1914.

188. **DiPersio LP, DiPersio JR.** 2007. Identification of an erm (T) gene in strains of inducibly clindamycin-resistant group B Streptococcus. Diagn Microbiol Infect Dis **57:**189-193.

189. **Marimón JM, Valiente A, Ercibengoa M, García-Arenzana JM, Pérez-Trallero E.** 2005. Erythromycin resistance and genetic elements carrying macrolide efflux genes in Streptococcus agalactiae. Antimicrob Agents Chemother **49:**5069-5074.

190. **Malbruny B, Werno AM, Anderson TP, Murdoch DR, Leclercq R.** 2004. A new phenotype of resistance to lincosamide and streptogramin A-type antibiotics in Streptococcus agalactiae in New Zealand. Journal of antimicrobial chemotherapy **54:**1040-1044.

191. **Wang Y-H, Su L-H, Hou J-N, Yang T-H, Lin T-Y, Chu C, Chiu C-H.** 2010. Group B streptococcal disease in nonpregnant patients: emergence of highly resistant strains of serotype Ib in Taiwan in 2006 to 2008. J Clin Microbiol **48:**2571-2574.

192. **Uh Y, Kim HY, Jang IH, Hwang GY, Yoon KJ.** 2005. Correlation of serotypes and genotypes of macrolide-resistant Streptococcus agalactiae. Yonsei Medical Journal **46:**480-483.

193. **Chopra I, Roberts M.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and molecular biology reviews **65:**232-260.

194. **Dogan B, Schukken Y, Santisteban C, Boor KJ.** 2005. Distribution of serotypes and antimicrobial resistance genes among Streptococcus agalactiae isolates from bovine and human hosts. J Clin Microbiol **43:**5899-5906.

195. **Duarte RS, Bellei BC, Miranda OP, Brito MA, Teixeira LM.** 2005. Distribution of antimicrobial resistance and virulence-related genes among Brazilian group B streptococci recovered from bovine and human sources. Antimicrob Agents Chemother **49:**97-103.

196. **Poyart C, Jardy L, Quesne G, Berche P, Trieu-Cuot P.** 2003. Genetic basis of antibiotic resistance in Streptococcus agalactiae strains isolated in a French hospital. Antimicrob Agents Chemother **47:**794-797.

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

198. **Culebras E, Rodriguez-Avial I, Betriu C, Redondo M, Picazo JJ.** 2002. Macrolide and tetracycline resistance and molecular relationships of clinical strains of Streptococcus agalactiae. Antimicrob Agents Chemother **46:**1574-1576.

199. **Heath PT.** 2011. An update on vaccination against group B streptococcus. Expert review of vaccines **10:**685-694.

200. **Sinha A, Lieu TA, Paoletti LC, Weinstein MC, Platt R.** 2005. The projected health benefits of maternal group B streptococcal vaccination in the era of chemoprophylaxis. Vaccine **23:**3187-3195.

201. **Lin F-YC, Weisman LE, Azimi PH, Philips III JB, Clark P, Regan J, Rhoads GG, Frasch CE, Gray BM, Troendle J.** 2004. Level of maternal IgG anti-group B streptococcus type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen. Journal of Infectious Diseases **190:**928-934.

202. **Baker CJ, Rench MA, Edwards MS, Carpenter RJ, Hays BM, Kasper DL.** 1988. Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus. New England Journal of Medicine **319:**1180-1185.

203. **Baker CJ, Paoletti LC, Wessels MR, Guttormsen H-K, Rench MA, Hickman ME, Kasper DL.** 1999. Safety and immunogenicity of capsular polysaccharide—tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. Journal of Infectious Diseases **179:**142-150.

204. **Baker CJ, Rench MA, McInnes P.** 2003. Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. Vaccine **21:**3468-3472.

205. **Baker C, Edwards M.** 2003. Group B streptococcal conjugate vaccines. Archives of disease in childhood **88:**375-378.

206. **Aguiar S, Serrano I, Pinto F, Melo-Cristino J, Ramirez M.** 2008. Changes in Streptococcus pneumoniae serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. Clinical Microbiology and Infection **14:**835-843.

207. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of invasive group B streptococcus strains from the greater Toronto area, Canada. J Clin Microbiol **52:**1441-1447.

208. **Franken C, Haase G, Brandt C, Weber-Heynemann J, Martin S, Lämmler C, Podbielski A, Lütticken R, Spellerberg B.** 2001. Horizontal gene transfer and host specificity of beta-haemolytic streptococci: the role of a putative composite transposon containing scpB and lmb. Molecular microbiology **41:**925-935.

209. **Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, Tettelin H, Brettoni C, Iacobini ET, Rosini R.** 2005. Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science **309:**148-150.

210. **Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, Rosini R, Runci Y, Mora M, Buccato S.** 2009. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. Journal of Infectious Diseases **199:**108-115.

211. **Santillan DA, Andracki ME, Hunter SK.** 2008. Protective immunization in mice against group B streptococci using encapsulated C5a peptidase. American journal of obstetrics and gynecology **198:**114. e111-114. e116.

212. **Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nature Reviews Microbiology **4:**932-942.

213. **Xue G, Yu L, Li S, Shen X.** 2010. Intranasal immunization with GBS surface protein Sip and ScpB induces specific mucosal and systemic immune responses in mice. FEMS Immunology & Medical Microbiology **58:**202-210.

214. **Baltimore RS, Huie SM, Meek JI, Schuchat A, O'Brien KL.** 2001. Early-onset neonatal sepsis in the era of group B streptococcal prevention. Pediatrics **108:**1094-1098.

215. **Palazzi DL, Rench MA, Edwards MS, Baker CJ.** 2004. Use of type V group B streptococcal conjugate vaccine in adults 65–85 years old. Journal of Infectious Diseases **190:**558-564.

216. **Maisey HC, Doran KS, Nizet V.** 2008. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert reviews in molecular medicine **10:**e27.

217. **Rajagopal L.** 2009. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiol **4:**201-221.

218. **Sheen TR, Jimenez A, Wang N-Y, Banerjee A, van Sorge NM, Doran KS.** 2011. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. J Bacteriol **193:**6834-6842.

219. **Rubens CE, Raff HV, Jackson JC, Chi EY, Bielitzki JT, Hillier SL.** 1991. Pathophysiology and histopathology of group B streptococcal sepsis in Macaca nemestrina primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. Journal of Infectious Diseases **164:**320-330.

220. **Rubens C, Smith S, Hulse M, Chi E, Van Belle G.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect Immun **60:**5157-5163.

221. **Tazi A, Bellais S, Tardieux I, Dramsi S, Trieu-Cuot P, Poyart C.** 2012. Group B Streptococcus surface proteins as major determinants for meningeal tropism. Current opinion in microbiology **15:**44-49.

222. **Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional Analysis in Type Ia Group B Streptococcusof a Cluster of Genes Involved in Extracellular Polysaccharide Production by Diverse Species of Streptococci. Journal of Biological Chemistry **276:**139-146.

223. **Brochet M, Couvé E, Zouine M, Vallaeys T, Rusniok C, Lamy M-C, Buchrieser C, Trieu-Cuot P, Kunst F, Poyart C.** 2006. Genomic diversity and evolution within the species Streptococcus agalactiae. Microbes and Infection **8:**1227-1243.

224. **Marques MB, Kasper DL, Pangburn MK, Wessels MR.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun **60:**3986-3993.

225. **Rubens C, Wessels M, Heggen L, Kasper D.** 1987. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. Proceedings of the National Academy of Sciences **84:**7208-7212.

226. **Wessels MR, Rubens CE, Benedi V-J, Kasper DL.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proceedings of the National Academy of Sciences **86:**8983-8987.

227. **Xia FD, Mallet A, Caliot E, Gao C, Trieu-Cuot P, Dramsi S.** 2015. Capsular polysaccharide of Group B Streptococcus mediates biofilm formation in the presence of human plasma. Microbes Infect **17:**71-76.

228. **Maisey HC, Doran KS, Nizet V.** 2008. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert reviews in molecular medicine **10**.

229. **Edwards M, Kasper D, Jennings H, Baker C, Nicholson-Weller A.** 1982. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. The Journal of Immunology **128:**1278-1283.

230. **Madoff LC, Paoletti LC, Kasper DL.** Surface structures of group B streptococci important in human immunity. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram-Positive Pathogens. Washington, DC: Am Soc Microbiol; 2006.

231. **Wessels MR, Rubens CE, Benedi VJ, Kasper DL.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc Natl Acad Sci U S A **86:**8983-8987.

232. **Maruvada R, Blom AM, Prasadarao NV.** 2008. Effects of complement regulators bound to Escherichia coli K1 and Group B Streptococcus on the interaction with host cells. Immunology **124:**265-276.

233. **Wessels MR, Haft RF, Heggen LM, Rubens CE.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun **60:**392-400.

234. **Lindahl G, Stålhammar-Carlemalm M, Areschoug T.** 2005. Surface proteins of Streptococcus agalactiae and related proteins in other bacterial pathogens. Clin Microbiol Rev **18:**102-127.

235. **Michel JL, Madoff LC, Kling DE, Kasper DL, Ausubel F.** 1991. Cloned alpha and beta C-protein antigens of group B streptococci elicit protective immunity. Infect Immun **59:**2023-2028.

236. **Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

237. **Schubert A, Zakikhany K, Schreiner M, Frank R, Spellerberg B, Eikmanns BJ, Reinscheid DJ.** 2002. A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol **46:**557-569.

238. **Brodeur BR, Boyer M, Charlebois I, Hamel J, Couture F, Rioux CR, Martin D.** 2000. Identification of group B streptococcal Sip protein, which elicits cross-protective immunity. Infect Immun **68:**5610-5618.

239. **van Sorge NM, Quach D, Gurney MA, Sullam PM, Nizet V, Doran KS.** 2009. The group B streptococcal serine-rich repeat 1 glycoprotein mediates penetration of the blood-brain barrier. Journal of Infectious Diseases **199:**1479-1487.

240. **Seifert KN, Adderson EE, Whiting AA, Bohnsack JF, Crowley PJ, Brady LJ.** 2006. A unique serine-rich repeat protein (Srr-2) and novel surface antigen (ε) associated with a virulent lineage of serotype III Streptococcus agalactiae. Microbiology **152:**1029-1040.

241. **Spellerberg B, Martin S, Franken C, Berner R, Lutticken R.** 2000. Identification of a novel insertion sequence element in Streptococcus agalactiae. bspeller@imib.rwth-aachen.de. Gene **241:**51-56.

242. **Santi I, Maione D, Galeotti CL, Grandi G, Telford JL, Soriani M.** 2009. BibA induces opsonizing antibodies conferring in vivo protection against group B Streptococcus. J Infect Dis **200:**564-570.

243. **Santi I, Scarselli M, Mariani M, Pezzicoli A, Masignani V, Taddei A, Grandi G, Telford JL, Soriani M.** 2007. BibA: a novel immunogenic bacterial adhesin contributing to group B Streptococcus survival in human blood. Mol Microbiol **63:**754-767.

244. **Lamy MC, Dramsi S, Billoët A, Réglier-Poupet H, Tazi A, Raymond J, Guérin F, Couvé E, Kunst F, Glaser P, Trieu-Cuot P, Poyart C.** 2006. Rapid detection of the "highly virulent" group B Streptococcus ST-17 clone. Microbes Infect **8:**1714-1722.

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

246. **Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, Taddei AR, Mora M, Rappuoli R, Grandi G, Telford JL.** 2005. Genome analysis reveals pili in Group B Streptococcus. Science **309:**105.

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

248. **Dramsi S, Caliot E, Bonne I, Guadagnini S, Prévost MC, Kojadinovic M, Lalioui L, Poyart C, Trieu-Cuot P.** 2006. Assembly and role of pili in group B streptococci. Molecular microbiology **60:**1401-1413.

249. **Konto-Ghiorghi Y, Mairey E, Mallet A, Duménil G, Caliot E, Trieu-Cuot P, Dramsi S.** 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in Streptococcus agalactiae. PLoS Pathog **5:**e1000422.

250. **Pezzicoli A, Santi I, Lauer P, Rosini R, Rinaudo D, Grandi G, Telford JL, Soriani M.** 2008. Pilus backbone contributes to group B Streptococcus paracellular translocation through epithelial cells. Journal of Infectious Diseases **198:**890-898.

251. **Maisey HC, Hensler M, Nizet V, Doran KS.** 2007. Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. J Bacteriol **189:**1464-1467.

252. **Mandlik A, Swierczynski A, Das A, Ton-That H.** 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends in microbiology **16:**33-40.

253. **Rinaudo CD, Rosini R, Galeotti CL, Berti F, Necchi F, Reguzzi V, Ghezzo C, Telford JL, Grandi G, Maione D.** 2010. Specific involvement of pilus type 2a in biofilm formation in group B Streptococcus. PLoS One **5:**e9216.

254. **Hensler ME, Quach D, Hsieh C-J, Doran KS, Nizet V.** 2008. CAMP factor is not essential for systemic virulence of Group B Streptococcus. Microbial pathogenesis **44:**84-88.

255. **Reiß A, Braun JS, Jäger K, Freyer D, Laube G, Bührer C, Felderhoff-Müser U, Stadelmann C, Nizet V, Weber JR.** 2011. Bacterial pore-forming cytolysins induce neuronal damage in a rat model of neonatal meningitis. Journal of Infectious Diseases **203:**393-400.

256. **Beier D, Gross R.** 2006. Regulation of bacterial virulence by two-component systems. Current opinion in microbiology **9:**143-152.

257. **Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, Angiuoli SV, Crabtree J, Jones AL, Durkin AS, Deboy RT, Davidsen TM, Mora M, Scarselli M, Margarit y Ros I, Peterson JD, Hauser CR, Sundaram JP, Nelson WC, Madupu R, Brinkac LM, Dodson RJ, Rosovitz MJ, Sullivan SA, Daugherty SC, Haft DH, Selengut J, Gwinn ML, Zhou L, Zafar N, Khouri H, Radune D, Dimitrov G, Watkins K, O'Connor KJ, Smith S, Utterback TR, White O, Rubens CE, Grandi G, Madoff LC, Kasper DL, Telford JL, Wessels MR, Rappuoli R, Fraser CM.** 2005. Genome analysis of multiple pathogenic isolates of

Streptococcus agalactiae: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A **102:**13950-13955.

258. **Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, Paulsen IT, Nelson KE, Margarit I, Read TD, Madoff LC, Wolf AM, Beanan MJ, Brinkac LM, Daugherty SC, DeBoy RT, Durkin AS, Kolonay JF, Madupu R, Lewis MR, Radune D, Fedorova NB, Scanlan D, Khouri H, Mulligan S, Carty HA, Cline RT, Van Aken SE, Gill J, Scarselli M, Mora M, Iacobini ET, Brettoni C, Galli G, Mariani M, Vegni F, Maione D, Rinaudo D, Rappuoli R, Telford JL, Kasper DL, Grandi G, Fraser CM.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. Proc Natl Acad Sci U S A **99:**12391-12396.

259. **Di Palo B, Rippa V, Santi I, Brettoni C, Muzzi A, Metruccio MM, Grifantini R, Telford JL, Paccani SR, Soriani M.** 2013. Adaptive response of Group B streptococcus to high glucose conditions: new insights on the CovRS regulation network. PLoS One **8:**e61294.

260. **Tran-Winkler HJ, Love JF, Gryllos I, Wessels MR.** 2011. Signal transduction through CsrRS confers an invasive phenotype in group A Streptococcus. PLoS Pathog **7:**e1002361.

261. **Pan X, Ge J, Li M, Wu B, Wang C, Wang J, Feng Y, Yin Z, Zheng F, Cheng G.** 2009. The orphan response regulator CovR: a globally negative modulator of virulence in Streptococcus suis serotype 2. J Bacteriol **191:**2601-2612.

262. **Lamy MC, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, Glaser P, Kunst F, Msadek T, Trieu-Cuot P, Poyart C.** 2004. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. Mol Microbiol **54:**1250-1268.

263. **Jiang S, Park SE, Yadav P, Paoletti LC, Wessels MR.** 2012. Regulation and function of pilus island 1 in group B Streptococcus. J Bacteriol **194:**2479-2490.

264. **Lembo A, Gurney MA, Burnside K, Banerjee A, De Los Reyes M, Connelly JE, Lin WJ, Jewell KA, Vo A, Renken CW.** 2010. Regulation of CovR expression in Group B Streptococcus impacts blood–brain barrier penetration. Molecular microbiology **77:**431-443.

265. **Mereghetti L, Sitkiewicz I, Green NM, Musser JM.** 2008. Remodeling of the Streptococcus agalactiae transcriptome in response to growth temperature. PLoS One **3:**e2785.

266. **Sitkiewicz I, Green NM, Guo N, Bongiovanni AM, Witkin SS, Musser JM.** 2009. Transcriptome adaptation of group B Streptococcus to growth in human amniotic fluid. PLoS One **4:**e6114.

267. **Yang Q, Porter AJ, Zhang M, Harrington DJ, Black GW, Sutcliffe IC.** 2012. The impact of pH and nutrient stress on the growth and survival of Streptococcus agalactiae. Antonie Van Leeuwenhoek **102:**277-287.

268. **Jiang SM, Ishmael N, Dunning Hotopp J, Puliti M, Tissi L, Kumar N, Cieslewicz MJ, Tettelin H, Wessels MR.** 2008. Variation in the group B Streptococcus CsrRS regulon and effects on pathogenicity. J Bacteriol **190:**1956-1965.

269. **Lupo A, Ruppen C, Hemphill A, Spellerberg B, Sendi P.** 2014. Phenotypic and molecular characterization of hyperpigmented group B Streptococci. Int J Med Microbiol **304:**717-724.

270. **Van Belkum A, Tassios P, Dijkshoorn L, Haeggman S, Cookson B, Fry N, Fussing V, Green J, Feil E, Gerner-Smidt P.** 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clinical Microbiology and Infection **13:**1-46.

271. **Savoia D, Gottimer C, Crocilla C, Zucca M.** 2008. Streptococcus agalactiae in pregnant women: phenotypic and genotypic characters. Journal of Infection **56:**120-125.

272. **Toresani I, Limansky A, Bogado I, Guardati MC, Viale A, Sutich EG.** 2001. Phenotypic and genotypic study of Streptococcus agalactiae in vagina of pregnant women in Argentina. MEDICINA-BUENOS AIRES- **61:**295-300.

273. **Foxman B, Zhang L, Koopman JS, Manning SD, Marrs CF.** 2005. Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiologic perspectives & innovations **2:**10.

274. **Selander RK, Musser JM, Caugant DA, Gilmour MN, Whittam TS.** 1987. Population genetics of pathogenic bacteria. Microbial pathogenesis **3:**1-7.

275. **Brochet M, Rusniok C, Couvé E, Dramsi S, Poyart C, Trieu-Cuot P, Kunst F, Glaser P.** 2008. Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of Streptococcus agalactiae. Proceedings of the National Academy of Sciences **105:**15961-15966.

276. **Lefébure T, Stanhope MJ.** 2007. Evolution of the core and pan-genome of Streptococcus: positive selection, recombination, and genome composition. Genome biology **8:**R71.

277. **Didelot X, Falush D.** 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics **175:**1251-1266.

278. **Schmidt H, Hensel M.** 2004. Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev **17:**14-56.

279. **Hacker J, Kaper JB.** 2000. Pathogenicity islands and the evolution of microbes. Annual Reviews in Microbiology **54:**641-679.

280. **Achtman M.** 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol **62:**53-70.

281. **Feil EJ, Enright MC.** 2004. Analyses of clonality and the evolution of bacterial pathogens. Current opinion in microbiology **7:**308-313.

282. **Quentin R, Huet H, Wang F-S, Geslin P, Goudeau A, Selander RK.** 1995. Characterization of Streptococcus agalactiae strains by multilocus enzyme genotype and serotype: identification of multiple virulent clone families that cause invasive neonatal disease. J Clin Microbiol **33:**2576-2581.

283. **Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179.

284. **Park CJ, Vandel NM, Ruprai DK, Martin EA, Gates KM, Coker D.** 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. J Clin Microbiol **39:**408-409.

285. **Blumberg HM, Stephens DS, Licitra C, Pigott N, Facklam R, Swaminathan B, Wachsmuth IK.** 1992. Molecular epidemiology of group B streptococcal infections: use

of restriction endonuclease analysis of chromosomal DNA and DNA restriction fragment length polymorphisms of ribosomal RNA genes (ribotyping). Journal of Infectious Diseases **166:**574-579.

286. **Limansky AS, Sutich EG, Guardati MC, Toresani IE, Viale AM.** 1998. Genomic diversity among Streptococcus agalactiae isolates detected by a degenerate oligonucleotide-primed amplification assay. Journal of Infectious Diseases **177:**1308-1313.

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

288. **Kalliola S, Vuopio-Varkila J, Takala AK, Eskola J.** 1999. Neonatal group B streptococcal disease in Finland: a ten-year nationwide study. The Pediatric Infectious Disease Journal **18:**806-810.

289. **Ekin IH, Gurturk K.** 2006. Characterization of bovine and human group B streptococci isolated in Turkey. J Med Microbiol **55:**517-521.

290. **Radhakrishnan S, Brahmadathan K, Mathai E, Jesudason M, Lalitha M.** 1995. Changing patterns of group B streptococcal serotypes associated with human infections. The Indian journal of medical research **102:**56-59.

291. **Ramaswamy SV, Ferrieri P, Flores AE, Paoletti LC.** 2006. Molecular characterization of nontypeable group B streptococcus. J Clin Microbiol **44:**2398-2403.

292. **Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC.** 2006. Identification of novel cps locus polymorphisms in nontypable group B Streptococcus. J Med Microbiol **55:**775-783.

293. **Ferrieri P, Baker C, Hillier S, Flores A.** 2004. Diversity of surface protein expression in group B streptococcal colonizing & invasive isolates. Indian Journal of Medical Research **119:**191.

294. **Moyo SR, Maeland JA, Bergh K.** 2002. Typing of human isolates of Streptococcus agalactiae (group B streptococcus, GBS) strains from Zimbabwe. J Med Microbiol **51:**595-662.

295. **Amundson NR, Flores AE, Hillier SL, Baker CJ, Ferrieri P.** 2005. DNA macrorestriction analysis of nontypeable group B streptococcal isolates: clonal evolution of nontypeable and type V isolates. J Clin Microbiol **43:**572-576.

296. **Zhao Z, Kong F, Martinez G, Zeng X, Gottschalk M, Gilbert GL.** 2006. Molecular serotype identification of Streptococcus agalactiae of bovine origin by multiplex PCR-based reverse line blot (mPCR/RLB) hybridization assay. FEMS microbiology letters **263:**236-239.

297. **Yao K, Poulsen K, Maione D, Rinaudo CD, Baldassarri L, Telford JL, Sorensen UB, Kilian M.** 2013. Capsular gene typing of Streptococcus agalactiae compared to serotyping by latex agglutination. J Clin Microbiol **51:**503-507.

298. **Creti R, Imperi M, Pataracchia M, Alfarone G, Recchia S, Baldassarri L.** 2012. Identification and molecular characterization of a S. agalactiae strain lacking the capsular locus. Eur J Clin Microbiol Infect Dis **31:**233-235.

299. **Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional analysis in type Ia group B Streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J Biol Chem **276:**139-146.

300. **Sanchez-Beato AR, Garcia E, López R, García JL.** 1997. Identification and characterization of IS1381, a new insertion sequence in Streptococcus pneumoniae. J Bacteriol **179:**2459-2463.

301. **Dmitriev A, Shen A, Shen X, Yang Y.** 2004. ISSa4-based differentiation of Streptococcus agalactiae strains and identification of multiple target sites for ISSa4 insertions. J Bacteriol **186:**1106-1109.

302. **Shakleina E, Dmitriev A, Tkacikova L, Suvorov A.** 2004. Presence of insertion sequences (IS elements) in group B streptococci of bovine origin. Indian Journal of Medical Research **119:**242.

303. **Tamura GS, Herndon M, Przekwas J, Rubens CE, Ferrieri P, Hillier SL.** 2000. Analysis of restriction fragment length polymorphisms of the insertion sequence IS1381 in group B streptococci. Journal of Infectious Diseases **181:**364-368.

304. **Granlund M, Öberg L, Sellin M, Norgren M.** 1998. Identification of a novel insertion element, IS1548, in group B streptococci, predominantly in strains causing endocarditis. Journal of Infectious Diseases **177:**967-976.

305. **Creti R, Imperi M, Pataracchia M, Alfarone G, Recchia S, Baldassarri L.** 2012. Identification and molecular characterization of a S. agalactiae strain lacking the capsular locus. European journal of clinical microbiology & infectious diseases **31:**233-235.

306. **Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. Journal of Infectious Diseases **173:**365-373.

307. **Lachenauer CS, Kasper DL, Shimada J, Ichiman Y, Ohtsuka H, Kaku M, Paoletti LC, Ferrieri P, Madoff LC.** 1999. Serotypes VI and VIII predominate among group B streptococci isolated from pregnant Japanese women. J Infect Dis **179:**1030-1033.

308. **Ippolito DL, James WA, Tinnemore D, Huang RR, Dehart MJ, Williams J, Wingerd MA, Demons ST.** 2010. Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. BMC Infect Dis **10:**336.

309. **Schuchat A, Balter S.** Epidemiology of group B streptococcal infections. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram-Positive Pathogens. Washington, DC: Am Soc Microbiol; 2006.

310. **Ippolito DL, James WA, Tinnemore D, Huang RR, Dehart MJ, Williams J, Wingerd MA, Demons ST.** 2010. Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. BMC Infect Dis **10:**336.

311. **Amin A, Abdulrazzaq Y, Uduman S.** 2002. Group B streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. Journal of Infection **45:**42-46.

312. **Palmeiro JK, De Carvalho NS, Botelho AC, Fracalanzza SE, Madeira HM, Dalla-Costa LM.** 2011. Maternal group B streptococcal immunization: capsular polysaccharide (CPS)-based vaccines and their implications on prevention. Vaccine **29:**3729-3730.

313. **Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-**

**Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P.** 2015. Corrigendum: Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **6:**6108.

314. **Bisharat N, Crook DW, Leigh J, Harding RM, Ward PN, Coffey TJ, Maiden MC, Peto T, Jones N.** 2004. Hyperinvasive neonatal group B streptococcus has arisen from a bovine ancestor. J Clin Microbiol **42:**2161-2167.

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

316. **Bergseng H, Afset J, Radtke A, Loeseth K, Lyng R, Rygg M, Bergh K.** 2009. Molecular and phenotypic characterization of invasive group B streptococcus strains from infants in Norway 2006–2007. Clinical Microbiology and Infection **15:**1182-1185.

317. **Matsubara K, Yamamoto G.** 2009. Invasive group B streptococcal infections in a tertiary care hospital between 1998 and 2007 in Japan. International Journal of Infectious Diseases **13:**679-684.

318. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518-1530.

319. **Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ.** 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett **241:**129-134.

320. **Cooper JE, Feil EJ.** 2004. Multilocus sequence typing–what is resolved? Trends in microbiology **12:**373-377.

321. **Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG.** 2003. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype- and clone-specific differences in invasive disease potential. Journal of Infectious Diseases **187:**1424-1432.

322. **Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences **95:**3140-3145.

323. **Bohnsack JF, Whiting A, Gottschalk M, Dunn DM, Weiss R, Azimi PH, Philips JB, Weisman LE, Rhoads GG, Lin F-YC.** 2008. Population structure of invasive and colonizing strains of Streptococcus agalactiae from neonates of six US Academic Centers from 1995 to 1999. J Clin Microbiol **46:**1285-1291.

324. **Manning SD, Lewis MA, Springman AC, Lehotzky E, Whittam TS, Davies HD.** 2008. Genotypic diversity and serotype distribution of group B streptococcus isolated. Clin Infect Dis **46:**1829-1837.

325. **Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD.** 2009. Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47:**1143-1148.

326. **Parker RE, Laut C, Gaddy JA, Zadoks RN, Davies HD, Manning SD.** 2016. Association between genotypic diversity and biofilm production in group B Streptococcus. BMC Microbiol **16:**86.

327. **Jones N, Oliver K, Jones Y, Haines A, Crook D.** 2006. Carriage of group B streptococcus in pregnant women from Oxford, UK. Journal of clinical pathology **59:**363-366.

328. **Fluegge K, Wons J, Spellerberg B, Swoboda S, Siedler A, Hufnagel M, Berner R.** 2011. Genetic differences between invasive and noninvasive neonatal group B streptococcal isolates. The Pediatric Infectious Disease Journal **30:**1027-1031.

329. **Salloum M, Van Der Mee-marquet N, Valentin-Domelier A-S, Quentin R.** 2011. Diversity of prophage DNA regions of Streptococcus agalactiae clonal lineages from adults and neonates with invasive infectious disease. PLoS One **6:**e20256.

330. **Bohnsack JF, Whiting A, Gottschalk M, Dunn DM, Weiss R, Azimi PH, Philips JB, Weisman LE, Rhoads GG, Lin FY.** 2008. Population structure of invasive and colonizing strains of Streptococcus agalactiae from neonates of six U.S. Academic Centers from 1995 to 1999. J Clin Microbiol **46:**1285-1291.

331. **Marchaim D, Efrati S, Melamed R, Gortzak-Uzan L, Riesenberg K, Zaidenstein R, Schlaeffer F.** 2006. Clonal variability of group B Streptococcus among different groups of carriers in southern Israel. European Journal of Clinical Microbiology and Infectious Diseases **25:**443-448.

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

333. **Davies HD, Jones N, Whittam TS, Elsayed S, Bisharat N, Baker CJ.** 2004. Multilocus sequence typing of serotype III group B streptococcus and correlation with pathogenic potential. Journal of Infectious Diseases **189:**1097-1102.

334. **Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE.** 2001. Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone. Infect Immun **69:**2416-2427.

335. **Luan SL, Granlund M, Sellin M, Lagergard T, Spratt BG, Norgren M.** 2005. Multilocus sequence typing of Swedish invasive group B streptococcus isolates indicates a neonatally associated genetic lineage and capsule switching. J Clin Microbiol **43:**3727-3733.

336. **Tien N, Ho C-M, Lin H-J, Shih M-C, Ho M-W, Lin H-C, Lin H-S, Chang C-C, Lu J-J.** 2011. Multilocus sequence typing of invasive group B Streptococcus in central area of Taiwan. Journal of Microbiology, Immunology and Infection **44:**430-434.

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

338. **Bisharat N, Crook DW, Leigh J, Harding RM, Ward PN, Coffey TJ, Maiden MC, Peto T, Jones N.** 2004. Hyperinvasive neonatal group B streptococcus has arisen from a bovine ancestor. J Clin Microbiol **42:**2161-2167.

339. **Martins ER, Pessanha MA, Ramirez M, Melo-Cristino J, Infections PGftSoS.** 2007. Analysis of group B streptococcal isolates from infants and pregnant women in Portugal revealing two lineages with enhanced invasiveness. J Clin Microbiol **45:**3224-3229.

340. **Brochet M, Couvé E, Glaser P, Guédon G, Payot S.** 2008. Integrative conjugative elements and related elements are major contributors to the genome diversity of Streptococcus agalactiae. J Bacteriol **190:**6913-6917.

# Chapter 2: Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada, from 2003 to 2013[ab]

[a] A version of this chapter has been published as

**Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol **54**: 7 1774-1781.

[b] A correction to the 2016 paper has been published as

**Alhhazmi A, Hurteau D, Tyrrell GJ.** 2017. A correction for Alhhazmi et al., Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol **55**: 1 342-343.

## 2.1 Introduction

Group B streptococcus (GBS) is Gram positive coccus bacteria primarily found in the human urogential tract and gastrointestinal tract. Since the 1960's, GBS has become recognized as important pathogens in both the neonatal and adult populations. In neonate, invasive disease includes sepsis and/or meningitis and in adults, invasive GBS (iGBS) disease can include meningitis, endocarditis, osteoarticular and soft tissue infections. To cause invasive disease in these groups, GBS use an array of virulence factors one of the most important being a polysaccharide capsule (1-4). There are currently ten recognized GBS capsular polysaccharide serotypes (CPS's) designated Ia, Ib, and II through IX. The CPS has been demonstrated to be associated with the level of pathogenicity ascribed to various CPS's. For example, previous work has found that there is a significant increase in the number of iGBS disease cases due to CPS III in neonates, specifically in late onset disease (LOD) patients (7-90 days old) (5-9) while in adult patients, CPS V and Ia are more common in North America than other CPS's (9, 10). These observations suggest GBS epidemiology is dynamic in nature with various CPS's having a higher tropism for specific populations.

According to the Canadian Taskforce on preventive health care, there are strategies to the prevention of early onset disease (EOD) in Canada. These strategies involve universal culture based screening at 35-37 weeks of gestation with the intrapartum administration of antibiotics, usually benzylpenicillin or ampicillin, to women who are colonised with GBS and have identified risk factors (11).

From a global perspective, GBS can also be typed via multi-locus sequence typing (MLST) (12). A major hypervirulent MLST clone currently circulating globally is the clonal complex 17 (CC17) (13-17). This lineage is strongly associated with the majority of GBS LOD infections, which are characterized by meningitis in infants after the first week of life (13). CC17 clones possess hypervirulent GBS adhesion gene, hvgA, which is usually present in CPS III strains (13-17). There have been reports of CPS IV isolates that contain CC17 specific *hvgA* (15); therefore, the PCR amplification of hvgA which had been used to assign GBS strains to the CC17 (17) was found inconclusive (15). However, PCR amplification could be used as initial screening step to assign strains to CC-17 with confirmation via MLST typing.

Monitoring the ever-changing epidemiology of GBS invasive disease in a large population provides important information regarding disease rates, polysaccharide capsule changes, sequence type variations, antimicrobial susceptibility trends. This documentation is critical as it provides data that can guide clinicians in treating patients with iGBS and for public health officials with respect to understanding changes in iGBS disease trends. Also, this information takes on added value as more attention is placed on GBS vaccine development especially for protection against neonatal iGBS disease. The objective of this report is to describe the epidemiology of iGBS disease in the Alberta population with respect to CPS distribution and GBS antimicrobial susceptibility from 2003 to 2013.

## 2.2 Materials and methods

### 2.2.1 Epidemiologic, demographic information and definitions

Neonatal iGBS disease is a notifiable disease to the Provincial Public Health Authorities in Alberta, Canada. This requires all neonatal GBS isolates collected from sterile site specimens in

Alberta to be forwarded to the Provincial Public Health Laboratory (PPHL) for capsular serotyping. All other isolates from iGBS disease cases other than neonatal disease are forwarded to the PPHL at the discretion of the submitting diagnostic laboratory in Alberta for capsule serotyping. The time period for the study was January 1, 2003 to December 31, 2013 (an eleven year period). The population of Alberta in 2003 was 3,134,337 and in 2013, 4,107,762 (http://www.ahw.gov.ab.ca/IHDA_Retrieval/[accessed 15 June 2015]).

EOD and LOD were defined as cases occurring in neonates between 0-7 days of age and 8-90 days of age, respectively. Children were defined as between age 91 days-14 years and adults were defined as being ≥15 years of age. iGBS disease was defined as GBS infection isolated from a positive sterile site, such as soft tissue (necrotic tissues, abscesses, ulcers, wounds, and cellulitis), blood, cerebrospinal fluid, and pleural, articular, or peritoneal fluid. In addition to the submission of iGBS isolate to the PPHL for serotyping, minimal demographic data was collected, including date of collection of specimen, age of case, anatomically collection site and sex of the cases.

### 2.2.2 GBS CPS typing

D. Hurteau assisted with the data collection and GBS typing. Isolates submitted to the PPHL were confirmed as GBS prior to CPS typing. CPS typing was performed using the Lancefield heat-acid extraction followed by a double immunodiffusion method as described previously (18, 19). The immunodiffusion CPS typing assay used for this study was based on reactions with antisera raised against CPS Ia, Ib, II, III, IV, V, VI, VII and VIII. The type-specific antisera panel was prepared in rabbits within the laboratory. PCR CPS typing was used for the identification of CPS IX using primer pair cpsI-7-9-F (5'-CTGTAATTGGAGGAATGTGGATCG) and cpsI-9-R (5'-

AATCATCTTCATAATTTATCTCCCATT) which amplify target regions specific to CPS IX as described previously (20).

### 2.2.3 PCR amplification of *hvgA*

A previously described PCR assay that targets a region (210 bp) of the *hvgA* gene was used to identify *hvgA* positive GBS isolates (14). The *hvgA* region was amplified by PCR using primer pair ST-17S (5'-TTAAATCCTTCCTGACCA TTCC) and ST-17AS (5'-ATACAAATTCTGCTGACTACCG) (17).

### 2.2.4 Multilocus sequence typing (MLST) assay and assignment to clonal clusters

Multilocus sequence typing (MLST) was carried out as described previously (12). Briefly, PCR was used to amplify internal (500-bp) fragments from seven housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) which were chosen based on their chromosomal location and sequence diversity. The seven PCR products were purified and sequenced and an allele number assigned to each fragment based on its sequence. Each isolate was assigned a sequence type (ST) based on the allelic profile of the seven amplicons based on STs in http://pubmlst.org/sagalactiae/ for MLST analysis.

Strains were grouped into CCs using the eBURST software program (21, 22). The default eBURST setting identified groups of related STs using the most stringent (conservative) definition, such that all members assigned to the same group shared identical alleles at six of the seven loci with at least one other member of the group. The term singleton ST refers to an ST that did not cluster into a CC.

### 2.2.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed by the disc diffusion method, as described by the National Committee for Clinical Laboratory Standards (NCCLS) (23). The antimicrobial agents assayed were penicillin, erythromycin, clindamycin, vancomycin, chlorampenicol. All antimicrobial discs were obtained from Oxoid Company (Nepean, Canada). Interpretative standards published by NCCLS were used to categorize the MIC results as susceptible, intermediate or resistant (24).

### 2.2.6 Statistical analyses

CPS distributions with respect to age group, gender, and specimen source were compared using the $X^2$ test. Incidence rates, i.e., the frequency with which GBS diseases occurred in a population over a period of time, with 95% confidence intervals (CIs) were calculated for each age group and gender. For categorical data such as the distribution of erythromycin- or clindamycin-nonsusceptible isolates among CPSs and age groups, analysis of variance (ANOVA) was performed. For nonparametric analysis, the Kruskal-Wallis test was used to compare the distribution of *hvgA* among CPSs. For trend assessment, multiple linear regression (polynomial regression) was applied using Excel 2012. P values of 0.05 were considered statistically significant. Data were analyzed using SPSS version 23 (IBM SPSS Statistics).

## 2.3 Results

### 2.3.1 CPS distribution and incidence rates

From 2003 to 2013, 1683 non-duplicate GBS isolates from cases of iGBS disease in Alberta were submitted to the PPHL for CPS typing. Table 2.1 shows the distribution of iGBS isolates presented by year and CPS. The overall isolate submission number increased from 123 isolates

in 2003 to 234 isolates in 2013. CPS III (20.3% of total), CPS V (19.1%) and CPS Ia (18.9%) were the most predominant types accounting for 58.3% of the total followed by CPS types: Ib (12.7%), II (11.1 %), IV (6.3%), VI (1.4%), IX (1.0%) and VIII (0.2%) ($X^2$ test, $p$<0.001). CPS VII was not detected in the 11-year period surveyed. There were 159 isolates (9.4%) that were found to be as nontypable (Table 2.1.).

The overall incidence of iGBS disease by year increased over the 11 years surveyed from 3.92 cases /100,000 in 2003 to 5.99 cases /100,000 in 2013 (Table 2.2.). The incidence rate for all years combined were 4.59 cases/100000 population (CI 95%, 4.31-4.9 cases/100000 population) and 3.99 cases/100000 population (CI 95%, 3.72-4.28 cases/100,000 population) among males and females, respectively, with 895/1668 (53.65%) cases for which sex was not indicated at the time of submission of the isolate to the PPHL.

A total of 264 cases of iGBS in neonates (≤90 days of age) were identified, of which 134 were categorized as EOD and 130 were categorized as LOD (Tables 2.2. & 2.3). The rate of EOD and LOD combined was 0.31 (12 cases, CI 95% 0.2-0.5) in 2003 and the rate was 0.73 (39, CI 95% 0.5-1.0) in 2013 (Table 2.3). For both EOD and LOD, the number of cases showed a gradual increase from 2003 to 2013, with an average incidence for all years combined of 0.26 cases/1000 live births (CI 95% 0.23-0.28)/1000 live births). In 2013, the final year of the survey, the incidence of EOD was 0.34 cases/1000 live births and that of LOD was 0.39 cases/1000 live births (Table 2.2). The CPS distribution by age showed that CPS III (28.4%, n=38) and CPS Ia (18.7%, n=25) ($X^2$ test, p<0.001) were the most common CPSs for EOD cases. This was mirrored for LOD cases, for which CPS III (65.4%, n=85) predominated followed by CPS Ia (18.5%, n=24) ($X^2$ test, $p$<0.001) (Table 2.4).

In total, CPS III accounted for 46.7% (123/264) strains causing invasive disease in neonates. Few isolates were identified from children 91 days to 14 years (18/1668, 1.1%). Eight of the cases were in children aged 91 days to 1 year old and five of the cases were in children aged 1- 14 years. Among the children cases, CPS III and Ib accounted for 46.2% and 30.8% (6/13 and 5/13 isolates), respectively (Table 2.4).

There were 425 cases among persons aged 15-50 years of age and 961 cases among persons >50 years of age. Among persons aged 15 to 50 years of age, incidence rate ranged from 1.28 to 2.84 cases/100,000 population over the eleven years surveyed. For cases among > 50 years of age, the incidence rate ranged from 2.8 to 5.99 cases /100,000 population from 2003 to 2013. For 15 to 50-year-old persons, CPS Ia and V were the most common 21.4% (91/425 isolates) and 18.4% (78/425 isolates), respectively ($X^2$ test, $p<0.001$). For adults >50 years of age, CPS V was the most common (22.0% (211/961 isolates), followed by CPS Ia (17.3%, 166/961 isolates) ($X^2$ test, $p<0.001$) (Table 2.4).

There is a requirement for EOD and LOD isolates in Alberta to be forwarded to the PPHL for capsular serotyping. All other isolates from iGBS disease cases other than neonatal disease are forwarded to the PPHL at the discretion of the submitting diagnostic laboratory in Alberta for capsule serotyping. For trend of iGBS rate over the survey period of time, neonatal (EOD, LOD, and neonate rate) and all ages patients (neonatal and non-neonatal rate) showed overall increase. Non-neonatal rate was underrepresented in our data whereas neonatal rate was more reliable. In this study, polynomial linear regression was proposed to predict iGBS rate over the study period for EOD, LOD, neonate, and all age patients. The linear polynomial (fifth degree polynomial for EOD and neonate, sixth degree polynomial for LOD, and cubic degree for

all age patients) is more stable than other linear regression models based on predication accuracy. Figure 2.1 showed the prediction accuracy of the linear polynomial of the iGBS rate (EOD; 84.8%, LOD; 85.6%, neonate; 90.1%, and all age patients; 89.7%). However, the polynomial linear regression model for EOD, LOD, and neonate data is more reliable due to the obligation of iGBS neonatal isolate submission in Alberta to PPHL, unlike the non-neonatal iGBS isolates. The proposed polynomial linear regression model in this work demonstrated a reliable increased trend of neonatal iGBS disease in Alberta.

### 2.3.2 Specimen Source

The majority of iGBS isolates were submitted from blood specimens (72.4%, $X^2$ test, $p$<0.001), Table 2.5), this occurred for all age groups. Very few isolates were collected from CSF (16 cases [1.0%]). Joint/synovial fluid plus soft tissue infections accounted for 8.3% of other iGBS sources. Together blood, joint/synovial fluid and soft tissue infections represented 87.5% of iGBS cases (Table 2.5). For EOD isolates, 79.9% were from blood and for LOD isolates, 90% were from blood. Among the iGBS cases from children, most (77.8% [14/18 isolates]) were obtained from blood (Table 2.5). The majority of iGBS cases involving persons ≥15 years of age (70% [n=970]) were diagnosed by blood cultures alone.

### 2.3.3 CPS IV strains

A total of 106 IV CPS type strains were identified during the survey period (Table 2.1). It was noted that CPS IV was rarely reported during 2003-2007; from 2008 to 2013, however, the number of CPS IV cases increased from a low of 12 cases (2003 to 2007) to a high of 94 cases (2008 to 2013) (Table 2.1). This rapid increase in the number of CPS type IV cases prompted further characterization of these isolates. HvgA is a surface-anchored adhesin that enables

persistent colonization by CC-17 GBS clones and contributes to meningitis in neonates (13-17). HvgA has also been observed previously in CPS IV strains (14, 15). I screened for the presence of *hvgA* positive GBS strains in our collection of neonatal GBS isolates as well as CPS IV isolates from all age groups using a hvgA PCR assay (17). For the neonatal cases, the majority of *hvgA*-positive isolates reported here were CPS III (74.4% [*n* = 32]), followed by CPS IV (11.6% [*n* = 5]), CPS Ia (7% [*n* = 3]), CPS Ib (4.7% [*n*= 2]), and CPS V (2.3% [*n* = 1]) (χ2 test, $P < 0.001$) (Fig. 2.2). Of the CPS IV isolates from all ages, 6.6% were *hvgA* positive (7 *hvgA*-positive isolates/106 CPS IV isolates) ($X^2$ test, $P < 0.001$) (Fig 2.3) ($X^2$ test, $p<0.001$). In the last year of the study (2013), the twenty-two of the viable CPS IV iGBS isolates were further characterized using MLST analysis. MLST typing of the 2013 CPS IV isolates identified 13 ST-459 isolates (the most common ST), followed by 2 isolates each of ST-2, ST-3, ST-671 and 1 isolate each of ST-136, ST-196 and ST-711 ($X^2$ test, $p<0.001$). Twenty of 22 isolates clustered in CC1. To determine whether the increase in CPS type IV is due to global expansion of ST-459, four isolates were randomly selected from different time points 2003, 2009, 2010 and 2011 and their STs and CCs were identified. The four isolates were typed as ST-459, CC-1.

### 2.3.4 iGBS Antimicrobial Resistance rates 2003 to 2013

Antimicrobial susceptibility assays penicillin, erythromycin, clindamycin, vancomycin, and chlorampenicol were performed for 98.5% (1658/1683 isolates), 98.3% (1656/1683 isolates), 98.6% (1659/1683 isolates), 98.6% (1660/1683 isolates), and 98.5% (1657/1683) of the isolates, respectively, over the eleven-year period. All iGBS isolates were susceptible to penicillin, vancomycin, and chlorampenicol. Fig 2.4 displays a gradual increase in erythromycin resistance from 23.6% in 2003 to 43.8% in 2013. There was no significant difference between the number

of erythromycin resistance iGBS isolates derived from all age groups. The number of erythromycin resistance iGBS isolates was significantly greater among CPS IV strains (85/106 [80.2%,]) (ANOVA test, p <0.001) (Table 2.6).

The proportion of clindamycin resistance iGBS isolates increased from 12.2% in 2003 to 32.3% in 2013 (Figure 2.4). The majority of clindamycin resistance strains were derived from 15 to 50 years in comparison to EOD, LOD, children, and > 50 years of age (ANOVA, p<0.001) (Figure 2.4). The clindamycin resistance phenotype was most predominant in CPS IV (82/108 [77.4%,]; p<0.001). Most of the iGBS isolates that were erythromycin resistant (63%) were clindamycin resistant (94.6%) (375 isolates) (Pearson $X^2$ test, p<0.001) (Table 2.6).

## 2.4 Discussion

GBS is one of the most important causal agents of bacterial pneumonia, meningitis, and sepsis in newborns in North America (25-27). The estimated incidence rate of neonatal GBS disease in Canada and the United States has decreased from 1-3 per 1000 live birth in the early 1990s to 0.35-0.5 per 1000 live birth with the application of intrapartum chemoprophylaxis prevention measures (28-31). Our screening study took place post the implementation of the preventive measures in Canada. Overall, the incidence rate of EOD and LOD in Alberta in 2003 to 2013 was 0.26 per 1000 live births, which is lower than the aforementioned rate in North America. However, there was twofold increases in EOD from 2003-2013 (0.15-0.34 per 1000 live birth, respectively) even with implementation of the preventive measures. It is not clear why Alberta experienced this increase. The LOD incidence rate increased from 0.15 to 0.39 cases per 1000 live birth (>2.5-fold change) between 2003 and 2013, respectively. Although the interventions guidelines are designed only to prevent EOD iGBS diseases (28), the increases in

incidence was documented for both EOD and LOD cases. This is similar to the reported increase in the United States 2004-2005 (10). Thus, surveillance of EOD and LOD rate in the upcoming years is important to assess whether the upturn is sustained.

The most common GBS CPS's seen in Alberta over the eleven-year surveillance period were CPS III, V and Ia. This is not unusual as CPS III has been documented to be the most common CPS type identified from cases of both invasive disease and in carriage in studies elsewhere (Toronto, Beijing, Ireland, England, Portugal, Global) (9, 15, 16, 32-35). CPS III accounts for one quarter to one half of the total GBS isolates serotyped in past epidemiological surveys. CPS V ranked a close second in our study in Alberta, Canada study and in the recently reported study from Toronto, Canada indicating CPS V is common in Canada (9).

Interestingly, in the meta-analysis study by Edmond et al., CPS Ia was found to be more common than CPS V (32). CPS V was not as common in the 1990's (and earlier) as CPS Ia, CPS V became more prevalent in the 2000's indicating GBS CPS changes in the population can gradually occur (32, 33, 36, 37). Closer examination by age showed that CPS III was the most predominant type in cases of EOD, LOD and disease in person up to 14 years of age. The predominance of CPS III as a neonatal pathogen is well documented (8, 13, 32, 33, 38, 39). For our study, CPS III was the most common CPS followed by CPS Ia, for cases of EOD (28%) and for LOD (65%). These findings are similar to a meta-analysis reported by Edmond et al. (32). These investigators collected data from previous publications on GBS disease in infants up to three months of age and found that CPS III accounted for 49% of cases in this age group followed by CPS Ia at 23% (together accounting for 72% of iGBS). The remaining CPS reported in this meta-analysis were all under 10% each (32).

It is interesting to note that the incidence of iGBS disease for all ages over the 11-year surveillance period increased 1.5-fold from 3.92 cases/100,000 in 2003 to 5.99 cases/100,000 in 2013 (123 cases to 234 cases). The explanation for this increase may be multifactorial. It is possible that this increase is due to elevated rates of iGBS disease and/or an increased interest in submission of invasive GBS isolates for CPS typing from diagnostic microbiology laboratories in the province. It is difficult to determine if elevated rates of iGBS disease are the cause of the increase as iGBS cases in persons over the age of 91 days (i.e. not EOD or LOD) are not included in the Alberta Health Notifiable Disease Regulations. Therefore, there is no requirement for diagnostic laboratories to forward GBS isolates from cases of iGBS disease to the Public Health Laboratory for analysis in these age groups. Elevated rates of GBS isolate submission first became apparent in 2009 with an incidence rate of 4.15 cases/100,000 peaking in 2013 at 5.99 cases/100,000. It may be theorized that if the incidence rate increases were due to increased interest from diagnostic laboratories sending GBS isolates to PPHL for CPS typing, then it is likely that the increase numbers should be fairly consistent for all CPSs from year to year. The data from Table 2.1 for the six most common CPS suggests that this is not the case. The case numbers for CPS Ia, Ib, III and IV showed increases, whereas the case numbers for CPS II and V remained fairly constant. Whether the increases seen over the 11-year period for CPS Ia, Ib, III and IV are due to simply increased submission rates or due to increased numbers of active cases, the data is important information on circulating CPS causing iGBS in Alberta.

While the increases in CPS Ia, Ib, and III may not be clearly understood, the increases seen for CPS IV are likely due to the introduction of a number CPS IV strains into the Alberta population. From 2003 to 2013, the identification of CPS type IV gradually increased 12-fold (2 cases to 24

cases). This increase is likely part of the global increase of CPS IV reported previously in North America, South America, Europe, and Asia (16, 40-44). Recently, a study from Toronto, Canada by Teatero et al. (9) described a collection of 600 GBS isolates collected from cases of iGBS between 2009 to 2012 in the greater Toronto area, more than 6% (37 isolates) were identified as CPS IV. This is nearly identical to our CPS IV proportion of 6.3%. The Toronto study identified six STs, representing three CCs, among the 37 isolates examined (9). The major sequence type identified in the Toronto analysis was ST459 (51%), which is similar to our findings (60%, ST459) for the year assayed (2013). These authors published a follow-up report in 2015 that described increased number of cases of CPS IV in Saskatchewan (19% of total) and Manitoba (16% of total), two other provinces in Canada (45). These isolates were collected January 2010 to May 2014. These investigators also showed that the vast majority of CPS IV isolates causing adult disease were ST-459 (94% in Saskatchewan and 87% in Manitoba) (45). Interestingly, another ST, ST-425, accounted for 30% of the sequence types in the Toronto study and was represented by 4 isolates in the Saskatchewan and Manitoba surveillance, but this ST was not identified in our Alberta collection in 2013. This may be due to the small sample size of CPS IV identified in 2013 (22 isolates) for which MLST analysis was performed (22 isolates) or the absence of this ST in Alberta during the year assayed. With respect to previous reports for STs in Alberta, Manning et al. described the ST's in a collection of GBS isolates collected in Alberta from 1995 to 2002 (37). These investigators reported 47 different ST from a collection of 424 GBS strains (37). In this collection, only ST-2 was a sequence type also reported in our 2013 CPS IV collection. ST-2 is part of CC1 of which only 0.9% were CPS IV in the report by Manning et al. suggesting the introduction of the other CPS IV sequence types in Alberta occurred after 2002 (46). Our results

showed that ST459 was identified in Alberta first time in 2003 and ten in the subsequent years of 2009, 2010, and 2011. In 2013, ST-459 was the predominant sequence type among CPS type IV isolates. A previous survey of MLST types in Alberta did not identify ST459 (47).

A concern associated with the increase of CPS type IV is the CC \17 lineage, which has been identified among CPS type IV in a number of studies such as in Portugal, France, and Canada (9, 14, 16). The increase of CPS type IV is not exclusively associate with CC17 lineages, but was also associated with CC1 and CC23 as well (9, 16). CPS type IV has emerged as a colonizing strain among certain human populations (48, 49); possibly increasing the probability of its involvement in horizontal DNA transfer among GBS strains. A surface adhesion gene, *hvgA*, was thought to be exclusive to the CC17 lineage; however, it was identified among a CPS type IV/CC1 isolate by Teatero et al. (9). This is similar to our results, suggesting the acquisition of the *hvgA* gene from an unknown CC17 donor (9).

CPS IV is not one of the components of a GBS vaccine under review, which includes CPS type Ia, Ib, II, III, and V; this is due to the low prevalence of CPS IV in European countries and in the United States (50, 51) . This exclusion may need to be rethought because of the increase of serotype IV strains combined with antibiotic resistance, as reported in this study and other studies, such as reports from the United States (41) and Portugal (16). In the United States, erythromycin resistance rates increased from 1.2% among isolates in 1980 to 1993 to 18% in 1997 and 1998. The increase of macrolide resistance presents a high risk on pregnant women who are allergic to penicillin and may require a macrolide for prophylaxis. Dynamic changes in CPS distribution and the emergence of antibiotic resistance emphasize the need for constant monitoring, in order to develop accurate GBS prevention strategies.

As CPSs have increased, there have been a concomitant increase in both erythromycin and clindamycin resistance. These increases are primarily seen among patients ≥ 15 years of age, as opposed to neonates or children. A number of reports have raised a concern regarding the increased numbers of erythromycin and or clindmycin resistant GBS isolates in North America and Europe (52-56). Resistance rates of over 20% for both erythromycin and clindamycin should preclude the use of these antibiotics in cases of iGBS.

In summary, we have reported an increase in iGBS disease in Alberta over an eleven-year period as measured by isolates submitted to the Provincial Laboratory. These increases have involved CPSs Ia Ib, III and IV. Increases are evident in cases both of EOD and LOD as well as the overall population. The increased rates of iGBS disease have been accompanied by elevated rates of erythromycin and clindamycin resistance.

**Table 2.1. Total iGBS isolates presented by year and CPS, 2003-2013.**

| | No. (%) of isolates | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Year** | **CPS Ia** | **CPS Ib** | **CPS II** | **CPS III** | **CPS IV** | **CPS V** | **CPS VI** | **CPS VII** | **CPSVIII** | **CPS IX** | **NT** | **Total** |
| 2003 | 23 | 13 | 14 | 16 | 2 | 41 | 1 | 0 | 0 | 0 | 13 | 123 |
| 2004 | 8 | 11 | 12 | 25 | 4 | 21 | 0 | 0 | 0 | 0 | 15 | 96 |
| 2005 | 23 | 6 | 14 | 21 | 2 | 28 | 0 | 0 | 0 | 0 | 15 | 109 |
| 2006 | 22 | 8 | 21 | 18 | 1 | 33 | 0 | 0 | 0 | 0 | 12 | 115 |
| 2007 | 22 | 17 | 12 | 26 | 3 | 25 | 0 | 0 | 0 | 0 | 8 | 113 |
| 2008 | 26 | 18 | 15 | 27 | 7 | 27 | 4 | 0 | 0 | 0 | 10 | 134 |
| 2009 | 30 | 23 | 16 | 36 | 10 | 16 | 4 | 0 | 1 | 0 | 14 | 150 |
| 2010 | 44 | 21 | 17 | 39 | 10 | 44 | 4 | 0 | 0 | 4 | 22 | 205 |
| 2011 | 33 | 27 | 23 | 34 | 19 | 33 | 0 | 0 | 1 | 3 | 8 | 181 |
| 2012 | 35 | 27 | 22 | 47 | 25 | 31 | 9 | 0 | 0 | 8 | 19 | 222 |
| 2013 | 46 | 41 | 21 | 52 | 24 | 23 | 2 | 0 | 1 | 1 | 23 | 235 |
| **Total** | 312 | 213 | 187 | 341 | 106 | 322 | 24 | 0 | 3 | 16 | 159 | 1683 |
| **(%)** | (18.9) | (12.7) | (11.1) | (20.3) | (6.3) | (19.1) | (1.4) | (0) | (0.2) | (1.0) | (9.4) | (100) |

**Table 2.2. Incidence rate of iGBS/100,000 for all ages and incidence rate/1000 live births for EOD and LOD by year.**

| Year | Total no. of cases/ 100000 (95% CI) (no. of cases) | No. of cases/1000 live births (95% CI) (no. of cases) | |
|---|---|---|---|
| | | EOD | LOD |
| 2003 | 3.92 (3.2-4.6) (123) | 0.15 (0.06-0.33) (6) | 0.15 (0.06-0.33) (6) |
| 2004 | 3.02 (2.4-3.7) (96) | 0.34 (0.18-0.58) (14) | 0.25 (0.09-0.39) (10) |
| 2005 | 3.38 (2.8-4.1) (109) | 0.27 (0.13-0.48) (11) | 0.17 (0.05-0.32) (7) |
| 2006 | 3.49 (2.9-4.2) (115) | 0.09 (0.03-0.24) (4) | 0.14 (0.05-0.3) (6) |
| 2007 | 3.32 (2.7-4.0) (113) | 0.21 (0.09-0.36) (10) | 0.17 (0.06-0.3) (8) |
| 2008 | 3.83 (3.2-4.5) (134) | 0.16 (0.06-0.29) (8) | 0.30 (0.14-0.45) (15) |
| 2009 | 4.15 (3.5-4.9) (150) | 0.27 (0.15-0.46) (14) | 0.26 (0.12-0.4) (12) |
| 2010 | 5.52 (4.8-6.3) (206) | 0.39 (0.15-0.46) (20) | 0.22 (0.08-0.33) (11) |
| 2011 | 4.75 (4.1-5.5) (181) | 0.35 (0.2-0.56) (18) | 0.33 (0.2-0.56) (17) |
| 2012 | 5.63 (4.9-6.4) (222) | 0.25 (0.06-0.28) (13) | 0.33 (0.22-0.58) (17) |
| 2013 | 5.63 (5.3-6.8) (234) | 0.34 (0.14-0.44) (18) | 0.39 (0.3-0.69) (21) |
| Total | 1683 | 134 | 130 |

**Table 2.3. Incidence rate of iGBS /1000 live births for neonates (EOD and LOD) by year.**

| Year | Neonates (EOD and LOD)<br><br>No. of cases/1000 live births (95% CI) (no. of cases) |
|---|---|
| 2003 | 0.31 (0.2-0.5) (12) |
| 2004 | 0.6 (0.4-0.9) (24) |
| 2005 | 0.44 (0.3-0.7) (18) |
| 2006 | 0.23 (0.1-0.4) (10) |
| 2007 | 0.38 (0.2-0.6) (18) |
| 2008 | 0.47 (0.3-0.7) (23) |
| 2009 | 0.51 (0.3-0.7) (26) |
| 2010 | 0.61 (0.4-0.9) (31) |
| 2011 | 0.69 (0.5-1.0) (35) |
| 2012 | 0.59 (0.4-0.8) (30) |
| 2013 | 0.73 (0.5-1.0) (39) |
| Total | 264 |

**Table 2.4**. **Numbers of iGBS cases presented by CPS type and age.**

| | No. (%) of cases | | | | | |
|---|---|---|---|---|---|---|
| **CPS** | **EOD** | **LOD** | **91days-14yr** | **15yr-50yr** | **>50yr** | **Total** |
| **Ia** | 25 (18.7) | 24 (18.5) | 2 (11.1) | 91 (21.4) | 166 (17.3) | 308 |
| **Ib** | 16 (11.9) | 8 (6.2) | 5 (27.8) | 53 (12.5) | 127 (13.2) | 209 |
| **II** | 19 (14.2) | 3 (2.3) | 2 (11.1) | 52 (12.2) | 108 (11.2) | 184 |
| **III** | 38 (28.4) | 85 (65.4) | 6 (33.3) | 69 (16.2) | 147 (15.3) | 345 |
| **IV** | 9 (6.7) | 2 (1.5) | 1 (5.6) | 34 (8.0) | 59 (6.1) | 106 |
| **V** | 19 (14.1) | 7 (5.4) | 2 (11.1) | 78 (18.4) | 211 (22.0) | 317 |
| **VI** | 2 (1.5) | 0 | 0 | 6 (1.4) | 16 (1.7) | 24 |
| **VII** | 0 | 0 | 0 | 0 | 0 | 0 |
| **VIII** | 0 | 0 | 0 | 1 (0.2) | 2 (0.2) | 3 |
| **IX** | 0 | 0 | 0 | 1 (0.2) | 15 (1.6) | 16 |
| **NT**[a] | 6 (4.5) | 1 (0.8) | 0 | 40 (9.4) | 110 (11.4) | 157 |
| Total | 134 | 130 | 18 | 425 | 961 | 1668[a] |

[a] Fifteen of the 1,683 isolates were of unknown specimen source.

**Table 2.5. Numbers of iGBS cases by specimen source and age.**

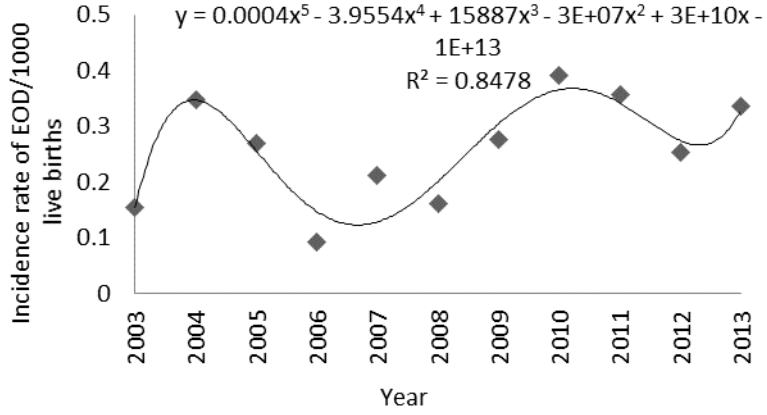
| Source | No.(%) of cases | | | | | |
|---|---|---|---|---|---|---|
| | **EOD** | **LOD** | **91days-14yr** | **15-50yrs** | **>50yrs** | **Total** |
| Blood | 107 (79.9) | 117 (90.0) | 14 (77.8) | 264 (62.1) | 706 (73.5) | 1208 (72.4) |
| Joint/synovial fluid | 0 | 0 | 0 | 33 (7.8) | 105 (11.0) | 138 (8.3) |
| Soft tissue | 3 (2.2) | 1 (0.8) | 0 | 45 (10.6) | 65 (6.7) | 114 (6.8) |
| Peritoneum/ dialysate | 0 | 0 | 0 | 19 (4.4) | 16 (1.2) | 35 (2.1) |
| Placental/ cord | 11 (8.2) | 2 (1.5) | 0 | 14 (3.3) | 0 | 27 (1.6) |
| CSF | 4 (3.0) | 7 (5.4) | 1 (5.6) | 3 (0.7) | 1 (0.1) | 16 (1.0) |
| Pleura | 0 | 0 | 0 | 2 (0.5) | 9 (0.9) | 11 (0.7) |
| Other | 9 (6.7) | 3 (2.3) | 3 (16.6) | 45 (10.6) | 59 (6.1) | 119 (7.1) |
| Total | 134 (8.0) | 130 (7.8) | 18 (1.1) | 425 (25.5) | 961 (57.6) | *1668A |

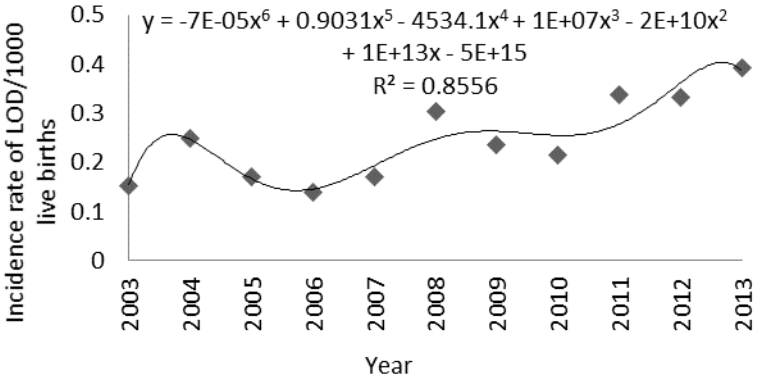
[a] NT, nontypeable

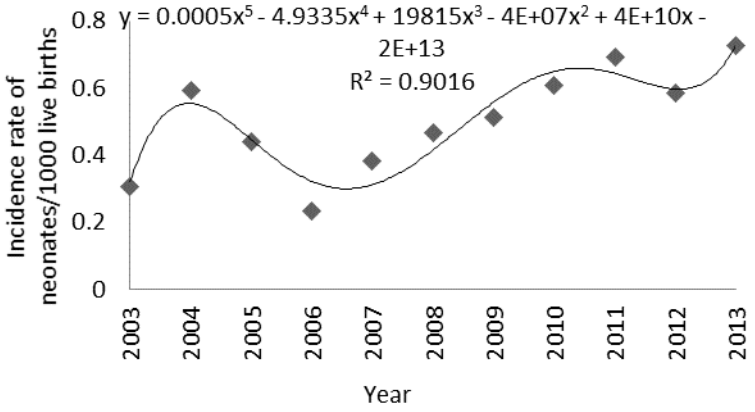
[b] Fifteen of the 1683 isolates were from patients of unknown age and therefore are not included in the table.

**Table 2.6. CPS type distribution among erythromycin and clindamycin resistant iGBS isolates, 2003-2013.**

| CPS | No. (%) of isolates | |
|---|---|---|
| | Erythromycin resistant (%) | Clindamycin resistant (%) |
| **Ia** | 92 (29.5) | 14 (4.5) |
| **Ib** | 71 (33.4) | 54 (26.4) |
| **II** | 78 (41.7) | 58 (31.0) |
| **III** | 91 (26.7) | 58 (17.0) |
| **IV** | 85 (80.2) | 82 (77.4) |
| **V** | 133 (41.3) | 83 (25.8) |
| **VI** | 9 (37.5) | 7 (29.2) |
| **VII** | 0 | 0 |
| **VIII** | 0 | 0 |
| **IX** | 4 (25.0) | 5 (31.3) |
| **NT** | 53 (33.3) | 35 (22.0) |
| **Total** | 616 | 396 |


y = 0.0004x5 - 3.9554x4 + 15887x3 - 3E+07x2 + 3E+10x - 1E+13
R² = 0.8478
Incidence rate of EOD/1000 live births
0.5
0.4
0.3
0.2
0.1
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
Year


y = -7E-05x6 + 0.9031x5 - 4534.1x4 + 1E+07x3 - 2E+10x2 + 1E+13x - 5E+15
R² = 0.8556
Incidence rate of LOD/1000 live births
0.5
0.4
0.3
0.2
0.1
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
Year


y = 0.0005x5 - 4.9335x4 + 19815x3 - 4E+07x2 + 4E+10x - 2E+13
R² = 0.9016
Incidence rate of neonates/1000 live births
0.8
0.6
0.4
0.2
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
Year

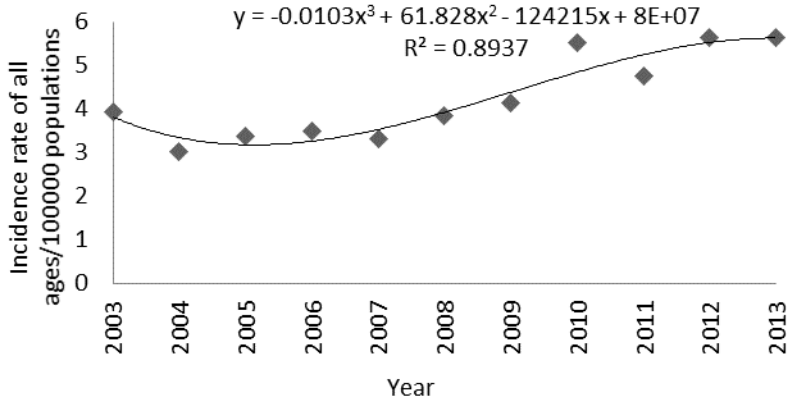

y = -0.0103x3 + 61.828x2 - 124215x + 8E+07
R² = 0.8937
Incidence rate of all ages/100000 populations
6
5
4
3
2
1
0
2003
2004
2005
2006
2007
2008
2009
2010
2011
2012
2013
Year

**Figure 2.1. Trend of iGBS incidence rate in EOD, LOD, neonates, and all age patients.**
EOD, LOD, and neonate (EOD and LOD combined) rates were calculated using the number of live births as denominator. Incidence rate of all ages was calculated using the number of population as denominator. The data fit a polynomial linear regression with fifth degree for EOD and neonates, sixth degree for LOD, and cubic degree for all age patients. Polynomial equation and predication accuracy ($R^2$) was indicated in each graph.

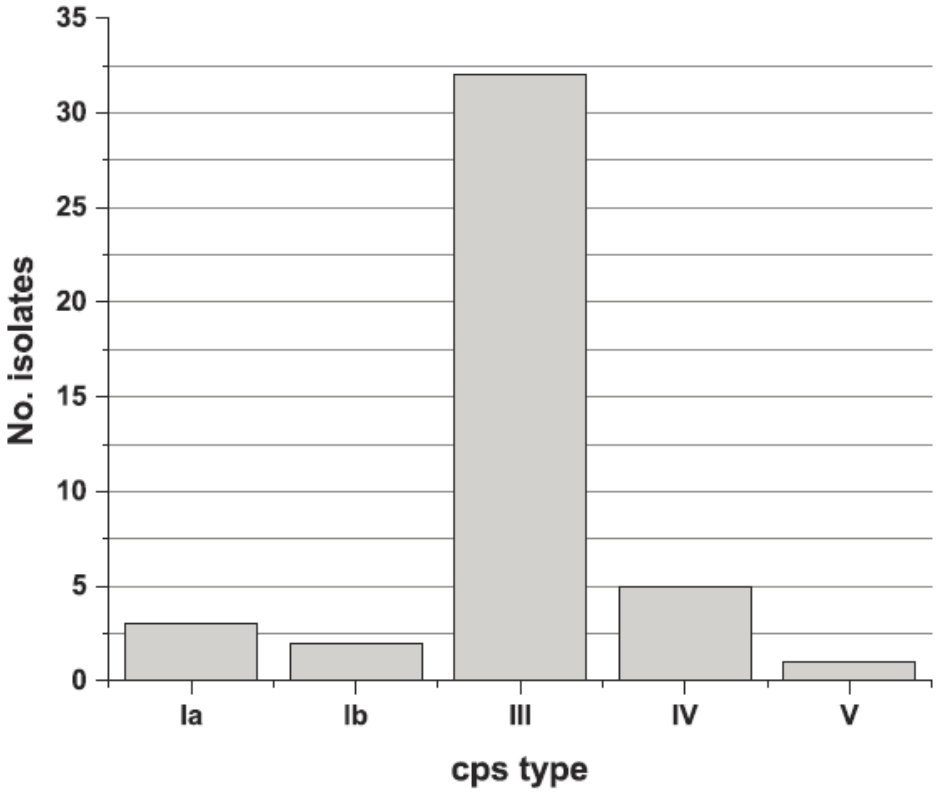



**Figure 2.2. Distribution of CPS types among *hvgA*-positive neonatal strains, 2003-2013.**

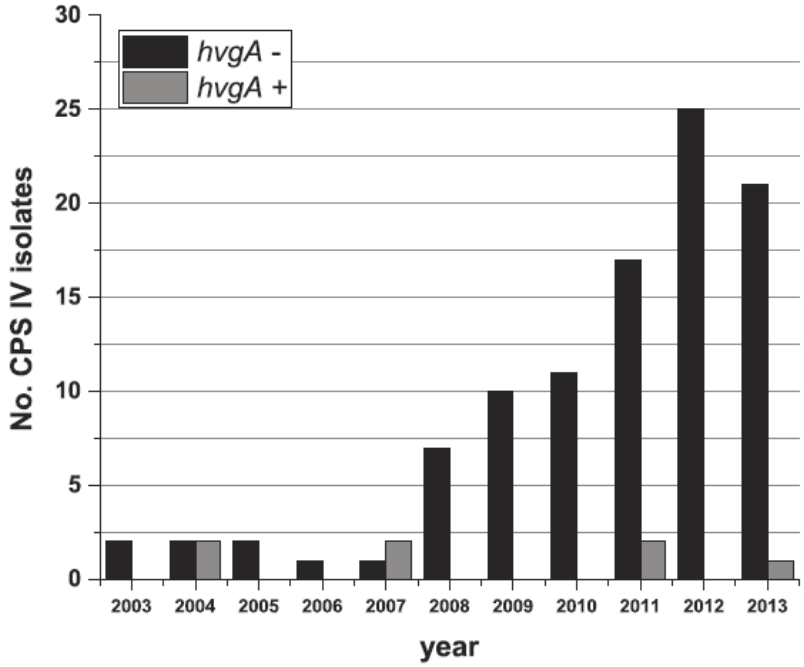



Figure 2.3. Distribution of *hvgA*-positive and *hvgA*-negative GBS CPS IV isolates for all ages, 2003-2013.

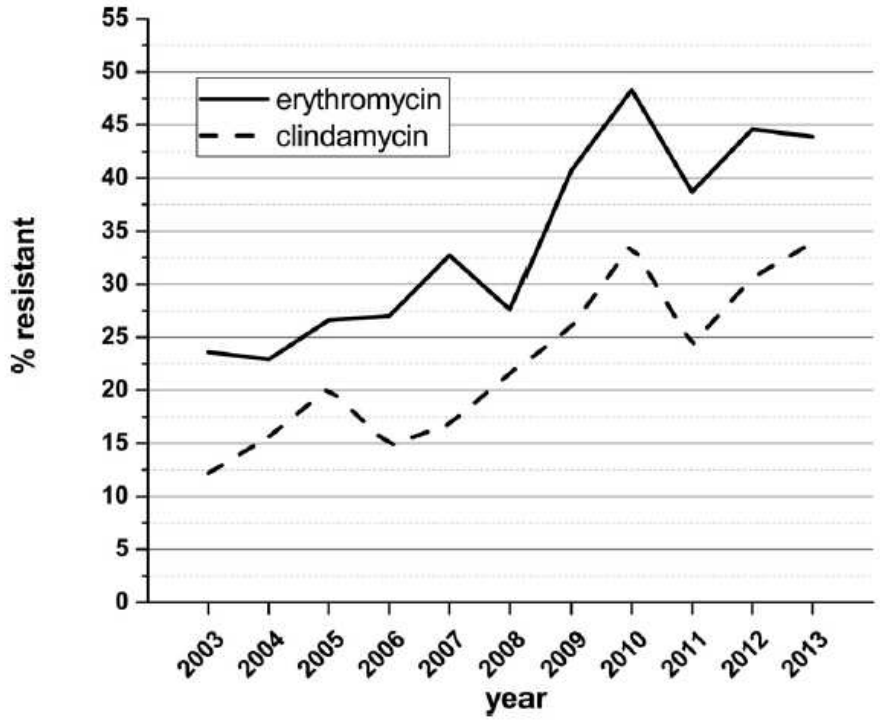



Figure 2.4. Erythromycin and clindamycin resistance among all iGBS isolates from 2003 to 2013.

## 2.5 References


1. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.
2. **Marques MB, Kasper DL, Pangburn MK, Wessels MR.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun **60:**3986-3993.
3. **Rubens CE, Wessels MR, Heggen LM, Kasper DL.** 1987. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. Proc Natl Acad Sci U S A **84:**7208-7212.
4. **Wessels MR, Rubens CE, Benedí VJ, Kasper DL.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc Natl Acad Sci U S A **86:**8983-8987.
5. **Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.
6. **Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.

7. **Bohnsack JF, Whiting A, Gottschalk M, Dunn DM, Weiss R, Azimi PH, Philips JB, 3rd, Weisman LE, Rhoads GG, Lin FY.** 2008. Population structure of invasive and colonizing strains of Streptococcus agalactiae from neonates of six U.S. Academic Centers from 1995 to 1999. J Clin Microbiol **46:**1285-1291.

8. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.

9. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of invasive group B streptococcus strains from the greater Toronto area, Canada. J Clin Microbiol **52:**1441-1447.

10. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ, Network ABCsEIP.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299:**2056-2065.

11. **Money D, Allen VM.** 2013. The prevention of early-onset neonatal group B streptococcal disease. J Obstet Gynaecol Can **35:**939-951.

12. **Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

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

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

15. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol doi:10.1128/JCM.03554-13.

16. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

17. **Lamy MC, Dramsi S, Billoët A, Réglier-Poupet H, Tazi A, Raymond J, Guérin F, Couvé E, Kunst F, Glaser P, Trieu-Cuot P, Poyart C.** 2006. Rapid detection of the "highly virulent" group B Streptococcus ST-17 clone. Microbes Infect **8:**1714-1722.

18. **Haug RH.** 1972. Type classification of the group B streptococci by means of Lancefield's precipitin method. Classification of strains of bovine and human origin; isolated during the year 1971 in Norway. Nord Vet Med **24:**631-638.

19. **Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179.

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

21. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518-1530.

22. **Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ.** 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett **241:**129-134.

23. **NCCLS.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. NCCLS, Villanova, PA.

24. **NCCLS.** 1997. Performance standards for antimicrobial susceptibility testing; 7th informational supplement, 7th informational supplement. NCCLS, Wayne, PA.

25. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and. J Clin Microbiol **45:**2909-2916.

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

27. **Manning SD, Lewis MA, Springman AC, Lehotzky E, Whittam TS, Davies HD.** 2008. Genotypic diversity and serotype distribution of group B streptococcus isolated. Clin Infect Dis **46:**1829-1837.

28. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

29. **Davies HD, Leblanc J, Bortolussi R, McGeer A, PICNIC.** 1999. The Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) study of neonatal group B streptococcal infections in Canada. Paediatr Child Health **4:**257-263.

30. **Davies HD, Adair CE, Schuchat A, Low DE, Sauve RS, McGeer A, Network ANGBSNatTIBD.** 2001. Physicians' prevention practices and incidence of neonatal group B streptococcal disease in 2 Canadian regions. CMAJ **164:**479-485.

31. **Verani JR, McGee L, Schrag SJ; Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC).** 2010. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. **19:**1-36.

32. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.

33. **Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.

34. **Meehan M, Cunney R, Cafferkey M.** 2014. Molecular epidemiology of group B streptococci in Ireland reveals a diverse population with evidence of capsular switching. Eur J Clin Microbiol Infect Dis **33:**1155-1162.

35. **Wang P, Tong JJ, Ma XH, Song FL, Fan L, Guo CM, Shi W, Yu SJ, Yao KH, Yang YH.** 2015. Serotypes, antibiotic susceptibilities, and multi-locus sequence type profiles of Streptococcus agalactiae isolates circulating in Beijing, China. PLoS One **10:**e0120035.

36. **Ippolito DL, James WA, Tinnemore D, Huang RR, Dehart MJ, Williams J, Wingerd MA, Demons ST.** 2010. Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. BMC Infect Dis **10:**336.

37. **Melin P, Efstratiou A.** 2013. Group B streptococcal epidemiology and vaccine needs in developed countries. Vaccine **31 Suppl 4:**D31-42.

38. **Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK.** 1989. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A **86:**4731-4735.

39. **Takahashi S, Adderson EE, Nagano Y, Nagano N, Briesacher MR, Bohnsack JF.** 1998. Identification of a highly encapsulated, genetically related group of invasive type III group B streptococci. J Infect Dis **177:**1116-1119.

40. **Darenberg J, Luca-Harari B, Jasir A, Sandgren A, Pettersson H, Schalén C, Norgren M, Romanus V, Norrby-Teglund A, Normark BH.** 2007. Molecular and clinical characteristics of invasive group A streptococcal infection in Sweden. Clin Infect Dis **45:**450-458.

41. **Ferrieri P, Lynfield R, Creti R, Flores AE.** 2013. Serotype IV and invasive group B Streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis **19:**551-558.

42. **Martins ER, Melo-Cristino J, Ramirez M, Infections PGftSoS.** 2012. Dominance of serotype Ia among group B Streptococci causing invasive infections in nonpregnant adults in Portugal. J Clin Microbiol **50:**1219-1227.

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

44. **Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B.** 2011. Emergence of group B Streptococcus serotype IV in women of child-bearing age in Ireland. Epidemiol Infect **139:**236-238.

45. **Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

46. **Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD.** 2009. Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47:**1143-1148.

47. **Manning SD.** 2003. Molecular epidemiology of Streptococcus agalactiae (group B Streptococcus). Front Biosci **8:**s1-18.

48. **Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P.** 2010. Clonal analysis of colonizing group B Streptococcus, serotype IV, an emerging pathogen in the United States. J Clin Microbiol **48:**3100-3104.

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

50. **Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nat Rev Microbiol **4:**932-942.

51. **Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P, Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M.** 2012. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis **31:**2097-2104.

52. **Borchardt SM, DeBusscher JH, Tallman PA, Manning SD, Marrs CF, Kurzynski TA, Foxman B.** 2006. Frequency of antimicrobial resistance among invasive and colonizing Group B streptococcal isolates. BMC Infect Dis **6:**57.

53. **Castor ML, Whitney CG, Como-Sabetti K, Facklam RR, Ferrieri P, Bartkus JM, Juni BA, Cieslak PR, Farley MM, Dumas NB, Schrag SJ, Lynfield R.** 2008. Antibiotic resistance

patterns in invasive group B streptococcal isolates. Infect Dis Obstet Gynecol **2008:**727505.

54. **Domelier AS, van der Mee-Marquet N, Arnault L, Mereghetti L, Lanotte P, Rosenau A, Lartigue MF, Quentin R.** 2008. Molecular characterization of erythromycin-resistant Streptococcus agalactiae. J Antimicrob Chemother **62:**1227-1233.

55. **Betriu C, Culebras E, Gómez M, Rodríguez-Avial I, Sánchez BA, Agreda MC, Picazo JJ.** 2003. Erythromycin and clindamycin resistance and telithromycin susceptibility in Streptococcus agalactiae. Antimicrob Agents Chemother **47:**1112-1114.

56. **Heelan JS, Hasenbein ME, McAdam AJ.** 2004. Resistance of group B streptococcus to selected antibiotics, including erythromycin and clindamycin. J Clin Microbiol **42:**1263-1264.

# Chapter 3: Identification of the Group B Streptococcus Capsule Type using a Dual Phenotypic/Genotypic Assay[a]

[a] A version of this chapter has been published as

**Alhhazmi A, Pandey A, Tyrrell GJ.** 2017. Identification of the Group B Streptococcus Capsule Type using a Dual Phenotypic/Genotypic Assay. J Clin Microbiol **55**:2637-2650.

## 3.1 Introduction

Group B Streptococci, (GBS) are recognized as a leading cause of neonatal invasive diseases as well as invasive disease in immunocompromised patients and in elderly individuals (1-7). An important virulence factor of GBS is a capsular polysaccharide (CPS) of which there are ten antigenic variants designated Ia, Ib and II to IX (8-11). In North America and a number of European countries, five CPS types (Ia, Ib, II, III, and V) cause the bulk of invasive disease cases, with CPS III causing a higher rate disease among neonates and CPS type Ia and V higher rates among adult patients (12-23). Interestingly, recent studies have noted the emergence and circulation of CPS IV, a previously uncommon serotype, as an important cause of both neonatal and adult infections (14, 19, 24-28).

As attention is focused on invasive GBS disease (iGBS) with the emergence of new strains and increased antibiotic resistance, the potential to prevent iGBS disease via vaccination becomes more attractive (21, 29). If a GBS vaccine is developed for use that is based on selected capsule types, it will become important to monitor distribution patterns of CPS types in circulation in the target population using sensitive and specific methods for determining CPS types.

Common phenotypic methods of GBS CPS type identification are based on serological assays such as capillary precipitin (30), immunodiffusion (30, 31) latex agglutination (32, 33), coaggulation (34), or enzyme immunoassay (35) which have proven invaluable for identifying CPS types. However, these assays may have complicated interpretations resulting in a number of incorrect typing assignments due to poor capsule expression, capsule operon mutations, rearrangements or limited accuracy. Molecular typing based on PCR-assays has been already

developed; however, these assays target capsule gene detection rather than capsule expression. Moreover, they tend to involve a combination of two different techniques, e.g., PCR plus sequencing (36), PCR plus blot hybridization (37, 38), PCR plus enzymatic restriction (39), or multiplex PCR plus agarose gel electrophoresis (40, 41). A real-time PCR assay is a more attractive molecular method to assign CPS type compared to conventional PCR since it is usually more rapid.

The CPS type-specific epitopes of each GBS polysaccharide are created by different arrangements of four component sugars (glucose, galactose, N-acetylglucosamine and sialic acid) into a unique repeating unit. Interestingly, all these structures contain a terminal sialic acid (Neu5Ac) (40, 42, 43). The conservation of Neu5Ac among all known GBS capsular types suggests that this structural feature is essential to GBS capsular polysaccharide pathogenicity. We propose that sialic acid on the surfaces of GBS capsule positive cells can be used as a universal phenotypic method to ascertain capsule expression followed by a CPS specific real-time PCR assay.

## 3.2 Materials and Methods

### 3.2.1 Bacterial strains used.

Ten GBS strains with known CPS types were used as reference strains for CPS typing (Table 3.1.). Seventy GBS clinical isolates previously typed by the phenotypic immunodiffusion assay, and 159 GBS clinical isolates that previously failed to be typed (nontypable [NT]) by the phenotypic immunodiffusion assay, were included in this study (15). A S*treptococcus pneumoniae* serotype 14 isolate (spn14) and *Escherichia coli* ATC25922 were used as control strains (Table 3.1) (44, 45). GBS isolates were cultured on Columbia blood agar plates

(DALYNN Biologicals, Canada) containing 5% sheep blood overnight at 37°C. They subsequently were inoculated into Todd-Hewitt broth (Becton Dickinson, USA) and incubated overnight at 37°C for use in experiments described here.

### 3.2.2 Identification of GBS

Isolates were identified based on colony morphology, β-hemolysis, Gram stain, and Lancefield grouping with type B antisera (Oxoid, Canada) (46). GBS isolates were additionally confirmed as GBS using a conventional PCR-based assay targeting the *cfb* gene that encodes the Christie-Atkins-Munch-Petersen (CAMP) factor (47).

### 3.2.3 GBS CPS typing

#### 3.2.3.1 Double immunodiffusion assay

Phenotypic CPS typing was performed using the Lancefield heat-acid extraction assay followed by a double immunodiffusion method described previously (30, 31). The immunodiffusion assay of GBS CPS typing used for this study was based on reactions with antisera raised against CPS types Ia, Ib, II-VIII. The type-specific antisera panel was prepared in rabbits as previously described (30, 31).

#### 3.2.3.2 CPS type IX identification

PCR CPS typing was used for the identification of CPS IX using primer pair cpsI-7-9-F (5’-CTGTAATTGGAGGAATGTGGATCG) and cpsI-9-R (5’-AATCATCTTCATAATTTATCTCCCATT) which amplify target regions specific to CPS IX as previously described (40).

#### 3.2.3.3 CPS type II identification

To identify CPS type II, a previously described PCR assay (41) was performed using primers II-F (5’-TCCGTACTACAACAGACTCATCC) and

II-R (5’-TTCTCTAGGAAATCAAATAATTCTATAGGG) which amplify target region specific for CPS type II (397 bp).

### 3.2.4 Genomic DNA Extraction

A. Pandey assisted with DNA extraction. Genomic DNA extraction was performed as follows. Overnight broth cultures (1.5 ml) were centrifuged for 10 minutes at 3,000 × g. The pellet was resuspended in 500 µl of 1X phosphate-buffered saline (PBS, 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, and 1000 ml $H_2O$ [pH 7.2]) and washed two times with PBS. Genomic DNA was extracted using a DNA Mericon kit (Qiagen, Germany). Extracted genomic DNA was concentrated and dissolved in 30 µl Qiagen elution buffer or water and stored at –20°C. RNase pre-treatment was done prior to quantification of genomic DNA.

### 3.2.5 Isolation of CPS

CPS from GBS strains were isolated as previously described (43, 48-50) with modifications. Bacteria were grown overnight in 200 ml of THB with 3% neopeptone for 24 hours at 35°C, diluted to 2 liters in fresh THB, and grown to an optical density at 600 nm ($OD_{600}$) of 0.7. The cultures were chilled on ice, and the cells were pelleted and washed twice with ice-cold PBS. The cells were then resuspended in 200 ml lysis buffer (25 mM Na phosphate buffer, 10 mM $MgCl_2$, 40% [wt/vol] sucrose, 13.3 U/ml mutanolysin [Sigma-Aldrich, Canada], pH 7.0) followed by incubation for 19 hours at 37°C with end-over-end mixing. Protoplasts were removed by centrifugation, and the mutanolysin extract was treated with DNase buffer (3.5 ml, 400 mM Tris, 60 mM $MgCl_2$, 20 mM $CaCl_2$, pH 7.5), along with sodium azide to a 0.05% final concentration. DNase (300 U) and RNase (200 µg) were added, and the sample was incubated for 24 hours at 37°C with rocking. After centrifugation at 3200×g for 30

minutes at 4°C to pellet precipitated material, pronase (0.5 mg predigested for 2 hours at 50°C to destroy glycosidases in the preparation) was added along with 0.1 ml $CaCl_2$ (10 mM final concentration), and the sample was incubated for 17 hours at 37°C with rocking. The remaining insoluble cell wall fragments and cell bodies were removed by centrifugation. The supernatant was collected and stored at 4°C.

### 3.2.6 Lectin-enzyme-immunoassay (Lectin-EIA)

Capsule extract (200 µl) along with 0.15 M sodium carbonate was used to coat the wells of 96 well EIA plates (MP Biomedicals, USA) at 4°C overnight. The detection method was based on biotinylated lectin extracted from slug agglutinin *Limax flavus* (LFA) (EY laboratories, USA). The amount of bound biotinylated LFA was quantified using horse radish peroxidise (HRP)-labeled streptavidin (Vector Laboratories, USA). Streptavidin was selected as a detection method because it had no carbohydrate groups to which lectins may incidentally bind and it provided an amplification step to detect small amounts of siaylated CPS (43, 51-53). The plates were washed with PBS for 15 minutes, blocked with 1% bovine serum albumin (BSA) in PBS for 4 hours at 37°C, then 50 µl of three dilutions (10, 1, 0.1 µg/ml) of biotinylated LFA in PBS with 1% BSA and 0.05% Tween 20 (PBSAT) were added to the wells. After incubation for 1 hour at room temperature (RT), the plates were washed with PBS for 15 minutes, and 50 µl of horse radish peroxidise (HRP)-conjugated to streptavidin (1 µg/ml) in PBSAT was added to each well. After incubation for 1 hour at RT, the plates were washed with PBS for 15 minutes. Fifty µl of HRP substrate (1-step ™Ultra TMB-ELIZA, Thermo Scientific, USA) was added to each well, followed by incubation at 37°C for 15 minutes. Absorbances were read on a microplate reader (DYNEX Technologies, USA) at 450 nm. To identify isolates

that were sialic acid positive, the average absorbance (95%CI) value was calculated for the negative control spn14 (no sialic acid in its capsule), and this value was subtracted from the absorbance values of the assayed GBS isolates.

### 3.2.7 Lectin Dot-Blot Assay

Late exponentially growing bacteria were washed with PBS and resuspended in PBS to give an $OD_{600}$ of approximately 2. The bacterial suspension was spotted onto nitrocellulose membrane (20 µl/spot) using a biodot apparatus (Bio-Rad, USA) and dried for 30 minutes at room temerature. The membranes were washed for 15 min with TBS (6.05 g Tris, 8.76 g NaCl in 1000 mL of $H_2O$. [pH 7.5]) and then incubated with blocking buffer (5% skim milk, 0.1% Tween 20 in TBS) for 1 hour at 37°C. Membranes were subsequently washed with TBS for 15 min, incubated with biotinylated lectin LFA (10 µg/ml in blocking buffer) for 1 h, washed three times (15 min each time) with TBS for 15 min and then incubated with HRP-conjugated streptavidin (1 µg/mL in blocking buffer). After incubation for 1 h at room temperature, the membranes were washed with TBST three times (15 min each time) and then with TBS (15 min each time). Detection was performed using 4-chloro-1-naphthol solution (Sigma-Aldrich, Canada). A positive spot was identified as a clearly defined spot at the site where the bacteria were applied, and a negative result was identified as a trace reaction or the absence of any reaction.

### 3.2.8 Primer design for real-time PCR GBS typing assay

Representative isolates of GBS CPS types with complete cps operon sequences available in GenBank were included in this study (Ia, CP000114.1; Ib, AB050723.1; II, AY375362.1; III, HG939456.1; IV, AF355776.1; V, AE009948.1; VI, AF337958.1; VII, AY376403.1; and VIII,

AY375363.1). The deduced nucleotide sequences of GBS capsular genes *cpsG*, *-H*, *-I*, *-J*, *-K*, *-M*, *-N*, and *-O* available for serotypes Ia, Ib, and II to VIII in the data bank were aligned by the Muscle program (http://www.ebi.ac.uk/Tools/msa/muscle/help/)(54) and then distinctive genes or regions were chosen to infer the primer and probe sequences listed in Table 3.2. The partial CPS IX sequence was also included in the multiple-sequence alignment (GenBank accession no. GQ499301.1). CPS types Ia, Ib, and II to IX were identified based on the ability of the primer pairs to amplify an amplicon and the probe to bind the cps genes *cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO-V*, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX. Probes used in the singleplex and duplex real-time PCR assays are labeled with a fluorescent reporter dye and a quencher dye. The sequences of the 10 primer pairs and 10 TaqMan probes used are given in Table 3.2 a&b

### 3.2.9 Singleplex real-time PCR assay

A real-time PCR mixture was prepared in a final volume of 20 µl. TaqMan Fast Universal PCR master mix (2X) (Applied Biosystems, USA) was used. Reaction mixtures included 0.18 mM reverse primer, 0.18 mM forward primer, and 0.5 mM probe. Two microliters of extracted DNA were used for each reaction. A real-time PCR assay was performed in a Applied Biosystems 7500 Real-Time PCR System. The cycling conditions were denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The rate of temperature increase was 1°C/s (or 0.5°C/s), and the fluorescence was acquired once.

### 3.2.10 Duplex real-time PCR assay

Two fluorogenic probes were utilized in each duplex reaction as in Table 3.2 a & b. The first probe was covalently labeled at the 5’-terminal nucleotide with the FAM (6-carboxyfluorescein) reporter dye and at the 3’-terminal nucleotide with the BHQ1 (Black Hole

Quencher 1) quencher. The second probe was labeled with Cal Fluor 540 or 560 (Integrated DNA Technologies [IDT], USA) reporter dye at the 5'-terminal nucleotide and again with the BHQ1 quencher dye at the 3'-terminal nucleotide. The duplex real-time PCR mixture was prepared in a final volume of 20 µl. TaqMan Fast Universal PCR master mix (2X; Applied Biosystems) was used, and a PCR was performed in the Applied Biosystems 7500 Real-Time PCR System as in the singleplex reaction. However, the duplex mixtures include two reverse primers (0.18 M), two forward primers (0.18 M), and two probes (0.5 mM) that target two CPS-specific regions. Four microliters of extracted DNA were used for each reaction. The cycling conditions were denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 3 s and 60°C for 30 s. The rate of temperature increase was 1°C/s (or 0.5°C/s), and the fluorescence was acquired once.

### 3.2.11 Standard curve and efficiency measurements

A standard curve was established for each primer pair and probe targeting cps genes (*cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO*-V, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX) in the PCR assay. Tenfold dilutions of the template were generated and a plot of the threshold cycle (CT) versus the DNA concentration was constructed. From this standard curve, information about the smallest amount of DNA detected and the CT slope were determined. The efficiency values were measured for each primer pair using the CT slope method. The amplification efficiency was calculated according to the following equation: $Ex = 10^{(-1/slope)} - 1$, where Ex is the efficiency.

## 3.3 Results

### 3.3.1 Detection of sialic acid from 10 GBS CPS types using a lectin enzyme immunoassay (EIA).

Understanding that sialylation is essential for full GBS capsule biosynthesis and loss of sialylated capsule reduces the amount of CPS expressed on the bacterial cell surface by 80% (43), I hypothesized that sialic acid could be used as a recognition moiety for capsule expression. The commercially available biotinylated lectin from the slug *Limax flavus* was selected to be used in our sialo-lectin binding assay. *L. flavus* lectin was selected because it reacts with sialic acid in any linkage (55, 56), whereas other sialic acid-specific lectins recognize only specific glycosidic linkages of sialic acid or other carbohydrate moieties (51, 56-58). Sialic acid is conserved at the terminus of the side chain of all GBS capsule types. To examine the use of sialo-lectin binding to detect GBS CPS expression, 10 GBS strains were selected representing all recognized GBS CPS types (Ia, Ib, and II to IX) for CPS extraction (Table 3.1). Recognition of the immobilized sialic acid from the 10 assayed CPS type bacterial isolates by biotinylated L. flavus lectin validated the presence of CPS. It also provided a dose-dependent signal that exhibited a saturating signal at a lectin concentration of 10 µg/ml (Fig. 3.1). Therefore, the working concentration of biotinylated *L. flavus* lectin with all the CPS types in the study was restricted to 10 µg/ml. The average absorbance at 450 nm for all CPS types was 0.85 with a 95% confidence interval (CI) of 0.77 to 0.93 (Fig. 3.1). For the asialo-CPS (spn14) isolate with no sialic acid in its capsule and the well with no capsule material, average absorbance of 0.45 (95% CI 0.44 to 0.45) and 0.022 (95% CI 0.02 to 0.03) were detected, respectively. The upper absorbance limit of the 95% CI of the negative control (spn14)

preparation was 0.45. This value was used as a cutoff for detecting the presence of sialic acid in further experiments.

### 3.3.2 Detection of sialic acid from the 10 GBS CPS types using a lectin dot blot assay

To verify that the sialo-lectin binding phenotypes from the 10 assayed CPS type isolates by the lectin EIA was not due to endogenous sialic acids coextracted from the bacteria during the capsule extraction process, whole bacteria were assayed in a lectin dot blot assay. As shown in Fig. 3.2, sialic acid was uniformly expressed in all the assayed strains, whereas no signal was detected on the spot corresponding to the asialo-CPS (spn14) isolate. The results suggested that both the lectin EIA and the lectin dot blot assays could be used as simple phenotypic assays to determine CPS expression for GBS.

### 3.3.3 GBS CPS typing assay

#### 3.3.3.1 Assignment of CPS types for types Ia, Ib, and II to IX using a singleplex real-time PCR CPS typing assay

Representative CPS DNA operon sequences for CPS Ia, Ib, and II to VIII and a partial CPS DNA operon sequence for IX were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/). The sequences of these strains were analyzed to generate CPS-specific gene primer pairs and dually labeled probes, which enabled the amplification of DNA amplicons to be easily discriminated by specific probes. A CPS-specific gene scheme was developed based on comparison analyses of *cps* genes. A unique region(s) of the *cps* gene(s) was identified for each CPS type based on the nucleotide sequence comparison with other CPS types. From this information, a set of primers for each CPS type, Ia, Ib, and II to IX, was

designed to uniquely target each of the 10 CPS types (Table 3.2 a & b). The identification of CPS types Ia, Ib, and II to IX depends on the ability of the primer pairs to amplify and probe.

To identify a specific fragment for the following *cps* genes: *cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO*-V, *cpsI*, *cpsM*, *cpsR*, and *cpsO*-IX, respectively. To verify the specificity of the designed primer pairs and probes, they were BLAST searched against the NCBI nonredundant sequence database to confirm the absence of serendipitous similarities. Based on this analysis, the designed primer pairs and probes were determined to have 100% specificity for the identification of GBS CPS types, except for CPS type III. The primers and probe targeting *cpsG* were similar to CPS types V and VI. However, the primers for CPS V and VI are specific for the identification of these CPS types. An algorithm was constructed to allow identification of the 10 CPS types, as shown in Fig. 3.3. The specificity and efficiency of each primer pair used separately were determined by singleplex real-time PCR with DNA extracted from 10 GBS strains representing all GBS recognized CPS types (Table 3.3) that were used as reference strains. Specific and characteristic PCR patterns were obtained with all primer pairs and probes for each CPS type (Table 3.4). No signal was obtained for the negative controls, isolates spn14 and *Escherichia coli* ATCC 2592 (data not shown). The standard curve and efficiency of each primer pair was determined by using a series of diluted genomic DNA extracts from the recognized CPS types to determine the smallest amount of DNA detected while maintaining a desirable efficiency. The minimum concentration of purified genomic DNA required for the *cpsH*, *cpsK*, *cpsJ*, *cpsN*, and *cpsI* primer pairs was 30 ng, whereas for the *cpsG*, *cpsM*, *cpsO*-V, and *cpsO*-IX primers 20 ng was required (Fig. 3.4). For the *cpsR* primer set, 40 ng was required for the detection. The designed primers have high amplification

efficiency ranging between 90.9 and 99.9%, as shown in Table 3.3, indicating that the assay is robust and reproducible.

#### 3.3.3.2 Assignment of CPS types using a duplex real-time PCR GBS typing assay

Five duplex reactions (1 to 5) (Table 3.2 a & b) containing primer pairs and probes specific for CPS types V/VI, III/II, VII/VIII, Ia/IV, and Ib/IX were used in a real-time PCR platform. The 10 GBS CPS reference strains previously assayed in the singleplex PCR typing assay were analyzed using the duplex real-time PCR assay to assign CPS type. The duplex real-time PCR assay allowed identification of the CPS type for the GBS strains shown in Table 3.5. A collection of 70 previously serotyped GBS clinical isolates representing all recognized CPS types were analyzed to determine the reliability of the duplex real-time PCR typing with the exception of CPS VII. No CPS type VII except for the reference strain was present in our collection; therefore, no clinical CPS VII isolates were assayed(15). We compared the CPS typing by the duplex real-time PCR assay against serotyping by the immunodiffusion assay for CPS types Ia, Ib, and II to VIII or a previously described PCR assay (40) for CPS type IX. A concordance of 97.2% for the duplex real-time PCR GBS typing and the phenotypic serotyping assay (immunodiffusion) was observed. No discordant results were obtained between the molecular assay and the serological assay for all CPS types, with the exception of CPS type Ib. Two isolates were typed by the serological assay as CPS type Ib, and yet the molecular assay assigned CPS type II for these isolates (Table 3.6). To confirm CPS genotyping of these two isolates as CPS type II, a previously described conventional PCR assay (41) was performed to distinguish between CPS types II and Ib. The PCR assay verified these isolates as CPS type II (Fig. 3.5).

### 3.3.4 Assignment of CPS type to a collection of clinical NT isolates using lectin EIA/lectin dot assays and a duplex real-time PCR GBS typing assay.

To determine the robustness of our assay algorithm, we assayed 159 GBS clinical isolates from Alberta, Canada, collected from 2003 to 2013 that were determined to be nontypeable (NT) in a double immunodiffusion assay. These GBS isolates were assayed for the presence of sialo-CPS using the lectin EIA after CPS extraction. A total of 47.2% (75/159) of the capsule preparations from the NT isolates reacted positively with *L. flavus* lectin, suggesting the presence of capsule (data are not shown). The lectin dot blot assay supported the results obtained from the lectin EIA since all 75 isolates spotted in the lectin dot blot assay displayed spots darker than the spots from isolates with an optical density (OD) below 0.45 in the lectin EIA (Fig. 3.6), suggesting the presence of sialic acid. To prove the stability and veracity of the duplex real-time PCR typing assay, the capsule types of the sialo-CPS-positive NT strains were assayed. DNA was extracted, and PCR amplification with the *cfb* primer pair was performed. All strains yielded the expected PCR product, which confirmed that the DNA preparations were devoid of PCR inhibitors and that the corresponding strains were GBS (data not shown). A duplex RT-PCR CPS typing assay was then performed to identify CPS genotypes. A capsular genotype was assigned for 71 of 75 isolates (94.7%). The majority of the strains were typed as CPS typeV (53.3%, 40/75). A total of 14.7% (11/75) of the isolates were typed as CPS type III, 8% (6/75) as type II, 5% (4/75) as type Ia, 5% (4/75) as Ib, 4% (3/75) as type VI, 2.7% (2/75) as type IV, and 1.3% (1/75) as type IX. Four isolates failed to be assigned a CPS type. To test the ability of the duplex PCR assay to assign CPS types to GBS isolates, we randomly selected 32 of the remaining 84 GBS isolates that did not react with *L. flavus* lectin in EIAs and dot blot

assays and assayed them in the duplex real-time PCR assay. The capsule types identified were CPS types Ia (3.1%, 1/32), Ib (31.3%, 10/32), II (3.1%, 1/32), III (6.3%, 2/32), V (18.3%, 6/32), VI (3.1%, 1/32), and IX (28.1%, 9/32). Two isolates failed to be assigned a capsule type. This demonstrated the ability of the duplex real-time PCR assay to genotypically identify CPS types which failed to express sialic acid on their surfaces, suggesting the absence of capsule.

### 3.4 Discussion

GBS capsular polysaccharide is a well-known protective antigen against GBS (29, 59). Past efforts to develop GBS vaccines have focused primarily on the use of capsular polysaccharides from more common types associated with GBS disease (29, 59). For this avenue of vaccine development to be effective requires inclusion of the most relevant CPS types in the target population (29, 59). Here, I present a dual GBS typing system that provides information regarding capsule expression and identification of capsule type.

The first step of the proposed GBS typing system involves detection of sialic acid and confirmation of capsule expression. GBS CPS is terminally linked to sialic acid. Sialyation is an essential process for full GBS capsule polysaccharide biosynthesis and expression. A past study reported sialylation of GBS capsular polysaccharide is required for full synthesis of CPS by GBS (43). These investigators found a 80% reduction in surface associated CPS produced by asialo mutant strains that had a deletion in the *cpsK* gene encoding sialyltransferase compared to the parental strain (43). This group also reported the same scenario with an asialo mutant with a deletion in *neuA* encoding CMP-sialic acid synthase (60). The lectin from *L. flavus* agglutinin was chosen in our study to detect sialic acid from GBS CPS types for two reasons. First, LFA binds to Neu5Ac regardless of its linkage. This was optimal for our assay

since sialic acid in the CPS among the known GBS CPS types has different linkages. Second, to date, nonspecific binding has not been identified for LFA (61). For these reasons, biotinylated LFA lectin was selected.

Serological CPS typing is the most common method for GBS CPS assignment, but the proportion of NT isolates is significant and has increased over time (11, 37, 62). This leads to misrepresentation of some of the CPS types. The sensitivity of the serological assays to detect GBS capsule polysaccharide can vary. These methods depend on the quality of the antibodies, the technical experience of the operator, and expression of detectable amount of capsule (63). A study by Kilian *et al.,* identified limitations associated with GBS latex agglutination serotyping (64). The agglutination assay was found less sensitive than the flow cytometric assay. Approximately one-half of the strains assigned as NT in the agglutination test were found to express type-specific polysaccharides by the flow cytometric method (64). To overcome these limitations, we developed a sialo-lectin assay for which sialic acid could be used as a universal approach to detect capsule expression without the need for antibodies. Moreover, the use of biotin-lectin in a streptavidin-HRP detection and amplification system has the advantage of detecting a small amount of siaylated capsule (43, 51-53). Two assays, lectin-EIA and lectin-dot blot assays, were described in our work to identify isolates with capsule. We also used these assays to characterize isolates in our collection that were identified as NT by immunodiffussion assay. Based on our results, there were 52.8% (84/159) NT isolates that were absent for sialic acid expression on the bacterial surface. However, 47.2% (75/159) of our serologically NT isolates expressed sialic acid on the surface, suggesting the presence of the capsule. The immunodiffussion assay is less sensitive than the sialo-lectin

methods as confirmed by our observation that 47.2% of the strains assigned as NT in this assay were found to express sialyated capsule polysaccharide when examined by the sialo-lectin assays. The 84 sialic acid negative isolates in the lectin-ElA and lectin dot assays presumably do not express capsular polysaccharides. Mechanisms of capsule loss could potentially include genetic alterations such as insertion or deletion of DNA fragments in the *cps* operon or mutations in specific conserved genes such as *cpsE*, *cpsF*, *cpsA* and *cpsG* located in *cps* locus (37, 65, 66).

The second step of our proposed GBS typing system is identification of the CPS type by PCR. Several molecular typing based PCR-assays have been previously developed (36, 40, 41, 67, 68). These assays target CPS-determining genes in the *cps* operon. PCR-assays are an attractive approach to GBS CPS typing because of their high discriminatory power for identifying CPS types. Such PCR assays were able to assign a CPS type to isolates that failed to be identified using serological assay methods (36, 40, 41, 68). Although PCR-based assays for typing GBS worked well, a number of limitations have been identified. Generally, these assays target capsule genes rather than expression, hence, the identified *cps* locus by the PCR-based assays does not confirm if the capsule polysaccharide is expressed or not. Moreover, to identify CPS type by the available PCR-based assays, two different techniques are required, such as PCR plus sequencing (36), PCR plus blot hybridization (37, 38), PCR plus enzymatic restriction (39), or multiplex PCR plus agarose gel electrophoresis (40, 41). The involvement of two molecular techniques to identify CPS type only at the genotypic level can consume time and may involve increased expense when techniques such as sequencing and blot hybridization are used. Also, these assays may not always be accurate. For example, the

multiplex PCR assay described by Imperi *et al.*, that was designed to distinguish between (Ia, Ib, II-IX) (40) was found to misidentify some CPS type Ib and IV strains as CPS type Ia (64, 69). In addition, the two-set of a multiplex PCR assay (set 1 - Ia, Ib, II, III, IV) and (set 2 -V, VI, VII, VIII) developed by Poyart *et al.,* (41) failed to distinguish between CPS types VII and IX (64). Also, the CPS-specific PCR assays described by Kong *et al.,* (36, 67), did not include a PCR specific for CPS types II, VII, or VIII (64). To address these issues, we developed a duplex real-time PCR assay which identifies CPS type based on detecting the presence of capsular genes. The real-time PCR technique used in our typing system is a more attractive approach to assign CPS type compared to conventional PCR. The main advantages of TaqMan real-time PCR assay are its high sensitivity and reliability (one step and gel free) with specific set of primers and probes to identify each CPS type. The assay is easy to perform and does not require the use of antisera. The method also has time advantages over previously described molecular methods (36, 40, 41, 68), because it requires only a single PCR reaction for amplification and detection and can be completed within one hour. To overcome GBS typing inaccuracies using PCR, previous investigators have developed a GBS CPS typing algorithm that includes three previously described PCR assays (36, 40, 41, 67). This approach resulted in a GBS CPS typing accuracy of 99% (69). The duplex real-time PCR assay had a level of accuracy of 100%. We believe that our assay would be ideally suited for high throughput GBS epidemiological studies in which a large collection of GBS isolates required CPS typing. A limitation of my assay was the inability to type a small subset of isolates. Four isolates of the 75 isolates that are sialic acid positive and 2 isolates of the 32 randomly selected sialic acid negative isolates, failed to be typed by our PCR assay. Possible reasons for this may be the presence of

insertion(s), mutation(s) impairing primer and/or probe binding. Alternatively, these isolates may represent new CPS types resulting from capsular switching events, in which GBS has acquired new capsule genes by horizontal gene transfer followed by genetic recombination. Recently, Breeding *et al.,* described a short report regarding real-time PCR CPS typing assay for GBS (70). These authors examined a collection of 21 clinical GBS isolates using their real-time PCR assay compared to latex agglutination and found 100% concordance for their assay suggesting the real-time PCR assay performs well. Our algorithm differs in that the presence of capsule is also determined phenotypically as well as assignment of CPS type by real-time PCR. Nonetheless, the assay described by Breeding *et al.,* and our assay algorithm, demonstrate the strength of the real-time PCR for GBS CPS typing.

In summary, I propose a new GBS typing system for which the first step is to confirm capsule expression using sialo-lectin assays followed by duplex real-time PCR typing that identifies the cps genotype. The combination of sialo-CPS lectin binding and duplex real-time PCR typing assays provides a simple and reliable tool for CPS expression confirmation and GBS CPS typing, respectively. This sensitive and specific method enables the characterization of GBS CPSs Ia, Ib, II-VIII, thereby reducing the rate of detection of NT isolates. It is therefore particularly well adapted for GBS CPS typing in large-scale epidemiological studies.

**Table3.1. Strains used in study.**

| Strain type | Designation | Source or reference |
|---|---|---|
| **GBS** | | |
| **Ia** | A909 | (71) |
| **Ib** | NCS4 | (72) |
| **II** | NCS6 | (72) |
| **III** | COH1 | (73) |
| **IV** | NCS11 | (72) |
| **V** | NCS13 | (72) |
| **VI** | NCS14 | (72) |
| **VII** | 7271 | (74) |
| **VIII** | JM9 | (75) |
| **IX** | ATCC27412 | |
| ***S. pneumoniae* serotype 14** | 10SR3072 | This study |
| ***E. coli*** | ATCC25922 | This study |

**Table 3.2a. Primer pairs used in the singleplex and multiplex real-time PCR assays .**

| Identifier | CPS type | Sequence (5'-3') | Duplex Reaction |
|---|---|---|---|
| **Primers** | | | |
| **cpsO-F** | V | AACAGAGGCCAATCAGTTGCA | 1 |
| **cpsO-R** | | CGGCATTGGTAGCTTTCTGTATG | |
| **cpsI-F** | VI | TTCACCTTCTGCCATCTCAA | 1 |
| **cpsI-R** | | AAGGGATAGTCGCGTAAAAGTC | |
| **cpsG-F** | III | AAACGGGTTACTCAGACTTCG | 2 |
| **cpsG-R** | | TCACCAAACTGCTTTCTCCTAG | |
| **cpsK-F** | II | GCTATTCCCTACATGGAAGATGG | 2 |
| **cpsK-R** | | TTACTGAAGCCATGATATCGGG | |
| **cpsM-F** | VII | CCTTTGAGAGTTCATAACTGTT | 3 |
| **cpsM-R** | | GTCCTCTAATTGCACCAATAAT | |
| **cpsR-F** | VIII | CCAGATGGGCATGAGTGGTTAC | 3 |
| **cpsR-R** | | CAGTCCCATAGGCGATGTAGG | |
| **cpsN-F** | IV | GTATGCTTTCGTGTCTGATTATGC | 4 |
| **cpsN-R** | | ATTGATCCAAAACCCAAACCTG | |
| **cpsH-F** | Ia | TTAATTTGCGATCCGGGAGTAG | 4 |
| **cpsH-R** | | GCAGGCCACTTTTGTAGAAATAG | |
| **cpsJ-F** | Ib | TGGGATATAGAGATTTAGTACCTGTTG | 5 |
| **cpsJ-R** | | ATTGGTTTGTGATATTCCATTCTCG | |
| **cpsO-F** | IX | CTGATGATCTTTGTTCGCCATTT | 5 |
| **cpsO-R** | | ACAAGGGTGATCCTCAATTCC | |

**Table 3.2b. Probes used in the singleplex and multiplex real-time PCR assays .**

| Probes identifier | CPS type | Sequence (5'-3') | Duplex Reaction |
|---|---|---|---|
| **cpsO** | V | 5' cal fluor 560-CAACGGAGTACTTAGGTGTACAGGAGA-3'BHQ1 | 1 |
| **cpsI** | VI | 5'FAM-ACAATGGAGGTGCATCATCAGCA-3'BHQ1 | 1 |
| **cpsG** | III | 5' cal fluor 560-TCTTGTCACAAAGACCATCTGGAGCG3'BHQ1 | 2 |
| **cpsK** | II | 5'FAM-ATTGTTATCACACATGGCGGCCC-3'BHQ1 | 2 |
| **cpsM** | VII | 5' cal fluor 540-CTAGGGAGTTAAGTTATGATGTGA-3'BHQ1 | 3 |
| **cpsR** | VIII | 5'FAM-TGGAGTATTCCTGTACGTCGCTATTGGA-3'BHQ1 | 3 |
| **cpsN** | IV | 5' cal fluor 560-CCTCTCCAGGTAGCTCACAAGCAAA-3'BHQ1 | 4 |
| **cpsH** | Ia | 5'FAM-TTGAATGCGACCCCAAAGGGAGA-3'BHQ1 | 4 |
| **cpsJ** | Ib | 5' FAM-CCGATTTTGAAATCAGCCAGAGCTCCT-3'BHQ1 | 5 |
| **cpsO** | IX | 5' cal fluor CAACATGAAACTGGTGCTGACCTTGT 3'BHQ1 | 5 |

**Table 3.3. Efficiency calculated for primers for real-time PCR GBS CPS typing**

| cps gene target | efficiency |
|---|---|
| *cpsH* | 92.60% |
| *cpsK* | 99.87% |
| *cpsJ* | 90.94% |
| *cpsG* | 92.31% |
| *cpsN* | 99.79% |
| *cpsO*-V | 98.56% |
| *cpsI* | 96.14% |
| *cpsM* | 91.84% |
| *cpsR* | 98.60% |
| *cpsO*-IX | 96.68% |

**Table 3.4. Singleplex real-time PCR for GBS typing.**

| | Mean $C_{T \pm SD}$ cps type [a] | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Gene target | Ia | Ib | II | III | IV | V | VI | VII | VIII | IX |
| *cpsH* | 23.9±0.2 | - | - | - | - | - | - | - | - | - |
| *cpsJ* | - | 24.1±0.5 | - | - | - | - | - | - | - | - |
| *cpsK* | - | - | 26.8±1.5 | - | - | - | - | - | - | - |
| *cpsG* | - | - | - | 18.4±1.6 | - | 23.2±2.2 | 26.4±2.7 | - | - | - |
| *cpsN* | - | - | - | - | 18.5±0.0 | - | - | - | - | - |
| *cpsO*-V | - | - | - | - | - | 20.0±1.4 | - | - | - | - |
| *cpsI* | - | - | - | - | - | - | 21.5±0.6 | - | - | - |
| *cpsM* | - | - | - | - | - | - | - | 26.2±2.3 | - | - |
| *cpsR* | - | - | - | - | - | - | - | - | 23.5±1.1 | - |
| *cpaO*-IX | | | | | | | | | | 17.6±0.1 |

[a]Data are expressed as mean CT values for at least three independent experiments.

**Table 3.5. Duplex real-time PCR for GBS CPS typing[a].**

| Reaction | cps genes-cps type | CPS type | Mean $C_T$ ± SD |
|---|---|---|---|
| 1 | *cpsO*-type V | V | 18.04 ± 1.33 |
| 1 | *cpsI*-type VI | VI | 18.07 ± 0.07 |
| 2 | *cpsG*-type III | III | 16.77 ± 0.70 |
| 2 | *cpsK*-type II | II | 24.88 ± 0.50 |
| 3 | *cpsM*-type VII | VII | 19.70 ± 1.34 |
| 3 | *cpsR*-type VIII | VIII | 22.44 ± 0.22 |
| 4 | *cpsH*-type Ia | Ia | 24.55 ± 0.04 |
| 4 | *cpsN*-type IV | IV | 18.12 ± 0.02 |
| 5 | *cpsJ*-type Ib | Ib | 19.97± 1.41 |
| 5 | *cpsO*-type IX | IX | 17.84±0.06 |

[a] Data are expressed as mean of CT with SD for at least three independent experiments

**Table 3.6. Comparison of typing results obtained with the immunodiffusion assay (the serological method) and the duplex real-time PCR assay using a collection of GBS clinical strains.**

| CPS type determined by PCR | Results for CPS type determined by immunediffusion assay | | | | | | | | | | Concordance % |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ia | Ib | II | III | IV | V | VI | VIII | IX[a] | Total | |
| **Ia** | 8/8 | | | | | | | | | 8 | 100 |
| **Ib** | | 6/8 | | | | | | | | 6 | 75 |
| **II** | | 2 | 6/6 | | | | | | | 8 | 100 |
| **III** | | | | 10/10 | | | | | | 10 | 100 |
| **IV** | | | | | 8/8 | | | | | 8 | 100 |
| **V** | | | | | | 11/11 | | | | 11 | 100 |
| **VI** | | | | | | | 5/5 | | | 5 | 100 |
| **VIII** | | | | | | | | 4/4 | | 4 | 100 |
| **IX** | | | | | | | | | 10/10 | 10 | 100 |
| Total | 8 | 8 | 6 | 10 | 8 | 11 | 5 | 4 | 10 | 70 | 97.2 |

[a]CPS type IX isolates were typed by previously described PCR assay (40)

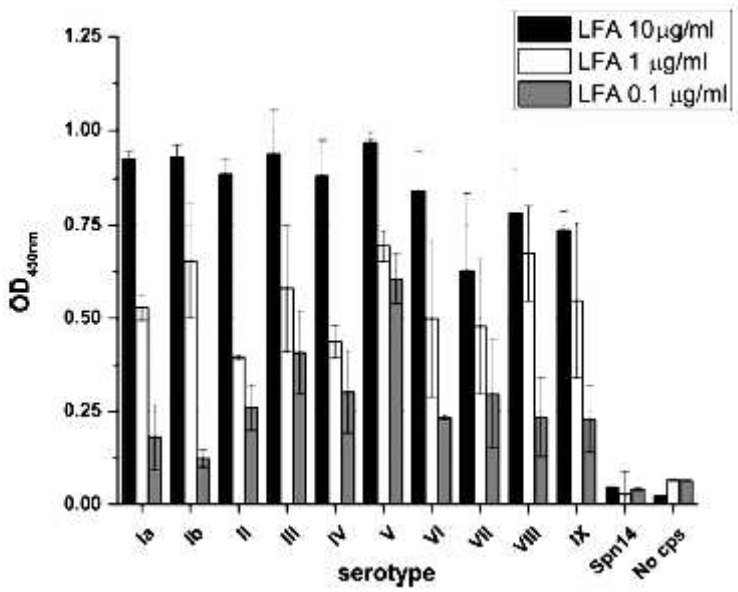



Figure 3.1. Recognition of GBS CPS by the lectin-EIA.

Crude GBS CPS preparations (Ia, Ib, II-IX) were incubated with LFA specific for Neu5Ac. Three concentrations of biotin-LFA lectin were used (10, 1, 0.1 μg/ml) while 1 μg/ml of HRP-labeled streptavidin was used. Data are expressed as mean $OD_{450}$ with SD for at least three independent experiments.

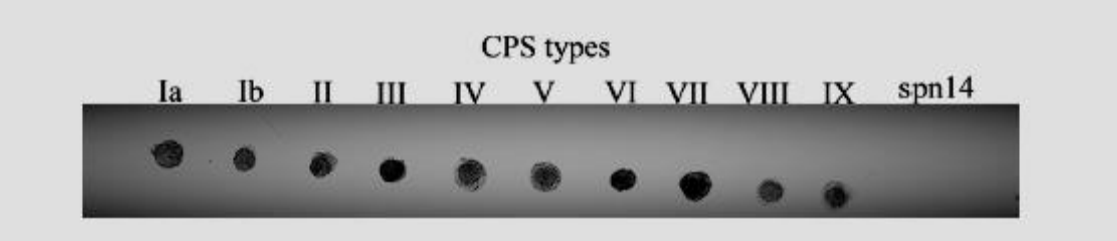



Figure 3.2. Recognition of GBS CPS by dot-ELISA.
GBS types Ia, Ib, II –IX whole bacteria ($10^7$ CFU) were incubated with LFA. Spn14 was used as a negative control.

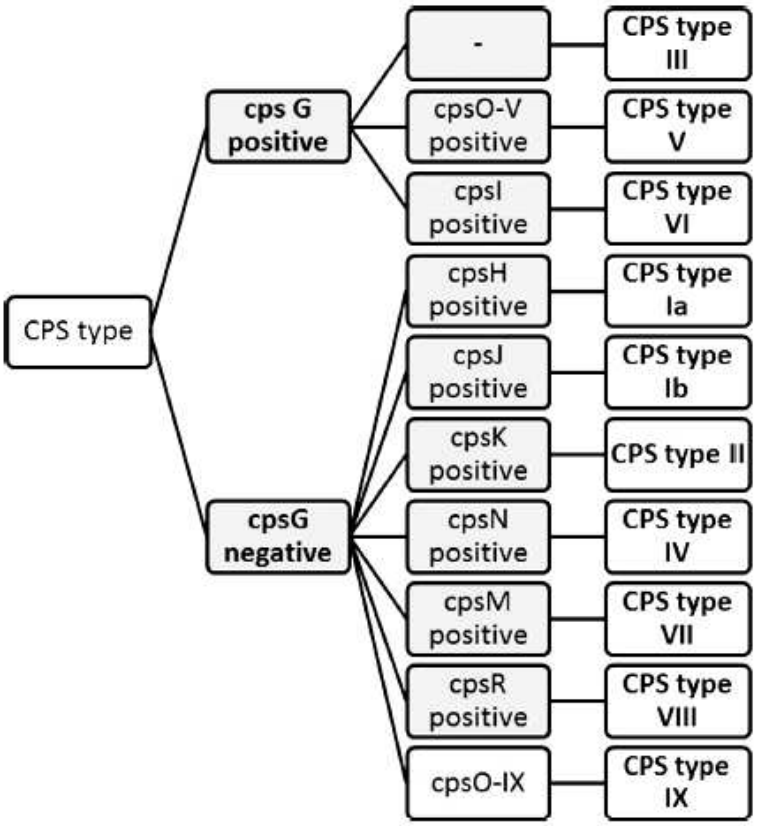



**Figure 3.3. Algorithm to identify GBS CPS types Ia, Ib, II-IX.**

Grey boxes are CPS target genes and White boxes are GBS CPS types

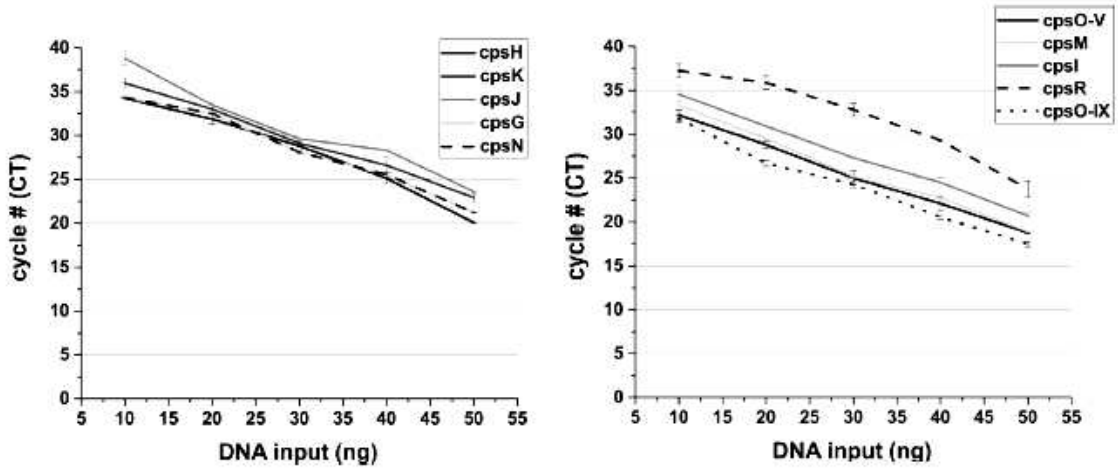



**Figure 3.4. Standard curves and efficiencies calculated for primers for real-time PCR GBS CPS typing.**

Tenfold dilutions of DNA template (10, 20, 30, 40, 50 ng) were plotted against $C_T$ value for each dilution to generate a standard curve. From this standard curve, the smallest amount of DNA detected and $C_T$ slope were determined for the primers cpsH, cpsK, cpsJ, cpsG, cpsN, cpsO-V, cpsI, cpsM, cpsR, and cpsO-IX to identify CPS type Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX respectively. The efficiency values were measured for each primer pair using the $C_T$ slope method. The amplification efficiency was calculated according to the following equation: $Ex = 10^{(-1/slope)} - 1$, where EX is the efficiency. Data are expressed as mean of $C_T$ with SD for at least three independent experiments.

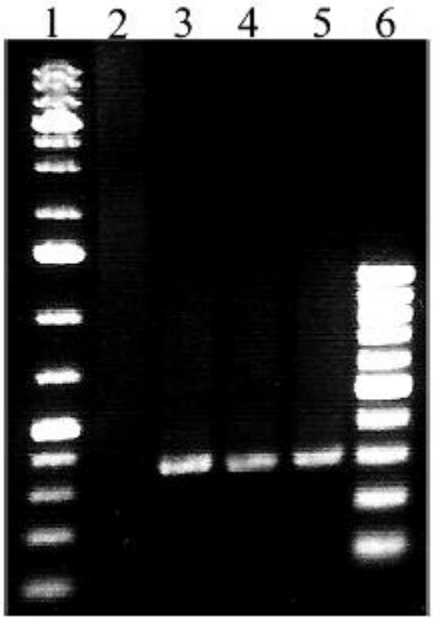



**Figure 3.5. GBS PCR typing of the two isolates that displayed a discrepancy between the immunodiffusion assay (CPS type Ib) and our duplex real-time PCR typing (CPS type II).**
To identify CPS type II a previously described PCR assay (41) was used lanes: 1, 1-kb DNA ladder, 2, CPS type Ib genomic DNA (gDNA), 3, CPS type II gDNA, 4, 04SR421 gDNA , 5, 13SR567 gDNA, and 6, 100 bp DNA ladder.

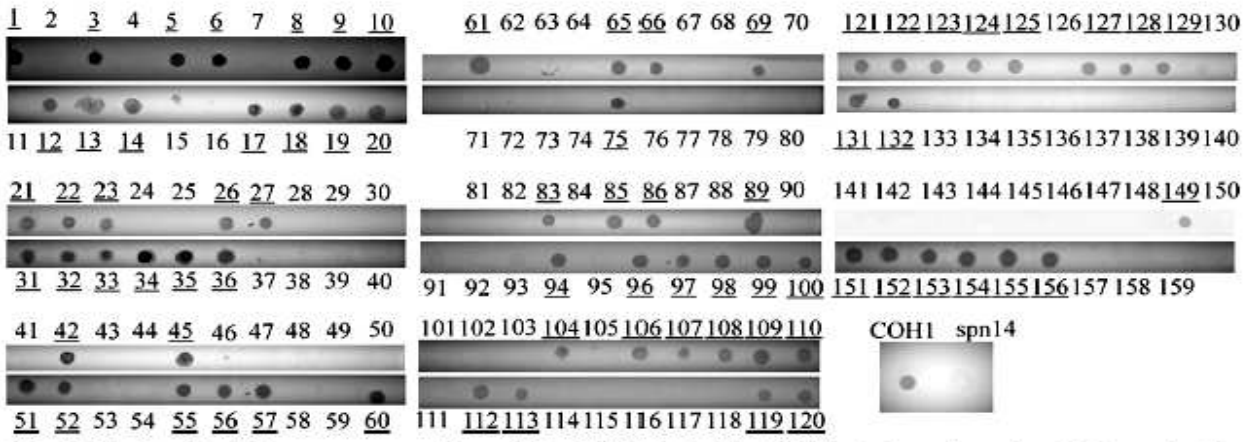



**Figure 3.6. Lectin dot blot assay for GBS isolates that failed to be assigned a CPS type by the immunodiffusion assay (NT isolates).**

Underlined dots are positive for sialic acid. There are 75 positive spots. GBS whole bacteria ($10^7$ CFU) were incubated with LFA, the positive control was the CPS type III GBS strain COHI. The negative control was spn14 isolate.

## 3.5 References


1. **Schuchat A.** 1999. Group B streptococcus. Lancet **353:**51-56.
2. **Baker CJ, Barrett FF.** 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr **83:**919-925.
3. **Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.
4. **Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.
5. **Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol **96:**498-503.
6. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.
7. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.
8. **Doran KS, Nizet V.** 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol **54:**23-31.

9. **Herbert MA, Beveridge CJ, Saunders NJ.** 2004. Bacterial virulence factors in neonatal sepsis: group B streptococcus. Curr Opin Infect Dis **17:**225-229.

10. **Rubens CE, Wessels MR, Kuypers JM, Kasper DL, Weiser JN.** 1990. Molecular analysis of two group B streptococcal virulence factors. Semin Perinatol **14:**22-29.

11. **Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL.** 2007. Serotype IX, a Proposed New Streptococcus agalactiae Serotype. J Clin Microbiol **45:**2929-2936.

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

13. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

14. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol doi:10.1128/JCM.03554-13.

15. **Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol doi:10.1128/JCM.00355-16.

16. **Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand **87:**260-271.

17. **Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis **173:**365-373.

18. **Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microb Drug Resist **10:**31-36.

19. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

20. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.

21. **Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A.** 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis **177:**998-1002.

22. **Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.

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

24. **Ferrieri P, Lynfield R, Creti R, Flores AE.** 2013. Serotype IV and invasive group B Streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis **19:**551-558.

25. **Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B.** 2011. Emergence of group B Streptococcus serotype IV in women of child-bearing age in Ireland. Epidemiol Infect **139:**236-238.

26. **Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P.** 2010. Clonal analysis of colonizing group B Streptococcus, serotype IV, an emerging pathogen in the United States. J Clin Microbiol **48:**3100-3104.

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

28. **Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba

and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

29. **Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nat Rev Microbiol **4:**932-942.

30. **Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

31. **Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179.

32. **Slotved HC, Elliott J, Thompson T, Konradsen HB.** 2003. Latex assay for serotyping of group B Streptococcus isolates. J Clin Microbiol **41:**4445-4447.

33. **Park CJ, Vandel NM, Ruprai DK, Martin EA, Gates KM, Coker D.** 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. J Clin Microbiol **39:**408-409.

34. **Håkansson S, Burman LG, Henrichsen J, Holm SE.** 1992. Novel coagglutination method for serotyping group B streptococci. J Clin Microbiol **30:**3268-3269.

35. **Arakere G, Flores AE, Ferrieri P, Frasch CE.** 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. J Clin Microbiol **37:**2564-2567.

36. **Kong F, Gowan S, Martin D, James G, Gilbert GL.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol **40:**216-226.

37. **Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL.** 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol **46:**2745-2750.

38. **Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae using multiplex PCR and reverse line blot hybridization. J Med Microbiol **54:**1133-1138.

39. **Sellin M, Olofsson C, Håkansson S, Norgren M.** 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol **38:**3420-3428.

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

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

42. **Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

43. **Chaffin DO, Mentele LM, Rubens CE.** 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol **187:**4615-4626.

44. **Kolkman MA, Morrison DA, Van Der Zeijst BA, Nuijten PJ.** 1996. The capsule polysaccharide synthesis locus of streptococcus pneumoniae serotype 14: Identification of the glycosyl transferase gene cps14E. J Bacteriol **178:**3736-3741.

45. **Kolkman MA, Wakarchuk W, Nuijten PJ, van der Zeijst BA.** 1997. Capsular polysaccharide synthesis in Streptococcus pneumoniae serotype 14: molecular analysis of the complete cps locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit. Mol Microbiol **26:**197-208.

46. **Facklam RR, Padula JF, Wortham EC, Cooksey RC, Rountree HA.** 1979. Presumptive identification of group A, B, and D streptococci on agar plate media. J Clin Microbiol **9:**665-672.

47. **Ke D, Ménard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, Bergeron MG.** 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem **46:**324-331.

48. **Calandra GB, Cole RM.** 1980. Lysis and protoplast formation of group B streptococci by mutanolysin. Infect Immun **28:**1033-1037.

49. **Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional analysis in type Ia group B Streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J Biol Chem **276:**139-146.

50. **Wessels MR, Benedí WJ, Jennings HJ, Michon F, DiFabio JL, Kasper DL.** 1989. Isolation and characterization of type IV group B Streptococcus capsular polysaccharide. Infect Immun **57:**1089-1094.

51. **Rogerieux F, Belaise M, Terzidis-Trabelsi H, Greffard A, Pilatte Y, Lambré CR.** 1993. Determination of the sialic acid linkage specificity of sialidases using lectins in a solid phase assay. Anal Biochem **211:**200-204.

52. **Duk M, Lisowska E, Wu JH, Wu AM.** 1994. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Anal Biochem **221:**266-272.

53. **Diamandis EP, Christopoulos TK.** 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem **37:**625-636.

54. **Edgar RC.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.

55. **Knibbs RN, Osborne SE, Glick GD, Goldstein IJ.** 1993. Binding determinants of the sialic acid-specific lectin from the slug Limax flavus. J Biol Chem **268:**18524-18531.

56. **Wasylnka JA, Simmer MI, Moore MM.** 2001. Differences in sialic acid density in pathogenic and non-pathogenic Aspergillus species. Microbiology **147:**869-877.

57. **Imberty A, Gautier C, Lescar J, Pérez S, Wyns L, Loris R.** 2000. An unusual carbohydrate binding site revealed by the structures of two Maackia amurensis lectins complexed with sialic acid-containing oligosaccharides. J Biol Chem **275:**17541-17548.

58. **Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ.** 1987. The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem **262:**1596-1601.

59. **Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P,**

**Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M.** 2012. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis **31:**2097-2104.

60. **Wessels MR, Haft RF, Heggen LM, Rubens CE.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun **60:**392-400.

61. **Geisler C, Jarvis DL.** 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology **21:**988-993.

62. **Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

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

64. **Yao K, Poulsen K, Maione D, Rinaudo CD, Baldassarri L, Telford JL, Sorensen UB, Kilian M.** 2013. Capsular gene typing of Streptococcus agalactiae compared to serotyping by latex agglutination. J Clin Microbiol **51:**503-507.

65. **Creti R, Imperi M, Pataracchia M, Alfarone G, Recchia S, Baldassarri L.** 2012. Identification and molecular characterization of a S. agalactiae strain lacking the capsular locus. Eur J Clin Microbiol Infect Dis **31:**233-235.

66. **Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC.** 2006. Identification of novel cps locus polymorphisms in nontypable group B Streptococcus. J Med Microbiol **55:**775-783.

67. **Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae. J Med Microbiol **54:**1133-1138.

68. **Wen L, Wang Q, Li Y, Kong F, Gilbert GL, Cao B, Wang L, Feng L.** 2006. Use of a serotype-specific DNA microarray for identification of group B Streptococcus (Streptococcus agalactiae). J Clin Microbiol **44:**1447-1452.

69. **Brigtsen AK, Dedi L, Melby KK, Holberg-Petersen M, Radtke A, Lyng RV, Andresen LL, Jacobsen AF, Fugelseth D, Whitelaw A.** 2015. Comparison of PCR and serotyping of Group B Streptococcus in pregnant women: the Oslo GBS-study. J Microbiol Methods **108:**31-35.

70. **Breeding KM, Ragipani B, Lee KD, Malik M, Randis TM, Ratner AJ.** 2016. Real-time PCR-based serotyping of Streptococcus agalactiae. Sci Rep **6:**38523.

71. **Madoff LC, Michel JL, Kasper DL.** 1991. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun **59:**204-210.

72. **Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK.** 2002. Binding and invasion of HeLa and MRC-5 cells by Streptococcus agalactiae. Microbiology **148:**3921-3931.

73. **Rubens CE, Heggen LM, Haft RF, Wessels MR.** 1993. Identification of cpsD, a gene essential for type III capsule expression in group B streptococci. Mol Microbiol **8:**843-855.

74. **Perch B, Kjems E, Henrichsen.** 1979. New serotypes of group B streptococci isolated from human sources. J Clin Microbiol **10:**109-110.

75. **Wagner M, Murai T, Wagner B, Günther E, Jelinkova J.** 1994. JM9 strains, a new type of group B streptococci from Japan. Zentralbl Bakteriol **280:**488-496.

# Chapter 4: Phenotypic and molecular analysis of nontypeable Group B streptococci from cases of invasive disease: identification of novel Group B streptococcal capsule gene clusters[a]

[a] A version of this chapter has been published as

**Alhhazmi A, Tyrrell GJ.** 2018. Phenotypic and molecular analysis of nontypeable Group B streptococci: identification of CPSIIa and hybrid CPSIIa/CPSVGroup B streptococcal capsule gene clusters. J Emerg Microb **i**n press.

## 4.1 Introduction

Group B streptococci, GBS, (also referred as *Streptococcus agalactiae*) are a leading cause of invasive infections which can manifest as pneumonia, septicaemia and meningitis in neonates and a serious cause of morbidity and/or mortality in adults with underlying diseases (1-7). GBS produce an array of virulence factors which include a beta hemolysin/cytolysin (beta-h/c) which can cause narrow zone of β-hemolysis on 5% sheep blood agar, a CAMP factor that lyses sheep red blood cells previously sensitized with a sphingomyelinase produced by some *Staphylococcus aureus* strains (8, 9), and a capsular polysaccharide (CPS) of which there are ten types (Ia, Ib, II-IX) (10-13). These 10 types of CPS can either be identified serologically or by various molecular assays. Those GBS that cannot be CPS typed are termed nontypable (NT). The NT phenotype of some GBS isolates may be due to the expression of undetectable amount of CPS by serological methods, lack of CPS expression, or production of uncharacterized capsular polysaccharide for which antibody are not yet available(14).

Functions of CPS include protecting GBS from being killed by host immune cells such as macrophages. It is a key component in the process of biofilm formation in the presence of human plasma (15). Inactivation of CPS biosynthesis gene(s), reduces resistance toward phagocytic killing (29) and inhibiting biofilm formation (15).

GBS CPS of the 10 CPS variants are formed by different arrangements of four component sugars (glucose, galactose, N-acetylglucosamine and sialic acid) into a unique repeating unit. All recognized GBS CPS types have sialic acid attached to their CPS structure (16-19) that can interfere with complement-mediated killing by host immune cells (20, 21). Based on the conservation of sialic acid among all recognized GBS CPS types and the essentiality of its

presence for full capsule biosynthesis and expression (16, 17), sialic acid has been utilized as recognition marker for GBS CPS production (22).

Expression of GBS virulence factors is controlled by the two component system CovR/S. CovR/S is a major global regulatory system that is responsible for modulating the transcription of up to 7% of total GBS genomic genes (23) including genes that encode GBS virulence factors such as β-h/c (24-28), CAMP factor (24), cell surface proteins (24), capsule and surface sialic acid expression (39). Mutations in *covR/S* have been shown to alter the phenotypic expression of these virulence factors (10).

The first objective of this study was to characterize GBS NT sia (-) isolates and identify *covR/S* mutations that may affect GBS virulence phenotypes. The second objective was to characterize sia (+) isolates and identify potential new CPS types among our NT collection using whole genome sequencing.

## 4.2 Materials and methods

### 4.2.1 Bacterial strains, growth conditions, and oligonucleotides

The relevant characteristics of the bacterial strains used in this study are listed in Table 4.1. GBS was cultured in TH broth or Columbia blood agar plates (Dalynn Biologicals, Canada) containing 5% sheep blood or RPMI 1640 (Thermo Fisher Scientific, Canada) as a synthetic medium. GBS liquid cultures were grown in standing filled flasks. All incubations were at 37°C.

### 4.2.2 Assay for Hemolytic Activity

GBS hemolytic activity was assayed as previously described (24). Briefly, an overnight culture ($10^9$ cfu) in TH broth were centrifuged for 5 min at 3000xg, washed twice with phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS. In a 96-well conical-bottom

microtitre plate (MP Biomedicals, USA), 100 µl per well ($10^8$ cfu) of the bacterial resuspension was placed in the first well, and serial twofold dilutions in PBS were performed across the plate, each in a final volume of 100 µl. An equal volume of 1% sheep erythrocytes washed once with PBS (5 min by centrifugation at 3000xg to avoid non-specific red blood cell lysis), and re-suspended in PBS, was then added to each well, and the plate was incubated at 37°C for 60 min. PBS alone and 0.1% SDS were used as negative and positive controls for hemolysis respectively. After incubation, the plates were centrifuged at 3000xg for 10 min to pellet the unlysed red blood cells and GBS, and 100 µl of the supernatant was transferred to a replica plate. Hemoglobin release was assessed by measuring $A_{420}$, and the hemolytic capacity was relative to SDS control (100%). Fold change is measured by dividing the hemolytic capacity of an isolate and the control GBS strain (COHI) (24). All assays were performed in triplicate, repeated three times and the mean values ± standard deviation (SD) is indicated.

### 4.2.3 Assay for orange pigment production

The broth cultures were incubated to the stationary phase of growth (18 h) and then centrifuged. The pellet was spotted onto nitrocellulose membrane (20 µl/spot) using a biodot apparatus (Bio-Rad, USA), which was dried for 30 minutes at room temperature.

### 4.2.4 RNA isolation

Todd Hewitt (TH) broth (20 ml) was inoculated (1:20) with an overnight culture of GBS strains and incubated at 37°C. Exponentially growing cells ($OD_{600}$ 0.3–0.4) were harvested for 2 min at 6000xg at 4°C. The pellets were re-suspended by vortexing in 400 ml of resuspension buffer (12.5 mM Tris, 5 mM EDTA and 10% glucose). The supernatant was transferred to a fresh tube, and 1 ml of Trizol reagent (Thermo Fisher Scientific, Canada) was added. The sample was

incubated for 5 min at room temperature. Total RNA was extracted twice with chloroform-isoamyl alcohol (24:1, v/v) and precipitated in 0.7 volumes of isopropanol. After a washing step with 70% ethanol, the RNA pellet was dissolved in sterile DNase and RNase-free water (Thermo Fisher Scientific, Canada) and treated for 30 min at 37°C with RNase-free DNase I (1 unit per mg of total RNA) in 50 mM Tris-HCl (pH 7.5) and 10 mM $MnCl_2$. DNase was inactivated by phenol-chloroform extraction, and the RNA were precipitated and washed with 70% ethanol, re-dissolved in RNase-free water and quantified by absorbance at 260 and 280 nm. Purity and integrity of RNA were visualized on agarose gels, and RNA was stored at -20°C until use.

### 4.2.5 Detection of mRNA by reverse-transcription real-time PCR

Using a high capacity cDNA reverse transcription kit with RNAse inhibitor (Thermo Fisher Scientific, Canada), 0.8 µg RNA was reversed transcribed as recommend by manufacture. Reverse-transcription real-time PCRs were carried out by using Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Canada). Reactions were carried out in a final volume of 20 µl containing 0.5 µg of RNAs 0.5 µM of each forward and reverse primer (Integrated DNA technology, IDT, USA) for each *cpsE*, *cylE*, *cfb*, and *rpsL* and 10 µl of master mix. The real-time PCR was performed using a Applied Biosystem 7500 Real-Time PCR System. The cycling conditions were denaturation at 95°C for 1 min, followed by 40 cycles of amplification at 95°C for 3 sec, 60°C for 30 sec. The rate of temperature increase was 1°C/sec (or 0.5°C/sec), and fluorescence was acquired once. Each reverse-transcription real-time PCR was repeated three times. The list of oligonucleotides used in this study is indicated in Table 4.2.

### 4.2.6 Genomic DNA Extraction

Genomic DNA extraction was performed as follows. Overnight broth cultures (1.5 ml) were centrifuged for 10 min at 3000xg. Bacterial cells was resuspended in 500 µl of 1X PBS (8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, and 1000 ml $H_2O$ [pH 7.2]) and washed 2 times with PBS. The pellet was used to extract genomic DNA using the Qiagen DNA mericon kit (Qiagen, Germany). Extracted genomic DNA was concentrated and dissolved in 30 µl Qiagen elution buffer or water and stored at -20°C. RNase pre-treatment was done prior to quantification of genomic DNA.

### 4.2.7 PCR amplification and sequencing of *covR* and *covS*

PCR assays that target either *covR* or *covS* based on the sequences from GBS strain 2603V/R (Genbank accession AE009948.1) were developed (Table 4.2). The amplified fragments were sequenced using the same primers, covR-F, covR-R, covSA-F, covSA-R, covSB-F, and covSB-R (Integrated DNA Technology, IDT, USA) (Table 4.2.) used for amplification and compared to the GBS genome (Genbank accession AE009948.1) available in Genbank.

### 4.2.8 Biofilm forming Assay

The biofilm forming assay was performed as described previously (15). Briefly, GBS were grown in TH (Becton Dickinson, USA) broth. Human plasma was collected from a human volunteer. Overnight GBS cultures were used to inoculate RPMI-glucose or RPMI-glucose supplemented with human plasma at an $OD_{600}$ of 0.1, vortexed briefly and 180 µl volumes dispensed in 96 wells plate (MP Biomedicals, USA) and incubated for 18 h at 37°C. The $OD_{600}$ of each culture was measured to ensure that all cells had reached stationary phase with a similar density, and the cells were washed twice in PBS and air-dried for 15 min. Biofilms were stained

with 0.4% crystal violet for 30 min and the wells were washed twice with PBS and air-dried. The bacterial biomass was then rsuspended for quantification in ethanol/acetone (80/20) solution and $OD_{540}$ was measured. When OD values were above 1, 2-fold dilutions were performed for accuracy. The assay was performed in triplicate and at least 3 independent experiments were performed.

### 4.2.9 Human Whole Blood killing Assay

Human whole blood killing assay was performed as described previously (26, 29). Briefly, GBS was grown to early logarithmic phase, washed, and resuspended in PBS. Inocula of $10^3$ CFU in 100 ml were mixed with 300 ml of freshly drawn human blood in heparinised tubes, and incubated for 3 h with agitation at 37°C, and dilutions were plated on blood agar for enumeration of CFU. The survival index was calculated as follows: (CFU at the end of the assay)/(CFU at t = 0 h). The assay was performed in triplicate and repeated two times independently.

### 4.2.10 Whole Genome sequencing

PLGBS16, PLGBS17 and PLGBS18 sia (+) NT isolates were sequenced using a MiSeq instrument with an average sequencing depth of 120X. Library preparation was performed using a tagmentation process. 1 ng genomic DNA as input and the Illumina Nextera XT Library preparation kit (FC-131-1096) as per manufacturer's protocol. Resultant libraries were purified from free primers using Ampure beads (1:0.8 reaction mix:bead ratio). Libraries were qualified using the Agilent Bioanalyzer and quantified using a Qubit fluorimeter and Qubit HS DNA reagents. Libraries were loaded at 10 pM on an Illumina MiSeq v2 reagent kit (MS-102-2003) and processed at 2x250 cycles with 1% PhiX control DNA (FC-110-3001). Sequence data for the

3 genomes have been deposited in the NCBI Short Read Archive database (BioProject PRJNA433769). Illumina reads were assembled to contigs by CLC genomic workbench using *de novo* assembly. The assembled genome is annotated using online annotation websites Rapid Annotation using Subsystem Technology (RAST) (30). Contigs corresponding to the chromosomal region encompassing the cps operon were identified and aligned by MUSCLE using Geneious and by using as query sequence the sequence of strain 18SR21 (GenBank accession AAJO01000077.1) (31) or 2603V/R (GenBank accession AE009948.1) (32) cps region.

#### 4.2.11 Multilocus sequence typing (MLST) assay and assignment to clonal clusters

Multilocus sequence typing (MLST) was carried out as described previously (33). Briefly, the seven housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK*, and *tkt*) were assigned an allele number based on their sequences. Each isolate was assigned a sequence type (ST) based on the allelic profile of the seven amplicons for each strain were grouped into CCs using the eBURST software program (34, 35). eBURST was set at the default setting that identified groups of related STs using the most stringent (conservative) definition. All members assigned to the same group shared identical alleles at six of the seven loci with at least one other member of the group (http://pubmlst.org/sagalactiae/).

#### 4.2.12 Double immunodiffusion assay for GBS CPS typing

CPS typing was performed using the Lancefield heat-acid extraction followed by a double immunodiffusion method as described previously (36, 37) for sia (+) isolates, PLGBS16, PLGBS17, and PLGB18. The immunodiffusion assay of GBS CPS typing used for this study was based on reactions with antisera raised against CPS types Ia, Ib, II, III, IV, V, VI, VII and VIII. The type-specific antisera panel was prepared in rabbits within the laboratory (36, 37).

### 4.2.13 CPS Dot Blot Assay

A dot blot assay to detect CPS was performed as previously described with minor modifications (38) for the sia (+) NT GBS isolates, PLGBS16, PLGBS17, and PLGB18. Late exponentially growing bacteria were washed in phosphate-buffered saline (PBS) and re-suspended in PBS to give an optical density at 600 nm of approximately 2 (Beckman Coulter, UK). The bacterial suspension was spotted onto nitrocellulose membrane (20 µl/spot) using a bio-dot apparatus (Bio-Rad, USA), which was dried for 30 min at room temperature. The membranes were washed for 15 min with TBS (6.05 g Tris, 8.76 g NaCl in 1000 mL of $H_2O$. [pH 7.5]), incubated with blocking buffer (5% skim milk, 0.1% Tween 20 in TBS) for 60 min at 37°C. The membrane was subsequently washed with TBS for 15 min. CPS was detected using specific rabbit polyclonal antibodies raised against CPSII (in-house preparation), CPSIII or CPSV (Statens Serum Institute, Denmark) at 1:1000 dilution. The secondary horseradish peroxidase–coupled anti-rabbit secondary antibody (Promega, Canada) was used at 1:50000 dilution. The membrane was washed 3× 15 min with TBST and 2× 15min with TBS before developing with SigmaFast BCIP/NBT (Sigma-Aldrich, USA) for approximately 5 min. Development was stopped using 3 changes of distilled water.

## 4.3 Results

### 4.3.1 Characterization of GBS sia (-) NT isolates

GBS isolates without an identifiable serotype by serological based typing assays are considered NT isolates. We reported previously that 9% (159/ 1683 isolates) of GBS isolates in Alberta, Canada, collected from patients with invasive diseases between 2003-2013 were identified as NT (39) and of those, 52.8% (84/159 isolates) were sialic acid negative sia (-) (22).

The sia (-) phenotype in NT GBS isolates suggests loss of cps expression as sialic acid is present on all known GBS cps types.

The expression of GBS CPS is regulated by the CovR/S system (11). Inactivation of the *covR/S* genes has been shown to result in capsule and surface sialic acid expression loss suggesting that the NT GBS isolates that were sia (-) in our collection may have genetic mutations in the *covR/S* genes resulting in a loss of CPS expression (11, 13, 14). Knowing that CovR/S also regulates other phenotypic properties, we decided to assay for changes in beta hemolysis, CAMP factor activity and inability to grow on minimally defined media in the NT sia (-) GBS to allow us to potentially identify CovR/S mutants. All of these phenotypes are regulated by CovR/S (11-15).

#### 4.3.1.1 Phenotypic differences between GBS NT sia(-) isolates and GBS wild type strain COHI

Analysis of  hemolytic activity in our collection of sia(-) isolates found that 15 sia(-) NT isolates (18%) exhibited increased beta hemolytic activity on 5% sheep blood agar compared to the positive control GBS isolate COH1 (Figure 4.1). These were designated PLGBS1 to 15. The increase in the hemolytic activity on sheep blood agar plates was confirmed by a microtitre hemolysis assay which showed an average fold increase of 3.5X (±0.8) (ranged from 2.6-4.36) over the control GBS strain COHI (Table 4.3). In addition to the increased hemolytic activity, these 15 sia (-) isolates visually displayed greater orange pigmentation vs control (GBS strain COHI) as shown in culture pellets (Figure 4.2). This is expected as beta hemolysis and orange pigment production are caused by the same ornithine rhamnolipid (15). This data suggested that these sia (-) isolates may harbor mutations in their *covR/S* genes. Based on the enhanced

beta hemolysis which suggested possible CovR/S changes, we focussed attention on only these 15 sia(-) NT GBS isolates in our collection of sia(-) isolates for subsequent analysis.

To confirm that the enhanced hemolytic activity and increase in orange pigmentation are reflected at the expression level in those isolates, we assayed *cylE* transcription. *cylE* (a gene encoded in *cyl* operon) is essential for GBS beta-h/c production/orange pigmentation. Reverse-transcription real-time PCR using primers specific for the *cylE* gene revealed increases in *cylE* mRNA production in all sia(-) isolates assayed (average fold increase of 4.3X ± 2.3) (Table 4.4). This suggested the enhanced beta hemolysis/orange pigment was due to increases in *cyl* operon transcription.

Assays for CAMP factor activity showed that these 15 isolates displayed no reaction or weaker CAMP activity on 5% sheep blood than the control isolate (COHI) (Figure 4.3). Also, reverse-transcription real-time PCR with primers specific for *cfb*, the gene responsible for the CAMP factor phenotype (40), showed a reduction in *cfb* mRNA transcript in comparison to the control (Table 4.4).

In addition to beta-h/c activity/orange pigmentation production, and CAMP activity, we assayed the growth rates of the 15 sia (-) isolates in comparison to the GBS control isolate COHI in TH, (an enriched medium), and RPMI, (a minimal defined medium). In TH broth, the 15 sia (-) isolates displayed a faster growth rate compared to the control strain, with average division time of 57 minutes (±0.01) compared to 64 minutes for COH1 (Figure 4.4). In RPMI, all 15 sia(-) isolates with increased beta-h/c activity were unable to grow in RPMI unlike the control which reached an average OD of 0.5 (±0.06) after 16 hrs of incubation.

To further demonstrate that the 15 sia (-) isolates did not produce polysaccharide capsule, we assayed for *cpsE* gene transcription. *cpsE* is a gene encoding a glycosyltransferase that initiates the biosynthesis of polysaccharide repeating units contained in the capsule (41). Loss of *cpsE* gene expression can lead to lack of CPS expression. It was found that *cpsE* transcription was downregulated to very low levels in all sia(-) isolates in comparison to the COH1 control (Table 4.4). This suggested the decrease in expression of the *cpsE* gene has led to loss of capsule expression in these GBS isolates.

#### 4.3.1.2 Reduction in biofilm formation of the 15 sia(-)isolates

Previous research has shown that GBS cps plays a major role in biofilm formation which is important for GBS persistence and pathogenicity (15). I hypothesized that the sia(-) isolates in our collection would display a reduced ability to form biofilm. The unencapsulated and asialo mutants (negative controls) were impaired in their ability to form biofilm with an average absorbance at 600 nm of 0.2 (±0.09) and 0.1 (±0.03), respectively (Figure 4.5). The positive control, COHI, was able to form biofilm with an average absorbance at 540 nm of 0.695 (±0.002). GBS isolates that were sia(-) were unable to form biofilm having an average absorbance of 0.1 (±0.02;p >0.001) (Figure 4.5)

#### 4.3.1.3 Loss of resistance toward phagocytic killing by the 15 sia (-) isolates

It has been previously demonstrated that GBS CPS inhibits complement deposition, thereby reducing oposonophagocytotic clearance and contributing to immune resistance (21). In addition, GBS isolates that display low hemolytic activity and high CPS expression have an increased resistance toward host immune defense. In contrast, isolates that exhibited high hemolytic activity and low capsule expression were phagocytosed in an oposonophagocytotic

assay (24, 26). Based on these observations, we hypothesized that sia (-) isolates may be more susceptible to host immune defenses. To determine this, the survival of these GBS isolates in a human whole-blood killing assay was assessed (26, 29). The 15 sia (-) isolates displayed a low survival index with an average value of 1.5 (±0.4, p>0.001) (ranged from 0.8-2) compared to the positive control (survival index value of 3.8 [±0.3]). The negative controls, the unencapsulated and asialo GBS mutants, had low survival indices of 1.1 (±0.6) and 1.6 (±0.1), respectively (Figure 4.6)

#### 4.3.1.4 Genetic analysis of the covR/covS genes of sia(-) NT isolates with increased β-h/c activity

Enhanced β-h/c activity increased orange pigment production, loss of CAMP activity, inability to grow in minimal essential media and loss of capsule expression strongly suggested mutations in the *covR/S* two component regulatory system had occurred (24-28). To verify whether the changes in the phenotypes of the 15 sia (-) isolates in this study was due to mutations that could potentially alter the amino acid coding sequence of CovR or CovS, the *covR* and *covS* genes were sequenced.

DNA sequencing of *covR* and *covS* genes identified mutations in the *covR* and *covS* sequences leading to the predicted amino acid changes shown in Table 4.5 for 14 of the sia (-) isolates. Eight of the 15 sia (-) isolates displayed mutations that predicted protein truncations of CovR (PLGBS2, 5, 8, 10, 11, 12, 13, 15). Only 3 sia (-) isolates showed DNA sequence mutations in *covS* (PLGBS4, 12 and 14). Also, the DNA sequence of *covS* for PLGBS14 predicted an amino acid substitution of 21 amino acids in CovS. The remaining DNA sequence mutations predict 2 to 4 amino acids mutations in CovR (PLGBS 3, 6, 7, 9) (Table 4.5).

### 4.3.2 Characterization of GBS sia (+) NT isolates

Knowing that the presence of surface sialic acid can predict the presence of polysaccharide capsule, it was unusual to find in our collection 3 GBS isolates (PLGBS16, PLGBS17, and PLGBS18) which failed to react with antisera raised against the nine known CPS types (Ia, Ib, II-VIII) yet were sialic acid positive (22) (Figure 4.7). Additionally, these three isolates could not be genotyped by our previously described real-time PCR typing assay (which include Ia, Ib and II-IX) (22) suggesting novel mechanisms of encapsulation. A polysaccharide stain revealed that these 3 GBS isolates microscopically displayed CPS surrounding the cell wall (Figure 4.8). These observations prompted us to further analyze these 3 unknown polysaccharide capsules.

#### 4.3.2.1 Phenotypic properties of GBS sia(+) NT GBS isolates

PLGBS16, PLGBS17, and PLGBS18 isolates exhibited beta-h/c activity similar to the wild type control, COHI (Figure 4.1), grew as a white colony (Figure 4.2), and displayed enhanced CAMP activity (4.3). Growth rate comparisons in THB (enriched media) and RPMI (minimal media) of the 3 sia (+) NT GBS isolates compared to the control GBS COHI isolate showed that they have similar growth rates with division times of 66 (PLGBS16), 65 (PLGBS17) and 63 (PLGBS18) minutes compared to 64 minutes for COHI (Figure 4.4). The growth profile for the 3 isolates in RPMI were similar to the control with an average of OD of 0.55 (±0.12), 0.59 (±0.09) and 0.6 (±0.06), respectively, after 16 hours of incubation.

#### 4.3.2.2 Biofilm formation by sia (+) NT GBS isolates

Based on the important role of GBS capsule in biofilm formation (15), I hypothesized that the 3 sia (+) GBS isolates were able to form biofilm unlike the 15 previously characterized

sia (-) isolates. All 3 isolates were able to form a biofilm in RPMI media containing 20% human plasma and 1% glucose (15, 42-44) (average absorbance at 600nm 0.5, ±0.05) similar to the positive control COHI (0.7, ±0.002) (Figure 4.5). Negative controls, the unencapsulated and asialo mutants, were impaired in their ability to form biofilms with an average absorbance at 600nm of 0.2 (±0.09) and 0.1 (±0.03), respectively (Figure 4.5).

#### 4.3.2.3 Resistance of phagocytic killing by sia(+) NT GBS isolates

To assay resistance to phagocytic killing, the ability of the 3 sia (+) NT GBS isolates to survive in fresh human blood was assayed in a human whole-blood killing assay (26, 29). The 3 isolates displayed high survival indices with an average index of 3.5 (±0.2) similar to the positive control COHI strain (survival index 3.8, ±0.3) (Figure 4.6), indicating that 3 sia(+) isolates were able to resist killing likely due to the presence of the capsule.

#### 4.3.2.4 Genetic analysis of sia (+) NT GBS isolates

As PLGBS16, PLGBS17, and PLGBS18 represented potentially new GBS CPS variant(s), whole genome sequence analyses were done focusing on the MLST and cps gene comparative analysis. MLST data for PLGBS16, PLGBS17, and PLGBS18 were assigned a ST1 for PLGBS16 and PLGBS17 that were included in clonal complex (CC) 1. ST1/CC1 (http://pubmlst.org/sagalactiae/) has been reported to be commonly found among CPSV isolates (45). PLGBS18 was assigned ST2/CC1

To investigate whether isolates PLGBS16, PLGBS17, and PLGBS18 encode novel genes involved in capsular polysaccharide synthesis, we compared the DNA sequences of the *cps* gene cluster of these isolates to the *cps* gene cluster of known CPS types (49). Comparative analysis of the *cps* gene cluster revealed that PLGBS16 and PLGBS18 and the previously sequenced

CPSIIa (18SR21, GenBank accession AAJO01000077.1) shared highly homologous sequences across the *cps* locus (Figure 4.9 and Table 4.6). Poyart *et al.,* (31) have suggested that CPSII is encoded by two *cps* clusters, designated subtypes IIa and IIb represented by 18SR21 and AY375362 cps sequences, respectively. PLGBS16 and PLGBS18 shared low homology with *cps* sequences of CPSIIb (GenBank accession AY375362.1) (Table 4.6). A CPS dot blot assay using in-house rabbit antibody raised against CPSII did not identify these isolates as CPSII, suggesting that the in-house antibody preparation targeted either CPSIIa or IIb only (Figure 4.10). The PLGBS17 isolate shared highly homologous sequences with CPSIIa (18SR21) (31) and CPSV (2603V/R) (32) sequences (Figure 4.9 and Table 4.6). Two well conserved regions spanning from *cpsA* to *cpsF* and from *neuB* to *neuA*, flanking a central region between *cpsG –cpsL* were found in PLGBS17. In the central region, the PLGBS17 isolate showed a high similarity to CPS-determining region of CPS type V including the following genes *cpsG*, *cpsH*, *cpsM*, *cpsN*, *cpsO*, and *cpsL*. It also shared high similarity to CPSIIa-specific genes including a fragment of *cpsG* (228 bp), and *cpsH*, *cpsI*, *cpsQ*, *cpsS*, and a fragment of *cpsL* (725 bp) (Figure 4.9 and Table 4.6). These results suggest that CPS-specific region of PLGBS17 is a hybrid of CPSIIa and V. Further analysis using rabbit polyclonal antibodies raised against CPSII and commercial antibodies against CPSV failed to react in a CPS dot blot assay (Figure 4.10). Together, the data suggested a novel hybrid CPS type composed of CPSIIa and V genes.

## 4.4 Discussion

Capsular polysaccharides expressed by various GBS strains are major virulence factors (46-48). GBS isolates without identifiable CPS type by standard serotyping assays (37, 49-54)

and recent molecular typing methods (31, 47, 55-57) are considered NT. GBS CPS-based vaccine does not cover the NT isolates that can cause invasive disease.

A possible explanation of the NT phenotype of some GBS isolates could be related to inactivation of regulation system(s) that regulates capsule expression in GBS. CovR/S is a major global regulatory system of GBS virulence factors, including capsule expression. To begin to understand the contribution of CovR/S mutants to the burden of invasive NT GBS isolates in our sia (-) NT GBS isolates, we identified sia (-) NT isolates with potential *covR* mutations (15/84, 18%). We screened this collection targeting a number of the distinctive phenotypes associated with *covR* mutations, such as increased hemolysis and orange pigmentation, loss of CAMP activity, fast growth in THB (a rich medium) and no growth in RMPI (a chemically defined minimal medium). Isolates displayed CovR/S mutant phenotypes found to contain mutations in *covR*. Other studies identified mutations in *covR* associated with the aforementioned CovR/S mutant phenotypes (26, 27, 58, 59). Two-CPS–based functions, biofilm formation and antiphagocytic killing ability were also investigated among sia (-) and CovR mutant strains. Associations of the NT phenotype and CovR/S mutations with the loss of biofilm and antiphagocytic killing ability were documented here as described previously with unencapsualted and asialo mutants (29, 41). These results suggest role of *covR* mutations in GBS sia (-) and NT phenotypes. Inactivation of the CovR/S system has been associated with a decrease in the expression of *cps* gene cluster encoding enzymes required for capsular biosynthesis, particularly, *cpsE* (24) and *cpsG* (25), along with a reduction in sialic acid expression on GBS cell surface (39). The loss of surface sialic acid has been described previously to predict the loss of capsule on GBS surface(22). A previous study found that inactivation of

*sivS/R* that belong to a two component system (TCS) in *Streptococcus iniae* reduced the expression of *cpsA* (a gene in *cps* cluster gene) (60). The capsule gene cluster in *S. iniae* has been found to be highly homologous and similar in genetic organization to GBS capsule gene cluster (60). The other 80% of sia (-) NT isolates could be due to mutations in cps biosynthetic genes as reported previously (9, 61). A study by Ramaswamy *et al.,* (9)investigated the underlying reasons behind the NT phenotype of eight clinical GBS isolates. In this study, immunological analyses using concentrated HCL capsule extracts and antisera against GBS recognized CPS types revealed that these isolates expressed a small amount of GBS capsule or no capsule. Sequencing *cps* gene cluster of these isolates demonstrated that CPS biosynthetic genes harbored mutation(s). These mutations involved insertion or deletion in *cpsE* (9, 61), *cpsG* (9, 19, 61), *cpsF* (61) or *cpsA* (41).

The NT phenotype of GBS isolates could also be related to produce uncharacterized capsular polysaccharide for which antibodies not yet are available. Three GBS isolates that were sia (+) and failed to assign *cps* genotype were further investigated using advance genomic analyses. Two GBS isolates PLGBS16 and PLGBS18 were found to belong to CPSIIa. For CPSII, two different *cps*-specific gene sequences have been reported and are available in GenBank (19, 31, 56, 62). It has been thought that there are two subtypes of CPSII designated IIa and IIb as in CPSIa and Ib (31). On the other hand, only one isolate (PLGBS17) was found to be a hybrid between CPSIIa and CPSV. Similarly, it has previously been found that identification of new CPS types among GBS isolates from human occurs with only low frequency (19). GBS has only ten CPS variants whereas *S. pneumonia* comprises 91 serotypes (19). This is due to the fact that *S. pneumonia* has high capacity to acquire foreign DNA through natural competence (19) whereas

the conservation of particular capsular polysaccharide structure in GBS confers a survival advantage in its hosts (19). Identification of isolates with capsule on their surface using sialic acid as marker regardless of their capsule variants allows us to identify a possible new capsule variant. This isolate is a result of GBS having acquired new capsule genes by horizontal gene transfer (HGT) followed by genetic recombination (19). The *cps* gene cluster of PLGBS17 encoded a fragment of *cpsG* (228 bp) and all *cpsH*, *cpsI*, *cpsQ*, *cpsS*, *cpsK* genes, and a fragment of *cpsL* (725 bp) from CPSIIa recombined with *cpsG*, *cpsH*, *cpsN*, *cpsM*, *cpsO* and *cpsK* in CPSV/ST1/CC1 background genome. This isolate was found to be serologically distinct from other known CPS variants, suggesting a possible new CPS variant. It has been previously suggested that the diversity in the existing GBS 10 CPS types has arisen through the introduction of novel DNA sequences, restricted to CPS-specific gene(s) and genetic recombination (18, 19). In a study by Cieslewicz *et al*., (63) the diversity between CPSIa and III occurred in only two genes, the last 60 codons of *cpsG* and *cpsH* gene. *cpsH* conferred capsule specificity between CPSIa and III. The suggested model for CPSIa and III evolution is by HGT when a segment of DNA sequences (*cpsI*, *cpsJ*, and *cpsK*) recombined with a segment of DNA from an unknown source, replacing the 3' of *cpsG* and all of *cpsH* in either type Ia or III (19). Additionally, CPSVI could have arisen from CPSIII by the same mechanism. HGT of a segment of DNA sequences, *cpsJ* and part of *cpsK*, recombined with DNA sequences of *cpsG*, *cpsH*, and *cpsI* from type III (19). CPSIb emerged when *cpsH* and *cpsI* from CPSIa recombined with *cpsJ* and *cpsK* from CPSVI (19). The same scenario has occurred with CPSIX for which a recombination event explained the differentiation between CPSV and IX, specifically, in *cpsM*, *cpsO*, and *cpsI* genes. However, other studies have suggested that capsular switching events in GBS are more

likely to involve the transfer and acquisition of the entire *cps* locus and are not restricted to CPS-specific genes as previously described (38, 64). A study by Neemuchwala *et al*., reported several cases of capsular switching including the entire *cps* gene cluster as a result of recombination events among GBS ST 1 (45). NT strains are often isolated from the colonization stage than the invasive for which it is more likely the entire capsular switching event or CPS-specific gene acquisition occur. This is due to that GBS could have colonized a human gut or an urogenital tract with more than one CPS variants (65). However, this does not mean that GBS isolate with a NT phenotype could not cause invasive GBS infection. GBS isolates with insertional mutation in cps gene cluster have been isolated from invasive adult infection (66). Similarly, 84 isolates in our collection were sia(-) and NT, but caused invasive GBS diseases.

The use of surface sialic acid as universal marker to confirm capsule presence enabled the characterization of GBS NT isolates in to two groups sia (-), indicating capsule loss and sia (+) suggesting new CPS type(s). The association of sia (-) NT isolates and CovR mutants suggests the favorable role of these mutations in affecting capsule expression and the NT phenotypes of these isolates. Genomic analytical methods found one GBS isolate (PLGBS17) that were sia (+) and failed to assign *cps* genotype to encode a hybrid of CPS type V and IIa specific genes. This isolate may represent a potential new CPS type. The identification of the new CPS type provides additional knowledge that can be used for the development of CPS-based vaccine covering all GBS CPS types. The distribution of invasive GBS isolates without capsule suggests that an effective GBS vaccine is combined CPS expressed by all GBS serotypes and a conserved surface protein. This vaccine might offer enhanced protection.

**Table 4.1. Strains Used in the study**

| Strain designation | Genotype or phenotype | Reference or source |
|---|---|---|
| COHI | CPS type III | (67) |
| COHI-II | Emr[a] Tcr[b] Tn9J6AE, asialo[e] CPS III | (17) |
| COHI31-15 | Tcr[b] Rfr[c] Smr[d] Tn916 cap-[f], CPS III | (68) |
| NCS6 | CPS type II | (69) |
| NCS13 | CPS type V | (69) |
| PLGBS1-PLGBS15 | Sia (-) and NT | (22) |
| PLGBS16, PLGBS 17 & PLGBS18 | Sia (+) and NT | (22) |

[a]Emr, erythromycin resistant; [b]Tcr, tetracycline resistant; [c]Rfr, rifampin resistant; [d]Smr, streptomycin resistant; [e]asialo sialic acid negative and [f]cap-, capsule negative.

**Table 4.2. DNA oligonucleeotides used in this study**

| Primers | Sequences (5'-3') | Source |
|---|---|---|
| cpsE-F | GTTTGCTCATATGTGGCATTGT | This study |
| cpsE-R | ATCCTACCATTACGACCTACTCT | This study |
| cfb-F | TTTCACCAGCTGTATTAGAAGTA | (40) |
| cfb-R | GTTCCCTGAACATTATCTTTGAT | (40) |
| cylE-F | CTGAAGCTTCCTTAGAAG | (70) |
| cylE-R | TGCCATTTGGAGAGATAAG | (70) |
| rpsL-F | GGACGTTTAGCACCGTATTTAGAAC | (70) |
| rpsL-R | CCTAAAAAACCTAACTCTGCCCTTC | (70) |
| covR-F | ATGGGTAAAAAGATCTTAATAAT | This study |
| covR-R | TTTTTCACGAATCACATAGCC | This study |
| covS-F | TTATATTTCTTTAGTTTCTTCAAA | This study |
| covS-R | CACGACTTAATGCATCAGAT | This study |

**Table 4.3. The microtitre hemolysis assay for sia (-) NT isolates**

| GBS designation | Hemolytic capacity[a] mean % (SD) | Fold change[b] |
|---|---|---|
| PLGBS1 | 81.5 (±0.1) | 3.7 |
| PLGBS2 | 58 (±0.08) | 2.6 |
| PLGBS3 | 85.5 (±0.02) | 3.9 |
| PLGBS4 | 78 (±0.2) | 3.5 |
| PLGBS5 | 96.1(±0.3) | 4.4 |
| PLGBS6 | 83.7 (±0.4) | 3.8 |
| PLGBS7 | 73.4 (±0.1) | 3.3 |
| PLGBS8 | 74.8 (±0.04) | 3.4 |
| PLGBS9 | 76.3 (±0.1) | 3.5 |
| PLGBS10 | 94.9 (±0.1) | 4.3 |
| PLGBS11 | 67 (±0.1) | 3.0 |
| PLGBS12 | 58.1 (±0.5) | 2.6 |
| PLGBS13 | 74 (±0.01) | 3.4 |
| PLGBS14 | 94.7 (±0.2) | 4.3 |
| PLGBS15 | 93.1 (±0.03) | 4.2 |

[a]Hemolytic capacity: The results were related to 0.1 % SDS (100%).

[b] Fold change is measured by dividing the hemolytic capacity of an isolate and the control GBS strain (COHI) (24).

**Table 4.4. Expression of the messenger RNA of *cylE*, *cfb*, and *cpsE* genes for GBS sia (-) NT isolates**

| GBS designation | *cylE* | *cpsE* | *cfb* |
|---|---|---|---|
| | Fold change in mRNA expression[a] mean (SD) | | |
| PLGBS1 | 2 (±0.4) | 0.04 (±0.03) | 0.6 (±0.7) |
| PLGBS2 | 2.1 (±0.8) | 0.12 (±0.05) | 0.1 (±0.04) |
| PLGBS3 | 6.4 (±1.3) | 0.42 (±0.5) | 0.1 (±0.02) |
| PLGBS4 | 2.2 (±0.8) | 0.03 (±0.03) | 0.1 (±0.1) |
| PLGBS5 | 8 (±1) | 0.03 (±0.01) | 0.1 (±0) |
| PLGBS6 | 4.6 (±0.3) | 0.01 (±0) | 0.1 (±0) |
| PLGBS7 | 3.1 (±1.2) | 0.01 (±0) | 0.03 (±0) |
| PLGBS8 | 2.2 (±0.5) | 0 (±0) | 0.1 (±0.2) |
| PLGBS9 | 2.5 (±0.3) | 0.04(±0.01) | 0.1 (±0.04) |
| PLGBS10 | 5.1 (±3.7) | 0.03 (±0.03) | 0.7 (±0.03) |
| PLGBS11 | 2.1 (±0.2) | 0.04 (±0.04) | 0.1 (±0.1) |
| PLGBS12 | 7.4 (±1.9) | 0.03 (±0.01) | 0.2 (±0.24) |
| PLGBS13 | 2.4 (±0.2) | 0.04 (±0.02) | 0.1 (±0) |
| PLGBS14 | 7.4 (±0.1) | 0.02 (±0.1) | 0.03 (±0.01) |
| PLGBS15 | 6.2 (±1.8) | 0.04 (±0.02) | 0.1 (±0.01) |
| COHI | 1 | 1 | 1 |

[a] $C_T$ differences between the tested genes and the housekeeping genes (*rpsL*) in sia (-) NT isolates and the positive control COHI wer calculated. These are $\Delta C_T$ values for the tested genes ($\Delta C_T E$) and the control ($\Delta C_T C$) conditions. The Double Delta (dd) $C_T$ Value (ddCT) was calculated which equals the difference between ΔCTE and ΔCTC (ΔCTE-ΔCTC). Then, fold change was calculated based on log2-ddCT. The average and standard deviation of the fold change were calculated from three independent experiments.

**Table 4.5. The predicted amino acid changes in CovR and CovS based on nucleotide mutation(s) in the *covR* and *covS* genes identified among 15 sia(-) NT GBS isolates.**

| Isolate designation | Predicted amino acid changes in CovR/S |
|---|---|
| PLGBS1 | No *covR* or *covS* mutations detected therefore no changes |
| PLGBS2 | CovR: truncation at amino acid 2 |
| PLGBS3 | CovR: Leu20Phe, Glu21Gly |
| PLGBS4 | CovS: Val1Ile, Met185Thr, Leu225Phe |
| PLGBS5 | CovR: truncation at amino acid 2 |
| PLGBS6 | CovR: Asp210Glu, Ile215Leu |
| PLGBS7 | CovR: Ile209Met, Ile211Leu |
| PLGBS8 | CovR: truncation at amino acid 2 |
| PLGBS9 | CovR: Leu19Ser, Leu20Val |
| PLGBS10 | CovR: truncation at amino acid 2 |
| PLGBS11 | CovR: truncation at amino acid 2 |
| PLGBS12 | CovR: truncation at amino acid 2<br>CovS: Val333Met |
| PLGBS13 | CovR: Ile4Asp, Ile6Asn, Ile7Asn, Glu8Gly, Asp9Arg and truncation at amino acid 10 |
| PLGBS14 | CovS: substitution of amino acids 253 to 274 with Asp-Val-Ala-Val-Val-Lys-Gly-His-Ile-Gly-Leu-Leu-Gln-Arg-Trp-Gly-Lys-Asp-Asp-Pro-Asp |
| PLGBS15 | CovR: truncation at amino acid 2 |

**Table 4.6. Nucleotide sequence identity comparisons for individual CPS-determining genes between the three sia (+) NT GBS isolates (PLGBS16, PLGBS17, and PLGBS18) and control strains (18SR21 CPS type IIa, AY375362 CPS type IIb and AE009948 CPS type V)**

| **18SR21** | ***cpsG%*** | ***cpsH%*** | ***cpsI%*** | ***cpsJ%*** | ***cpsP%*** | ***cpsQ%*** | ***cpsK%*** | ***cpsL%*** |
|---|---|---|---|---|---|---|---|---|
| **Type IIa** | | | | | | | | |
| **PLGBS 16** | 100 | 100 | 100 | 99.9 | 61.5 | 100 | 100 | 100 |
| **PLGBS 17** | 100 | 100 | 100 | 99.9 | 100 | 100 | 100 | 98.2 |
| **PLGBS 18** | 100 | 100 | 100 | 99.9 | 99.9 | 100 | 100 | 100 |
| **AY375362** | ***cpsG %*** | ***cpsH %*** | ***cpsI %*** | ***cpsJ%*** | ***cpsP%*** | ***cpsQ%*** | ***cpsK%*** | ***cpsL%*** |
| **Type IIb** | | | | | | | | |
| **PLGBS 16** | 73.7 | 47.2 | 56.5 | 49.7 | absent | absent | 86.4 | 99.9 |
| **PLGBS 17** | 30.3 | 47.2 | 56.5 | 49.7 | absent | absent | 86.4 | 98.1 |
| **PLGBS 18** | 73.7 | 47.2 | 56.5 | 49.7 | absent | absent | 86.4 | 99.9 |
| **AE009948** | ***cpsG%*** | ***cpsH%*** | ***cpsJ%*** | ***cpsM%*** | ***cpsN%*** | ***cpsO%*** | ***cpsK%*** | ***cpsL%*** |
| **Type V** | | | | | | | | |
| **PLGBS 17** | 100 | 100 | 100 | 100 | 100 | 100 | 100 | 58.2 |

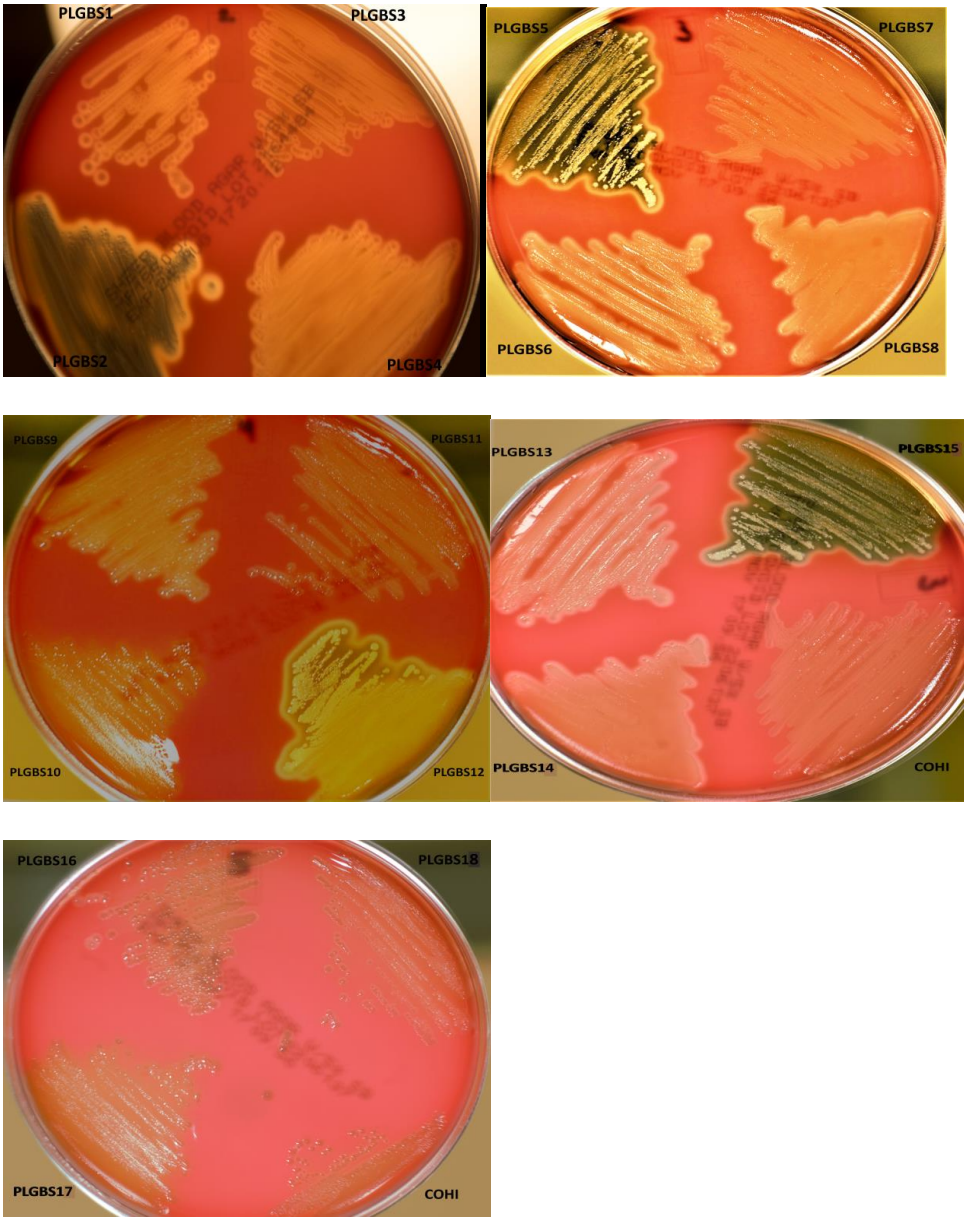



**Figure 4.1. Hemolytic activity of sia (-) NT and sia (+) NT isolates on blood agar with 5% sheep blood.**

Bacteria were streaked onto a 5% sheep blood agar plate and incubated for 24 h at 37°C. The GBS COHI isolate was included as control which displayed a narrow zone of hemolysis.

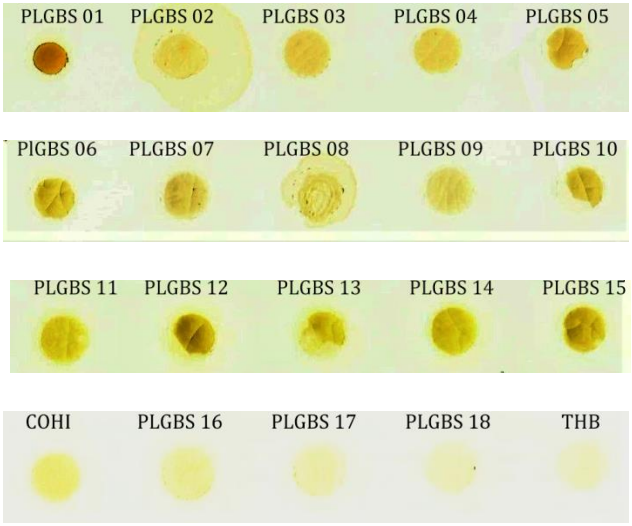



**Figure 4.2. Orange pigmentation of sia (-) NT and sia (+) NT isolates.**

In TH broth, a rich medium, late stationary phase cultures of sia(-)NT isolates were spotted on nitrocellulose membrane using bio-dot apparatus. GBS COHI was a control; and sia (+) NT isolates (PLGBS16, PLGBS17, and PLGBS18) displayed yellowish spots as the control.

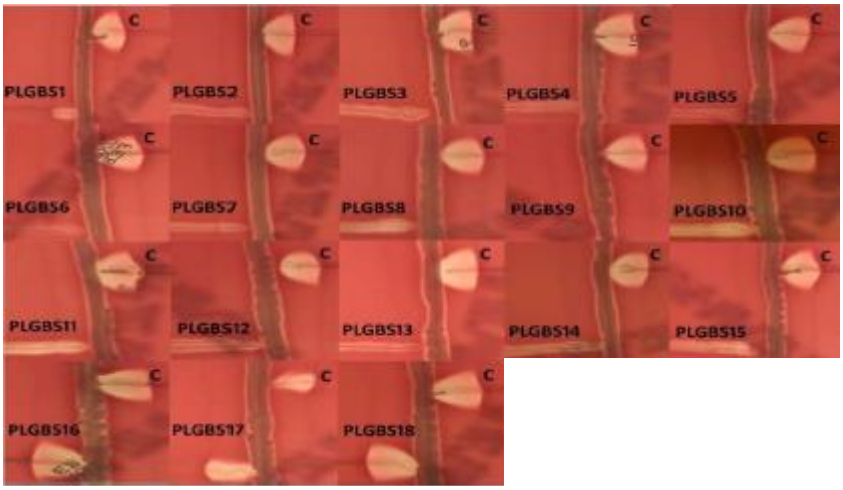



**Figure4.3. CAMP activity for sia (-) NT and sia (+) NT isolates.**

Bacteria were spotted on 5% sheep blood agar plates and incubated for 24 h at 37°C. The size of the area of complete lysis of red blood cells between *S. aureus* and GBS isolates reflected the level of CAMP expression. There was very faint lysis between *S. aureus* and sia (-) NT isolates as compared with the control. Isolates (PLGBS16, PLGBS 17, and PLGBS18) were sia (+) NT isolates and displayed CAMP activity similar to the control.

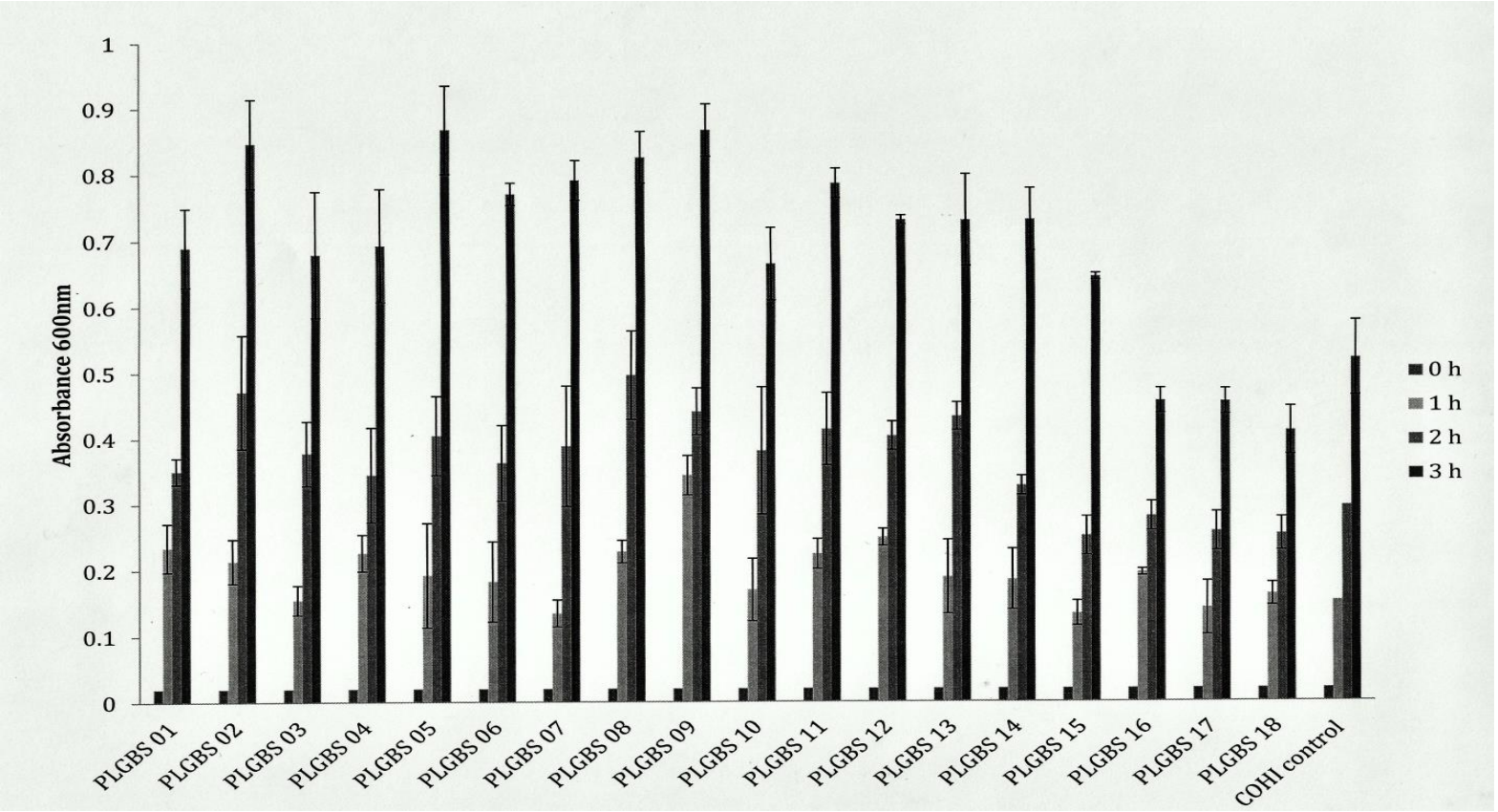



Figure 4.4. **Growth of sia (-) NT and sia (+) NT isolates.**

Bacteria were grown in TH broth at 37°C with shaking. GBS COHI was included as control.
Isolates (PLGBS 16, PLGBS 17, and PLGBS 18) were sia (+) NT GBS isolates.

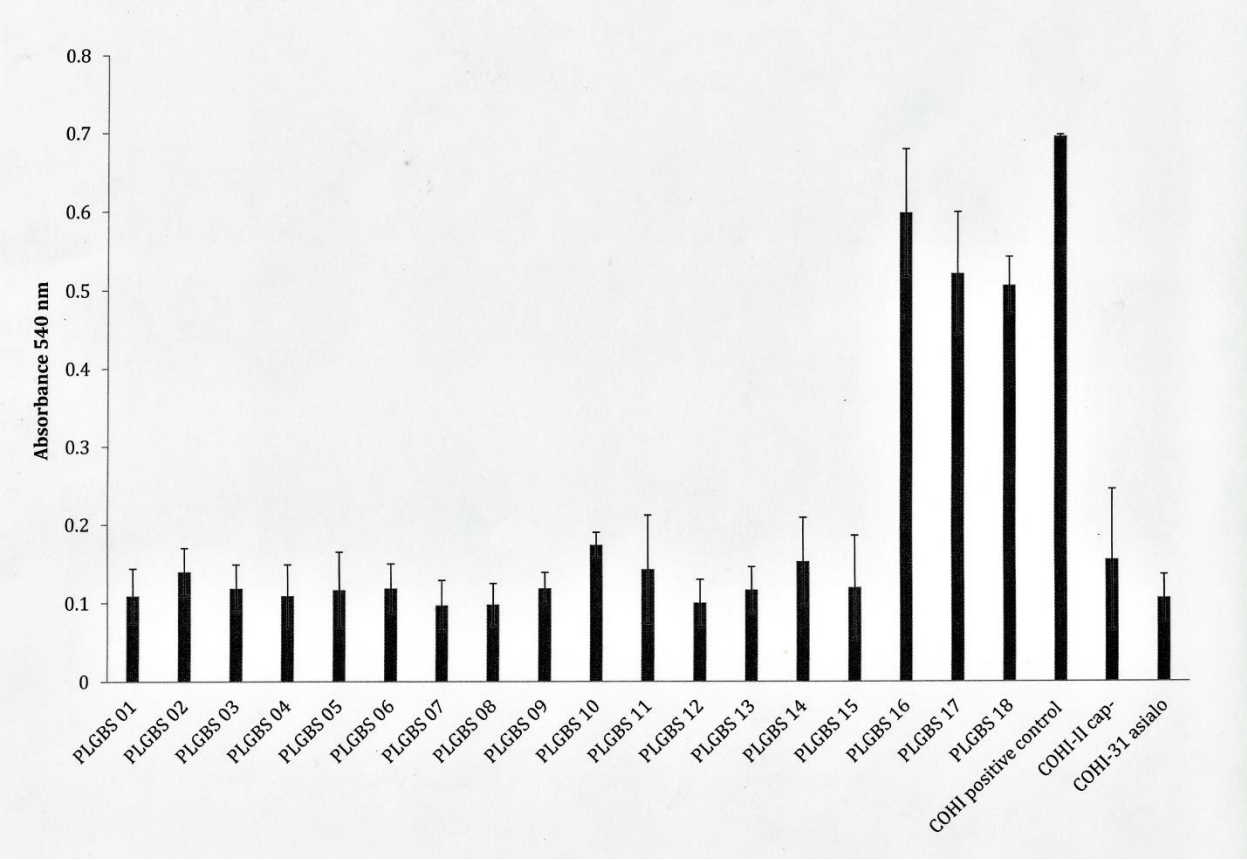



**Figure 4.5. Biofilm formation of sia (-) NT and sia (+) NT isolate.**

Bacteria were grown at in RPMI supplemented with 1% glucose and 20% human plasma (HP). Cells were stained with crystal violet. Quantification was performed by solubilization of the stained biomass in ethanol/acetone (80/20) and measuring the absorbance at 540nm. COHI was included as positive control while unencaspulated (COHI-II) and asialo (COHI-31-15) mutants were negative controls.

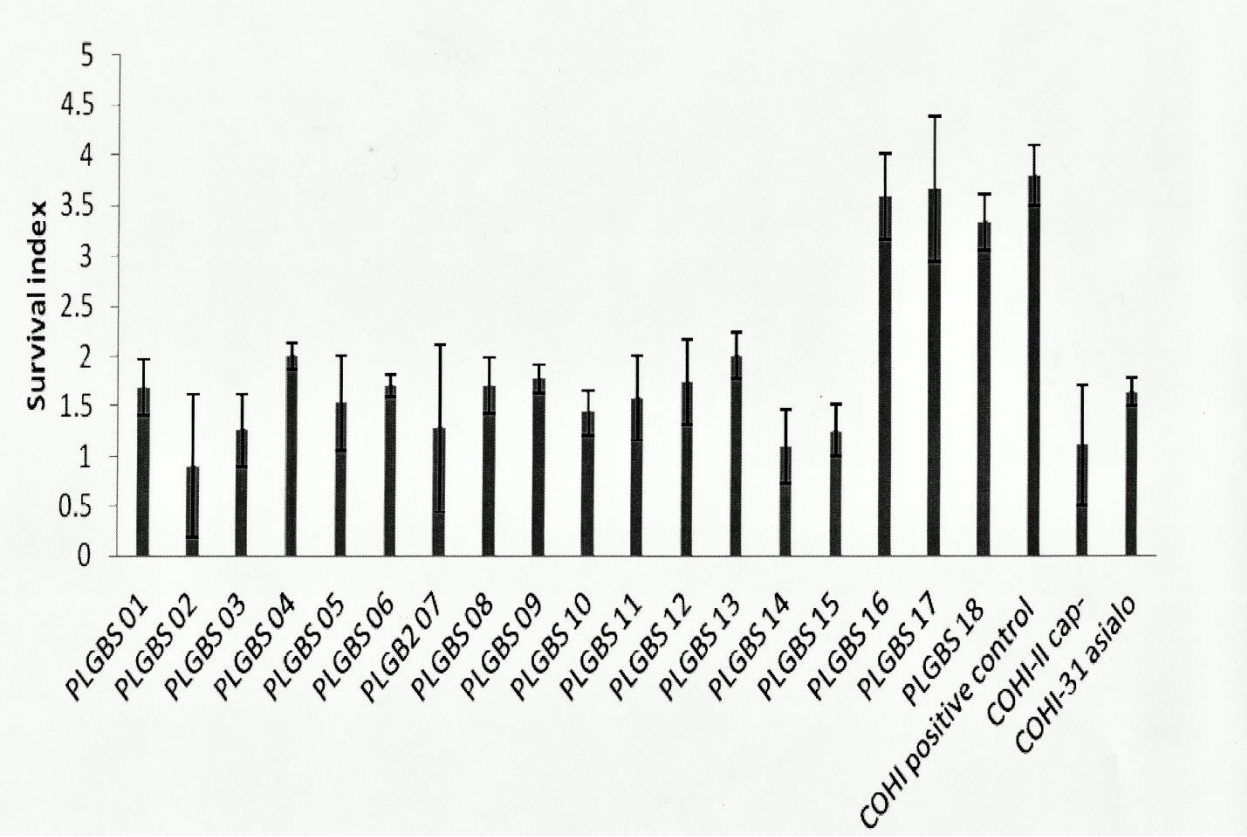
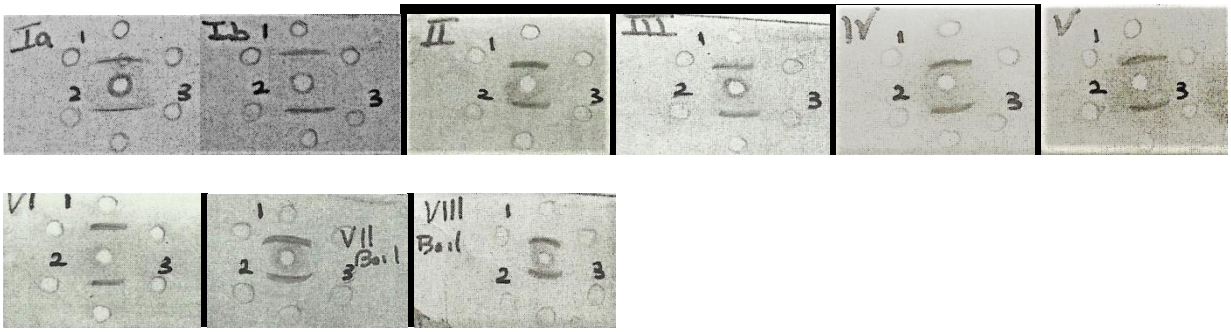



**Figure 4.6. Human whole-blood killing assay of sia (-) NT and sia (+) NT isolates.**

Bacteria ($10^3$ CFU/100 ml) were mixed with 300 ml of freshly drawn human blood in heparinised tubes, and incubated for 3h with agitation at 37°C, and dilutions were plated on blood agar for enumeration of CFU. The survival index was calculated as follows: (CFU at the end of the assay)/ (CFU at t = 0 h). GBS COHI was included as negative control while unencapsualted (COHI-II) and asialo (COHI-31-15) mutants were used as positive controls.

A. 100 ˚C




B. 50 ˚C

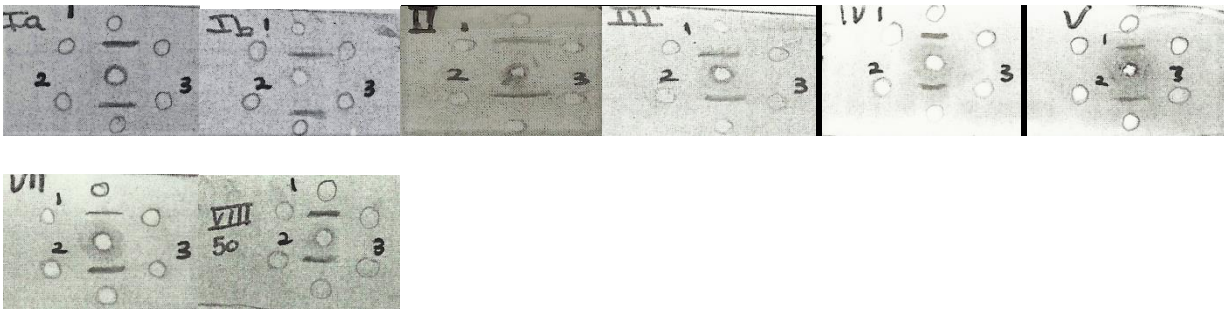



**Figure 4.7. Immunodiffussion assay for sia (+) NT isolates (well #1:PLGBS16, well #2: PLGBS18, and well #3: PLGBS17).**

Ten microliters antiserum was added in the center well, and 10 µl of either 100 ˚C (boiled as in panel A) or 50˚C (not boiled as in panel) B extract capsule was added in the surrounding wells. In each panel an antiserum raised against one of the serotype (Ia, Ib, II,-VIII) was added in the center hole. Reactions appeared in the up and the bottom wells were controls for each antiserum type.

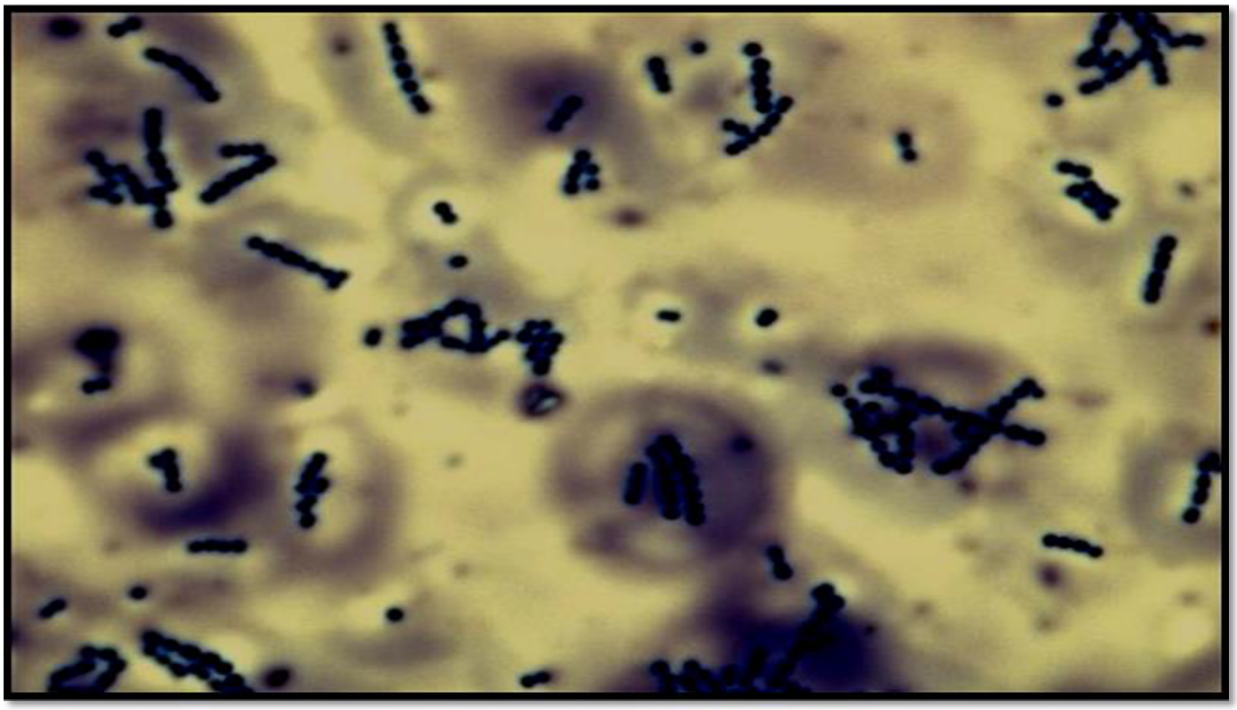


**Figure 4.8. . Capsules visualized using Anthony's capsular polysaccharide stain.**

PLGBS17 were used as an example. PLGBS16 and 18 demonstrated the same reactions. PLGBS17 was grown in milk broth for 18 h to provide a proteinaceous background for contrast. The smear was stained with 1% crystal violet for 2 minutes and then rinsed gently with a 20% solution of copper sulfate.

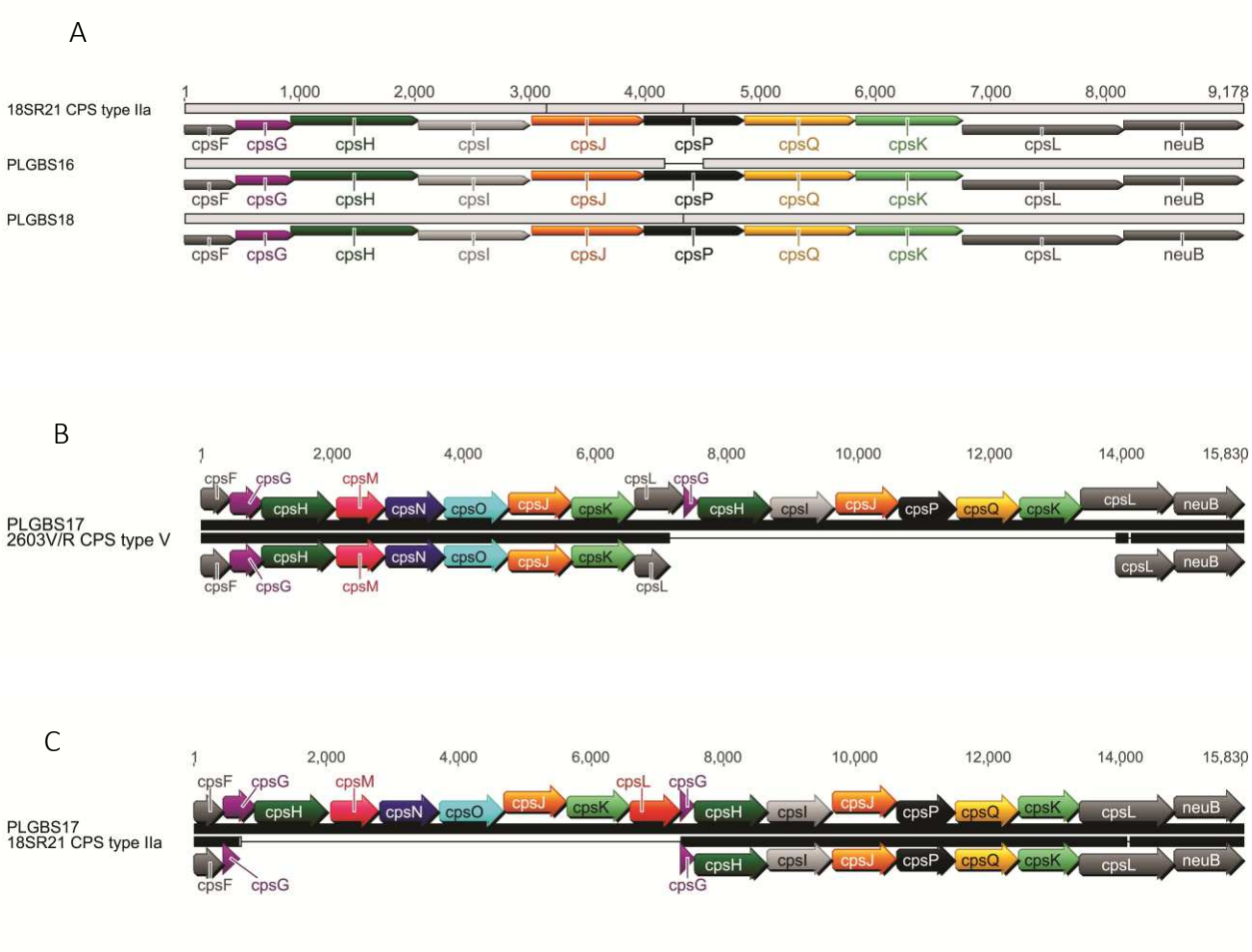



**Figure 4.9. Comparative analyses between CPS-specific gene sequences.**

A. PLGBS16 and PLGBS18 vs. 18SR21 (CPSIIa), B. PLGBS17 vs. 2603V/R (CPSV) and C. 18SR21 (CPSIIa)

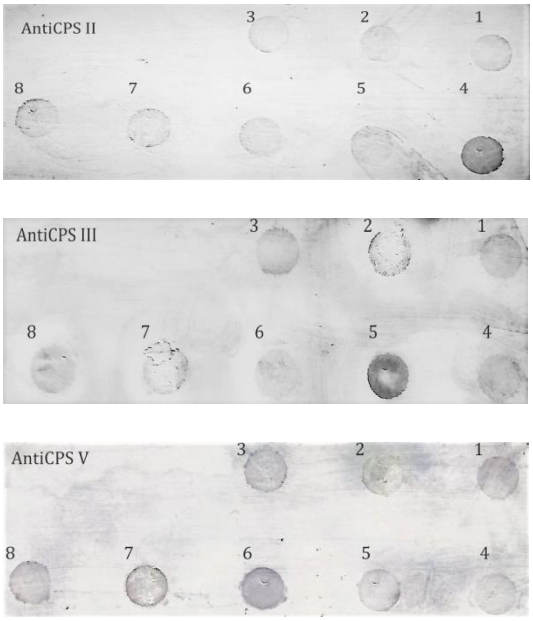



**Figure 4.10. CPS dot-blot analysis for CPS type II or CPS type V expression on GBS isolates PLGBS16, PLGBS17, and PLGBS18 that were sia (+) NT isolates.**

Bacterial suspensions of 1) PLGBS16, 2) PLGBS17, 3) PLGBS18, 4) CPSII control (NCS6), 5) control CPSIII (COHI), 6) CPSV control (NCS13), 7, unencapsualted mutant (COHI-II) as a negative control and 8) asialo mutant (COHI 31-15) as a negative control were spotted onto nitrocellulose membrane using bio-dot apparatus. CPS was detected using rabbit polyclonal antibodies raised against CPSII (in-house), CPSIII or CPSV (Statens Serum Institute) at 1:1000 dilution followed by a horseradish peroxidise-coupled anti-rabbit secondary antibody.

## 4.5 References


1. **Schuchat A.** 1999. Group B streptococcus. Lancet **353:**51-56.
2. **Baker CJ, Barrett FF.** 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr **83:**919-925.
3. **Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.
4. **Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.
5. **Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol **96:**498-503.
6. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.
7. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.
8. **Tapsall JW, Phillips EA.** 1987. Presumptive identification of group B streptococci by rapid detection of CAMP factor and pigment production. Diagn Microbiol Infect Dis **7:**225-228.

9. **Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC.** 2006. Identification of novel cps locus polymorphisms in nontypable group B Streptococcus. J Med Microbiol **55:**775-783.

10. **Doran KS, Nizet V.** 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Mol Microbiol **54:**23-31.

11. **Herbert MA, Beveridge CJ, Saunders NJ.** 2004. Bacterial virulence factors in neonatal sepsis: group B streptococcus. Curr Opin Infect Dis **17:**225-229.

12. **Rubens CE, Wessels MR, Kuypers JM, Kasper DL, Weiser JN.** 1990. Molecular analysis of two group B streptococcal virulence factors. Semin Perinatol **14:**22-29.

13. **Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL.** 2007. Serotype IX, a Proposed New Streptococcus agalactiae Serotype. J Clin Microbiol **45:**2929-2936.

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

15. **Xia FD, Mallet A, Caliot E, Gao C, Trieu-Cuot P, Dramsi S.** 2015. Capsular polysaccharide of Group B Streptococcus mediates biofilm formation in the presence of human plasma. Microbes Infect **17:**71-76.

16. **Chaffin DO, Mentele LM, Rubens CE.** 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol **187:**4615-4626.

17. **Wessels MR, Haft RF, Heggen LM, Rubens CE.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun **60:**392-400.

18. **Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, Romano MR, Pinto V, Brogioni B, Torricelli G, Janulczyk R, Grandi G, Margarit I.** 2014. Structure of the type IX group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem **289:**23437-23448.

19. **Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

20. **Carlin AF, Chang YC, Areschoug T, Lindahl G, Hurtado-Ziola N, King CC, Varki A, Nizet V.** 2009. Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. J Exp Med **206:**1691-1699.

21. **Marques MB, Kasper DL, Pangburn MK, Wessels MR.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun **60:**3986-3993.

22. **Alhhazmi A, Pandey A, Tyrrell GJ.** 2017. Identification of the Group B Streptococcus Capsule Type using a Dual Phenotypic/Genotypic Assay. J Clin Microbiol**:**JCM. 00300-00317.

23. **Di Palo B, Rippa V, Santi I, Brettoni C, Muzzi A, Metruccio MM, Grifantini R, Telford JL, Paccani SR, Soriani M.** 2013. Adaptive response of Group B streptococcus to high glucose conditions: new insights on the CovRS regulation network. PLoS One **8:**e61294.

24. **Lamy MC, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, Glaser P, Kunst F, Msadek T, Trieu-Cuot P, Poyart C.** 2004. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. Mol Microbiol **54:**1250-1268.

25. **Lupo A, Ruppen C, Hemphill A, Spellerberg B, Sendi P.** 2014. Phenotypic and molecular characterization of hyperpigmented group B Streptococci. Int J Med Microbiol **304:**717-724.

26. **Sendi P, Johansson L, Dahesh S, Van-Sorge NM, Darenberg J, Norgren M, Sjölin J, Nizet V, Norrby-Teglund A.** 2009. Bacterial phenotype variants in group B streptococcal toxic shock syndrome. Emerg Infect Dis **15:**223-232.

27. **Whidbey C, Burnside K, Martinez RM, Gendrin C, Vornhagen J, Frando A, Harrell MI, McAdams R, Rajagopal L.** 2015. A Hyperhemolytic/Hyperpigmented Group B Streptococcus Strain with a CovR Mutation Isolated from an Adolescent Patient with Sore Throat. Clin Res Infect Dis **2**.

28. **Whidbey C, Harrell MI, Burnside K, Ngo L, Becraft AK, Iyer LM, Aravind L, Hitti J, Waldorf KM, Rajagopal L.** 2013. A hemolytic pigment of Group B Streptococcus allows bacterial penetration of human placenta. J Exp Med **210:**1265-1281.

29. **Liu GY, Doran KS, Lawrence T, Turkson N, Puliti M, Tissi L, Nizet V.** 2004. Sword and shield: linked group B streptococcal beta-hemolysin/cytolysin and carotenoid pigment function to subvert host phagocyte defense. Proc Natl Acad Sci U S A **101:**14491-14496.

30. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the

Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206-214.

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

32. **Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, Paulsen IT, Nelson KE, Margarit I, Read TD, Madoff LC, Wolf AM, Beanan MJ, Brinkac LM, Daugherty SC, DeBoy RT, Durkin AS, Kolonay JF, Madupu R, Lewis MR, Radune D, Fedorova NB, Scanlan D, Khouri H, Mulligan S, Carty HA, Cline RT, Van Aken SE, Gill J, Scarselli M, Mora M, Iacobini ET, Brettoni C, Galli G, Mariani M, Vegni F, Maione D, Rinaudo D, Rappuoli R, Telford JL, Kasper DL, Grandi G, Fraser CM.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. Proc Natl Acad Sci U S A **99:**12391-12396.

33. **Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

34. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518-1530.

35. **Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ.** 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett **241:**129-134.

36. **Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179.

37. **Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

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

39. **Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol doi:10.1128/JCM.00355-16.

40. **Ke D, Ménard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, Bergeron MG.** 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem **46:**324-331.

41. **Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional analysis in type Ia group B Streptococcus of a cluster of genes involved in extracellular polysaccharide production by diverse species of streptococci. J Biol Chem **276:**139-146.

42. **D'Urzo N, Martinelli M, Pezzicoli A, De Cesare V, Pinto V, Margarit I, Telford JL, Maione D, Group MotDS.** 2014. Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 Streptococcus agalactiae strains. Appl Environ Microbiol **80:**2176-2185.

43. **Parker RE, Laut C, Gaddy JA, Zadoks RN, Davies HD, Manning SD.** 2016. Association between genotypic diversity and biofilm production in group B Streptococcus. BMC Microbiol **16:**86.

44. **Rosini R, Margarit I.** 2015. Biofilm formation by Streptococcus agalactiae: influence of environmental conditions and implicated virulence factors. Front Cell Infect Microbiol **5:**6.

45. **Neemuchwala A, Teatero S, Athey TB, McGeer A, Fittipaldi N.** 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B Streptococcus. Emerg Infect Dis **22:**1941-1944.

46. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and. J Clin Microbiol **45:**2909-2916.

47. **Kong F, Gowan S, Martin D, James G, Gilbert GL.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol **40:**216-226.

48. **Manning SD.** 2003. Molecular epidemiology of Streptococcus agalactiae (group B Streptococcus). Front Biosci **8:**s1-18.

49. **Holm SE, Håkansson S.** 1988. A simple and sensitive enzyme immunoassay for determination of soluble type-specific polysaccharide from group B streptococci. J Immunol Methods **106:**89-94.

50. **Pritchard DG, Egan ML, Gray BM, Dillon HC.** 1988. Immunochemical characterization of the polysaccharide antigens of group B streptococci. Rev Infect Dis **10 Suppl 2:**S367-371.

51. **Håkansson S, Burman LG, Henrichsen J, Holm SE.** 1992. Novel coagglutination method for serotyping group B streptococci. J Clin Microbiol **30:**3268-3269.

52. **Lancefield RC.** 1934. A SEROLOGICAL DIFFERENTIATION OF SPECIFIC TYPES OF BOVINE HEMOLYTIC STREPTOCOCCI (GROUP B). J Exp Med **59:**441-458.

53. **Lancefield RC, Hare R.** 1935. THE SEROLOGICAL DIFFERENTIATION OF PATHOGENIC AND NON-PATHOGENIC STRAINS OF HEMOLYTIC STREPTOCOCCI FROM PARTURIENT WOMEN. J Exp Med **61:**335-349.

54. **Slotved HC, Elliott J, Thompson T, Konradsen HB.** 2003. Latex assay for serotyping of group B Streptococcus isolates. J Clin Microbiol **41:**4445-4447.

55. **Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae. J Med Microbiol **54:**1133-1138.

56. **Wen L, Wang Q, Li Y, Kong F, Gilbert GL, Cao B, Wang L, Feng L.** 2006. Use of a serotype-specific DNA microarray for identification of group B Streptococcus (Streptococcus agalactiae). J Clin Microbiol **44:**1447-1452.

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

58. **Lembo A, Gurney MA, Burnside K, Banerjee A, De Los Reyes M, Connelly JE, Lin WJ, Jewell KA, Vo A, Renken CW.** 2010. Regulation of CovR expression in Group B Streptococcus impacts blood–brain barrier penetration. Molecular microbiology **77:**431-443.

59. **Lupo A, Ruppen C, Hemphill A, Spellerberg B, Sendi P.** 2014. Phenotypic and molecular characterization of hyperpigmented group B Streptococci. International journal of medical microbiology **304:**717-724.

60. **Bolotin S, Fuller JD, Bast DJ, Beveridge TJ, de Azavedo JC.** 2007. Capsule expression regulated by a two-component signal transduction system in Streptococcus iniae. FEMS Immunol Med Microbiol **50:**366-374.

61. **Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL.** 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol **46:**2745-2750.

62. **Manning SD, Lacher DW, Davies HD, Foxman B, Whittam TS.** 2005. DNA polymorphism and molecular subtyping of the capsular gene cluster of group B streptococcus. J Clin Microbiol **43:**6113-6116.

63. **Chaffin DO, Beres SB, Yim HH, Rubens CE.** 2000. The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. J Bacteriol **182:**4466-4477.

64. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol **52:** 1441-1447.

65. **Ferrieri P, Hillier SL, Krohn MA, Moore D, Paoletti LC, Flores AE.** 2004. Characterization of vaginal & rectal colonization with multiple serotypes of group B streptococci using multiple colony picks. Indian J Med Res **119 Suppl:**208-212.

66. **Sellin M, Olofsson C, Håkansson S, Norgren M.** 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol **38:**3420-3428.

67. **Rubens CE, Heggen LM, Haft RF, Wessels MR.** 1993. Identification of cpsD, a gene essential for type III capsule expression in group B streptococci. Mol Microbiol **8:**843-855.

68. **Rubens CE, Wessels MR, Heggen LM, Kasper DL.** 1987. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. Proc Natl Acad Sci U S A **84:**7208-7212.

69. **Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK.** 2002. Binding and invasion of HeLa and MRC-5 cells by Streptococcus agalactiae. Microbiology **148:**3921-3931.

70. **Jiang SM, Cieslewicz MJ, Kasper DL, Wessels MR.** 2005. Regulation of virulence by a two-component system in group B streptococcus. J Bacteriol **187:**1105-1113.

# Chapter 5: Complete Genome Sequence of *Streptococcus agalactiae* CPS type V PLGBS 13 Strain Sequence Type 1 (ST1) and Genome Analysis of Multiple Pathogenic ST1 Isolates

## 5.1 Introduction

Group B Streptococci (GBS) are recognized as a leading cause of neonatal invasive diseases as well as invasive diseases in immunocompromised patients and in elderly individuals worldwide (1-7). GBS are primarily found in the human urogential tract and gastrointestinal tracts. There are ten immunologically distinct serotypes have been described: serotypes Ia, Ib, and II through IX (8-10). From a global perspective, GBS can also be typed via multi-locus sequence typing (MLST) (11). MLST permits the classification of the majority of GBS strains caused human infections into more than 700 sequence types (STs), which are clustered in five major clonal complexes (CCs) (11).

Five CPS types (Ia, Ib, II, III, and V) are responsible for the majority of invasive GBS disease cases with CPS III causing a higher rate disease among neonates and CPS type Ia and V having higher rates among adult patients in North America and European countries, (12-23). Serotype V was rarely isolated from human before the mid-1980s (17), but it now accounts for the bulk of invasive GBS diseases in nonpregnant adults (14, 15, 17, 22, 24). The increase in GBS disease cases in nonpregnant adults over the past decade has been linked to the emergence of serotype V strains (14, 15, 17, 22, 24).

The majority (92%) of serotype V GBS strains isolated from the bloodstream of nonpregnant adults in North America between 1992 and 2013 was identified as ST 1 (25). A recent study of 229 GBS isolates identified only one ST 1 strain that was not a capsular type V (26). We recently reported two ST 1 GBS isolates that were identified as CPS type IIa (PLGBS16) and CPS hybrid of IIa and V (PLGBS17). There are limited studies regarding molecular

epidemiology of ST1 isolates associated with other serotypes than serotype V. Intraspecies genetic variability among GBS isolates is thought to account for the differences in their transmissibility, infection severity, and antimicrobial resistance (27-29). This genetic variability is mainly driven by large scale recombination events and phage-mediated horizontal gene transfer (27, 29, 30). It also can arise via small genetic changes such as short insertions, deletions, and/or single nucleotide changes (27, 29, 30). The rapid influx of whole genome sequence (WGS) data and the development of novel bioinformatics analyses and powerful computational tools have enabled studying the genetic variability among GBS isolates (25, 26, 31). As few complete genomes of ST 1 GBS strain isolated from a human had been determined previously (25, 32), I completed genome sequence of the ST 1/serotype V PLGBS13 strain and compared to these sequenced genomes. PLGBS13 has been used extensively in our laboratory experiments as it is a highly invasive GBS isolate. In addition, to gain a better understanding of the genomic makeup of PLGBS13 WGS was performed. To perform comparisons, two ST 1/nonserotype V (PLGBS16 and PLGBS17) (cps operon described in Chapter 4) and two ST 1/serotype V GBS (PLGBS19 and PLGBS20) genomes were also sequenced and compared to PLGBS 13 (ST 1 / serotype V).

## 5.2 Materials and Methods

### 5.2.1 Bacterial isolates and serotyping

The isolates used in this work are shown in Table 5.1. All the bacteria were isolated from patients with invasive GBS disease living in Alberta, Canada; two isolates (PLGBS19 and PLGBS20) were identified as serotype V and two isolates (PLGBS16 and PLGBS 17) were identified as serotype IIa and CPS hybrid of serotype IIa and V, respectively. These GBS isolates

were isolated from the blood of nonpregnant adults as described previously (33). PLGBS13 was isolated from a case of nonpregnant adult (skin and wound infection) living in Alberta, Canada in 1997 and has previously been shown to be highly invasive (33). This strain has been used extensively in our laboratory as a highly invasive GBS strain. Identification of GBS serotypes was performed using an immunodiffusion assay as described previously (34, 35) and confirmed by a real time PCR GBS typing assay previously described (36).

### 5.2.2 Multilocus sequence typing (MLST) assay and assignment to clonal clusters

Multilocus sequence typing (MLST) was carried out as described previously (11). Briefly, the sequences of seven GBS housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK, and tkt*) were assigned an allele number based on their sequences. Each isolate was assigned a sequence type (ST) based on the allelic profile of the seven sequences. Strains were grouped into CCs using the eBURST software program (37, 38). The default eBURST setting identified groups of related STs using the most stringent (conservative) definition, such that all members assigned to the same group shared identical alleles at six of the seven loci with at least one other member of the group (http://pubmlst.org/sagalactiae/)

### 5.2.3 Whole-Genome Sequencing (WGS)

PLGBS13 genome was determined using a combination of 454 Roche and Illumia MiSeq data. Genomic DNA from an overnight culture was isolated using an extraction protocol and subjected to library preparation according to the manufacturer's protocol. Subsequently, paired end sequencing reads derived from short-read Illumina MiSeq data were mapped to the newly assembled contig for error correction of the 454 Roche data (39). Annotation of the closed genome of PLGBS13 strain was performed using the online annotation website Rapid

Annotation using Subsystem Technology (RAST) (40). The genome of PLGBS13 was deposited in GenBank (BioProject PRJNA473176).

PLGBS16, PLGBS17, PLGBS19, and PLGBS20 isolates were sequenced using MiSeq instruments with an average sequencing depth of 120×.Library preparation was performed using a tagmentation process. 1 ng genomic DNA was used as input and the Illumina Nextera XT Library preparation kit (FC-131-1096) as per manufacturer's protocol. Resultant libraries were purified from free primers using Ampure beads (1:0.8 reaction mix:bead ratio). Libraries were qualified using the Agilent Bioanalyzer and quantified using a Qubit fluorimeter and Qubit HS DNA reagents. Libraries were loaded at 10pM on an Illumina MiSeq v2 reagent kit (MS-102-2003) and processed at 250 cycles with 1% PhiX control DNA (FC-110-3001). Sequence data for the four genomes have been deposited in the NCBI GenBank database (PLGBS16, BioProject PRJNA433769; PLGBS17, BioProject PRJNA434007; PLGBS19 and PLGBS20, BioProject PRJNA473176).

### 5.2.4 Comparative genomic analyses

The genome of PLGBS13 was compared to GBS genomes listed in Table 5.1 using progressive alignment Mauve (41). To identify polymorphisms in each strain relative to the genome of GBS strain PLGBS 13 ST 1/serotype V CLC genomic benchwork version 7 was used. To identify recombination, the genome data was analyzed with BRAtNextGene (42), run with 20 iterations, 100 replicates, and a significance cutoff of 0.05.

## 5.3 Results

### 5.3.1 Determination of the complete genome sequence of ST1 CPS type V PLGBS13

Two complete genomes of ST 1 GBS strains (one from human and the other from cow) have been determined previously (25, 32). The cow GBS is 09mas018883 (GenBank accession HF952104.1) and the genome was published in June 2014 (32) and SGBS001 (GenBank accession CP01011867.1) published in June 2015 (25). I first determined the complete genome sequence of a serotypeV/ST 1 (PLGBS13) strain. PLGBS13 was first described by Tyrrell et al., (33). I used a combination of two next generation sequencing technologies, a 454 Roche platform and paired-end Illumina short-read data to completely assemble the genome of PLGBS13 strain. The genome was 2,095,031 bp with 2,046 predicted ORFs, 101 RNA and GC% of 35.6 (Fig 5.1). The eighteen genes required for CPS V synthesis were identified in the GBS *cps* operon (cpsA:SAG1146-neuA:SAG1163) (Fig 5.2). A region of glycosyltransferases and related proteins (SAG1150- SAG1157) that direct the synthesis of the type V polysaccharide repeat unit is flanked on either side by genes that are conserved in all the known GBS capsule serotypes. Upstream of the serotype-determining region (*cpsG-cpsL*) are genes *cpsA-cpsF* (SAG1158-SAG1163) found in all the ten known GBS serotypes which contain genes that function in the regulation and the export of the polysaccharide capsule. Downstream of the variable region are genes that encode enzymes for the biosynthesis and activation of sialic acid (SAG1146-SAG1149), the terminal sugar on the side chain of all the ten serotypes (43). PLGBS13 encodes several genes identified as surface proteins and secreted products that are potential virulence factors or targets of protective immunity: CAMP factor (SAG1893), streptococcal enolase (SAG0564), hyaluronidase (SAG1187) (27) and hemolysin/cytolysin (*cylE*, SAG0668) (28). Two

genes, *lmb* (SAG1815 and SAG1224) encode laminin-binding proteins and three *scpB* genes (SAG1225, SAG1226, and SAG0416) encoding C5a peptidase. Two of the *scpB* genes are similar to SAG1236 in 2603V/R (44), these peptidases are frame-shifted near their C terminus suggesting that C5a is inactive. The third C5a peptidase gene (SAG0416) is 100% identical to SAG0416 in 2603V/R. The genome of PLGBS13 contains genes predicated to encode alpha-like protein (Alp) 3 (Alp3) (SAG0436), rib (SAG0640), AlpST1 (SAG0588) and pilus 1 (SAG0649) and 2a (SAG0650).

To determine the distribution of antimicrobial resistance (AR) genes in the PLGBS13 genome, the seven most common AR-encoding genes *erm*(A/TR), *erm*(B), *mef*(A/E), *tet*(M), *tet*(O), *aphA-3*, and *aad-6*, and two AR related genes, *int-Tn* and *mreA* were searched in the PLGBS13 genome. PLGBS13 contained *mreA* (SAG0891) as do all GBS isolates (45). Tetracycline resistance gene *tetM* (SAG0575) and *int-Tn* (SAG0572) carried by Tn916 were found in PLGBS13 genome. *tetM* and *int-Tn* were commonly located in the transposable element Tn916 inserted in mobile genetic element (MGE) region.2 of the majority of GBS isolates (Fig 5.1) as reported in Da Cunha *et al*.(46). Macrolide resistance elements *aphA* and *aad*6 were absent in PLGBS13; however they were commonly found among ST 1 strains (46, 47).

When I compared the PLGBS13 whole genome to other strains whose complete genomes are available from NCBI, PLGBS13 shared high homology (99.5%) to the only other complete human GBS genome of serotype V/ST 1, SGBS001 strain (25). PLGBS13 has 6 mobile genetic elements regions (1, 231,218-250,546 bp; 2, 591,097-660,672 bp; 3, 986,108-996,485 bp; 4, 1,268,316-1,283,211 bp; 5, 1,921,442-1,929,844 bp; and 6, 2,039,104-2,056,738 bp) similar to SGBS001 (25)(Fig 5.3). PLGBS13 shares high similarity to the serotype V/ST1 Swedish cow

mastitis strain 09mas018883(97.8%) (32) (Fig.5.3). The major difference between the two isolates is the presence of approximately of a 45 kb region that is unique to 09mas018883 strain (located between SAG1245 and SAG1246 in PLGBS 13), which includes genes encoding proteins involved in lactose utilization. The lac.2 operon is commonly present in bovine sourced isolates (48). With respect to the *cps* operon, PLGBS13 shares DNA homology of 89.5%,89.4%, 89%, and 80.1% with A909 (CPS type Ia) (49), GD201008-011 (CPS type Ia) (50), 2603V/R (CPS type V) (44), NEM316 (CPS type III) (51), respectively as shown in Fig 5.3.

### 5.3.2 Genomes of ST 1 GBS isolates

Recently, I described that serotype V GBS strains were responsible for 19.1 % of GBS invasive disease in Alberta, Canada (2003-2013) (15). Previously, it has been found that the vast majority of GBS serotype V isolates from nonpregnant adults are ST 1 (25). From our Alberta collection, two GBS isolates were identified previously as CPS IIa (PLGBS16) and a CPS hybrid of IIa and V (PLGBS17). Both of these isolates were typed via MLST as ST 1. Two other serotype V GBS isolates (PLGBS19 and PLGBS20) were randomly selected from our collection for genomic compassion to the ST 1 isolates PLGBS16 and PLGBS17. These isolates were also identified as ST 1. The four GBS isolates were recovered from the blood of nonpregnant adults in Alberta, Canada (2004). (Table 5.1).

To determine the genetic diversity among the four ST1 GBS strains, the sequences of these isolates were determined using a MiSeq instrument (Illumina). PLGBS19 and PLGBS20 harbored the complete *cps* gene cluster of serotype V whereas PLGBS 16 encoded the complete *cps* gene cluster of serotype IIa. PLGBS17 encoded a hybrid capsule gene cluster comprised of *cpsIIa* and *cpsV* as described in chapter 4. I identified a collection of the main virulence

determinants and pili content in these GBS isolates. The four isolates possessed CAMP factor, enolase, hyaluronidase, hemolysin/cytolysin, two *lmb* genes, and PI-1 and PI-2a.

However, there were differences among these isolates in the *scpB* and *alp*ha like/*rib* genes. PLGBS16, PLGBS17 harbored two and five *scpB* genes encoding C5a peptidase, respectively, but both PLGBS19, and PLGBS20 encoded 3 *scpB* genes. For *alpha*/*rib* gene content, PLGBS16 encoded *rib* and *alp*ha-like 3, whereas PLGBS20 encoded *rib*, *alpha*-like 3 genes, and *alp*ST1 gene. PLGBS17 contained four *alpha*/rib genes, two *rib* genes, one *alpha* like 3 gene, and one *alpST1* gene, but PLGBS 20 encoded only one *rib* gene.

### 5.3.3 Polymorphism identification among ST 1 isolates relative to PLGBS13

Polymorphisms in the genome sequences of the four ST 1 strains were identified relative to PLGBS13 strain. First, we aligned the short reads to the genome of serotype V / ST 1PLGBS13 strain and identified polymorphisms by using Geneious version 8.0.3. The four ST 1 isolates differed from the reference strain PLGBS13 by an average of 3669 single-nucleotide polymorphisms (SNPs). Serotype V GBS isolates (PLGBS19 and PLGBS20) had only a SNP average of 675 in relation to PLGBS13 whereas PLGBS16 (CPS type IIa) and PLGBS17 (a CPS hybrid of IIa and V) had a SNP average of 6663 and 8833 in comparison to PLGBS13 (Table 5. 2).

### 5.3.4 Recombination identification among ST 1 GBS isolates

To identify recombination regions among GBS isolates ST1 CPS type V and the other serotypes (CPS type IIa and a CPS hybrid IIa and V) with respect to PLGBS13, we used bayesian analysis of recombination (BRATNextGen) (42). CPS type IIa (PLGBS16) and the CPS hybrid of IIa and V (PLGBS17) isolates had experienced horizontal gene transfer of different sizes all of which include the cps gene cluster. PLGBS16 CPS type IIa had exchanged a DNA region of 169 Kb and

had acquired a *cps*IIa locus. The CPS hybrid of IIa and V (PLGBS17) isolate had gained a *cps*IIa locus of 77 kb beside the *cps*V locus in the background of ST 1 isolate (Table 5.3). A slightly narrower area of recombination was defined in other loci of the genomes of both PLGBS16 and PLGBS17 strains. Recombination in the serotype V isolates PLGBS19 and PLGBS20 was less extensive (Table 5.3)

In conclusion, the genetic diversity among ST 1 GBS isolates that are serotype V is mainly driven by small genetic changes such as insertions, deletions or mutations. However, ST 1 GBS isolates that have serotypes other than serotype V (IIa and CPS hybrid of IIa and V) reported here could be mainly explained by recombination in the cps gene cluster and few genes.

## 5.4 Discussion

GBS ST 1/Serotype V isolates have been found to be responsible for the vast majority of nonpregnant adult invasive GBS infections (25). It has been proposed that the acquisition of resistance to antibiotic classes is the main driver for the emergence of ST 1 GBS clones among adult cases (46). In our ST1 isolates, the *tetM* gene was present in a Tn916 transposon, but none of the isolates contained macrolide resistance elements. This is may be due to the small sample size included in our study. The *ermB* gene was reported as one of the most common macrolide resistance elements among macrolide resistant ST 1 GBS isolates (46). It was first described in *S. pneumonia* (52). Specifically, *ermB* is carried by the Tn917 transposon and inserted into Tn916 leading to Tn3872 transposon (52). This suggest that the single insertion of Tn917 in Tn916 resulting in to Tn3872 did not occur in the isolates included in this study. Da Cunha *et al*., discovered that a significant proportion of ST 1 strains contained the *ermB* (macrolide resistance element) that is present along with *tetM* in Tn3872 (46). In all our ST

1/serotype V GBS isolates, *tetM* and possible other colocalized genetic determinants have been acquired in their genomes which could allow for the increased capacity to cause disease in nonpregnant adults (25, 46).

Analysis of the two serotype V/ST 1 isolates causing disease in humans (strains PLGBS19 and PLGBS20) determined that these strains differed from PLGBS13 in 675 SNPs. In contrast, the other two ST 1 strains PLGBS 16 (serotype IIa) and PLGBS 17 (CPS hybrid IIa and V) differed from the reference strain by 6663 SNPs as results of mainly recombination within the cps gene cluster. It has been found that the diversity among GBS different serotypes is primarily driven by recombination (30, 46). In ST 1 GBS isolates associated with serotypes other than CPS type V, small scale genetic changes due to insertions, deletions, or mutations in genomic regions other than the *cps* gene cluster are the key players of genetic diversity rather than extensive recombination (25, 46). Similarly, other major CCs showed the same population structure, such as ST 17 (26). PLGBS16 and PLGBS17 ST 1 isolates reported here is an example of GBS evolution model suggested previously (25). This model resembles the antigenic shift/antigenic drift model of influenza in which recombination drives the emergence of new GBS CPS types, such as CPS type IIa as in PLGBS 16 or CPS hybrid of CPS type IIa and CPS type V as in PLGBS 17 followed by the accumulation of deletions, insertions or mutations resulting in new genetic polymorphisms over time. Multiple recombination events have been identified in GBS genomes that can donate GBS strains with an enhanced virulence arsenal (46, 53). In contrast, a single recombination event resulting in capsular switching that replaces only the cps locus encoding capsular biosynthetic genes with no change in the rest of the genome has also been characterized in GBS isolates (14, 30, 53, 54). It has been proposed that most of these recombination events most

likely occurred by conjugation and that DNA exchange took place in the human gut or urogenital tract, which can be cocolonized with multi-cps genotypes of GBS isolates (55).

This study provides information regarding the genetic diversity among GBS isolates with the same genotype, ST 1. The genetic diversity among GBS isolates identified as ST 1 and serotype V is mainly driven by small genetic changes such as insertions, deletions or mutations. In contrast, ST 1 GBS isolates that have serotypes other than serotype V (CPS type IIa and CPS hybrid of IIa and V) reported here could be mainly explained by recombination in the cps gene cluster and few genes. The genetic diversity can explain the phenotypic diversity which can be reflected in GBS clones' behavior in respect to the host innate and adaptive immune responses, escape clinical interventions, including antibiotic therapy and vaccine introduction

**Table 5.1. Strains used in this study.**

| Strain designation | CPS type | Reference |
|---|---|---|
| PLGBS 13 | V | (33) |
| PLGBS 16 | IIa | Chapter 4 |
| PLGBS 17 | IIa and V | Chapter 4 |
| PLGBS 19 | V | This study |
| PLGBS 20 | V | This study |
| SGBS001 | V | (25) |
| 09mas018883 | V | (32) |
| 2603V/R | V | (44) |
| A909 | Ia | (49) |
| GD201008-011 | Ia | (50) |
| NEM316 | III | (51) |

**Table 5.2. SNP analysis for GBS isolates ST1 in comparison to PLGBS13.**

| Strain designation | CPS type | SNPs compared to PLGBS13 |
|---|---|---|
| PLGBS16 | IIa | 4492 |
| PLGBS17 | CPS hybrid of IIa and V | 8833 |
| PLGBS19 | V | 713 |
| PLGBS20 | V | 637 |

**Table 5.3. Recombination events among GBS ST1 isolates (two isolates serotype V [PLGBS19 and PLGBS20], CPS type IIa {PLGBS16] and a CPS hybrid of CPS type IIa and CPS type V [PLGBS17])[a].**

| Designation | Recombingenic region (bp) | Size (bp) | Genes in the recombined region |
|---|---|---|---|
| **PLGBS16** | 940488-940913 | 425 | PLGBS16 genome |
| | 1178344-1180456 | 2,081 | PLGBS16 genome, hypothetical protein, Regulator of exopolysaccharide synthesis, competence and biofilm formation, *ftr* and prophage |
| | 1191402-1208363 | 16,961 | *cps* gene cluster, *cpsA-neuD* |
| | 1295946-1296270 | 324 | DNA modification methylase |
| **PLGBS17** | 457353-458837 | 1484 | surface protein, *rib* |
| | 613765-614391 | 626 | surface protein, *rib* |
| | 940153-940577 | 424 | PLGBS17 genome |
| | 188234-195811 | 7577 | CPS IIa-specific genes , *cpsG-cpsL* |
| **PLGBS19** | 940513-941136 | 623 | PLGBS19 genome |
| | 1007106-1007249 | 143 | Two-component system histidine kinase, tyrosine recombinase, *xerC* |
| | 1386491-1386793 | 302 | ABC-type Fe3+siderophore transport system, permease component |
| **PLGBS20** | 940481-940904 | 423 | PLGBS20 genome |
| | 724945-725171 | 226 | transcriptional regulator, *gntR* |
| | 1154718-1155570 | 852 | Glycine betaine transporter, *opuD* |

[a] BRATNextgene run with 20 iterations, 100 replicates, and a significance cutoff of 0.05.

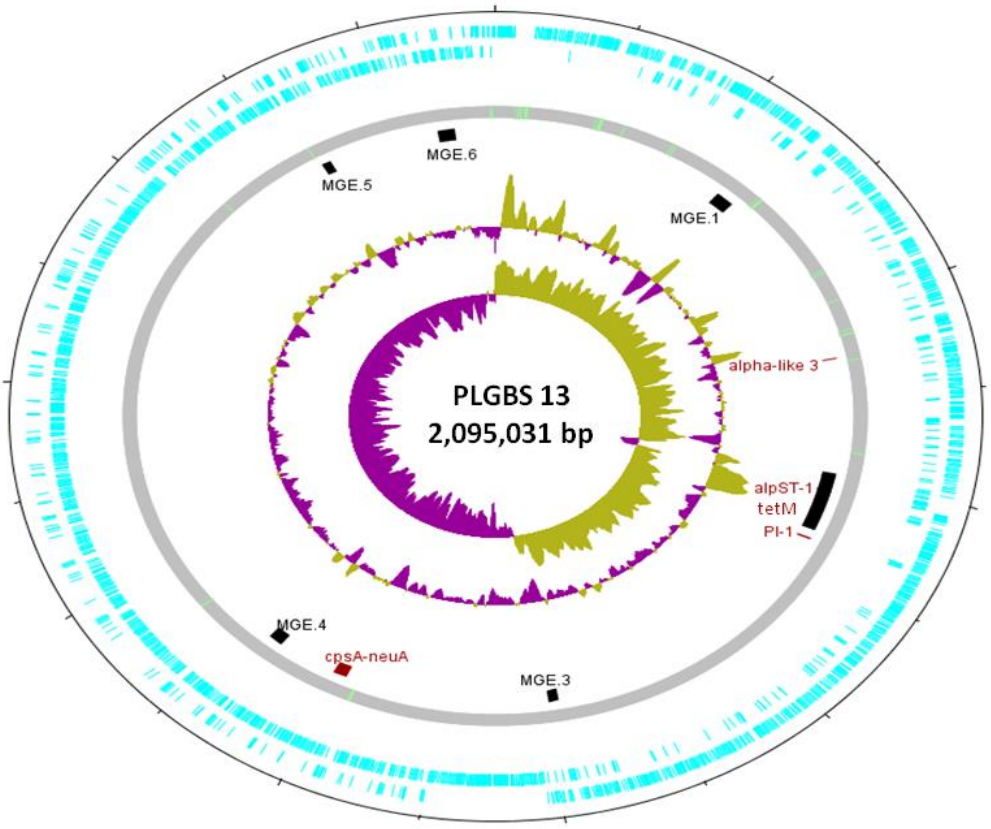



**Figure 5.1. Whole-genome analysis of a ST-1 serotype V PLGBS13 strain isolated from nonpregnant adult.**

Genome atlas for the reference serotype V ST-1 strain PLGBS13 strain. Genome scale in megabases is given in the innermost circle (circle 1). Circle 2 shows GC skew, calculated as (G − C)/(G + C) and averaged over a moving window of 10,000 bp showing excess G (green) and C (purple). GC content is displayed in circle 3 with values above average (outward directed) or below average (inward directed) indicated. Annotated coding sequences are shown in light blue (forward, clockwise and reverse, counter clockwise) in circle 4. Reference genome landmarks are displayed in circle 5 and include ribosomal operons (green), MGEs (black, MGE.1–6), pilus islands [red, pilus island 1 (PI-1) and 2a (PI-2a)], capsule biosynthesis operon (red, neuAcpsA), and Alp-encoding genes, and tetM


Figure 5.2. *cps* gene cluster of PLGBS 13 strain.

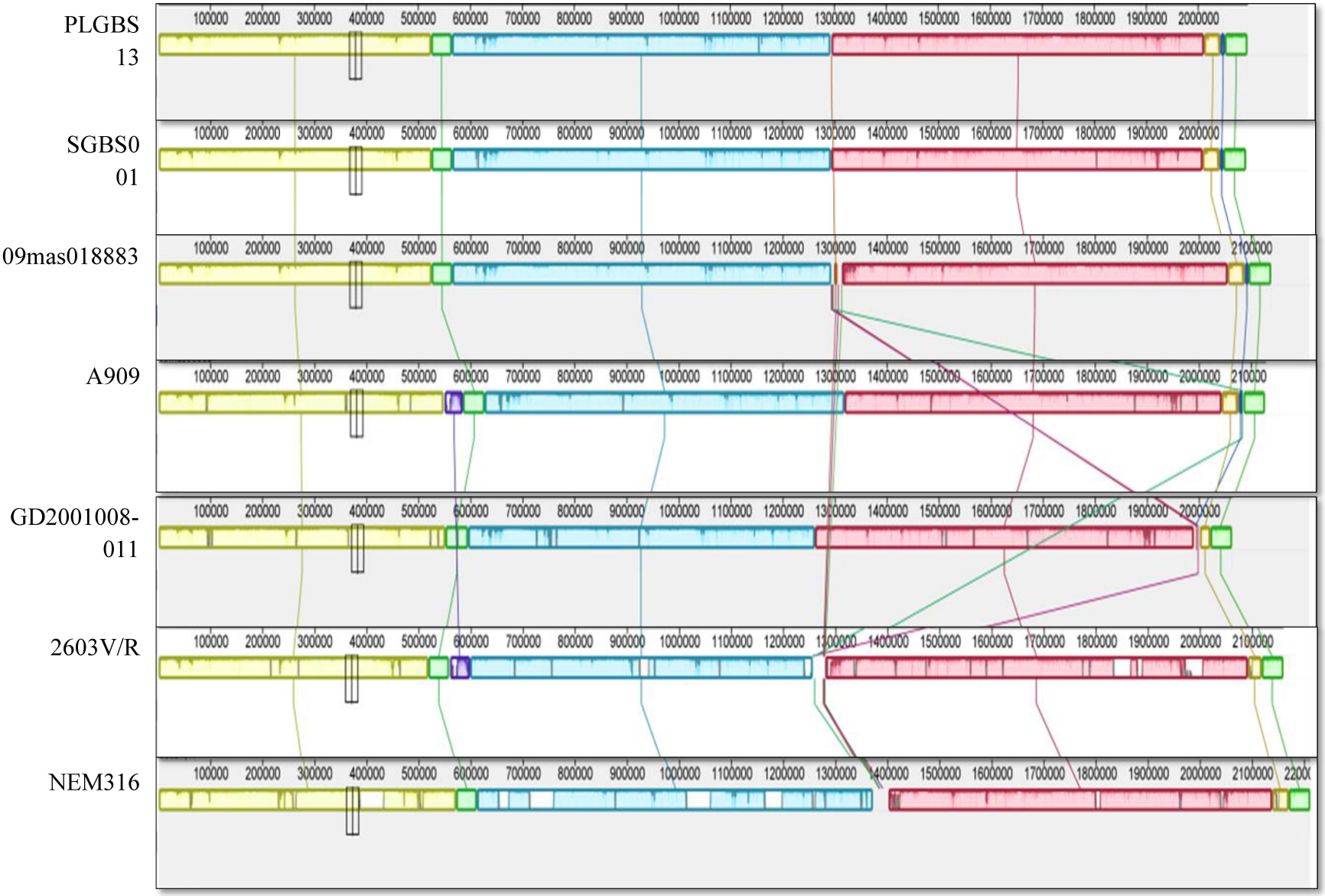

PLGBS 13
SGBS0 01
09mas018883
A909
GD2001008-011
2603V/R
NEM316

**Figure 5.3. Whole genome sequence comparisons of PLGBS 13 genome to publically available GBS genomes in NCBI.**

MAUVE alignment display of PLGBS13 to GBS genomes is organized into one horizontal panel per input genome sequence and in order of decreasing homology (sequences, serotype, NCBI accession no.) for SS1 (1, V, CP01086.1), 09mas018883 (1, V, NC_ 021485.1), A909 (2, 1a, NC_007432.1), GD201008-001 (3, 1a, NC_018646.1), 2603V/R (4, V, NC_004116.1), NEM316 (5, III, NC_004368.1). Each genome's panel contains the name of the genome sequence and a scale showing the sequence coordinates for that genome. Colored block surrounds a region of the genome sequence that aligned to part of another genome, and is homologous and internally free from genomic rearrangement. Colored blocks in PLGBS13 genome are connected by lines to similarly colored blocks in other genomes. These lines indicate which regions in each genome are homologous. The boundaries of colored blocks indicate the breakpoints of genome rearrangement. Regions outside blocks lack detectable homology among the input genomes. Areas that are completely white contain sequence elements specific to a particular genome.

## 5.5 References


1. **Schuchat A.** 1999. Group B streptococcus. Lancet **353:**51-56.
2. **Baker CJ, Barrett FF.** 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr **83:**919-925.
3. **Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.
4. **Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.
5. **Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol **96:**498-503.
6. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.
7. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.
8. **Dmitriev AV, Shakleina EV.** 2003. [Methods of molecular epidemiology of infections caused by group B streptococci]. Zh Mikrobiol Epidemiol Immunobiol**:**83-92.

9. **Domelier AS, van der Mee-Marquet N, Arnault L, Mereghetti L, Lanotte P, Rosenau A, Lartigue MF, Quentin R.** 2008. Molecular characterization of erythromycin-resistant Streptococcus agalactiae. J Antimicrob Chemother **62:**1227-1233.

10. **Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

11. **Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

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

13. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

14. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol **52:** 1441-1447.

15. **Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol **54**: 7 1774-1781.

16. **Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand **87:**260-271.

17. **Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis **173:**365-373.

18. **Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microb Drug Resist **10:**31-36.

19. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

20. **Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.

21. **Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A.** 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland:

implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis **177:**998-1002.

22. **Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.

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

24. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ, Network ABCsEIP.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299:**2056-2065.

25. **Flores AR, Galloway-Peña J, Sahasrabhojane P, Saldaña M, Yao H, Su X, Ajami NJ, Holder ME, Petrosino JF, Thompson E, Margarit Y Ros I, Rosini R, Grandi G, Horstmann N, Teatero S, McGeer A, Fittipaldi N, Rappuoli R, Baker CJ, Shelburne SA.** 2015. Sequence type 1 group B Streptococcus, an emerging cause of invasive disease in adults, evolves by small genetic changes. Proc Natl Acad Sci U S A **112:**6431-6436.

26. **Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT,**

**Poyart C, Glaser P, Consortium D.** 2014. Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **5:**4544.

27. **Beres SB, Carroll RK, Shea PR, Sitkiewicz I, Martinez-Gutierrez JC, Low DE, McGeer A, Willey BM, Green K, Tyrrell GJ, Goldman TD, Feldgarden M, Birren BW, Fofanov Y, Boos J, Wheaton WD, Honisch C, Musser JM.** 2010. Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. Proc Natl Acad Sci U S A **107:**4371-4376.

28. **Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, Corander J, Bryant J, Parkhill J, Nejentsev S, Horstmann RD, Brown T, Drobniewski F.** 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet **46:**279-286.

29. **Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, Pessia A, Aanensen DM, Mather AE, Page AJ, Salter SJ, Harris D, Nosten F, Goldblatt D, Corander J, Parkhill J, Turner P, Bentley SD.** 2014. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet **46:**305-309.

30. **Brochet M, Rusniok C, Couvé E, Dramsi S, Poyart C, Trieu-Cuot P, Kunst F, Glaser P.** 2008. Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of Streptococcus agalactiae. Proc Natl Acad Sci U S A **105:**15961-15966.

31. **Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba

and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

32. **Zubair S, de Villiers EP, Fuxelius HH, Andersson G, Johansson KE, Bishop RP, Bongcam-Rudloff E.** 2013. Genome Sequence of Streptococcus agalactiae Strain 09mas018883, Isolated from a Swedish Cow. Genome Announc **1**.

33. **Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK.** 2002. Binding and invasion of HeLa and MRC-5 cells by Streptococcus agalactiae. Microbiology **148:**3921-3931.

34. **Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179.

35. **Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

36. **Alhhazmi A, Pandey A, Tyrrell GJ.** 2017. Identification of the Group B Streptococcus Capsule Type using a Dual Phenotypic/Genotypic Assay. J Clin Microbiol **55**:2637-2650.

37. **Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518-1530.

38. **Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ.** 2004. Displaying the relatedness among isolates of bacterial species -- the eBURST approach. FEMS Microbiol Lett **241:**129-134.

39. **Koren S, Harhay GP, Smith TP, Bono JL, Harhay DM, Mcvey SD, Radune D, Bergman NH, Phillippy AM.** 2013. Reducing assembly complexity of microbial genomes with single-molecule sequencing. Genome Biol **14:**R101.

40. **Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206-214.

41. **Darling AE, Mau B, Perna NT.** 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One **5:**e11147.

42. **Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, Corander J.** 2012. Detection of recombination events in bacterial genomes from large population samples. Nucleic Acids Res **40:**e6.

43. **Alper I, Frenette M, Labrie S.** 2013. Genetic diversity of dairy Geotrichum candidum strains revealed by multilocus sequence typing. Appl Microbiol Biotechnol **97:**5907-5920.

44. **Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, Paulsen IT, Nelson KE, Margarit I, Read TD, Madoff LC, Wolf AM, Beanan MJ, Brinkac LM, Daugherty SC, DeBoy RT, Durkin AS, Kolonay JF, Madupu R, Lewis MR, Radune D, Fedorova NB, Scanlan D, Khouri H, Mulligan S, Carty HA, Cline RT, Van Aken SE, Gill J, Scarselli M, Mora M, Iacobini ET, Brettoni C, Galli G, Mariani M, Vegni F, Maione D, Rinaudo D, Rappuoli R, Telford JL, Kasper DL, Grandi G, Fraser CM.** 2002. Complete

genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. Proc Natl Acad Sci U S A **99:**12391-12396.

45. **Duarte RS, Bellei BC, Miranda OP, Brito MA, Teixeira LM.** 2005. Distribution of antimicrobial resistance and virulence-related genes among Brazilian group B streptococci recovered from bovine and human sources. Antimicrob Agents Chemother **49:**97-103.

46. **Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P.** 2015. Corrigendum: Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **6:**6108.

47. **Tazi A, Morand PC, Réglier-Poupet H, Dmytruk N, Billoët A, Antona D, Trieu-Cuot P, Poyart C.** 2011. Invasive group B streptococcal infections in adults, France (2007-2010). Clin Microbiol Infect **17:**1587-1589.

48. **Richards VP, Choi SC, Pavinski Bitar PD, Gurjar AA, Stanhope MJ.** 2013. Transcriptomic and genomic evidence for Streptococcus agalactiae adaptation to the bovine environment. BMC Genomics **14:**920.

49. **Madoff LC, Michel JL, Kasper DL.** 1991. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun **59:**204-210.

50. **Liu G, Zhang W, Lu C.** 2012. Complete genome sequence of Streptococcus agalactiae GD201008-001, isolated in China from tilapia with meningoencephalitis. J Bacteriol **194:**6653.

51. **Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, Msadek T, Zouine M, Couvé E, Lalioui L, Poyart C, Trieu-Cuot P, Kunst F.** 2002. Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease. Mol Microbiol **45:**1499-1513.

52. **McDougal LK, Tenover FC, Lee LN, Rasheed JK, Patterson JE, Jorgensen JH, LeBlanc DJ.** 1998. Detection of Tn917-like sequences within a Tn916-like conjugative transposon (Tn3872) in erythromycin-resistant isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother **42:**2312-2318.

53. **Neemuchwala A, Teatero S, Athey TB, McGeer A, Fittipaldi N.** 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B Streptococcus. Emerg Infect Dis **22:**1941-1944.

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

55. **Ferrieri P, Hillier SL, Krohn MA, Moore D, Paoletti LC, Flores AE.** 2004. Characterization of vaginal & rectal colonization with multiple serotypes of group B streptococci using multiple colony picks. Indian J Med Res **119 Suppl:**208-212.

# Chapter 6: Summarizing Discussion

## 6.1 General discussion

Implementation of screening guidelines and effective antimicrobial prophylaxis have contributed to significantly reduce early onset GBS invasive disease in the neonatal population; but late-onset GBS infections mostly remained unchanged (1). In Canada, neonatal GBS disease is nationally notifiable; however, invasive GBS is also an increasing health concern among adults leading to septicemia, meningitis, pneumonia, bone, joint and tissue infections. Adults most often are those with underlying medical conditions, pregnant women, and those residing in extended health care facilities (2, 3). All hospitals in Canada are expected to follow the recommendations for prevention of perinatal GBS infections from the Canadian Taskforce on preventive health care (4). To further reduce the incidence of EOD in Canada, updated recommendations for neonates with a risk of developing EOD have been issued in 2017 (5). However, these recommendations still only address EOD and not LOD and adult disease. The data in this thesis has clearly shown that invasive GBS disease is dynamic in nature over time and warrants continued surveillance to help guide future prevention and treatment recommendations.

## 6.2 GBS causing infections in Alberta population

A study addressing incidence rate in neonates and nonpregnant adults, the prevalence of capsular types and antimicrobial susceptibility patterns in Alberta in a period of 11 years from 2003-2013 (6) is available in chapter 2 of this thesis. Very little was known regarding the epidemiology of GBS infections in Alberta during this time period, and even less on the

molecular characteristics of GBS isolates. Two earlier population-based studies regarding invasive GBS disease in Alberta were previously published. One study by Manning et al., examined the genetic diversity of GBS and serotype distribution of GBS strains from only neonates with invasive disease in Alberta (1993-2003), and compared the data to the genotypes and serotypes identified among pregnant women from Alberta (7). The other study by Tyrrell et al., defined invasive GBS disease incidence rate, serotype distribution and antimicrobial resistant profile among only nonpregnant adult population for Alberta and parts of Canada over 20 years ago (1996). Little is known post publication of these two studies about the epidemiologic and microbiologic characteristics of iGBS after the implementation of preventative measures in Canada among infant populations and as well among nonpregnant adult population. This lack of information substantiated the importance of chapter 2. It presents a detailed description of the epidemiological characterization of GBS isolates causing neonatal infections in Alberta, as well as of those responsible for an increasing number of invasive infections in nonpregnant adults. One main limitation of our study is the fact that invasive GBS from cases over the age of 91 days (i.e. not EOD or LOD) are not included in the Alberta Health Notifiable Disease Regulations. This makes it likely that not all cases of iGBS from cases over the age of 91 days (children and adults) occurring in Alberta were reported for our surveillance period. The calculated incidence of GBS invasive disease in Alberta for patients over 91 days may have underestimated the actual incidence. Despite this limitation, we believe that the collection studied is large enough to reflect at least the circulating CPS types causing iGBS and the bacterial population diversity in Alberta. Another limitation of our study is the absence of clinical data, which prevents us from evaluating the associations between GBS

infection and patient history, including underlying medical conditions that were previously shown to be risk factors for GBS invasive infections (2, 3). Importantly, the surveillance study presented in chapter 2 identified an increase in the incidence rate of GBS disease from a low of 3.92/100,000 population in 2003 to a high of 5.99/100,000 population in 2013 (123 cases to 234 cases) for all age groups including nonpregnant adults. The increase was apparent in 2009 with an incidence rate of 4.15 cases /100,000 population reaching the highest rate in 2013 at 5.99 cases/100,000 opoulation. This increase could be due to multiple factors. It could be due to elevated rates of invasive GBS disease and/or an increased interest in submission of invasive GBS isolates for CPS typing from diagnostic microbiology laboratories in the province. However, there is no requirement for diagnostic laboratories to send GBS isolates to Provincial Public Health Laboratory (PPHL) for CPS typing in Edmonton, Alberta. Moreover, the increase in numbers for all CPS are not consistent from year to year, suggesting the increase is more likely due to elevated rates.

For EOD, the incidence rate increased approximately twofold from 0.26 cases (2003) to 0.34 cases (2013) per 1000 live birth even with implementation of the recommend preventive measures. The LOD incidence rate also increased fourfold from 0.15 cases to 0.56 cases per 1000 live births between 2003 and 2013, respectively. Although the intervention guidelines are only designed to prevent invasive EOD (8); the upturn in incidence rate was found for both EOD and LOD cases, similarly to the reported increase in the United States 2004-2005 (9). Thus, surveillance of EOD and LOD rates in the upcoming years is important to continue to monitor to see if this upturn is continued.

## 6.3 Distribution of Group B streptococci serotypes associated with invasive diseases in neonates and nonpregnant adults in Alberta

While all GBS serotypes are able to both colonize and cause invasive disease, the prevalence of the GBS serotypes is different according to disease presentation and age of patients To the best of my knowledge this is the first study providing in-depth analysis of the serotype epidemiology of invasive GBS isolates from all age groups including EOD, LOD and patients over the age of 90 days in Alberta. There has been extensive data on serotype distribution from other parts of Canada (3, 10, 11). The findings from this study are similar to those reported elsewhere (2, 12-16), but the serotype data of invasive disease isolates from this study are nevertheless important as it confirms the presence of temporal variation in serotype distribution of invasive disease isolates over 11 years in the study-setting. This has positive implications for the design of serotype-specific polysaccharide vaccines for prevention of GBS neonatal patients.

Serotypes III, V and Ia accounted for the majority of invasive cases in Alberta over the eleven year surveillance period. These findings are consistent with serotype distribution data from cases of both invasive disease and in carriage in studies elsewhere (Toronto, Beijing, Ireland, England, Portugal, Global). Serotype III was responsible anywhere for one quarter to one half of the total GBS isolates serotyped in past epidemiological surveys, which was consistent to our findings. Serotype V was the second most frequently identified invasive serotype in Alberta and in the recently reported study from Toronto, Canada indicating CPS V is common in Canada (10). However, CPS Ia was found to be more common than CPS V in the meta-analysis study by Edmond et al (13). Interestingly, CPS V was not as common in the 1990's

(and earlier) as CPS Ia. CPS V became more common in the 2000's indicating GBS CPS changes in the population can gradually occur (2, 13, 17, 18). In our study, CPS type IV gradually increased 12 fold (2 cases to 24 cases) over the years surveyed. This finding is likely part of the global increase of CPS IV previously reported in North America, South America, Europe, and Asia (12, 14, 19-23). This increase was also documented in other provinces in Canada, such as the Greater Toronto area (10), Manitoba and Saskatchewan (11). The high invasive index of this serotype can be attributed to a dominant clone sequence type (ST459), suggesting that the underlying genotype can influence the invasive potential. ST459 clone may potentially possess particular genetic features that facilitate the dissemination and adaption of this clone to cause diseases and could explain the increase in the number of CPS IV in Alberta and globally. CPS IV is not included in the potential vaccine that includes CPS Ia, Ib, II, III, and V due to its previous low prevalence in European countries and in the United States in the past decade (24, 25). This may become important because of the potential foe the increase in serotype IV cases to combine with antibiotic resistance, particularly, co-resistance to second-line macrolide antibiotics as we reported in chapter 2 as well as by other investigators (20) and Portuguese (14). Dynamic changes in CPS type distribution and emergence of resistance highlight the need for constant monitoring, of GBS diseases in order to develop accurate GBS prevention strategies.

Serotype distribution by age is also consistent with data of invasive disease isolates in young neonates and children from elsewhere (2, 13, 26-29). CPS III was the most predominant type in cases of EOD, LOD and up to 14 years followed by CPS Ia, for cases of EOD (28%) and for LOD (65%). These findings are also similar to the meta-analysis reported by Edmond et al. (13).

This study was based on collecting data from previous publications on GBS disease in neonates up to three months of age and found that CPS III accounted for 49% of cases in this age group followed by CPS Ia at 23% (together accounting for 72% of iGBS). Remaining CPS types reported in this meta-analysis were all under 10% each (13). These CPS types are included in a tri-valent GBS conjugate vaccine currently under evaluation for safety and immunogenicity in pregnant women (25). Such a trivalent serotype-specific GBS vaccine would therefore provide potential cover against at least 93% of invasive disease serotypes in young neonates.

Finally, important information drawn from chapter 2 is the number of nontypeable isolates being particularly high in invasive isolates among adult population (Fig 6.1). The polysaccharide capsule of GBS is an important virulence factor that plays a role in bacterial evasion of the innate immune system of the host. Accordingly, the majority of GBS strains causing invasive infections, particularly in newborns, are encapsulated. The data drawn in Fig 6.1 and adapted from chapter 2 are consistent with previously reported higher prevalence of nontypeable isolates among nonpregnant adults (30). According to Fig 6.1, the prevalence of nontypeable isolates among nonpregnant adults was documented (15-50 yrs [25.9%], and >50 yrs [69.6%]; altgother [95.6%]) as compared to the neonates (only 4.4% nontypeable). Nevertheless, nontypeable isolates still possess other virulence factors than the capsule, and it has been found that non capsulated isolates can more easily adhere to epithelial cells, because the capsule hinders the interaction of bacterial surface factors with the epithelial cell receptors (31).

## 6.4 Sialic acid as universal marker for GBS capsule production

Generally, sialic acids are typically found as terminal monosaccharides attached to cell surface of the glycan chains of glycolipids and glycoproteins. Sialic acids are well known for their important roles in many physiological and pathological processes, including microbe binding that leads to infections, regulation of the immune response, the progression and spread of human malignancies and in certain aspects of human evolution (32-34). GBS capsular polysaccharide is terminally linked to sialic acid. Importantly, sialyation is an essential process for full GBS capsule polysaccharide biosynthesis and expression. Sialylation of GBS capsular polysaccharide has been found to be required for full synthesis of CPS by GBS (35). A 80% reduction in surface associated CPS produced was associated with asialo mutant strains that had a deletion in the *cpsK* a gene encoding sialyltransferase (35) and *neuA* gene encoding CMP-sialic acid synthase (36) compared to the parental strain. Plant lectins have traditionally been powerful tools to explore glycan structures and GBS capsule structure (37). Their specificity is such that even isomeric glycans with identical sugar compositions can be distinguished. This work describes for the first time the use of sialo-lectin binding as a universal marker for GBS capsule expression and the development and application of sialo-lectin binding assays, new techniques, to investigate the GBS capsule expression. GBS capsular polysaccharide from all the recognized variants are characterized by the presence of covalently bound sialic acid linked to a polysaccharide backbone. The high resolution and the use of no CPS-specific antisera for these assays to confirm capsule expression are considered a major advantage over the classical serological typing techniques.

Chapter 3 describes two experiments in which crude capsule extracts (EIA assay) and whole bacteria (dot blot assay) were used to detect sialic acid from GBS isolates with known CPS types. According to experiments described in chapter 3, it is concluded that both assays using biotinylated lectin extracted from *Limax flavus* agglutinin (LFA) and streptavidin bound to horse radish peroxidease can identify isolates with cell surface sialic acid. The selectivity of the lectin in these assays is considered sufficient due to the ability of LFA to recognize multiple forms of sialic acid (38). This was optimal for our assays since sialic acid in CPS among the known GBS CPS types has different linkages (35, 39, 40). Still, the concept of using sialo-lectin binding to ascertain GBS capsule expression can be applied to techniques other than EIA and dot blot techniques. Flow cytometery is an example of these techniques.

Sialo-lectin methods described in chapter 3 give an overall impression of binding of the lectin used to the total amount of the sialyated capsule present in the sample. To investigate weak binding and strong binding of lectin to sialic acid, serial dilutions of a competing sugar to sialic acid could be added in EIA setting. However, to date, binding of LFA to other sugars has not been identified (38). This area could be further investigated in the future.

### 6.5 GBS capsular dual phenotypic and genotypic typing system

Typing of bacteria within a species is usually performed for a number of purposes such as surveillance, epidemiology or research. To select a typing method, the scientific question and the methods available need to be considered. Different methods have different resolution; i.e. the ability to classify a strain collection into groups. For GBS isolates, a common typing method is typing of the GBS polysaccharide capsule into ten different variants. In chapter 3, we aimed at and succeeded in developing a dual typing system for GBS isolates and compared its typing

results with serotyping of capsular polysaccharides, and applied it in an investigation of strains of GBS with an unidentifiable serotype. The first step of this system involves detection of sialic acid and confirmation of capsule expression. The second step of the typing system is identification of the CPS type by a PCR assay. The main objective of chapter 3 was to investigate whether sialic acid detection can ascertain capsule expression and assign a genotype for GBS capsular polysaccharide My results showed both methods produced complete concordance for GBS typing.

Generally, the development of new microbiological typing techniques needs to be compared with established methods in order to investigate their congruence and to ensure consistency in the typing of bacteria. Although serological CPS typing has been the most common method for GBS CPS assignment, there are limitations with these assays. Serological typing assays depend on the quality of the antibodies, the technical experience of the operator and expression of detectable amount of capsule by bacteria being assayed (41). Nontypeable strains are also a well known problem encountered with serological typing methods.

Immunologically, the phenomenon of non typability to some extent might reflect problems with the specificity of the antibodies as well. However, this could be overcome using sialic acid to first determine expression of capsule. Further the developed sialo-lectin assays in chapter 3 depend on the use of biotin-lectin in a streptavidin-HRP detection and amplification system. Such a system has the advantage of detecting a small amount of siaylated capsule (35, 42-44). Two assays, lectin-EIA and lectin-dot blot assays, were described in our work to identify isolates with capsule. Sialic acid could replace serological CPS typing methods by confirming capsule production.

CPS genotyping is usually a straightforward method and identifies the gene for the capsule type in question if the corresponding PCR is carefully designed. For typing of GBS capsular polysaccharide, chapter 3 indicates that GBS CPS typing-based on PCR method could be preferred, as it leaves only few strains nontypeable and gives usually unambiguous results. CPS genotyping uses PCR assisted amplification of *cps* loci and probe to identify a specific fragment for the following *cps* genes: *cpsH*, *cpsJ*, *cpsK*, *cpsG*, *cpsN*, *cpsO-V*, *cpsI*, *cpsM*, *cpsR, and cpsO-IX*. These probes used in the duplex real-time PCR assay with DNA are labelled with a fluorescent reporter dye and a quencher dye. The duplex real-time PCR of cps genotyping assay is fast and comparably straightforward method using less complicated laboratory protocols compared to, i.e. conventional PCR assay that needs follow up detection techniques, such as sequencing (45), blot hybridization (46, 47), enzymatic restriction (48), or agarose gel electrophoresis (49, 50). The equipment needed for real-time PCR is available in many microbiological laboratories. CPS genotyping overcomes some serological typing assay limitations, such as serotyping errors. As seen in Fig 6.2 adapted from chapter 2 &3, using our typing system, 71 isolates out of 159 nontypeable isolates that failed to be assigned a capsule type by a serological typing assay were sialic acid positive and assigned a capsule type by PCR. CPS V ranked in second in Alberta based on the used serological typing method in chapter 3, however, using our PCR typing system to assign a capsule type to GBS serologically nontypeable isolates, CPS V ranked in the first before CPS type III (now second) as a cause of invasive GBS disease.

My developed dual typing system in chapter 3 allows us to identify isolates that may be encoding a new CPS type. After screening a collection of nontypeable isolates, I identified 4

isolates that were found to express sialyated capsule on the surface and failed to assign a capsule genotype by real-time PCR assay. However, this represents a small fraction of GBS isolates (0.05%) that have undergone capsular recombinational or *cps* gene exchange. Our data also suggests that serotyping errors may account for a significant proportion of nontypeable isolates, emphasizing the high resolution of genetic approach in confirming all putative transformation events.

The resolution achieved by this dual method is dependent on the selection of cps loci for the identification of each CPS type and the specificity and sensitivity of the designed primers and probes. Mutations in the selected *cps* loci and acquisition of new *cps* genes as a result of recombination events (even though they are rare) could affect the resolution of this method. This method will need regular monitoring and finding additional *cps* gene loci which can be combined with previously described *cps* scheme in chapter 3 into an even more discriminating assay. These characteristics render the real-time PCR GBS typing as a tool which is especially well suited for investigation of outbreaks surveillance and epidemiology.

Three applications of the developed assays were investigated in chapter 3 and 4. First, these assays provided a complete description of GBS capsule and information regarding capsule production obtained from Sialo-lectin assays. In addition, the real-time PCR GBS typing assay assigned capsule type for GBS isolates. This system has a high resolution to classify GBS isolates into the 10 known GBS capsule variants (chapter 3). Another investigators group has recently described a short report regarding real-time PCR CPS typing assay for GBS (51). Interestingly, this previously reported assay and our real-time GBS PCR typing assay demonstrate the strength of the use of real-time PCR for GBS CPS typing. In addition, our typing system has the

advantage of ensuring the presence of GBS capsule using a sialo-lectin assay as well as assignment of CPS type by real-time PCR. In chapter 4, sialo-lectin binding was used to identify GBS isolates with capsule on their surface. The proposed CPS typing system in chapter 3 could theoretically be used to rescreen isolates that failed to be typed by serological typing methods to understand the underlying reasons of the nontypeable phenotype of these isolates. In chapter 3, we screened 159 nontypeable isolates based on serological assay for sialic acid presence on their surface using sialo-lectin assays. Seventy five strains (47.2%) were sialic acid positive suggesting the presence of the capsule, whereas 84 isolates (52.8%) were sialic acid negative indicating the absence of sialic acid on the bacterial surface. Approximately half of the nontypeable isolates express a surface of siaylated capsule and assign a CPS type except for only four isolates failed to be typed by the real-time PCR GBS typing assay. Such assays have high discrimination power that provides an opportunity to investigate the underlying reasons of the nontypeable phenotype of GBS isolates. Whether it is due to undetectable capsule expression by serological methods, technical problems of the serological assays, unproduced capsule, new capsule type or other unknown reasons.

Second, the developed typing system in chapter 3 provided us with a collection of GBS isolates with no capsule on their surface. Previous studies revealed that CovR/S regulatory system regulated a variety of GBS virulence factors, including the capsule (39, 46, 47, 48). This allowed putting forward a possible explanation for GBS capsule loss for which CovR/S system might have a role. To begin to understand the contribution of *covR/S* mutants to the number of nontypeable GBS isolates in our Alberta collection, I identified nontypeable GBS isolates with hemolysis and capsule loss suggesting potential *covR/S* mutation(s) (15 isolates). I screened this

collection targeting a number of the distinctive phenotypes associated with *covR/S* mutations, such as increased hemolysis and orange pigmentation, loss of CAMP activity, rapid growth in THB (a rich medium) and no growth in RPMI (a chemically defined minimal medium). Isolates that displayed *covRS* mutant phenotypes were found to contain mutation(s) in *covR* or *covS*. Two CPS–based functions, biofilm formation and antiphagocytic ability were also investigated among GBS nontypeable isolates that displayed *covR/S* mutant phenotypes and contained covR/S mutations. Associations of the nontypeable phenotype/*covRS* mutation(s) with the loss of biofilm and antiphagocytic ability were noted (chapter 4). These experiments are described in chapter 4. While understanding how *covR/S* gene mutations may lead to sialic acid loss is far from complete, my results lay the foundation for future research identifying how mutation(s) in covR/covS can affect sialic acid and capsule expression. My results focusing on determining the distribution of *covR/S* mutants among isolates with no sialic aicd on their surface provide a possible explanation of the nontypeable phenotypes of GBS isolates. To confirm that CovR and CovS are required for capsule and silaic acid expression and consequently plays a role in the nontypeable phenotype of GBS strains, in-frame deletion mutant of covR or covS could be first constructed in a GBS strain. To test whether the capsule and sialic acid are expressed on the surface of the *covR* or *covS* mutants, serological typing assays, such as double immunodiffusion, agglutination and sialo-lectin binding assays could be applied. Furthermore, to confirm that *covR*/*covS* are responsible for the nontypeable phenotype of the *covR*/*covS* mutants, capsule and sialic acid expression could be restored upon ectopic expression of CovR or CovS transcribed from its own promoter and capsule and sialic acid expression detected.

Third, the typing system algorithms have presented the advantage of identifying isolates with capsule on their surface regardless of their capsule variants. This can be potentially used for the identification of possible new capsule variants. We used genomic analyses, e.g. illumina whole genome sequencing, de novo assembly and cps comparative analysis on three clinical GBS sia (+) isolates, and found one isolate that encoded capsule genes from both CPS type IIa and type V. These isolates were found to be serologically distinct from other known CPS variants, suggesting a possible new CPS variant. These results present interesting avenues for future research.

The first research question is to investigate the chemical structure of the potential new capsular polysaccharide variant. The capsule polysaccharide preparation of the new variant could be structurally characterized by evaluating its molecular size, monosaccharide composition, and structural identity. To estimate the average molecular weight, size exclusion chromatography-HPLC could be done. High performance an ionic exchange chromatography-pulsed amperometric can be applied to detect monosaccharide residues composition in the capsule preparation. The configuration of the monosaccharides composing the repeating unit and sugar linkage-types analysis could be investigated.

Another research question is arising based on the genetic resembles of the *cps* gene cluster of the new variant to serotype IIa and V. “Does the chemical structure of CPS hybrid of IIa and V resemble both IIa and V?” The new variant polysaccharide repeating unit could be compared to other CPS types, specifically; type IIa and V to identify the new variant unique structure. At the genetic level, genes responsible for the capsular polysaccharide specificity among serotypes V, IIa, and the new variant need to be determined. As we reported in chapter

4, the *cps* operon of the CPS hybrid of IIa and V shared CPS-specific genes *cpsH*, *cpsJ*, and *cps*K and *cpsG*, *cpsH*, *cpsM*, *cpsN*, *cpsO*, *cpsK* and *cpsL* from both type IIa and V, respectively. The combination of CPS -specific genes from both types in one type is identified the first time in this study. This observation led me to hypothesize that the presence of these genes together could account for the structural diversity among serotypes IIa, V and the new variant. To test this hypothesis, episomal transfer of *cpsHJK* genes from type IIa to type V specific genes (*cpsHMNOK*) could be sufficient to drive the synthesis of the new variant GBS capsule polysaccharide. Expression of the new variant of CPS in the recipient and recombinant strain could be analyzed by latex agglutination and flow cytometry using sera raised against type IIa, V and the new variant.

### 6.6 Capsular switching among GBS *cps* loci

Horizontal gene transfer that is responsible for the synthesis and assembly of the CPS may occur en-bloc and cause a switch of GBS CPS type (10, 39, 52-54). It also has been proposed that capsular switching could be restricted to capsule-specific genes in GBS, by recombinational events. Genome comparative and MLST analysis presented in chapter 5 looked at the cps loci and MLST of seven housekeeping genes of the genome of the three GBS isolates sia (+)/ NT by real-time PCR GBS typing assay. This approach is expected to allow me to identify changes in the *cps* genes and genomic background of these isolates with high resolution, enabling the identification of new CPS type and capsular switching events. In our study, we identified both cases. PLGBS17 isolate, CPS hybrid of IIa and V, may have arisen through the introduction of CPSIIa-specific genes to replace CPS V-specific genes, but CPS IIa-specific genes integrated into CPS type V *cps* loci by recombination event. In our collection, only one isolate displayed this

hybrid *cps* genotype. This could be due to either a small rate of capsular transformation, the clearing of these variants from the population or a combination of both. The stability of GBS particular capsular polysaccharide structure may confer a survival advantage in its hosts (39). It also suggests that the rate of gene exchange in this species may be lower than that of other streptococci. It has been found that GBS has less diverse clonal structure compared to other streptococcal species, such as *S. pyogenes* (55) and *S. pneumoniae* (56). Genomic analysis of eight fully sequenced strains of GBS revealed that GBS has high rate of exchanging a large chromosomal fragments through conjugation (57). My research focusing on identifying new CPS types should allow future work to investigate whether this CPS type is more widely distributed. This could be achieved by examining a larger collection of GBS nontypeable isolates, through the use of new designed primers and probes to identify this new variant.

My analysis also provided evidence for the existence of capsular transformation in GBS and indicated that both capsular switching of the entire cps loci and the exchange of capsule-specific genes can occur. Taken together, our data support the existence of capsular gene exchange in GBS, but at a lower frequency. However, another study suggested that GBS capsular gene exchange occurs at high frequency among GBS isolates (58). The success of these new CPS types is possibly restrained by interactions between the new capsule and the ability of this isolate to survive and cause human infection. If immune pressure were an overwhelming selective force, we would expect to see the expansion of these capsule switching sub-lineages.

### 6.7 Complete genome of sequence type 1/serotype V isolates

Serotype V GBS isolates was first isolated from humans in 1975, and rates of invasive serotype V GBS disease significantly increased starting in the early 1990s (59). It has been found

that 92% of serotype V GBS strains isolated from the bloodstream of nonpregnant adults in the United States and Canada between 1992 and 2013 were ST 1. In chapter 5, elucidation of the complete genome of ST1/serotype V (PLGBS 13 strain) revealed that this strain had the highest homology with a GBS strain causing cow mastitis (60). The genome of this strain showed the highest homology to a ST1/serotype V published recently (61) and considered the second complete genome of ST 1 available in Genbank. The recent ST 1/serotype V strain is differs from serotype V strains isolated in the late 1970s by acquisition of cell surface proteins and antimicrobial resistance determinants (61).

### 6.8 Virulence gene diversity among Group B Streptococcal sequence type 1

While several studies have identified putative GBS virulence factors and their mechanisms in pathogenesis, little has been shown regarding the extent of genetic variability and the role of selection and recombination among diverse GBS strain populations. In my epidemiological survey, serotype V accounted for the majority of invasive GBS diseases among nonpregnat adult population (Fig 6.1). In chapter 5, single nucleotide polymorphism SNPs and recombinational analyses of GBS strains representing multilocus sequence types 1 were conducted. In chapter 5, I used PLGBS13 strain as a reference for whole-genome comparison, SNPs and recombinational analyses. Comparative methods were used to uncover allelic variation in a number of virulence genes and antimicrobial resistance profile. Then the genome of these isolates was analyzed for SNPs and for evidence of recombinations. SNP analysis was used to investigate the variability among ST1 with different CPS types. ST1 isolates with serotype V varied by a small number of SNPs whereas isolates with other serotypes (IIa and CPS hybrid of IIa and V) had a high number of SNPs. Recombinational analysis revealed that serotype IIa and CPS hybrid of IIa and V isolates

have experienced recombination regions including the *cps* gene cluster leading to the diversification of CPS types at the same lineages whereas ST1/serotype V isolates had small genetic changes in their genome. The extent of virulence gene diversity also differed among isolates with the same lineage revealing some extent of heterogeneity of the genomes in the same lineage. For instance, the studies ST1 lineage isolates contained differences in *scpB* and *alpha* like/*rib* genes, reflecting the independent divergence of this lineage. All ST 1 isolates studied in chapter 5 have the same GBS pilus type and presents a pilus as potential target in developing a vaccine-specific type V infection commonly among nonpregnant adults. Although vaccination remains an attractive approach to preventing disease, the inherent genetic variability of circulating GBS strains has made it challenging to identify universal vaccine targets. Continued efforts to identify novel antigenic proteins and to understand the mechanisms involved in pathogenesis is critical for developing new strategies for preventing GBS associated disease. Recombination of CPS type among different genotypes combined with the divergence of successful clones could potentially contribute to additional shifts, thereby influencing disease prevention protocols, particularly vaccination. Several GBS vaccine candidates have been investigated including the polysaccharide capsule [46], alpha [47] and beta [48] C proteins and Sip [49], which are promising alternatives to antibiotic chemoprophylaxis of women during childbirth. It is particularly important, however, for such vaccines to be multivalent and have an ability to recognize different genetic variants in order to effectively combat GBS disease.

As shown in chapter 5, *tetM* gene, was present in Tn916 in ST 1 isolates, but none of the isolates contained macrolide resistance elements. This is may be due to the small sample size included in our study. *ermB* gene was reported as one of the most found macrolide resistance

elements among macrolide resistant ST1 GBS isolates (62). On the other hand, all the isolates found to belong to ST 459/CPS type IV in chapter 2 were resistant to macrolide elements and tetracycline. This may explain the expansion of ST 459/CPS type IV clones in Alberta and globally (54, 63). It is believed that GBS has emerged as a successful human pathogen due in part to its ability to readily exchange genetic material and acquire virulence characteristics that have allowed it to persist in human populations. It has been proposed that the acquisition of resistance to antibiotic classes is the main driver for the emergence of ST1 GBS clones among adult cases. This also applies to other CCs which emerged as result of the expansion of tetracycline-resistant clones (62). It has been found that the increase in ST1/CPS type V GBS since the 1990s was primarily driven by the acquisition of genetic fragments including *tetM* and other genetic determinants that allowed for an increased capacity to cause disease in nonpregnant adults (61, 62). Da Cunha et al., discovered that a significant proportion of ST 1 strains contained the *ermB* (macrolide resistance element) present along with *tetM* in Tn3872 (62).

## 6.9 Conclusions

GBS infection surveillance studies that provide Information about the implementation and effectiveness of GBS preventive measures are critical. Hence, follow-up on GBS infections from all Canadian provinces is important. The developed typing system of GBS capsular polysaccharides by sialo-lectin and a real-time PCR GBS typing assay provides concordant results for typing GBS strains. Sialic acid detection performed with very good discrimination for GBS isolates with capsule on their surface. Real-time PCR for *cps* genotyping was shown to be superior to serological typing assays since it was able to type almost all strains. Applications of GBS typing system have the potential to lead to the identification of possible new CPS type. Genomic comparative and recombinational analyses of ST1, the most common type among nonpregnant adults revealed diversity among *cps* gene cluster within the same clone, affecting capsule-based vaccines.

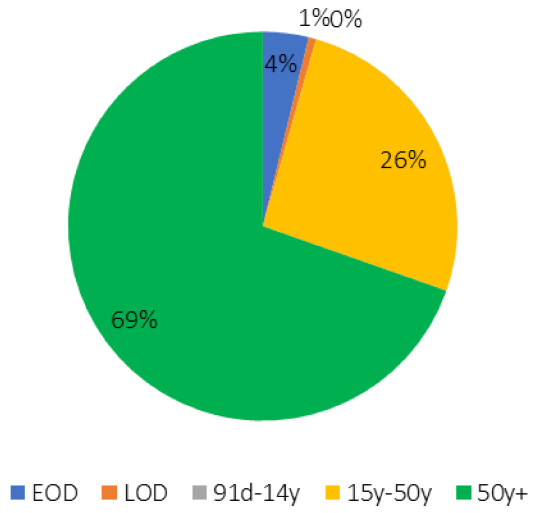



Figure 6.1. Distribution of nontypeable isolates among neonates (EOD and LOD), children (91d-14yrs), and adults (15y-50 yrs and >50 yrs)

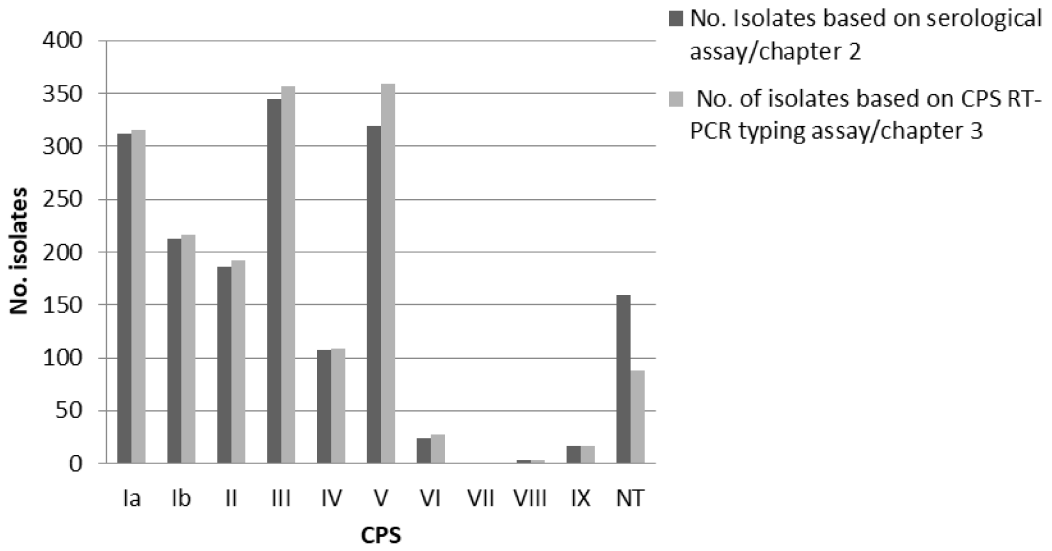



**Figure 6.2. Serotype distribution among iGBS isolates in Alberta.**
These isolates were assigned preliminary a capsule type by serological typing assay (chapter 2). Then, isolates that were identified as nontypeable isolates by serological assay were reassayed their CPS type using real-time PCR typing assay

## 6.10 References


1. **Verani JR, Schrag SJ.** 2010. Group B streptococcal disease in infants: progress in prevention and continued challenges. Clinics in perinatology **37:**375-392.
2. **Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.
3. **Tyrrell GJ, Senzilet LD, Spika JS, Kertesz DA, Alagaratnam M, Lovgren M, Talbot JA.** 2000. Invasive disease due to group B streptococcal infection in adults: results from a Canadian, population-based, active laboratory surveillance study--1996. Sentinel Health Unit Surveillance System Site Coordinators. J Infect Dis **182:**168-173.
4. **Money D, Allen VM.** 2013. The prevention of early-onset neonatal group B streptococcal disease. J Obstet Gynaecol Can **35:**939-951.
5. **Jefferies AL.** 2017. Management of term infants at increased risk for early-onset bacterial sepsis. Paediatrics & Child Health **22:**223-228.
6. **Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol **54**: 7 1774-1781.
7. **Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD.** 2009. Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47:**1143-1148.
8. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A,**

**Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

9. **Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ, Network ABCsEIP.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299:**2056-2065.

10. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol **52:** 1441-1447.

11. **Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

12. **Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of invasive group B streptococcus strains from the greater Toronto area, Canada. J Clin Microbiol **52:**1441-1447.

13. **Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556.

14. **Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological

surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

15. **Meehan M, Cunney R, Cafferkey M.** 2014. Molecular epidemiology of group B streptococci in Ireland reveals a diverse population with evidence of capsular switching. Eur J Clin Microbiol Infect Dis **33:**1155-1162.

16. **Wang P, Tong JJ, Ma XH, Song FL, Fan L, Guo CM, Shi W, Yu SJ, Yao KH, Yang YH.** 2015. Serotypes, antibiotic susceptibilities, and multi-locus sequence type profiles of Streptococcus agalactiae isolates circulating in Beijing, China. PLoS One **10:**e0120035.

17. **Ippolito DL, James WA, Tinnemore D, Huang RR, Dehart MJ, Williams J, Wingerd MA, Demons ST.** 2010. Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. BMC Infect Dis **10:**336.

18. **Melin P, Efstratiou A.** 2013. Group B streptococcal epidemiology and vaccine needs in developed countries. Vaccine **31 Suppl 4:**D31-42.

19. **Darenberg J, Luca-Harari B, Jasir A, Sandgren A, Pettersson H, Schalén C, Norgren M, Romanus V, Norrby-Teglund A, Normark BH.** 2007. Molecular and clinical characteristics of invasive group A streptococcal infection in Sweden. Clin Infect Dis **45:**450-458.

20. **Ferrieri P, Lynfield R, Creti R, Flores AE.** 2013. Serotype IV and invasive group B Streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis **19:**551-558.

21. **Martins ER, Melo-Cristino J, Ramirez M, Infections PGftSoS.** 2012. Dominance of serotype Ia among group B Streptococci causing invasive infections in nonpregnant adults in Portugal. J Clin Microbiol **50:**1219-1227.

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

23. **Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B.** 2011. Emergence of group B Streptococcus serotype IV in women of child-bearing age in Ireland. Epidemiol Infect **139:**236-238.

24. **Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nat Rev Microbiol **4:**932-942.

25. **Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P, Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M.** 2012. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis **31:**2097-2104.

26. **Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.

27. **Tazi A, Disson O, Bellais S, Bouaboud A, Dmytruk N, Dramsi S, Mistou MY, Khun H, Mechler C, Tardieux I, Trieu-Cuot P, Lecuit M, Poyart C.** 2010. The surface protein HvgA

mediates group B streptococcus hypervirulence and meningeal tropism in neonates. J Exp Med **207:**2313-2322.

28. **Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK.** 1989. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A **86:**4731-4735.

29. **Takahashi S, Adderson EE, Nagano Y, Nagano N, Briesacher MR, Bohnsack JF.** 1998. Identification of a highly encapsulated, genetically related group of invasive type III group B streptococci. J Infect Dis **177:**1116-1119.

30. **Ferrieri P, Baker C, Hillier S, Flores A.** 2004. Diversity of surface protein expression in group B streptococcal colonizing & invasive isolates. Indian Journal of Medical Research **119:**191.

31. **Nizet V, Ferrieri P, Rubens CE.** 2000. Molecular Pathogenesis of Group B Streptococcal. Streptococcal infections: clinical aspects, microbiology, and molecular pathogenesis**:**180.

32. **Bowers LD, Bigard X.** 2017. Achievements and Challenges in Anti-Doping Research, p 77-90, Acute Topics in Anti-Doping, vol 62. Karger Publishers.

33. **Wasik BR, Barnard KN, Parrish CR.** 2016. Effects of sialic acid modifications on virus binding and infection. Trends in microbiology **24:**991-1001.

34. **Varki NM, Varki A.** 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest **87:**851-857.

35. **Chaffin DO, Mentele LM, Rubens CE.** 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol **187:**4615-4626.

36. **Wessels MR, Haft RF, Heggen LM, Rubens CE.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun **60:**392-400.

37. **Brooks SA.** 2017. Lectin Histochemistry: Historical Perspectives, State of the Art, and the Future. Histochemistry of Single Molecules: Methods and Protocols**:**93-107.

38. **Geisler C, Jarvis DL.** 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology **21:**988-993.

39. **Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

40. **Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, Romano MR, Pinto V, Brogioni B, Torricelli G, Janulczyk R, Grandi G, Margarit I.** 2014. Structure of the type IX group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem **289:**23437-23448.

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

42. **Rogerieux F, Belaise M, Terzidis-Trabelsi H, Greffard A, Pilatte Y, Lambré CR.** 1993. Determination of the sialic acid linkage specificity of sialidases using lectins in a solid phase assay. Anal Biochem **211:**200-204.

43. **Duk M, Lisowska E, Wu JH, Wu AM.** 1994. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Anal Biochem **221:**266-272.

44. **Diamandis EP, Christopoulos TK.** 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem **37:**625-636.

45. **Kong F, Gowan S, Martin D, James G, Gilbert GL.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol **40:**216-226.

46. **Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL.** 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol **46:**2745-2750.

47. **Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae using multiplex PCR and reverse line blot hybridization. J Med Microbiol **54:**1133-1138.

48. **Sellin M, Olofsson C, Håkansson S, Norgren M.** 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol **38:**3420-3428.

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

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

51. **Breeding KM, Ragipani B, Lee KD, Malik M, Randis TM, Ratner AJ.** 2016. Real-time PCR-based serotyping of Streptococcus agalactiae. Sci Rep **6:**38523.

52. **Luan SL, Granlund M, Sellin M, Lagergard T, Spratt BG, Norgren M.** 2005. Multilocus sequence typing of Swedish invasive group B streptococcus isolates indicates a neonatally associated genetic lineage and capsule switching. J Clin Microbiol **43:**3727-3733.

53. **Neemuchwala A, Teatero S, Athey TB, McGeer A, Fittipaldi N.** 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B Streptococcus. Emerg Infect Dis **22:**1941-1944.

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

55. **Friães A, Ramirez M, Melo-Cristino J, Infections PGftSoS.** 2007. Nonoutbreak surveillance of group A streptococci causing invasive disease in Portugal identified internationally disseminated clones among members of a genetically heterogeneous population. J Clin Microbiol **45:**2044-2047.

56. **Serrano I, Melo-Cristino J, Carriço J, Ramirez M.** 2005. Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal. J Clin Microbiol **43:**1706-1715.

57. **Brochet M, Rusniok C, Couvé E, Dramsi S, Poyart C, Trieu-Cuot P, Kunst F, Glaser P.** 2008. Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of Streptococcus agalactiae. Proc Natl Acad Sci U S A **105:**15961-15966.

58. **Martins ER, Melo-Cristino J, Ramirez M.** 2010. Evidence for rare capsular switching in Streptococcus agalactiae. J Bacteriol **192:**1361-1369.

59. **Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis **173:**365-373.

60. **Zubair S, de Villiers EP, Fuxelius HH, Andersson G, Johansson KE, Bishop RP, Bongcam-Rudloff E.** 2013. Genome Sequence of Streptococcus agalactiae Strain 09mas018883, Isolated from a Swedish Cow. Genome Announc **1**.

61. **Flores AR, Galloway-Peña J, Sahasrabhojane P, Saldaña M, Yao H, Su X, Ajami NJ, Holder ME, Petrosino JF, Thompson E, Margarit Y Ros I, Rosini R, Grandi G, Horstmann N, Teatero S, McGeer A, Fittipaldi N, Rappuoli R, Baker CJ, Shelburne SA.** 2015. Sequence type 1 group B Streptococcus, an emerging cause of invasive disease in adults, evolves by small genetic changes. Proc Natl Acad Sci U S A **112:**6431-6436.

62. **Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P.** 2015. Corrigendum: Streptococcus agalactiae clones infecting

humans were selected and fixed through the extensive use of tetracycline. Nat Commun **6:**6108.

63. **Campisi E, Rinaudo CD, Donati C, Barucco M, Torricelli G, Edwards MS, Baker CJ, Margarit I, Rosini R.** 2016. Serotype IV Streptococcus agalactiae ST-452 has arisen from large genomic recombination events between CC23 and the hypervirulent CC17 lineages. Sci Rep **6:**29799.

## Bibliography

**Afshar B, Broughton K, Creti R, Decheva A, Hufnagel M, Kriz P, Lambertsen L, Lovgren M, Melin P, Orefici G, Poyart C, Radtke A, Rodriguez-Granger J, Sørensen UB, Telford J, Valinsky L, Zachariadou L, Efstratiou A, Group MotDS.** 2011. International external quality assurance for laboratory identification and 264

**Achtman M.** 2008. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. Annu Rev Microbiol **62:**53-70.

**Afshar B, Broughton K, Creti R, Decheva A, Hufnagel M, Kriz P, Lambertsen L, Lovgren M, Melin P, Orefici G, Poyart C, Radtke A, Rodriguez-Granger J, Sørensen UB, Telford J, Valinsky L, Zachariadou L, Efstratiou A, Group MotDS.** 2011. International external quality assurance for laboratory identification and typing of Streptococcus

**Aguiar S, Serrano I, Pinto F, Melo-Cristino J, Ramirez M.** 2008. Changes in Streptococcus pneumoniae serotypes causing invasive disease with non-universal vaccination coverage of the seven-valent conjugate vaccine. Clinical Microbiology and Infection **14:**835-843.

**Alhhazmi A, Hurteau D, Tyrrell GJ.** 2016. Epidemiology of Invasive Group B Streptococcal Disease in Alberta, Canada 2003-2013. J Clin Microbiol **54**: 7 1774-1781.

**Alhhazmi A, Pandey A, Tyrrell GJ.** 2017. Identification of the Group B Streptococcus Capsule Type using a Dual Phenotypic/Genotypic Assay. J Clin Microbiol **55**:2637-2650.

**Alper I, Frenette M, Labrie S.** 2013. Genetic diversity of dairy Geotrichum candidum strains revealed by multilocus sequence typing. Appl Microbiol Biotechnol **97:**5907-5920.

**Amin A, Abdulrazzaq Y, Uduman S.** 2002. Group B streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. Journal of Infection **45:**42-46. 72

**Amundson NR, Flores AE, Hillier SL, Baker CJ, Ferrieri P.** 2005. DNA macrorestriction analysis of nontypeable group B streptococcal isolates: clonal evolution of nontypeable and type V isolates. J Clin Microbiol **43:**572-576.

and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. Journal of clinical microbiology. 2015;53(9):2919-26.

**Arakere G, Flores AE, Ferrieri P, Frasch CE.** 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B streptococcal isolates. J Clin Microbiol **37:**2564-2567.

**Baker C, Edwards M.** 2003. Group B streptococcal conjugate vaccines. Archives of disease in childhood **88:**375-378.

**Baker CJ, Barrett FF.** 1973. Transmission of group B streptococci among parturient women and their neonates. J Pediatr **83:**919-925.

**Baker CJ, Barrett FF.** 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA **230:**1158-1160.

**Baker CJ, Byington CL, Polin RA.** 2011. Policy statement-Recommendations for the prevention of perinatal group B streptococcal (GBS) disease. Pediatrics **128:**611-616.

**Baker CJ, Paoletti LC, Wessels MR, Guttormsen H-K, Rench MA, Hickman ME, Kasper DL.** 1999. Safety and immunogenicity of capsular polysaccharide—tetanus toxoid conjugate vaccines for group B streptococcal types Ia and Ib. Journal of Infectious Diseases **179:**142-150.

**Baker CJ, Rench MA, Edwards MS, Carpenter RJ, Hays BM, Kasper DL.** 1988. Immunization of pregnant women with a polysaccharide vaccine of group B streptococcus. New England Journal of Medicine **319:**1180-1185.

**Baker CJ, Rench MA, McInnes P.** 2003. Immunization of pregnant women with group B streptococcal type III capsular polysaccharide-tetanus toxoid conjugate vaccine. Vaccine **21:**3468-3472.

**Baltimore RS, Huie SM, Meek JI, Schuchat A, O'Brien KL.** 2001. Early-onset neonatal sepsis in the era of group B streptococcal prevention. Pediatrics **108:**1094-1098.

**Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta obstetricia et gynecologica Scandinavica **87:**260-271.

**Barcaite E, Bartusevicius A, Tameliene R, Kliucinskas M, Maleckiene L, Nadisauskiene R.** 2008. Prevalence of maternal group B streptococcal colonisation in European countries. Acta Obstet Gynecol Scand **87:**260-271.

**Bedford H, de Louvois J, Halket S, Peckham C, Hurley R, Harvey D.** 2001. Meningitis in infancy in England and Wales: follow up at age 5 years. BMJ **323:**533-536.

**Beier D, Gross R.** 2006. Regulation of bacterial virulence by two-component systems. Current opinion in microbiology **9:**143-152. 68

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

**Berardi A, Rossi C, Lugli L, Creti R, Bacchi Reggiani ML, Lanari M, Memo L, Pedna MF, Venturelli C, Perrone E, Ciccia M, Tridapalli E, Piepoli M, Contiero R, Ferrari F, GBS Prevention Working Group Ei-R.** 2013. Group B streptococcus late-onset disease: 2003-2010. Pediatrics **131:**e361-368.

**Beres SB, Carroll RK, Shea PR, Sitkiewicz I, Martinez-Gutierrez JC, Low DE, McGeer A, Willey BM, Green K, Tyrrell GJ, Goldman TD, Feldgarden M, Birren BW, Fofanov Y, Boos J, Wheaton WD, Honisch C, Musser JM.** 2010. Molecular complexity of successive bacterial epidemics deconvoluted by comparative pathogenomics. Proc Natl Acad Sci U S A **107:**4371-4376.

**Bergh K, Stoelhaug A, Loeseth K, Bevanger L.** 2004. Detection of group B streptococci (GBS) in vaginal swabs using real-time PCR with TaqMan probe hybridization. Indian J Med Res **119 Suppl:**221-223.

**Bergseng H, Afset J, Radtke A, Loeseth K, Lyng R, Rygg M, Bergh K.** 2009. Molecular and phenotypic characterization of invasive group B streptococcus strains from infants in Norway 2006–2007. Clinical Microbiology and Infection **15:**1182-1185.

**Bergseng H, Bevanger L, Rygg M, Bergh K.** 2007. Real-time PCR targeting the sip gene for detection of group B Streptococcus colonization in pregnant women at delivery. J Med Microbiol **56:**223-228.

**Berkley JA, Lowe BS, Mwangi I, Williams T, Bauni E, Mwarumba S, Ngetsa C, Slack MP, Njenga S, Hart CA, Maitland K, English M, Marsh K, Scott JA.** 2005. Bacteremia among children admitted to a rural hospital in Kenya. N Engl J Med **352:**39-47.

**Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, et al.** Structure of the type IX group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. The Journal of biological chemistry. 2014;289(34):23437-48.

**Berti F, Campisi E, Toniolo C, Morelli L, Crotti S, Rosini R, Romano MR, Pinto V, Brogioni B, Torricelli G, Janulczyk R, Grandi G, Margarit I.** 2014. Structure of the type IX group B Streptococcus capsular polysaccharide and its evolutionary relationship with types V and VII. J Biol Chem **289:**23437-23448.

**Betriu C, Culebras E, Gómez M, Rodríguez-Avial I, Sánchez BA, Agreda MC, Picazo JJ.** 2003. Erythromycin and clindamycin resistance and telithromycin susceptibility in Streptococcus agalactiae. Antimicrob Agents Chemother **47:**1112-1114.

**Bisharat N, Crook DW, Leigh J, Harding RM, Ward PN, Coffey TJ, Maiden MC, Peto T, Jones N.** 2004. Hyperinvasive neonatal group B streptococcus has arisen from a bovine ancestor. J Clin Microbiol **42:**2161-2167.

**Blumberg HM, Stephens DS, Licitra C, Pigott N, Facklam R, Swaminathan B, Wachsmuth IK.** 1992. Molecular epidemiology of group B streptococcal infections: use of restriction endonuclease analysis of chromosomal DNA and DNA restriction fragment length polymorphisms of ribosomal RNA genes (ribotyping). Journal of Infectious Diseases **166:**574-579.

**Blumberg HM, Stephens DS, Modansky M, Erwin M, Elliot J, Facklam RR, Schuchat A, Baughman W, Farley MM.** 1996. Invasive group B streptococcal disease: the emergence of serotype V. J Infect Dis **173:**365-373. 146

**Bohnsack JF, Whiting A, Gottschalk M, Dunn DM, Weiss R, Azimi PH, Philips JB, Weisman LE, Rhoads GG, Lin F-YC.** 2008. Population structure of invasive and colonizing strains of Streptococcus agalactiae from neonates of six US Academic Centers from 1995 to 1999. J Clin Microbiol **46:**1285-1291.

**Bolotin S, Fuller JD, Bast DJ, Beveridge TJ, de Azavedo JC.** Capsule expression regulated by a two-component signal transduction system in Streptococcus iniae. FEMS Immunol Med Microbiol. 2007;50(3):366-74. Epub 2007/05/30.

**Borchardt SM, DeBusscher JH, Tallman PA, Manning SD, Marrs CF, Kurzynski TA, Foxman B.** 2006. Frequency of antimicrobial resistance among invasive and colonizing Group B streptococcal isolates. BMC Infect Dis **6:**57.

**Bowers LD, Bigard X.** 2017. Achievements and Challenges in Anti-Doping Research, p 77-90, Acute Topics in Anti-Doping, vol 62. Karger Publishers.

**Boyer KM, Gotoff SP.** 1986. Prevention of early-onset neonatal group B streptococcal disease with selective intrapartum chemoprophylaxis. New England Journal of Medicine **314:**1665-1669.

**Breeding KM, Ragipani B, Lee KD, Malik M, Randis TM, Ratner AJ.** 2016. Real-time PCR-based serotyping of Streptococcus agalactiae. Sci Rep **6:**38523.

**Brigtsen AK, Dedi L, Melby KK, Holberg-Petersen M, Radtke A, Lyng RV, Andresen LL, Jacobsen AF, Fugelseth D, Whitelaw A.** 2015. Comparison of PCR and serotyping of Group B Streptococcus in pregnant women: the Oslo GBS-study. J Microbiol Methods **108:**31-35.

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

**Brochet M, Couvé E, Glaser P, Guédon G, Payot S.** 2008. Integrative conjugative elements and related elements are major contributors to the genome diversity of Streptococcus agalactiae. J Bacteriol **190:**6913-6917.

**Brochet M, Couvé E, Zouine M, Vallaeys T, Rusniok C, Lamy M-C, Buchrieser C, Trieu-Cuot P, Kunst F, Poyart C.** 2006. Genomic diversity and evolution within the species Streptococcus agalactiae. Microbes and Infection **8:**1227-1243.

**Brochet M, Rusniok C, Couvé E, Dramsi S, Poyart C, Trieu-Cuot P, Kunst F, Glaser P.** 2008. Shaping a bacterial genome by large chromosomal replacements, the evolutionary history of Streptococcus agalactiae. Proceedings of the National Academy of Sciences **105:**15961-15966.

**Brodeur BR, Boyer M, Charlebois I, Hamel J, Couture F, Rioux CR, Martin D.** 2000. Identification of group B streptococcal Sip protein, which elicits cross-protective immunity. Infect Immun **68:**5610-5618.

**Brooks SA.** 2017. Lectin Histochemistry: Historical Perspectives, State of the Art, and the Future. Histochemistry of Single Molecules: Methods and Protocols**:**93-107.

**Brueggemann AB, Griffiths DT, Meats E, Peto T, Crook DW, Spratt BG.** 2003. Clonal relationships between invasive and carriage Streptococcus pneumoniae and serotype-and clone-specific differences in invasive disease potential. Journal of Infectious Diseases **187:**1424-1432.

**Brzychczy-Włoch M, Gosiewski T, Bodaszewska M, Pabian W, Bulanda M, Kochan P, Strus M, Heczko PB.** 2010. Genetic characterization and diversity of Streptococcus agalactiae isolates with macrolide resistance. J Med Microbiol **59:**780-786.

**Calandra GB, Cole RM.** 1980. Lysis and protoplast formation of group B streptococci by mutanolysin. Infect Immun **28:**1033-1037.

**Campbell JR, Hillier SL, Krohn MA, Ferrieri P, Zaleznik DF, Baker CJ.** 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery. Obstet Gynecol **96:**498-503.

**Campisi E, Rinaudo CD, Donati C, Barucco M, Torricelli G, Edwards MS, Baker CJ, Margarit I, Rosini R.** 2016. Serotype IV Streptococcus agalactiae ST-452 has arisen from large genomic recombination events between CC23 and the hypervirulent CC17 lineages. Sci Rep **6:**29799.

**Campisi E, Rinaudo CD, Donati C, Barucco M, Torricelli G, Edwards MS, Baker CJ, Margarit I, Rosini R.** 2016. Serotype IV Streptococcus agalactiae ST-452 has arisen from large genomic recombination events between CC23 and the hypervirulent CC17 lineages. Sci Rep **6:**29799.

**Carlin AF, Chang YC, Areschoug T, Lindahl G, Hurtado-Ziola N, King CC**. 2009. Group B Streptococcus suppression of phagocyte functions by protein-mediated engagement of human Siglec-5. The Journal of experimental medicine. 206(8):1691-9.

**Casali N, Nikolayevskyy V, Balabanova Y, Harris SR, Ignatyeva O, Kontsevaya I, Corander J, Bryant J, Parkhill J, Nejentsev S, Horstmann RD, Brown T, Drobniewski F.** 2014. Evolution and transmission of drug-resistant tuberculosis in a Russian population. Nat Genet **46:**279-286.

**Castor ML, Whitney CG, Como-Sabetti K, Facklam RR, Ferrieri P, Bartkus JM, Juni BA, Cieslak PR, Farley MM, Dumas NB.** 2009. Antibiotic resistance patterns in invasive group B streptococcal isolates. Infect Dis Obstet Gynecol. **2008**:727505.

**CDC.** 2005. Early-onset and late-onset neonatal group B streptococcal disease--United States, 1996-2004. MMWR Morb Mortal Wkly Rep **54:**1205-1208.

**Chaffin DO, Beres SB, Yim HH, Rubens CE.** 2000.The serotype of type Ia and III group B streptococci is determined by the polymerase gene within the polycistronic capsule operon. J Bacteriol.;182(16):4466-4477.

**Chaffin DO, Mentele LM, Rubens CE.** 2005. Sialylation of group B streptococcal capsular polysaccharide is mediated by cpsK and is required for optimal capsule polymerization and expression. J Bacteriol **187:**4615-4626.

**Chen KT, Puopolo KM, Eichenwald EC, Onderdonk AB, Lieberman E.** 2005. No increase in rates of early-onset neonatal sepsis by antibiotic-resistant group B 61

**Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, Pessia A, Aanensen DM, Mather AE, Page AJ, Salter SJ, Harris D, Nosten F, Goldblatt D, Corander J, Parkhill J, Turner P, Bentley SD.** 2014. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet **46:**305-309.

**Chewapreecha C, Harris SR, Croucher NJ, Turner C, Marttinen P, Cheng L, Pessia A, Aanensen DM, Mather AE, Page AJ, Salter SJ, Harris D, Nosten F, Goldblatt D, Corander J, Parkhill J, Turner P, Bentley SD.** 2014. Dense genomic sampling identifies highways of pneumococcal recombination. Nat Genet **46:**305-309.

**Chopra I, Roberts M.** 2001. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. Microbiology and molecular biology reviews **65:**232-260.

**Cieslewicz MJ, Chaffin D, Glusman G, Kasper D, Madan A, Rodrigues S, Fahey J, Wessels MR, Rubens CE.** 2005. Structural and genetic diversity of group B streptococcus capsular polysaccharides. Infect Immun **73:**3096-3103.

**Cieslewicz MJ, Kasper DL, Wang Y, Wessels MR.** 2001. Functional Analysis in Type Ia Group B Streptococcusof a Cluster of Genes Involved in Extracellular Polysaccharide Production by Diverse Species of Streptococci. Journal of Biological Chemistry **276:**139-146.

**Cooper JE, Feil EJ.** 2004. Multilocus sequence typing–what is resolved? Trends in microbiology **12:**373-377.

**Creti R, Imperi M, Pataracchia M, Alfarone G, Recchia S, Baldassarri L.** 2012. Identification and molecular characterization of a S. agalactiae strain lacking the capsular locus. Eur J Clin Microbiol Infect Dis **31:**233-235.

**Culebras E, Rodriguez-Avial I, Betriu C, Redondo M, Picazo JJ.** 2002. Macrolide and tetracycline resistance and molecular relationships of clinical strains of Streptococcus agalactiae. Antimicrob Agents Chemother **46:**1574-1576.

**Cutland CL, Madhi SA, Zell ER, Kuwanda L, Laque M, Groome M, Gorwitz R, Thigpen MC, Patel R, Velaphi SC, Adrian P, Klugman K, Schuchat A, Schrag SJ, Team PT.** 2009. Chlorhexidine maternal-vaginal and neonate body wipes in sepsis and vertical transmission of pathogenic bacteria in South Africa: a randomised, controlled trial. Lancet **374:**1909-1916. 58

**Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P, Consortium D.**

2014. Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **5:**4544.

**Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P.** 2015. Corrigendum: Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **6:**6108.

**Da Cunha V, Davies MR, Douarre PE, Rosinski-Chupin I, Margarit I, Spinali S, Perkins T, Lechat P, Dmytruk N, Sauvage E, Ma L, Romi B, Tichit M, Lopez-Dahesh S, Hensler ME, Van Sorge NM, Gertz RE, Schrag S, Nizet V, Beall BW.** 2008. Point mutation in the group B streptococcal pbp2x gene conferring decreased susceptibility to β-lactam antibiotics. Antimicrob Agents Chemother **52:**2915-2918.

**Daly JA, Seskin KC.** 1988. Evaluation of rapid, commercial latex techniques for serogrouping beta-hemolytic streptococci. J Clin Microbiol **26:**2429-2431.

**Daniels JP, Gray J, Pattison HM, Gray R, Hills RK, Khan KS, Group GC.** 2011. Intrapartum tests for group B streptococcus: accuracy and acceptability of screening. BJOG **118:**257-265. 59

**Darenberg J, Luca-Harari B, Jasir A, Sandgren A, Pettersson H, Schalén C, Norgren M, Romanus V, Norrby-Teglund A, Normark BH.** 2007. Molecular and clinical characteristics of invasive group A streptococcal infection in Sweden. Clin Infect Dis **45:**450-458.

**Darling AE, Mau B, Perna NT.** 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One **5:**e11147.

**Darlow B, Campbell N, Austin N, Chin A, Grigg C, Skidmore C, Voss L, Walls T, Wise M, Werno A.** 2015. The prevention of early-onset neonatal group B streptococcus infection: New Zealand Consensus Guidelines 2014.

**Davies HD, Adair CE, Schuchat A, Low DE, Sauve RS, McGeer A.** 2001. Physicians' prevention practices and incidence of neonatal group B streptococcal disease in 2 Canadian regions. CMAJ **164:**479-485. .

**Davies HD, Jones N, Whittam TS, Elsayed S, Bisharat N, Baker CJ.** 2004. Multilocus sequence typing of serotype III group B streptococcus and correlation with pathogenic potential. Journal of Infectious Diseases **189:**1097-1102.

**Davies HD, Leblanc J, Bortolussi R, McGeer A, PICNIC.** 1999. The Pediatric Investigators Collaborative Network on Infections in Canada (PICNIC) study of neonatal group B streptococcal infections in Canada. Paediatr Child Health **4:**257-263.

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

**de Azavedo JC, McGavin M, Duncan C, Low DE, McGeer A.** 2001. Prevalence and mechanisms of macrolide resistance in invasive and noninvasive group B streptococcus isolates from Ontario, Canada. Antimicrob Agents Chemother **45:**3504-3508.

**de Steenwinkel FD, Tak HV, Muller AE, Nouwen JL, Oostvogel PM, Mocumbi SM.** 2008. Low carriage rate of group B streptococcus in pregnant women in Maputo, Mozambique. Trop Med Int Health **13:**427-429.

**Di Palo B, Rippa V, Santi I, Brettoni C, Muzzi A, Metruccio MM, Grifantini R, Telford JL, Paccani SR, Soriani M.** 2013. Adaptive response of Group B streptococcus to high glucose conditions: new insights on the CovRS regulation network. PLoS One **8:**e61294.

**Diamandis EP, Christopoulos TK.** 1991. The biotin-(strept)avidin system: principles and applications in biotechnology. Clin Chem **37:**625-636.

**Didelot X, Falush D.** 2007. Inference of bacterial microevolution using multilocus sequence data. Genetics **175:**1251-1266.

**Diedrick MJ, Flores AE, Hillier SL, Creti R, Ferrieri P.** 2010. Clonal analysis of colonizing group B Streptococcus, serotype IV, an emerging pathogen in the United States. J Clin Microbiol **48:**3100-3104.

**Dillon HC, Gray E, Pass MA, Gray BM.** 1982. Anorectal and vaginal carriage of group B streptococci during pregnancy. J Infect Dis **145:**794-799.

**DiPersio LP, DiPersio JR.** 2007. Identification of an erm (T) gene in strains of inducibly clindamycin-resistant group B Streptococcus. Diagn Microbiol Infect Dis **57:**189-193.

**Diseases CoI, Fetus Co, Newborn.** 1997. Revised guidelines for prevention of early-onset group B streptococcal (GBS) infection. Pediatrics **99:**489-496.

**Dmitriev A, Shen A, Shen X, Yang Y.** 2004. ISSa4-based differentiation of Streptococcus agalactiae strains and identification of multiple target sites for ISSa4 insertions. J Bacteriol **186:**1106-1109.

**Dmitriev AV, Shakleina EV.** 2003. [Methods of molecular epidemiology of infections caused by group B streptococci]. Zh Mikrobiol Epidemiol Immunobiol**:**83-92.

**Dogan B, Schukken Y, Santisteban C, Boor KJ.** 2005. Distribution of serotypes and antimicrobial resistance genes among Streptococcus agalactiae isolates from bovine and human hosts. J Clin Microbiol **43:**5899-5906.

**Domelier AS, van der Mee-Marquet N, Arnault L, Mereghetti L, Lanotte P, Rosenau A, Lartigue MF, Quentin R.** 2008. Molecular characterization of erythromycin-resistant Streptococcus agalactiae. J Antimicrob Chemother **62:**1227-1233.

**Doran KS, Nizet V.** 2004. Molecular pathogenesis of neonatal group B streptococcal infection: no longer in its infancy. Molecular microbiology **54:**23-31.

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

**Duarte RS, Bellei BC, Miranda OP, Brito MA, Teixeira LM.** 2005. Distribution of antimicrobial resistance and virulence-related genes among Brazilian group B streptococci recovered from bovine and human sources. Antimicrob Agents Chemother **49:**97-103.

**Duk M, Lisowska E, Wu JH, Wu AM.** 1994. The biotin/avidin-mediated microtiter plate lectin assay with the use of chemically modified glycoprotein ligand. Anal Biochem **221:**266-272. 151

**D'Urzo N, Martinelli M, Pezzicoli A, De Cesare V, Pinto V, Margarit I.** 2014. Acidic pH strongly enhances in vitro biofilm formation by a subset of hypervirulent ST-17 Streptococcus agalactiae strains. Appl Environ Microbiol. 80(7):2176-85.

**Edgar RC.** 2004. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics **5:**113.

**Edmond KM, Kortsalioudaki C, Scott S, Schrag SJ, Zaidi AK, Cousens S, Heath PT.** 2012. Group B streptococcal disease in infants aged younger than 3 months: systematic review and meta-analysis. Lancet **379:**547-556. 260

**Edwards M, Kasper D, Jennings H, Baker C, Nicholson-Weller A.** 1982. Capsular sialic acid prevents activation of the alternative complement pathway by type III, group B streptococci. The Journal of Immunology **128:**1278-1283.

**Edwards MS, Baker CJ.** 2005. Group B streptococcal infections in elderly adults. Clin Infect Dis **41:**839-847.

**EDWARDS MS.** 1990. Group B streptococcal infections. The Pediatric Infectious Disease Journal **9:**779-780.

**Ekin IH, Gurturk K.** 2006. Characterization of bovine and human group B streptococci isolated in Turkey. J Med Microbiol **55:**517-521.

**Elliott JA, Facklam RR, Richter CB.** 1990. Whole-cell protein patterns of nonhemolytic group B, type Ib, streptococci isolated from humans, mice, cattle, frogs, and fish. J Clin Microbiol **28:**628-630.

**Enright MC, Spratt BG, Kalia A, Cross JH, Bessen DE.** 2001. Multilocus sequence typing of Streptococcus pyogenes and the relationships between emm type and clone. Infect Immun **69:**2416-2427.

**Evans JJ, Klesius PH, Pasnik DJ, Bohnsack JF.** 2009. Human Streptococcus agalactiae isolate in Nile tilapia (Oreochromis niloticus). Emerg Infect Dis **15:**774-776.

**Evans JJ, Pasnik DJ, Klesius PH, Al-Ablani S.** 2006. First report of Streptococcus agalactiae and Lactococcus garvieae from a wild bottlenose dolphin (Tursiops truncatus). J Wildl Dis **42:**561-569.

**Facklam RR, Padula JF, Wortham EC, Cooksey RC, Rountree HA.** 1979. Presumptive identification of group A, B, and D streptococci on agar plate media. J Clin Microbiol **9:**665-672.

**Farley MM, Harvey RC, Stull T, Smith JD, Schuchat A, Wenger JD, Stephens DS.** 1993. A population-based assessment of invasive disease due to group B Streptococcus in nonpregnant adults. N Engl J Med **328:**1807-1811.

**Farley MM.** 2001. Group B streptococcal disease in nonpregnant adults. Clin Infect Dis **33:**556-561.

**Feil EJ, Enright MC.** 2004. Analyses of clonality and the evolution of bacterial pathogens. Current opinion in microbiology **7:**308-313.

**Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG.** 2004. eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data. J Bacteriol **186:**1518-1530.

**Ferrieri P, Baker C, Hillier S, Flores A.** 2004. Diversity of surface protein expression in group B streptococcal colonizing & invasive isolates. Indian Journal of Medical Research **119:**191.

**Ferrieri P, Blair LL.** 1977. Pharyngeal carriage of group B streptococci: detection by three methods. J Clin Microbiol **6:**136-139.

**Ferrieri P, Hillier SL, Krohn MA, Moore D, Paoletti LC, Flores AE**. 2004.Characterization of vaginal & rectal colonization with multiple serotypes of group B streptococci using multiple colony picks. Indian J Med Res.119:208-212.

**Ferrieri P, Lynfield R, Creti R, Flores AE.** 2013. Serotype IV and invasive group B Streptococcus disease in neonates, Minnesota, USA, 2000-2010. Emerg Infect Dis **19:**551-558.

**Figueira-Coelho J, Ramirez M, Salgado M, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microbial Drug Resistance **10:**31-36.

**Figueira-Coelho J, Ramirez M, Salgado MJ, Melo-Cristino J.** 2004. Streptococcus agalactiae in a large Portuguese teaching hospital: antimicrobial susceptibility, serotype distribution, and clonal analysis of macrolide-resistant isolates. Microb Drug Resist **10:**31-36.

**Flores AR, Galloway-Peña J, Sahasrabhojane P, Saldaña M, Yao H, Su X, Ajami NJ, Holder ME, Petrosino JF, Thompson E, Margarit Y Ros I, Rosini R, Grandi G, Horstmann N, Teatero S, McGeer A, Fittipaldi N, Rappuoli R, Baker CJ, Shelburne SA.** 2015. Sequence type 1 group B Streptococcus, an emerging cause of invasive disease in adults, evolves by small genetic changes. Proc Natl Acad Sci U S A **112:**6431-6436.

**Florindo C, Damiao V, Silvestre I, Farinha C, Rodrigues F, Nogueira F, Martins-Pereira F, Castro R, Borrego MJ, Santos-Sanches I, Infection GftPoNG.** 2014. Epidemiological surveillance of colonising group B Streptococcus epidemiology in the Lisbon and Tagus Valley regions, Portugal (2005 to 2012): emergence of a new epidemic type IV/clonal complex 17 clone. Euro Surveill **19**.

**Fluegge K, Supper S, Siedler A, Berner R.** 2004. Antibiotic susceptibility in neonatal invasive isolates of Streptococcus agalactiae in a 2-year nationwide surveillance study in Germany. Antimicrob Agents Chemother **48:**4444-4446. 63

**Fluegge K, Wons J, Spellerberg B, Swoboda S, Siedler A, Hufnagel M, Berner R.** 2011. Genetic differences between invasive and noninvasive neonatal group B streptococcal isolates. The Pediatric Infectious Disease Journal **30:**1027-1031.

**Foxman B, Gillespie BW, Manning SD, Marrs CF.** 2007. Risk factors for group B streptococcal colonization: potential for different transmission systems by capsular type. Ann Epidemiol **17:**854-862.

**Foxman B, Zhang L, Koopman JS, Manning SD, Marrs CF.** 2005. Choosing an appropriate bacterial typing technique for epidemiologic studies. Epidemiologic perspectives & innovations **2:**10.

**Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718.

**Franciosi RA, Knostman JD, Zimmerman RA.** 1973. Group B streptococcal neonatal and infant infections. J Pediatr **82:**707-718. 262

**Franken C, Haase G, Brandt C, Weber-Heynemann J, Martin S, Lämmler C, Podbielski A, Lütticken R, Spellerberg B.** 2001. Horizontal gene transfer and host specificity of beta-haemolytic streptococci: the role of a putative composite transposon containing scpB and lmb. Molecular microbiology **41:**925-935.

**Friães A, Ramirez M, Melo-Cristino J, Infections PGftSoS.** 2007. Nonoutbreak surveillance of group A streptococci causing invasive disease in Portugal identified internationally disseminated clones among members of a genetically heterogeneous population. J Clin Microbiol **45:**2044-2047. 266

**Garland SM, Kelly N, Ugoni AM.** 2000. Is antenatal group B streptococcal carriage a predictor of adverse obstetric outcome? Infect Dis Obstet Gynecol **8:**138-142.

**Geisler C, Jarvis DL.** 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology **21:**988-993.

**Geisler C, Jarvis DL.** 2011. Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology **21:**988-993.

**Geng Y, Wang KY, Huang XL, Chen DF, Li CW, Ren SY, Liao YT, Zhou ZY, Liu QF, Du ZJ, Lai WM.** 2012. Streptococcus agalactiae, an emerging pathogen for cultured ya-fish, Schizothorax prenanti, in China. Transbound Emerg Dis **59:**369-375.

**Gherardi G, Imperi M, Baldassarri L, Pataracchia M, Alfarone G, Recchia S, Orefici G, Dicuonzo G, Creti R.** 2007. Molecular epidemiology and distribution of serotypes, surface proteins, and antibiotic resistance among group B streptococci in Italy. J Clin Microbiol **45:**2909-2916.

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

**Glaser P, Rusniok C, Buchrieser C, Chevalier F, Frangeul L, Msadek T, Zouine M, Couvé E, Lalioui L, Poyart C, Trieu-Cuot P, Kunst F.** 2002. Genome sequence of Streptococcus agalactiae, a pathogen causing invasive neonatal disease. Mol Microbiol **45:**1499-1513.

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

**Granlund M, Öberg L, Sellin M, Norgren M.** 1998. Identification of a novel insertion element, IS1548, in group B streptococci, predominantly in strains causing endocarditis. Journal of Infectious Diseases **177:**967-976.

**Gray KJ, Bennett SL, French N, Phiri AJ, Graham SM.** 2007. Invasive group B streptococcal infection in infants, Malawi. Emerg Infect Dis **13:**223-229. 60

**Green PA, Singh KV, Murray BE, Baker CJ.** 1994. Recurrent group B streptococcal infections in infants: clinical and microbiologic aspects. J Pediatr **125:**931-938.

**Grimwood K, Stone PR, Gosling IA, Green R, Darlow BA, Lennon DR, Martin DR.** 2002. Late antenatal carriage of group B Streptococcus by New Zealand women. Aust N Z J Obstet Gynaecol **42:**182-186.

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

**Gygax SE, Schuyler JA, Kimmel LE, Trama JP, Mordechai E, Adelson ME.** 2006. Erythromycin and clindamycin resistance in group B streptococcal clinical isolates. Antimicrob Agents Chemother **50:**1875-1877.

**Hacker J, Kaper JB.** 2000. Pathogenicity islands and the evolution of microbes. Annual Reviews in Microbiology **54:**641-679.

**Håkansson S, Burman LG, Henrichsen J, Holm SE.** 1992. Novel coagglutination method for serotyping group B streptococci. J Clin Microbiol **30:**3268-3269.

**Hansen SM, Uldbjerg N, Kilian M, Sørensen UB.** 2004. Dynamics of Streptococcus agalactiae colonization in women during and after pregnancy and in their infants. J Clin Microbiol **42:**83-89.

**Harper IA.** 1971. The importance of group B streptococci as human pathogens in the British Isles. J Clin Pathol **24:**438-441.

**Harrison LH, Ali A, Dwyer DM, Libonati JP, Reeves MW, Elliott JA, Billmann L, Lashkerwala T, Johnson JA.** 1995. Relapsing invasive group B streptococcal infection in adults. Annals of internal medicine **123:**421-427.

**Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A.** 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis **177:**998-1002.

**Harrison LH, Elliott JA, Dwyer DM, Libonati JP, Ferrieri P, Billmann L, Schuchat A.** 1998. Serotype distribution of invasive group B streptococcal isolates in Maryland: implications for vaccine formulation. Maryland Emerging Infections Program. J Infect Dis **177:**998-1002.

**Haug RH.** 1972. Type classification of the group B streptococci by means of Lancefield's precipitin method. Classification of strains of bovine and human origin; isolated during the year 1971 in Norway. Nord Vet Med **24:**631-638.

**Heath PT, Schuchat A.** 2007. Perinatal group B streptococcal disease. Best Pract Res Clin Obstet Gynaecol **21:**411-424.

**Heath PT.** 2011. An update on vaccination against group B streptococcus. Expert review of vaccines **10:**685-694.

**Heelan JS, Hasenbein ME, McAdam AJ.** 2004. Resistance of group B streptococcus to selected antibiotics, including erythromycin and clindamycin. J Clin Microbiol **42:**1263-1264.

**Henning KJ, Hall EL, Dwyer DM, Billmann L, Schuchat A, Johnson JA, Harrison LH, Program MEI.** 2001. Invasive group B streptococcal disease in Maryland nursing home residents. J Infect Dis **183:**1138-1142.

**Hensler ME, Quach D, Hsieh C-J, Doran KS, Nizet V.** 2008. CAMP factor is not essential for systemic virulence of Group B Streptococcus. Microbial pathogenesis **44:**84-88.

**Herbert MA, Beveridge CJ, Saunders NJ.** 2004. Bacterial virulence factors in neonatal sepsis: group B streptococcus. Curr Opin Infect Dis **17:**225-229.

**Hickman ME, Rench MA, Ferrieri P, Baker CJ.** 1999. Changing epidemiology of group B streptococcal colonization. Pediatrics **104:**203-209.

**Holm SE, Håkansson S.** A simple and sensitive enzyme immunoassay for determination of soluble type-specific polysaccharide from group B streptococci. J Immunol Methods. 1988;106(1):89-94.

**Imberty A, Gautier C, Lescar J, Pérez S, Wyns L, Loris R.** 2000. An unusual carbohydrate binding site revealed by the structures of two Maackia amurensis lectins complexed with sialic acid-containing oligosaccharides. J Biol Chem **275:**17541-17548.

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

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

**Ippolito DL, James WA, Tinnemore D, Huang RR, Dehart MJ, Williams J, Wingerd MA, Demons ST.** 2010. Group B streptococcus serotype prevalence in reproductive-age women at a tertiary care military medical center relative to global serotype distribution. BMC Infect Dis **10:**336.

**Jackson LA, Hilsdon R, Farley MM, Harrison LH, Reingold AL, Plikaytis BD, Wenger JD, Schuchat A.** 1995. Risk factors for group B streptococcal disease in adults. Annals of internal medicine **123:**415-420.

**Janapatla RP, Ho Y-R, Yan J-J, Wu H-M, Wu J-J.** 2008. The prevalence of erythromycin resistance in group B streptococcal isolates at a University Hospital in Taiwan. Microbial Drug Resistance **14:**293-297.

**Jefferies AL.** 2017. Management of term infants at increased risk for early-onset bacterial sepsis. Paediatrics & Child Health **22:**223-228.

**Jiang S, Park SE, Yadav P, Paoletti LC, Wessels MR.** 2012. Regulation and function of pilus island 1 in group B Streptococcus. J Bacteriol **194:**2479-2490.

**Jiang SM, Ishmael N, Dunning Hotopp J, Puliti M, Tissi L, Kumar N, Cieslewicz MJ, Tettelin H, Wessels MR.** 2008. Variation in the group B Streptococcus CsrRS regulon and effects on pathogenicity. J Bacteriol **190:**1956-1965.

**Joachim A, Matee MI, Massawe FA, Lyamuya EF.** 2009. Maternal and neonatal colonisation of group B streptococcus at Muhimbili National Hospital in Dar es Salaam, Tanzania: prevalence, risk factors and antimicrobial resistance. BMC Public Health **9:**437.

**Johnson DR, Ferrieri P.** 1984. Group B streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J Clin Microbiol **19:**506-510.

**Johri AK, Paoletti LC, Glaser P, Dua M, Sharma PK, Grandi G, Rappuoli R.** 2006. Group B Streptococcus: global incidence and vaccine development. Nat Rev Micro **4:**932-942.

**Jones AL, Knoll KM, Rubens CE.** 2000. Identification of Streptococcus agalactiae virulence genes in the neonatal rat sepsis model using signature-tagged mutagenesis. Mol Microbiol **37:**1444-1455.

**Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM, Bisharat N, Spratt BG.** 2003. Multilocus sequence typing system for group B streptococcus. J Clin Microbiol **41:**2530-2536.

**Jones N, Oliver K, Jones Y, Haines A, Crook D.** 2006. Carriage of group B streptococcus in pregnant women from Oxford, UK. Journal of clinical pathology **59:**363-366.

**Jordan HT, Farley MM, Craig A, Mohle-Boetani J, Harrison LH, Petit S, Lynfield R, Thomas A, Zansky S, Gershman K, Albanese BA, Schaffner W, Schrag SJ, Active Bacterial Core Surveillance (ABCs)/Emerging Infections Program Network CDC.** 2008. Revisiting the need for vaccine prevention of late-onset neonatal group B streptococcal disease: a multistate, population-based analysis. Pediatr Infect Dis J **27:**1057-1064.

**Kalliola S, Vuopio-Varkila J, Takala AK, Eskola J.** 1999. Neonatal group B streptococcal disease in Finland: a ten-year nationwide study. The Pediatric Infectious Disease Journal **18:**806-810.

**Ke D, Ménard C, Picard FJ, Boissinot M, Ouellette M, Roy PH, Bergeron MG.** 2000. Development of conventional and real-time PCR assays for the rapid detection of group B streptococci. Clin Chem **46:**324-331.

**Kiely RA, Cotter L, Mollaghan AM, Cryan B, Coffey A, Lucey B.** 2011. Emergence of group B Streptococcus serotype IV in women of child-bearing age in Ireland. Epidemiol Infect **139:**236-238. 53

**Kimura K, Suzuki S, Wachino J-i, Kurokawa H, Yamane K, Shibata N, Nagano N, Kato H, Shibayama K, Arakawa Y.** 2008. First molecular characterization of group B streptococci with reduced penicillin susceptibility. Antimicrob Agents Chemother **52:**2890-2897.

**Knibbs RN, Osborne SE, Glick GD, Goldstein IJ.** 1993. Binding determinants of the sialic acid-specific lectin from the slug Limax flavus. J Biol Chem **268:**18524-18531.

**Kolkman MA, Morrison DA, Van Der Zeijst BA, Nuijten PJ.** 1996. The capsule polysaccharide synthesis locus of streptococcus pneumoniae serotype 14: Identification of the glycosyl transferase gene cps14E. J Bacteriol **178:**3736-3741.

**Kolkman MA, Wakarchuk W, Nuijten PJ, van der Zeijst BA.** 1997. Capsular polysaccharide synthesis in Streptococcus pneumoniae serotype 14: molecular analysis of 150

**Kong F, Gowan S, Martin D, James G, Gilbert GL.** 2002. Serotype identification of group B streptococci by PCR and sequencing. J Clin Microbiol **40:**216-226.

**Kong F, Lambertsen LM, Slotved HC, Ko D, Wang H, Gilbert GL.** 2008. Use of phenotypic and molecular serotype identification methods to characterize previously nonserotypeable group B streptococci. J Clin Microbiol **46:**2745-2750.

**Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae using multiplex PCR and reverse line blot hybridization. J Med Microbiol **54:**1133-1138. 54

**Kong F, Ma L, Gilbert GL.** 2005. Simultaneous detection and serotype identification of Streptococcus agalactiae using multiplex PCR and reverse line blot hybridization. J Med Microbiol **54:**1133-1138.

**Konto-Ghiorghi Y, Mairey E, Mallet A, Duménil G, Caliot E, Trieu-Cuot P, Dramsi S.** 2009. Dual role for pilus in adherence to epithelial cells and biofilm formation in Streptococcus agalactiae. PLoS Pathog **5:**e1000422.

**Koren S, Harhay GP, Smith TP, Bono JL, Harhay DM, Mcvey SD, Radune D, Bergman NH, Phillippy AM.** 2013. Reducing assembly complexity of microbial genomes with single-molecule sequencing. Genome Biol **14:**R101. 233

**Kotiw M, Zhang GW, Daggard G, Reiss-Levy E, Tapsall JW, Numa A.** 2003. Late-onset and Recurrent Neonatal Group B Streptococcal Disease Associated with Breast-milk Transmission. Pediatric and Developmental Pathology **6:**251-256.

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

**Krohn MA, Hillier SL, Baker CJ.** 1999. Maternal peripartum complications associated with vaginal group B streptococci colonization. J Infect Dis **179:**1410-1415.

**Kuruvilla K, Thomas N, Jesudasan M, Jana A.** 1999. Neonatal group B streptococcal bacteraemia in India: ten years' experience. Acta Paediatrica **88:**1031-1032.

**Lachenauer CS, Kasper DL, Shimada J, Ichiman Y, Ohtsuka H, Kaku M, Paoletti LC, Ferrieri P, Madoff LC.** 1999. Serotypes VI and VIII predominate among group B streptococci isolated from pregnant Japanese women. J Infect Dis **179:**1030-1033.

**Lamagni TL, Keshishian C, Efstratiou A, Guy R, Henderson KL, Broughton K, Sheridan E.** 2013. Emerging trends in the epidemiology of invasive group B streptococcal disease in England and Wales, 1991-2010. Clin Infect Dis **57:**682-688.

**Lamy MC, Dramsi S, Billoët A, Réglier-Poupet H, Tazi A, Raymond J, Guérin F, Couvé E, Kunst F, Glaser P, Trieu-Cuot P, Poyart C.** 2006. Rapid detection of the "highly virulent" group B Streptococcus ST-17 clone. Microbes Infect **8:**1714-1722.

**Lamy MC, Zouine M, Fert J, Vergassola M, Couve E, Pellegrini E, Glaser P, Kunst F, Msadek T, Trieu-Cuot P, Poyart C.** 2004. CovS/CovR of group B streptococcus: a two-component global regulatory system involved in virulence. Mol Microbiol **54:**1250-1268.

**Lancefield RC, Hare R.** 1935. The serological differentiation of pathogenic and non-pathogenic strains of hemolytic streptococci from parturient women. J Exp Med **61:**335-349. 55

**Lancefield RC, McCarty M, Everly WN.** 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J Exp Med **142:**165-179. .

**Lancefield RC.** 1933. A serological differentiation of human and other groups of hemolytic streptococci. J Exp Med **57:**571-595.

**Lancefield RC.** 1934. A serological differentiation of specific types of bovine hemolytic streptococci (GROUP B). J Exp Med **59:**441-458.

**Lauer P, Rinaudo CD, Soriani M, Margarit I, Maione D, Rosini R, Taddei AR, Mora M, Rappuoli R, Grandi G, Telford JL.** 2005. Genome analysis reveals pili in Group B Streptococcus. Science **309:**105.

**Leclercq R.** 2002. Mechanisms of resistance to macrolides and lincosamides: nature of the resistance elements and their clinical implications. Clinical Infectious Diseases **34:**482-492.

**Lefébure T, Stanhope MJ.** 2007. Evolution of the core and pan-genome of Streptococcus: positive selection, recombination, and genome composition. Genome biology **8:**R71.

**Lembo A, Gurney MA, Burnside K, Banerjee A, De Los Reyes M, Connelly JE, Lin WJ, Jewell KA, Vo A, Renken CW.** 2010. Regulation of CovR expression in Group B Streptococcus impacts blood–brain barrier penetration. Molecular microbiology **77:**431-443.

**Liddy H, Holliman R.** 2002. Group B Streptococcus highly resistant to gentamicin. J Antimicrob Chemother **50:**142-143.

**Limansky AS, Sutich EG, Guardati MC, Toresani IE, Viale AM.** 1998. Genomic diversity among Streptococcus agalactiae isolates detected by a degenerate oligonucleotide-primed amplification assay. Journal of Infectious Diseases **177:**1308-1313.

**Lin F-YC, Weisman LE, Azimi PH, Philips III JB, Clark P, Regan J, Rhoads GG, Frasch CE, Gray BM, Troendle J.** 2004. Level of maternal IgG anti-group B streptococcus type III antibody correlated with protection of neonates against early-onset disease caused by this pathogen. Journal of Infectious Diseases **190:**928-934.

**Lindahl G, Stålhammar-Carlemalm M, Areschoug T.** 2005. Surface proteins of Streptococcus agalactiae and related proteins in other bacterial pathogens. Clin Microbiol Rev **18:**102-127.

**Liu G, Zhang W, Lu C.** 2012. Complete genome sequence of Streptococcus agalactiae GD201008-001, isolated in China from tilapia with meningoencephalitis. J Bacteriol **194:**6653.

**Liu GY, Doran KS, Lawrence T, Turkson N, Puliti M, Tissi L, et al**. Sword and shield: linked group B streptococcal beta-hemolysin/cytolysin and carotenoid pigment 201

**López Sastre JB, Fernández Colomer B, Coto Cotallo GD, Ramos Aparicio A.** 2005. Trends in the epidemiology of neonatal sepsis of vertical transmission in the era of group B streptococcal prevention. Acta Paediatrica **94:**451-457.

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

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

**Lupo A, Ruppen C, Hemphill A, Spellerberg B, Sendi P.** 2014. Phenotypic and molecular characterization of hyperpigmented group B Streptococci. Int J Med Microbiol **304:**717-724.

**Lyytikäinen O, Nuorti JP, Halmesmäki E, Carlson P, Uotila J, Vuento R, Ranta T, Sarkkinen H, Ämmälä M, Kostiala A.** 2003. Invasive group B streptococcal infections in Finland: a population-based study. Emerging infectious diseases **9:**470.

**Madhi SA, Kuwanda L, Cutland C, Klugman KP.** 2005. The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and-uninfected children. Clinical Infectious Diseases **40:**1511-1518.

**Madhi SA, Radebe K, Crewe-Brown H, Frasch CE, Arakere G, Mokhachane M, Kimura A.** 2003. High burden of invasive Streptococcus agalactiae disease in South African infants. Ann Trop Paediatr **23:**15-23.

**Madoff LC, Michel JL, Kasper DL.** 1991. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun **59:**204-210.

**Madoff LC, Paoletti LC, Kasper DL.** Surface structures of group B streptococci important in human immunity. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram-Positive Pathogens. Washington, DC: Am Soc Microbiol; 2006.

**Maiden MC, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA.** 1998. Multilocus sequence typing: a portable approach to the identification of clones within populations of pathogenic microorganisms. Proceedings of the National Academy of Sciences **95:**3140-3145.

**Maione D, Margarit I, Rinaudo CD, Masignani V, Mora M, Scarselli M, Tettelin H, Brettoni C, Iacobini ET, Rosini R.** 2005. Identification of a universal Group B streptococcus vaccine by multiple genome screen. Science **309:**148-150.

**Maisey HC, Doran KS, Nizet V.** 2008. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert reviews in molecular medicine **10:**e27.

**Maisey HC, Hensler M, Nizet V, Doran KS.** 2007. Group B streptococcal pilus proteins contribute to adherence to and invasion of brain microvascular endothelial cells. J Bacteriol **189:**1464-1467.

**Malbruny B, Werno AM, Anderson TP, Murdoch DR, Leclercq R.** 2004. A new phenotype of resistance to lincosamide and streptogramin A-type antibiotics in Streptococcus agalactiae in New Zealand. Journal of antimicrobial chemotherapy **54:**1040-1044.

**Mandlik A, Swierczynski A, Das A, Ton-That H.** 2008. Pili in Gram-positive bacteria: assembly, involvement in colonization and biofilm development. Trends in microbiology **16:**33-40.

**Maniatis AN, Palermos J, Kantzanou M, Maniatis NA, Christodoulou C, Legakis NJ.** 1996. Streptococcus agalactiae: a vaginal pathogen? J Med Microbiol **44:**199-202.

**Manning SD, Lacher DW, Davies HD, Foxman B, Whittam TS.** 2005.DNA polymorphism and molecular subtyping of the capsular gene cluster of group B streptococcus. Journal of clinical microbiology.43(12):6113-6.

**Manning SD, Lewis MA, Springman AC, Lehotzky E, Whittam TS, Davies HD.** 2008. Genotypic diversity and serotype distribution of group B streptococcus isolated. Clin Infect Dis **46:**1829-1837. 73

**Manning SD, Springman AC, Lehotzky E, Lewis MA, Whittam TS, Davies HD.** 2009. Multilocus sequence types associated with neonatal group B streptococcal sepsis and meningitis in Canada. J Clin Microbiol **47:**1143-1148.

**Manning SD.** 2003. Molecular epidemiology of Streptococcus agalactiae (group B Streptococcus). Front Biosci **8:**s1-18.

**Marchaim D, Efrati S, Melamed R, Gortzak-Uzan L, Riesenberg K, Zaidenstein R, Schlaeffer F.** 2006. Clonal variability of group B Streptococcus among different groups of carriers in southern Israel. European Journal of Clinical Microbiology and Infectious Diseases **25:**443-448.

**Margarit I, Rinaudo CD, Galeotti CL, Maione D, Ghezzo C, Buttazzoni E, Rosini R, Runci Y, Mora M, Buccato S.** 2009. Preventing bacterial infections with pilus-based vaccines: the group B streptococcus paradigm. Journal of Infectious Diseases **199:**108-115. 65

**Marimón JM, Valiente A, Ercibengoa M, García-Arenzana JM, Pérez-Trallero E.** 2005. Erythromycin resistance and genetic elements carrying macrolide efflux genes in Streptococcus agalactiae. Antimicrob Agents Chemother **49:**5069-5074.

**Marques MB, Kasper DL, Pangburn MK, Wessels MR.** 1992. Prevention of C3 deposition by capsular polysaccharide is a virulence mechanism of type III group B streptococci. Infect Immun **60:**3986-3993.

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

**Martins E, Melo-Cristino J, Ramirez M.** 2007. Reevaluating the serotype II capsular locus of Streptococcus agalactiae. J Clin Microbiol **45:**3384-3386.

**Martins ER, Melo-Cristino J, Ramirez M, Infections PGftSoS.** 2012. Dominance of serotype Ia among group B Streptococci causing invasive infections in nonpregnant adults in Portugal. J Clin Microbiol **50:**1219-1227.

**Martins ER, Melo-Cristino J, Ramirez M.** 2010. Evidence for rare capsular switching in Streptococcus agalactiae. J Bacteriol **192:**1361-1369.

**Martins ER, Melo-Cristino J, Ramirez M.** 2012. Dominance of serotype Ia among group B Streptococci causing invasive infections in nonpregnant adults in Portugal. J Clin Microbiol **50:**1219-1227.

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

**Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, Corander J.** 2012. Detection of recombination events in bacterial genomes from large population samples. Nucleic Acids Res **40:** e6.

**Maruvada R, Blom AM, Prasadarao NV.** 2008. Effects of complement regulators bound to Escherichia coli K1 and Group B Streptococcus on the interaction with host cells. Immunology **124:**265-276.

**Matsubara K, Katayama K, Baba K, Nigami H, Harigaya H, Sugiyama M.** 2002. Seroepidemiologic studies of serotype VIII group B Streptococcus in Japan. Journal of Infectious Diseases **186:**855-858.

**Matsubara K, Yamamoto G.** 2009. Invasive group B streptococcal infections in a tertiary care hospital between 1998 and 2007 in Japan. International Journal of Infectious Diseases **13:**679-684.

**McDougal LK, Tenover FC, Lee LN, Rasheed JK, Patterson JE, Jorgensen JH, LeBlanc DJ.** 1998. Detection of Tn917-like sequences within a Tn916-like conjugative transposon (Tn3872) in erythromycin-resistant isolates of Streptococcus pneumoniae. Antimicrob Agents Chemother **42:**2312-2318.

**Meehan M, Cunney R, Cafferkey M.** 2014. Molecular epidemiology of group B streptococci in Ireland reveals a diverse population with evidence of capsular switching. Eur J Clin Microbiol Infect Dis **33:**1155-1162.

**Meehan M, Cunney R, Cafferkey M.** 2014. Molecular epidemiology of group B streptococci in Ireland reveals a diverse population with evidence of capsular switching. Eur J Clin Microbiol Infect Dis **33:**1155-1162.

**Melin P, Efstratiou A.** 2013. Group B streptococcal epidemiology and vaccine needs in developed countries. Vaccine **31 Suppl 4:**D31-42.

**Melin P.** 2011. Neonatal group B streptococcal disease: from pathogenesis to preventive strategies. Clin Microbiol Infect **17:**1294-1303. 57

**Mereghetti L, Sitkiewicz I, Green NM, Musser JM.** 2008. Remodeling of the Streptococcus agalactiae transcriptome in response to growth temperature. PLoS One **3:**e2785.

**Michel JL, Madoff LC, Kling DE, Kasper DL, Ausubel F.** 1991. Cloned alpha and beta C-protein antigens of group B streptococci elicit protective immunity. Infect Immun **59:**2023-2028.

**Money D, Allen VM.** 2013. The prevention of early-onset neonatal group B streptococcal disease. J Obstet Gynaecol Can **35:**939-951.

**Moyo SR, Maeland JA, Bergh K.** 2002. Typing of human isolates of Streptococcus agalactiae (group B streptococcus, GBS) strains from Zimbabwe. J Med Microbiol **51:**595-662.

**Musser JM, Mattingly SJ, Quentin R, Goudeau A, Selander RK.** 1989. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc Natl Acad Sci U S A **86:**4731-4735.

**NCCLS.** 1997. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, 4th ed. NCCLS, Villanova, PA.

**NCCLS.** 1997. Performance standards for antimicrobial susceptibility testing; 7th informational supplement, 7th informational supplement. NCCLS, Wayne, PA.

**Neemuchwala A, Teatero S, Athey TB, McGeer A, Fittipaldi N.** 2016. Capsular Switching and Other Large-Scale Recombination Events in Invasive Sequence Type 1 Group B Streptococcus. Emerg Infect Dis **22:**1941-1944.

**NIZET V, FERRIERI P, RUBENS CE.** 2000. Molecular Pathogenesis of Group B Streptococcal. Streptococcal infections: clinical aspects, microbiology, and molecular pathogenesis**:**180.

**Ojukwu JU, Abonyi LE, Ugwu J, Orji IK.** 2006. Neonatal septicemia in high risk babies in South-Eastern Nigeria. Journal of perinatal medicine **34:**166-172.

**Osman NB, Folgosa E, Bergström S.** 1995. An incident case-referent study of threatening preterm birth and genital infection. J Trop Pediatr **41:**267-272.

**Overbeek R, Olson R, Pusch GD, Olsen GJ, Davis JJ, Disz T, Edwards RA, Gerdes S, Parrello B, Shukla M, Vonstein V, Wattam AR, Xia F, Stevens R.** 2014. The SEED and the Rapid Annotation of microbial genomes using Subsystems Technology (RAST). Nucleic Acids Res **42:**D206-214.

**Palazzi DL, Rench MA, Edwards MS, Baker CJ.** 2004. Use of type V group B streptococcal conjugate vaccine in adults 65–85 years old. Journal of Infectious Diseases **190:**558-564.

**Palmeiro JK, De Carvalho NS, Botelho AC, Fracalanzza SE, Madeira HM, Dalla-Costa LM.** 2011. Maternal group B streptococcal immunization: capsular polysaccharide (CPS)-based vaccines and their implications on prevention. Vaccine **29:**3729-3730.

**Pan X, Ge J, Li M, Wu B, Wang C, Wang J, Feng Y, Yin Z, Zheng F, Cheng G.** 2009. The orphan response regulator CovR: a globally negative modulator of virulence in Streptococcus suis serotype 2. J Bacteriol **191:**2601-2612.

**Park CJ, Vandel NM, Ruprai DK, Martin EA, Gates KM, Coker D.** 2001. Detection of group B streptococcal colonization in pregnant women using direct latex agglutination testing of selective broth. J Clin Microbiol **39:**408-409.

**Parker RE, Laut C, Gaddy JA, Zadoks RN, Davies HD, Manning SD.** 2016. Association between genotypic diversity and biofilm production in group B Streptococcus. BMC Microbiol **16:**86.

**Pearlman M.** 2003. Prevention of Early-Onset Group B Streptococcal Disease in Newborns. Obstetrics & Gynecology **102:**414-415.

**Perch B, Kjems E, Henrichsen.** 1979. New serotypes of group B streptococci isolated from human sources. J Clin Microbiol **10:**109-110.

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

**Pezzicoli A, Santi I, Lauer P, Rosini R, Rinaudo D, Grandi G, Telford JL, Soriani M.** 2008. Pilus backbone contributes to group B Streptococcus paracellular translocation through epithelial cells. Journal of Infectious Diseases **198:**890-898.

**Phares CR, Lynfield R, Farley MM, Mohle-Boetani J, Harrison LH, Petit S, Craig AS, Schaffner W, Zansky SM, Gershman K, Stefonek KR, Albanese BA, Zell ER, Schuchat A, Schrag SJ, Network ABCsEIP.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. JAMA **299:**2056-2065.

**Pinheiro S, Radhouani H, Coelho C, Gonçalves A, Carvalho E, Carvalho JA, Ruiz-Larrea F, Torres C, Igrejas G, Poeta P.** 2009. Prevalence and mechanisms of erythromycin resistance in Streptococcus agalactiae from healthy pregnant women. Microbial Drug Resistance **15:**121-124.

**Poyart C, Jardy L, Quesne G, Berche P, Trieu-Cuot P.** 2003. Genetic basis of antibiotic resistance in Streptococcus agalactiae strains isolated in a French hospital. Antimicrob Agents Chemother **47:**794-797. 64

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

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

**Pritchard DG, Egan ML, Gray BM, Dillon HC.** Immunochemical characterization of the polysaccharide antigens of group B streptococci. Rev Infect Dis. 1988;10 Suppl 2:S367-71.

**Puopolo KM, Madoff LC, Eichenwald EC.** 2005. Early-onset group B streptococcal disease in the era of maternal screening. Pediatrics **115:**1240-1246.

**Quentin R, Huet H, Wang F-S, Geslin P, Goudeau A, Selander RK.** 1995. Characterization of Streptococcus agalactiae strains by multilocus enzyme genotype and serotype: identification of multiple virulent clone families that cause invasive neonatal disease. J Clin Microbiol **33:**2576-2581. 70

**Quinlan JD, Hill DA, Maxwell BD, Boone S, Hoover F, Lense JJ.** 2000. The necessity of both anorectal and vaginal cultures for group B streptococcus screening during pregnancy. J Fam Pract **49:**447-448.

**Radhakrishnan S, Brahmadathan K, Mathai E, Jesudason M, Lalitha M.** 1995. Changing patterns of group B streptococcal serotypes associated with human infections. The Indian journal of medical research **102:**56-59.

**Rajagopal L.** 2009. Understanding the regulation of Group B Streptococcal virulence factors. Future Microbiol **4:**201-221.

**Ramaswamy SV, Ferrieri P, Flores AE, Paoletti LC.** 2006. Molecular characterization of nontypeable group B streptococcus. J Clin Microbiol **44:**2398-2403.

**Ramaswamy SV, Ferrieri P, Madoff LC, Flores AE, Kumar N, Tettelin H, Paoletti LC.** 2006. Identification of novel cps locus polymorphisms in nontypable group B Streptococcus. J Med Microbiol **55:**775-783.

**Regan JA, Klebanoff MA, Nugent RP.** 1991. The epidemiology of group B streptococcal colonization in pregnancy. Vaginal Infections and Prematurity Study Group. Obstet Gynecol **77:**604-610.

**Reiß A, Braun JS, Jäger K, Freyer D, Laube G, Bührer C, Felderhoff-Müser U, Stadelmann C, Nizet V, Weber JR.** 2011. Bacterial pore-forming cytolysins induce neuronal damage in a rat model of neonatal meningitis. Journal of Infectious Diseases **203:**393-400.

**Richards VP, Choi SC, Pavinski Bitar PD, Gurjar AA, Stanhope MJ.** 2013. Transcriptomic and genomic evidence for Streptococcus agalactiae adaptation to the bovine environment. BMC Genomics **14:**920.

**Rinaudo CD, Rosini R, Galeotti CL, Berti F, Necchi F, Reguzzi V, Ghezzo C, Telford JL, Grandi G, Maione D.** 2010. Specific involvement of pilus type 2a in biofilm formation in group B Streptococcus. PLoS One **5:**e9216.

**Rocchetti TT, Marconi C, Rall VL, Borges VT, Corrente JE, da Silva MG.** 2011. Group B streptococci colonization in pregnant women: risk factors and evaluation of the vaginal flora. Arch Gynecol Obstet **283:**717-721.

**Rodriguez-Granger J, Alvargonzalez JC, Berardi A, Berner R, Kunze M, Hufnagel M, Melin P, Decheva A, Orefici G, Poyart C, Telford J, Efstratiou A, Killian M, Krizova P, Baldassarri L, Spellerberg B, Puertas A, Rosa-Fraile M.** 2012. Prevention of group B streptococcal neonatal disease revisited. The DEVANI European project. Eur J Clin Microbiol Infect Dis **31:**2097-2104.

**Rogerieux F, Belaise M, Terzidis-Trabelsi H, Greffard A, Pilatte Y, Lambré CR.** 1993. Determination of the sialic acid linkage specificity of sialidases using lectins in a solid phase assay. Anal Biochem **211:**200-204.

**Rosini R, Margarit I.** 2015.Biofilm formation by Streptococcus agalactiae: influence of environmental conditions and implicated virulence factors. Front Cell Infect Microbiol.5:6.

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

**Rubens C, Smith S, Hulse M, Chi E, Van Belle G.** 1992. Respiratory epithelial cell invasion by group B streptococci. Infect Immun **60:**5157-5163.

**Rubens CE, Heggen LM, Haft RF, Wessels MR.** 1993. Identification of cpsD, a gene essential for type III capsule expression in group B streptococci. Mol Microbiol **8:**843-855.

**Rubens CE, Raff HV, Jackson JC, Chi EY, Bielitzki JT, Hillier SL.** 1991. Pathophysiology and histopathology of group B streptococcal sepsis in Macaca nemestrina primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. Journal of Infectious Diseases **164:**320-330.

**Rubens CE, Wessels MR, Heggen LM, Kasper DL.** 1987. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. Proc Natl Acad Sci U S A **84:**7208-7212.

**Rubens CE, Wessels MR, Kuypers JM, Kasper DL, Weiser JN.** 1990. Molecular analysis of two group B streptococcal virulence factors. Semin Perinatol **14:**22-29.

**Sadowy E, Matynia B, Hryniewicz W.** 2010. Population structure, virulence factors and resistance determinants of invasive, non-invasive and colonizing Streptococcus agalactiae in Poland. Journal of antimicrobial chemotherapy **65:**1907-1914.

**Salloum M, Van Der Mee-marquet N, Valentin-Domelier A-S, Quentin R.** 2011. Diversity of prophage DNA regions of Streptococcus agalactiae clonal lineages from adults and neonates with invasive infectious disease. PLoS One **6:**e20256.

**Sanchez MJ, Descorps-Declere S, Souche E, Buchrieser C, Trieu-Cuot P, Moszer I, Clermont D, Maione D, Bouchier C, McMillan DJ, Parkhill J, Telford JL, Dougan G, Walker MJ, Holden MT, Poyart C, Glaser P.** 2015. Corrigendum: Streptococcus agalactiae clones infecting humans were selected and fixed through the extensive use of tetracycline. Nat Commun **6:**6108.

**Sanchez-Beato AR, Garcia E, López R, García JL.** 1997. Identification and characterization of IS1381, a new insertion sequence in Streptococcus pneumoniae. J Bacteriol **179:**2459-2463.

**Santi I, Maione D, Galeotti CL, Grandi G, Telford JL, Soriani M.** 2009. BibA induces opsonizing antibodies conferring in vivo protection against group B Streptococcus. J Infect Dis **200:**564-570.

**Santi I, Scarselli M, Mariani M, Pezzicoli A, Masignani V, Taddei A, Grandi G, Telford JL, Soriani M.** 2007. BibA: a novel immunogenic bacterial adhesin contributing to group B Streptococcus survival in human blood. Mol Microbiol **63:**754-767.

**Santillan DA, Andracki ME, Hunter SK.** 2008. Protective immunization in mice against group B streptococci using encapsulated C5a peptidase. American journal of obstetrics and gynecology **198:**114. e111-114. e116.

**Savoia D, Gottimer C, Crocilla C, Zucca M.** 2008. Streptococcus agalactiae in pregnant women: phenotypic and genotypic characters. Journal of Infection **56:**120-125.

**Schmidt H, Hensel M.** 2004. Pathogenicity islands in bacterial pathogenesis. Clin Microbiol Rev **17:**14-56.

**Schoening T, Wagner J, Arvand M.** 2005. Prevalence of erythromycin and clindamycin resistance among Streptococcus agalactiae isolates in Germany. Clinical Microbiology and Infection **11:**579-582.

**Schrag S, Gorwitz R, Fultz-Butts K, Schuchat A.** 2002. Prevention of perinatal group B streptococcal disease. Revised guidelines from CDC. MMWR Recomm Rep **51:**1-22. 56

**Schrag SJ, Zywicki S, Farley MM, Reingold AL, Harrison LH, Lefkowitz LB, Hadler JL, Danila R, Cieslak PR, Schuchat A.** 2000. Group B streptococcal disease in the era of intrapartum antibiotic prophylaxis. N Engl J Med **342:**15-20.

**Schubert A, Zakikhany K, Schreiner M, Frank R, Spellerberg B, Eikmanns BJ, Reinscheid DJ.** 2002. A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol **46:**557-569.

**Schuchat A, Balter S.** Epidemiology of group B streptococcal infections. In: Fischetti VA, Novick RP, Ferretti JJ, Portnoy DA, Rood JI, editors. Gram-Positive Pathogens. Washington, DC: Am Soc Microbiol; 2006.

**Schuchat A, Schrag SJ.** 2008. Epidemiology of invasive group B streptococcal disease in the United States, 1999-2005. Jama **299:**2056-2065.

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

**Schuchat A.** 1999. Group B streptococcus. Lancet **353:**51-56.

**Seifert KN, Adderson EE, Whiting AA, Bohnsack JF, Crowley PJ, Brady LJ.** 2006. A unique serine-rich repeat protein (Srr-2) and novel surface antigen (ε) associated with a virulent lineage of serotype III Streptococcus agalactiae. Microbiology **152:**1029-1040.

**Selander RK, Musser JM, Caugant DA, Gilmour MN, Whittam TS.** 1987. Population genetics of pathogenic bacteria. Microbial pathogenesis **3:**1-7.

**Sellin M, Olofsson C, Håkansson S, Norgren M.** 2000. Genotyping of the capsule gene cluster (cps) in nontypeable group B streptococci reveals two major cps allelic variants of serotypes III and VII. J Clin Microbiol **38:**3420-3428.

**Sendi P, Johansson L, Dahesh S, Van-Sorge NM, Darenberg J, Norgren M, Sjölin J, Nizet V, Norrby-Teglund A.** 2009. Bacterial phenotype variants in group B streptococcal toxic shock syndrome. Emerging infectious diseases **15:**223-232.

**Sendi P, Johansson L, Norrby-Teglund A.** 2008. Invasive group B Streptococcal disease in non-pregnant adults : a review with emphasis on skin and soft-tissue infections. Infection **36:**100-111.

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

**Seo YS, Srinivasan U, Oh K-Y, Shin J-H, Chae JD, Kim MY, Yang JH, Yoon H-R, Miller B, DeBusscher J.** 2010. Changing molecular epidemiology of group B streptococcus in Korea. Journal of Korean medical science **25:**817-823.

**Serrano I, Melo-Cristino J, Carriço J, Ramirez M.** 2005. Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal. J Clin Microbiol **43:**1706-1715.

**Shakleina E, Dmitriev A, Tkacikova L, Suvorov A.** 2004. Presence of insertion sequences (IS elements) in group B streptococci of bovine origin. Indian Journal of Medical Research **119:**242.

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

**Sheen TR, Jimenez A, Wang N-Y, Banerjee A, van Sorge NM, Doran KS.** 2011. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. J Bacteriol **193:**6834-6842.

**Shet A, Ferrieri P.** 2004. Neonatal & maternal group B streptococcal infections: a comprehensive review. Indian Journal of Medical Research **120:**141.

**Shibuya N, Goldstein IJ, Broekaert WF, Nsimba-Lubaki M, Peeters B, Peumans WJ.** 1987. The elderberry (Sambucus nigra L.) bark lectin recognizes the Neu5Ac(alpha 2-6)Gal/GalNAc sequence. J Biol Chem **262:**1596-1601.

**Sinha A, Lieu TA, Paoletti LC, Weinstein MC, Platt R.** 2005. The projected health benefits of maternal group B streptococcal vaccination in the era of chemoprophylaxis. Vaccine **23:**3187-3195.

**Sitkiewicz I, Green NM, Guo N, Bongiovanni AM, Witkin SS, Musser JM.** 2009. Transcriptome adaptation of group B Streptococcus to growth in human amniotic fluid. PLoS One **4:**e6114. 69

**Skoff TH, Farley MM, Petit S, Craig AS, Schaffner W, Gershman K, Harrison LH, Lynfield R, Mohle-Boetani J, Zansky S, Albanese BA, Stefonek K, Zell ER, Jackson D, Thompson T, Schrag**

**SJ.** 2009. Increasing burden of invasive group B streptococcal disease in nonpregnant adults, 1990-2007. Clin Infect Dis **49:**85-92.

**Slotved HC, Elliott J, Thompson T, Konradsen HB.** 2003. Latex assay for serotyping of group B Streptococcus isolates. J Clin Microbiol **41:**4445-4447.

**Slotved HC, Kong F, Lambertsen L, Sauer S, Gilbert GL.** 2007. Serotype IX, a Proposed New Streptococcus agalactiae Serotype. J Clin Microbiol **45:**2929-2936.

**Sørensen UB, Poulsen K, Ghezzo C, Margarit I, Kilian M.** 2010. Emergence and global dissemination of host-specific Streptococcus agalactiae clones. MBio **1**.

**Spellerberg B, Martin S, Franken C, Berner R, Lutticken R.** 2000. Identification of a novel insertion sequence element in Streptococcus agalactiae. bspeller@imib.rwth-aachen.de. Gene **241:**51-56. 67

**Spratt BG, Hanage WP, Li B, Aanensen DM, Feil EJ.** 2004. Displaying the relatedness among isolates of bacterial species the eBURST approach. FEMS Microbiol Lett **241:**129-134.

**Strakova L, Motlova J.** 2004. Active surveillance of early onset disease due to group B streptococci in newborns. Indian Journal of Medical Research **119:**205.

**Suara RO, Adegbola RA, Baker CJ, Secka O, Mulholland EK, Greenwood BM.** 1994. Carriage of group B Streptococci in pregnant Gambian mothers and their infants. J Infect Dis **170:**1316-1319.

**Takahashi S, Adderson EE, Nagano Y, Nagano N, Briesacher MR, Bohnsack JF.** 1998. Identification of a highly encapsulated, genetically related group of invasive type III group B streptococci. J Infect Dis **177:**1116-1119.

**Tamura GS, Herndon M, Przekwas J, Rubens CE, Ferrieri P, Hillier SL.** 2000. Analysis of restriction fragment length polymorphisms of the insertion sequence IS1381 in group B streptococci. Journal of Infectious Diseases **181:**364-368.

**Tapsall JW, Phillips EA.** 1987. Presumptive identification of group B streptococci by rapid detection of CAMP factor and pigment production. Diagn Microbiol Infect Dis **7:**225-228.

**Tazi A, Bellais S, Tardieux I, Dramsi S, Trieu-Cuot P, Poyart C.** 2012. Group B Streptococcus surface proteins as major determinants for meningeal tropism. Curr Opin Microbiol **15:**44-49.

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

**Tazi A, Morand PC, Réglier-Poupet H, Dmytruk N, Billoët A, Antona D, Trieu-Cuot P, Poyart C.** 2011. Invasive group B streptococcal infections in adults, France (2007-2010). Clin Microbiol Infect **17:**1587-1589.

**Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

**Teatero S, Athey TB, Van Caeseele P, Horsman G, Alexander DC, Melano RG, Li A, Flores AR, Shelburne SA, McGeer A, Demczuk W, Martin I, Fittipaldi N.** 2015. Emergence of Serotype IV Group B Streptococcus Adult Invasive Disease in Manitoba and Saskatchewan, Canada, Is Driven by Clonal Sequence Type 459 Strains. J Clin Microbiol **53:**2919-2926.

**Teatero S, McGeer A, Low DE, Li A, Demczuk W, Martin I, Fittipaldi N.** 2014. Characterization of Invasive Group B Streptococcus from the Greater Toronto Area, Canada. J Clin Microbiol 52: 1441-1447.

**Tettelin H, Masignani V, Cieslewicz MJ, Donati C, Medini D, Ward NL, Angiuoli SV, Crabtree J, Jones AL, Durkin AS, Deboy RT, Davidsen TM, Mora M, Scarselli M, Margarit y Ros I, Peterson JD, Hauser CR, Sundaram JP, Nelson WC, Madupu R, Brinkac LM, Dodson RJ, Rosovitz MJ, Sullivan SA, Daugherty SC, Haft DH, Selengut J, Gwinn ML, Zhou L, Zafar N, Khouri H, Radune D, Dimitrov G, Watkins K, O'Connor KJ, Smith S, Utterback TR, White O, Rubens CE, Grandi G, Madoff LC, Kasper DL, Telford JL, Wessels MR, Rappuoli R, Fraser CM.** 2005. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial "pan-genome". Proc Natl Acad Sci U S A **102:**13950-13955.

**Tettelin H, Masignani V, Cieslewicz MJ, Eisen JA, Peterson S, Wessels MR, Paulsen IT, Nelson KE, Margarit I, Read TD, Madoff LC, Wolf AM, Beanan MJ, Brinkac LM, Daugherty SC, DeBoy RT, Durkin AS, Kolonay JF, Madupu R, Lewis MR, Radune D, Fedorova NB, Scanlan D, Khouri H, Mulligan S, Carty HA, Cline RT, Van Aken SE, Gill J, Scarselli M, Mora M, Iacobini ET, Brettoni C, Galli G, Mariani M, Vegni F, Maione D, Rinaudo D, Rappuoli R, Telford JL, Kasper DL, Grandi G, Fraser CM.** 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V Streptococcus agalactiae. Proc Natl Acad Sci U S A **99:**12391-12396.

**Thigpen MC, Whitney CG, Messonnier NE, Zell ER, Lynfield R, Hadler JL, Harrison LH, Farley MM, Reingold A, Bennett NM, Craig AS, Schaffner W, Thomas A, Lewis MM, Scallan E,**

**Schuchat A, Network EIP.** 2011. Bacterial meningitis in the United States, 1998-2007. N Engl J Med **364:**2016-2025.

**Tien N, Ho C-M, Lin H-J, Shih M-C, Ho M-W, Lin H-C, Lin H-S, Chang C-C, Lu J-J.** 2011. Multilocus sequence typing of invasive group B Streptococcus in central area of Taiwan. Journal of Microbiology, Immunology and Infection **44:**430-434.

**Toresani I, Limansky A, Bogado I, Guardati MC, Viale A, Sutich EG.** 2001. Phenotypic and genotypic study of Streptococcus agalactiae in vagina of pregnant women in Argentina. MEDICINA-BUENOS AIRES- **61:**295-300.

**Tran-Winkler HJ, Love JF, Gryllos I, Wessels MR.** 2011. Signal transduction through CsrRS confers an invasive phenotype in group A Streptococcus. PLoS Pathog **7:**e1002361.

**Tyrrell GJ, Kennedy A, Shokoples SE, Sherburne RK.** 2002. Binding and invasion of HeLa and MRC-5 cells by Streptococcus agalactiae. Microbiology **148:**3921-3931.

**Tyrrell GJ, Senzilet LD, Spika JS, Kertesz DA, Alagaratnam M, Lovgren M, Talbot JA.** 2000. Invasive disease due to group B streptococcal infection in adults: results from a Canadian, population-based, active laboratory surveillance study--1996. Sentinel Health Unit Surveillance System Site Coordinators. J Infect Dis **182:**168-173.

**Uh Y, Kim HY, Jang IH, Hwang GY, Yoon KJ.** 2005. Correlation of serotypes and genotypes of macrolide-resistant Streptococcus agalactiae. Yonsei Medical Journal **46:**480-483.

**Uhl JR, Vetter EA, Boldt KL, Johnston BW, Ramin KD, Adams MJ, Ferrieri P, Reischl U, Cockerill FR.** 2005. Use of the Roche LightCycler Strep B assay for detection of group B Streptococcus from vaginal and rectal swabs. J Clin Microbiol **43:**4046-4051.

**Van Belkum A, Tassios P, Dijkshoorn L, Haeggman S, Cookson B, Fry N, Fussing V, Green J, Feil E, Gerner-Smidt P.** 2007. Guidelines for the validation and application of typing methods for use in bacterial epidemiology. Clinical Microbiology and Infection **13:**1-46.

**van der Mee-Marquet N, Jouannet C, Domelier AS, Arnault L, Lartigue MF, Quentin R.** 2009. Genetic diversity of Streptococcus agalactiae strains and density of vaginal carriage. J Med Microbiol **58:**169-173.

**van Sorge NM, Quach D, Gurney MA, Sullam PM, Nizet V, Doran KS.** 2009. The group B streptococcal serine-rich repeat 1 glycoprotein mediates penetration of the blood-brain barrier. Journal of Infectious Diseases **199:**1479-1487.

**Varaldo PE, Montanari MP, Giovanetti E.** 2009. Genetic elements responsible for erythromycin resistance in streptococci. Antimicrob Agents Chemother **53:**343-353.

**Varki NM, Varki A.** 2007. Diversity in cell surface sialic acid presentations: implications for biology and disease. Lab Invest **87:**851-857. 263

**Verani JR, Schrag SJ.** 2010. Group B streptococcal disease in infants: progress in prevention and continued challenges. Clinics in perinatology **37:**375-392.

**Verani JR, McGee L, Schrag SJ; Division of Bacterial Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention (CDC).** 2010. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recomm Rep. **19:**1-36.

**Verhoeven PO, Noyel P, Bonneau J, Carricajo A, Fonsale N, Ros A, Pozzetto B, Grattard F.** 2014. Evaluation of the new brilliance GBS chromogenic medium for screening of Streptococcus agalactiae vaginal colonization in pregnant women. J Clin Microbiol **52:**991-993.

**Wagner M, Kaatz M.** 1997. Virulence of strains of new types and type candidates of group B streptococci (Streptococcus agalactiae). Comparative evaluation using mice and a chicken embryo model. Adv Exp Med Biol **418:**819-821.

**Wagner M, Murai T, Wagner B, Günther E, Jelinkova J.** 1994. JM9 strains, a new type of group B streptococci from Japan. Zentralbl Bakteriol **280:**488-496. 154

**Wang P, Tong JJ, Ma XH, Song FL, Fan L, Guo CM, Shi W, Yu SJ, Yao KH, Yang YH.** 2015. Serotypes, antibiotic susceptibilities, and multi-locus sequence type profiles of 104

**Wang P, Tong JJ, Ma XH, Song FL, Fan L, Guo CM, Shi W, Yu SJ, Yao KH, Yang YH.** 2015. Serotypes, antibiotic susceptibilities, and multi-locus sequence type profiles of Streptococcus agalactiae isolates circulating in Beijing, China. PLoS One **10:**e0120035.

**Wang Y-H, Su L-H, Hou J-N, Yang T-H, Lin T-Y, Chu C, Chiu C-H.** 2010. Group B streptococcal disease in nonpregnant patients: emergence of highly resistant strains of serotype Ib in Taiwan in 2006 to 2008. J Clin Microbiol **48:**2571-2574.

**Wasik BR, Barnard KN, Parrish CR.** 2016. Effects of sialic acid modifications on virus binding and infection. Trends in microbiology **24:**991-1001.

**Wasylnka JA, Simmer MI, Moore MM.** 2001. Differences in sialic acid density in pathogenic and non-pathogenic Aspergillus species. Microbiology **147:**869-877.

**Wen L, Wang Q, Li Y, Kong F, Gilbert GL, Cao B, Wang L, Feng L.** 2006. Use of a serotype-specific DNA microarray for identification of group B Streptococcus (Streptococcus agalactiae). J Clin Microbiol **44:**1447-1452.

**Wessels MR, Benedí WJ, Jennings HJ, Michon F, DiFabio JL, Kasper DL.** 1989. Isolation and characterization of type IV group B Streptococcus capsular polysaccharide. Infect Immun **57:**1089-1094.

**Wessels MR, Haft RF, Heggen LM, Rubens CE.** 1992. Identification of a genetic locus essential for capsule sialylation in type III group B streptococci. Infect Immun **60:**392-400.

**Wessels MR, Rubens CE, Benedi VJ, Kasper DL.** 1989. Definition of a bacterial virulence factor: sialylation of the group B streptococcal capsule. Proc Natl Acad Sci U S A **86:**8983-8987.

**Wheeler SM, Foley GE.** 1943. A Note on Non-Group-A Streptococci Associated with Human Infection. J Bacteriol **46:**391-392.

**Whidbey C, Burnside K, Martinez RM, Gendrin C, Vornhagen J, Frando A**. 2015. A Hyperhemolytic/Hyperpigmented Group B Streptococcus Strain with a CovR Mutation Isolated from an Adolescent Patient with Sore Throat. Clin Res Infect Dis.2(2).

**Whidbey C, Harrell MI, Burnside K, Ngo L, Becraft AK, Iyer LM.** 2013. A hemolytic pigment of Group B Streptococcus allows bacterial penetration of human placenta. The Journal of experimental medicine.210(6):1265-81.

**Whitney CG, Daly S, Limpongsanurak S, Festin MR, Thinn KK, Chipato T, Lumbiganon P, Sauvarin J, Andrews W, Tolosa JE, Health GNFPAR.** 2004. The international infections in pregnancy study: group B streptococcal colonization in pregnant women. J Matern Fetal Neonatal Med **15:**267-274.

**Wilkinson HW, Thacker LG, Facklam RR.** 1973. Nonhemolytic group B streptococci of human, bovine, and ichthyic origin. Infect Immun **7:**496-498.

**Xia FD, Mallet A, Caliot E, Gao C, Trieu-Cuot P, Dramsi S.** 2015. Capsular polysaccharide of Group B Streptococcus mediates biofilm formation in the presence of human plasma. Microbes Infect **17:**71-76.

**Xue G, Yu L, Li S, Shen X.** 2010. Intranasal immunization with GBS surface protein Sip and ScpB induces specific mucosal and systemic immune responses in mice. FEMS Immunology & Medical Microbiology **58:**202-210.

**Yancey MK, Duff P, Clark P, Kurtzer T, Frentzen BH, Kubilis P.** 1994. Peripartum infection associated with vaginal group B streptococcal colonization. Obstet Gynecol **84:**816-819.

**Yang Q, Porter AJ, Zhang M, Harrington DJ, Black GW, Sutcliffe IC.** 2012. The impact of pH and nutrient stress on the growth and survival of Streptococcus agalactiae. Antonie Van Leeuwenhoek **102:**277-287.

**Yao K, Poulsen K, Maione D, Rinaudo CD, Baldassarri L, Telford JL, Sorensen UB, Kilian M.** 2013. Capsular gene typing of Streptococcus agalactiae compared to serotyping by latex agglutination. J Clin Microbiol **51:**503-507.

**Yildirim AO, Lämmler C, Weiss R.** 2002. Identification and characterization of Streptococcus agalactiae isolated from horses. Vet Microbiol **85:**31-35.

**Yu H-W, Lin H-C, Yang P-H, Hsu C-H, Hsieh W-S, Tsao L-Y, Chen C-H, Lin H-C, Tseng Y-C.** 2011. Group B streptococcal infection in Taiwan: maternal colonization and neonatal infection. Pediatrics & Neonatology **52:**190-195.

**Zamzami TY, Marzouki AM, Nasrat HA.** 2011. Prevalence rate of group B streptococcal colonization among women in labor at King Abdul-Aziz University Hospital. Arch Gynecol Obstet **284:**677-679.

**Zappulli V, Mazzariol S, Cavicchioli L, Petterino C, Bargelloni L, Castagnaro M.** 2005. Fatal necrotizing fasciitis and myositis in a captive common bottlenose dolphin (Tursiops truncatus) associated with Streptococcus agalactiae. J Vet Diagn Invest **17:**617-622.

**Zhao Z, Kong F, Martinez G, Zeng X, Gottschalk M, Gilbert GL.** 2006. Molecular serotype identification of Streptococcus agalactiae of bovine origin by multiplex PCR-based reverse line blot (mPCR/RLB) hybridization assay. FEMS microbiology letters **263:**236-239. 71

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

**Zubair S, de Villiers EP, Fuxelius HH, Andersson G, Johansson KE, Bishop RP, Bongcam-Rudloff E.** 2013. Genome Sequence of Streptococcus agalactiae Strain 09mas018883, Isolated from a Swedish Cow. Genome Announcment.