Group B streptococci and other Neonatal infections

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Abstract

The main objectives of this thesis were to estimate the incidence and etiology of invasive infections in neonates and to characterize invasive strains of group B streptococci from a defined geographic area.

All infants aged 0-120 days with a bacterial or fungal isolate from blood or CSF in the defined area were identified. Invasive GBS isolates from neonates and adults were collected from normally sterile sites. All relevant clinical information was available for all patients. The GBS isolates were characterized by coagglutination with type specific antisera for serotypes Ia, Ib and II-VIII. Indirect whole cell based fluorescent antibody test was used for typing of the surface proteins, alpha c protein, beta c protein and rib. Multiplex and specific PCR were used for genotyping of the surface protein encoding genes, *bca, bac, epsilon/alp1, rib, alp2* and *alp3*. All strains were tested with E-test against 12 antibiotics.

The incidence of invasive infections day 0-27 was found to be 3.7/1 000 live births with aerobic Gram-negative rods, GBS and *Staphylococcus aureus* dominating. The incidence of very late onset infections was 20 times higher in preterm than in term neonates. The total incidence of CoNS infections was 1.1/1 000 live births. The most common serotypes in neonates were serotypes III (60 %), V (22 %) and Ia (10 %) and from adults V (42 %) and III (25 %). Surface proteins were detected in 51 %. The genes were identified alone or in combinations in 99 % of the strains. Both surface proteins and encoding genes were significantly related to certain serotypes. Two GBS strains were resistant to penicillin G. Intermediate susceptibility to erythromycin and clindamycin increased over the study period.

The incidence of invasive neonatal infections increased but the case fatality rate decreased compared to a preceding study from the same area. CoNS are important pathogens in preterm neonates. Serotype V had doubled its frequency in both neonates and adults. Demonstration of serotypes, genotypes and surface proteins in GBS strains are useful in epidemiological studies and in formulation of vaccines and should continuously be followed. No genotype or surface protein was so common that it could be a GBS vaccine candidate alone. Penicillin remains the drug of choice for GBS in the investigated geographic area.

Key words: Neonatal infections, sepsis, incidence, Group B streptococcus, serotype, epidemiology, genotype, surface protein, antibiotic susceptibility

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The thesis is based on the following papers, which will be referred to in the text by their roman numerals.

I Persson E, Trollfors B, Lind Brandberg L, Tessin I (2002) *Septicaemia and meningitis in neonates and during early infancy in the Göteborg area of Sweden*. Acta Paediatr; 91: 1087-1092

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

III Persson E, Berg S, Bevanger L, Bergh K, Valsö-Lyng R, Trollfors B (2007) *Characterization of invasive group B streptococci (GBS) based on demonstration of surface proteins and of genes encoding surface proteins.* Accepted for publication; Clin Microbiol Infect

IV Persson E, Berg S, Bevanger L, Bergh K, Valsö-Lyng R, Trollfors B (2007) *Antimicrobial susceptibility of invasive group B streptococcal isolates.* Submitted

Abbreviations

BPS	The group B protective surface protein
CDC	Centers for Disease Control
CLSI	Clinical and Laboratory Standard Institute
CoNS	Coagulase Negative Staphylococci
CPS	Capsular Polysaccharide
CSF	Cerebral Spinal Fluid
ELBW	Extremely Low Birth Weight (< 1 000g)
EOS	Early Onset Sepsis
EOD	Early Onset Disease
GBS	Group B Streptococcus
I	Intermediate
LOS	Late Onset Sepsis
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NICU	Neonatal Intensive Care Unit
PCR	Polymerase Chain Reaction
PFGE	Pulse-field Gel Electrophoresis
PROM	Premature Rupture Of Membranes
R	Resistant
S	Sensitive
SRGA	Swedish Reference Group for Antibiotics
Trim-sulfa	Trimethoprim-sulphamethoxazole
VLBW	Very Low Birth Weight (< 1 500g)
Y-NHH	Yale New Heaven Hospital

1 Introduction

1.1 Neonatal sepsis

Invasive neonatal infections are important causes of mortality and morbidity in newborn infants all over the world including industrialized countries with high hygienic standards, deliveries at hospitals, access to antimicrobial agents for prophylaxis, treatment and advanced intensive care. Most studies of the incidence and etiology of neonatal sepsis and meningitis come from these countries, while there is a lack of data from developing countries, where the morbidity and mortality probably are immense.

There is a strong association between infection in the amniotic cavity and premature delivery [Gold, Rom]. Infections with clinical symptoms in the mother, or more often, subclinical infections may cause up to 80 % of premature deliveries. Development of advanced neonatal intensive care has made it possible for the survival of ELBW and VLBW neonates, patients that are highly susceptible to invasive infections for a long time during their hospital period. Most infections in these children are clinically suspected, and only a part of all cases of neonatal sepsis are also verified by culture.

1.1.1 Definitions of neonatal sepsis and meningitis

Hippocrates introduced the word sepsis 2 400 years ago to denote a condition where an overwhelming infection leads to tissue breakdown with rotting, foul odor, and disease. The word “bacteremia” is used when bacteria are isolated from the blood whether clinical symptoms are present or not while the words “sepsis” or “septicemia” are used for the isolation of bacteria in blood in combination with clinical symptoms and signs of infections. Neonatal sepsis traditionally refers to this syndrome in newborn babies during the first month of life. During the last 15-20 years more premature/immature children survive and this has resulted in a large group of neonates with a high susceptibility to infections for a long period after birth. There is no universally agreed definition of the length of this period. The inclusion period for neonatal sepsis and meningitis often covers the whole hospital period [I, Bal 88, Fan, Gla, Rön 98] but sometimes the period of one month after fullterm pregnancy at 40 weeks is used. Neonatal sepsis and meningitis include both bacterial and fungal infections.

1.1.2 Very early, early, late and very late neonatal sepsis

There is no consensus on how to classify neonatal sepsis and meningitis in periods after birth. Early and late onset sepsis has been reported as occurring before or after 48 hours of age [Isa 96], 72 hours of age [Sto 02a], or 96 hours of age [Biz, Gla]. The first week of life is often reported as early onset sepsis with a subgroup of infections that develop during the first 24 hours of life called very early onset infections [I, Rön 98, Rön 05, Tes, Ves]. Late onset infections occur during the second to fourth weeks of life while infections from day 28-30 to day 120-180 are called very late onset infections [I, Biz].

1.1.3 Susceptibility to infections

Newborns are highly susceptible to bacterial and fungal infections. Several immunological systems are immature and in premature neonates even less developed with reduced level of maternally derived immunoglobulins, complement factors and decreased function of the neutrofil phagocyte system. IgG is the only immunoglobulin class that crosses the placenta but the major fraction is being transferred after 32 weeks of gestation. At term the IgG level is approximately the same as in the mother with all subclasses present. Premature infants have lower IgG levels, at 28 weeks of gestational age the IgG level is less than 50 % of the levels at term [Fle, Gar].

1.1.4 Bacterial colonization of newborns

The intrauterine environment of the fetus is sterile and the colonization starts after rupture of the membranes. The baby then rapidly becomes colonized during the passage through the birth canal and later from the surrounding environment. In the birth canal the newborn encounters several potential pathogens including Gram-negative *Enterobacteriacae*, staphylococci and most importantly GBS. Healthy newborns acquire their own unique normal flora within a few days after birth [Gol 89]. Neonates who require care in NICUs have different microbiological colonization compared to healthy newborns. These newborns are often treated with broad-spectrum antibiotics, which wipe out whatever bacteria the infant might have acquired during birth. In the first week of life virtually all infants in NICUs are heavily colonized with a multiplicity of staphylococcal strains, Gram-negative organisms (often multiresistant) and *Candida* species [Gol 81, Men]. There is, however, considerable day-to-day variation in numbers and species

present. Several investigators have linked the hands of hospital staff to colonization, antibiotic resistance and infections [Boyc, Kli 01]. It has been demonstrated that patients in NICUs become colonized and infected with the same strains of staphylococci that are isolated from hospital staff and that nurses and neonates shared the same clone of *Staphylococcus epidermidis* [Mil]. Vaginal colonization with GBS occurs in 15-30 % of all pregnant women [Dav 01a, Cam, Håk 07] and 50-70 % of their infants become colonized during or after birth but only 1-2 % of the colonized infants will develop an early onset GBS sepsis [Ben 99a].

1.1.5 Risk factors for neonatal infection

Newborns that develop early-onset sepsis usually have one or more risk factors associated with obstetric complications, such as PROM, premature onset of labor, chorioamnionitis, peripartum maternal fever, traumatic delivery and fetal hypoxia [Rem (ch 21)]. Risk factors for development of late-onset infections are low birth weight and low gestational age, prolonged hospital stay, antibiotic treatment, central venous catheters, total parenteral nutrition, malformations, operations and other invasive interventions [Jia, Fan].

1.1.6 Causative agents in neonatal infections

Changing patterns of organisms responsible for neonatal sepsis have been reported from neonatal centra in USA and Europe. Before the antibiotic era Gram-positive cocci, eg group A streptococci and *Staphylococcus aureus* caused most of the neonatal sepsis cases. Following the introduction of sulfonamides and penicillin, Gram-negative enteric organisms, particularly *Escherichia coli* became the predominant cause of serious neonatal infections but *Staphylococcus aureus* was also an important pathogen during this period. During the late 1960s and 1970s GBS emerged and was the dominating organism together with Gram-negative enteric organisms [I, Biz, Fre, Gla, Tes, Ves]. Although GBS continues to be a major pathogen in neonatal sepsis, *Escherichia coli* has outnumbered GBS as the leading cause of EOS in preterm babies in some regions, probably due to the increasing use of perinatal antibiotic prophylaxis with ampicillin and the emergence of ampicillin resistant strains of *Escherichia coli* [Bal 01, Hyd]. CoNS have during the last decade emerged to become the most significant pathogens in LOS [I, Biz, Cla, Gla, Jia].

1.1.6.1 Early and very early onset infections

GBS and Gram-negative bacteria (eg *Escherichia coli*) but also *Staphylococcus aureus* are the dominating pathogens in very early and early onset infections [I, Biz, Jia, Tes, Ves].

1.1.6.2 Late and very late onset infections

CoNS are the most commonly isolated organisms in late and very late onset infections followed by Gram-positive and Gram-negative organisms which are equally often isolated [Biz, Cla, Jia, Käl]. Common organisms are Klebsiella species, enterococci and Candida species [I, Biz, Cla, Jia, Mak].

1.1.7 Incidence

The true incidence of neonatal sepsis and meningitis is hard to estimate and studies from different centra are difficult to compare. Routines for obtaining cultures from blood and CSF vary and are often not described in epidemiological studies. There is no general agreement on how to include commensal organisms in the incidence rate and the definitions of the subgroups very early, early, late and very late infection periods differ between studies. In most studies the incidence has been calculated as the number of cases per live births in a hospital, (often a tertiary hospital with a large number of high-risk deliveries) or as the number of admissions or hospital days [Biz, Cla, Gla, Jia, Rön 98]. This will result in higher incidence rates in this kind of studies compared with population based incidence studies, which are based on numbers of cases and number of live births in a defined area [I, Gre, Käl, Tes, Ves]. The difficulties to compare different incidence studies make it important to follow the incidence and the etiological panorama in a defined hospital or geographic area. One objective in this thesis [I] was to study the incidence of neonatal infections in a defined geographic area under the same circumstances as in an earlier publication from the same geographic area [Tes].

The reported incidence of GBS infections, the dominating pathogen for neonatal sepsis and meningitis ranges from 0.7-3.7/1 000 live births but has declined since the introduction of antibiotic prophylaxis [Dal, Eke, Sch 00, Tri 04]. In the prophylaxis program mothers are identified either on the basis of clinical risk factors or on positive lower vaginal/rectal swabs obtained late in pregnancy. In USA where the incidence of early onset GBS infection used

to be high, early onset GBS disease has declined with more than 50 % after introduction of the prophylaxis program [Sch 00].

1.1.8 Coagulase negative staphylococci (CoNS): pathogens or contaminations

CoNS form an important part of the normal bacterial flora of the skin, the nasal mucosa and the umbilicus of all humans including newborns. It is therefore unavoidable that these organisms often contaminate blood and CSF cultures since many procedures involve penetration of the skin. There is, however, convincing evidence that CoNS also cause invasive infections in neonates. The first study in which CoNS were isolated from blood of symptomatic newborns and assumed to be the causative organism of the infection was published in 1971 [Cly]. Since then CoNS have emerged to be the most common agents in late onset sepsis among neonates. Prematurity, prolonged hospital stay and invasive interventions are known risk factors for CoNS sepsis. Preterm neonates are often colonized with the same bacteria that cause invasive disease and it has been assumed that most invasive CoNS infections mainly derive from the skin and that indwelling vascular lines is the major port of entry for the infection. The ability of CoNS to form an adherent multilayered biofilm on polymer surfaces is considered their main virulence determinant [Ott]. In a study from Australia 50 % of neonates with CoNS sepsis had a central venous catheter [Isa 03]. There are, however, authors who have proposed an alternative hypothesis regarding the pathogenesis of CoNS bacteremia. They suggested that CoNS bacteremia may be primarily due to mucosal colonization and that bacterial translocation occurs through an immature or damaged bowel-mucosa, rather than to vascular lines [Luo]. The associations between total parenteral nutrition and intestinal mucosa atrophy may be the link between total parenteral nutrition and CoNS infection in this hypothesis [San].

There is no general agreement on how to define CoNS sepsis in neonates but most authors define neonatal CoNS sepsis as bacteremia with CoNS, clinical symptoms of sepsis and laboratory signs of infection [Isa 96, Jia, Rön 98, Rön 05, Käl, Sto 02a]. One objective of this thesis was to apply the criteria according to the Y-NHH on a Swedish population and to estimate the incidence of CoNS sepsis [I].

1.2 Group B streptococci

Streptococcus agalactiae, also known as group B *streptococcus* (GBS), is a Gram-positive coccus of the genus Streptococcus; it is an opportunistic pathogen that colonizes the gastrointestinal and genitourinary tracts in healthy adults. Rebecca Lancefield classified streptococci into different serogroups according to the group specific polysaccharides in the cell wall [Lan 33]. GBS is then divided into serotypes according to the CPS [Lan 34, Lan 38]. Nine serotypes (Ia, Ib, II-VIII) have been identified until now [Kog]. GBS was rarely mentioned as a cause of neonatal sepsis until 1964, when the first study of perinatal GBS infections was published [Eic]. Before this time, it was mainly recognized as a cause of bovine mastitis. GBS has since the early 1970s remained as the leading cause of mortality and morbidity among neonates [I, II, Sch 00, Puo] and is also a common pathogen among pregnant women and an important cause of premature delivery [Fei]. The incidence among nonpregnant adults has increased over the past decade, particularly among adults with underlying severe diseases [II, Edw 05b, Far]. The CPS has been shown to be a target for protective antibodies. Most GBS strains also express surface proteins that can elicit protective immunity. Both surface proteins and CPS have therefore been evaluated as possible components in a vaccine against GBS.

The incidence of neonatal early onset GBS disease has declined during the 1990s in the industrialized world since the introduction of surveillance programs and intrapartum antibiotic prophylaxis [CDC, Sch 02]. This positive development is, however, partly counteracted by the increased survival of premature neonates with a high susceptibility to infections. Furthermore, widespread use of antibiotic prophylaxis rise concerns about selection of resistant bacteria and risk of allergic reactions in the mother.

1.2.1 GBS infections in neonates

A GBS infection in neonates divides into early and late onset disease depending on the neonates age at the onset of the disease. In early onset sepsis, presenting during the first week of life, the neonate is infected by exposure to GBS before birth through ruptured membranes or during passage through the birth canal [Rem (ch 26)]. The organism is spread from the maternal genital tract through ruptured membranes into the fetus. After colonization of the respiratory tract, disease may develop and GBS can further disseminate into the blood stream. Transmission of GBS into the amniotic fluid can even occur through intact membranes. The disease

manifestations are sepsis, meningitis and pneumonia, often with rapid progress and multiorgan involvement. Despite significant progress in neonatal intensive care in recent decades, GBS sepsis still carries a case fatality rate between 5-15 % [II, Dav 01b, Tri 04].

In late onset GBS disease the predominant manifestation is sepsis with meningitis. The case fatality rate is lower than in early onset disease (2-6 %) but long-term neurodevelopment sequels of varying severity appear in about 50 % of patients with meningitis. The pathogenesis in late onset disease is less well understood but vertical transmission from the mother to the child probably explains most of the infections and breast milk can be a source of infection [Bin]. The infection can also occur as nosocomial, acquired by horizontal transfer from nursery staff.

1.2.2 GBS infections in adults

In pregnant and postpartum women, GBS cause a variety of different clinical manifestations, from mild urinary tract infections to severe sepsis, chorioamnionitis, endometritis and septic abortion [Sch 00]. In nonpregnant adults, GBS infections are an increasing cause of invasive disease particularly in elderly people and among those with underlying medical conditions [II, Bol, Dah, Far]. The most common manifestations in nonpregnant adults are sepsis, erysipelas, endocarditis, urosepsis, and meningitis. Diabetes mellitus and malignant diseases are the most prevalent underlying diseases [II, Sch 00].

1.2.3 The group B carbohydrate of GBS

Rebecca Lancefield developed a serological classification for streptococci based on the antigenic differences in cell wall carbohydrates. The letters A-G designated the different serogroups [Lan 33]. The group B carbohydrate does not seem to be important for natural immunity to GBS infection. It does not induce protective antibodies against GBS infection [Lan 75] and maternal antibodies against the group B specific carbohydrate do not protect against neonatal infection [Ant].

1.2.4 The polysacharide capsule

Lancefield classified the type specific CPS into four serotypes Ia, Ib, II, and III [Lan 34, Lan 38]. So far nine serotypes have been defined Ia, Ib and II-VIII [Kog]. CPS protects GBS by down regulating the complement system and preventing phagocytosis and plays therefore an important role in the pathogenesis of GBS. The CPS has been shown to elicit type specific antibodies against GBS, which protect against invasive infections [Lan 75, Bak 76]. Protective immunity can be elicited in animals by passive administration of type specific antibodies or by active immunization with the type specific CPS [Lan 75, Bak 76]. The CPS has therefore been investigated as components in GBS vaccines [Bak 85]. A survey of the serotype distribution of invasive GBS isolates with special emphasis on differences between age groups and changes over time is an important part of epidemiological studies of GBS disease and one of the objectives of this thesis [II].

1.2.5 Serotype distribution of GBS

In epidemiological studies from the 1970s and 1980s serotypes Ia, Ib, II, and III were evenly distributed among neonates with early-onset disease [Dil, Wil 73]. In neonates with meningitis serotype III was the dominating serotype. In adults serotype II dominated [Schu 98]. A shift in serotype distribution was reported in several studies from the 1990s when serotype V emerged as an important serotype among both neonates and adults [II, Ber, Har, Lin 98, Ren]. Further studies from both Europe and North America show that serotype III still is the most important serotype in neonatal GBS infections and that the rate of serotype V continues to increase and now account for 4-21 % [II, Eke, Tri 06, Wei]. Serotypes VI and VIII predominate in colonizing GBS strains among pregnant women in Japan [Lac] but are rare among isolates from Europe and North America. In adults serotype V has increased and is now the most common serotype [II, Edw 05a, Edw 05b, Far, Har].

1.2.6 Alpha c protein and beta c protein

The first surface protein antigen described was the c antigen in 1971, which was found to consist of two fractions. One fraction was sensitive to digestion with trypsin and the other was resistant to digestion with trypsin [Wil 71]. The proteins were called the “Ibc proteins”. These proteins were later

designed alpha and beta, where the alpha antigen corresponded to the trypsin resistant protein fraction and beta to the trypsin sensitive fraction [Bal 79]. The two proteins have then been designed alpha c protein and beta c protein. A GBS strain can express one or both of the alpha and beta c proteins. The alpha c protein and beta c protein are primarily expressed by GBS strains of serotypes Ia, Ib and II. Both the alpha and the beta component of the protein c have been shown to elicit protective immunity in animal models [Bev 85b, Lan 75]. The gene *bca* encodes alpha c protein and the gene *bac* encode beta c protein.

*1.2.7 The alpha like protein (*alp*) family of surface proteins*

The alpha like family of surface proteins includes the alpha c protein, rib, alp2, alp3, alp4 and epsilon/alp1 proteins. They exhibit a ladder like pattern when analyzed with Western blot and are also named "ladder like" proteins. The proteins have regions of identical repetitions and show ability to elicit protective immunity in animal models. The alp2 and alp3 proteins have identical sequences in the N-terminals. They are considered to be variants of the classical R1 protein. The encoding gene for alp3, *alp3* is possessed in GBS serotype V strains indicating that the protein is expressed. [Lau 00]. Alp2 is more rare than the other ladder-forming proteins and the gene *alp2* has been identified in serotypes Ia, III and V [III, Lau 00, Zha].

Protein rib was first described as a novel group B streptococcal surface protein [Stå] but was later shown to be identical to protein R4 [Bev 95, Smi]. Protein rib is expressed by almost all type III strains and antibodies to rib confer protective immunity against lethal infection with rib expressing strains in a mouse model [Stå].

1.2.8 Other surface proteins

The group B protective surface protein (BPS) is the latest R-like protein antigen discovered [Erd].

Several other surface proteins of GBS have been identified and some of them, like the sip protein induce protective immunity. The sip protein has been identified in all nine serotypes of GBS [Rio]. The nomenclature of surface proteins and genes encoding surface proteins has been different and somewhat confusing in the literature. In Table 1 the most recent nomenclature of proteins and encoding genes is given.

Nomenclature protein	*Nomenclature gene*	*Year authors and [Ref]*
c alpha protein, Cα	*bca*	2000 Maelandet [Mae 00]
C alpha protein	*bca*	2002 Kong [Kon 02a]
Alpha-C protein	*Alpha-C*	2004 Creti [Cre]
Cα	*bca*	2004 Zeng [Zen]
		2006 Zhao [Zha]
alpha C protein	*bca*	2007 Ho [Ho]
epsilon	*epsilon*	2004 Creti [Cre]
[Alp1/Alp5/Epsilon]	*Alp1*	2004 Zeng [Zen]
epsilon/alp1	*epsilon/alp1*	2007 Ho [Ho]
C alpha like protein 2	*alp2*	2002 Kong [Kon 02a]
Alp2	*alp2*	2000 Lauchenauer [Lau 00]
		2004 Maeland [Mae 04]
		2006 Zhao [Zha]
C alpha like protein 3	*alp3*	2002 Kong [Kon 02a]
Alp3	*alp3*	2004 Maeland [Mae 04]
		2004 Zeng [Zen]
		2006 Zhao [Zha]
Alp3/R28	*alp3*	2005 Lindahl [Lin]
R4	*rib*	2004 Maeland [Mae 04]
R4	*r4*	2004 Smith [Smi]
Rib	*rib*	1993 Stålhammar-Carlemalm [Stå]
		2006 Zhao [Zha]
		2007 Ho [Ho]
C beta protein	*bac*	2002 Kong [Kon 02a]
c beta protein, cβ	*bac*	2004 Maeland [Mae 04]
cβ	*bac*	2004 Zeng [Zen]
BPS	*sar5*	2002 Erdogan [Erd]
Sip	*sip*	2001 Rioux [Rio]

Table 1. Nomenclature for some of the GBS surface proteins and encoding genes.

1.2.9 Neonatal susceptibility and pathogenesis of GBS infection

Recognition of bacteria as foreign material can be done indirectly via opsonization with antibodies or the complement system. Neonates have low levels of both antibodies and complement proteins and this negatively affects recognition, chemotaxis and phagocytocis of the bacteria. The CPS is a major virulence factor since it surrounds the organism and the cell wall antigen will be covered. CPS protects the bacteria from opsonization and phagocytocis but there will also be a balance between the need to adhere and the immune evasive capacity conferred by the capsule. The role of the surface proteins in bacterial virulence is not yet fully understood but

attachment to the epithelial surface via the surface proteins is a necessary component of colonization and further invasiveness. It has been shown that strains of GBS undergo mutations in the repeated region of the alpha c protein encoding gene *bca* during the passage from mother to infant. These mutations coincide with a loss of susceptibility to antibody-mediated killing and the GBS strain become less well recognized by antibodies and less susceptible to phaghocytic killing [Mad 96, Grav 98]. The beta c protein has been shown to increase the binding of the complement factor H that inhibits the alternative pathway of the complement system [Are]. GBS is a very potent inflammatory agent and the infected host suffers from all consequences of hyper-inflammation and inflammatory cytokines. In animal models of early-onset sepsis it has been shown that GBS infections cause an early hemodynamic phase with pulmonary hypertension, reduced cardiac output, and hypoxemia followed by a late phase characterized by a progressive fall in cardiac output, systemic hypotension, granulocytopenia, granulocyte trapping in the lungs and increased pulmonary vascular permeability [Roj, Rem (ch 26)].

1.2.10 Prevention of GBS disease

Strategies to prevent GBS infection can be obtained by elimination of exposure to GBS or enhancement of the resistance of the host to the organism. Chemoprophylaxis and vaccines are the possibilities available.

1.2.10.1 Chemoprophylaxis to prevent GBS disease

In the 1980s, it was found that effective treatment with chemoprophylaxis of GBS colonized women resulted in reduced rates of neonatal colonization and early-onset sepsis [Boy]. In USA the CDC stated in 1996 that one of two preventive strategies should be used: (1) universal prenatal GBS screening of all women at 35-37 weeks of gestation followed by intrapartum chemoprophylaxis of all GBS carriers, or (2) treatment of women in labor who have risk factors and whose GBS status is unknown [CDC]. Hospitals that followed the recommendations had fewer neonates with early-onset disease [Fac]. The risk factors are shown in Table 2. In USA it was shown that early-onset GBS sepsis was reduced by 78 % when using the screening protocol compared with 41 % when using the risk based method [Ben 99b]. From 2002 USA uses the screening program according to the revised guidelines from the CDC [Sch 02]. In Sweden there exist routines for regular check-ups of women during pregnancy at antenatal clinics and there is no

national program yet but most centers use a risk-based program (www.infpreg.se). Also in other parts of Europe a risk-based program is used [Tri 04, Col]. If all colonized women receive chemoprophylaxis during labor it will be approximately 25 % of all laboring women. The possibility of emerging resistance to the most commonly used antibiotics is a concern, especially if penicillin allergy is present. Several patterns of antimicrobial resistance in GBS have emerged especially to clindamycin and erythromycin [Bor, Flu, Mou]. Another shortcoming of intrapartum chemoprophylaxis is that it does not prevent late-onset disease. One objective of this thesis was to study the antimicrobial susceptibility among invasive GBS isolates, changes over time and differences related to the age of the patient and to capsular serotype [IV].

Chorioamnionitis
GBS bacteriuria in current pregnancy
Maternal rectovaginal colonization
Maternal temperature of ≥ 38° C
Preterm labor or preterm rupture of membranes < 37 weeks of gestation
Previous delivery of infant with early-onset GBS sepsis
Prolonged (> 18 hours) interval between rupture of membranes and delivery

Table 2. Clinical risk factors for GBS transmission to neonates [Sch 02]

1.2.10.2 Immunoprophylaxis to prevent GBS disease

Active immunization with a GBS vaccine for prevention of GBS disease should have many advantages; it would protect neonates against early and late-onset disease as well as maternal and adult disease. The vaccine should preferably reduce or eliminate maternal gastrointestinal/genital carriage of GBS as this will reduce neonatal exposure at times when maternal transfer of antibody is inadequate, for example when the baby is born prematurely. Vaccines against GBS have been studied almost since the time when it was shown that capsular serum antibodies were protective [Lan 34] but no vaccine is, however, as yet available. In the 1970s and 1980s several immunization studies with purified CPS from the major serotypes were performed but disappointingly they were found to be poorly immunogenic in adult volunteers [Bak 88, Kot]. To enhance the immunogenicity, CPSs have been conjugated to a carrier protein. This conjugation leads to activation and clonal expansion of carrier-specific T-cells, which leads to induction of immunologic memory and the ability to respond to the CPS antigens already

in infancy. Polysaccharides have been coupled to tetanus toxoid (TT) and immunization studies in animals showed that they were more immunogenic than uncoupled CPS [Lag, Pao]. Further studies with conjugated CPS vaccines in adult volunteers and pregnant women have showed a good response to the vaccines [Bak 03a, Bak 03b] Correlation between IgG antibodies in maternal and cord sera after immunization indicates efficient transport of specific antibodies to the fetus via placenta [Bak 03b].

Several GBS surface proteins elicit protective immunity and it has been shown in animal models that they confer protective immunity to the offspring [Bev 85b, Mad 92, Stå]. Some surface proteins conjugated to CPS were immunogenic and also simultaneously enhanced the immunogenicity of the CPS [Grav 99, Mad 94]. Surface proteins can also act as vaccine components alone or in combination. A combined rib and alpha c protein vaccine was shown to protect against a majority of infections with GBS strains in a mouse model [Lar]. One objective of this thesis was to identify surface protein antigens in invasive GBS isolates that could be used in GBS vaccines [III].

1.2.11 Methods for epidemiological typing

Knowledge of the epidemiology of GBS infections requires typing methods that can identify changes of virulence or emergence of new serotypes of GBS. Serotyping of GBS can be done by immunoprecipitation, latex agglutination, coagglutination, double immunodiffusion, enzyme immunoassays and recently by molecular methods [Bev 85a, Håk 92, Kon 02b, Lan 34, Wen, Zen]. Genotyping methods, including PCR, PFGE, MLEE and MLST, can be used to characterize bacterial genes and distinguish specific bacterial clones as well as emerge and spread of new clones [Kon 02a, Jon 03, Que, Skj]. One aim of this thesis was to characterize invasive GBS based on genes encoding surface proteins in samples from a defined geographic area [III].

2 Objectives of the study

The main objectives of this thesis were to estimate the incidence and etiology of invasive infections in neonates and to characterize invasive strains of group B streptococci from a defined geographic area.

2.1 Paper I

The main objectives of paper I were to study the incidence, etiology and prognosis of neonatal septicaemia and meningitis caused by traditional pathogens during the first 120 days of life and to evaluate if CoNS found in cultures from blood and cerebrospinal fluid were true pathogens or contaminants according to criteria from the Yale-New Haven Hospital.

2.2 Paper II

In paper II the specific objectives were to survey the serotype distribution of invasive GBS isolates in a Swedish population, and to detect changes in serotype distribution over time and differences between age groups and patients with different clinical manifestations.

2.3 Paper III

The objectives of paper III were to analyze the distribution of three surface proteins and six genes encoding for surface proteins in invasive GBS isolates from a defined geographic area over a 13-year span, to compare the distribution in different age groups and capsular serotypes and to identify surface protein antigens which might be used in GBS vaccines.

2.4 Paper IV

The specific objectives of paper IV were to survey the susceptibility against 12 antimicrobial agents among invasive GBS isolates in southwest Sweden during 1988-2001, to monitor changes over time and to study differences related to the age of the patient and to capsular serotypes.

3 Material and methods

3.1 Patients and material

3.1.1 Paper I

All infants aged 0 through 120 days with a bacterial or fungal isolate from blood or cerebrospinal fluid during 1987-1996 were identified from the files of the departments of clinical bacteriology at Sahlgrenska and Östra Hospitals, Göteborg, Sweden. These two laboratories served the two departments of pediatrics with three neonatal wards in the area. The infant's mother should be living in Göteborg or one of the surrounding communities of Mölndal, Härryda, Partille, Kungälv and Öckerö at the time of delivery. The total number of live births was 83 550 during the study period and 1 209 infants had a positive culture.

3.1.2 Paper II

Invasive GBS isolates from both neonates and adults were prospectively collected from normally sterile sites (blood, CSF and synovial fluid). Strains were collected from the six laboratories of clinical bacteriology, which served all 13 hospitals in the two counties Västra Götaland and Halland in western Sweden between 1998 and 2001. The laboratories were: the Department of Bacteriology, Sahlgrenska University Hospital, Göteborg (n=25), the Department of Bacteriology, Sahlgrenska University Hospital Östra, Göteborg (n=29), the Departments of Clinical Microbiology at Borås Hospital (n=30), Halmstad Hospital (n=26), Skövde Hospital (n=23) and Uddevalla Hospital (n=34). The strains were obtained from 52 neonates, 33 males and 19 females aged 0-86 days and from 115 adults, 55 males and 60 females with a median age of 68 years (range 19-96 years). Only one isolate from each infectious episode was included in the study. In total, 161 GBS strains were available for typing 50 from neonates and 111 from adults.

3.1.3 Paper III and IV

Invasive GBS isolates were available from two studies performed in southwest Sweden. In the first study, a total of 136 invasive GBS isolates from 1988-1997 had been collected [Ber]. The participating laboratories were the departments of bacteriology, Sahlgrenska Hospital Sahlgrenska, Göteborg (n=76), Sahlgrenska Hospital Östra, Göteborg (n=12), Borås

Hospital (n=6), Halmstad Hospital (n=25) and Karlstad Hospital (n=17). From the four latter laboratories only strains collected 1995-1997 were available. The second study included the same patients and GBS strains as those described in paper II. Only one isolate from each infectious episode was included. In the second study period, 1998-2001, most labor wards in the area agreed with the risk based program for intrapartum antimicrobial prophylaxis that was recommended by the American College of Obstetricians and Gynecologists 1996 [CDC].

3.2 Methods

3.2.1 Paper I

Organisms were divided in ”traditional neonatal pathogens” and ”commensals” according to guidelines from the Y-NHH [Gla]. Traditional pathogens were always considered as the cause of the infections. They include group A, B and D streptococci, *Streptococcus pneumoniae*, *Staphylococcus aureus, Listeria monocytogenes, Haemophilus influenzae* and *parainfluenzae* and all other Gram-negative rods and Candida species. Commensal species include CoNS, Gram-positive rods other than Listeria, Gram-negative cocci (other than *Neisseria meningitides* and *Neisseria gonorrhoea,* which were not isolated from any culture), and Gram-positive anaerobes.

CoNS was considered the cause of the infection if another blood culture obtained within 24 hours after the first yielded the same organism *or* if an intravascular access device was in place before symptoms developed *and* if at least one of the following symptoms were documented: apnea, bradycardia, temperature > 38.0^{o}C, temperature < 36.5^{o} C [Gla]. Commensal species other than CoNS were not included in this study. ”Aerobic Gram-negative rods” is used as a term for all Gram-negative rods except *Haemophilus* species. Multiple positive blood and/or CSF cultures in the same infant growing the same species with the same antibiotic susceptibility were considered as a single case. If different species were isolated from the same infant on different occasions, each infectious episode was included as a separate case.

Very early, early, late and very late onset infections were defined as infections with onset of symptoms < 24 hours, 1-6 days, 7-27 days and 28-120 days after birth, respectively. Clinical data were obtained from the hospital records from all infants with an isolate and all relevant information were available for all patients.

Information on number of live births, birth weight and gestational age in the six communities was obtained from the Centre for Epidemiology at the National Board of Health and Welfare, Stockholm, Sweden.

3.2.2 Papers II, III and IV

GBS isolates were collected from normally sterile sites (blood, CSF and synovial fluid). Only one isolate from each infectious episode was included in the study. Isolates were identified as GBS by colony morphology, microscopy following Gram's stain of smears, and coagglutination with group specific reagents (Streptest; Murex Biotec, Dartford, UK). The isolates where then stored in broth at -70° C. Clinical data (age, sex, gestational age, underlying medical conditions, clinical manifestations and outcome) were obtained from individual hospital notes. All hospital notes were found and the information was available for all patients.

3.2.3 Paper II

Serotyping was performed by coagglutination (Group B Streptococcus, Serotyping Test, ESSUM®, Bacterum AB, Umeå, Sweden [Håk 92]) with type specific antisera for serotypes Ia, Ib, II, III, IV, V, VI, VII and VIII. The only strain which was not typeable by coagglutination was examined with precipitation techniques (ring test and diffusion test) [Rot, Wil] for serotypes I through VIII by Dr Jitka Motlová, National Streptococcus and Enterococcus Reference Laboratory, National Institute of Public Health, Prague, Czech Republic.

3.2.4 Papers III and IV

The isolates were stored in broth at -70° C prior to lyophilization and then transported to St Olav's University Hospital, Trondheim, Norway.

3.2.5 Paper III

Antibody-based surface protein typing was performed using murine monoclonal antibodies against the GBS proteins alpha c protein, beta c protein, and rib in an indirect whole cell based fluorescent antibody test (FAT). The fluorescence, recorded in a Nikon epifluorescence microscope,

was graded from 0 to 3+, with scores of 2+ and 3+ indicative of a positive test. For molecular genotyping a multiplex PCR was used to detect the genes *bca, epsilon/alp1, rib* and *alp2/alp3,* encoding the proteins alpha c protein, epsilon/alp1, rib and alp2/alp3. The primers were constructed (Eurogentech SA, Liege, Belgium) and used as described by Creti [Cre]. All *alp2*/*alp3* PCR positive isolates were further tested by *alp2* and *alp3* specific PCR. All strains were examined for the gene *bac* using primer pairs as specified by Kong et al [Kon 02a]. The test was performed and the PCR products were detected using Agilent 2100 Bioanalyzer as recommended by the manufacturer (Agilent Technologies, St Clara, CA, USA). Only one isolate from each infectious episode was included in the study.

3.2.6 Paper IV

The epsilometer test (E-test) was used to determine the minimum inhibitory concentrations (MICs) to 12 antibiotics. The strains were cultured overnight on either blood or Columbia agar plates (5 % ox blood). The inoculum was prepared according to the instructions of the manufacturer using Brain Heart broth with a density of approximately McFarland 0.5. Using cotton swabs and swabbing rotator (EPA inoculator, AES laboratorium) the bacterial suspension was transferred to the plates. The handling of the E-test strips was done according to the instructions of the producer. Plates with E-test strips were incubated at 35-37° C for 20 h in 5 % CO_2. After inspecting the plates with semiconfluent growth, the MIC results were read where growth merged with the strip at the sharp end of the pear-shaped inhibition zone. Bacteriostatic drugs such as clindamycin, doxycycline, erythromycin, linezolid, trim-sulfa and quinupristin-dalfopristin can give diffuse edges and were read at 80 % inhibition. An E-test MIC value that fell between the conventional two-fold dilutions was rounded up to the next upper two-fold dilution value before interpretation. Isolates were classified as sensitive (S), intermediate (I), or resistant (R) according to the CLSI guidelines to interpret MIC results [CLSI]. CLSI guidelines for breakpoints are derived from zone diameters in the disc diffusion test. For antibiotics without MIC values for GBS in the CLSI guidelines, MIC values for *Streptococcus pneumoniae* were used. For trim-sulfa MIC values on the E-strips refer to the trimethoprim component of the combination. Reported MIC values for the combination refer to the sum of the two substances' concentrations in the ratio 1:19. The trim-sulfa MIC values were therefore obtained by multiplying the trimethoprim concentration with 20. A phenotypic approach was employed to detect inducible clindamycin resistance. Erythromycin-resistant and clindamycin sensitive strains were tested with clindamycin and erythromycin

disks 25 mm apart. Strains displaying blunting of the clindamycin zone proximal to the to the erythromycin disk were classified as D-zone positive (inducible resistance) and classified as clindamycin resistant and assigned a MIC 256 μg/ml.

4 Summary of main results

4.1 Paper I

Septicaemia and meningitis in neonates and during early infancy in the Göteborg area of Sweden

The incidence of invasive infections verified with blood or CSF culture with "traditional neonatal pathogens" in the first 28 days of life 1987-1996 was 3.7/1 000 live births. The yearly incidence ranged from 2.7 to 4.5 with no tendency to increase or decrease over time. In 90 % of the cases only the blood culture was positive. Meningitis occurred in 10 %, most often together with a positive blood culture (72 %). The overall case fatality rate in infections day 0-27 was 9 %.

Only 64 cases (21 %) with invasive infections day 0-27 had a gestational age ≥ 37 weeks, birth weight ≥ 2 500 g and no known predisposing conditions. Premature birth and VLBW resulted in an increased risk of invasive infections with an incidence in the 28 first days of life of 162.8/1 000 live births among neonates < 29 weeks of gestational age. The case fatality rate in patients with culture verified infections was 23 % in neonates with a gestational age < 29 weeks and 3 % among neonates ≥ 37 weeks.

The most common isolates day 0-27 were aerobic Gram-negative rods, GBS and *Staphylococcus aureus*. These organisms were isolated alone or in mixed infections in 239 cases (78 %). The incidence rates were 1.0/1 000 live births for each of these organisms. The incidence of enterococcal infections was 0.7/1 000 live births (Table 3).

Very late onset infections from day 28 through day 120 after birth with "traditional neonatal pathogens" were diagnosed in 69 neonates/infants with 83 infectious episodes. The incidence was 10.1/1 000 live births in preterm and 0.5/1 000 live births in fullterm infants. There were no major differences in the etiologic panorama between neonatal infections seen in preterm and fullterm neonates. The species are shown in Table 3.

Organism	*day 0-27*		*day 28-120*	
Group B streptococci	73	20 %	4	3 %
Staphylococcus aureus	61	17 %	12	10 %
Aerobic Gram-negative rods				
Escherichia coli	36	10 %	9	8 %
Klebsiella pneumoniae	25	7 %	4	3 %
Klebsiella oxytoca	2	1 %	1	1 %
Pseudomonas species	5	1 %	1	1 %
Serratia marcescens	1	0.3 %	1	1 %
Enterobacter species	7	2 %	7	6 %
Xanthomonas maltophilia	1	0.3 %		
Enterococci	37	10 %	13	11 %
Streptococcus pneumoniae	4	1 %	6	5 %
Group A streptococci			1	1 %
Listeria monocytogenes	2	1 %		
Haemophilus influenzae	5	1 %	2	2 %
Haemophilus parainfluenzae	1	0.3 %		
Prevotella species	1	0.3 %		
Candida albicans	14	4 %	7	6 %
Candida parapsilosis	2	1 %	3	3 %
Candida glabrata			1	1 %
Ureaplasma urealyticum			1	1 %
Mixed bacterial infections *	28	8 %	10	9 %
CoNS	60	16 %	32	28 %

Table 3. Organisms isolated from blood and/or CFS day 0-27 and 28-120

**GBS , Staphylococcus aureus, Escherichia coli, Klebsiella, Pseudomonas, Enterobacter, Enterococci, Group A streptococci* and *Candida albicans.*

VLBW and premature babies were overrepresented in late onset infections and term babies dominated in very early and early infections (Table 4). GBS was overrepresented (52 %) in very early onset infections. Aerobic Gram-negative rods were overrepresented (47 %) in late onset infections.

Infections (%)

Onset of infection days	*< 1 500g*	*1 500 - 2 499g*	*> 2 499g*	*< 30 weeks*	*30-36 weeks*	*> 36 weeks*	*Total*
0	12 (15)	17 (21)	52 (65)	12 (15)	20 (25)	49 (60)	81 (17)
1-6	20 (15)	29 (22)	84 (64)	16 (12)	34 (26)	83 (62)	133 (28)
7-27	76 (50)	22 (15)	53 (35)	78 (52)	24 (16)	49 (32)	151 (31)
28-120	45 (39)	26 (23)	44 (38)	44 (38)	31 (27)	40 (35)	115 (24)
total	153 (32)	94 (19)	233 (49)	150 (31)	109 (23)	221 (46)	480 (100)

Table 4. Number and (%) of all infections in relation to birth weight and gestational weeks.

CoNS were isolated together with traditional pathogens in many infections. CoNS were isolated alone or together with other commensals from 487 cases during the first 28 days of life and from 146 cases between day 28 and 120 after birth. Using the criteria from Y-NHH, 85 % of all CoNS isolates were contaminants. After exclusion of these cases, the incidence of CoNS infections was 1.1/1 000 live births. CoNS infections occurred most frequently in premature infants with VLBW in late and very late infections (Table 5). Predisposing factors were found in 89 of the 92 CoNS infections. The most common were preterm delivery and/or low birth weight, malformation, umbilical catheter, caesarian section, idiopathic respiratory distress syndrome and mechanical ventilation.

CoNS infections (%)

onset of infection days	*< 1 500g*	*1 500- 2 499g*	*> 2 499g*	*< 30 weeks*	*30-36 weeks*	*> 36 weeks*	*Total*
0	1 (17)	4 (67)	1 (17)	1 (17)	4 (67)	1 (17)	6 (7)
1-6	6 (43)	4 (29)	4 (29)	5 (36)	5 (36)	4 (29)	14 (15)
7-27	28 (70)	9 (23)	3 (8)	30 (75)	8 (20)	2 (5)	40 (43)
28-120	20 (63)	5 (16)	7 (22)	22 (69)	6 (19)	4 (13)	32 (35)
total	55 (60)	22 (24)	15 (16)	58 (63)	23 (25)	11 (12)	92 (100)

Table 5. CoNS infections in relation to onset of infection and birth weight and gestational age.

4.2 Paper II

Serotypes and clinical manifestations of invasive group B streptococcal infections in western Sweden 1998-2001

During the study period, 52 invasive GBS infections in neonates and infants aged 0-86 days (33 boys, 19 girls) were documented. No patient with invasive GBS infection was found in the age group 3 months to 18 years. 115 invasive GBS infections were identified in adults (55 males, 60 females). Their median age was 68 years (range 19-96 years). The case fatality rate was 8 % among neonates and 9 % among adults.

Of the neonatal cases 80 % were early onset infections, 16 % were late onset (7-27 days after birth) infections and 2 % were very late onset (28 days to 4 months after birth) infections. Preterm neonates with EOS had known risk factors for infection in 64 %.

Most adults had a known underlying medical condition (82 %), the most common being diabetes mellitus and malignant disease.

A total of 161 invasive strains were available for serotyping. Fifty of these strains (31 %) were obtained from neonates and infants. The distribution is shown in (Table 6). There were no differences in neonates related to gestational age, postnatal age, manifestation or outcome and serotype distribution.

Neonates		
Serotype	*Number*	*Percent*
Ia	5	10 %
Ib	2	4 %
II	1	2 %
III	30	60 %
IV	1	2 %
V	11	22 %
NT	0	0 %

Table 6. Serotype distributions among neonates aged 0-86 days.

From adults 111 strains were available for serotyping.the distribution is shown in (Table 7).

	Adults	
Serotype	*Number*	*Percent*
Ia	10	9 %
Ib	10	9 %
II	7	6 %
III	28	25 %
IV	8	7 %
V	47	42 %
NT	1	1 %

Table 7. Serotype distribution among adults aged 19-96 years.

A difference in the serotype distribution between adults and neonates was found ($p < 0.002$) (Figure 1). The differences in serotypes III and V contributed most to this difference.

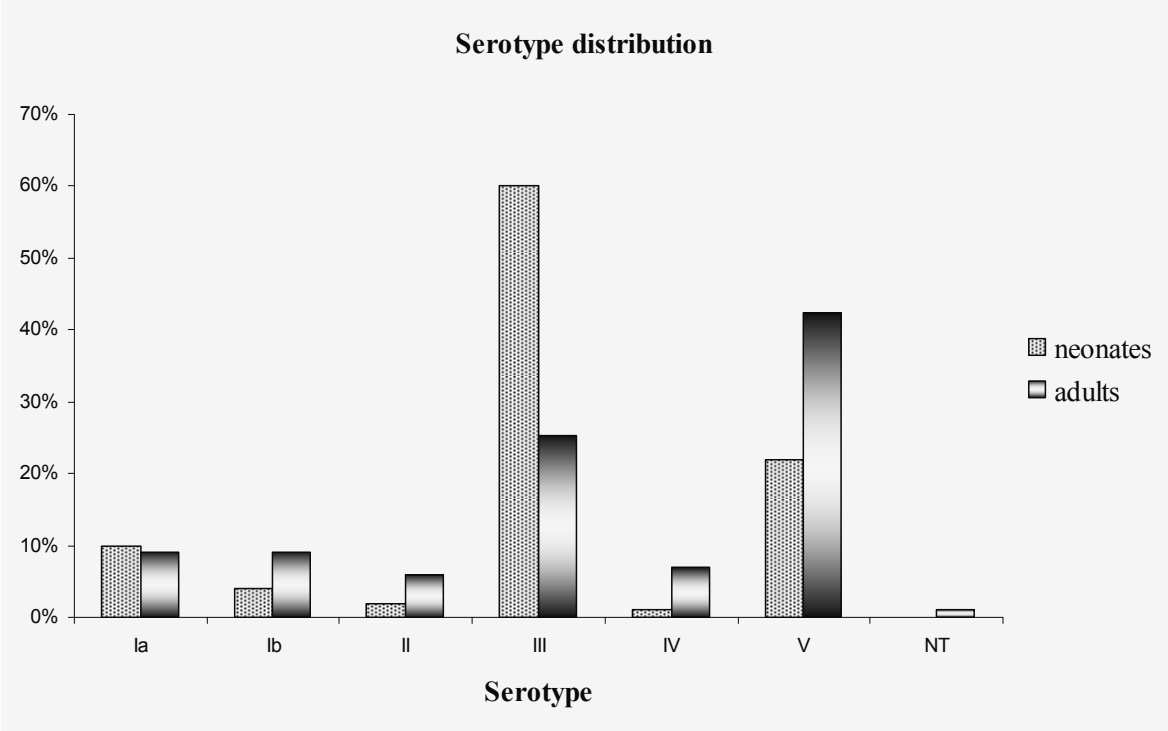


Figure 1. Serotype distribution in adults and neonates

4.3 Paper III

Characterization of invasive group B streptococci (GBS) based on demonstration of surface proteins and of genes encoding surface proteins

The surface proteins, alpha c protein, beta c protein and rib were detected alone or in combinations in 51 % of the strains. The most commonly detected protein was rib followed by alpha c protein. The most common combination was alpha and beta c protein.

The 6 genes *bca*, *bac*, *rib*, *epsilon/alp1*, *alp2* and *alp3* were identified alone or in combinations in 99 % of the GBS strains. The most common genes identified alone were *rib* followed by *alp3* and *epsilon/alp1*. *Alp2* could only be detected in 4 strains. All combinations of genes included the *bac* gene.

The *epsilon/alp1* and *bca* genes were more common in strains in which alpha c protein was found. *Bac* was more common in strains where beta c protein was detected and *rib* in strains where rib was detected. All of the 8 rib positive, *rib* negative strains had the *alp3* gene.

Most genotypes and surface proteins were detected in strains of all capsular serotypes. *Epsilon/alp1* was significantly related to serotype Ia, *bca* and *bac* to serotype Ib and II, *rib* to serotype III and *alp3* to serotype V.

Alpha c protein was significantly related to serotypes Ia, Ib and II. Beta c protein to serotypes Ib and II and rib to serotype III, respectively.

The *rib* genotype was more common in neonates than in adults and the *alp3* genotype was more common in adults than in neonates. There were no differences in protein expression between the age groups. The differences in genotypes between the two age groups could be explained by differences in serotypes between the two age groups.

There were no significant changes of genotypes and surface proteins during the 13-year period.

No genotype or surface protein that was studied was so common that it could be a successful GBS vaccine candidate alone.

4.4 Paper IV

Antimicrobial susceptibility of invasive group B streptococcal isolates from southwest Sweden 1988-2001

All isolates were sensitive to cefotaxime, meropenem, linezolid, vancomycin, moxifloxacin and quinupristin-dalfopristin.

Two strains were classified as R to penicillin G with MIC values of 0.25 μg/ml. Both strains were sensitive to the other two β-lactam antibiotics that were tested: cefotaxime and meropenem. According to the SRGA breakpoints these strains would have been classified as S.

The proportions of strains with intermediate susceptibility to erythromycin and clindamycin increased over the two study periods; for erythromycin from 10 % to 79 % and for clindamycin from 10 % to 55 %.

All strains were resistant to gentamycin but no strain showed high-level resistance. No strain was resistant to trim-sulfa.

There were no differences in susceptibility to any agents between strains isolated from neonates and from adults.

Serotype V dominated among strains with intermediate susceptibility to erythromycin and clindamycin.

Penicillin remains the drug of choice in the region but we suggest that antibiotic sensitivity analysis should be performed on the GBS isolates from penicillin-allergic patients.

5 Discussion

The studies included in this thesis provide information on invasive neonatal infections in general and on different aspects of GBS infections and the GBS organisms as part of management and prevention of GBS infections.

5.1 Incidence of neonatal sepsis and meningitis

The incidence of invasive neonatal infections is usually defined as blood or CSF verified sepsis/meningitis in population based surveillance or admissions to neonatal care units. Culture proven sepsis is the main criterium used to study the incidence of sepsis and differences in etiology that can be studied over time and in defined geographic areas. A methodological consideration in studies based on culture proven sepsis is that the true incidence of sepsis is much higher since up to 70 % of clinically diagnosed sepsis episodes can be culture negative [deL, vdZ]. This is due to reasons such as suboptimal sample volumes, low pathogenic bacteria or concurrent antibiotic use. In a French study only 3.9 % of all neonates who received antibiotics during the first 3 days of life had a positive blood culture [Lab]. The total incidence of culture proven neonatal sepsis and meningitis in the industrialized world varies between 2.7-7.1/1 000 live born [I, Biz, Gla, Her, Jia, Rön 98, Sang, Ves]. This may reflect true differences in the incidence but may also be due to several methodological differences, where the most important issues are whether CoNS are included or not, blood sample volume and how often blood cultures are obtained, when sepsis is suspected. The symptoms of severe infections in neonates are often very discrete and mild at the beginning but a fulminate sepsis with adverse outcome can occur within a few hours. Therefore antibiotic therapy must be started immediately when infection is suspected. Excessive duration of antibiotic therapy is shown to be independently associated with LOS together with VLBW, mechanical ventilation and central venous catheterization [Lab]. Third generation cephalosporins have also been related to the emergence and spread of antibiotic resistance among Gram-negative bacilli [Jai, Cor]. In a Dutch crossover study it was shown that the relative risk for colonization with strains resistant to the empiric therapy was 18 times higher for neonates at units using amoxicillin-cefotaxime regimes than those at units using penicillin-tobramycin regimes [DeM].

In the Göteborg area of Sweden the incidence of neonatal culture proven sepsis and meningitis has been followed in population based studies since

1975 [I, Tes]. In paper I we divided neonatal sepsis according to the criteria of Y-NHH [Gla] into separate groups; sepsis with traditional neonatal agents and sepsis with CoNS, respectively. The total incidence for these both groups during the first 28 days of life was 4.4/1 000 live births and for the whole period of 120 days 5.7/1 000 lives births. This incidence is nearly the same as in a study [Her] from Mallorca, Spain, in which the incidence was 4.9/1 000 live births in the first 60 days of life for both traditional pathogens and CoNS. The inclusion criteria for CoNS [Gla] were the same as those used in paper I. The incidence rate is also similar to other studies covering these years from USA, Spain, Norway, Taiwan and Australia [Biz, Her, Jia, Rön 98, Sang] (table 8). Combined, these studies demonstrate that the neonatal period carries a higher incidence of invasive infections than any other age group.

Authour year of publication [ref]	*Study years*	*Study design*	*Study number*	*Age days*	*Total incidence*	*CoNS % of study number*	*Case fataliy rate %*
Persson E 2002 [I]	1987-1996	population based	480	0-120	5.7/1 000 live births	19	7
Bizzaro M 2005 [Biz]	1989-2003	hospital based	647	0-	7.1/1 000 live births	29	12
Gladstone IM 1990 [Gla]	1979-1988	hospital based	270	0-30	2.7/1 000 live births (no CS)	19	15.9
Rönnestad A 1998 [Rön 98]	1989-1994	hospital based	206	0-	4.7/100 admissions	41	11.2
Hervas JA 1992 [Her]	1977-1991	hospital based	334	0-60	4.9/1 000 live births	16	7.5
Källman J 1997 [Käl]	1981-1994	hospital based	132	0-27	2.9/1 000 live births	15	11
Sanghvi KP 1996 [Sang]	1989-1993	hospital based	214	0-	3.8/1 000 live births	39	11.1
Jiang JH 2004 [Jia]	1992-2001	hospital based	325	0-	3.29/1 000 hospital days	20.1	16.3

Table 8. Incidence of neonatal sepsis and meningitis in studies covering the same years as in paper I.

Comparison of the 10 years in paper I of neonatal sepsis with the previous 10 years [Tes] demonstrated an increasing incidence of sepsis among premature

neonates < 29 weeks of gestational age from 63.5/1 000 live birth to 162/ 1 000 live births but with advanced neonatal intensive care, there has been a substantial improvement in neonatal mortality in this group of infants. Between these two 10-year periods the case fatality rate decreased from 44 % to 23 % even though the incidence of sepsis increased (Table 9).

	No of births	*No of cases*	*Incidence**	*Mortality**	*Case fatality rate*
			Study: Persson et al 2000 [I]		
Gestational age					
≤ 29 weeks	430	70	162.8	37.2	23 %
30-36 weeks	4 236	61	14.4	1.4	10 %
≥ 37 weeks	78 884	174	2.2	0.1	3 %
Birth weight					
≤ 1 499 g	645	73	113.2	24.8	22 %
1 500-2 499 g	2 978	51	17.1	1.7	10 %
≥ 2 500 g	79 927	181	2.3	0.1	3 %
	No of births	*No of cases*	*Incidence**	*Mortality**	*Case fatality rate*
			Study: Tessin et al 1990 [Tes]		
Gestational age					
≤ 29 weeks	397	25	63.5	27.9	44 %
30-36 weeks	5 538	72	12.9	3.4	26 %
≥ 37 weeks	70 526	134	1.9	0.1	5 %
Birth weight					
≤ 1 499 g	567	39	68.8	24.7	36 %
1 500-2 499 g	3 200	48	15.4	3.5	23 %
≥ 2 500 g	80 000	144	1.8	0.1	6 %

Table 9. Comparison between the study of Tessin et al [Tes] and paper I concerning incidence, mortality and case fatality rate for neonates with invasive infections caused by traditional pathogens with onset during the 28 first days of life related to gestational age and birth weight
* Number of cases/1 000 live births.

The proportion of early onset sepsis with the most common agents (GBS, Gram-negative rods and *Staphylococcus aureus*) was the same over the two

periods for both premature and term infants. The incidence of GBS sepsis increased from 0.8 to 1.0/1 000 live births between the two periods. During this time periods there were no general guidelines of prophylaxis to prevent early onset GBS disease in the Göteborg area. In the following study period covering the years 1997-2006 the GBS incidence hopefully has dropped since guidelines of a risk based screening program has been more implemented (www.infpreg.se).

Early onset sepsis is an important cause of neonatal disease and the majority of these infections result from vertical transmissions of bacteria from the mother to the newborn. VLBW infants are a small group of infants in whom early onset sepsis stands for a high percentage of mortality and morbidity. It has been suggested that as many as 85 % of early preterm births are associated with intrauterine infection before rupture of membranes [Gold, Rom]. Early-onset sepsis is also associated with an increased risk of several complications of prematurity, including bronchopulmonary dysplasia, severe intraventricular hemorrhage or periventricular leucomalacia. The effect of the cytokine response to infection on these adverse outcomes for VLBW infants still requires further studies. In paper I early onset sepsis represented 64 % of the infections with traditional neonatal pathogens during day 0-27. The total case fatality rate day 0-27 was 9 %. Of these deaths 65 % were due to early-onset disease and 35 % of these infections belong to VLBW infants. This was nearly the same as compared with a study from Norwegian where the case fatality rate was 40 % among VLBW neonates in very early onset infections [Rön 05]. The widespread use of intrapartum antibiotics to prevent GBS disease has lead to concern over the possible selection for more virulent organisms which may cause early onset sepsis in the future [Bal 01, Hyd, Sch 06a, Sch 06b, Sto 02b]. However, currently available data show different results. Stable trends were found on increasing non-GBS sepsis in neonates and among preterm infants the incidence of *Escherichia coli* infections increased significantly [Bal 01, Hyd, Lev, Rön 05, Sto 05]. On the other hand, intrapartum antibiotic exposure was not associated with increased non-GBS early onset sepsis in other studies [Sto 02b, Sch 06a, Sch 06b].

As late onset sepsis is known to increase with decreasing birth weight and with increasing survival of ELBW neonates, late onset sepsis will continue to be a challenging complication that affects mortality, other morbidities, length of hospital stay and costs of care. Overuse of antibiotics in NICUs is a well-recognized problem and the liberal use of broad-spectrum antibiotics promotes colonization and overgrowth with pathogenic organisms by altering the fecal microflora. Many infants are already on antibiotics when the late onset sepsis occurs. The rising increase of commensal species in late

and very late onset infections are observed in several studies and CoNS have increased to be the most common organisms associated with late-onset infections [Biz, Gla, Hal, Her, Isa 96, Jia, Käl, Mak, Par, Rön 98, Sto 02a]. It remains difficult to determine whether blood culture isolates with CoNS reflect true infections or are contaminants. In paper I we used the criteria from CDC as were used in Y-NHH [Garn, Gla]. Nearly the same rate of contaminations 83 % was found in another Swedish study [Käl] that used criteria based on laboratory parameters. In 2005,Y-NHH revised the criteria for CoNS infections with addition of glucose level and treatment with antistaphylococcal antibiotic (table 10) [Biz]. The majority of infants with CoNS infection in paper I had a late onset or a very late onset infection (43 % and 35 % respectively) and 67 % of the infants had a birth weight below 1500 grams. In a study from the Netherlands from 2007 [Hir] CoNS accounted for 66 % of all neonatal bloodstream infections and 70 % of the bloodstream infections among VLBW infants.

Currently, there is no possibility to determine whether an isolate of CoNS from the blood or CSF of a neonate is a true pathogen or a commensal. The criteria of Y-NHH are an important effort in separating true infections from contaminants. Other efforts include comparisons of biological characteristics and genetics between different strains of CoNS. Biofilm forming strains are associated with decreases of the inflammatory response, which limited the immune system to counteract the infection [Hir, Kli 05, Kli 06]. Strains with the *isa* gene which is a predictor for biofilm forming phenotype were found in only 51 % and 48 % of NICU strains indicating that several other virulence factors than biofilm are important for CoNS isolates [Kli 05, Hir]. Antibiotic resistance among clinical CoNS isolates is a worldwide problem [Kre, Mon, Vil]. CoNS sepsis with methicillin/gentamycin resistant strains was also found to be associated with increased host inflammatory response [Kli 05]. It remains an important challenge to determine the true importance of CoNS infections and to diagnose them in neonates. To lower the incidence of CoNS in NICU it is generally agreed that improved hygiene could counteract cross-infection and thus lower the incidence of CoNS LOS infections.

	Criteria for the diagnoses of CoNS from Yale New Haven Hospital 2005
	Sepsis caused by a commensal species required isolation of the organism from a blood culture and both criteria below to be fulfilled
1.	At least 2 of these 6 symptoms documented at the time the blood culture was taken
	a. Apnea b. Bradycardia c. Core temperature > 38.0° C d. Core temperature < 36.5° C e. Serum glucose > 140 mg/dL (7.8 mmol/l) f. Serum glucose < 40mg/dL (2.3 mmol/l)
2	Either 1 of the following 2:
	a. Another blood culture positive for the same organism taken within 24 h of the first b. A central access device in place before symptoms developed, followed by treatment with an appropriate antistaphylococcal antibiotic for > 120 h

Table 10. Criteria for CoNS infection according to Y-NHH 2005 [Biz]

A new study covering the incidence of neonatal infections in the Göteborg area 1997-2006 has recently been started. When the results of this study are available data from the same defined region for more than 30 years will be available.

5.2 Group B streptococci (paper II-IV)

Efforts have been made during a long period of time to find an effective vaccine against GBS in humans. Current vaccine strategies have focused on antigens presented on the surface of GBS. This comprises the antigens from type specific CPS and a number of surface exposed protein antigens. The surface proteins include the proteins described in Table 1 and the new proteins that continue to be described as the GBS genome sequences are analyzed. The CPS serotypes of GBS are known to vary both over time and geographic areas. A challenge to GBS vaccines based on CPS serotypes has been that shifts in serotypes of strains occur. It is possible that after the introduction of a multivalent serotype based vaccine, serotypes that are not included in the vaccine become dominating. Studies from both Europe and USA show similar serotype distribution among neonates and adults (Table 11). An experimental tetravalent conjugate vaccine containing serotypes I-III

would theoretically prevent 80 % of all neonatal and infant cases with a gestational age over 34 weeks, and 57 % of all adult cases in western Sweden. To prevent 98 % of the adults and 99 % of neonates over 34 weeks gestation age it would today be needed a sixvalent conjugate vaccine with serotypes I-V.

				Serotypes neonate								
Author	*Study years*	*patients number*	*case fatality %*	Ia %	Ib %	II %	III %	IV %	V %	VI %	VII%	NT %
Persson [II]	1998-2001	50	8	10	4	2	60	2	22			0
Kalliola [Kal]	1985-1994	395	8	23	11	6	47	3	1			7
Berg [Ber]	1988-1997	78	3	18	3	5	63	3	9			0
Harrison [Har]	1992-1993	210		33	2	9	44		12	1		1
Lin [Lin]	1995-1998	67		40	9	6	27		15			3
Davies [Dav 01b]	1993-1999	168	9	18	5	5	53		14			4
Andrews [And]	1997-1999	122		25	6	5	52		9			3
Trijbels-Smeulders [Tri06]	1997-1999	184	7	19	3	8	55		7			7
Weisner [Wei]	2000-2001	353	8	27	6	4	48	1	1	1		3
Strakova [Str]	2001-2002	105		13	8	14	42	3	13	1		1
Ekelund [Eke]	1984-2002	472	9	17	7	6	59	1	4			6
Fluegge Flu]	2001-2003	296		15	5	5	65	1	8			
				Serotypes adult								
Author	*Study years*	*patients number*	*case fatality %*	Ia %	Ib %	II %	III %	IV %	V %	VI %	VII%	NT %
Persson [II]	1998-2001	111	9	9	9	6	25	7	42			1
Berg [Ber]	1988-1997	66		6	23	15	29	2	21			5
Tyrerell [Tyr]	1996	106		17	10	9	19	59	31		1	13
Harrison [Har]	1991-1993	478	12	27	12	12	14		29			5

Table 11. Comparisons between serotype distribution and case fatality rate of invasive GBS infections among neonates and adults in different studies.

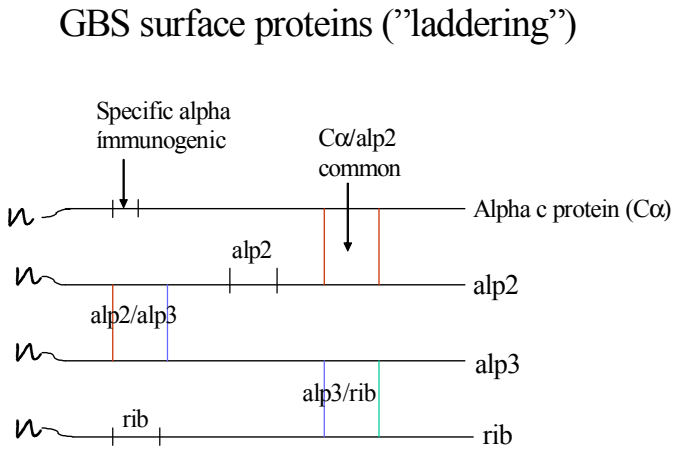
Among colonized women in Asia serotypes VI and VIII dominate [Lac] in contrast to serotype III, V and Ia in Europe and USA [Ber, Har, Mot]. These differences describe one of the difficulties in developing a globally effective CPS conjugate GBS vaccine. A vaccine suitable for Asian or European populations might not be suitable for African populations. We showed in paper II significant changes in serotype distribution in both neonates and adults during a relatively short period of time. In both groups there were pronounced increases in serotype V. This increase was not followed by a

decrease of a certain serotype. Instead there were smaller decreases in the proportions of several serotypes. The increase in serotype V was also seen in other studies covering approximately the same years [Dav 01b, Lin 98, Str]. Serotype V was first reported in 1985 [Jel] and it has been proposed that the emergence of this serotype depends on serotype switching. Non-typeable strains, which probably are non-capsulated, were found in a lower degree in paper II than in several other studies (Table 11). We found only one non-typeable strain in the study described in paper II. This strain was sent to the National Streptococcus and Enterococcus Reference Laboratorium in Prague, Czech Republic for typing with the precipitation method but could neither be typed there. In the work of paper III we retyped all isolates with fluorescent antibody test for control and the non-typeable strain in paper II was retyped as serotype IV, four other strains were also retyped to other serotypes. The retyped strains were then sent to Bacterum AB in Umeå [Håk 92] for control and showed similar results as the fluorescent antibody test for three strains and different serotype for two strains, which also were different from the original. In paper III and IV the serotype results from the coagglutination test performed in paper II were used.

Several of the typing methods available are labor intensive and require high titer of the serotype specific antisera, which are expensive and difficult to make. Molecular serotype identification methods are theoretically attractive because of their potentially high discriminatory powers and reproducibility [Sel]. In a study from Australia a PCR method for CPS serotyping was conducted which was accurate and reproducible and a DNA microarray method was also reported in 2006 [Kon 02b, Wen].

In paper III we identified six genes encoding immunologic surface proteins and three immunologic proteins that are known to elicit protective immunity. The six genes could be identified in 294 of the 297 invasive GBS strains and the distributions of the genes were similar to what has been reported in other studies from USA and Australia [Kon 02a, Man 06, Zha] This kind of molecular method will be useful in epidemiological studies and can easily be combined with identification of CPSs.

The three proteins were identified by an indirect whole cell based fluorescent antibody test. There is a high degree of cross-reaction between the surface proteins, which contributes to the difficulties of identification with antibody-based methods. The antigen sites in alpha like proteins can be identical or nearly identical in immunological specificity (Figure 2). Results from different studies of protein expression are therefore difficult to compare as the antisera used for detection may contain antibodies against more than one of the antigenic determinants of the alpha-like proteins.



The areas called common shows partly immunologic similarity between the proteins
The *n*-terminals between alp2 and alp3 are identical
The reptitions between alp3 and rib are identical

Figure 2. Relationship between the “laddering forming” proteins that contribute to cross-reaction between the proteins (with permission from Professor Maeland, Trondheim, Norway)

To use an immunologic surface protein as the carrier protein in a multivalent conjugate CPS vaccine is an attractive way to enhance the vaccine efficacy. The proteins most commonly used as carrier proteins in such vaccines are tetanus and diphtheria toxoids, to which the vaccine recipients most likely will have antibodies and an immunologic memory due to vaccination with these toxoids in infancy. In people who have strong immunity to tetanus toxoid the immune reaction can lead to significant adverse effects and may also suppress the immune response to the polysaccharide antigen. The surface proteins in the alp family of proteins are proposed to have abilities to act as carrier proteins. They are known to be immunologic and present at the surface of GBS in large quantities. The disadvantage is that they are quite variable and it is uncertain whether they cross-react between strains [Mae 05]. Furthermore the extent of surface accessibility of antigen may vary from strain to strain and even if the encoding genes are present the expression of the surface proteins are uncertain. Unfortunately none of the investigated proteins in paper III were so commonly detected that it showed the ability to act as a protein vaccine component alone. The genes *rib* and *alp3* were most

common in our study and theoretically a combination of protein rib, alp3 and alpha c protein would be interesting as protein vaccine against GBS.

For epidemiological studies of bacteria there are several methods that can be used. MLEE detects genetic variations by differences in protein mobility via gel electrophoresis; the method is used mostly for research since it is expensive and demanding. PFGE is a method where digestion of whole chromosomal DNA with cutting enzyme is followed by separation in agarose gel, the method has been used in several GBS studies [Amu, Han, Ram, Skj]. MLST is the method that has been increasingly used during recent years; the method is based on genetic variation at seven housekeeping genes that are identified by DNA sequencing. DNA fragments of the genes are amplified and sequenced. The sequences of these genes are compared to known alleles at each locus at the MLST database and each isolate can be given a sequences type (ST). The ST is then designed with a digital number. A dominant clone in serotype III, ST17 has been shown to be strongly associated with neonatal invasive infections [Jon 06, Lua]. Isolates with different capsular serotypes can belong to the same ST suggesting that recombination occurs at the capsular locus. A MLST scheme for GBS was developed in 2003 [Jon 03, Lua]. Since MLST uses sequence data, which are stored in an Internet database, the results are unambiguous, highly reproducible and comparisons between geographic areas and over time can easily be done.

Widespread use of antibiotics in hospitals and in the community carries with it the potential for emergence of antibiotic resistance. This issue was the focus of paper IV. Since guidelines for intrapartum antibiotics to protect from early onset GBS infections have been implemented several studies from both Europe and Nord-America showed increasingly frequencies of in vitro resistance of GBS to erythromycin and clindamycin [Flu, Lin 00]. Resistance to penicillin has not yet been reported but it has been shown that intermediate resistance for penicillin occurs among GBS strains [And, Aza, Bet, Sim]. The European Committee on Antimicrobial Susceptibility Testing (EUCAST) has issued guidelines for breakpoints for a few antibiotics and is expected to issue further guidelines during 2007–2008. These breakpoints will be similar to guidelines from SRGA. In paper IV we used the guidelines from CLSI because they are the most widely used and referred to [CLSI].

The breakpoints in CLSI and EUCAST/SRGA differ in the matter that breakpoints from CLSI general are lower than the European/Swedish breakpoints. In paper IV, two strains were, according to the definitions of CLSI resistant to penicillin. In the CLSI guidelines there are however, no breakpoints for intermediate and resistant susceptibility against penicillin for

GBS. Thus, all strains with MIC values over the breakpoint are considered resistant. According to SRGA recommendations the two strains would have been sensitive to penicillin. Penicillin still remains the antibiotic of choice for intrapartum antibiotic prophylaxis because of its narrow antimicrobial activity spectrum and little adverse effect on the enteric bacterial species. With the use of high doses of penicillin for treatment of neonatal sepsis these strains can be considered as accessible to treatment. In paper IV we found a significant increase in the rate of intermediate susceptibility against both erythromycin and clindamycin over the two study periods, indicating that resistance to these antibiotics may occur in a near future. There was also a significant association between these intermediately susceptible strains and serotype V. The associations between serotype V and resistance to erythromycin and clindamycin have been found earlier in several studies [Bor, Man 03, vBot, Lin 00]. Results from PGFE analyses of macrolide resistant strains indicate that there has been an emergence of a specific macrolide clone family that acquired resistance at a certain point of evolution and then subsequently increased in number [vBot, Die].

6 Conclusions

The incidence of neonatal sepsis and meningitis in the Göteborg area of Sweden was 3.7/1 000 live born during the first 28 days of life. The incidence had risen from 2.8/1 000 during a ten-year period. In the group of premature infants < 29 weeks of gestational age the increased rate was most pronounced. VLBW and premature babies were overrepresented in late onset infections and term babies in early onset infections. In spite of increased infection rate among prematures there has been a substantial decrease in the case fatality rate due to infections. The most common agents were Gram-negative rods, GBS, and *Staphylococcus aureus.* Using the criteria from Y-NHH, 85 % of all CoNS infections were contaminants. The incidence of CoNS infections was 1.1/1 000 lives born during day 0-120 of life.

The distribution of GBS capsular serotypes had changed significantly in both neonates and adults during a short period of time. There was a pronounced increase of serotype V in both neonates and adults. A difference in serotype distribution between neonates and adults was found. A conjugated CPS vaccine including serotype Ia, III and V would theoretically cover 80 % of the neonatal cases in babies over 34 gestational weeks.

The genes *bca, bac, rib, epsilon/alp1, alp2* and *alp3* were identified alone or in combinations in 99% of the GBS strains. The most common genes identified were *rib* followed by *alp3*. Three surface proteins were detected in 51 % of the strains. Most genotypes and surface proteins were detected in strains of all capsular serotypes but there were also several significant relationships between genotypes, surface proteins and capsular serotypes. None of the studied immunological surface proteins were so commonly detected that it could be a successful GBS vaccine candidate alone.

Penicillin remains the drug of choice for treatment of GBS infections in the area studied. Two strains had MIC values over the breakpoint according to the CLSI guidelines but were sensitive to the other two β-lactam antibiotics studied. The proportions of strains with intermediate susceptibility to erythromycin and clindamycin increased significantly over the study period indicating a supposed increase also in the resistance rate for these antibiotics. There were no differences found in the susceptibility among GBS strains from neonates or adults.

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