IDENTIFICATION, SEROTYPING, INVASIVENESS AND TRANSCRIPTIONAL PROFILING OF GROUP B STREPTOCOCCUS

Thesis Submitted to Jawaharlal Nehru University

For the Award of the Degree of

DOCTOR OF PHILOSOPHY

By

HEMLATA

SCHOOL OF LIFE SCIENCES

JAWAHARLAL NEHRU UNIVERSITY

NEW DELHI-110067, INDIA

JULY, 2009

School of Life Sciences, Jawaharlal Nehru University, New Delhi-110067

Atul Kumar Johri, Ph.D
Assistance Professor of Microbiolgy
Email: akjohri14@yahoo.com
Phone: 91-11-26744511
Fax: 91-11-26742558

Warden, Chandrabhaga Hostel

CERTIFICATE

The research work embodied in this thesis entitled **"Identification, Serotyping, Invasiveness and Transcriptional Profiling of Group B Streptococcus"** has been carried out by **Hemlata** for the degree of Doctorate of Philosophy. It is certified that the work presented is original and has not been submitted in part or full for any degree or diploma of any university or institute.

Hemlata
(Candidate)

Dr. Atul Kumar Johri
(Supervisor)

Prof. R. Madhubala
(Dean)

Dedicated to My Parents

ACKNOWLEDGEMENT

It is a matter of great pleasure to convey my deep sense of acknowledgement and sincere thanks to my teachers and friends. As I pick my pen to write this page a galaxy of names hovers over my mind.

At the very outset, I sincerely acknowledge with deep faith the omnipotent help of God the Almighty without whose grace and blessing all my endeavors borne no fruit.

My utmost sense of gratitude goes to my supervisor, **Dr. Atul Kumar Johri**, who has always been a source of encouragement and support. I am especially indebted to him for his patience and guidance through the course of my thesis work. Working under him has been a great and joyful experience in many ways.

I thank present Dean **Prof. R. Madhubala** and former Dean(s) **Prof. P. K. Yadava, Prof. R. K. Kale, Prof. R.N.K. Bamzai,** School of Life Sciences for providing with necessary facility to carry out this investigation.

A special world of thanks to **Prof. Rajendra Prasad, Rector, JNU** for allowing us to use the capacity-build up fund for the DNA microarray work and **Department of Biotechnology Govt. of India** for providing the grant to conduct the research.

I sincerely express my deep gratitude to **Dr. Meenakshi Dua,** School of Environmental Sciences, for her time to time suggestions and for her caring nature.

I am grateful to all my lab mates, **Dr. Vikas, Dr. Puja, Abhinay, Hemant, Manoj, Deepak, Pratap, Naveen, Pallavi, Takshila, Ruby, Alvina, Parul, Nidhi** for their consistent help, suggestions and enlightened friendly company and all trainees attended lab time to time, for their ever-ready and helpful presence.

I express my thanks to our laboratory attendant **Kamlesh, Sohanlal, Bijendra** and **Babita** for their help.

I salute all my friends, **Mandavi, Sharmistha, Sarita, Manju, Lovely, Sumita, Monika, Anuradha, Veerendera, Ankush, Soumya** and others for all the

exciting moments and experiences and making my stay in Jawaharlal Nehru University a joyful one.

I am also thankful to unending list of people belonging to SLS family Like **Mr. R. N. Saini, Mr. B.A. Khan, Mr. A.C. Alexander, Mr. S. K. Mishra, Dr. S.P. Sharma, Rajendera, Aslam** and **Balwant** of Central Instruments Facility, Central Computer Instrument Facility and the administrative staff of School of Life Sciences has a share in the success of this endeavor.

I would also like to thank **Meenu madam** and **Shiney madam** for their friendly and caring attitude.

I would like to thank to all my batchmates, seniors and juniors for giving me nice company during my stay in JNU.

I owe my most sincere gratitude to my Parents and sister for all their love and concern. Their support shall forever remain an immeasurable source of strength for me.

Last but not least, my thanks also go to the Council of Scientific and Industrial Research (CSIR) for providing Fellowship.

Hemlata

ABBREVIATIONS

BSA	:	bovine serum albumin
CTAB	:	hexadecyltrimethyl ammoniu bromide
DNA	:	deoxyribonucleic acid
DTT	:	dithiothreitol
EDTA	:	ethylene diamine tetra acetic acid
g	:	gram
h	:	hour
kb	:	kilo base
kD	:	kilo Dalton
mm	:	millimeter (10^{-3} meter)
M	:	molar (molar per liter)
mM	:	millimolar (10^{-3} molar)
mg	:	milligram (10^{-3} gram)
ml	:	milliliter (10^{-3} liter)
µg	:	microgram (10^{-6} gram)
µl	:	microliter (10^{-6} litre)
µm	:	micrometer
µM	:	micromolar
nm	:	nanometer
pH	:	hydrogen ion concentration
RNA	:	ribonucleic acid
rpm	:	rotations per minute
sec	:	seconds
min	:	minute (s)
TE	:	tris-EDTA
UV	:	ultraviolet
v/v	:	volume/volume
w/v	:	weight/volume
w/w	:	weight/weight
DMSO	:	dimethyl sulfoxide
Fig.	:	figure
GAS	:	group A streptococcus
GBS	:	group B streptococcus
OD	:	optical density
RT	:	room temperature
FBS	:	fetal bovine serum
ATCC	:	american type culture collection
THB	:	todd hewitt broth
^{0}C	:	digree celcius
SDS	:	sodium dodecyl sulfate
dNTP	:	deoxynucleotide tri phosphate
MQ	:	milli Q

PCR	:	polymerase chain reaction
CFU	:	colony forming unit
RPMI	:	Roswell Park Memorial Institute
PBS	:	phosphate buffer saline
MOI	:	multiplicity of infection
ORF	:	open reading frame
IL	:	interleukin
TNF	:	tumor necrosis factor

CONTENTS

Introduction

One Introduction

The ability of bacterial pathogen to adapt to often changing environment within a single host is critical to their growth and survival. Nutrient such as carbon, oxygen (Johri et al, 2003) and environmental stimuli such as temperature and pH may regulate certain traits including those involved the ability of a commensal to become a frank pathogen. Group B *Streptococcus* (GBS), also referred to as *Streptococcus agalactiae*, is a Gram-positive, β–hemolytic opportunistic pathogen that colonizes the gastrointestinal and genitourinary tracts of up to 50 % of healthy adults (Johri et al, 2006). The pathogenesis of neonatal GBS infection begins with the asymptomatic colonization of the female genital tract. Approximately 20–30 % of healthy women are colonized rectovaginally with GBS, and 50–70 % of infants born to these women will themselves become colonized with the bacterium and at risk of developing sepsis, pneumonia and meningitis. Centers for Disease Control (CDC, USA) issued guidelines recommending antibiotic treatment before birth for babies at high risk (CDC, 2002). However, GBS still remains a leading cause of sepsis and meningitis in newborns, as well as of severe invasive diseases in adults.

Presumptive tests for identification of GBS are often used by clinical laboratories because of their relative simplicity and cost effectiveness compared with serological identification (e.g., the Lancefield precipitin, latex agglutination, or coagglutination tests). Of the several presumptive tests, the CAMP (Christie-Atkins- Munch-Petersen) test, originally described by Christie (Christie et al, 1944) is often used because it requires minimal reagents, employs a simple methodology, is inexpensive, and rarely gives false-positive reactions with other streptococcal groups. Latex agglutination tests (hereinafter called latex tests) have been used for identification and grouping of many clinically important microorganisms, including different streptococcal groups (Slotved et al, 2003).

GBS are differentiated from other β-hemolytic streptococci by Lancefield serological typing. GBS have been classified into nine serotypes (Ia Ib, II, III, IV, V, VI, VII and VIII) on the basis of immunological specificity of cell wall capsular polysaccharides present that are antigenically and structurally unique (Johri et al, 2006).

Recently type IX serotype of GBS has also been identified (Slotved, 2007). Some Isolates may be reported as Nontypeable (NT) due to the expression of an uncharacterized polysaccharide for which antibodies are not yet available.

Multilocus sequence typing (MLST) is an unambiguous sequence- based typing method that involves sequencing approximately 500-bp fragments of seven housekeeping genes and has been used successfully to type strains and investigate the population structure of a number of human bacterial pathogens, including *Neisseria meningitidis* (Maiden et al, 1998) and *Streptococcus pneumonia* (Enright et al, 1998). MLST is particularly suitable for epidemiological studies because it provides data that can easily be compared between laboratories over the Internet.

Neonatal GBS infection comes in two forms: early onset disease (EOD) and late onset disease (LOD). EODs are classified epidemiologically through 7 days of age, but have a median onset of only 6–8 h of life, with pneumonia and respiratory failure complicated by bloodstream infection and septicaemia. These cases result from ascending infection of the bacterium through the placental membranes to initiate infection *in utero*, or, alternatively, by aspiration of infected vaginal fluids during the birth process. In contrast, LOD infection occurs in infants up to 7 months of age, with gradual symptoms related to bacteremia (Baker and Edwards, 2001).

The pathogenesis of GBS infections is thought to be a multistep process. Adherence of GBS to epithelial cells may be integral to several of these steps. Prospective studies have shown that colonization of the rectum and vagina of the mother by GBS is correlated with GBS sepsis in newborn infants (Baker and Edward, 1991), indicating that colonization of these sites is a prerequisite for infection. GBS adherence to vaginal and rectal epithelial cells may allow GBS to colonize these sites. Infection of the fetus occurs following infection of the amniotic cavity and often begins as pneumonia, implicating the lung as the site of initial infection. Bacterial invasion of respiratory epithelial cells is thought to be important in infection of the infant. Previous work with other invasive organisms has shown that bacteria which were deficient in adherence were also unable to invade eukaryotic cells (Donnenberg et al, 1990; Falkow, 1991) implicating adherence as a necessary step in the invasion process. These observations led us to hypothesize that GBS adherence might play an important role in infection as a first

step in the invasion of epithelial cells. Human isolates of GBS express a capsular polysaccharide (CPS), a major virulence factor that helps the microorganism evade host defence mechanisms (Rubens et al, 1987).

However, in many regions of the world, the serotypes that cause GBS infections are not restricted to those most prevalent in the United States (Walsh and Hutchins, 1989). Several studies revealed the prevalence of GBS serotypes VI and VIII among pregnant women in Japan (Hoshina, 1997; Lachenauer et al, 1999; Matsubara et al, 2000, Walsh and Hutchins, 1989). Of particular interest is the fact that GBS serotype VIII, the serotype most frequently isolated from colonized women in Japan, is rarely associated with neonatal disease. Serotyping is important for our understanding of the epidemiology of GBS disease and in determining the prognosis of the disease in infants. 30 % of GBS disease in non-pregnant adults is caused by serotype V (Harrison et al, 1998), while serotype III causes more than 70 % of infant meningitis and most late-onset (7–89 days of age) disease (Davies et al, 2001). Vaccines currently under development target the most prevalent GBS serotypes (Baker and Edwards, 2003).

Ongoing monitoring of the distribution of GBS serotypes is important for charting changes in serotype prevalence. In addition, in order to effectively formulate a multivalent vaccine, we need to understand the serotype distributions prevalent in different parts of the world. The differences in serotype distribution among various populations also may reflect differences in pathogenesis among the serotypes.

Several promising vaccines based on CPS, surface protein, such as α and β components of the C protein complex, Rib, Sip and C5a peptidase (Mikamo et al, 2004; Carlemalm et al, 1993; Brodeur et al, 2000; Cheng et al, 2001) have been developed against GBS. CPS-tetanus toxoid conjugate vaccines effective against all nine currently identified GBS serotypes have been prepared and were shown to induce functionally active CPS-specific IgG (Paoletti and Madoff, 2002). Clinical trials of conjugate vaccines prepared with purified CPS types Ia, Ib, II, III and V have demonstrated that these preparations are safe and immunogenic (Paoletti and Kasper, 2002; paoletti et al, 2000). Not unexpectedly, these preparations do not offer protection against other GBS serotypes, such as type VIII, prevalent in other regions of the world (Lachenauer et al, 1999). The identification of proteins located on the outer surface of GBS cells is an approach that has

been used to find new potential protein-based vaccine candidates. Despite the many studies that are focused on developing a GBS vaccine using conventional approaches, including the cultivation of pathogens and the identification of highly immunogenic and protective antigens using standard biochemical and microbiological techniques, little success has been achieved in terms of developing a vaccine that is globally effective.

These days new emerging technologies such as genomics, proteomics, *in silico* are presenting exciting new opportunities in the hunt for an effective and globally relevant GBS vaccine. To understand the mechanism by which pathogens cause disease, it is necessary to identify the genes that are required for the establishment and maintenance of an infection. Genomics has revolutionized the way in which novel vaccine candidates are identified for the development of efficacious vaccines. Reverse vaccinology, whereby all candidates of interest are identified by analysis of a pathogen's genome, enables characterization of many candidates simultaneously. The analysis of multiple genomes of GBS revealed tremendous diversity and identified candidates that are not shared by all the strains sequenced, but provide general protection when combined (Tettelin et al, 2006). The sequencing of GBS genomes from several serotypes (Tettelin et al, 2005), including types V and III (Glaser et al, 2002; Tettelin et al, 2002) and technologies such as DNA microarray and proteomic analysis now allow global approaches to revealing the complex nature of GBS pathogenicity (Lauer et al, 2005) as well as new approaches to vaccine development (Maione et al, 2005, Johri et al 2007). Interestingly, at least 18% of the GBS-specific genes were either absent or divergent from these 19 other strains, including those of the same serotype. It seems there is an enormous amount of genetic heterogeneity within these bacterial serotypes. Genome of GBS serotypes exhibit endless diversity. A vaccine suitable for African, European, and USA may/may not be suitable for Asian populations (WHO report 2003). The distribution pattern of different serotypes of GAS in the world population including developed and developing countries is essential to be explored

Keeping in mind all the background, the following objectives were designed for the present work.

- Collection of samples from different clinical laboratories and hospitals of Delhi and surrounding areas as well as other states.
- Identification of GBS.
- Serotyping of GBS positive samples using typing antisera kit.
- Interaction of most prevalent and invasive *vs* noninvasive serotype of GBS with different human cell lines to study adherence and invasion.

  Following human cell lines would be used to study invasion assay.

  a. Human type II alveolar epithelial carcinoma (A549)
  b. Human cervical epithelial cells line (ME180)
- Transcriptional profiling and comparative genomics of GBS using genomic approach (DNA microarray) to identify virulent factors/gene which were differentially expressed in invasive vs noninvasive serotype or in prevalent serotypes.
- Data analysis to identify the genes up regulated or down regulated

Review of Literature

From the plagues of biblical times to the HIV and H1N1 pandemic of today, infectious disease have played a major role in human history and are responsible for millions of deaths, primarily in developing countries (WHO, 2004). Amongst diseases pathogenic bacteria are responsible for about half of this burden and are a major threat to human health.

Most bacteria that live on the skin or in the gut of humans are harmless or beneficial. Some are usually harmless, but can cause disease under certain conditions. Some species are highly pathogenic and are lethal for a high percentage of the humans they infect. *Streptococci* is one of the major group of pathogenic bacteria, causing so many diseases in humans ranging from highly fatal diseases like meningitis, toxic shock syndrome, pneumonia, sepsis and rheumatic fever to common infections like pharyngitis, erysipelas, impetigo and necrotizing fascitis.

2.1. *Streptococcus*

Streptococci is the general term for a diverse collection of gram positive cocci with characteristically growing in chains or pairs (from the Greek *Streptos*, pliant or chain; and *coccos,* a grain or berry) resembling a string of beads." Virtually all *Streptococci* that are important in human medicine fall in to the genera *Streptococcus* and *Enterococcus*. The genus *Streptococcus* are very heterogeneous group of nonmotile nonsporeforming, facultative bacteria, some members are part of normal flora and others are potent pathogens. The genus consists of six clusters of species **(Table 1)** each of which is characterized by distinct pathogenic potential and other properties.

- The *pyogenic* group includes most species that are overt pathogens of human and animals.
- The *mitis* group includes commensuals of human oral cavity and pharynx, although one of the species, *Streptococcus pneumoniae*, is also an important human pathogen.
- The *anginosus* group and *salivarius* group are part of commensual microflora of the oral cavity and pharynx.

- The *bovis* group belongs in the colon
- The *mutans* group of streptococci colonizes exclusively tooth surface of man and some animals; some species belonging to this cluster are involved in the development of dental caries.

Virtually all the commensally species, including the *enterococci,* are opportunistic pathogen, primarily if they gain access to the bloodstream from the oral cavity or from the gut.

2.2. Classification of *Streptococcus*

An early attempt at differentiating the streptococci was probably made in 1903 by Shottmuller, who used blood agar to differentiate strains that were β-hemolytic from those that were not. Colonies of streptococci belonging to pyogenic group are generally surrounded by a clear zone, usually 3-4 mm in diameter, caused by the lysis of red blood cells in the agar medium induced by bacterial hemolysin.

2.2.1. α-hemolytic

Some commensuals *Streptococci* give rise to a green discoloration around colonies on blood agar. This phenomenon is termed **α-hemolysis**. This factor causing the green discoloration is not a hemolysin, but hydrogen peroxide, which oxidizes hemoglobin to the green methaemoglobin. For e.g. *S. pneumoniae*, .*S mitis* and *S. oralis* (**Table 2.1**).

2.2.2. β-hemolytic

This is called **β-hemolysis** and constitutes the principal marker for potentially pathogenic streptococci in cultures of throat swab, vaginal swab and other clinical samples. For e.g. *S. agalactiae, S. pyogenes*

2.2.2.1. Group A Streptococcus (GAS)

Streptococcus pyogenes is the most important member of GAS which causes so many pyogenic diseases in humans with a characteristic tendency to spread, as opposed to staphylococcal lesions which are typically localized (Ananthnarayan, 2000). *Streptococcus* species may also possess the Group A surface antigen (composed of N-acetyl-b-D glucosamine linked to a polymeric rhamnose backbone),

but human infections by non *S. pyogenes* GAS strains (some *S. dysgalactiae* subsp. *S. equisimilis* and *S. anginosus* Group strains) appear to be uncommon.

2.2.2.2 Group B Streptococcus (GBS)

S. agalactiae causes pneumonia and meningitis in neonates and the elderly, with occasional systemic bacteremia. They can also colonize the intestines and the female reproductive tract, increasing the risk for premature rupture of membranes and transmission to the infant.

2.2.2.3 Group C Streptococcus

S. equi which causes strangles in horses and *S. zooepidemicus* which causes infections in several species of mammals including cattle and horses. This can also cause death in chickens and moose.

2.2.2.4. Group D Streptococcus

Many former Group D streptococci have been reclassified and placed in the genus Enterococcus (includes *S. faecalis, S. faciem, S. durans,* and *S. avium*). For example, *S. faecalis* is now *Enterococcus faecalis*. The remaining non-enterococcal Group D strains include *S. bovis* and *S. equinus*.

2.2.3. γ-hemolytic

Some of the streptococci do not show any hemolytic pattern when cultures on blood agar. These are called **γ-hemolytic**. They include the fecal *streptococci enterococci* and related species. They are called the 'enterococcus' group. For e.g. *S. mutans, S. faecalis* (**Fig 2.1**).

Table 2.1: *Streptococcus* spp. of clinical importance			
Phylogenetic group	Species	Lancefield group	Types of hemolysis
Pyogenic	*S. pyogenes*	A	β
	S. agalactiae	B	β
	S. equisimilis	C	β
Mitis group	*S. pneumoniae*	O	α
	S. mitis	O	α
	S. aralis	Not identified	α
	S. sanguis	H	α
	S. gordonii	H	α
Anginosus	*S. anginosus*	G, F (and A)	α
	S. intermedius		α
Salivarius	*S. salivarius*	K	None
Bovis	*S. bovis*	D	α or none
Mutans	*S. mutans*	Not designated	None
	S. sobrinus	Not designated	None

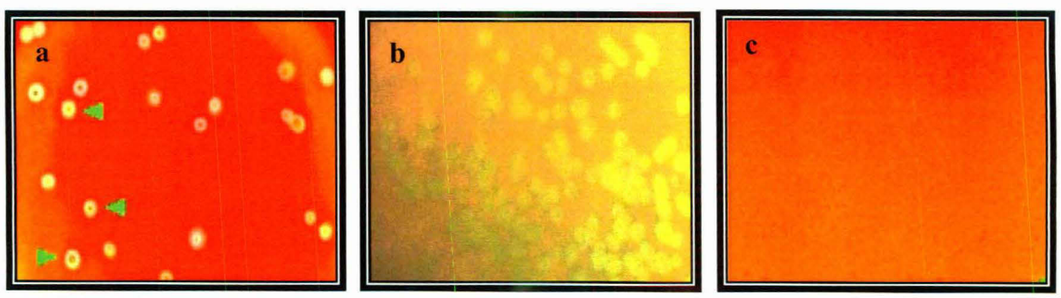

Fig.2.1: Hemolytic activity on blood agar plates: (a) Beta hemolysis e.g. *S. agalactiae* and *S. pyogenes* (b) Alpha hemolytic e.g. *S. penumoniae* (c) Gamma hemolysis e.g *E. faecalis*. Green arrows show clear zone of hemolytic area around the colonies.

2.3. GBS pathogenesis

Until the 1960s, GBS disease in neonates was rare. During the 1960s and 1970s, several investigators recognized the emergence of this disease and its profound impact on neonatal mortality and morbidity (Hood et al, 1961; Eickhoff et al, 1964). *S. agalactiae,* once were considered pathogens only of domestic animals, causing mastitis in cows but now is best known as a cause of postpartum infection and most common cause of neonatal sepsis. More recently, the role of this organism as a cause of infection in nonpregnant adults has been described in a number of series. The physiopathology of GBS infections suggests that this opportunistic pathogen can adapt to various environmental factors, including pH, osmolarity, temperature, and the presence of nutrients such as oxygen (Johri et al, 2007).

GBS is an opportunistic pathogen and harmlessly colonizes vaginal and gastrointestinal tract in 50% of the healthy adults (Schuchat, 1998) from the maternal genital tract to the infant *in utero* or at delivery. This microorganism causes pneumonia, septicaemia and meningitis in neonates, is responsible for significant morbidity in pregnant women and the elderly, and is a serious cause of mortality in immune-compromised adults (Dermer et al, 2004; Edward and Baker, 2005). GBS has two surface carbohydrates, the group B carbohydrate and the capsular polysachharides (CPS). Whereas the group B antigen is common to all GBS, the CPS antigens are the basis for serological distinction (Paoletti et al, 2002). Isolates of GBS are divided into nine CPS serotypes (Ia Ib, II, III, IV, V, VI, VII and VIII) each antigenically and structurally unique and varies in their distribution among geographic areas. The difference in serotype distribution among various populations also may reflect differences in pathogenesis among the serotypes (Johri et al, 2006). Recently type IX serotype of GBS has also been identified (Slotved et al, 2007).

Neonatal GBS infection comes in two forms: early onset (EOD) and late onset (LOD). Early-onset infections are classified epidemiologically through 7 days of age, but have a median onset of only 6–8 h of life, with pneumonia and respiratory failure complicated by bloodstream infection and septicemia. These cases result from ascending infection of the bacterium through the placental membranes to initiate infection *in utero*, or, alternatively, by aspiration of infected vaginal fluids during the birth process. Premature, low-birth-weight infants are at increased risk of developing early-onset infection, with GBS placental infection itself often triggering premature

labor. In contrast, late-onset GBS infection occurs in infants up to 7 months of age, with gradual symptoms related to bacteremia, no lung involvement and a high incidence (~50 %) of meningitis (Baker and Edwards, 2001). Maternal intrapartum antibiotic prophylaxis (IAP) against GBS has significantly decreased the incidence of neonatal early-onset sepsis (EOS) caused by GBS (CDC, 2005). EOD has not been eliminated, however, with persistent disease primarily occurring in premature infants and in infants born to women who have falsely screened GBS negative (Puopolo et al, 2005).

LOD is less common than EOD but is becoming relatively more important because its incident is not declined as a result of prophylactic measure that have caused a decrease in incidence of early onset infections. Little is known about the pathogenesis of late onset infections, but vertical transmission of the organism from the mother to the infants probably explains most infection during this period. Some of the disease caused by late onset is mental retardation, cortical blindness, deafness, uncontrolled seizures, hydrocephalus, hearing loss, speech and language delay (Schrag et al, 2000; Edward et al, 2001). IAP has had no effect on the incidence of late onset neonatal GBS sepsis (LOS) (CDC, 2005).

While the incidence of group B streptococcal disease in neonates appears to be decreasing, the rate in nonpregnant adults appears to be increasing, with an overall increase of 32 % between 1999 and 2005 (Phares et al, 2008). A recently published study of surveillance data from 10 states in USA found that the incidence of group B streptococcal infection in persons aged 15-64 years increased from 3.4 per 100,000 population in 1999 to 5 per 100,000 in 2005. In adults aged 65 years and older, the incidence increased from 21.5 per 100,000 population in 1999 to 26 per 100,000 in 2005 (Phares et al, 2008).

Other clinical manifestation GBS osteomyelitis, arthritis, discitis, and colonization of diabetic foot infections and decubitus ulcers. Chorioamnionitis, endometritis, and the full spectrum of urinary tract infections (from asymptomatic bacteruria to cystitis and pyelonephritis with bacteremia) are observed with group B streptococcal infection. Group B streptococcal infections leading to necrotizing fasciitis and toxic shock syndrome have been documented (Gardam et al, 1998; Sendi et al, 2008).

2.4. Epidemiology of GBS

During the 1970s and 1980s, GBS emerged as a significant neonatal and maternal pathogen in the United States and Western Europe with reported mortality rates of 15 to 50 % (Pass et al, 1979; Baker and Edward, 2001). The burden of prenatal GBS disease varies between countries and represents a very significant cause of neonatal morbidity and mortality (Franciosi et al, 1973). Vaginal colonization of GBS has been reported to occur in about 12–27 % of women in North Africa, India, Middle East, Pakistan, Thailand, Saudi Arabia and the US (WHO, 2005). In the US, approximately 10-30 % of pregnant women are colonized with GBS in the vagina or rectum (Regan et al, 1991; CDC, 2002). Two recent studies of invasive disease isolates from neonates and pregnant women both found that serotypes Ia, III, and V were predominant, with the remaining isolates comprising serotypes Ib and II and nontypeable GBS (Andrews et al, 2000; Ferrieri et al, 2004). Serotypes Ia, Ib, II, III, and V are most commonly associated with colonization and disease in the US (Lachenauer et al, 1991). Type IV GBS was reported in a surveillance study in Maryland in 1992 (Harrison et al, 2005). Type IV has been reported as the dominant colonizing serotype in a recent study of pregnant women in the United Arab Emirates (Amin et al, 2002) and the second most common colonizing serotype in a study of pregnant women in Turkey (Ekin et al, 2006). Other reports from Kuwait (Sweih et al, 2005), Israel (Marchaim et al, 2003), and Turkey (Eren et al, 2005) have not found a significant proportion of type IV GBS isolates in studies of maternal GBS colonization, suggesting that type IV GBS transmission is found in highly localized populations even in similar geographic regions. In colonization studies from Japan, serotypes VI and VIII strains are detected most frequently (Lachenauer et al, 1999).

The prevalence of GBS colonization during pregnancy is variable; in one study, among women who had positive GBS cultures between 26 and 28 week gestation, only 65 % remained colonized, while 8 % of those with negative prenatal cultures were positive for GBS at term (Boyer et al, 1983). Treatment of colonized mothers succeeded in temporarily eradicating the organism, but most of the women were re-colonized within 6 weeks. At birth, 50 to 65 % of infants who are born to colonized mothers have positive GBS cultures from mucus membranes. (external ear canal, throat, umbilicus, anorectal sites) (Ferrieri et al, 1977; Hoogkamp-Korstanje et al, 1982). Approximately 98 % of colonized newborns remain healthy, but 1-2 %

develops invasive GBS infection (Baker and Edwards, 2001). The overall incidence of neonatal GBS infection was approximately 2 per 1000 live births in the US prior to the introduction of intrapartum prophylaxis (Baker and Edwards, 2001). In Germany a high prevalence rate of *S. agalactiae* colonization was found i.e. 16 % (Brimil et al, 2006).

GBS has been classified in to nine antigenically distinct serotypes based on their capsular polysaccharide structure (types Ia, Ib, II, III, IV, V, VI, VII and VIII) identified to date. In the US and Western Europe, types Ia, II, and III accounted for 85% of the isolates from infants (Baker and Barrett, 1974; Dillon et al, 1987). It was observed that 78-87 % of EOD in US mainly caused by serotypes Ia, III, and V (in descending frequency) in newborn infants and parturient women (Harrison et al, 1998; Zaleznik et al, 2000). LOD GBS disease in infants 7-90 days of age is dominated by serotype III, followed by serotypes Ia and V (Harrison et al, 1998). Studies from India show a variable distribution of serotypes, but the most common isolates belong to types Ia, III, II and Ib (Prakash et al, 1976; Chaudhary et al, 1981; Mani et al, 1984; Lakshmi et al, 1988) For the past two decades no significant data are available on epidemiology of GBS in India.

2.5. Indian scenario of GBS

Epidemiological studies in India have shown lower colonization and infection rates in general (Chaudhary et al, 1981; Mani et al, 1984; Lakshmi et al, 1988; Dalal et al, 1998; Stoll and Schuchat, 1998). However on closer analysis, taking into consideration use of adequate culture techniques and microbiological media, some of the GBS colonization rates reported from India and other developing countries are similar to those reported in the United States (Stoll and Schuchat, 1998).

In a study done in 507 pregnant Indian women, 12 % were reported to have GBS isolated from the throat and vagina, and 10 % had positive vaginal cultures alone (Dalal et al, 1998). Similarly, another study showed the overall carriage rate in pregnant women to be 16% (Chaudhary et al, 1981). Although both these studies used selective broth media, culture sites did not include the anorectum and this might have lowered the yield of positive cultures. Another study reported colonization rates of 5-6 %, but no selective broth media were used. (Mani et al, 1984; Lakshmi et al, 1988). Colonization rates in infants born to asymptomatic maternal carriers of GBS are 53-56

% and are consistent with rates reported in other parts of the world (Chaudhary et al, 1981; Mani et al, 1984). Despite significant GBS colonization rates, reports of invasive neonatal GBS disease in India are infrequent. In a 10-yr study between 1988 and 1997 in Vellore, only 10 cases of neonatal GBS infection were identified, giving an incidence of 0.17 per 1000 live births (Kuruvilla, 1999). However, this number represents only the cases occurring among deliveries in a tertiary care hospital located in a predominantly rural community. In India where 65 % of women give birth at home, the true incidence of invasive GBS disease in the newborn is largely unknown (Health, 2003). In addition, blood cultures from ill neonates are not always done in many rural primary health care centres, which may contribute to the underestimation of the number of GBS cases. Preterm births and stillbirths are also usually not investigated, and thus the total burden of perinatal GBS disease remains unrecognized.

The estimated incidence of neonatal GBS infection in India can be calculated from Indian epidemiological data reporting maternal and infant GBS colonization rates as 10 and 50 % respectively (Chaudhary et al, 1981; Mani et al, 1984; Dalal et al, 1998). Since about 2 % of colonized neonates develop true infection (Pass et al, 1979), the attack rate of neonatal GBS infection in India was calculated to be approximately 1 per 1000 live births.

The role of GBS in the developing world is not well defined. Carriage rates and serotypes in women in underdeveloped countries are similar to those observed in the industrial world. However, for unknown reasons, early group B streptococcal disease in infants is not well documented in technologically less-developed countries (Johri et al 2006, Woods et al, 2009).

2.6. GBS serotyping

Serotyping the organism is important in studying the epidemiology of the disease as well as deciding a course of treatment. Capsular serotyping has been one of the mainstays in the descriptive epidemiology of GBS. GBS have been classified into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, X) on the basis of immunological specificity of cell wall CPS present on its surface. Capillary precipitation test (Lancefield, 1934; Heard and Mawn, 1993) is one method to serotype GBS but it is a time consuming and may not be sensitive enough to detect very small quantities of antigen. Later latex agglutination was reported (shown to be serotype specific)

(Slotved et al, 2003) and able to serotype those GBS that were previously considered as nontypeable (NT) by Capillary precipitation test. There are several possibilities for GBS to be NT. They may not be producing enough of the serotype antigen for our test to detect (Palacios et al, 1997) or test may not be sensitive enough to detect very small quantities of antigen. Another possibility is that the GBS have some genetic defect in the production of the serotype antigen and may not be producing any serotype antigens. A third possibility is that a new serotype has been introduced and our observed increase in NT-GBS may simply be due to a serotype that we are not able to detect with our antisera or our serotyping methods (Elliott et al, 2004). Some of the proportion of GBS serotypes still was designated as NT by latex agglutination. These NT-GBS were then characterized by genotypic techniques such as PCR of gene fragments of the variable region of the *cps* locus (Kong et al, 2002), Aside from serotyping and multilocus enzyme electrophoresis (MLEE) (Musser et al, 1989), a method based on primary structures of proteins, other typing methods used are DNA based. These include ribotyping (Blumberg et al, 1992), random amplified polymorphism (Limansky et al, 1998), pulsed field gel electrophoresis (Gordillo et al, 1993; Rolland et al, 1999; Benson and Ferrieri, 2001) and more recently, multilocus sequence typing (MLST) (Jones et al, 2003). Among these methods, only MLST enables one to infer phylogenetic relationships between clones. Epidemiological studies using MLEE and MLST for *S. agalactiae* identified hypervirulent lineages (enzyme type-1 and sequence type ST-17, respectively) strongly associated with infections in neonates compared to carriage strains (Musser et al, 1989; Jones et al, 2003) or to strains associated with infections in adults (Luan et al, 2005). One purpose of the surveillance study is to monitor the serotype distribution of GBS so that the most common serotypes will be included in the potential vaccine. These previously NT-GBS can now be included in the data set and the percentages of each serotype can be more accurately determined

2.6.1. GBS CPS

CPS is a major virulence factor that helps the microorganism evades host defense mechanisms (Rubens et al, 1987). It also serves as an epidemiological marker and is main component of conjugate vaccine. For studies of epidemiology and pathogenesis, it is important to identify as many phenotypic or molecular as possible to increase discriminatory power of typing system (Hauge et al, 1996). Identification

of surface protein antigens, combined with CPS serotyping, allows subdivision of GBS strains into a large number of serovariants, which can facilitate epidemiological, pathogenetic, and other related studies of GBS infection (Kvam et al, 1995). The group B carbohydrate is an antigen common to all strains and serotypes of GBS, positioned proximal to the cell wall (Wagner et al, 1980). The group B carbohydrate is composed of rhamnose, galactose, N-acetylglucosamine, and glucitol (**Fig 2.3**). CPS gene contains 18 open reading frames (ORFs) (**Fig 2.2**).

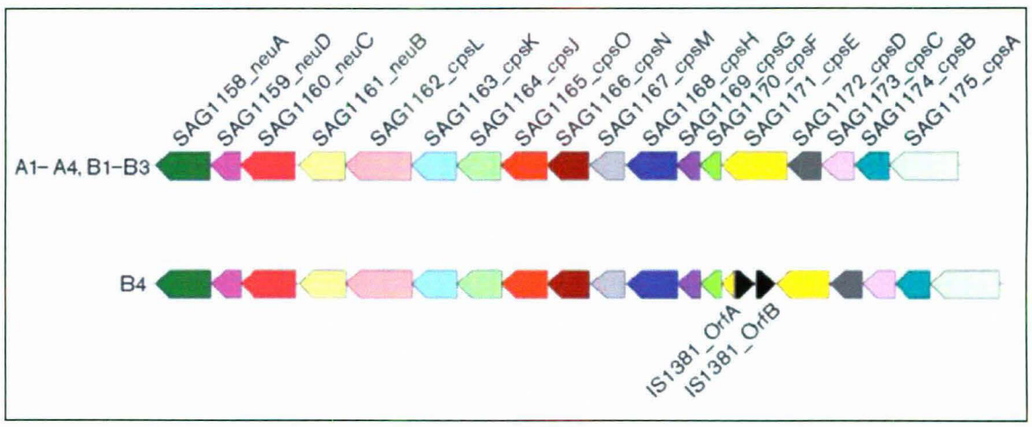

Fig.2.2: Schematic of the CPS genes sequenced (Ramaswamy et al, 2006).

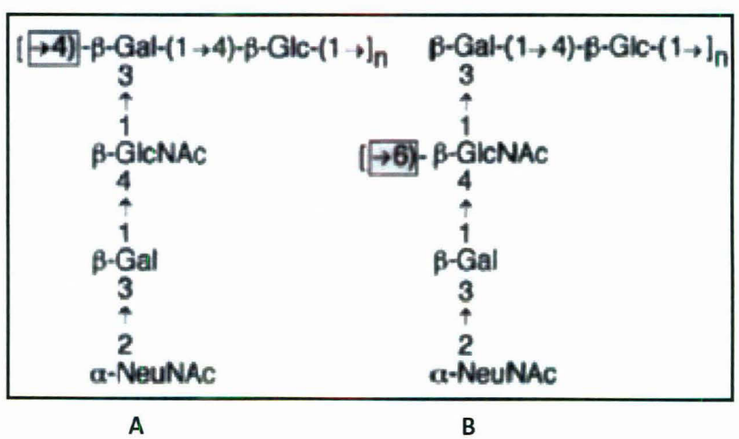

Fig.2.3: GBS type Ia and III CPS repeating unit structures. The critical linkages differentiating the type Ia and III CPS are shown in the shaded boxes (Chaffin et al, 2000).

These sugars form four different oligosaccharide units linked by phosphodiester bonds to create a complex and highly branched tetra antennary structure (Michon et al, 1997). Description of arrangement of saccharide in the repeating of each CPS is shown in **Fig 2.4, 2.5**. The terminal position and abundance of rhamnose suggested that this sugar would constitute or be part an immunodominant epitope. (Chalifour et al, 1991).

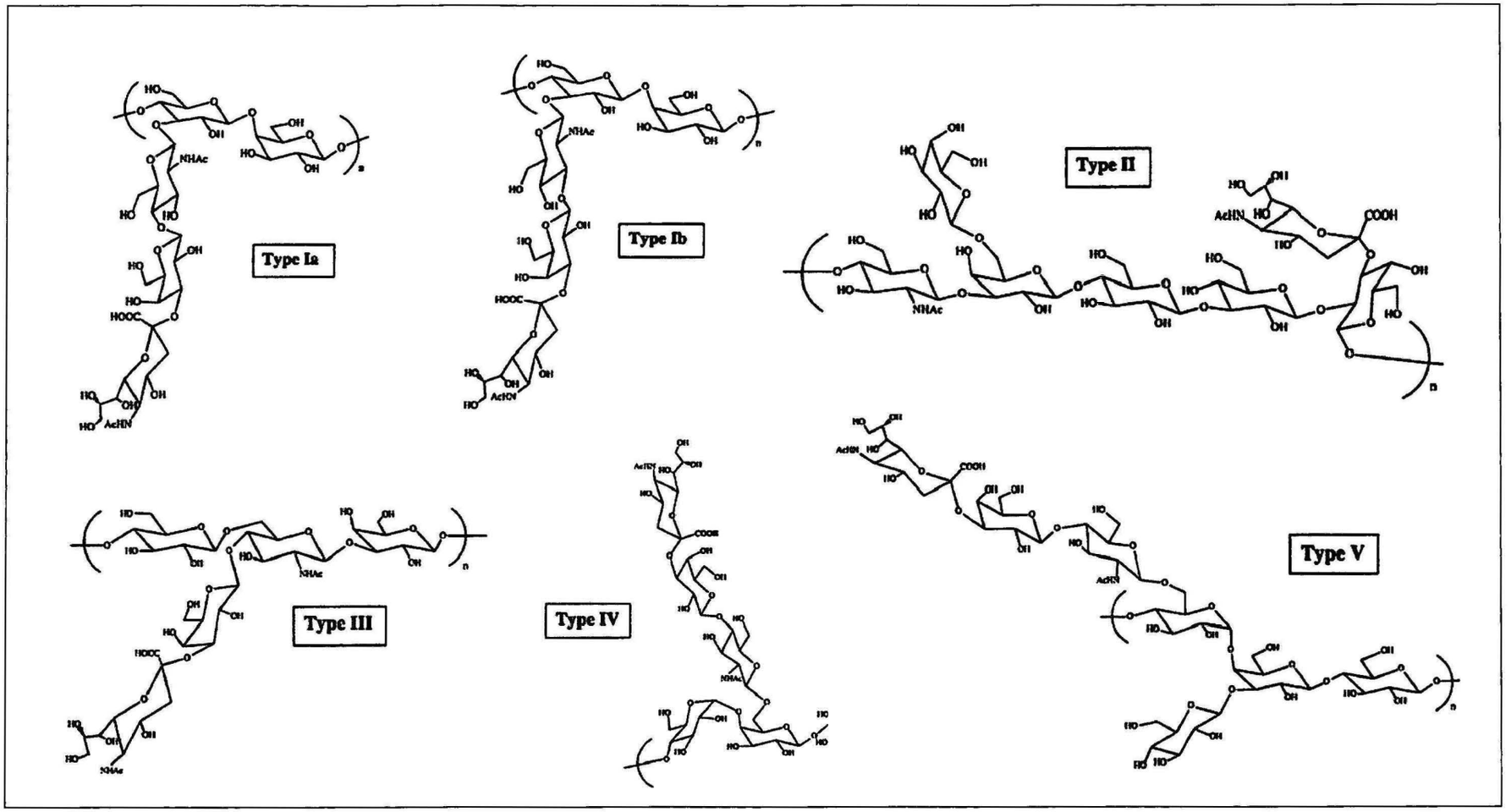

Fig.2.4: Arrangement of saccharides in CPS of GBS type Ia, Ib, II, III, IV and V (Madoff et al, 2006).

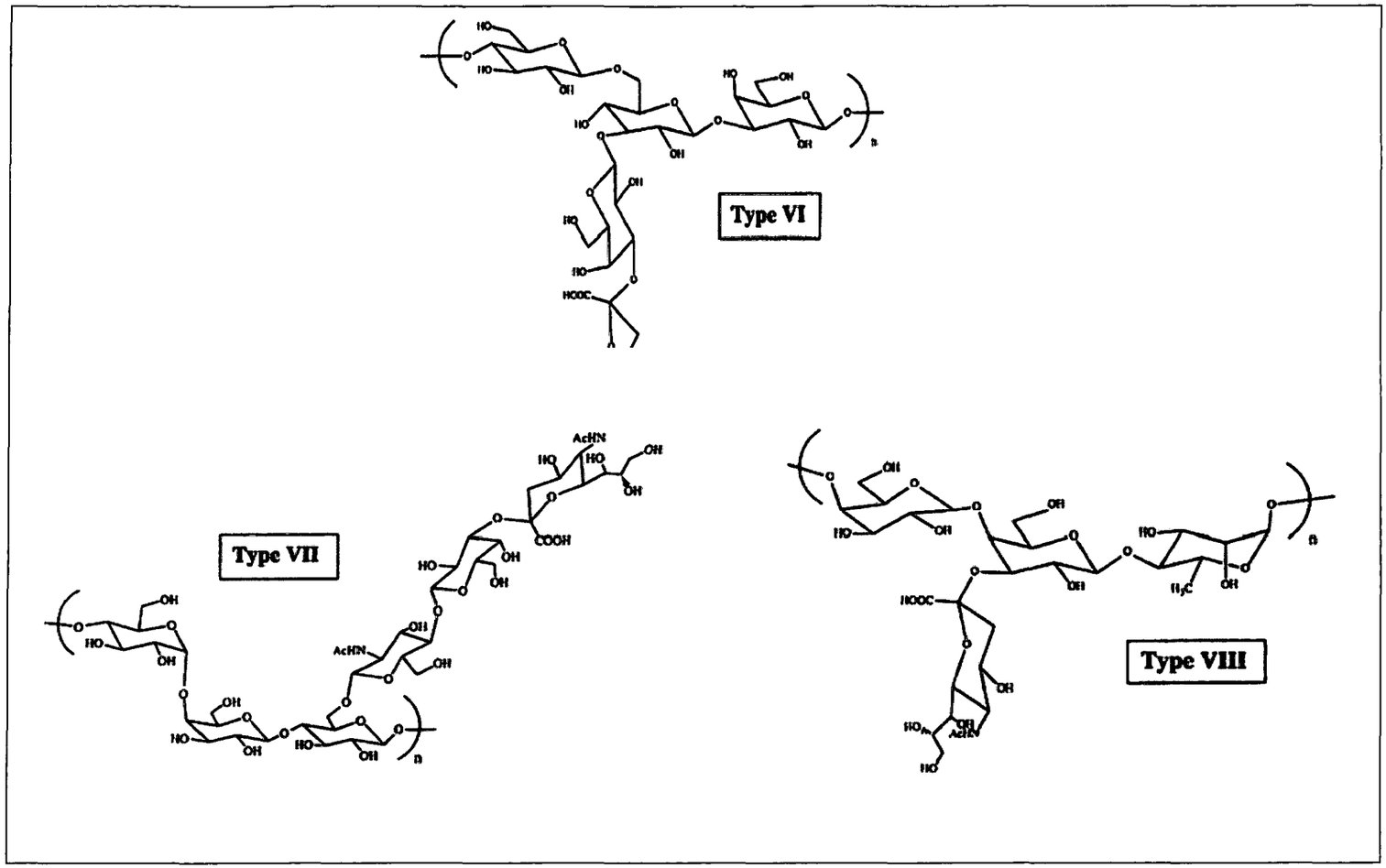

Fig.2.5: Arrangement of saccharides in CPS of GBS type VI, VII and VII (Madoff et al, 2006).

2.7. Interaction (Colonization, Adherence and Invasion) of GBS with host cells

Successful establishment of infection by bacterial pathogens requires (1) mechanisms for colonization (adherence and initial multiplication), (2) production of extracellular substances ("invasins"), that promote the immediate invasion of tissues and (3) ability to bypass or overcome host defense mechanisms which facilitate the actual invasive process (**Fig 2.6**).

The molecular events underlying GBS pathogenesis are poorly understood. The pathogenic process can be viewed as a series of hurdles that the organism must overcome to make access to host. The tools used to accomplish these tasks include products that bacteria may express on the surface and/or secrete into the surrounding environment. In many cases, especially when a particular step in pathogenesis is vital to organism survival, bacteria are equipped with multiple apparatuses with seemingly overlapping function. For example, several GBS surface components interact with epithelial cells. Including the CPS, whose structure determines serotype; the CPS impairs internalization by host epithelial cells but does not influence adhesion (Tamura et al, 1994). Lipoteichoic acid (LTA) mediates adherence to adult and neonatal epithelial cells (Teti et al, 1987). Bacterial proteins also play a role: adhesion of GBS to epithelial cells decreases by up to 75 % after treatment of bacteria with proteases, which degrade bacterial proteins (Bulgakova et al, 1986; Tamura et al, 1994).

2.7.1. Adherence

A low-affinity GBS interaction with epithelial cells is mediated by its amphiphilic cell wall-associated lipotechoic acid, while higher affinity interactions with host cells are mediated by a series of size-variable, pronase-sensitive; hydrophobic GBS surface proteins (Wibawan et al, 1992). Recent investigations have revealed that these high-affinity protein-mediated interactions with epithelium generally proceed through an intermediary: GBS effectively binds the extracellular matrix components fibronectin, fibrinogen and laminin. These proteins are known to interact with host cell-anchored proteins such as integrins and have been demonstrated to mediate adherence of related Gram-positive pathogens (Schwartz-Linek et al, 2004).

Attachment of GBS to fibrinogen is mediated by repetitive motifs within surface anchored protein fibronictin binding protein A (FbsA) (Schubert et al, 2002). The transcriptional regulator RogB positively regulates the ability of GBS to bind fibrinogen and fibronectin by increasing expression of downstream genes with extracellular matrix binding motifs as well as *fbs* A (Gutekunst et al, 2003). GBS fibronectin binding is also dependent on cellular glutamine transport encoded by the *gln* PQ operon (Tamura et al, 2002). Finally, Rib (a surface protein; resistance to protease, immunity, group B) confers protective immunity and is expressed by most invasive GBS isolates (Stalhammar-Carlemalm et al, 1993); Rib is closely related to the R28 protein of GAS that promotes epithelial cell binding (Stalhammar-Carlemalm et al, 1999).

GBS virulence determinants play a role in penetration of host cellular barriers. Bloodstream isolates of GBS secrete high levels of an enzyme that degrades hyaluronic acid, the main polysaccharide component of host connective tissue (Kjems et al, 1980; Pritchard et al, 1994). CAMP factor is a GBS extracellular protein that is toxic when injected intravenously in rabbits (Skalka and Smola, 1981). Recent electronmicroscopy and chemical cross-linking studies have shown that CAMP factor oligomerizes in the target membrane to form discrete pores and trigger cell lysis (Lang and Palmer, 2003).

2.7.2. Invasion

While attachment mechanisms allow GBS to compete with other microflora for a niche on the gastrointestinal and vaginal mucosa, the ability of the organism to penetratehost cellular barriers is a first distinguishing feature of its pathogenicity. In tissue culture, GBS are able to invade chorionic epithelial cells but not amniotic cells (Winram et al, 1998). Nevertheless, GBS can traverse placental membranes and weaken their tensile strength, a process that is speculated to involve local generation of oxygen radicals and prostaglandin E2 (Bennett et al, 1987). As a result of these processes, GBS may access the fetus within the amniotic cavity, induce placental membrane rupture or trigger premature delivery. After aspiration of infected amniotic or vaginal fluid, the newborn lung is the initial focus of GBS infection. From there, the organism rapidly gains access to the bloodstream and is circulated through other organs and tissues. GBS disruption of the lung barrier to infection appears to be a

combination of three processes: intracellular invasion, direct cytolytic injury and damage induced by the inflammatory response of the newborn host.

Intracellular invasion of both alveolar epithelial and pulmonary endothelial cells by GBS was first noted in newborn macaques after intramniotic challenge (Rubens et al, 1991), and later confirmed in human tissue culture lines derived from both cellular barriers (Rubens et al, 1992; Gibson et al, 1993). GBS cellular invasion occurs when the organism triggers its own endocytotic uptake and enters the cell within a membrane-bound vacuole, a process that requires microfilament components of the host cytoskeleton and is now appreciated to involve host signalling pathways mediated by PI 3-kinase (Tyrrell et al, 2002).

Cellular invasion is correlated to GBS virulence potential, as clinical isolates from infants with bloodstream infections invade epithelial cells better than strains from the vaginal mucosa of asymptomatic women (Valentin-Weigand and Chhatwal, 1995). Genetic phenotyping of type III GBS strains identified a particular restriction digest pattern (RDP III-3) characteristic of the vast majority of isolates from invasive neonatal infection (Takahashi et al, 1998). Subsequent subtractive hybridization studies identified a gene unique to RDP III-3 strains encoding the surface-anchored protein, Spb1, required for maximal epithelial cell invasion (Adderson et al, 2003). Similarly, elimination of the genes encoding the fibronectin-binding C5a peptidase ScpB or the alpha C surface protein each significantly reduced GBS epithelial cell invasion (Bolduc et al, 2002; Cheng et al, 2002).

Early-onset GBS pneumonia is characterized by widespread damage to lung epithelium and endothelium, with haemorrhage, proteinaceous fluid and neutrophils entering the alveolar airspaces. The loss of barrier integrity allows GBS direct entry to the circulation, and appears to result largely from the actions of the GBS b -haemolysin/cytolysin (b -H/C). Mutagenesis and heterologous expression studies have identified a single ORF, *cyl* E, as necessary and sufficient for GBS b -H/C expression (Pritzlaff et al, 2001). This pore-forming toxin lyses lung epithelial and endothelial cells and compromises their barrier function (Nizet et al, 1996; Gibson et al, 1999). At subcytolytic doses, the GBS b -H/C promotes GBS intracellular invasion and triggers the release of interleukin-8 (IL-8), the principal hemoattractant for human neutrophils (Doran et al, 2002). The cytolytic, proinvasive and proinflammatory effects of the GBS are all neutralized by dipalmotyl phosphatidylcholine (DPPC), the major phospholipid constituent of human lung surfactant (Nizet et al, 1996; Doran et al,

2002). This finding may in part explain the greatly elevated risk of premature, surfactant-deficient neonates to suffer severe GBS lung injury and invasive disease.

Because GBS is the leading cause of bacterial meningitis in newborns, it is evident that a further propensity exists for the bacterium to breech the specialized endothelium comprising the human blood–brain barrier. GBS have been shown to invade and trancytose polar monolayers of human brain microvascular endothelial cells, with serotype III strains doing so most efficiently (Nizet et a, 1997). As seen with epithelial cell barriers, the GBS b -H/C is directly cytolytic for human brain endothelial cells and b -H/C knockout mutants show decreased blood–brain barrier penetration and lethality in a mouse model of haematogenous meningitis (Doran et al, 2003).

2.8. Environmental factors affecting GBS invasiveness and its gene expression

Bacteria monitor the environment and alter gene expression in response to many factors, including temperature, pH, osmotic activity, oxygen levels, nutrient sources, and ion concentrations. Presence of oxygen during growth is positively associated with the ability of GBS to invade human cells was recently demonstrated *in vitro* with GBS grown in a chemostat, where nutrient conditions were precisely controlled (Johri et al, 2003). In addition to oxygen, the rate of growth also influences GBS invasiveness, as shown by the fact that GBS grown in a chemostat at a cell mass-doubling time (t_d) of 1.8 h invaded respiratory epithelial cells in significantly greater numbers than did those grown at a relatively slower t_d of 11.0 h (Malin and Paoletti, 2001). It has been shown previously that the hemolytic activity of GBS increases with temperature (Marchlewicz and Duncan, 1981; Vanberg et al, 2007) (**Fig 2.7**). Study by Musser et al, (2008) demonstrated that temperature is able to trigger extensive modifications in GBS transcriptome. It would be interesting to study the influence of temperature in *in vivo* conditions, especially when variation of temperature between physiological and pathological conditions is of lower magnitude.

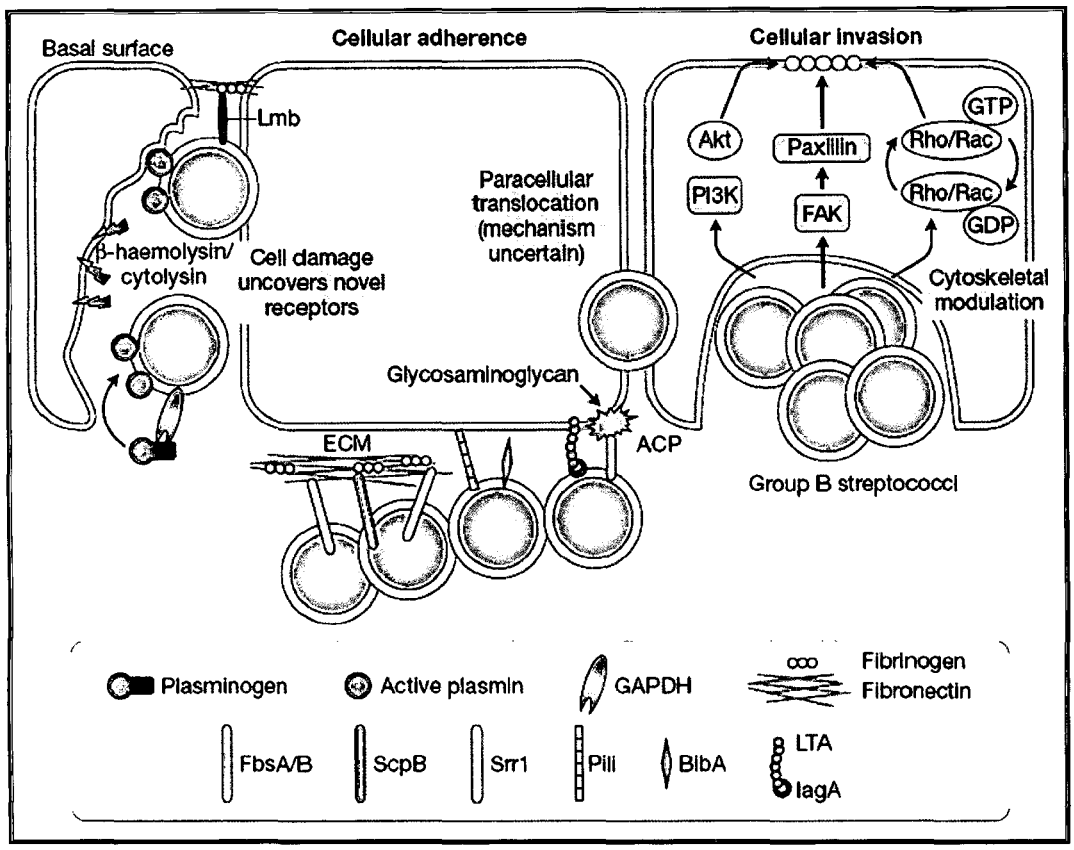

Fig.2.6: Molecular mechanisms of GBS cellular adherence and invasion. (Maisey et al, 2008).

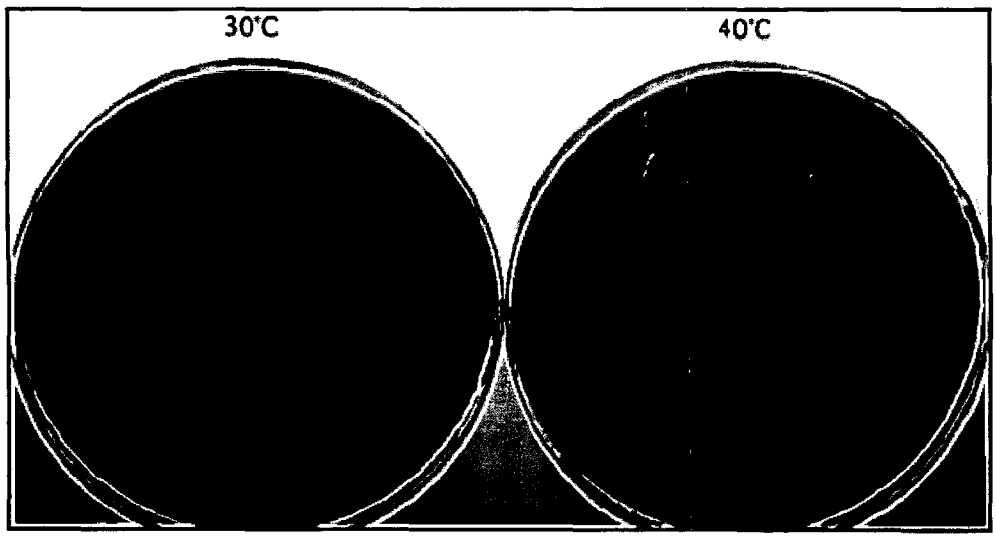

Fig.2.7. Difference in hemolysis and hemolytic activity of GBS between incubation at 30°C and 40°C (Musser et al, 2008).

Adherence of GBS was found markedly increased at acidic pH. Adherence at pH 4 was 10- to 20- fold higher than it was at pH 7 for both respiratory (A549) and urogenital squamous (ME180) epithelial cells (Tamura et al, 1994). Recent studies (Mereghetti et al, 2008a; Mereghetti et al, 2008b; Sitkiewicz and Musser, 2009) have shown that the transcriptome of GBS responds extensively to environmental changes. Musser et al, (2009), studied the differential expression of GBS grown in THB and human amniotic fluid (AF) and discovered that GBS significantly remodels its transcriptome in response to exposure to human amniotic fluid by growing rapidly in AF.

Recently Johri et al (2007) have conducted comparative genomics and proteomics and found that genes and proteins related to virulence up-regulated. They have further found that these proteins and genes were cell wall and membrane-associated which are expressed by GBS grown in chemostat at high and low level of oxygen conditions that resulted in high level *in vitro* invasion and low-level invasion respectively. It was hypothesized that novel proteins would be expressed by GBS under growth conditions that support high-level invasion (Johri et al, 2007).

2.9. Host immune response

Killing of GBS by these cells requires opsonization of the bacterium by specific antibodies or serum complement. Neonates are particularly prone to GBS invasive disease because of quantitative or qualitative deficiencies in phagocytic cell function, specific anti-GBS immunoglobulin, or the classic and alternate complement pathways (Maisey et al, 2008)

Once GBS penetrates cellular barriers to reach the bloodstream or deep tissues, a broader immunological response is activated to clear the infection, in which host phagocytic cells including neutrophils and macrophages play a critical role. Effective uptake and clearance of GBS by these cells depends upon opsonisation by specific antibodies or serum complement, factors that can be quantitatively and qualitatively deficient in newborns, especially those born prematurely. The propensity of GBS to produce invasive infections further reflects many virulence factors that allow the bacteria to resist opsonophagocytosis or neutralise the bactericidal activities of neutrophils and macrophages.

Upon penetration of GBS into the lung tissue or bloodstream of the newborn infant, an immunological response is recruited to clear the microorganism. Central to this response are host phagocytic cells, including neutrophils and macrophages. Effective uptake and killing by neutrophils requires opsonisation of the bacterium by specific antibodies in the presence of complement. However, complement deposition does not affect GBS survival or uptake by macrophages, probably because GBS protect themselves by binding factor H, a host counter-regulator of complement (Maruvada et al, 2008). Neonates are particularly prone to invasive disease because of their quantitative or qualitative deficiencies in phagocytic cell function, specific antibody, or the classic and alternative complement pathways. In addition to these newborn host susceptibilities, GBS possess a number of virulence determinants that seek to thwart each of the key components of effective opsonophagocytic killing. The sialylated GBS-CPS represents one such defence factors. Complement is a system of enzymatic reactions used by the innate immune system to recognize microbes and coat their surfaces with host proteins, making them more easily detected and engulfed by phagocytic cells bearing complement receptors, while simultaneously amplifying other aspects of the inflammatory response. The thick CPS is critical for limiting the effectiveness of host complement defence. The serotype-specific epitopes of ten known GBS CPSs (Ia, Ib, II–VIII and more recently IX) are created by different arrangements of four monosaccharides (glucose, galactose, N-acetylglucosamine and sialic acid) into unique repeating units, but unfailingly these structures contain a terminal sialic acid bound to galactose in an a2!3 linkage (Jennings et al, 1983; Wessels et al 1987; Wessels et al, 1989; Jennings et al 1993; Kogan et al, 1995; Kogan et al,1996; Slotved et al, 2007). This sialic acid molecule provides antiphagocytic protection by impairing surface deposition of opsonically active complement C3 on the bacterial surface. GBS subjected to sialidase treatment, or isogenic GBS mutants lacking capsular sialylation, are more susceptible to neutrophil killing and are less virulent in animal models of infection (Campbell et al, 1991; Marques et al, 1992). However, since others have shown that encapsulated and unencapsulated GBS are equally susceptible to macrophage uptake, the role of CPS in resisting phagocytosis per se versus other aspects of immune cell killing remains unclear (Segura et al, 1998). Sialic-acid-dependent reduction in C3 deposition is correlated with diminished production of C5a, an important complementderived chemoattractant (Takahashi et al, 1999) which works synergistically with ScpB-

mediated proteolytic inactivation of C5a to reduce host neutrophil mobilisation. Additionally, a new cell-surface GBS immunogenic bacterial adhesin (BibA) was recently determined to mediate inhibition of other complement components. BibA binds human C3bp, a component of the classical complement pathway, promotes resistance to phagocytic killing, mediates adherence to epithelial cells and contributes to virulence in a mouse model of infection (Santi et al, 2007). GBS b-protein was shown to prevent opsonophagocytosis by binding short consensus repeats found in the middle region of factor H, enabling the unbound active region to block C3b deposition on the bacterial cell surface (Jarva et al, 2004). The beta antigen of C protein binds human IgA antibody (Jerlstrom et al, 1991), and IgA deposited nonspecifically on the bacterial surface probably inhibits interactions with complement. Finally, a cell-surface protease, CspA, targets host fibrinogen, producing adherent fibrin-like cleavage products that coat the bacterial (**Fig 2.8**). Several studies confirms that immune response of human cell lines (Mikamo et al, 2004) and neonatal rat (Teti et al, 1993) causes the production of TNF-a, IL-1, IL-6, and gamma interferon when challenged with GBS.

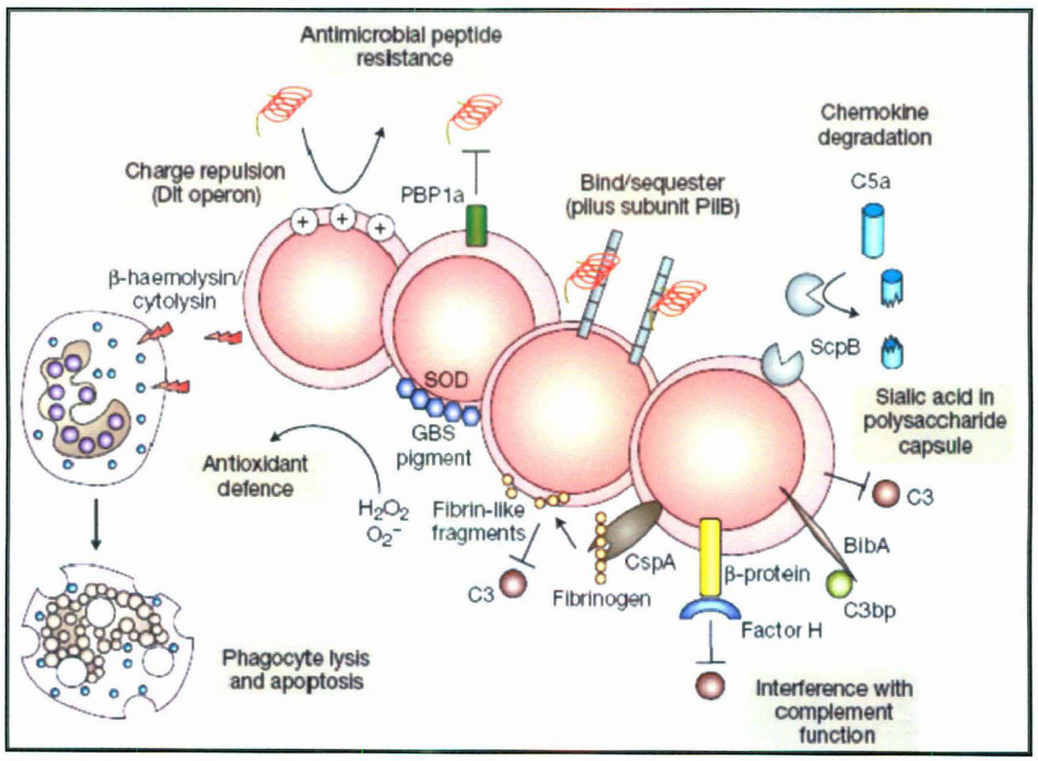

Fig.2.8: Mechanisms of GBS immune evasion (Maisey et al, 2008).

Table: 2.2. Key virulence factors of GBS				
Virulence Factor	**Genetic Nature**	**Chemical basis**	**Function**	**Proposed contribution(s) to disease pathogenesis**
Exo-polysaccharide surface capsule	*cps*A-L, *neu*A-D	High-molecular-weight polymer with terminal sialic acid residues	Impairs complement C3deposition and activationDecreases immune recognition, perhaps through molecular imicry of host sialic acid epitopes	Blocks opsonophagocytic clearance Delays neutrophil recruitment
β-hemolysin/ cytolysin	*cyl*E	CylE protein (79 Kd)	Forms pores in cell membranes Induces apoptosis Promotes cellular invasion Triggers iNOS, cytokine release	Direct tissue injury Penetration of epithelial barriers Induction of sepsis syndrome Phagocytic resistance
Hyaluronate Lyase	*hyl*B	HylB enzyme (110 kD)	Cleaves human C5a Binds fibronectin	Inhibit PMN recruitments Extracelluar matrix attachment Epithelial adherence and invasion
CAMP Factor	*cf*b	CAMP protein (24 kD)	CAMP reaction (co-haemolysin) Binds to Fc portion of IgG, IgM	Direct tissue injury Impairment of antibody function
Lipotechoic acid	Complex	Amphiphilic glycerol phosphate polymer of complex lipids and short-chain fatty acids	Amphiphilic glycerol phosphate polymer of complex lipids and short-chain fatty acids	Epithelial cell attachment activation of the sepsis syndrome Resistance to neutrophil killing
C protein (α and β components)	*bca* (alpha) *cba* (beta)	Alpha: protein with mutiple identical tandem repeats (14–145 kD); beta:84–94 kD variants	Binds cervical epithelial cells Blocks intracellular killing by neutrophils non-immune binding of IgA	Epithelial cell adherence Epithelial cell invasion Resistance to phagocytic clearance
Serine protease	*csp*A	CspA protein (142 kD)	Cleaves fibrinogen to fibrin-like fragments	Resistance to phagocytic clearance? Promotes tissue spread
Fibrinogen receptor	*fbs*A	FbsA protein (44.2 kD)	Binds fibrinogen through repetitive structure motifs	Extracellular matrix attachment Epithelial adherence Resistance to opsonophagocytic killing

2.10. Regulation of virulence

Recently completed GBS genomes revealed a relatively large number of two-component regulatory systems, including one that displayed a particularly high degree of similarity to CsrRS, a multigene regulatory system in *S. pyogenes*. Orthologous CsrRS system in GBS controls expression of at least three GBS products implicated in virulence and is the first description of a two component system shown to regulate expression of multiple GBS virulence factors and to affect virulence during experimental infection.

The results by Jiang et al, (2005), indicate evidence of both up- and down regulation of different virulence determinants by GBS CsrRS. This imply that the GBS CsrRS system has divergent effects on different target genes or that it has regulatory activity on another regulator that, in turn, acts on one or more regulated structural genes. Interaction of the CsrRS system with other regulators could be part of a regulatory cascade or network in which multiple elements link an environmental stimulus with a series of downstream responses. The possibility of one or more intermediate regulators could explain the observed up (CAMP factor)- and down (beta-hemolysin and C5a peptidase)-regulatory effects on different target genes. Certain well-characterized systems involve both activation and repression of target genes by a single response regulator, for example OmpR in *Escherichia coli* and BvgA in *Bordetella pertussis* (Cotter et al, 2000; Stock et al, 2000). By contrast, the *S. pyogenes* CsrR protein appears to act primarily or exclusively as a repressor by binding to promoter regions upstream of regulated genes (Federle et al, 1999; Miller et al, 2001; Graham et al, 2002). While it is possible that the GBS CsrR protein regulates target promoters directly but with opposite effects, it seems at least equally likely that one or more of the regulated genes might be controlled by another (CsrR-regulated) regulatory system. The more complex pattern of regulation may explain why GBS CsrR mutants were attenuated in experimental infection, while *S. pyogenes* CsrR mutants were more virulent than the wild type (Heath et al, 1999; Levin et al 1998).

The repertoire of regulated genes is also different in the two species-both CAMP factor and C5a peptidase are also produced by *S. pyogenes*, but in contrast to GBS, their expression is not significantly altered by inactivation of *csrR* (Graham et al, 2002). Conversely, CsrR mutants in *S. pyogenes* display increased expression of

the *has* operon that directs synthesis of the hyaluronic acid capsule, whereas expression of the capsular polysaccharide biosynthesis operon in GBS appears not to be controlled by the CsrRS system. Therefore, although CsrRS appears to be an important multigene regulator for both *S. pyogenes* and GBS, the GBS CsrRS system differs significantly with respect to its dual roles as an activator and repressor of virulence factor expression, the repertoire of regulated genes, and its overall role in pathogenesis.

An important but still unanswered question is identification of the environmental signal(s) to which the GBS CsrRS system responds. Extracellular magnesium concentration was recently shown to signal through the *S. pyogenes* CsrRS system, presumably by binding to the extracellular domain of the CsrS protein (Gryllos et al, 2003). However, since the extracellular domain of the GBS CsrS protein has only limited similarity to that of the *S. pyogenes* protein, it is possible, and perhaps likely, that the extracellular ligand(s) for the GBS system is distinct from that for *S. pyogenes*. The fact that the CsrRS system controls expression of several products implicated in infection suggests that the system may serve as a means for GBS to perceive the local host environment and adapt to it by altering expression of multiple factors that modulate bacterium-host interactions. Further investigation of this novel regulatory system is warranted to identify the environmental signals that interact with CsrS and to characterize further the role of the CsrRS system in pathogenesis of GBS infection.

2.11. Heterogeneity of GBS genome

S. agalactiae genome is 2,211,488 bp long. The complete genome sequences of three of the nine GBS serotypes Ia (Tettelin et al, 2005) III (Glaser et al, 2002) and V (Tettelin et al, 2002) have been reported. Of particular interest was the finding that most genes apparently unique to specific strains of the same serotype were found clustered in regions (islands). These islands not only contained atypical nucleotide compositions differing from the 35.7% G+C content of the entire genome, but also contained most of the known or putative GBS virulence factors. Interestingly, all of these islands also contained sequences known to be associated with mobile genetic elements, e.g., insertion sequences, proteins of phages, plasmids, and transposons, suggesting that these islands correspond to horizontal gene transfer events. A large

number of phage and plasmid-related genes were identified in the chromosome of the serotype III strain, however no complete temperate phage genomes were found. Additionally, three copies of an approximate 50 kb sequence were present that had the characteristics of an integrative plasmid. The association of mobile elements and virulence factors in chromosomal islands suggests that they may be pathogenicity islands and thus have an important role in virulence acquisition and genetic diversity (Ferretti et al, 2004).

To fully explore gene variability within the GBS species Tettelin et al, (2005) have determined the complete genome sequence of the type Ia strain A909 and draft genome sequences of five additional strains, representing the five major serotypes. Comparative analysis of the six newly sequenced genomes and the two genomes already available in the databases suggests that a bacterial species can be described by its ''pan-genome'' (pan, from the Greek word *παν*, meaning whole), which includes a core genome containing genes present in all strains and a dispensable genome composed of genes absent from one or more strains and genes that are unique to each strain. Surprisingly, unique genes were still detected after eight genomes were sequenced, and mathematical extrapolation predicts that new genes will still be found after sequencing many more strains. Thus, the genomes of multiple, independent isolates are required to understand the global complexity of bacterial species. Analysis of multiple GBS genomes was found to be instrumental for the development of vaccines (Maione et al, 2005) and for the functional characterization of important genetic determinants (Lauer et al, 2005). Johri et al, (2007) have studied both comparative genomics and comparative proteomics of genes upregulated and cell wall-associated and membrane proteins expressed by GBS grown in a chemostat in conditions that resulted in high level *in vitro* invasiveness with those of GBS held at conditions that resulted in low-level invasion and they have hypothesized that novel proteins would be expressed by GBS under growth conditions that support high-level invasion. Further, Johri et al (2007) selected GBS type V because of its recent emergence and current prevalence in adult human disease (Rench et al, 1993 and Zaleznik et al, 2000). It was the first study that integrates bacterial physiology (controlled and balanced growth in a chemostat) with proteomics and genomics to investigate factors involved with bacterium host cell interactions.

2.12. GBS vaccines

Despite the many studies that are focused on developing a GBS vaccine using conventional approaches, including the cultivation of pathogens and the identification of highly immunogenic and protective antigens using standard biochemical and microbiological techniques, little success has been achieved in terms of developing a vaccine that is globally effective.

Several approaches have been used for the development of GBS vaccine ranging from the immunization with live-attenuated bacteria up to the formulation of the safer sub-unit vaccines. As a first approach to vaccine development, CPS-tetanus toxoid conjugates against all nine GBS serotypes were shown to induce CPS-specific IgG that is functionally active against GBS of the homologous serotype (Paoletti and Madoff, 2002). Clinical phase 1 and phase 2 trials of conjugate vaccines prepared with CPS from GBS types Ia, Ib, II, III, and V revealed that these preparations are safe and highly immunogenic in healthy adults (Paoletti and Kasper, 2003). Although these vaccines are likely to provide coverage against the majority of GBS serotypes that currently cause disease in the US, they do not offer protection against pathogenic serotypes that are more prevalent in other parts of the world (e.g., serotypes VI and VIII, which predominate among GBS isolates from Japanese women) (Lachenauer, 1999).

Cell surface proteins of GBS have also attracted attention as possible vaccine candidates since they confer protective immunity in animal models (Bevanger et al, 1985; Madoff et al, 1992; Stalhammar-Carlemalm et al, 1993; Larsson et al, 1996). To date, a few protein-based vaccines against GBS have been described. These include the tandem repeat–containing α and β antigens of the C protein complex (Madoff et al, 1992) and Rib (Stalhammar-Carlemalm et al, 1993); surface immunogenic protein, Sip (Brodeur et al, 2000) and C5a-ase, a serine protease that inactivates complement factor C5a (Cheng etal, 2001). However, of these proteins, only Sip and C5a-ase are conserved at the gene level in the majority of GBS isolates (Brodeur et al, 2000; Tettelin et al, 2002) and no systematic analysis on the extent of cross-protection is available. Unfortunately, these proteins were not found to be present in all clinical isolates (Stalhammar-Carlemalm et al, 1993; Ferrieri et al, 1997)

However, recent years have witnessed the emergence of genomics, proteomics, gene expression and *in silico* technologies that are presenting exciting new opportunities in the hunt for an effective and globally relevant GBS vaccine.

2.12.1. CPS vaccines

Clinical data and experimental observation supports an important role of CPS specific antibodies in the prevention or control of GBS disease. Native GBS type Ia, Ib, II and III CPSs have been tested as vaccines in adults. First clinical trials with GBS enrolled 33 healthy adults to evaluate the safety and immunogicity of type III CPS extracted by two methods (Baker et al, 1978). Although both preparations were well tolerated, the extraction methods produced a type III CPS that was more immunogenic than that obtained by tricarboxylic acid (TCA) extraction. By 1980s, more than 300 healthy adults, including 40 third trimester pregnant women, were safely vaccinated with type III CPS or other GBS CPS vaccines at CPS doses ranging from 10 to 150 μg (Baker and Kasper, 1985; Baker et al, 1988). Although well tolerated these antigens failed to induce a strong specific antibody response in the target population. The class of human antibody among responders to GBS type III CPS (Baker and Kasper, 1985) and to GBS type II CPS (Guttormsen et al, 1996) was predominantly IgG. The response to CPS antigens differed with the serotype tested; native type II CPS was the most immunogenic (88% rate of response) and type Ia CPS the least (40%) (Baker and Kasper, 1985). GBS CPS vaccines induced antibodies that were against homologous GBS serotypes in opsonophagocytic assays and were passively protective in animal studies (Baker and Kasper, 1985). By the late 1980s, preclinical and clinical results showed that, although the CPS structures were critical vaccine components, they were not sufficiently immunogenic by themselves to be effective GBS vaccines. Reserachers then begin to focus on the development of GBS CPS vaccines whose immunogenicity was enhanced by methods that preserved their native antigenic structures (**Table 2.3**).

2.12.2. Polysaccharide-Protein conjugate vaccines

The first GBS CPS conjugate vaccines were prepared in 1990 by three different coupling strategies. Native type III CPS was coupled to TT (III-TT) using adipic acid dihydrazide as a spacer molecule (Michel et al, 1994) or directly via aldehyde formed on a selected number of silaic acid residues. In a different approach,

coupling of a type II oligosaccharide of 14 pentasaccharide repeating units to TT using a synthetic 6-C spacer molecule resulted in a single site attachment of the reducing end of the oligosaccharide (Wessels et al, 1987). All three conjugates were better immunogen in laboratory animals than was uncoupled to type III CPS. Moreover, antibodies elicited by these GBS conjugate vaccines were of the IgG class and were functionally active when tested in *vitro* and *in vivo*. Further progress was made in developing conjugate vaccine against GBS serotype other than type III (Wessels et al, 1995). Immunity in mice to multiple GBS serotype was demonstrated in studies of a mixture of a Ia-TT, Ib-TT, II-TT, and III-TT conjugate administered as single tetravalent vaccine (wessels et al, 1993). The lack of interference of one serotype with another in the tetravalent vaccine opened the way to the development of a multivalent GBS vaccine for use in humans. However clinical observation of an increased prevalence of serotype V dictated its inclusion in a multivalent vaccine (Koskiniemi et al, 1998).

To broaden coverage with a single vaccine construct, the beta C protein of GBS has been used both as carrier of type III CPS as well as to non type III GBS strains that contain this protein (Legergard et al, 1990). Although the results with this vaccine proved that border coverage could be attained with the relevant carrier protein, the alpha C protein of GBS would be a better carrier for GBS CPSs because it is present in larger number of serotypes (Rench et al, 1993). A GBS type III alpha C protein conjugate vaccine could potentially provide coverage against up to 90% of disease causing strains of GBS. Conjugate vaccine utilizing both the full length 9 repeat alpha C protein and 2 repeat alpha C protein as carriers for type III polysaccharide elicited antibodies in mice that protected neonatal mouse pups against lethal intraperitoneal challenge with either alpha positive or type III GBS (Kling et al, 1997) C5a peptidase as potential carrier protein in conjugates vaccines. A conjugates vaccine using the C5a peptidase coupled to type III polysaccharide was able to reduce the severity of pneumonia in intranasally inoculated mouse model of GBS infection using a single challenge strain of GBS with a different capsular serotype (Haft et al, 1996).

2.12.3. Protein antigens

Efforts to develop polysaccharide vaccines against such GBS infections have, in several cases, met with difficulties, e.g. poor immunogenicity of the antigens and

immunological cross-reactivity with human tissue. Therefore, it is of interest to analyze the possibility of using bacterial surface proteins as vaccines against these infections. Indeed, protein antigens may have several advantages: most proteins elicit a T-cell-dependent antibody response resulting in long-term memory, proteins are immunogenic without being conjugated to other molecules, and by employing recombinant DNA techniques production of large amounts of antigens for vaccine preparation is possible. Moreover, the emerging technology of DNA vaccination requires that the immunogen is a protein (Charlotte et al, 1999). One advantage with a vaccine based on proteins, rather than on polysaccharides, might be absence of immunological crossreactions with human glycoproteins (Pritchard et al, 1992) (**Table 2.3**).

2.12.3.1. Alpha C protein

Because the alpha C protein found in about 50 % of clinical isolates of GBS and in 70 % of non type III GBS strain, this laddering protein is an attractive candidate for use in a GBS vaccine (Johnson et al, 1984; Manning et al, 2006). The alpha C protein is the prototype of a family of streptococcal surface proteins that are characterized by the presence of: (1) conserved amino terminal domains, (2) long tandem repeating elements, and (3) carboxy-terminal domains containing the highly conserved consensus sequence LPXTGX associated with attachment of these proteins to the cell wall. The most frequent form of α C protein found in nature contains 9 identical 246 bp repeating elements and a 33 bp partial repeat and has a predicted molecular weight of 108,705 Da. Alpha C protein binds host cell surface glycosaminoglycan and mediates translocation of GBS across epithelial barriers, facilitating invasive GBS infection (Baron et al, 2004; Baron et al, 2007) (**Table 2.3**).

2.12.3.2. Rib protein

Two cell surface proteins, alpha and beta, have also been studied in detail since they confer protective immunity (Lancefield et al, 1975; Bevanger et al, 1985; Michel et al, 1991), but these proteins are usually not expressed by type III (Lancefield et al, 1975; Bevange et al, 1983; Johnson et al, 1984) strains. They describe that a cell surface protein, designated protein Rib (resistance to proteases, immunity, group B), that confers protective immunity and is expressed by most strains of type III. Protein Rib

was first identified as a distinct 95-kD protein in extracts of a type III strain, and was purified to homogeneity from that strain. Rabbit antiserum to protein Rib was used to demonstrate that it is expressed on the cell surface of 31 out of 33 type III strains, but only on 1 out of 25 strains representing the other three serotypes. Mouse protection tests showed that antiserum to protein Rib protects against lethal infection with three different strains expressing this antigen, including a strain representing a recently identified high virulence type III clone (Musser et al, 1985). Protein Rib is immunologically unrelated to the alpha and beta proteins, but shares several features with the alpha protein. Most importantly, the NH2-terminal amino acid sequences of the Rib and alpha proteins are identical at 6 out of 12 positions. In addition, both protein Rib and the alpha protein are relatively resistant to trypsin (and Rib is also resistant to pepsin) and both proteins vary greatly in size between different clinical isolates (Margaretha et al, 1993). Finally, both protein Rib and the alpha protein exhibit a regular ladderlike pattern in immunoblotting experiments (Jerlstrgm et al, 1991), which may reflect a repetitive structure. Taken together, these data suggest that the Rib and alpha proteins are members of a family of proteins with related structure and function. Since protein Rib confers protective immunity, it may be valuable for the development of a protein vaccine against the GBS, an encapsulated bacterium (**Table 2.3**).

2.12.3.3. Surface immunogenic protein (Sip)

The localization and accessibility of the GBS Sip at the surface of intact GBS cells were studied by flow cytometric assay and immunogold electron microscopy (Rioux et al, 2001). Comparison of the predicted amino acid sequences of Sip proteins from six serologically distinct strains clearly indicated that this protein is highly conserved. Immunoblot assays using a Sip-specific monoclonal antibody also indicated that a protein band with an approximate molecular mass of 53 kDa was present in every GBS strain tested, which included representative isolates of all serotypes (Brodeur et al, 2000). In addition, the immune response induced after immunization with recombinant Sip (rSip) efficiently protected mice against experimental infection with GBS strains representing serotypes Ia/c, Ib, II/R, III, V, and VI (Brodeur et al, 2000). Most importantly, the surface exposure of Sip is not hindered by other surface antigens. These observations further intensify the interest in

Sip as a potential vaccine candidate. Current status of various vaccine candidates against GBS has been summarized in **Table 2.3**.

2.12.3.4. C5a peptidase

Streptococcal C5a peptidase (SCPB) is a highly conserved surface protein among strains of GBS (Suvorov et al, 1991). Enzymatic activity is highly specific for C5a, cleaving the chemotaxin at its polymorpho nuclear leucocyte (PMN) binding site (Wexler et al, 1985). Although little is known about the impact of the peptidase on the virulence of GBS, Bohnsack et al, (1997), showed that SCPB reduces the acute neutrophil response to infections by GBS in C5a knockout mice supplemented with human recombinant C5a. Based on studies of GAS, there is also reason to believe that SCPB may contribute to the organism's ability to colonize mucosal surfaces. The sequence of SCPB is 98% identical to that expressed by GAS (Chmouryguina et al, 1997). In GAS, the peptidase has been shown to retard clearance of streptococci from the oral mucosa of mice (Ji et al, 1998). Moreover, mice immunized with recombinant peptidase clear streptococci more rapidly following intranasal challenge (Ji et al, 1997). Antibody directed toward SCPB can neutralize peptidase activity, but because the protein protrudes from the surface, it could also be opsonic or could induce antibody-dependent killing by macrophages. Further authors have investigated the potential use of SCPB as a stand-alone vaccine antigen and as a protein carrier for polysaccharide vaccines.

2.12.4. Pilus based GBS vaccine

Pili appear to play a key role in the adhesion and attachment of gram-negative and gram-positive pathogens to host cells. With the idea of eliciting immune responses that counteract the essential biological role of pili, components of pili have been tested as vaccine candidates against different pathogens.

In GBS, the 2 pili encoded by pilus island 1 (PI-1) and pilus island 2a (PI-2a) and localized in 2 distinct loci (PI-1 and PI-2) were first identified (Lauer et al, 2005). The overall gene organization of the 2 islands consists of 3 genes that encode LPXTG motif– carrying proteins and correspond to the major pilus subunit (i.e., backbone protein [BP]) and the 2 ancillary proteins (AP1 and AP2) and 2 sortase genes that are involved in the assembly of pili. BP and AP1 of both pilus islands were shown to elicit opsonophagocytic antibodies that protected mice in the active maternal

immunization model (Maione et al, 2005). More recently, PI-2a was shown to have an allele, pilus island 2b (PI-2b) that displayed a similar genetic organization but varied substantially in gene sequence (Dramsi et al, 2006; Rosini et al, 2006). The protective activity of the backbone and the ancillary proteins of PI-2b was not investigated.

The high degree of conservation of GBS pili was unexpected, when one considers the variability observed in other pathogenic bacteria. One possible explanation is that pili expression may be regulated such that they appear on the bacterial surface only transiently, thus avoiding the selective pressure of the host immune system. However, no substantial regulation was detected in GBS grown in a chemostat under different conditions (Johri et al, 2007) or isolated from the blood of infected mice. A second explanation for pilus conservation is that GBS occupies environmental niches (e.g., lower gastrointestinal and vaginal mucosa) that are relatively inert from an immunological standpoint. In fact, a considerable proportion of women heavily colonized with GBS have low CPS IgG serum concentrations, and these women pose a high risk of early-onset infection to their newborns. On the other hand, in *S. pyogenes,* which colonizes the immunologically reactive nasopharynx and tonsils, 9 pilus islands and ~20 serologically distinct alleles have been identified (Lancefield and Dole, 1946; Kratovac, 2007). When pilus components from the 3 islands are combined, a vaccine conferring protection against 94% of contemporary GBS strains circulating in the US and Italy can be formulated. This provides a rationale for the development of a universal GBS vaccine that is potentially capable of preventing GBS disease in all groups. Furthermore, because the role of pili is to promote bacterial adhesion to host tissue, it is tempting to speculate that pilus-based vaccines might also elicit antibodies capable of preventing GBS colonization.

2.12.5. Genomic and gene-expression approaches

Genomics allows antigen candidates to be identified on the basis of sequence conservation in different serotypes and strains of a given pathogen, and by predicting the surface exposure of a protein. Tettelin et al, (2005), compared the predicted protein sets of *S. agalactiae*, *S. pyogenes* and *S. pneumoniae.* This analysis revealed that approximately 50% of the genes are homologous, indicating substantial overlap in the virulence mechanisms used by these pathogens (Rappuoli, 2000). In terms of vaccine development, the identification of shared virulence factors and protective antigens could support a concept of combined vaccination approaches. Although, the

sequence of a single genome does not reflect how genetic variability drives pathogenesis within a bacterial species, and is a limitation regarding genome wide screens for vaccine candidates or for antimicrobial targets, the identification of universal GBS vaccine candidates by multigenome analysis and screening has been reported (Maione et al 2005). In this approach the genome sequences of eight GBS strains belonging to different serotypes of GBS were analysed and compared. This study revealed that 1,811 genes (~80% of each genome) were shared by all strains - the 'core' genome - and 765 genes were not present in all strains - the 'variable' genome. Using *in silico* analysis, genes encoding putative surface-associated and secreted proteins were identified from these two subgenomes. A total of 589 proteins were identified (396 'core' genes and 193 'variable' genes), of which 312 were successfully expressed, purified and used to immunize mice. A combination of four proteins, Sip (SAG0032), present in the core subgenome, and three other putative, surface-associated proteins (SAG1408, SAG0645, SAG0649), from the variable subgenome, elicited protection in infant mice and their combination proved highly protective against a large panel of GBS strains, including all circulating serotypes. Mathematical extrapolation of the GBS genomic data indicates that the gene reservoir available for inclusion in the GBS pan-genome is vast, and that unique genes will continue to be identified even after the sequencing of hundreds of genomes (Tettelin et al, 2005). Diversity among isolates also arises by homologous recombination leading to the exchange of complete loci encoding surface proteins, or of the internal part of genes encoding putative antigens, as was first described for the α-C/Rib family (Lachenauer et al, 2000). The combination of the different alleles at these multiple loci allows GBS strains to express different combinations of surface proteins, a strategy used by the pathogen to evade host immune mechanisms; however, this versatility will also have to be taken into consideration when designing a universal vaccine that is effective against GBS (Brochet et al, 2006). Use of genomics and proteomics for the development has been summarized in **Table 2.4**.

Table 2.3: Current status of GBS vaccine research and development (Johri et al, 2006)				
S.No.	Vaccine Target	Advantage and Approach	Limitation	References
1. Capsular carbohydrate				
a	Unmodified polysaccharide vaccine (type III serotype)	Phase I trials indicated that the vaccine was safe and well tolerated	60% of the recipients showed an immune response; Requirement to improve immunogenicity of the CPS	Baker et al, 1988
b	Conjugate polysaccharide vaccine	Type III serotype: increase in immunogenicity when coupled to an immunogenic protein (tetanus toxoid (TT)); Conjugate vaccine with all nine currently identified GBS serotypes (Ia, Ib, II, III, IV, V, VI, VII and VIII) prepared and tested clinically	Capsular conjugate vaccines of this type need to be multivalent in order to provide sufficient coverage against prevalent serotypes	Paoletti and Kasper, 2002; Paoletti et al, 1994; Wessels et al, 1990; Paoletti et al, 1992
c	Conjugate bivalent polysaccharide vaccine	Bivalent vaccine (GBS type II-TT and type III-TT) combined and administered; Well tolerated	Further testing is warranted to investigate immune interference when more than two GBS CPS conjugate vaccines are simultaneously dministered	Paoletti and Madoff, 2002
d	Conjugate multivalent polysaccharide vaccine	Proposed that effective GBS vaccine in the United States includes five major serotypes (Ia, Ib, II, III and V); It is anticipated that multivalent vaccines will include each conjugate vaccine prepared separately	Formulation of a GBS conjugate vaccine for use in the United States might not be effective in other regions	Paoletti et al, 1999; Paoletti and Madoff, 2002
2. Proteins				
a	C5a peptidase	Present on all strains and serotypes of GBS; Little or no antigenic variability; Capable of inducing antibodies that are opsonically activ; Immunization induces serotypeindependent protection	Progress as a potential vaccine is unknown	Cheng et al, 2001
b	β-Component of the C protein	Elicits protective immunity in animal models	This protein is only present in a minority of strains that cause infection (~20%)	Madoff et al, 1992
c	LmbP	Expressed by most GBS strains	Progress as a potential vaccine is unknown	Heath et al, 2005
d	Sip	Present on all GBS strains; Induces protective antibodies; Recombinant SIP protein protected mice infected with numerous GBS strains	Biological function is not well understood; No recent reports of progress towards the development of a vaccine	Brodeur et al, 2000 Lindahl et al, 2005; Spellerberg et al, 1994; Heath et al, 2005
e	LrrG	Highly conserved protein antigen that induces protection	Progress as a potential vaccine is unknown	Seepersaud et al, 2003

Table 2.4: Application of genomics/proteomics approaches to identify potential GBS vaccine candidates (Johri et al, 2006)

Methods/ Technique	Vaccine candidate Identified	Chracterstics	Limitation	References
Comparative genome analysis	Sip, CAMP factor, R5 protein, enolase, Hyaluronidase, Hemolysin/cytolysin (cylE)	Identifies conserved genes; Detects putative virulence factors	None observed	Tettelin et al, 2002
Multiple genome Screening	Three cell-wall surface-anchor proteins (components of a pilus-like structure), Sip	Identifies conserved genes; Detects putative virulence factors	None observed	Maione, 2005
STM	LmbP, a permease, yaluronate-associated proteins, Clp protease homologue	Identifies genes that are essential for virulence, based on a negative selection method; Direct selection for antigenicity	Genes that are essential for growth are not identified	Jones et al, 2000
Proteomic approach	Ornithine arbamoyl transferase, Phosphoglycerate kinase, Non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase, Purine nucleoside phosphorylase, Enolase, Glucose-6-phosphate isomerase	Identifies cell-surface-expressed proteins using 2-dimensional electrophoresis (2DE) and MALDI-MS	Proteins expressed only in vivo are not identified	Hughes, 2002
Comparative genomics and proteomics	ATP synthase Fo, beta subunit,6-Phosphofructokinase,L-Lactate dehydrogenase,Pyruvate kinase,ATP-dependent Clp protease, ATP-binding subunit,ATP-dependent RNA helicase, DEAD/DEAH box family,Glutamine ABC transporter, glutamine-binding,Amino acid ABC transporter, permease protein,Iron-compound ABC transporter, iron-compound-binding protein,Glutamine ABC transporter, glutamine-binding protein/permease,Amino acid ABC transporter, permease protein	Idenfies surface associated genes and proteins using Microarray and proteomics		Johri et al 2007

CAMP, Christie, Atkins and Munch-Petersen; Clp protease, class III heat shock protein; GBS, group B Streptococcus; LmbP, laminin binding protein; MALDI-MS,matrix-assisted laser desorption/ionization mass spectrometry; Sip, surface immunogenic protein; STM, signature tag mutagenesis.

It is clear from the literature presented herein that over the past two decades no authentic GBS epidemiology, serotyping has been done in India. Further, no invasion, comparative genomics study was conducted. Therefore for the present Ph.D. work these tasks have been taken into consideration to find out prevalent serotype in India as well as good vaccine candidate against GBS of India origin using comparative genomics.

Materials and Methods

Materials and Methods

3.1. Bacterial Cultures

S.agalactiae (GBS) samples were collected from different pathological laboratories and hospitals in Delhi and surrounding areas are listed in **Table 3.1**. After serotyping analysis we have found Ia, III, V and VII are the prevalent serotypes in the total samples collected therefore were further used for invasion and transcriptional profiling. *Staphylococcus aureus* was used as control for Catalase test. *S.pyogenes* (GAS) was used as control in CAMP test.

Table 3.1. Hospitals/laboratories from where samples were collected

S No	Name of hospitals/laboratories
1	Lady Hardinge Medical College, New Delhi
2	All India Institute of Medical Sciences, New Delhi
3	Safdarjung Hospital, New Delhi
4	Sawai Mansingh General Hospital and Medical College, Jaipur, Rajasthan
5	Dr. Dang's Pathology Laboratory, New Delhi
6	Dr. Lal's Pathology Laboratory, New Delhi, India*

***They also have samples collection centers in small as well as big towns of North India.**

3.2. Mammalian cell Cultures

Human cell lines that were used throughout in the study are mentioned in **Table 3.2**

Table 3.2: List of Human Cell lines used in this study

S.No.	Cell line Name	Organ/tissue	ATCC No.	Source
1.	A549	Human type II alveolar epithelial carcinoma	ATCC CCl-185	Prof. R. K. Saxena, School of Life Sciences, JNU, New Delhi, India
2.	ME-180	Human cervical epithelial cells	ATCC HTB-33	National Centre for Cell Science (NCCS) Pune, India

3.3. Kits

All kits used throughout in this study are mentioned in **Table 3.3.**

Table 3.3: Kits used in this Study

S.No.	Kit	Source
1.	Streptex Murex	Remel Europe Ltd, UK
2	GBS typing antisera	Denka Seiken, Japan
3	RNeasy Midi kit	Qiagen USA
4.	Gel Extracion kit	Qiagen USA

3.4. Enzymes and Chemicals.

All important chemicals and enzymes used throughout in this study are mentioned in **Table 3.4.**

Table 3.4: List of Enzymes and Chemicals used in this study

S.No	Chemicals	Source
1	Penicillin	Himedia
2	Gentamycin	Himedia
3	Taq polymerase	Biolabs (NEB)
4	Lysozyme	Sigma Aldrich
5	Proteinase K	Sigma Aldrich
6	RNAse	Sigma Aldrich
7	Todd Hewitt Broth (THB)	Himedia
8	Blood agar base-2	Himedia
9	0.25% Trypsin solution	Himedia
10	Fetal Bovine Serum (FBS)	Gibco
11	RPMI-1640	Gibco, Pan Biotech
12	DMSO	Sigma Aldrich
13	Mutanolysin	Sigma Aldrich
14	Phenol	Sigma Aldrich/SRL
15	Trypsin (0.25%)	Himedia
16	DNA 1Kb ladder	NEB
17	Agarose	SERVA
18	SDS (Sodium dodecyl sulfate)	Sigma Aldrich
19	EDTA (ethylene diamine tetra acetic acid)	SD Fine

20	Tris-HCl	Merck
21	CTAB (Cetyl trimethyl ammonium bromide)	Sigma Aldrich
22	dNTP	Biotool
23	Glycerol	Merck
24	Sheep blood	Bajaj Blood supplier
25	Chloroform	Fisher Scientific
26	Isoamyl Alcohol	Merck
27	Hydrochloric acid	SD Fine
28	Sodium Hydroxide	Qualigens
29	Sodium Chloride	Merck
30	Sodium acetate	SRL
31	Ethanol	Merck
32	Ethedium Bromide (EtBr)	Sigma Aldrich
33	RNA Protection Buffer	Qiagen
34	β-mercaptoethanol	Sigma
35	Trizol	Invitrogen
36	Isopropanol	Qualigens
37	Glass bead (0.1mm)	BioSpec. Products
38	MOPS	Himedia
39	DEPC	Sigma
40	Formaldehyde (37-40%)	Merck

3.5. Instruments, Equipments and Accessories

All instruments, equipments and accessories used in the study are mentioned in **Table 3.5**.

Table 3.5: Instruments, Equipment and Accessories used in this study

S.No.	Instruments/Equipments	Source
1	Shaker Incubator with cooling and heating	Infors Multitron
2	Shaker incubator	Hicon
3	Colony counter	Scientific system
4	Vertical Laminar air flow	Scientific syetem
5	Horizontal Laminar air flow	UCS
6	Fluorescent and compound light microscope	Leica
7	Inverted microscope	Olympus
8	CO_2 Incubator	Shel l ab
9	Gel Documentation system	Alpha Innotech

10	Spectrophotometer (visible range)	Thermo electronic
11	4^0C freezers	LG
12	Microwave oven	BPL SANYO
13	Rocker	Neolab
14	Minispin	Eppendorf
15	Eppendorf centrifuge with freezing facility	Eppendorf
16	Centrifuges	Hitachi and Sorvall
17	Hot plates	Neolab
18	Water baths	Neolab
19	Incubators (37^0C)	Scientific system
20	-80^0C deep freezer	Thermo Forma
21	-20^0C refrigerators	Vestfrost and Bluestar
22		
23	Microarray machine	Applied biosystem
24	Eppendorf mastercycler PCR machine	Eppendorf
25	protein gel assembly not used in your case	BioRad
26	DNA gel assembly	Genei
27	pH meters	Scientific system
28	Weighing balance	Sortorius and Citizen
29	Hot air oven	Scientific system
30	Micropipettes (0.1 to 10, 5-50, 20-200 and 200-1000 µl)	Eppendorf
31	Easypet dispenser	Eppendorf
32	Multipett	Eppendorf
33	Combitips	Eppendorf
34	Vacuum pump	Scientific system
35	Pasteur pipettes	Scientific system
36	Serological pipettes (5 ml, 10 ml and 25 ml)	Cellstar

3.6. Database

Database used in this study are listed in **Table 3.6.**

Table 3.6: Database List

S.No.	Database	Website
1	Virulence Factor Database	http://www.mgc.ac.cn/VFs/main.htm

3.7. Collection and purification and Identification of GBS

GBS samples were provided in the form of already cultured bacterial colonies on blood agar plates. All the given details of patients such as sex, age, locality, anatomical site of isolation were noted. These plates were considered as master plates and stored at 4^0C over night. For isolation and purification of β-hemolytic streptococci, one β hemolytic colony was transferred and streaked on fresh blood agar plate with the help of disposable loops and incubated in 37°C temp overnight in incubator. The colonies were analyzed for further isolation and purification process. After every streak, the purity status was checked by observing gram stained slides. Purified culture was further studied.

3.8. Identification of GBS

3.8.1. Colony morphology

Streaked Blood agar plates containing pure β-hemolytic streptococcal colonies were examined for colony morphology; including shape, size, elevation margin and pigmentation. For better resolution of colony morphology, colonies were examined under stereoscopic microscope with zoom facility. A loop full bacterial culture was taken from pure culture plates and inoculated in THB and incubated in an metabolic shaker at 37°C for 2 to 2.5 h till OD_{650nm} reached 0.5-0.7. This culture was used for Gram staining to check purity, size, shape and arrangement of β-hemolytic colonies.

3.8.2. Gram Reaction

One loop full of log phase bacterial culture was put on clear micro slide and a smear was made by spreading the bacterial culture using the loop. Staining of the smear was made and heat fixed then the smear was stained with Crystal violet for 45 sec followed by Lugol's Iodine for 1 min then washed with 95 % Alcohol until the extra stain were removed from the slide after that stained with saffranine. After washing with distilled water slide was observed under oil immersion on a Compound light microscope

3.8.3. Catalase Test

This was performed in order to separate GBS from *S. aureus* and other β-hemolytic colonies. A drop of hydrogen peroxide was put on fresh pure culture taken from blood agar plates. Degradation of Hydrogen peroxide due to Presence of

Catalase enzyme in *S.aureus* was confirmed by release of O_2 in the form of bubbles. In case of GBS there was no evolution of bubbles as it lacks Catalase enzyme.

3.8.4. CAMP test

In laboratory GBS can be identified on the basis of CAMP test. CAMP is an acronym for the authors of this test (Christie, Atkinson, Munch, Peterson). The CAMP test takes advantage of the capacity of GBS to produce this CAMP factor; most other hemolytic streptococci do not produce CAMP factor. Inoculate Staphylococcus strain (Horizontal streak) and then inoculate the GBS (on left) and GAS (on right) perpendicular to Staphylococcus streak. Blood agar plates were inoculated in such a way so as not to touch two different organisms (Staphylococcus and Streptococcus) but to come close to each other. The Staphylococcus is used because it produces a lysin that only partially lyses the red blood cells (called beta-lysin). The CAMP factor reacts with the partially lysed area of the blood agar plate to enhance the hemolytic activity. A arrowhead shape zone of enhanced hemolytic activity by the GBS (on left) near the Staphylococcus streak differentiates it from GAS (on right). This means that the bacterium on the left is GBS because it is producing a CAMP factor.

3.9. STREPTEX IDENTIFICATION FOR GBS

'Streptex' identification antisera kit was used for identification of GBS isolates. This kit is used for qualitative detection and identification of the Lancefield group of streptococci. Reagents are provided for groups A, B, C, D, F and G covering the majority of streptococcal clinical isolates.

3.9.1. Principle

Majority of species of *Streptococcus* possesses group-specific antigens which are usually carbohydrate structural components of the cell wall. These antigens can be extracted in soluble form and identified by agglutination reactions with homologous antisera. Latex suspension and extraction were provided with the kit.

(a) Latex suspension

The polystyrene latex particles, which are coated with purified rabbit antibody to the appropriate group antigen, are suspended at a concentration of 0.5% in phosphate buffer pH 7.4 containing 0.1% sodium azide.

(b) Extraction enzyme

Freeze-dried proteolytic fraction obtained from *Streptomyces griseus* cultures containing calcium chloride. When reconstituted, the working strength solution contains 0.01% bronopol as preservative.

3.9.2. Identification

3-5 colonies were picked from pure culture and 400 µl of extraction enzyme was added in an Eppendorf. The light suspension was made of culture in enzyme sol^{n}. The suspension (by shaking) was incubated at 37°C in a water bath for 10 min or any time up to 1 hr (Eppendorf were vortexed after 5 min incubation). Resuspend the GBS latex vial by shaking vigorously for few seconds. From the vial 20µl was placed vertically on the reaction card. After this 40 µl of extract was put in one circle on reaction card. The contents were mixed in circle with a mixing stick and spread to cover the complete area of circle. The card was rocked gently for 1 min. Positive result showing agglutination reaction will confirm the GBS presence.

In this way, all GBS isolates were identified by using 'Streptex' identification kit. All identified GBS and other streptococcal isolates were preserved in deep freezer at -80^{0}C temperature by preparing 10% glycerol stock in the following manner

3.10. Preservation

GBS culture was preserved with 10 % glycerol. These glycerol stocks were preserved in deep freeze at –80°C in the following way.

A loop full GBS culture was taken from pure culture plates and inoculated in THB. It was incubated in an incubator shaker at 200 rpm and 37^{0}C temperature for 2 to 2.5 h until OD_{650} reaches 0.5 to 0.7 (log phase). 1.2 ml of log phase broth culture was mixed with 300 µl of 50 % autoclaved glycerol in Cryovials, labeled and was kept in the deep freeze at –80°C for further use.

3.11. Serotyping of GBS through latex agglutination test

All isolates identified as GBS positive were serotyped by using GBS typing antisera kit (Denka Seiken Kit, Japan). The typing antisera used in this study were Ia, Ib, II, III, IV, V, VI, VII and VIII. To do this few colonies (2-3) of GBS were incubated overnight (16-20 hr.) in 5 ml of THB at 29-30°C. Centrifuged at 3000 rpm for 5 min. and pellet obtained was resuspended for digestion in a mixture of THB (0.5 ml) + swine pancreatic extract (0.2ml) + pH indicator. The pH was adjusted to range from 8.0 to 8.5 and the mixture was incubated for 1 hour at 37° C for digestion. After digestion, the mixture was centrifuged and suspended in 0.5 ml of PBS. This suspension was further heated at 121° C for 30 minutes, and then agglutination tests were carried out on a glass slide with each of the nine antisera. Serotype was determined by a strong agglutination reaction within 1 minute. If there was no agglutination, the isolate was assigned as "nontypeable" (NT).

3.12. Growth pattern study

Frozen Glycerol stock of GBS was taken from -80°C. Then 200µl of the sample was inoculated in 5 ml of three tubes containing fresh THB media and incubated at 37°C in incubator shaker at 200 RPM till the O.D. reaches 0.5 at 650 nm. 200 µl from this culture was further inoculated into three tubes containing 5 ml fresh THB. 5 ml THB without GBS culture was used as a control. Tubes 1, 2 and 3 were used to take OD at 650nm after every 15 min time interval starting from 0 min till the steady phase is obtained. Then the growth curves were plotted by taking average OD_{650nm} against time.

3.13. CFU counts

Frozen Glycerol stock of GBS was taken from -80°C. 400 µl of the sample was inoculated in 5 ml of two fresh THB media in 15 ml tubes and incubated at 37°C in incubator shaker till the O.D. reaches 0.5 at 650 nm. 200 µl from this revived culture was inoculated into two 5 ml fresh THB media. Again the two tubes were incubated at 37°C in incubator shaker till the O.D becomes 0.5 at 650nm. Serial dilutions were made from 10^{-1} to 10^{-7} using autoclaved MQ water. 50 µl of the culture was taken from dilution 10^{-4}, 10^{-5}, and 10^{-6} and was plated on blood agar

plates in duplicates. The plates were incubated at 37°C over night and CFU count was taken. CFU was calculated according to the following formula.

CFU count per ml = Average CFU on blood agar plate X dilution factor x 20

3.14. Maintenance of Human cell lines

Human type II alveolar epithelial carcinoma cells (A549) ATCC CCl-185 and Human cervical epithelial cells (ME-180) ATCC HTB-33 were maintained at 37°C with 5 % CO_2 in Roswell Park Memorial Institute (RPMI)-1640 culture medium (HiMedia) with 10 % fetal bovine serum (FBS) (HiMedia) without antibiotics as described (Ruben et al., 1992, Purushothaman et al., 2004).

3.14.1. Sub culturing cells from frozen stock

Frozen stock of cell line (A549 and ME-180) from -80°C freezer were taken out and thawed for 1-2 minutes in the laminar hood by rubbing the Cryo tube. Cryo tubes were wiped with 70 % ethanol before opening. Cells were transferred to a 15 ml falcon tube and 10 ml of cell culture media (RPMI 1640 media with 10 % FBS) was added to wash cells. Cells were centrifuged at 500 rpm for 5 minutes. Supernatant was sucked. Pellet was suspended in 6 ml of cell culture media (RPMI 1640 media with 10% FBS). 3 ml of media with cells were transferred into each of the two 60 mm cell culture dishes (Cell star) and incubated at 37°C temperature in CO_2 incubator (5% CO_2) and were checked on next day. The humidity in CO_2 incubator was maintained up to 95 %. The cells divide and grow with time to reach 95% confluency (covering 95 % of surface area of culture dish) in 5-8 days (ME-180 cells take 4-5 days and A549 cells take 5-8 days). After that the cells were sub cultured in the following way.

3.14.2. Sub culturing cells from confluent monolayer (Splitting)

Media was sucked and cells were washed with 3 ml PBS (pH 7.4). After sucking the PBS, 200 µl of 0.25 % Trypsin-EDTA solution was added. Then it was incubated at 37°C for 5-10 min or until cells had detached. Once the cells were attached, 12 ml of RPMI with 10 % FBS was added and gently pipetted the cells in and out against the wall of the dish to disaggregate cells. It was repeated for 3 times. 3

ml each to 4 new 60 mm cell culture dishes were transferred and these were incubated in CO_2 incubator at 37°C and checked on the next day. Media was sucked and cells were washed with PBS (pH 7.4) once. 3 ml of RPMI with 10 % FBS was added to each dish and incubated in CO_2 incubator at 37^0C and were checked on next day under inverted microscope.

Old Media was discarded by suction pump and again 3ml of Fresh media (RPMI-1640 + 10 % FBS) was fed to cells after every 2 day to keep them growing healthy. Colour of the media was changed in culture dishes containing cells due to release of some chemical in the media by cells. To preserve the cells for further use, DMSO stocks were prepared and stored at -80°C temperature.

3.14.3. Freezing and preservation mammalian cells

Freezing media for freezing the cells was prepared by adding 10 % DMSO into RPMI with 10 % FBS. Media was sucked from the cell culture dishes. It was washed once with 3 ml PBS. 200 ul of 0.25 % trypsin-EDTA solution was added and incubated at 37°C for 4-7 minutes for trypsonization. Once detached, 3 ml of freezing media to each culture dish was added and gently mixed up and down and against the wall of culture dish to disaggregate cells. 1 ml to each cryo tube (total of 3 cryo tubes per dish) was transferred and each tube was labeled and frozen at -80°C.

3.14.4. Viability and cell count

Cell viability and cell count were determined by 0.4 % trypan blue (Sigma-Aldrich, USA) exclusion and hemocytometer counts as described (Johri et al 2007). Adherent cells were brought into suspension using 200 µl 0.25 % trypsin–EDTA and were resuspended in 800 µL of fresh RPMI-1640. For cells that grow in clumps centrifuged and were resuspended in a small volume and gently pipetted to break up clumps. Under sterile conditions 100 µl of cell suspension was removed and an equal volume of 0.4% trypan Blue (dilution factor =2) and mix by gentle pipetting. Using approx. 10 µl of this suspension chambers of hemocytometer were filled and under a compound light microscope using 20X magnification. Viable cells (seen as bright cells) were counted. Non-viable cells were stained blue and were not counted. The actual number of viable cell count was estimated using dilution factors.

3.15. Interaction assay of GBS serotypes Ia, III, V and VII with mammalian cell lines

To check the invasion efficiency of North Indian isolates of GBS (Type Ia, III, V and VII) with A549 and ME-180 cells invasion and adherence assays were performed as originally described by LaPenta et al., (1994) as mentioned below.

3.15.1. Invasion assay

GBS (Type Ia, III V and VII) were grown in THB at 37°C till OD_{650} reached up to 0.5. 1 ml of GBS culture (Type Ia, III V and VII) was centrifuged at 8000 rpm for 10 min. Supernatant was discarded carefully and pellet was washed twice in phosphate-buffered saline (PBS, pH 7.0). This pellet was resuspended in RPMI-1640 cell culture medium without FBS. GBS (Type Ia, III, V and VII) was inoculated onto A549 and ME-180 monolayers with a multiplicity of infection (MOI) of 1:1 (one bacteria for one mammalian cell) and allowed to incubate for 2 h at 37°C in 5% CO_2 in CO_2 incubator. During this incubation GBS gets attached and invades the cells. After 2 h incubation extracellular bacteria were removed by washing four times with PBS (pH 7.4). Then 200 µl of 0.25% trypsin-EDTA solution was added on monolayer of cells. These dishes were incubated at 37°C and 5% CO_2 for 5-7 minutes to detach the cells. Then 800 µl of 0.025% Triton X-100 in distilled H_20 was added to break the cells having invaded bacteria. The lysate was then transferred to 1.5 ml eppendorf tubes and vortexed gently for 1 min to disrupt streptococcal chains.

Aliquots of the lysates were quantitatively (50 µl) plated on BAP to determine the number of intracellular bacteria. For this, serial dilutions (10x, 50x, 100x and 500x) of lysates were prepared. Spread plating of 50 µl from each dilution on blood agar plates was done with the help of disposable sterile L-shaped spreaders. Spread plating was done in duplicate from each dilution. These blood agar plates were incubated at 37°C temperature for 18-20 h. The colonies were counted on next day. Experiments were performed in Quadruplicate every time (four cell culture plates were taken for one experiment). The total number of invaded GBS was counted in following way.

Total number of invading GBS = total colonies x dilution factor x 20

Percentage invasion efficiency was calculated by using following formula.

$$\% \text{ Invasion efficiency} = \frac{\text{Total invaded bacteria}}{\text{Total no of bacteria added}} \times 100$$

3.15.2. Adherence assay

During adherence assay after 2 hr. incubation the infected monolayers (without any antibiotic treatment) were then washed 5 times with PBS, detached from the dishes with 200 µl of Trypsin-EDTA, lysed with 800 µl of 0.025% Triton X-100, transferred quantitatively to microtubes, and vortexed vigorously for 1 min. Aliquots diluted in 0.025% Triton X-100 were plated on blood agar plates. The plates were incubated at 37°C for overnight, and GBS CFU were counted. The number of attached GBS was calculated as total (attached and invaded) CFU minus invaded CFU. Each test was done in quadruplicate, and the number of CFU recovered per well was determined.

$$\% \text{ Adherence efficiency} = \frac{\text{Total adhered bacteria}}{\text{Total no of bacteria added}} \times 100$$

3.16. Comparative genomics using DNA microarray

3.16.1. Selection of Virulence Factors genes

Total 140 Virulence factors genes were selected from the VFDB **(http://www.mgc.ac.cn/VFs/main.htm)** that were previously identified or predicted to be involved in virulence **Table 3.7.**

3.16.2. Oligo designing

Expression microarray analysis was performed with a custom made chip formulated on the virulence factor gene sequence of GBS type Ia, III and V. Gene list for putative virulence factor for GBS type Ia, III and V was downloaded from site Virulence Factor Database (VFDB) **(http://www.mgc.ac.cn/VFs/main.htm.).** Oligos were designed against respective gene by Ocimum Biosolutions (Hyderabad) **Table 3.8.** Oligos mentioned in green colored rows were used as productive control.

3.16.3. Isolation of RNA

This was done as described by Johri et al (2007) as follows. A 20 ml volume of GBS type Ia, III, V and VII was grown in THB media at 37^0C till the O.D. reaches 0.5. After getting the desired OD RNA protect bacterial reagent (40 ml; QIAGEN) was immediately added, and the sample was incubated for 5 min at room temperature. GBS cells were then pelleted by centrifugation at 7000 rpm for 15 min, supernatant was discarded. Pellet obtained was resuspended in 1 ml of lysozyme (30 mg/ml) in Tris-EDTA buffer and 2,000 U of mutanolysin, and the mixture was incubated for 15 min at 37°C. 3 ml RTL buffer (Qiagen) and 30 µl of β-mercaptoethanol was added and the contents was mixed and stored at −80°C. Total RNA was extracted from this lysate by using RNeasy spin columns as described in the manual (QIAGEN). RNA samples were on 1.5 % formaldehyde agarose gel to check the RNA integrity and purity **Fig 3.1**. We have found that RNA was pure and therefore further used for the microarray analysis.

Table 3.7: List of GBS virulence factors genes used for Comparative Microarray analysis					
S.No	Virulence factors	Related genes	*S. agalactiae* A909 (serotype Ia)	*S. agalactiae* NEM316 (serotype III)	*S. agalactiae* 2603V/R (serotype V)
			NC_007432 (2127839 bp)	NC_004368 (2211485 bp)	NC_004116 (2160267 bp)
1	Agglutinin receptor	-		gbs1356	SAG1283
2	Fibronectin Binding protein	pavA	SAK_1277	gbs1263	SAG1190
3			SAK_1142	gbs1087	SAG1052*
4			SAK_0955	gbs0850	SAG0832
5	Laminin-binding protein	Lmb	SAK_1319	gbs1307	SAG1234
6	Glucan Binding Protein	Grab			
7	Pilus Island1		SAK_0776	gbs0628	SAG0645
8			SAK_0777	gbs0629	SAG0646
9			SAK_0778	gbs0630	SAG0647
10			SAK_0779	gbs0631	SAG0648
11			SAK_0780	gbs0632	SAG0649
12	Pilus Island2			gbs1474	SAG1404
13				gbs1475	SAG1405
14				gbs1476	SAG1406
15				gbs1477	SAG1407
16		-		gbs1478	SAG1408
17	Streptococcal plasmin receptor/GAPDH	plr/gapA	SAK_1790	gbs1811	SAG1768
18	Hyaluronidase	hylB	SAK_1284	gbs1270	SAG1197
19	Streptococcal enolase	Eno	SAK_0713	gbs0608	SAG0628
20	Capsule	-	SAK_1247	gbs1233	SAG1158
21			SAK_1248	gbs1234	SAG1159
22			SAK_1249	gbs1235	SAG1160
23			SAK_1250	gbs1236	SAG1161
24			SAK_1251	gbs1237	SAG1162
25			SAK_1252	gbs1237.1	SAG1163

26			SAK_1253	gbs1238	
27					SAG1164
28			SAK_1254	gbs1239	
29					SAG1165
30			SAK_1255		
31					
32				gbs1240	
33					SAG1166
34					SAG1167
35					SAG1168
36			SAK_1256	gbs1241	SAG1169
37			SAK_1257	gbs1242	SAG1170
38			SAK_1258	gbs1243	SAG1171
39			SAK_1259	gbs1244	SAG1172
40			SAK_1260	gbs1245	SAG1173
41			SAK_1261	gbs1246	SAG1174
42			SAK_1262	gbs1247	SAG1175
43			SAK_1263	gbs1248	SAG1176
44	Alpha C protein	Bca	SAK_0517		
45	Alpha-like protein	alp2		gbs0470	
46	Beta C protein	Cba	SAK_0186		
47	Rib	Rib			SAG0433
48	Surface immunogenic protein	Sip	SAK_0065	gbs0031	SAG0032
49	Pneumococcal surface antigen A / Metal binding protein SloC	psaA	SAK_1556	gbs1589	SAG1533
50	C3-degrading protease	cppA	SAK_1738	gbs1775	SAG1730
51	C5a peptidase	scpA/scpB	SAK_1320	gbs1308	SAG1236*
52	Serine protease	htrA/degP	SAK_2135	gbs2133	SAG2174
53	Trigger factor	tig/ropA	SAK_0155	gbs0104	SAG0105
54	Beta-hemolysin/cytolysin	cylX	SAK_0790	gbs0644	SAG0662

55		cylD	SAK_0791	gbs0645	SAG0663
56		cylG	SAK_0792	gbs0646	SAG0664
57		acpC	SAK_0793	gbs0647	SAG0665
58		cylZ	SAK_0794	gbs0648	SAG0666
59		cylA	SAK_0795	gbs0649	SAG0667
60		cylB	SAK_0796	gbs0650	SAG0668
61		cylE	SAK_0797	gbs0651	SAG0669
62		cylF	SAK_0798	gbs0652	SAG0670
63		cylI	SAK_0799	gbs0653	SAG0671
64		cylJ	SAK_0800	gbs0654	SAG0672
65		cylK	SAK_0801	gbs0655	SAG0673
66	CAMP factor	cfa/cfb	SAK_1983	gbs2000	SAG2043

Table 3.8: List of Oligo designed for GBS virulence factor genes					
Row 384	Column 384	Gene name	Oligo name	Oligo sequence	Productive control
A	1	Agglutinin receptor	ObsCa031#0050	GTTTGATATGACGAAGACACAAGCGGAGAATAGCGACTATGACTTAACCT	
A	2	Agglutinin receptor	ObsCa031#0104	CAAACCCTGTATCTGAACAAACTGAAAGTATGGCATCAACTGGACAATCT	
A	3	Fibronectin Binding protein	ObsCa031#0001	TCACCTACCTTGAATCTGTTGAAACATCGCTAAATCATGCTTCTATGGAA	
A	4	Fibronectin Binding protein	ObsCa031#0106	CCTAAAACAGGTGATGATCAAAATGTCATTTTTAAACTTGTAGGTTTTGG	
A	5	Fibronectin Binding protein	ObsCa031#0053	AGAGAAGATACAGCTCGTCTTGAGAATATGATTTGGAATCGTGCTTACCA	
A	6	Laminin-binding protein	ObsCa031#0054	GACACTAGATAGAGTCAAAGGGCTAGAAGATATGGAAGTCACACAAGGCA	
A	7	Glucan binding protein	ObsCa031#0055	GTTCATACTGGTGGGAAACGATTTGTAAAGAAAGACTCAACAGAAACACA	
A	8		obsprodcntrl#033	CATATCAAGTGTTATGAGGGCAATTCGCAGCCATACTCAGATTTCGCCCG	Alien 2
A	9	Pilus island 1	ObsCa031#0004	CCACTACTAATCAACAAGGAAAGGCTACATTTAACCAACTACCAGATGGA	
A	10	Pilus island 1	ObsCa031#0005	TGAACACATCGGCGGAAAGATTGCTTATCAGGTAGACCAAATCAAAGTTA	
A	11	Pilus island 1	ObsCa031#0006	TTTGTGATGGGAAGTCTGATTCTCTTATTTCCGATTGTGAGCCAGGTAAG	
A	12	Pilus island 1	ObsCa031#0007	CTATTCGTGATTTCCCAATTCCCAAAATTCGTGATGTTCGTGAGTTTCCG	
A	13	Pilus island 2	ObsCa031#0060	ACTGGTTACCGTATATCTATGAAGGATGCTGTAGTTGCTGTAGTTGCTAA	
A	14	Pilus island 2	ObsCa031#0061	CTTATAATAGAACACTGGACCCAAGCCGCCTATCAGATCCCTATACTGAA	
A	15	Pilus island 2	ObsCa031#0062	ACACCCGATAACTTTTCAGATTTGTTGGTTGTTCCTGGACATGATTATGC	
A	16	Pilus island 2	ObsCa031#0063	GTAATAACCCAACGGAAGAAAGTGAACCACAAGAAGGTACTCCAGCTAAC	
A	17	Pilus island 2	ObsCa031#0117	AATACCAAAGATTCTGATAACCCAACACCATTGAACCCAACTGAACCAAA	
A	18	Pilus island 2	ObsCa031#0064	TTGTCGCAAATACCGCTTAATACCAATGTTTTAGGGGAAAGTACCGTACC	
A	19	Streptococcalplasmin receptor/GAPDH	ObsCa031#0009	GTTATCTCAGGTGCTTCATGTACTACAAACTGTCTTGCTCCAATGGCTAA	
A	20	Hyaluronidase	ObsCa031#0010	GCACTATTGATATTGAACGCAAAGAGCAAACAGGTACTTGGAACAGCATT	

A	21	Streptococcal enolase	ObsCa031#0011	AACCCAACACTTGAAGTAGAAGTTTATACAGAATCAGGTGCTTTCGGACG	
A	22	Capsule	ObsCa031#0012	AGGAAGCAATGGAGTATTATTCTTCACATGATGTTGACAATGTTGTAAGT	
A	23	Capsule	ObsCa031#0013	CGCTCATTGAACATCATACTGTTGTAGAATCACACTGTAATATAGCACCT	
A	24	Capsule	ObsCa031#0014	ACCAGTTGTGAATGTAGCGTTGATTTATAATGTCCCAGTATGCCACCTTC	
B	1	Capsule	ObsCa031#0015	TATTCGGATCATTCAATTGGATCAGAAGTACCTATCGCAGCAGCAGCAAT	
B	2	Capsule	ObsCa031#0016	ACGACATGGATTCCTATTGGGACATTATTAGCTGCTGGTGTAAATTGGTT	
B	3	Capsule	ObsCa031#0017	ACTATAACTCCAAAGTCAGTATTATTGTTGACACAGCCACTTGCACAAGA	
B	4	Capsule	ObsCa031#0018	TGGTGGGTTGTCAGAAGCTAGAAACTATGGAATTTATCATTCAAAGGGAA	
B	5	Capsule	ObsCa031#0128	AAGAGAGAATTATTTTCTACGCTGTGCTATCCAGTAGGGAAGTTACACGA	
B	6	Capsule	ObsCa031#0019	ATACCTATATACAACTCAGAAGCATACCTTAAAGAATGTGTGCAATCCGT	
B	7	Capsule	ObsCa031#0129	ACTTTAGAGGTGGCGTTGGAAGAAATGTATTATGGGAAAACTTATGGAGT	
B	8	[redacted]	obsprodcntrl#034	CAATCAGTTAGAAACAGTGGCTTGCGATATAAGCGTATCCACGCGGCACA	Alien 7
B	9	Capsule	ObsCa031#0020	AGGAGTTAAAGAGTTATGGTTAAATAGTGATCTACCTTTGGGGTCGCATT	
B	10	Capsule	ObsCa031#0076	TGCTGAAGCCAACTTTATTTGGAAGAGAATTGTTTTCAATAGAGTGGTTT	
B	11	Capsule	ObsCa031#0130	TGGCGGTAAGCATTCAGCCTATAATCTAGCAATGAGATATATGCAACCAA	
B	12	Capsule	ObsCa031#0131	TCTAGCAAGGGAGATTAGTTGTGATGTGAATACAGGATTAATAATTGGCG	
B	13	Capsule	ObsCa031#0132	ACTGGGTTTAGGCGAGGGAAACTCAGCTTACAAAATAGTGATGTTAGTTG	
B	14	Capsule	ObsCa031#0021	TTTTGTCACAGTGGGGACACATGAACAGCAGTTCAACCGTCTTATTAAAG	
B	15	Capsule	ObsCa031#0022	TTGGGTAACCTTTGATAAAGAAGATGCTAGGAGTATTCTAAGAGAAGAGA	
B	16	Capsule	ObsCa031#0023	TTTGGATATCACGGGTGCTATTATAGGTTTGCTCATATGTGGCATTGTGG	
B	17	Capsule	ObsCa031#0024	ATACTCCACCTATTGGTTTAGTTGTTGATGCCGCAATAATCGCTAATGCT	
B	18	Capsule	ObsCa031#0025	AGAGGATATCACTACTCTTGAGAAGGGAAATTTACCTAAAGCACCATCTT	
B	19	Capsule	ObsCa031#0026	ATACAGCTTTGTCAAATGTTTTAATGCTTGGTATTACACCAGTCGTTGCG	
B	20	Capsule	ObsCa031#0027	AGCATTACCTCACAGCTCCAAACGTCCGTTCAAACTAATATGACTATTGA	
B	21	Capsule	ObsCa031#0028	ACAACTCTACATTACCCAACCTTCACTTTCAAACGCTGTTAGGAACCTTG	
B	22	α C protein	ObsCa031#0029	GGGATGTTTCTCAGTTGCAGAGTACAGGAAGGGCTAGTCTTACCTATAAT	

B	23	α like protein	ObsCa031#0084	AAGACGGAATTGTTGAAGTTCACTATCCAGATGGTACTGTTGATGATGTG	
B	24	β C protein	ObsCa031#0030	ATTGTATAAGGCTATGAGTGAGAGCTTGGAGCAGGTTGAGAAGGAATTAA	
C	1	Rib	ObsCa031#0138	ATTATACAGTATTGAAGACAGATGGAAGTCCTCATACGAAGCCTGATGGA	
C	2	Surface immunogenic protein	ObsCa031#0031	AAGTTTCTCTCAATACAATTTCGGAAGGTATGACACCAGAAGCAGCAACA	
C	3	Pneumococcal surface antigen A/metal binding protein SloC	ObsCa031#0032	TTGCGGTTAGCGATGGAGTTGATGTTATTTATCTAAATGGTCAAAGCGGA	
C	4	C3 degrading protease	ObsCa031#0033	ATCGTACTCGTGCGGTGAATGGTACTAAGAAGTTAGCAAAGATTATTGTT	
C	5	C5a peptidase	ObsCa031#0034	TTCAGCAGCAACGATGTATGTGACAGATAAGGATAATACCTCAAGCAAGG	
C	6	Serine protease	ObsCa031#0035	ACTGATAAGGGCTTACAAGTTTACGGTGAAGGCTCTGGAGTCATCTATAA	
C	7	Trigger factor	ObsCa031#0036	TGACTTTGTAGGTTCAGTTGATGGTGTTGAATTTGATGGTGGTAAAGGAG	
C	8	β hemolysin/ cytolysin	ObsCa031#0037	CAACTGGTTGGAAATATTAGGTATTCTGAGTTTCTTACGGAAGGTGGTGT	
C	9	β hemolysin/ cytolysin	ObsCa031#0038	AGTGGACCAATCTCTTTAACTGGCGTGCGATTAAGGAAACTATCATAGAA	
C	10	β hemolysin/ cytolysin	ObsCa031#0039	TGGCGGTATTGATTGTTTAGTTAATAATGCTGGTATTGTTAGAGATGGCT	
D	1	β hemolysin/ cytolysin	ObsCa031#0040	AGACTAGCAGATTTTGTGATTGAAAATAGTGAGGATATAGATGACCAAGC	
D	2	β hemolysin/ cytolysin	ObsCa031#0041	ATTTTAGATGCACTTGATGGTAGCCTCCCCTCTCTTGATGAATTAACAGC	
D	3	β hemolysin/ cytolysin	ObsCa031#0042	ATTTATGGGATGCTATCGCACAACTAAATCAACAATCGCAGATGACAGTT	
D	4	β hemolysin/ cytolysin	ObsCa031#0043	TCTTAATGAAGGGTTCTATTGCGAAGGTATTTGACAAAGCCAACGAAGCC	
D	5	β hemolysin/ cytolysin	ObsCa031#0044	GAAGGTTATCTTGTGAATGTTCCAGGAAGTTACCCGATTGAGCATGATGA	
D	6	β hemolysin/ cytolysin	ObsCa031#0045	ATGAAATTACTGGTGAAGAATGTGAAGAAGCCTTAGACCTTGTGATTCCT	
D	7	β hemolysin/ cytolysin	ObsCa031#0046	AAGGTTTCGTTTGGAATGAGCGGAAGAAGGCAGTTAGTTCAGATTATGAT	
D	8	β hemolysin/ cytolysin	ObsCa031#0047	GACTGTACTGCTCAGCTTTGGTACTTCATTTAAACGAGATTGGGTGGAAT	
D	9	β hemolysin/ cytolysin	ObsCa031#0048	ACTCTAATTCTGGTGACTATCATATTCTAACCTATGCTCCCAGTGGTTCA	
D	10	CAMP factor	ObsCa031#0049	AATTCTATTGGTAGTCGTGTAGAAGCCTTAACAGATGTGATTGAAGCAAT	

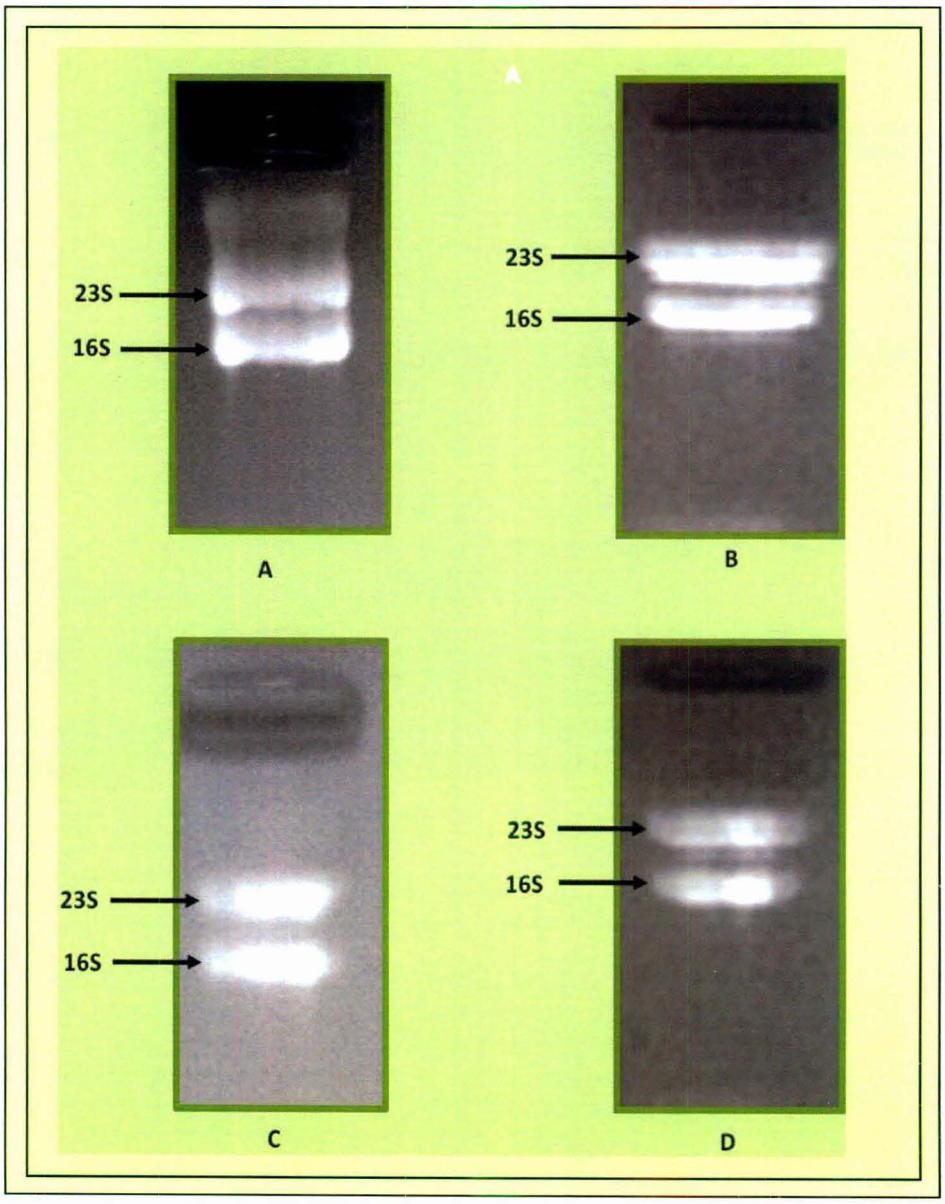

Fig 3.1: Total RNA isolated from GBS Serotypes. (A) Type Ia, (B) Type III, (C) Type V and (D) Type VII

3.16.4. Dnase treatment and Quality check

Total RNA was treated with Dnase (Rnase free) for 30 min at 37°C for removing contaminating genomic DNA followed by RNA purification using Rneasy kit (Qiagen) **Fig 3.2.** RNA concentration was checked by taking absorbance at 260 and 280 nm using NanoDrop (NanoDrop, USA) and quality of the RNA was checked using electrophoretic analysis with Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Palo Alto, CA). Total RNA with the quality standards was released for probe generation **Fig 3.3** and **Fig 3.4**.

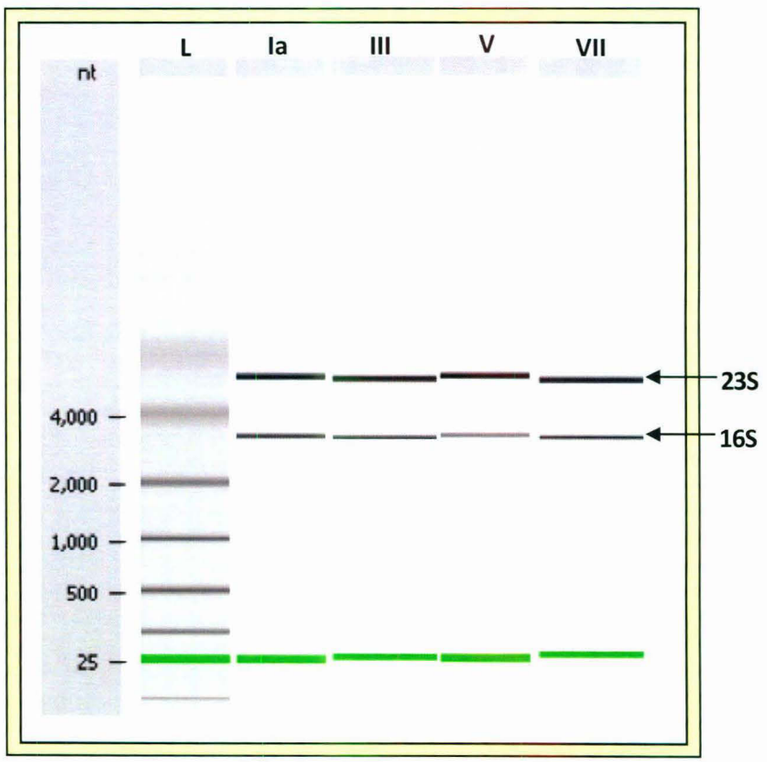

Fig 3.2: 100ng of total RNA of GBSIa, GBSIII, GBSV, GBSVII after DNase treatment. L is Ladder

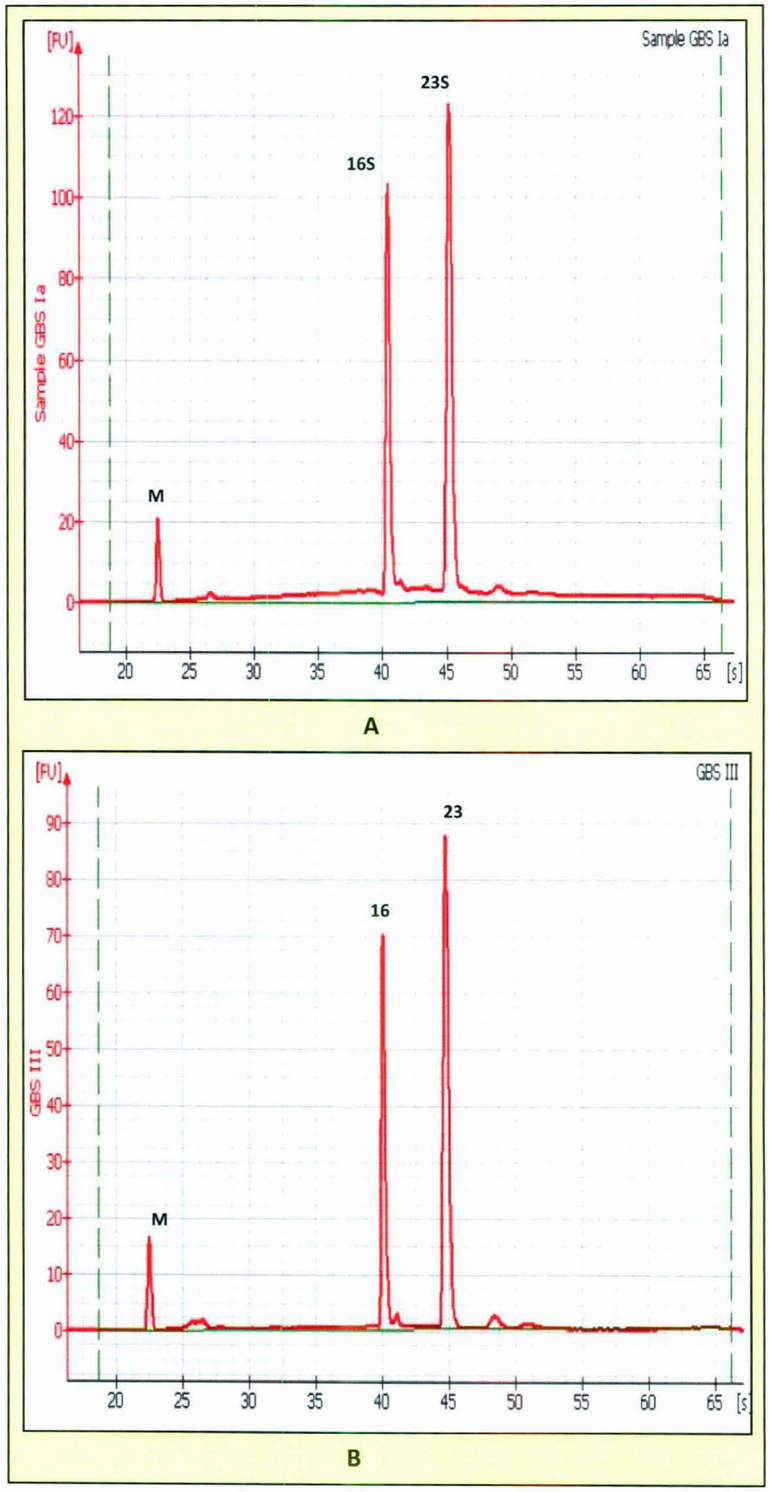

Fig 3.3: Electropherograms image of total RNA samples of GBS serotypes showing RNA free from DNA contamination. (A) GBS type Ia, (B) GBS type III

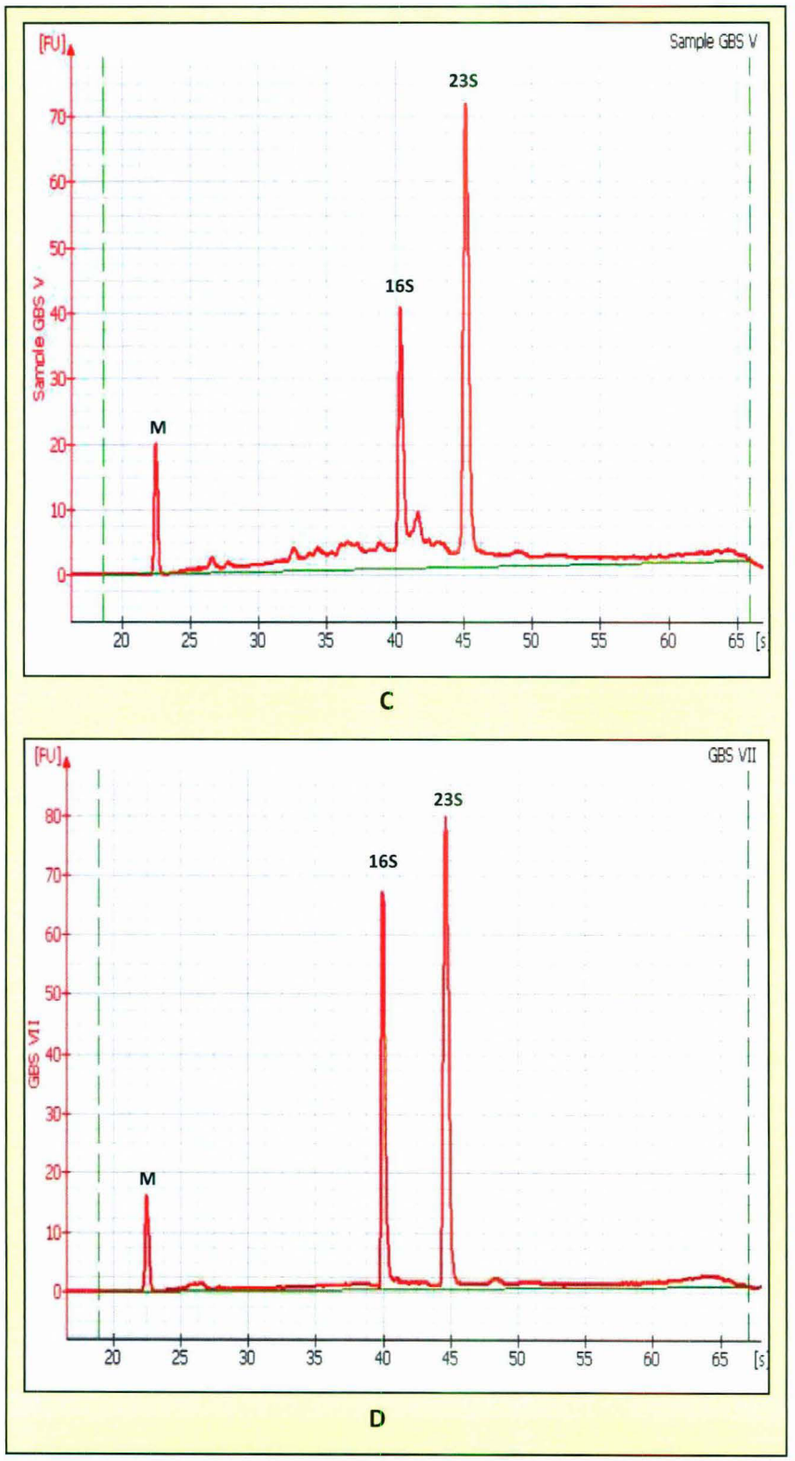

Fig. 3.4: Electropherograms image of total RNA samples of GBS serotypes showing RNA free from DNA contamination (C) GBS type V, (D) GBS type VII

3.16.5. Chip designing

68 oligos were synthesized at 50 nmol scale and were printed on Epoxysilane coated Nexterion® slide E from SCHOTT, Germany. **Fig 3.5.** showing the chip designing and the location of the oligos. Each probe was checked for its performance by standard QC process.

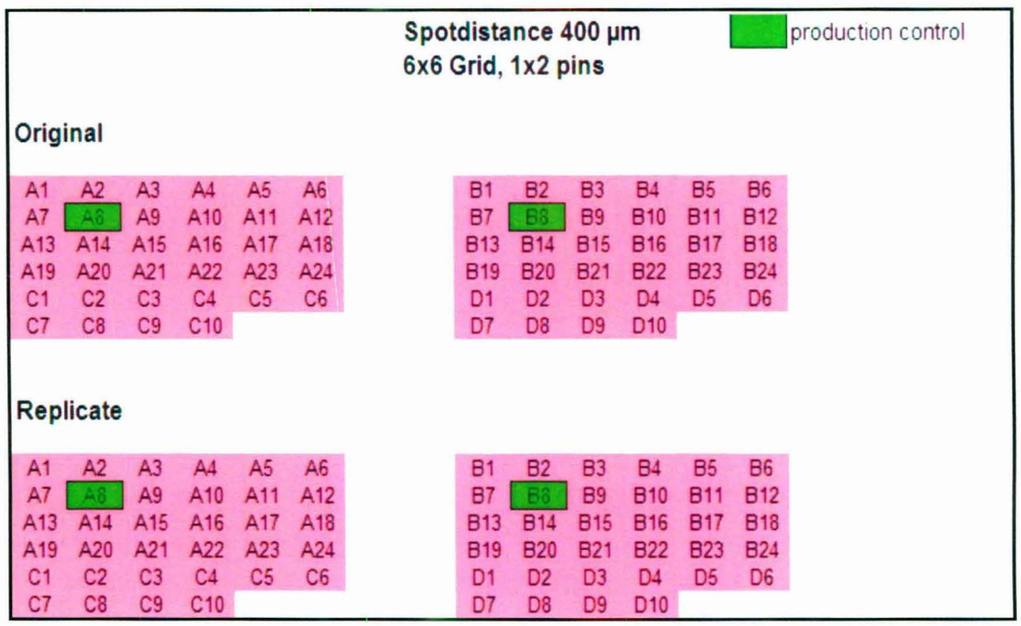

Fig 3.5: Cstom OciChip CA031_*S.agalactiae* Spot Localization

3.16.6. Probe generation

1µg of total RNA was used for amplification using Message AmpTMII-Bacteria, prokaryotic RNA amplification kit from Ambion by linear transcription based RNA amplification system to produce cRNA. Briefly, mRNA is polyadenylated using poly(A) polymerase(PAP) followed by reverse transcription primed with a oligo (dT) primer bearing T7 promoter and second strand cDNA synthesis. The resulting cDNA is then transcribed with T7 RNA polymerase to generate multiple copies of aminoallyl antisense RNA. aRNA is then labelled with Cy3 dye and unincorporated Cy3 molecules were removed by purification process.

3.16.7. Hybridization and Analysis

7.5 mg of the labeled aRNA in 75 ml of Ocimum's Hyb buffer was used for hybridization with the CA031 custom array chip for *S. agalactiae* designed and printed at Ocimum Biosolutions, Hyderabad. Hybridized chips were scanned using Affymetrix 428TMarray scanner at three different PMT gains and the images were analyzed using Genowiz software (Ocimum Biosolutions). Analysis was performed to detect differentially expressed genes among the groups (Fold change cut-off of 2). Signal value for each gene/probe was taken by averaging intensity among its replicate. Also, functional classification (Gene Ontology based) was carried out for differentially expressed genes.

3.16.8. Expression Analysis and experimental design

For each slide, the image analysis carried out for extracting the data from 40, 50 and 60 PMT settings. Each slide produced three datasets corresponding to each PMT settings. The data analysis involved three major steps viz. preprocessing, differential expression analysis and gene enrichment analysis. Each analysis has been described in details below:

The pre-processing was carried out for all samples of GBS Ia, GBS III, GBS V, and GBS VII. The main steps involved were filtration, transformation and normalization. The signal intensities were obtained by subtracting mean intensities from background corrected values for each PMT settings for each slide. The control probes, empty spots, and negative intensities were eliminated from each array leaving behind 130 probes per array. The replicated genes averaged out, which resulted into 65 probes per array.

Intensity values obtained from image analysis were log2 transformed and were normalized using quantile normalization technique present in Affy package of Bioconductor. Data before and after normalization is shown through box plots and density plots in **Fig. 3.6.** As the objective of the experiment was to find out differential gene regulation patterns in different serotypes of *S.agalactiae*, each serotype was compared with other three serotypes. In each comparison, genes with 1.5-fold difference (log fold change of 0.5849) were considered to be differentially expressed. The genes with log fold change value >= 0.5849 (FC >= 1.5) were declared as up-regulated while genes with log fold change <= -0.5849 (FC <= 0.66) were declared as down-regulated. The interest was to compare the expression levels

for selected virulent genes in GBS Ia, GBS III, GBS V, and GBSVII. The expression data was generated on 140 probes (genes), in which each probe was biologically replicated. The samples and experimental details are shown in **Table 3.9**. Each sample is to be compared with other three samples. As stated above, the primary interest of the study is to determine genes that are differentially regulated between samples. The differentially expressed genes further subjected to biological analysis to study their functional relevance.

In order to facilitate comparison between samples, and keeping in view the experimental layout, it was imperative to overcome the bias at chip level data due to variation from sample to sample. To reduce variation across samples the quantile normalization was performed on log transformed data across samples.

Table 3.9: Sample description and experimental design

S.No	Slide No.	Sample Type
1	SL6	GBS Ia
2	SL7	GBS III
3	SL8	GBS V
4	SL9	GBS VII

Table.3.10: The list of sample comparisons considered in the study

S.No	Comparison
1	GSB Ia vs GSB III
2	GSB Ia vs GSB V
3	GSB Ia vs GSB VII
4	GSB III vs GSB V
5	GSB III vs GSB VII
6	GSB V vs GSB VII

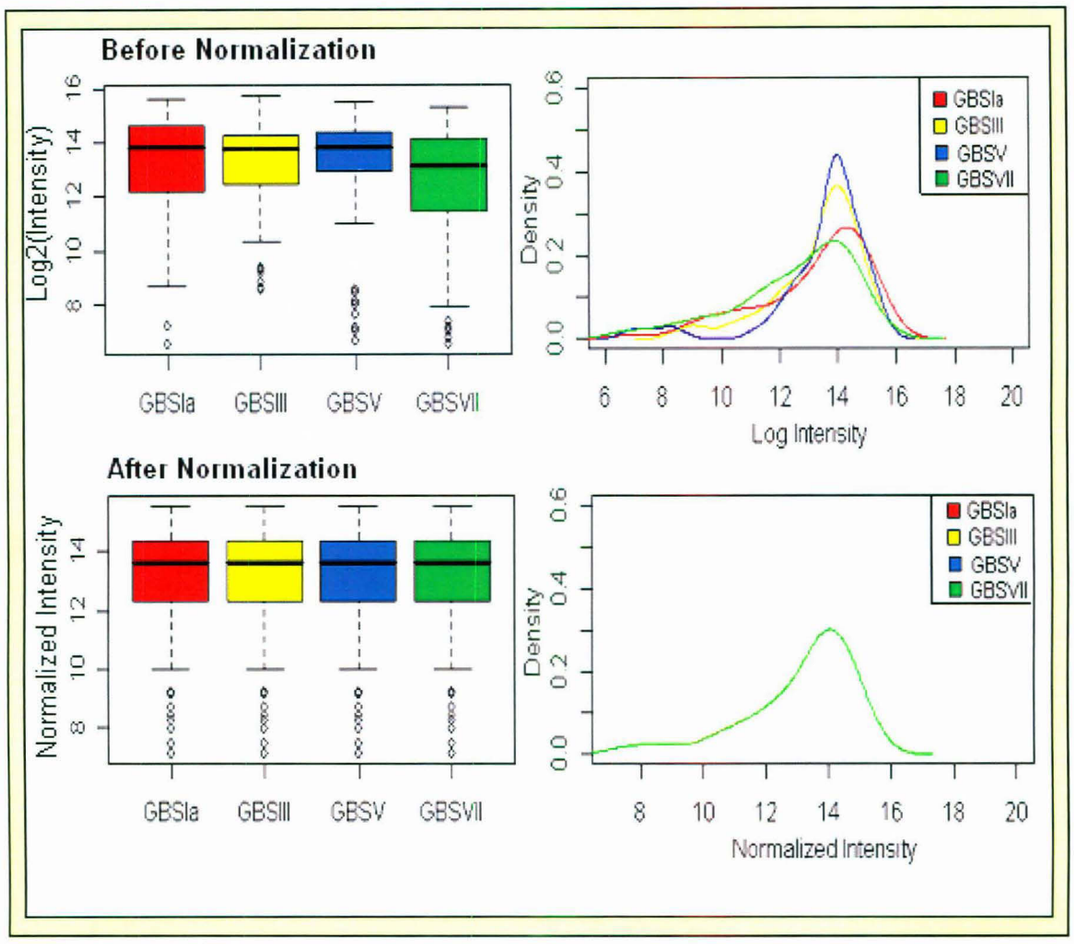

Fig 3.6: Boxplot and density plot view for all samples before and after normalization

3.16.9. Differential expression analysis and generation of heat map

Simple fold change technique was employed in order to detect differentially expressed genes by fixing the threshold. The genes with log fold change value >= 0.5849 (FC >= 1.5) were declared as up-regulated while genes with log fold change <= -0.5849 (FC <= 0.66) were declared as down-regulated. Each comparison carried out as shown in **Table 3.10**.

3.16.10. Heat Map and gene enrichment analysis

In order to generate heat map for all samples, the agglomerative hierarchical clustering method was employed to obtain similarity matrix for all samples by using Euclidean distance.

Heat map generated for differentially expressed genes as shown in colours. The color code ranges from the dark red (which has lowest value of intensity) to light yellow (which has highest value of intensity). Due to lack of annotated gene information, direct gene ontology and pathway information could not be obtained for Streptococcus agalactiae. Considerable time and efforts would be required build this knowledge base with current unigene database and corresponding ESTs.

Note: For fold change of genes that are up and down regulated as shown in heat maps please see corresponding tables and coding numbers e.g., Ca031#0009 has been shown in fig 4.17 heat map and corresponding fold change has been shown in table 4.5 and same pattern has been followed in all cases). In case of heat maps the color code ranges from the dark red (which has lowest value of intensity) to light yellow, white (which have highest value of intensity).

Results

4.1. Sample Collection

Total 250 GBS samples were collected from different pathological laboratories and hospitals of Delhi and surrounding areas. In order to narrow down the GBS identification colony morphology, gram staining, catalase test and CAMP test were used for screening of samples. Out of total samples 30 samples (12 %) were identified as GBS. Details of all serotypes of GBS positive samples shown in **Table 4.1**.

4.2. Identification of GBS

4.2.1. Colony Morphology and β-hemolytic pattern

All 250 samples were analyzed for β-hemolytic pattern and colony morphology on Blood agar plates (BAP). Due to hemolysis on BAP clear zone lysis was observed around the colonies as shown by arrow in **Fig 4.1**. Most of the bacterial colonies were smooth and glistening surface with light to cream yellow in color. Among these 250 samples analyzed we have observed that 64 samples showed β-hemolysis. These 64 samples further analysed for identification.

4.2.2. Gram Staining

Samples showing β- hemolysis were checked for purity of the culture and identification as gram positive. Typical purple colored cocci cells arranged in long chain, a characteristic feature of streptococci, were observed as shown in **Fig 4.2.** All 64 samples showing β-hemolytic pattern were identified as gram positive.

4.2.3. CAMP test

Samples were again analyzed for identification for the presence of GBS by CAMP test (Characteristic feature of GBS). GBS positive samples shows zone of enhanced hemolysis (positive) as shown by green arrow in **Fig 4.3**. Among 64 samples 30 samples were identified as positive for CAMP test when tested against *S.aureus*. This test was also used to differentiate between group B and group A streptococcus as the CAMP factor produced by GBS in association with the lytic factor produced by *S.aureus* causes increased lytic pattern.

4.2.4. Catalase Test

GBS is Catalase negative and when tested against *S. aureus* in presence of H_2O_2 (Hydrogen peroxide), it does does not produce oxygen in the form of bubble as shown by arrow (**Fig 4.3**) however formation of bubble was observed in case of *S. aureus*.

4.2.5. Identification of GBS by Streptex antisera kit

Further final confirmation of GBS positive samples identification was done by using Streptex identification antisera kit. All positive samples screened after colony morphology, gram staining, Catalase test and CAMP test were identified as strong agglutination appears due to the reaction of extracted group B carbohydrate antigens (structural component of the cell wall of bacterial isolate) with respective antisera provided with the kit. We observed that GBS culture shows formation of strong agglutination after 2-3. All samples showing agglutination with GBS antisera were Identified as GBS positive (**Fig 4.5**)

As a result among 250 samples collected, 30 samples were identified as GBS positive contributing to 12 % of the total sample collection.

4.3. Serotyping of GBS using typing antisera kit

The serotype distributions of GBS have been reported to have changed over the period of time. In order to figure out the serotypes distribution pattern of different GBS serotypes, serotyping was carried out by using Denka Seiken Kit (Japan). Data from this study indicates that among the total collected samples i.e., 250 samples, 12 % were found to be GBS positive. Among the GBS positive samples 66.6 % were isolated from urine 6.6 % were isolated from vagina. However for 30 % GBS positive samples origin were not provided.

GBS was isolated more frequently from urine (20 positive cultures) than vaginal swabs (2 positive). Some of the samples were not assigned any serotype and designated as nontypable. We found that in all the GBS positive samples Type Ia and III followed by II were found highest i.e., 23, 16 % and 16 % respectively. Amongst

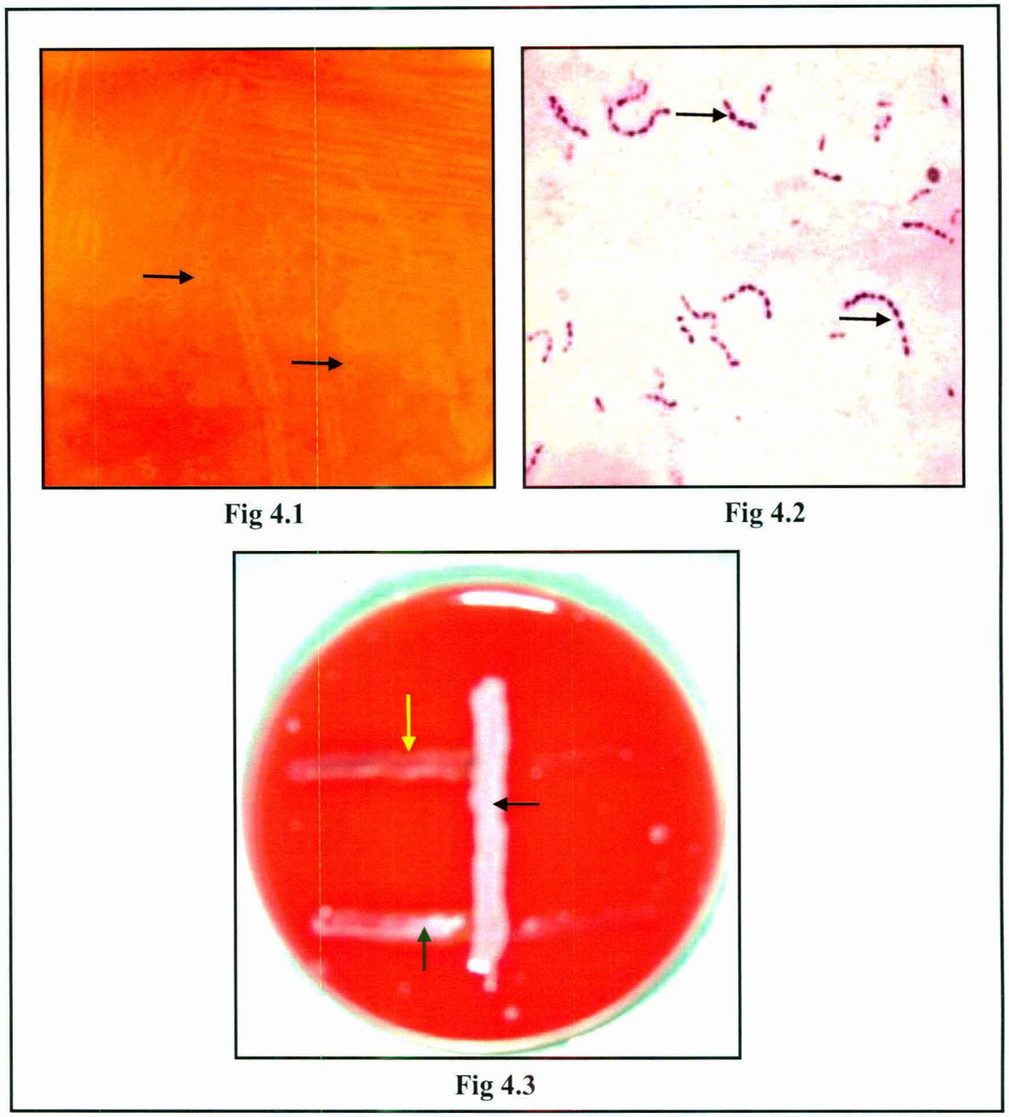

Fig 4.1

Fig 4.2

Fig 4.3

Fig 4.1: Colonies of GBS on Blood agar plates (BAP). β-hemolytic zones around the colonies are represented by black arrows.

Fig 4.2: Gram staining of GBS showing purple colored cells arranged in chain as shown by black arrows.

Fig 4.3: Green arrow showing CAMP positive GBS isolates streaked at right angle to the test organism *S. aureus* (Black arrow). Enhanced lysis can be seen in GBS as shown by green arrow. GAS was used as negative control.

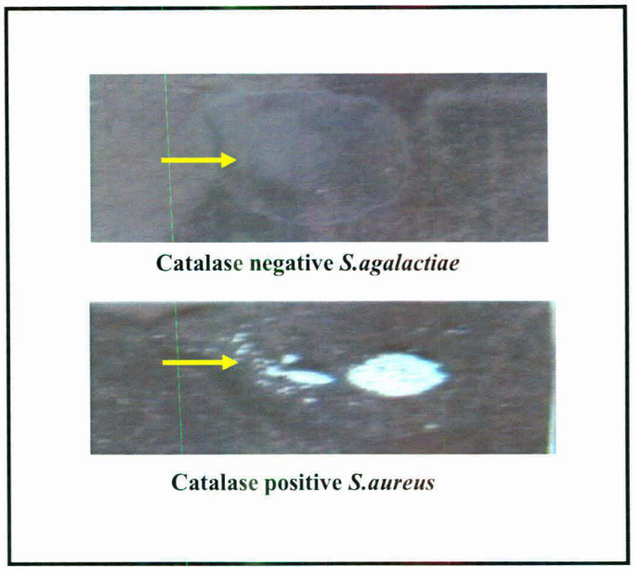

Fig 4.4: In *S. aureus*, release of bubbles as shown by arrow confirms it catalase positive. In GBS, there is no release of bubbles as indicated by arrow confirms it catalase negative

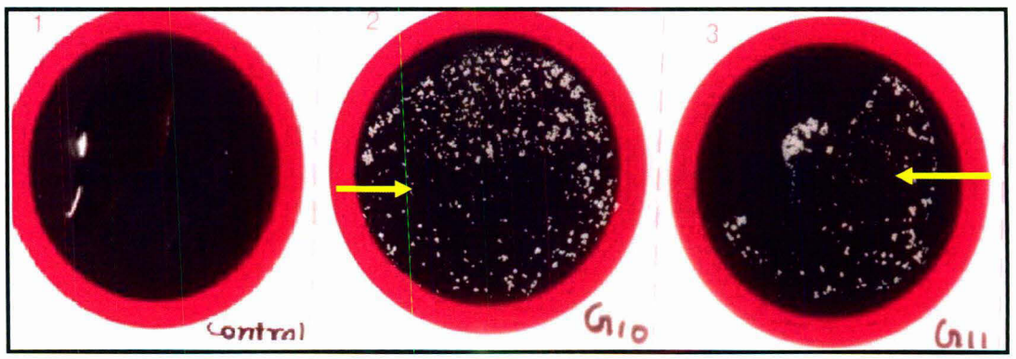

Fig 4.5: Identification of GBS using Streptex Identification kit. (1) Negative Control is GAS. (2) Sample number G10 identified as GBS positive. (3) Positive control. Arrows shows the formation of strong agglutination in GBS positive samples.

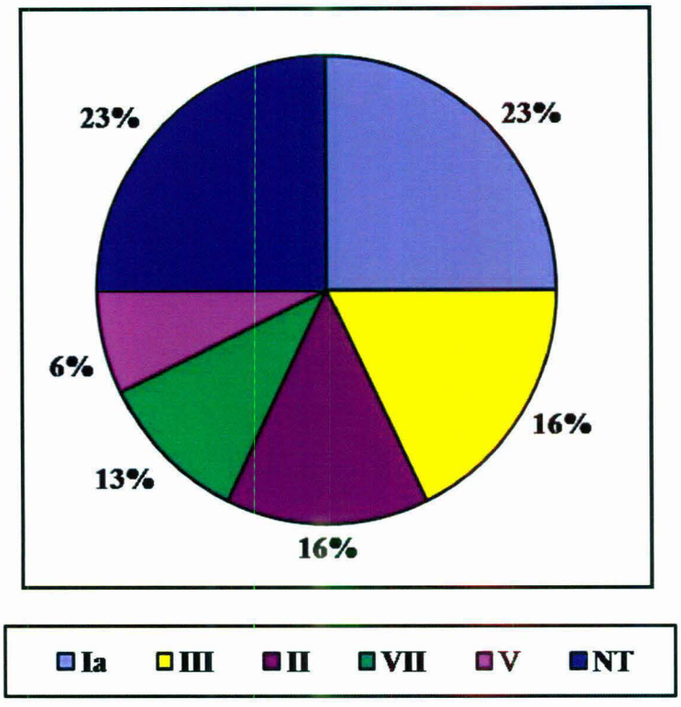

Fig 4.6: Distribution pattern of Serotypes identified among the 30 GBS isolates. Serotype Ia was the most prevalent serotype followed by Type III.

Table 4.1: Details of GBS positive samples			
Site of Sample collection	**Serotype Distribution**		**Total samples (GBS +)**
Urine (n=20)	**15 females**	**3 (NT), 3(VII), 1 (II), 3 (III), 4 (Ia), 1 (V)**	**30 (No details were provided for 9 GBS positive samples)**
	5 males	**3(NT), 1 (Ia), 1 (II)**	
Semen (n=1)	**1 male**	**1 (III)**	
Pus (n=1)	**1 baby**	**-**	
Vaginal swab (n=2)	**2 females**	**-**	

Abbreviation: NT, Nontypable.
Samples isolated from vaginal swabs and pus were identified as Group G and Group F respectively. One Sample was isolated from male semen designate GBS as type III.

Table 4.2: Details of GBS samples Serotyping					
S.No	Sample No.	Site of sample collection	Sex	Age	Serotype
1	G1	-		-	NT
2	G5	Urine	F	24	NT
3	G6	Urine	M	45	NT
4	G7	Urine	F	47	NT
5	G15	Urine	M	68	NT
6	G23	Urine	F	23	NT
7	G28	Urine	M	26	NT
8	G3	-	-	-	Ia
9	G11	-	F	44	Ia
10	G14	Urine	M	-	Ia
11	G18	Urine	F	70	Ia
12	G24	Urine	F	83	Ia
13	G26	Urine	F	51	Ia
14	G29	Urine	F	13	Ia
15	G2	-	-	-	II
16	G4	-	-	-	II
17	G9	Urine	F	54	II
18	G27	Urine	M	56	II
19	G35	-	-	-	II
20	G10	-	-	-	III
21	G13	Semen	-	-	III
22	G16	Urine	-	-	III
23	G17	Urine	-	-	III
24	G30	Urine	-	-	III
25	G12	-	F	50	V
26	G32	Urine	F	24	V
27	G8	-	-	-	VII
28	G25	-	-	-	VII
29	G31	-	-	-	VII
30	G36	-	-	-	VII

4.4. Growth pattern

Growth pattern of GBS type Ia, III, V and VII was studied at optimum temperature i.e. 37°C with shaking at 200 rpm. By analyzing growth pattern, we can determine the time lapse taken by the bacteria to reach 0.5 OD_{650} (log phase) **Fig. 4.12**. Growth curve of all four GBS serotypes shows same pattern for lag, log and stationary phases and this growth of serotype has no correlation which serotypes it belongs. In case of type V and VII time taken to attain log phase is less as compared to time taken by type Ia and III. In exponential phase growth of GBS type V and VII faster as compared to growth of type Ia and III. Around 5 h, we have observed that in all four cases decline phase has started. CFU for GBS Ia, III, V and VII was calculated at OD_{650nm} 0.5. $8x10^6$, $8x10^6$, $8x10^7$ and $2.3x10^8$.

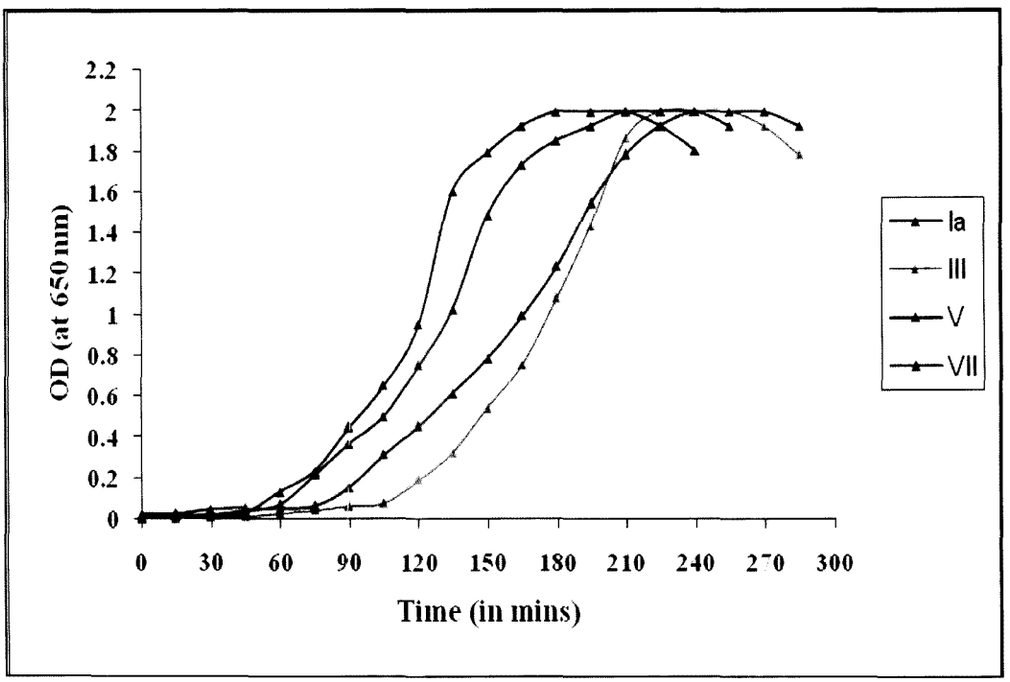

Fig 4.12: Growth pattern of GBS type Ia, III, V and VII

4.5. Interaction of GBS with human cell lines A549 and ME-180

In epidemiological part of this study we found GBS type Ia most prevalent followed by type III, V and VII. These serotypes were further selected to compare the invasiveness by interacting with A549 and ME-180 cell lines. To start with invasion assays, growth pattern of all these selected serotypes were standardized at OD_{650nm} of 0.5, i.e., exponential phases as mentioned above.

4.6.1. Maintenance of Cell lines

Both the human cells were maintained 37°C in 5 % CO_2 incubator in healthy condition. The viability assay was done by using trypan blue exclusion method and cells were counted using hemocytometer as mentioned in the materials and method. The count of A549 cells (confluency ~ 95%) per dish (60 mm) was 4.5 x 10^6 and for ME-180 cells (confluency ~95 %), it was 2.1 x 10^6 per dish (60 mm). A549 cells required 6 days to achieve ~95 % confluency while ME-180 cells required 5 days to reach up to ~95 % confluency Both the cell lines were spindle shaped in morphology **Fig 4.13 (A, B)**.

4.6.2. Adherence and invasion

The adherence and invasion of the human epithelial cell lines A549 and ME-180 by prevalent GBS serotypes Ia and III were compared with those of less prevalent serotype V and VII by a conventional invasion assay (Johri et al, 2003). With the A549 cells a maximum of 2 and minimum 0.08 % invasion was found by type Ia and type V respectively (**Fig. 4.14**). However with ME-180 cells maximum invasion shown by type Ia and III i.e. 2 % and 1.8 % respectively followed by type V i.e. 1.6 % **(Fig. 4.14)**. A minimum of 0.07 % invasion has been found by type VII with ME-180 cells as compared to other serotypes. Type Ia also shown highest adherence with A549 cells i.e., 2.3 % as compared to other serotypes, however minimum adherence has been shown by type VII with ME-180 cells i.e., 0.5 % **(Fig 4.15).**

(A) (B)

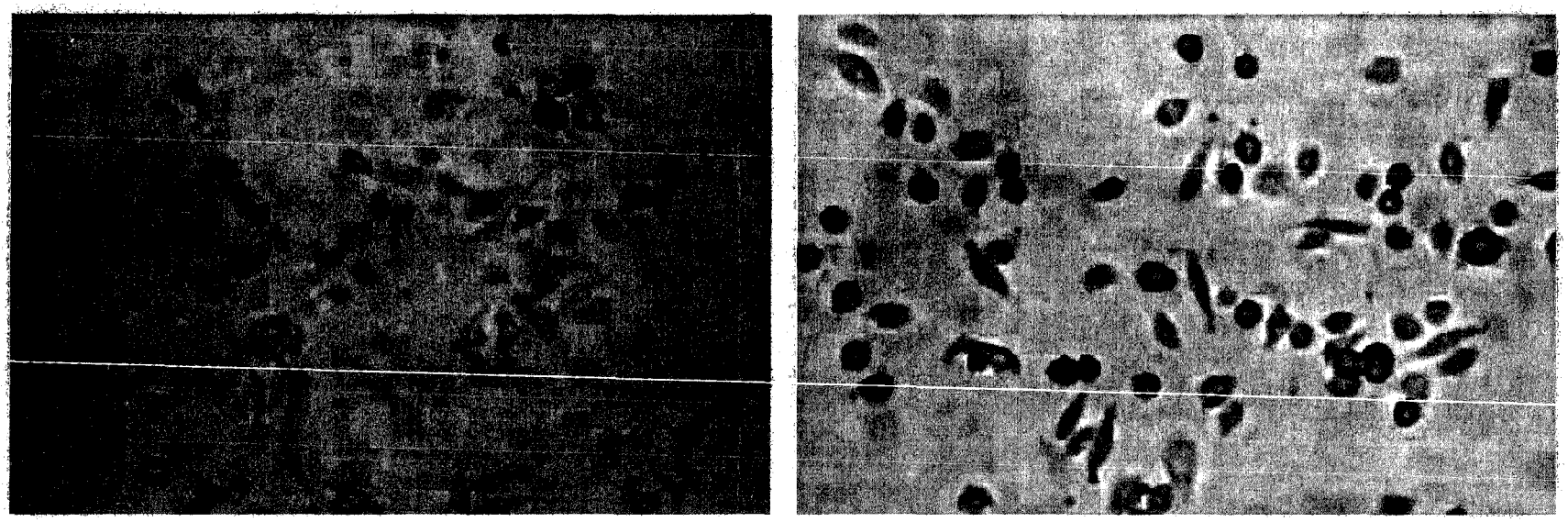

Fig 4.13: Inverted microscope picture of (A) Human lung alveolar carcinoma epithelial cell line type II pneumocytes; ATCC CCl-185 (A549) (B) Human cervix epithelial cell line ATCC HTB-33 (ME-180). (20x).

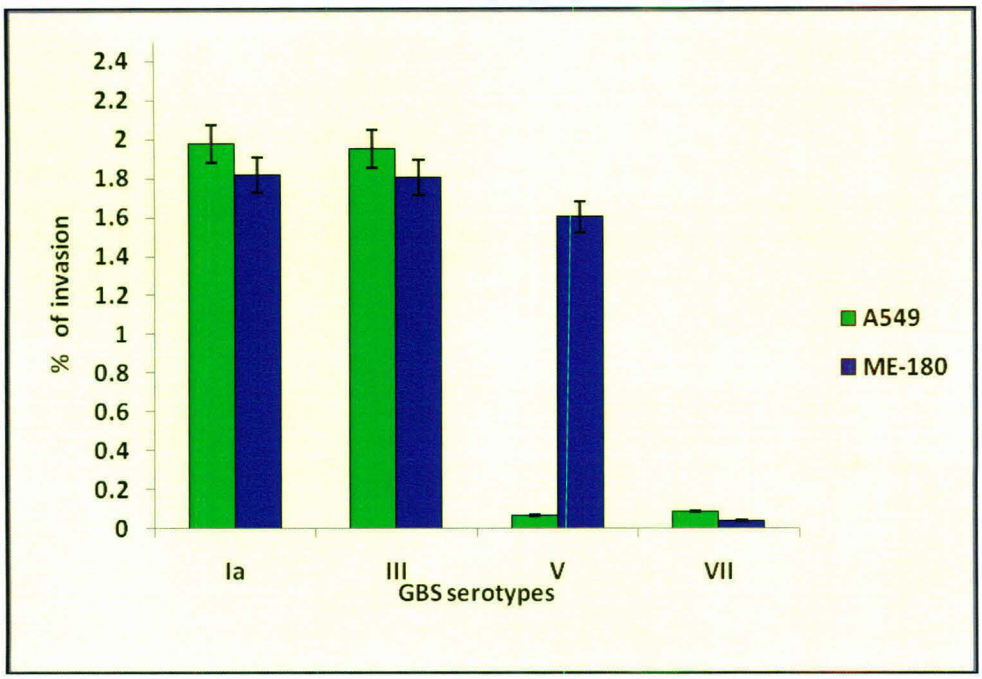

Fig 4.14: Invasion of GBS type Ia, III, V and VII with A549 and ME-180 cell lines. Error bars indicate standard deviations.

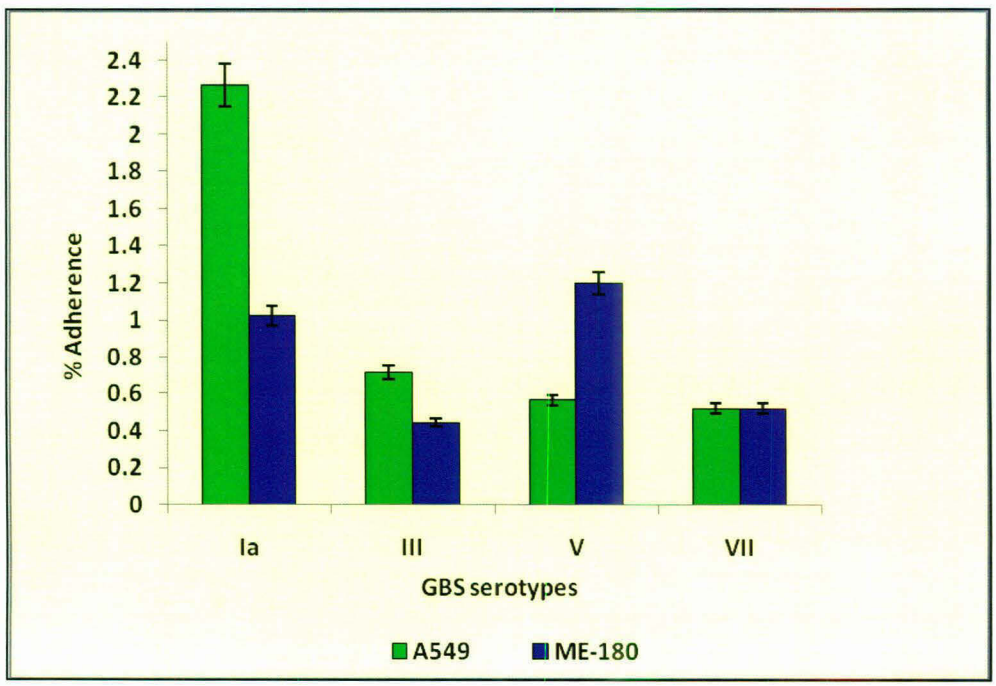

Fig 4.15: Adherence of GBS type Ia, III, V and VII with A549 and ME-180 cell lines. Error bars indicate standard deviations

4.6. Comparative transcriptional profiling of GBS type Ia, III, V and VII

As mentioned before for this study highly invasive GBS types Ia, III and less invasive VII and V were selected to compare the differential virulence gene(s) expression profiling between invasive and non/less invasive GBS types to find out the up-regulation of genes related to virulence.

Note: For fold change of genes that are up and down regulated as shown in heat maps please see corresponding tables and coding numbers e.g., Ca031#0009 has been shown in fig 4.17 heat map and corresponding fold change has been shown in table 4.5 and same pattern has been followed in all cases). In case of heat maps the color code ranges from the dark red (which has lowest value of intensity) to light yellow, white (which have highest value of intensity).

4.6.1. GBS Ia vs GBS III

In GBS III total 14 genes were found up-regulated and 23 were found down regulated as compared to type 1a. **Table 4.5 and 4.6** shows the up-regulated and downregulated genes in GBS type III as compared to GBS type Ia. The differentially expressed genes are shown through bivariate scatter plot in **Fig 4.16**. The up and down regulated probes are highlighted in blue points and the cut-off value (0.58) is shown in red lines. Heat map of GBS Ia vs GBS III is shown in **Fig. 4.17** and corresponding genes are shown in tables.

Level of transcripts of four genes gbs1474, gbs1475, gbs1476, gbs1478 encoding for Pilus islands (PI-2) were found 11-22 fold up-regulated. Agglutinin receptor and glucan binding protein (gpbB) predicted to be involved in adherence were found 30.7 and 37.4 fold up-regulated. Maximum up-regulation was found for cpsI (involved in cell wall synthesis) i.e. 55 folds as compared to 1a **(Table 4.5).**

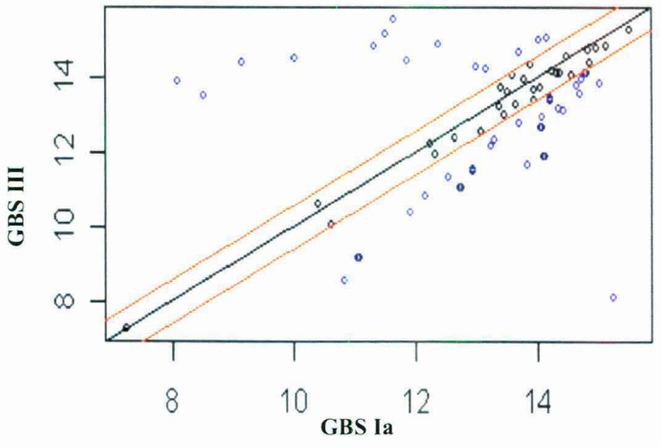

Fig 4.16: Bivariate scatter plot for GBS Ia and GBS III

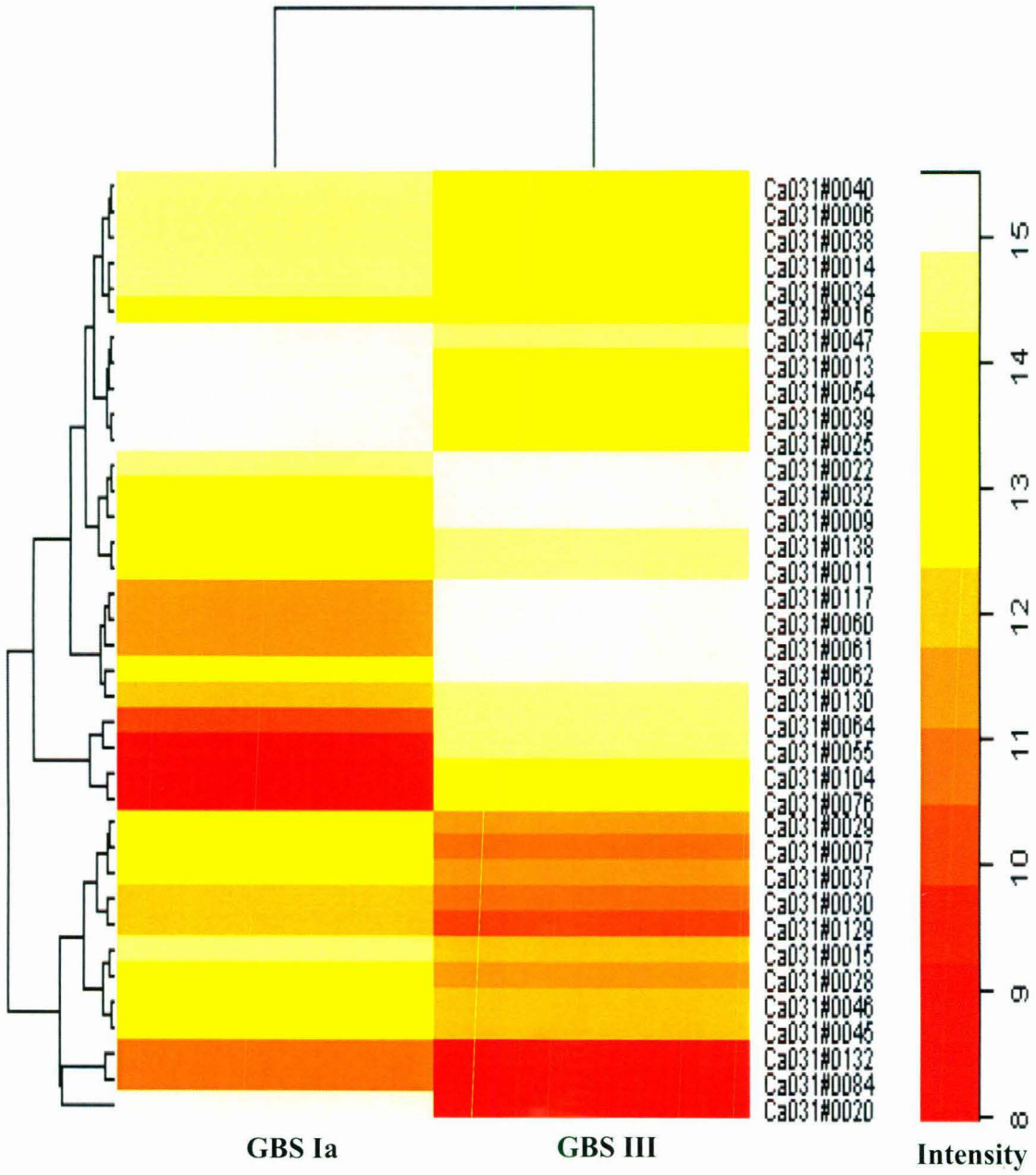

Fig 4.17: Heatmap for samples GBS Ia and GBSIII. Each Oligo number represents a particular gene

Table 4.5: Gene up-regulated in GBS type III compared to GBS type Ia (n=14)

S. No	locus name on Chip	Gene locus	Gene product name	Gene	Fold change	Function
1	Ca031#0009	gbs1811	Streptococcal plasmin receptor/GAPDH	plr/gapA	1.9	GAPDH, glycolytic enzyme used for bacterial energy generation, bind several host proteins, confer resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006)
2	Ca031#0011	gbs0608	Streptococcal enolase	eno	2.3	Glycolytic enzyme α-enolase as a plasmin binding protein on the outside of the bacterial cell (Pancholi and Fischetti, 1998)
3	Ca031#0022	gbs1242	Capsule	cpsF	1.8	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
4	Ca031#0032	gbs1589	Pneumococcal surface antigen A / Metal binding protein SloC	psaA	1.9	Essential virulence factor, a metal (Mn^{2+} and Zn^{2}) Binding protein (ABC-type), Potential drug target and a candidate vaccine component
5	Ca031#0055		Glucan binding protein	gbpC/gbpD /grab	37.4	GbpB may be involved in cell wall synthesis (Mattos-Graner et al, 2006)
6	Ca031#0060	gbs1474	Pilus island 2	-	11.4	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation (Lauer et al, 2005)
7	Ca031#0061	gbs1475	Pilus island 2	-	14.7	do
8	Ca031#0062	gbs1476	Pilus island 2	-	5.6	do
9	Ca031#0064	gbs1478	Pilus island 2	-	22.2	do
10	Ca031#0076	gbs1240	Capsule	cpsI	55.5	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
11	Ca031#0104	-	agglutinin receptor	-	30.7	Mediates specific adhesion and aggregation (Prakobphol et al, 2000)
12	Ca031#0117	-	Pilus island 2	-	12.3	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005)
13	Ca031#0130	-	Capsule	-	5.9	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
14	Ca031#0138	-	Rib	-	2.0	GBS surface protein involved in adhesion (Larsson et al, 2004)

Table 4.6: Gene downregulated in GBS type III compared to GBS type Ia (n=23)

S.No	locus name	Gene locus	Gene product name	Gene name	Fold change	Functions
1	Ca031#0006	gbs0631	Pilus island 1	-	0.3	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0007	gbs0632	Pilus island 1	-	0.3	do
3	Ca031#0013	gbs1234	capsule	neuD	0.5	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
4	Ca031#0014	gbs1235	capsule	neuC,	0.4	do
5	Ca031#0015	gbs1236	capsule	neuB	0.2	do
6	Ca031#0016	gbs1237	capsule	cpsM	0.5	do
7		-	capsule		0.00	do
8	Ca031#0025	gbs1245	capsule	cpsC	0.4	do
9	Ca031#0028	gbs1248	capsule	-	0.2	do
10		-	alpha C protein	bca	0.4	An important virulence factor, plays role in interaction with epithelial surfaces and initiation of infection (Bolduc et al, 2002; Baron et al, 2004).
11	Ca031#0030	-	beta C protein	cba	0.3	The beta protein interacts with two components of the human immune system, IgA-Fc and factor H (FH), suggesting that it plays a role in immune (Lindahl, 2005)
12	Ca031#0034	gbs1308	C5a peptidase	scpA/scpB	0.3	Serine protease that inactivates human C5a(Bohnsack et al, 1991)
13	Ca031#0037	gbs0644	β hemolysin	cylX	0.3	Pore-forming exotoxin (Hensler et al, 2008)
14	Ca031#0038	gbs0645	β hemolysin	cylD	0.5	do
15	Ca031#0039	gbs0646	β hemolysin	cylG	0.5	do
16	Ca031#0040	gbs0647	β hemolysin	acpC	0.4	do
17	Ca031#0045	gbs0652	β hemolysin	cylF	0.4	do
18	Ca031#0046	gbs0653	β hemolysin	cylI	0.5	do
19	Ca031#0047	gbs0654	β hemolysin	cylJ	0.6	do
20	Ca031#0054	gbs1307	Laminin-binding protein	lmb	0.4	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)

21	Ca031#0084	gbs0470	α like proteins	alp2	0.2	surface-anchored proteins, inducers of protective antibodies (Maeland et al, 2004; Lachenauer et al, 2000
22	Ca031#0129	-	capsule	-	0.3	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
23	Ca031#0132	-	capsule	-	0.2	do

4.6.2. GBS Ia vs GBS V

Similarly, GBS Ia vs GBS VII comparison was carried out. Table **4.7** and **4.8** shows the up and down regulated genes in GBS type V as compared to GBS type Ia. In **Fig 4.18** the up and down regulated genes are highlighted in blue points and cut-off value (0.58) is shown in red lines. Heat map of GBS Ia vs GBS V is shown in **Fig. 4.19.**

Microarray analysis of GBS V compared with GBS Ia reveals total 15 genes were up-regulated. Transcript level of three genes encoding proteins involved in binding i.e. SAG1405, SAG1406, SAG1407, SAG1408 encoding pilus island (PI-2) was found 21.4 fold up-regulated, SAG1164, SAG1165, SAG1166, SAG1167, SAG1168 encoding capsule was found 71.6 fold up-regulated and gbpB encoding glucan binding protein homologous to peptidoglycan hydrolase from *S. pneumonia* was found 23.2 fold up-regulated as compared to GBS Ia **Table 4.7**. A total of 26 genes were found down regulated as compared to1a **(Table 4.8).** Amongst genes encodes for capsule biosynthesis were found down regulated.

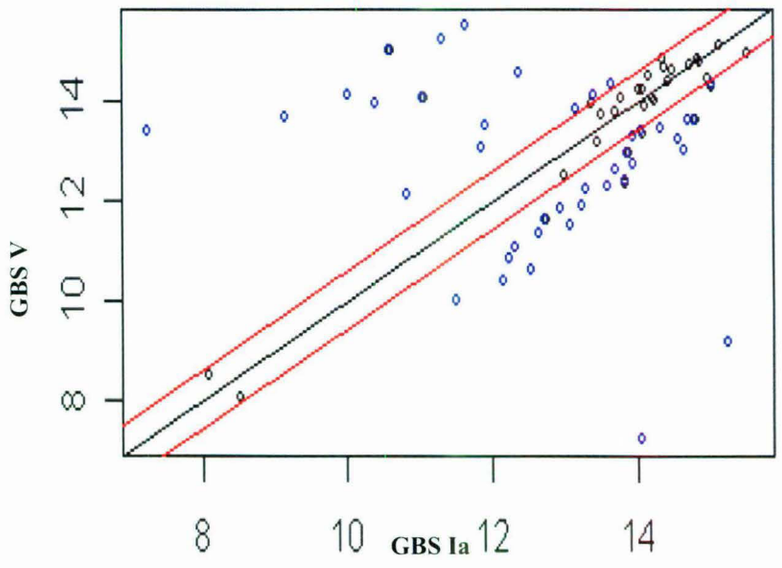

Fig 4.18: Bivariate scatter plot for GBS Ia and GBS V

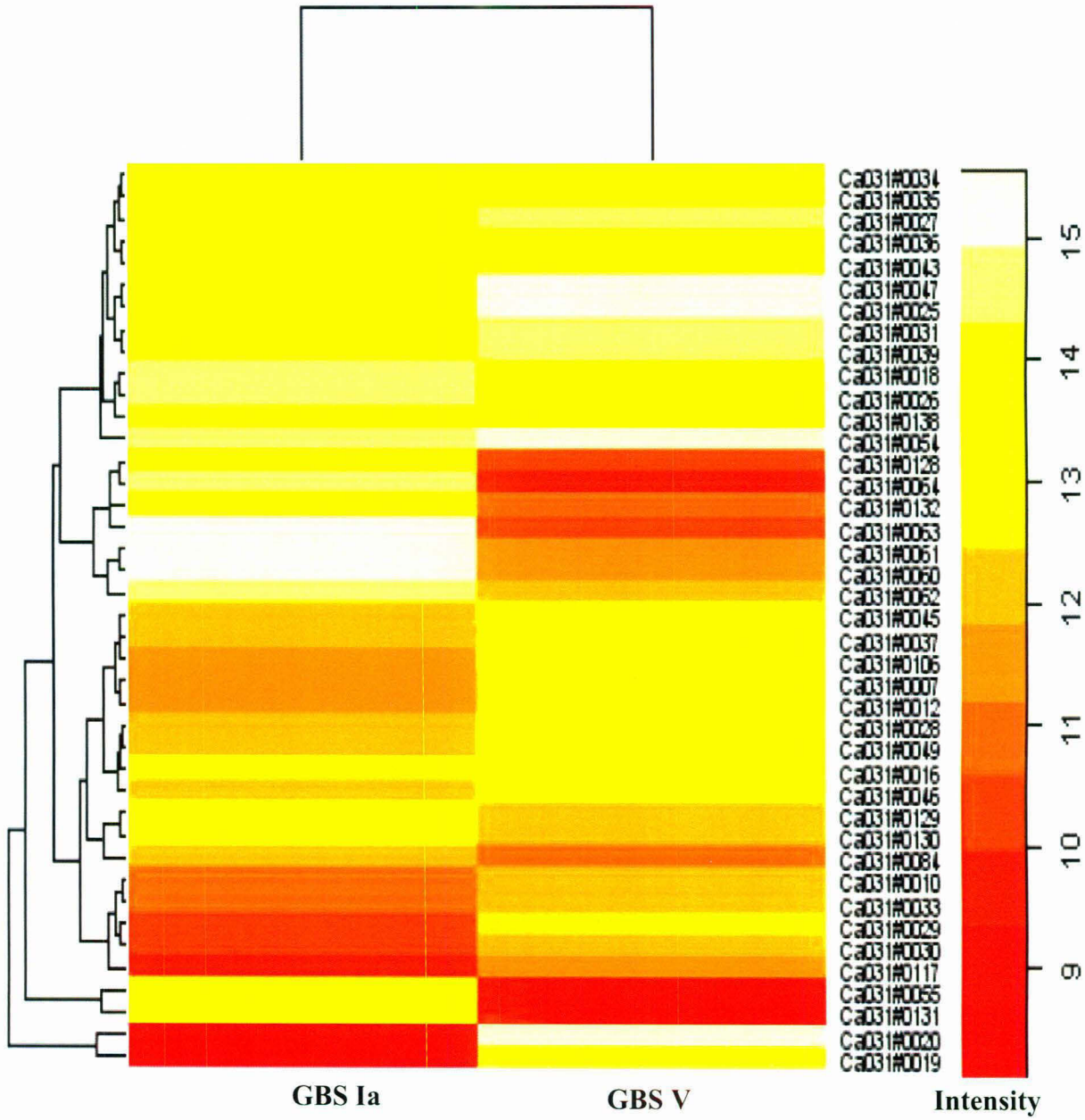

Fig 4.19: Heatmap for samples GBS Ia and GBS V. Each Oligo number represents a particular gene

Table 4.7: Gene upregulated in GBS type V compared to GBS type Ia (n=15)						
S.No	**locus name on Chip**	**Gene locus**	**Gene product name**	**Gene**	**Fold change**	**Functions**
1	Ca031#0018	-	Capsule		1.6	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
2	Ca031#0026	-	Capsule		1.6	do
3	Ca031#0055	-	Glucan binding protein		23.2	GbpB may be involved in cell wall synthesis (Mattos-Graner et al, 2006)
4	Ca031#0060	SAG1404	Pilus island 2		15.2	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005)
5	Ca031#0061	SAG1405	Pilus island 2		14.7	do
6	Ca031#0062	SAG1406	Pilus island 2		4.5	do
7	Ca031#0063	SAG1407	Pilus island 2		21.4	do
8	Ca031#0064	SAG1408	Pilus island 2		17.2	do
9	Ca031#0084	-	α like proteins	Alp2	2.5	Surface-anchored proteins, inducers of protective antibodies Maeland et al, 2004; Lachenauer et al, 2000
10	Ca031#0128	SAG1164	Capsule	cpsJ	11.7	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
11	Ca031#0129	SAG1165	capsule	cpsO	3.0	do
12	Ca031#0130	SAG1166	capsule	cpsN	2.3	do
13	Ca031#0131	SAG1167	capsule	cpsM	71.6	do
14	Ca031#0132	SAG1168	capsule	cpsH	8.0	do
15	Ca031#0138	SAG0433	Rib	rib	1.5	GBS surface protein involved in adhesion (Larsson et al, 2004)

Table 4.8: Gene downregulated in GBS type V compared to GBS type Ia (n=26)						
S.No	**locus name**	**Gene locus**	**Gene product name**	**Gene**	**Fold change**	**Functions**
1	Ca031#0007	SAG0649	Pilus island 1		0.4	Pilus oligomerization and polymerization formation(Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0010	SAG1197	Hyaluronidase	hylB	0.4	Facilitates bacterial invasion by degrading extracellular hyaluronan and may promote persistent colonization of the vagina by GBS (Sukhnanand et al, 2005)
3	Ca031#0012	SAG1158	capsule	neuA	0.4	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
4	Ca031#0016	SAG1162	capsule	cpsL	0.4	do
5	Ca031#0019	-	capsule	cpsI	0.0	do
6	Ca031#0020	-	capsule	-	0.0	do
7	Ca031#0025	SAG1173	capsule	cpsC	0.4	do
8	Ca031#0027	-	capsule		0.5	do
9	Ca031#0028	-	capsule		0.3	do
10	Ca031#0029	-	α C proteins	Bca	0.2	Interact with epithelial surfaces and initiation of infection (Bolduc et al, 2002; Baron et al, 2004).
11	Ca031#0030	-	β- C protein	cba	0.2	Interacts with two components of the human immune system, plays a role in immune (Lindahl, 2005)
12	Ca031#0031	SAG0032	Surface immunogenic protein	Sip	0.3	Highly conserved protein, potential vaccine candidate, (Rioux et al, 2001; Brodeur et al, 2000).
13	Ca031#0033	SAG1730	C3 degrading protease	cppA	0.3	Escapes bacteria from ingestion and killing by PMN cells (Rainard and Boulard, 1992)
14	Ca031#0034	SAG1236	C5a peptidase	-	0.6	Serine protease that inactivates human C5a(Bohnsack et al, 1991)
15	Ca031#0035	SAG2174	Serine protease	-	0.6	Diverse role (eg., heat shock protein) (Nair et al, 2003)
16	Ca031#0036	SAG0105	Trigger factor	-	0.5	Protein folding of newely synthesized protein (Deuerling et al, 1999)

17	Ca031#0037	SAG0662	β hemolysin	-	0.4	Pore-forming exotoxin (Hensler et al., 2008)
18	Ca031#0039	SAG0664	β hemolysin	-	0.3	do
19	Ca031#0043	SAG0668	β hemolysin	-	0.4	do
20	Ca031#0045	SAG0670	β hemolysin	-	0.3	do
21	Ca031#0046	SAG0671	β hemolysin	-	0.4	do
23	Ca031#0049	SAG2043	CAMP factor	-	0.4	Pore-forming toxin, useful in identification of GBS in the clinical laboratory (Hensler et al., 2008)
24	Ca031#0054	SAG1234	Laminin-binding protein	-	0.6	and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)
25	Ca031#0106	SAG1052	Fibronectin Binding protein	-	0.3	Binds to human plasma fibronectin (Butler et al, 1987)
26	Ca031#0117	-	Pilus island 2	-	0.3	Pilus oligomerization and polymerization formation(Lauer et al, 2005; Rosini et al, 2006)

4.6.3. GBS Ia vs GBS VII

Table **4.9 and 4.10** shows the up and downregulated genes in GBS type VII as compared to type Ia. In **Fig 4.20**., the up and down regulated genes are highlighted in blue points and the cut-off value (0.58) is shown in red lines. Heat map of GBS Ia vs GBS VII is shown in **Fig. 4.21.**

Microarray analysis of GBS VII reveals up-regulation of 16 genes as compared to type 1a. Transcript level of gene lmb encoding laminin binding protein which helps in adhesion of GBS to host cell, was found highly up-regulated i.e., 27.5 folds in GBS VII as compared to GBS Ia **(Table 4.9)**. In case of GBS VII transcript plr/gap encoding streptococal plasmin receptor/GAPDH (important for evasion of GBS from host immune response) was found 2.8 fold up-regulated (**Table 4.9**). A total of 11 genes were found down regulated in type VII as compared to type 1a **(Table 4.10).** Amongst genes related to capsule formation and fibronecting binding proteins were found down regulated.

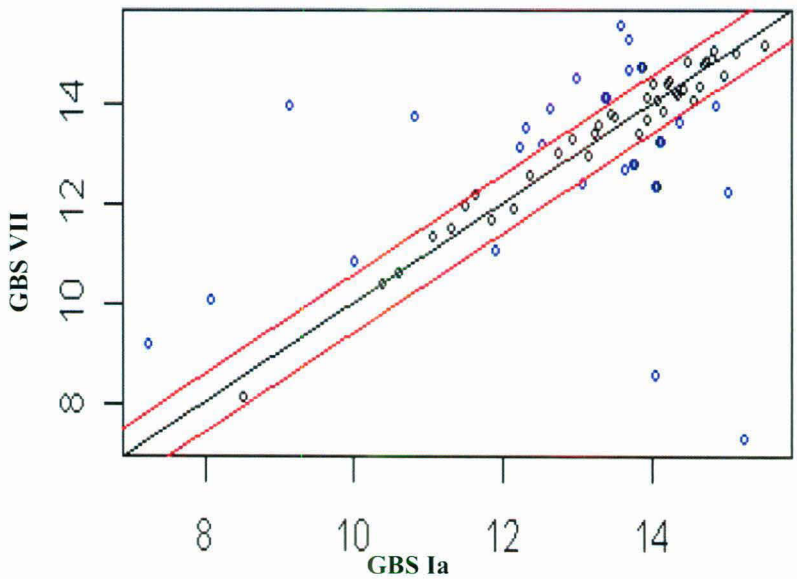

Fig 4.20: Bivariate scatter plot for GBS Ia and GBS VII

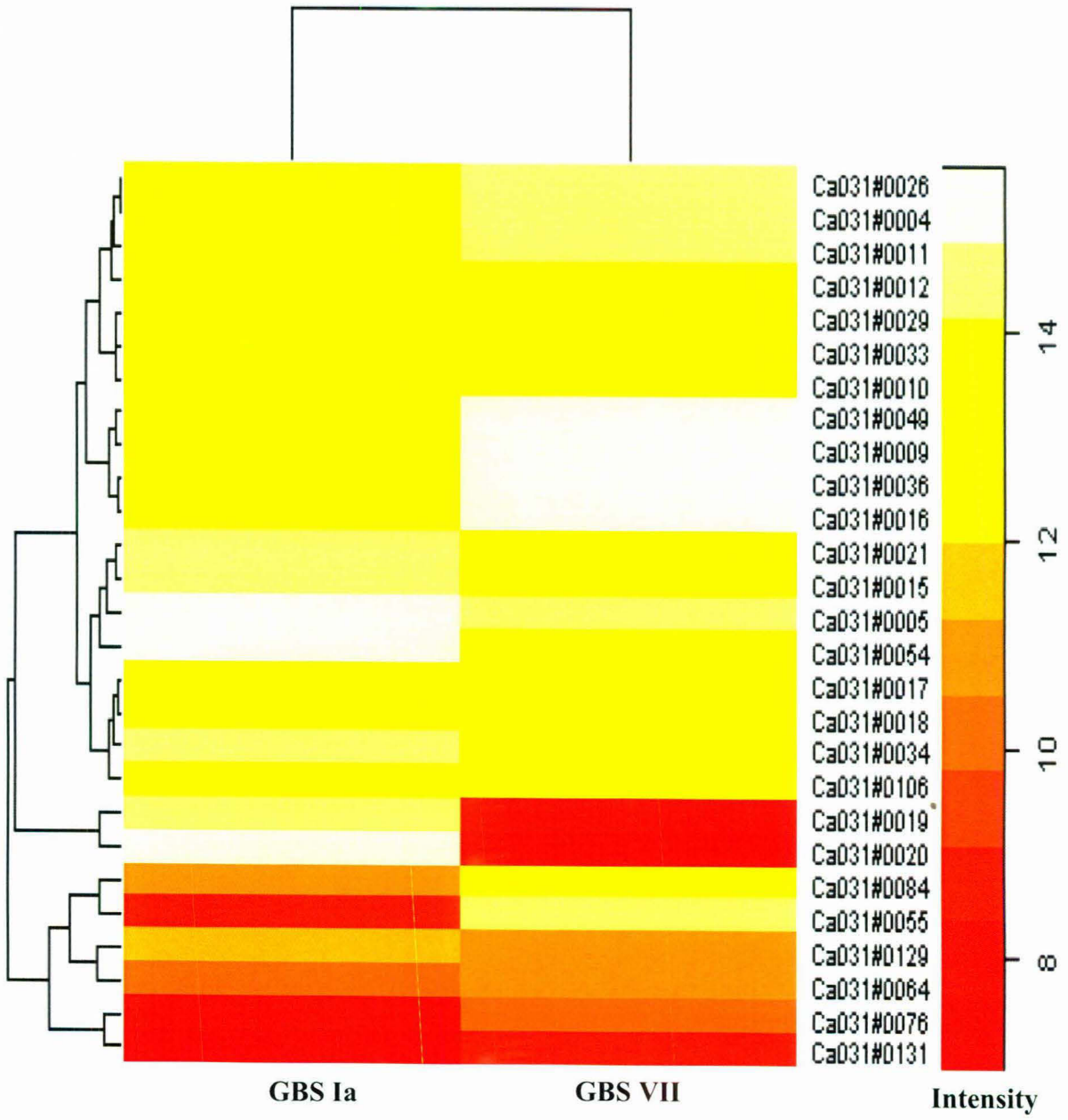

Fig 4.21: Heatmap for samples GBS Ia and GBS VII. Each Oligo number represents a particular gene

Table 4.9: Gene upregulated in GBS type VII compared to GBS type Ia (n=16)

S.No	locus name	Gene product name	Gene	Fold change	Functions
1	Ca031#0004	Pilus island 1	-	1.6	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation (Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0009	Streptococcal plasmin receptor/GAPDH	plr/gap A	2.8	GAPDH, glycolytic enzyme used for bacterial energy generation, bind several host proteins, confer resistance against reactive oxygen species produced byhost phagocytic cells (Madureira et al, 2006)
3	Ca031#0010	Hyaluronidase	hylB	2.2	Facilitates bacterial invasion by degrading extracellular hyaluronan and may promote persistent colonization of the vagina by GBS (Sukhnanand et al., 2005)
4	Ca031#0011	Streptococcal enolase	eno	2.8	Glycolytic enzyme α-enolase as a plasmin binding protein on the outside of the bacterial cell (Pancholi and Fischetti, 1998)
5	Ca031#0012	Capsule	neuA	2.3	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
6	Ca031#0016	Capsule	cpsL	1.9	Do
7	Ca031#0026	Capsule	cpsB	1.6	Do
8	Ca031#0029	α C protein	bca	1.5	An important virulence factor, plays role in interaction with epithelial surfaces and initiation of infection (Bolduc et al, 2002; Baron et al, 2004.)
9	Ca031#0033	C3-degrading protease	cppA	1.8	Stops C3 deposition on bacterial cells and thereby escapes bacteria from ingestion and killing by PMN cells (Rainard and Boulard, 1992)
10	Ca031#0036	Trigger factor	tig/ropA	1.7	Protein folding of newely synthesized protein (Deuerling et al, 1999)
11	Ca031#0049	CAMP factor	cfa/cfb	3.7	Pore-forming toxin, useful in identification of GBS in the clinical laboratory (Hensler et al, 2008)
12	Ca031#0055	Glucan binding protein	gbpB	27.5	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)
13	Ca031#0064	Pilus island 2	-	1.7	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006)
14	Ca031#0076	Capsule	-	3.8	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
15	Ca031#0084	α-like protein	alp2	7.3	surface-anchored proteins, inducers of protective antibodies (Maeland et al, 2004; Lachenauer et al, 2000)
16	Ca031#0131	Capsule	-	3.7	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)

Table 4.10: Gene downregulated in GBS type VII compared to GBS type Ia (n=11)					
S.No	**locus name**	**Gene product name**	**Gene**	**Fold change**	**Function**
1	Ca031#0005	Pilus island 1	-	0.5	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0015	capsule	neuB	0.5	do
3	Ca031#0017	capsule	cpsK	0.4	do
4	Ca031#0018	capsule	cpsJ	0.4	do
5	Ca031#0019	capsule	cpsI	0.0	do
6	Ca031#0020	capsule	cpsH	0.0	do
7	Ca031#0021	capsule	cpsG	0.5	do
8	Ca031#0034	C5a peptidase	scpA/scpB	0.2	Serine protease that inactivates human C5a (Bohnsack et al, 1991)
9	Ca031#0054	Fibronectin binding protein	fbsA	0.1	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)
10	Ca031#0106	Fibronectin binding protein	fbsB	0.6	Binds to human plasma fibronectin (Butler et al., 1987)
11	Ca031#0129	capsule	-	0.5	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)

4.6.4. GBS III vs GBS V

Table **4.11 and 4.12** shows the up and downregulated genes in GBS type V as compared to type III. In **Fig 4.22** up and down regulated genes are highlighted in blue points and cut-off value (0.58) is shown in red lines. Heat map of GBS III vs GBS V is shown in **Fig. 4.23.**

Comparisons of transcriptional profiling of GBS V with GBS III by microarray analysis reveals up-regulation of 19 genes. In case of type V a maximum up-regulation of 71.6 fold was found in case of cpsM gene which encodes for capsule and cell wall synthesis as compared to type III **(Table 4.11)**. Pilus island-2 (PI-2) coding for pilus protein and helps in attachment of GBS to host cell by involving in synthesis of plii was 31.2 fold up-regulated. Cluster of Pilus island 1 (PI-1) was found 1.5-2.4 folds up-regulated in GBS V as compared to GBS Ia (**Table 4.11)**. A total number of 22 genes (important genes includes enolase, sip and CAMP factor) were found down regulated in type V as compared to type III **(Table 4.12)**.

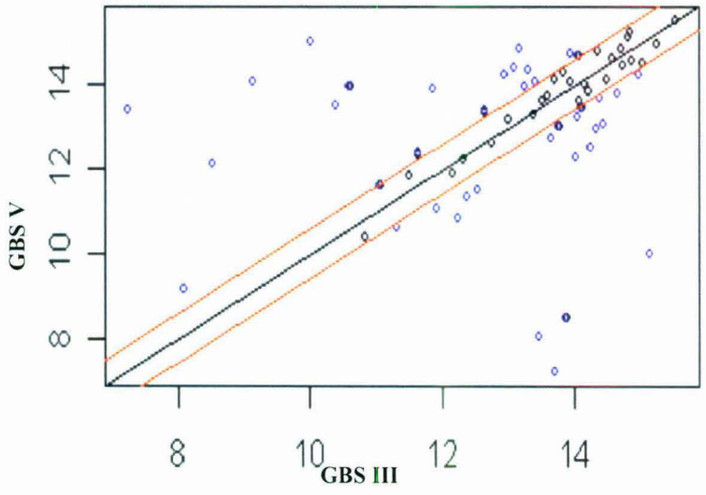

Fig 4.22: Bivariate scatter plot for GBS III and GBS V

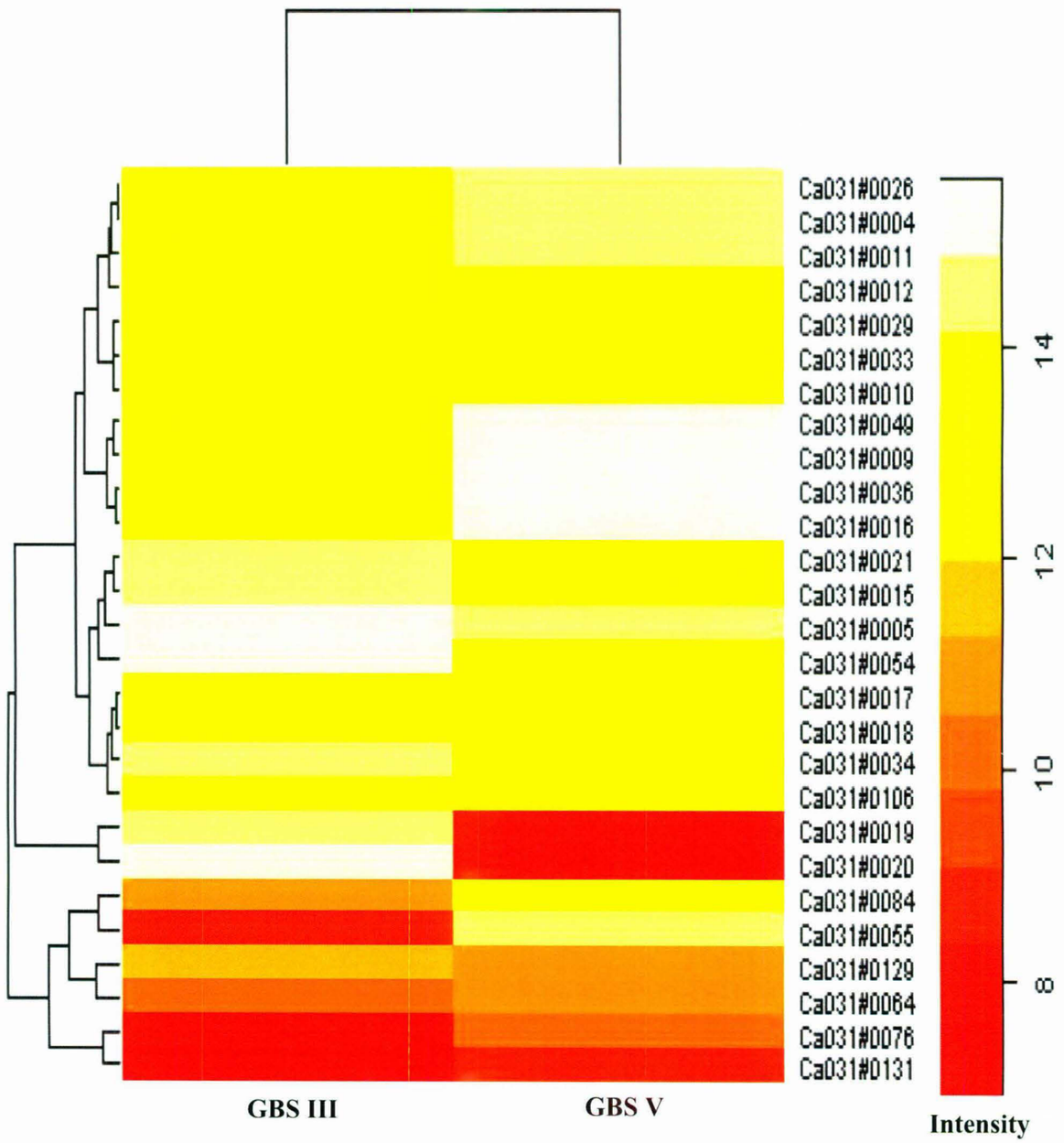

Fig 4.23: Heatmap for samples GBS III and GBS V. Each Oligo number represents a particular gene

Table 4.11: Gene upregulated in GBS type V compared to GBS type III (n=19)						
S.No	**locus name on Chip**	**Gene locus**	**Gene product name**	**Gene**	**Fold change**	**Functions**
1	Ca031#0004	SAG0646	Pilus island 1	-	1.6	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0006	SAG0648	Pilus island 1	-	2.4	do
3	Ca031#0007	SAG0649	Pilus island 1	-	1.5	do
4	Ca031#0013	SAG1159	capsule	neuD	1.7	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
5	Ca031#0014	SAG1160	capsule	neuC	2.4	do
6	Ca031#0015	SAG1161	capsule	neuB	4.0	do
7	Ca031#0018	-	capsule	-	2.0	do
8	Ca031#0020	-	capsule	-	2.1	do
9	Ca031#0021	-	capsule	cpsG	1.5	do
10	Ca031#0028	-	capsule	-	1.6	do
11	Ca031#0034	SAG1236	C5a peptidase	scpA/scpB	1.6	Serine protease that inactivates human C5a (Bohnsack et al, 1991)
12	Ca031#0038	SAG0663	β-hemolysin/cytolysin	cylD	1.5	Pore-forming exotoxin (Hensler et al, 2008)
13	Ca031#0040	SAG0665	β-hemolysin/cytolysin	acpC	3.1	do
14	Ca031#0063	-	Pilus island 2	-	31.9	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation (Lauer et al, 2005; Rosini et al, 2006)
15	Ca031#0084	-	α-like protein	alp2	12.3	Surface-anchored proteins, inducers of protective antibodies (Maeland et al, 2004; Lachenauer et al, 2000)
16	Ca031#0128	SAG1164	capsule	cpsJ	10.2	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
17	Ca031#0129	SAG1165	capsule	cpsO	8.7	do
18	Ca031#0131	SAG1167	capsule	cpsM	71.6	do
19	Ca031#0132	SAG1168	capsule	cpsH	29.9	do

Table 4.12: Gene downregulated in GBS type V compared to GBS type III (n=20)

S.No	locus name	Gene locus	Gene product name	Gene	Fold change	Functions
1	Ca031#0009	SAG1768	Streptococcal plasmin receptor/GAPDH	plr/gap A	0.5	GAPDH, glycolytic enzyme used for bacterial energy generation, bind several host proteins, confer resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006)
2	Ca031#0010	SAG1197	Hyaluronidase	hylB	0.5	Facilitates bacterial invasion by degrading extracellular hyaluronan and may promote persistent colonization of the vagina by GBS (Sukhnanand et al., 2005)
3	Ca031#0010	SAG0628	Streptococcal enolase	eno	0.3	Glycolytic enzyme α-enolase as a plasmin binding protein on the outside of the bacterial cell (Pancholi and Fischetti, 1998
4	Ca031#0012	SAG1158	capsule	neuA	0.4	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
5	Ca031#0019	-	capsule	-	0.0	do
6	Ca031#0027	-	capsule	-	0.6	do
7	Ca031#0029	-	α- C protein	bca	0.6	An important virulence factor, plays role in interaction with epithelial surfaces and initiation of infection (Bolduc et al, 2002;Baron et al, 2004).
8	Ca031#0031	SAG0032	Surface immunogenic protein	sip	0.5	Highly conserved protein, potential vaccine candidate, (Rioux et al, 2001;Brodeur et al, 2000)
9	Ca031#0032	SAG1533	Pneumococcal surface antigen A / Metal binding protein SloC	psaA	0.5	Essential virulence factor, a metal (Mn^{2+} and Zn^{2}) Binding protein (ABC-type), Potential drug target and a candidate vaccine component (Lawrence et al, 1998)
10	Ca031#0033	SAG1730	C3-degrading protease	cppA	0.3	Stops C3 deposition on bacterial cells and thereby escapes bacteria from ingestion and killing by PMN cells (Rainard and Boulard, 1992)
11	Ca031#0036	SAG0105	Trigger factor	tig/ropA	0.3	Protein folding of newely synthesized protein (Deuerling et al, 1999)
12	Ca031#0039	SAG0664	β-hemolysin/cytolysin	cylG	0.5	Pore-forming exotoxin (Hensler et al, 2008)
13	Ca031#0043	SAG0668	β-hemolysin/cytolysin	cylB	0.5	do
14	Ca031#0049	SAG2043	CAMP factor	cfa/cfb	0.3	Pore-forming toxin, useful in identification of GBS in the clinical laboratory (Hensler et al., 2008)
15	Ca031#0055	-	Glucan binding protein	lmb	0.6	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)

16	Ca031#0076	-	capsule	-	0.02	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
17	Ca031#0104	-	agglutinin receptor	-	0.02	Mediates specific adhesion and aggregation (Prakobphol et al, 2000)
18	Ca031#0106	SAG1052	Fibronectin binding protein	fbs A	0.4	Binds to human plasma fibronectin (Butler et al., 1987)
19	Ca031#0117	-	Pilus island 2	-	0.0	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006)
20	Ca031#0130	SAG1166	capsule	cpsN	0.3	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)

4.6.5. GBS III vs GBS VII

Table 4.13 and 4.14 shows the up-regulated and downregulated genes in GBS type VII as compared to GBS type III. In **Fig 4.24** the up and down regulated genes are highlighted in blue points and cut-off value (0.58) is shown in red lines. Heat map of GBS III vs GBS VII is shown in **Fig. 4.25.**

Comparative microarray analysis of GBS VII with III reveals 19 up-regulating genes. Genes like alp2 (encodes for surface anchored alpha like proteins) and implicated to be involved in adherence was found 36.2 fold up-regulated in type VII as compared to type III. Pilus island (PI-1) involved in oligomerization and polymerization of pilus protein and Alpha C protein predicted to be involved in initiation of infection was found 3.8 and 3.6 fold up-regulated respectively in GBS VII as compared to GBS III (**Table 4.13)**. A total of 16 genes which includes pilus, rib were found down regulated **(Table 4.14).**

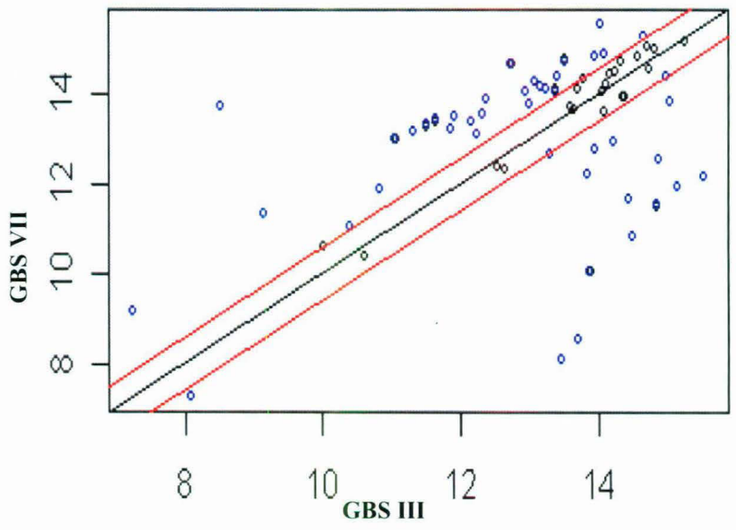

Fig 4.24: Bivariate scatter plot for GBS III and GBS VII

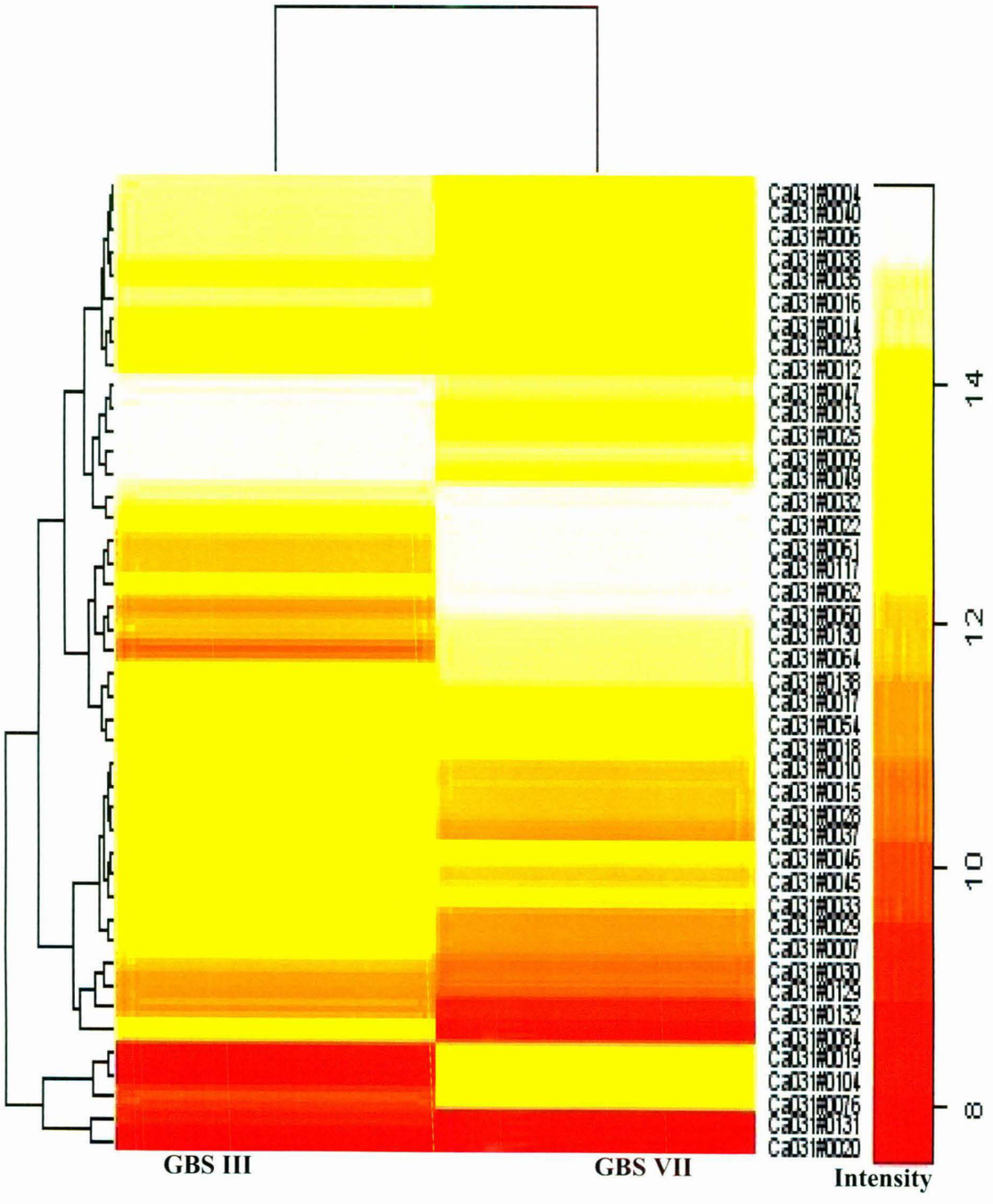

Fig 4.25: Heatmap for samples GBS III and GBS VII. Each Oligo number represents a particular gene

Table 4.13: Gene up-regulated in GBS type VII compared to GBS type III (n=28)

S.No.	locus name	Gene locus	Gene product name	Gene	Fold Change	Function
1	Ca031#0004	gbs0629	Pilus Island1	-	1.8	Pilus oligomerization and polymerization (Lauer et al, 2005; Rosini et al, 2006)
2	Ca031#0006	gbs0631	Pilus Island1	-	2.2	do
3	Ca031#0007	gbs0632	Pilus Island1	-	3.8	do
4	Ca031#0009	gbs1811	Streptococcal plasmin receptor/GAPDH	plr/gapA	1.5	GAPDH, bind several host proteins, confer resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006)
5	Ca031#0010	gbs1270	Hyaluronidase	hylB	2.9	Facilitates bacterial invasion by degrading extracellular hyaluronan and may promote persistent colonization of the vagina by GBS (Sukhnanand et al., 2005)
6	Ca031#0012	gbs1233	Capsule	neuA	2.8	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
7	Ca031#0013	gbs1234	Capsule	neuD	1.8	do
8	Ca031#0014	gbs1235	Capsule	neuC,	2.1	do
9	Ca031#0015	gbs1236	Capsule	neuB	2.5	do
10	Ca031#0016	gbs1237	Capsule	cpsM	3.7	do
11	Ca031#0023	gbs1243	Capsule	cpsE	1.7	do
12	Ca031#0025	gbs1245	Capsule	cpsC	2.3	do
13	Ca031#0028	-	Capsule	-	3.3	do
14	Ca031#0029	-	Alpha C protein	bca	3.6	In interaction with epithelial surfaces and initiation of infection (Baron et al, 2004)
15	Ca031#0030	-	Beta C protein	cba	2.0	The beta protein interacts with two components of the human immune system, IgA-Fc and factor H (FH), suggesting that it plays a role in immune (Lindahl, 2005)
16	Ca031#0033	gbs1775	C3-degrading protease	cppA	1.8	Escapes bacteria from ingestion and killing by PMN cells (Rainard and Boulard, 1992)
17	Ca031#0035	gbs2133	Serine protease	htrA/degP	1.6	Diverse role (eg., heat shock protein) (Nair et al, 2003)

18	Ca031#0037	gbs0644	Beta-hemolysin/cytolysin	cylX	3.4	Pore-forming exotoxin (Hensler et al, 2008)
19	Ca031#0038	gbs0645	Beta-hemolysin/cytolysin	cylD	1.9	do
20	Ca031#0040	gbs0647	Beta-hemolysin/cytolysin	acpC	1.9	do
21	Ca031#0045	gbs0652	Beta-hemolysin/cytolysin	cylF	2.3	do
22	Ca031#0046	gbs0653	Beta-hemolysin/cytolysin	cylI	2.3	do
23	Ca031#0047	gbs0654	Beta-hemolysin/cytolysin	cylJ	1.7	do
24	Ca031#0049	gbs2000	CAMP factor	cfa/cfb	2.8	Pore-forming toxin, useful in identification of GBS in the clinical laboratory (Hensler et al., 2008)
25	Ca031#0084	-	Alpha-like protein	alp2	36.2	Surface-anchored proteins, inducers of protective antibodies (Maeland et al, 2004)
26	Ca031#0129	-	Capsule	cpsO	1.5	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
27	Ca031#0131	-	Capsule	cpsM	3.7	do
28	Ca031#0132	-	Capsule	cpsH	4.4	do

Table 4.14: Gene downregulated in GBS type VII compared to GBS type III (n=16)

S.No.	locus name on Chip	Gene locus	Gene product name	Gene	Fold change	Functions
1	Ca031#0017	gbs1237.1	Capsule	cpsL	0.4	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
2	Ca031#0018	gbs1238	Capsule	cpsIaJ	0.6	do
3	Ca031#0019	gbs1239	Capsule	cpsJ	0.0	do
4	Ca031#0020		Capsule	-	0.5	do
5	Ca031#0022	gbs1242	Capsule	cpsF	0.4	do
6	Ca031#0032	gbs1589	Pneumococcal surface antigen A / Metal binding protein SloC	psaA	0.6	Essential virulence factor, a metal (Mn^{2+} and Zn^{2}) Binding protein (ABC-type), Potential drug target and a candidate vaccine component (Lawrence et al, 1998)
7	Ca031#0054	gbs1307	Laminin-binding protein	lmb	0.3	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)
8	Ca031#0060	gbs1474	Pilus Island2	-	0.0	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005; Rosini et al, 2006))
9	Ca031#0061	gbs1475	Pilus Island2	-	0.0	do
10	Ca031#0062	gbs1476	Pilus Island2	-	0.1	do
11	Ca031#0064	gbs1478	Pilus Island2	-	0.0	do
12	Ca031#0076	gbs1240	Capsule	cpsI	0.0	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
13	Ca031#0104	-	Agglutinin receptor	-	0.0	Mediates specific adhesion and aggregation (Prakobphol et al, 2000)
14	Ca031#0117	-	Pilus Island2	-	0.1	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005)
15	Ca031#0130	-	Capsule	-	0.1	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
16	Ca031#0138	-	Rib	-	0.4	GBS surface protein involved in adhesion (Larsson et al, 2004)

4.6.6. GBS V vs GBS VII

Table 4.15 and **4.16** shows the up and downregulated genes in GBS type V as compared to type VII. In **Fig 4.26** up and down regulated genes are highlighted in blue points and cut-off value (0.58) is shown in red lines. Heat map of GBS III vs GBS VII is shown in **Fig 4.27.**

When transcriptional profiling of GBS V was compared with VII, we have found that a total 28 genes were found up-regulated in VII. Amongst these genes cfa/cfb encoding for CAMP factor which is a pore forming toxin and also used for identification of GBS in clinical laboratory was found 9.3 fold up-regulated in type VII. Alpha C protein Involved in initiation of infection and neuA gene encoding capsule and involved in cell wall synthesis was found 5.9 and 5.8 folds up-regulated in VII as compared to V (**Table 4.15)**. We have found 21 genes (mostly related to capsule formation and pilus island) down regulated in type VII as compared to type V **(Table 4.16)**.

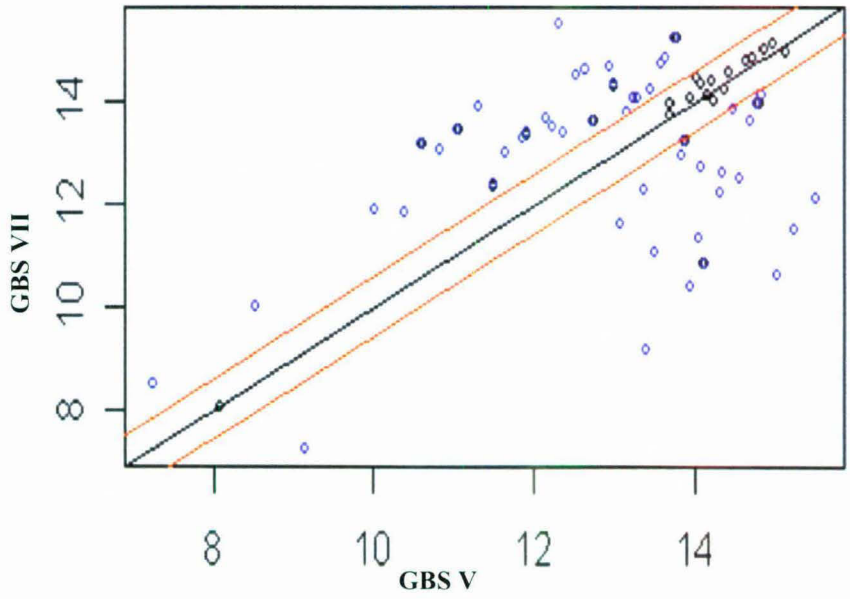

Fig 4.26: Bivariate scatter plot for GBS V and GBS VII

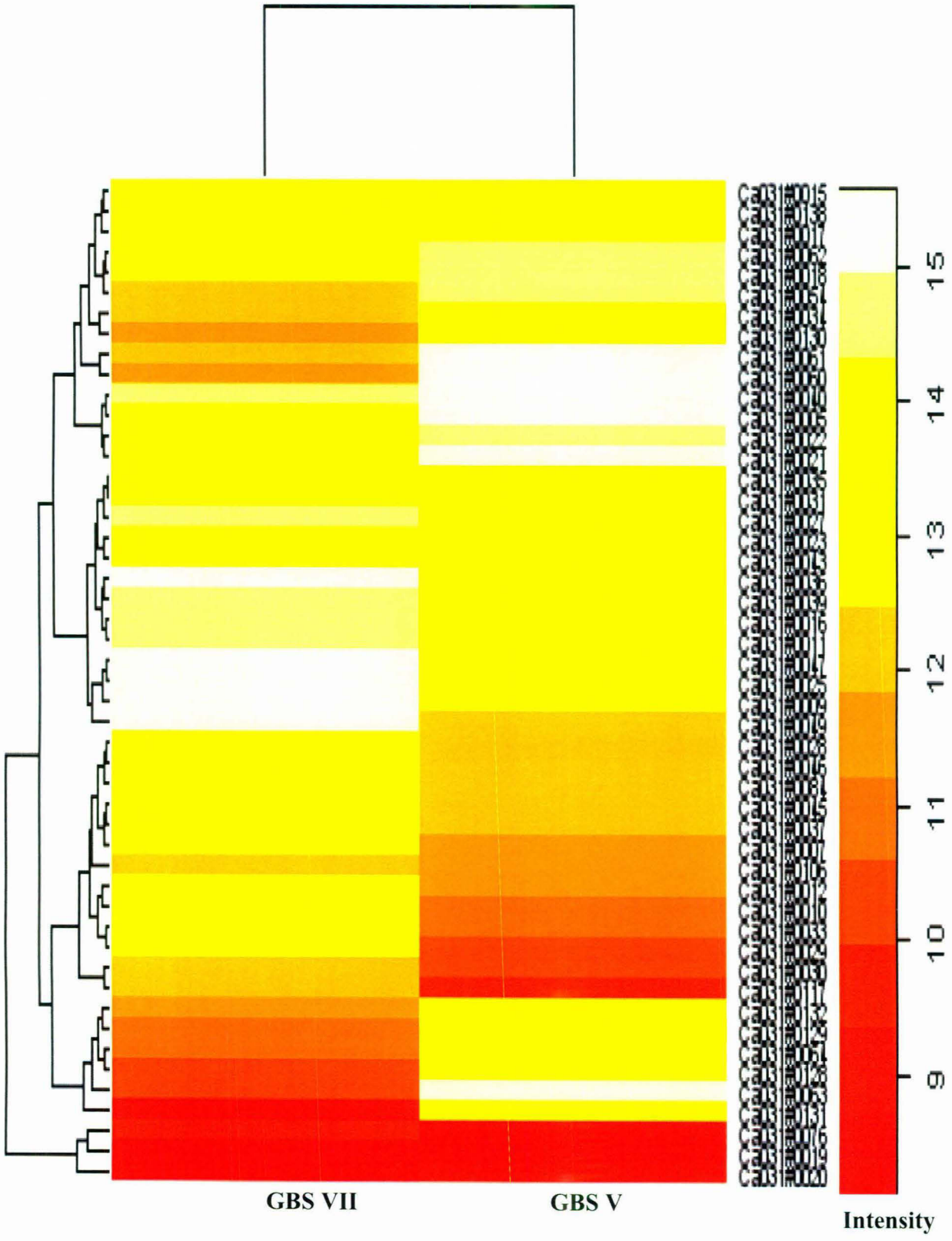

Fig 4.27: Heatmap for samples GBS VII and GBS V. Each Oligo number represents a particular gene

Table 4.15: Gene up-regulated in GBS type VII compared to GBS type V (n=28)

S.No	locus name	Gene locus	Gene product name	Gene	Fold change	Functions
1	Ca031#0007	SAG0649	Pilus Island1		2.5	Pilus oligomerization and polymerization (Lauer et al, 2005; Rosini et al, 2006))
2	Ca031#0009	SAG1768	Streptococcal plasmin receptor/GAPDH	plr/gapA	2.7	GAPDH, glycolytic enzyme used for bacterial energy generation, bind several host proteins, confer resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006)
3	Ca031#0010	SAG1197	Hyaluronidase	hylB	5.2	Facilitates bacterial invasion by degrading extracellular hyaluronan and may promote persistent colonization of the vagina by GBS (Sukhnanand et al., 2005)
4	Ca031#0011	SAG0628	Streptococcal enolase	eno	3.9	Glycolytic enzyme α-enolase as a plasmin binding protein on the outside of the bacterial cell (Pancholi and Fischetti, 1998
5	Ca031#0012	SAG1158	capsule	neuA	5.8	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
6	Ca031#0016	SAG1162	capsule	neuB	4.0	do
7	Ca031#0019		capsule	cpsI	2.4	do
8	Ca031#0023	SAG1171	capsule	cpsE	1.5	do
9	Ca031#0025	SAG1173	capsule	cpsC	2.1	do
10	Ca031#0027	-	capsule	cpsX	1.7	do
11	Ca031#0028	-	capsule	cpsY	2.0	do
12	Ca031#0029	-	Alpha C protein	bca	5.9	Interaction with epithelial surfaces and initiation of infection (Bolduc et al, 2002; Baron et al, 2004)
13	Ca031#0030	-	Beta C protein	cba	2.7	The β protein interacts with two components of the human immune system, IgA-Fc and factor H (FH), suggesting that it plays a role in immune (Lindahl, 2005)
14	Ca031#0031	SAG0032	Surface immunogenic protein	sip	1.7	Highly conserved protein, potential vaccine candidate, (Rioux et al., 2001; Brodeur et al., 2000)
15	Ca031#0033	SAG1730	C3-degrading protease	cppA	4.7	Escapes bacteria from ingestion and killing by PMN cells (Rainard and Boulard, 1992)
16	Ca031#0035	SAG2174	Serine protease	htrA/degP	1.7	Diverse role (eg., heat shock protein) (Nair et al, 2003)
17	Ca031#0036	SAG0105	Trigger factor	tig/ropA	3.3	Protein folding of newely synthesized protein (Deuerling et al, 1999)

18	Ca031#0037	SAG0662	Beta-hemolysin/cytolysin	cylX	2.6	Pore-forming exotoxin (Hensler et al, 2008)
19	Ca031#0039	SAG0664	Beta-hemolysin/cytolysin	cylG	2.5	do
20	Ca031#0043	SAG0668	Beta-hemolysin/cytolysin	cylB	1.8	do
21	Ca031#0045	SAG0670	Beta-hemolysin/cytolysin	cylF	2.7	do
22	Ca031#0046	SAG0671	Beta-hemolysin/cytolysin	cylI	2.4	do
23	Ca031#0047	SAG0672	Beta-hemolysin/cytolysin	cylJ	2.3	do
24	Ca031#0049	SAG2043	CAMP factor	cfa/cfb	9.3	Pore-forming toxin, useful in identification of GBS in the clinical laboratory (Hensler et al., 2008)
25	Ca031#0076	-	capsule	alp2	2.8	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
26	Ca031#0084	-	Alpha-like protein	fbsA	2.9	Surface-anchored proteins, inducers of protective antibodies (Maeland et al, 2004)
27	Ca031#0106	SAG1052	Fibronectin Binding protein	-	1.8	Binds to human plasma fibronectin (Butler et al., 1987)
28	Ca031#0117	-	Pilus Island2	-	3.6	Pilus oligomerization and polymerization (Lauer et al, 2005; Rosini et al, 2006)

Table 4.16: Gene downregulated in GBS type VII compared to GBS type V (n=21)

S.No	Locus name on Chip	Gene locus	Gene product name	Gene	Fold change	Functions
1	Ca031#0005	SAG0647	Pilus Island1	-	0.5	Pilus oligomerization and polymerization (Lauer et al, 2005)
2	Ca031#0015	SAG1161	capsule	cpsL	0.6	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
3	Ca031#0017	SAG1163	capsule	cpsK	0.3	do
4	Ca031#0018	-	capsule	cpsJ	0.3	do
5	Ca031#0020	-	capsule	cpsH	0.2	do
6	Ca031#0021	SAG1169	capsule	cpsG	0.4	do
7	Ca031#0022	SAG1170	capsule	cpsF	0.6	do
8	Ca031#0034	SAG1236	C5a peptidase	scpA/scpB	0.4	Serine protease that inactivates human C5a(Bohnsack et al, 1991)
9	Ca031#0040	SAG0665	Beta-hemolysin/cytolysin	acpC	0.6	Pore-forming exotoxin (Hensler et al, 2008)
10	Ca031#0054	SAG1234	Laminin-binding protein	lmb	0.2	Mediates attachment to human laminin and plays a crucial role in the adhesion/invasion of eukaryotic host cells (Ragunathan et al, 2009)
11	Ca031#0060	SAG1404	Pilus Island2	-	0.0	Pilus oligomerization and polymerization and sortase enzyme for anchoring at the memebrane formation(Lauer et al, 2005)
12	Ca031#0061	SAG1405	Pilus Island2	-	0.0	do
13	Ca031#0062	SAG1406	Pilus Island2	-	0.2	do
14	Ca031#0063	SAG1407	Pilus Island2	-	0.0	do
15	Ca031#0064	SAG1408	Pilus Island2	cpsJ	0.1	do
16	Ca031#0128	SAG1164	capsule	cpsO	0.0	Synthesis of capsular polysaccharide (Cieslewicz et al, 2001)
17	Ca031#0129	SAG1165	capsule	cpsN	0.1	do
18	Ca031#0130	SAG1166	capsule	cpsM	0.3	do
19	Ca031#0131	SAG1167	capsule	cpsH	0.0	do
20	Ca031#0132	SAG1168	capsule	rib	0.1	do
21	Ca031#0138	SAG0433	Rib	-	0.5	GBS surface protein involved in adhesion (Larsson et al, 2004)

4.7. Distribution of upregulated and downregulated genes

Up- and down-regulated genes obtained in different sample comparisons were used to find out unique and common genes across the comparisons. Venn diagrams depicting the number of unique and common down-regulated genes in GBS Ia vs III, V and VII are shown in **Figs 4.28, 4.29, 4.30,** and **4.31**. Total two genes were found commonly up-regulated in GBS III, V and VII as compared to GBS Ia. These include gbpB and Pilus protein 2 as also shown in **Table 4.17**.

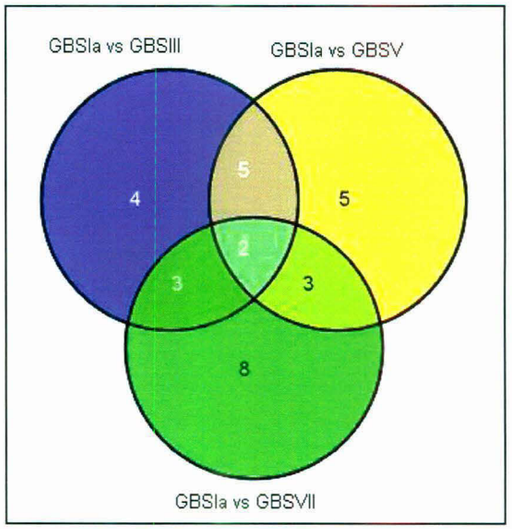

Fig 4.28. Venn Diagram depicting the common and unique up-regulated genes GBS1a vs V,III and VII.

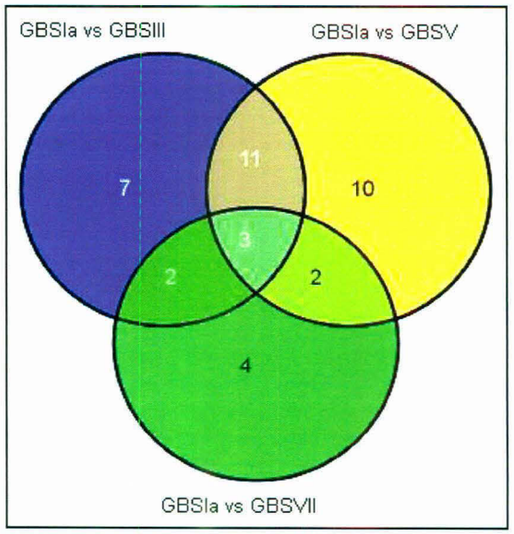

Fig 4.29. Venn Diagram depicting the common and unique down-regulated genes in GBS 1a vs V, VII and III.

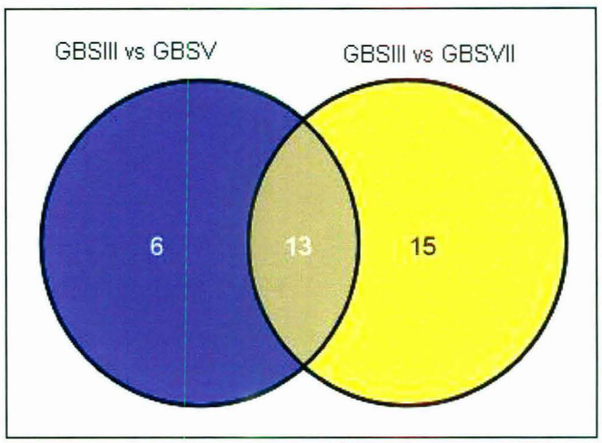

Fig 4.30: Venn diagram depicting up-regulated genes in GBS III vs others

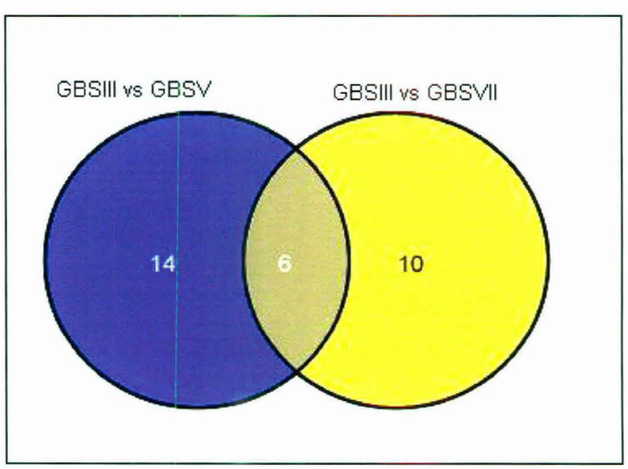

Fig 4.31: Venn diagram depicting down-regulated genes in GBSIII vs others

Among these common genes gbpB was highly up-regulated in GBS III, i.e., 37.4. folds as compared to type 1a. Expression of PI-2, i.e., gbs1478 was found highest in GBS III as compared to type 1a i.e. 22.2 folds (**Table 4.17**).

Genes for cpsH, rib and Pilus were the only genes that were common as well found up-regulated between GBS III and V compared to GBS Ia. Expression of rib (encoding surface anchored Rib protein involved in adhesion and cpsH encoding capsule and implicated to be involved in cell wall synthesis) were found more up-regulated in GBS III i.e. 2.0 and 55.5 fold respectively when compared with GBS Ia (**Table 4.18**).

Genes gbpB, plr/gapA and eno encoding glucan binding protein, streptococcal plasmin recptor/GAPDH and streptococcal enolase were found commonly up-regulated between III and VII when compared with GBS Ia. The expression of plr/gapA and eno (both are glycolutic enzymes) were found more up-regulated in i.e. 2.8 folds in GBS VII as compared to 1a but gbpB was more up-regulated in GBS III i.e. 37.4 folds (involved in adherence) as compared to 1a **(Table 4.19)**.

Transcript of genes eno, PI-2 and cpsI encoding streptococcal enolase, Pilus island 2 and capsule was found commonly up-regulated between GBS V and VII when compared with Ia. Expression of PI-2 encoding pilus protein involved in pili synthesis was found more up-regulated i.e. 17.2 fold in GBS V and expression of gbpB was found more up-regulated i.e. 27.5 fold when GBS V and VII were compared against GBS Ia (**Table 4.20**).

Five genes commonly found down-regulated in GBS V and VII include cpsI, cpsH, scpA/scpB, lmb and fbsA as compared to Ia **(Table 4.21)**. Genes found commonly down regulated (n=4) in GBS III and VII includes cpsH, neuB, scpA/scpB and lmb as compared to Ia **(Table 4.22)**. Some of the genes that were exclusively up-regulated in GBS III include cpsF, psaA (agglutinin receptor) and PI-2. Gene coding for agglutinin receptor implicated to be involved in adhesion and aggregation was found 30.7 fold up-regulated (**Table 4.23).** Gene of Pilus island PI-2 that was exclusively and differentially expressed in GBS V i.e. SAG1407 was found 21.4 fold up-regulated (**Table 4.24**).

Genes exclusively found in GBS VII include Pilus island 1,hylB encoding Hyaluronidase, cpsL and neuA encoding capsule and tig/ropA and cfa/cfb encoding CAMP. Expression of gene neuA for capsule implicated to be involved in cell wall synthesis was found 2.3 folds up-regulated (**Table 4.25).**

Table 4.17: Gene commonly up-regulated in GBS type III , V and VII compared to GBS type Ia (n=2)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0055	Glucan binding protein	gbpB
2	Ca031#0064	Pilus island 2	-

Table 4.18: Gene commonly up-regulated in GBS type III and V compared to GBS type Ia (n=7)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
2	Ca031#0060	Pilus island 2	-
3	Ca031#0061	Pilus island 2	-
4	Ca031#0062	Pilus island 2	-
6	Ca031#0130	capsule	cpsH
7	Ca031#0138	Rib	rib

Table 4.19: Gene commonly up-regulated in GBS type III and VII compared to GBS type Ia (n=3)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0055	Glucan binding protein	gbpB
2	Ca031#0009	Streptococcal plasmin receptor	Plr/gapA
3	Ca031#0011	Streptococcal enolase	eno

Table 4.20: Gene commonly up-regulated in GBS type V and VII compared to GBS type Ia (n=3)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0026	capsule	-
2	Ca031#0064	Pilus island 2	-
3	Ca031#0055	Glucan binding protein	gbpB

Table 4.21: Gene commonly down-regulated in GBS type V and III compared to GBS type Ia (n=5)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0019	capsule	cpsI
2	Ca031#0020	capsule	cpsH
3	Ca031#0034	C5a peptidase	scpA/scpB
4	Ca031#0054	Laminin binding protein	lmb
5	Ca031#0106	Fibronectin binding protein	fbs A

Table 4.22: Gene commonly down-regulated in GBS type III and VII compared to GBS type Ia (n=5)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0015	capsule	neuB
2	Ca031#0020	capsule	cpsH
3	Ca031#0034	C5a peptidase	scpA/scpB
4	Ca031#0054	Laminin binding protein	lmb
5	Ca031#0129	capsule	-

Table 4.23: Gene exclusively up-regulated in GBS type III compared with V and VII (n=4)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0022	capsule	cpsF
2	Ca031#0032	Pneumococcal surface antigen A / Metal binding protein SloC	psaA
3	Ca031#0104	agglutinin receptor	-
4	Ca031#0117	Pilus island 2	-

Table 4.24: Gene exclusively up-regulated in GBS type V compared with III and VII (n=5)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0018	Capsule	-
2	Ca031#0063	Pilus island 2	-
3	Ca031#0128	Capsule	cpsJ
4	Ca031#0129	capsule	cpsO
5	Ca031#0132	capsule	cpsH

Table 4.25: Gene exclusively up-regulated in GBS type VII compared with III and V (n=8)			
S.No	**Locus name on Chip**	**Gene product name**	**Gene**
1	Ca031#0004	Pilus island 1	-
2	Ca031#0010	Hyaluronidase	hylB
3	Ca031#0012	capsule	neuA
4	Ca031#0016	capsule	cpsL
5	Ca031#0029	Alpha C protein	bca
6	Ca031#0033	C3-degrading protease	cppA
7	Ca031#0036	Trigger factor	tig/ropA
8	Ca031#0049	CAMP factor	cfa/cfb

Discussion

Group B Streptococcus (GBS), or *S.agalactiae* is a gram positive facultative anaerobe and is a leading cause of pneumonia, septicaemia and meningitis in neonates and responsible for significant morbidity and mortality in pregnant women and immune-compromised adults (Dermer et al, 2004; Edward and Baker, 2005). The prevalence of GBS serotypes fluctuate over time and by geographical location. Heterogeneity in serotypes distribution pattern of GBS is a great hindrance in development of universal vaccine against it (WHO, 2005; Johri et al, 2006). A lot of efforts have been made on GBS serotyping in developed countries, but very less information is available in developing countries like India. Although there is significant geographical variation in the proportion of women colonized with GBS, the range of colonization reported from developing countries i.e., India is similar to that identified in populations studied in the United States (Stoll and Schuchat, 1998). Before the introduction of vaccines, extensive epidemiological studies will be required to assess not only the burden of disease but also the distributions of GBS serotypes to determine the optimal formulations of vaccine antigens (Harrison et al, 1998). Therefore for the present Ph.D. work an initiative was launched to do epidemiological study of GBS in Northern India by collecting samples from hospitals and pathological laboratories from Delhi and surrounding areas. In this study total 250 samples were collected from Delhi and surrounding areas to have an idea about the incidence of GBS in the population of northern region of India. All Samples were plated on blood agar plates to check the β-hemolytic pattern. Sample found β-hemolytic were tested as gram positive.

For further identification we narrowed down the number of GBS to 30 based on Catalase, CAMP and gram stain tests. CAMP test was conducted as GBS produces extracellular diffusible protein CAMP factor (most other hemolytic streptococci do not produce CAMP factor) which interacts with Staphylococcal beta-hemolysin on sheep RBC and shows enhanced lytic zone (Christie et al, 1986). We have also observed enhanced lytic zone in all the 30 GBS positive samples. Catalase test was also done and we found that all 30 GBS were catalase negative. GBS positive samples were finally confirmed by using Streptx identification kit for the identification of streptococci in human samples. Positive agglutination reaction on mixing latex coated

with the GBS cell wall carbohydrate specific antibody with the sample confirms it as GBS positive. In the present work, incidence of GBS among the samples collected from urine, vagina, pus, semen was found 12 % (30 samples was found as GBS positive out of 250) as it was also reported in previous study that in United States of America, approximately 10-30 % of pregnant women are colonized with GBS in the vagina or rectum (Regan et al, 1991; CDC, 2002) therefore we conclude that rate of GBS incidence is similar in India and USA and in turn support our data on incidence report. Moreover according to WHO, vaginal colonization of GBS has been reported to occur in about 12–27 % of women in North Africa, Middle East, Pakistan, Thailand, Saudi Arabia and the US (WHO, 2005). Although we also detected Group C, D, F and G other than B in the samples but the incidence of other groups were less in comparison to GBS therefore not included in this study.

In a study done on 507 pregnant Indian women, 12 % were reported to have GBS isolated from the throat and vagina, and 10 % had positive vaginal cultures alone (Dalal et al, 1998). Similarly, another study showed the overall carriage rate in pregnant women was 16 % (Chaudhary et al, 1981). There are few reports which revealed that GBS harmlessly colonizes vaginal and gastrointestinal tract in 50 % of the healthy adults (Schuchat, 1998) and the late-onset GBS infection occurs in infants up to 7 months of age and a high incidence (~50 %) of meningitis (Baker and Edwards, 2001). In a more recent studies it has been reported that the incidence of GBS disease in neonates is decreasing and the rate in non-pregnant adults appears to be increasing, with an overall increase of 32 % between 1999 and 2005 (Phares et al, 2008). A recently published study of surveillance data from 10 states in USA found that the incidence of GBS infection in persons aged 15-64 years increased from 3.4 per 100,000 population in 1999 to 5 per 100,000 in 2005. In adults aged 65 years and older, the incidence increased from 21.5 per 100,000 population in 1999 to 26 per 100,000 in 2005 (Phares et al, 2008).

To study the distribution pattern of GBS serotypes, 30 samples were found as GBS positive were further subjected to serotyping by using the GBS typing antisera kit (Denka Seiken Kit) to study the distribution pattern of GBS serotypes. Capsular serotyping has been one of the mainstays in the descriptive epidemiology of GBS. GBS have been classified into nine serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII) on the basis of immunological specificity of cell wall capsular polysaccharide (CPS)

present on its surface which is considered as a major virulence factor and main component of glycoconjugate vaccine (Paoletti et al, 2002). Any GBS isolate for which no serotype could not be assigned by using typing antisera kit were recorded as Nontypeable (NT). A considerable number of GBS isolates were designated as NT in a previous study (Gherardi et al, 2007). In the present work, we also observed that NT accounted for 23% of the all serotypes. In our study type Ia (23 %) was found most prevalent followed by GBS III (16 %). Other serotypes were 16 % type II, 6% type V, 13 % type VII. Serotyping of GBS indicates the heterogeneous distribution pattern of GBS in Delhi and surrounding areas. Maximum number of GBS isolates was isolated from urine samples. This study reported the prevalence of GBS Ia followed by GBS III. Previous Studies from India show a variable distribution of serotypes, but the most common isolates belong to types III, II and Ib (Kuruvilla et al, 1999). Comparison of the distribution of serotypes identified in previous studies with that of the present study showed a great increase in GBS Ia. Ongoing monitoring of the distribution of GBS serotypes is important for charting changes in serotype prevalence.

The differences in serotype distribution among various populations also may reflect differences in pathogenesis among the serotypes (Mikamo et al, 2004). Adherence of bacteria and their invasion into host tissue is mediated through a complex series of events that involve changes in surface constituents. Stages of pathogeensis involve colonization and adherence to epithelial cells that allow GBS to invade the cells and cause disease in neonates and adults. Several studies has been done in order to elucidate the GBS pathogenesis with different epithelial cells (Johri et al, 2003; Johri et al, 2007; Tamura & Nittayajarn 2000; Mikamo et al, 2004; Nizet et al, 1997; Rubens et al, 1992).

We also compared the adherence and invasion of prevalent GBS serotypes Ia, III and less prevalent GBS serotypes V and VII. To fulfill this objective two cell lines A549 (Human alveolar epithelial cell line) and ME-180 (Human cervix epithelial cell line) were selected. Adherence and invasion of GBS type Ia and III was compared with GBS type V and VII to study their invasiveness using conventional method (Malin et al, 2001).

We have found that with the A549 cells a maximum of 2.0 and minimum 0.08 % invasion was found by type Ia and type V respectively. However with ME-180

cells maximum invasion shown by type Ia and III i.e. 2% and 1.8 % respectively followed by type V i.e. 1.6%. A minimum of 0.07 % invasion has been found by type VII with ME-180 cells as compared to other serotypes. Similarly adherence was carried out with both the cell line. GBS Ia shows highest adherence with A549 cells i.e., 2.3 % as compared to other serotypes, however minimum adherence has been shown by type VII with ME-180 cells i.e., 0.5 %. Interaction assay results indicates that GBS Ia was found as the most invasive as well as most adhering when compared with other serotypes of GBS followed by GBS III.

During interaction process certain changes in the surface proteome *vis a vis* gene expression profile of host and GBS may influence the invasive potential of GBS. Therefore we emphasize on differential expression of genes, the blueprints of surface proteins and virulence factors of GBS in this study, we compared the expression of virulence genes in invasive i.e. Ia and III with less invasive GBS serotypes i.e. V and VII by using DNA microarray and comparative genomics. To conduct DNA microarray, total 66 genes were targeted because these genes either reported or predicted to be involved in pathogenesis of GBS. The expression profiles of these genes in case of GBS III, V and VII were compared against most invasive GBS Ia.

Microarray data showed that out of 66 genes, 14 were up-regulated **(Table 4.5)** and 20 were down-regulated **(Table 4.6)** in GBS type III in comparison to GBS Ia. In GBS III maximum up-regulation was found 71.6 fold for the gene cpsM. CpsM Among the up-regulated genes, we identified clusters of pilus island 2 (PI-2) including gbs 1474, gbs 1475, gbs 1476, gbs1478 (PI-2) codes for pilus-like structures (Rosini et al, 2006) that extend out from the surface of the bacteria and helps in adherence of bacteria to host cell. As pilus islands have been reported in the attachment process and in turn make the GBS invasive in nature and we also observed same pilus island in GBS III (from this study) was found up-regulated therefore we predict that as this GBS III also reported as second highest invasive serotype in this study may be this pilus island helping the GBS III a invasion process. We also observed that among cluster of pilus island, gbs 1478 shows highest expression i.e. 22.2.fold as compared to other pilus gene. GBS PilA homologue (GBS 1478) promoted adhesion to human pulmonary epithelial cells (Dramsi et al, 2006). However, the role of the PilB protein in GBS disease progression and survival within the host has not been characterized. Recent study shows that pilus protein expression

by GBS could also play a role in the bacterium's initial interactions with hBMEC (Maisey et al, 2007). Increase in treanscript level PI-2 gene in GBS III from this study can be associated with increase virulence of GBS III. Glucan binding protein B (GbpB) was found 37.4 fold up-regulated in GBS III from this study. This protein has been reported as an immunologically dominant protein (Mattos-Graner et al, 2006). Its biological function is unclear, although GbpB shares homology with a putative peptidoglycan hydrolase from *S.pneumoniae*, indicative of a role in murein biosynthesis (Mattos-Graner et al, 2006). GbpB function is essential for growth and thus it was concluded that *gbpB* is a vital gene (Mattos-Graner et al, 2006). Another gene plr/gapA (GAPDH) was also found up-regulated in GBS III. Madureira et al, (2007) recently shown that GAPDH is virulence associated protein at the bacterial surface that interacts with the Fibrinogen and plsaminogen. We may conclude that differential up-regulation of all these virulence gene may be responsible for virulence of GBS III.

Increased expression of PI-2 in GBS III may be responsible for its invasive nature. Many of these GBS–host-cell interactions involve attachment of the bacterium to extracellular matrix (ECM) molecules such as agglutinin, fibronectin, fibrinogen and laminin, which in turn bind host-cell-surface proteins such as integrins. We observed the expression of agglutinin receptor was highly up-regulated i.e. 30.7 fold in type III as compared to 1a. This agglutinin protein is involved in adhesion and aggregation of GBS on the host cell surface and contributes to its virulence (Prakobphol et al 2000). Some of the genes that were exclusively up-regulated in GBS III include cpsF, psaA agglutinin receptor and PI-2. Gene coding for agglutinin receptor implicated to be involved in adhesion and aggregation **(Table 4.23).** We hypothesize that these up-regulated genes are contributing towards the invasive nature of GBS III.

Our comparative microarray data showed that out of 31 genes, 15 were up-regulated **(Table 4.7)** and 16 were down-regulated **(Table 4.8)** in GBS type V in comparison to GBS Ia. Among the 15 up-regulated genes in GBS V we identified clusters of PI-2 and capsule gene was 21.4, 71.6 fold up-regulated respectively. In addition, up-regulation gbpB i.e., 23.2 fold was also observed. Increased expression of capsule may also be responsible for the virulent nature of GBS as cps expression on GBS surface helps it evade the human immune response. Expression of CPS by

GBS, is not constitutive but varies during growth in vitro and in primary cultures isolated from different sites of infection (Paoletti et al, 1996).

Microarray analysis of GBS V compared with GBS Ia reveals total 15 genes were up-regulated **(Table 4.7)**. Transcript level of three genes encoding protein involved in binding were higher i.e. SAG1405, SAG1406, SAG1407,SAG1408 encoding pilus island (PI-2), SAG1164, SAG1165, SAG1166, SAG1167, SAG1168 encoding capsule and gbpB encoding glucan binding protein homologous to peptidoglycan hydrolase from *S. pneumonia.* Other up-regulating genes includes cpsJ, cpsO, cpsN,cpsM, cpsH involved in cell wall synthesis as reported by Cieslewicz et al, (2001), α like proteins (alp2) and Rib (rib) a surface anchored protein involved in attachment of bacteria to the host cell (Maeland et al., 2004, Larsson et al., 2004). Gene that are highly expressed in GBS V mainly involves adhesion proteins. We can conclude that increased production of adhering molecule may be responsible for its increased virulence. We observed that gene of pilus island PI-2 that was exclusively and differentially expressed in GBS V i.e. SAG1407 was found 21.4 fold up-regulated. Several down-regulating genes were also noticed/reported in type V as compared to 1a. These include Pilus island (PI-1), SAG0032, encoding surface immunogenic protein, SAG1730 encoding C3 degrading protease, SAG1236 encoding C5 peptidase, SAG2174 encoding Serine protease, and 0105 encoding Trigger factor. As in the present study we observed that type V was less invasive as compared to type 1a. As mentioned surface immunogenic protein, serine protease were found down regulated therefore making this type V less invasive as compared to type 1a.

Microarray data revealed that 16 genes were up-regulated **(Table 4.9)** in GBS type VII in comparison to GBS Ia. Transcript level of gene lmb encoding laminin binding protein was found highly up-regulated (27.5 folds) in GBS VII as compared to GBS Ia. It has been reported that binding of GBS to human laminin is mediated by the lipoprotein Lmb, which has been studied on the molecular level by Spellerberg et al, (1999). As this gene was found up-regulated in type VII we hypothesize that this gene is helping the GBS VII in adherence process. In case of GBS VII transcript level of gene cspK in cps locus of GBS down-regulated as compared GBS Ia. cpsK has been reported to play a role in sialylation of sialic acid residue on GBS surface and lowers the deposition of C3 on GBS surface and aids in evasion of host immune

response. Decreased expression of this gene may attribute to its less virulence compared to GBS Ia.

In case of GBS VII, plr/gap genes which encode streptococal plasmin receptor/GAPDH and important for evasion of GBS from host immune response was found 2.8 fold up-regulated. C5 peptidase encoded by scpA/scpB was predicted to be involved in inactivation of human C5a (Bohnsack et al, 1991) resulting evasion from host immune system. Genes fbsA, fbsB coding for fibronectin binding protein plays an important role in adherence/invasion of eukaryotic cells (Ragunathan et al, 2009).

Microarray analysis showed that out of 39 genes, 19 were up-regulated **(Table 4.11)** and 20 were down-regulated **(Table 4.12)** in GBS type V in comparison to GBS III. Among the capsule encoding gene cpsM was found to be up-regulated to 71.76 folds, Other up-regulating genes include cluster of pilus island (PI-1) SAG0646, involved in the attachment of GBS to host cell (Rosini et al, 2006). We have observed that these pilus proteins were 31.9 fold up-regulated in type V as compared to type III. Other genes neuD, neuC, neuB, cpsJ, cpsO, cpsM and cpsH which encode capsule and cell wall synthesis have been observed up regulated in type V as compared to type III. Additionally we also observed the up-regulation of scpA/scpB which encode C5a peptidase and this gene has been reported in inactivation of Human C5 and therefore its role in evasion from the host immune system has been suggested by Bohnsack et al, (1991). We also observed the up-regulation of cylD and acpP genes. These genes encodes pore forming toxin i.e, β-hemolysin/cytolusin (Hensler et al, 2008).

Microarray analysis of GBS V reveals 15 down-regulating genes. Important down-regulated genes in type V as reported in this study are hylB, eno, neuA, bca, sip and cppA as compared to GBS III. These genes were reported to involved in persistence and colonization of GBS to host cells, in cell wall synthesis, adherence, helps GBS to escape from ingestion and killing by PMN.

Comparative genomics between GBS type VII and III reveals that a total of 19 genes were up-regulated **(Table 4.13)** and 20 were down-regulated **(Table 4.14)** in GBS type VII in comparison to GBS III. Up-regulating genes mainly include gbs 0629, gbs0631, gb0032 which encode Pilus islands 1 and 2 (PI1 and PI-2) and have been reported to be involved in pilus formation (Lauer et al, 2005; Rosini et al, 2006).

We also observed other important genes i.e, plr/gapA up-regulated. These genes encode streptococcal plasmin receptor/GAPDH that confers resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006). We also observed the up-regulation of hylB that encodes hyaluronidase which helps in degradation of hyaluran and persistence colonization of GBS (Sukhnanand et al., 2005). Capsule forming genes ie, neuA, neuD, neuC, neuB, cpsM, cpsE, cpsC were also reported up-regulated (Cieslewicz et al, 2001). α and β component of C-protein encoding genes, bca and cba were predicted to be involved in virulence by playing a role in adhesion (Baron et al, 2004; Lindahl, 2005) were also found up-regulated in type VII as compared to type III . We also observed the up-regulation of cylJ, cylX , cylD, cylC, cylF, cylI which encode pore forming toxic β-hemolysin/cytolysin protein (Hensler et al, 2008a; Hensler et al, 2008b).

Down-regulating genes mainly includes pneumococcal surface antigen A/ Metal binding protein SloC belongs to the lipoprotein receptor antigen I family (LraI) (Kitten et al, 2000; Lawrence et al, 1998) and is contained within an operon that encodes an ATP-binding cassette (ABC) transport system. All clusters of pilus encoding genes (PI-2) (Rosini et al, 2006) were found downregulated in GBS VII as compared to type III. Rib encoding Rib protein, a GBS surface protein involved in adherence (Larsson et al, 2004) of GBS was also found down-regulated in type VII as compared to III.

Microarray analysis showed that 28 were up-regulated **(Table 4.15)** and 21 were down-regulated **(Table 4.16)** in GBS type VII in comparison to GBS V. Out of 21 up-regulating genes single gene of pilus island 1 (PI-1) and single gene of pilus island 2 (PI-2) (Lauer et al, 2005; Rosini et al, 2006) encoding for pilus protein of GBS were found up-regulating. Hyaluronidase encoded by gene hylB helps in degradation of hyaluran and persistence colonization of GBS, plr/gapA encoding streptococcal plasmin receptor/GAPDH that confers resistance against reactive oxygen species produced by host phagocytic cells (Madureira et al, 2006), bca and cba encoding α and β component of C-protein that helps in interaction of GBS surface with the host cell (Baron et al, 2004; Lindahl, 2005), cppA encoding C3 degrading protease that helps in evasion from host immune system (Rainard and Boulard, 1992), cylX, cylG, cylB, cylF, cylI,cylJ encoding clusters of β-hemolysin toxin protein

involves in pore formation and invasion of GBS in to the host cell (Hensler et al, 2008) were found up-regulating in GBS VII.

Down-regulating genes in GBS VII involve clusters of Pilus island 2 (PI-2). The expression of surface adhesion molecules i.e. rib and lmb encoding Rib and laminin binding protein was also found down-regulating.

By studying the comparative transcriptional profiling of GBS Ia, III, V and VII, we have found that genes have been reported previously involved either in invasion or adherence are also found up-regulated in invasive serotypes like type III and 1a from this study.

We found that 2 genes i.e., gbpB and PI-2 were commonly up-regulated in GBS III,V and VII as compared to type 1a (**Table 4.17**). Glucan binding proteins shows several fold up-regulation followed by up-regulation of Pilus island 2. In GBS V also shows increased expression of Pilus island 2. These genes can further be targeted at protein level to check the inhibition and may serve as potential vaccine candidates. Recently it has been shown that pilus island in each GBS strain are conserved and all strain carried at least 1 of the 3 island, and a combination of three pilus component conferred protection against all tested GBS strains (Rosini et al, 2006).

Additionally several other genes involved in evasion of GBS from host immune response, helps GBS in evasion from host immune system and adherence/invasion of eukaryotic cells, capsule formation and have been observed up-regulated in selected invasive serotype i.e., type III, 1a from this study. We suggest that these genes can be targeted as vaccine candidate against GBS of Indian origin. It can be concluded that GBS is a leading pathogen in the world and improved vaccine is essential to eradicate it. Present study is the first and comprehensive effort to reveal the distribution pattern of GBS in north Indian population. GBS type Ia was found as the most prevalent and invasive serotype among all. Findings of DNA microarray analysis by doing comparative genomics (using a chip having 66 genes which encodes virulence factors) also supported that virulent genes were up-regulated in GBS type Ia and III in comparison to other serotypes. These up-regulated genes may further be tested as potential vaccine candidates to develop and improve the vaccine against GBS.

Summary

Group B *Streptococcus* (GBS), also referred to as *Streptococcus agalactiae*, is a Gram-positive, β–hemolytic opportunistic pathogen that colonizes the gastrointestinal and genitourinary tracts of up to 50 % of healthy adults. The pathogenesis of neonatal GBS infection begins with the asymptomatic colonization of the female genital tract. Approximately 20–30 % of healthy women are colonized rectovaginally with GBS, and 50–70 % of infants born to these women will themselves become colonized with the bacterium and at risk of developing sepsis, pneumonia and meningitis. Centers for Disease Control (CDC, USA) issued guidelines recommending antibiotic treatment before birth for babies at high risk. However, GBS still remains a leading cause of sepsis and meningitis in newborns, as well as of severe invasive diseases in immune-compromised adults.

GBS are differentiated from other β-hemolytic streptococci by Lancefield serological typing. GBS have been classified into nine serotypes (Ia Ib, II, III, IV, V, VI, VII and VIII) on the basis of immunological specificity of cell wall capsular polysaccharides present that are antigenically and structurally unique. Recently type IX serotype of GBS has also been identified (Slotved, 2007). Some isolates may be reported as Nontypeable (NT) due to the expression of an uncharacterized polysaccharide for which antibodies are not yet available.

The present study was designed to compare the transcription profiles of prevalent GBS serotypes circulating in north Indian human population. For this purpose, epidemiological study of GBS was conducted and total of 250 samples were collected from different pathological laboratories and hospitals of Delhi and surrounding areas. GBS isolates were identified using gram staining, catalase and CAMP test. Out of total 250 samples 30 samples (12 %) were identified as GBS. All 30 samples were found gram positive, catalase negative and CAMP positive. Streptex agglutination test was further used for the confirmation of GBS in 30 samples.

In order to figure out the serotypes distribution pattern of different GBS serotypes, serotyping was carried out by using Denka Seiken Kit (Japan). GBS was isolated more frequently from urine (20 positive cultures) than vaginal swabs (2 positive). Some of the samples were not assigned any serotype and designated as nontypable.

In epidemiological part of this study we found GBS type Ia most prevalent followed by type III, V and VII. These serotypes were further selected to compare the invasiveness by interacting with A549 and ME-180 cell lines. To start with invasion assays, growth pattern of all these selected serotypes were standardized at OD_{650nm} of 0.5 i.e. exponential phases. Growth curve of all four GBS serotypes shows same pattern for lag, log and stationary phases and this growth of serotype has no correlation which serotypes it belongs. In case of type V and VII time taken to attain log phase is less as compared to time taken by type Ia and III. In exponential phase growth of GBS type V and VII faster as compared to growth of type Ia and III. Around 5 h, we have observed that in all four cases decline phase has started. CFU for GBS Ia, III, V and VII was calculated at OD_{650nm} 0.5 and were found $8x10^6$, $8x10^6$, $8x10^7$ and $2.3x10^8$ respectively.

Both the human cells were maintained 37°C in 5 % CO_2 incubator in healthy condition. The viability assay was done by using trypan blue exclusion method and cells were counted using hemocytometer. The count of A549 cells (confluency ~ 95%) per dish (60 mm) was 4.5×10^6 and for ME-180 cells (confluency ~95%), it was 2.1×10^6 per dish (60 mm). Both the cells took 5-8 days to reach confluency.

The adherence and invasion of the human epithelial cell lines A549 and ME-180 by prevalent GBS serotypes Ia and III were compared with those of less prevalent serotype V and VII by a conventional invasion assay. In our samples type Ia showed maximum invasion and adherence followed by type III with both A549 and ME-180 cell lines.

Our comparative genomics analyses using DNA microarray reveals the up-regulation of several important genes that are involved in evasion of GBS from host immune response, helps GBS in evasion from host immune system and adherence/invasion of eukaryotic cells, capsule formation in selected invasive serotypes i.e., type III, 1a from this study. We suggest that these genes can be targeted as vaccine candidate against GBS of Indian origin as currently available vaccine preparation may not work against the currently circulating GBS serotypes from India. As GBS is one of the leading causes of neonatal sepsis, pneumonia and meningitis therefore we suggest that we should develop an indigenous vaccine or a universally effective vaccine against this bacterium.

References

Adderson, E.E., Takahashi, S., Wang, Y., Armstrong, J., Miller, D.V. Bohnsack, J.F. 2003. Subtractive hybridization identifies a novel predicted protein mediating epithelial cell invasion by virulent serotype III group B *Streptococcus agalactiae*. Infect. Immun. 71:6857–6863.

Amin, A., Abdulrazzaq, Y.M., Uduman, S., 2002. Group B Streptococcal serotype distribution of isolates from colonized pregnant women at the time of delivery in United Arab Emirates. J. Infect. 45:42–46.

Andrews, J.I., Diekema, D.J., Hunter, S.K., Rhomberg, P.R., Pfaller, M.A., Jones, R.N., Doern, G.V., 2000. Group B streptococci causing neonatal bloodstream infection: antimicrobial susceptibility and serotyping results from SENTRY centers in the Western Hemisphere. Am. J. Obstet. Gynecol. 183:859-862.

Arakere, G., Flores, A.E., Ferrieri, P., Fraschi, C.E., 1999. Inhibition enzyme-linked immunosorbent assay for serotyping of group B Streptococcal Isolates. J. Clin. Microbiol. 37(8):2564-2567.

Baker C.J., Edwards M.S., 2003. Group B Streptococcal conjugate vaccines. Arch. Dis. Child. 88:375-378.

Baker, C.J., Barrett, F.F., 1974. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA. 230:1158-60.

Baker, C.J., Edward, M.S., Kasper, D.L., 1978. Immunogenicity of polysaccharides from type III group B Streptococcus. J. Clin. Invest. 61:1107-1110.

Baker, C.J., Edwards, M.S., 1991. Group B Streptococcal infections, In: J. S. Remington and J. Klein (ed.), Infectious diseases of the fetus and newborn infant. W. B. Saunders Co., Philadelphia. p. 820-881.

Baker, C.J., Edwards, M.S., 2001. Group B Streptococcal infections. In: Infectious diseases of the fetus and newborn infant. Remington, J.S., and Klein, J.O. (eds). Philadelphia, PA: W.B. Saunders, 1091–1156.

Baker, C.J., Kasper, D.L., 1985. Group B Streptococcus vaccines. Rev. Infect. Dis. 7:458-467.

Baker, C.J., Rench, M.A., Edward, R.J., Carpenter, R.J., Hays, B.M., Kasper, D.L., 1988. Immunization of pregnant women with a polysaccharide vaccine of group B Streptococcus. N. Eng. J. Med. 319:1180-1220.

Baker, J.R., Pritchard, D.G. 2000. Action pattern and substrate specificity of the hyaluronan lyase from group B Streptococci. Biochem. J. 348(2):465-471.

Baron, M.J., Bolduc, G.R., Goldberg, M.B., Auperin, T.C, Madoff, L.C., 2004. Alpha C protein of group B Streptococcus binds host cell surface glycosaminoglycan and enters cells by an actin-dependent mechanism. J. Biol. Chem. 279(23):24714-24723.

Baron, M.J., Filman, D.J., Prophete, G.A., Hogle, J.M., Madoff, L.C., 2007. Identification of a glycosaminoglycan binding region of the alpha C protein that mediates entry of group B Streptococci into host cells. J. Biol. Chem. 282:10526–10536.

Bennett, P.R., Rose, M.P., Myatt, L., and Elder, M.G., 1987. Preterm labor: stimulation of arachidonic acid metabolism in human amnion cells by bacterial products. Am. J. Obstet. Gynecol. 156:649–655.

Benson, J.A., Ferrieri, P., 2001. Rapid pulsed-field gel electrophoresis method for group B Streptococcus isolates. J. Clin. Microbiol. 39:3006-3008.

Bernheimer, A.W., Linder, R., Avigad L.S. 1979. Nature and mechanism of action of the CAMP protein of group B streptococci. Infect. Immun. 23:838-844.

Bevanger, L. 1983. Ibc proteins as serotype markers of group B Streptococci. Acta Pathol. Microbiol. Iramunol. Scand. Sect. 91:231.

Bevanger, L., Naess, A.I., 1985. Mouse-protective antibodies against the Ibc proteins of group B Streptococci. Acta. Pathol. Microbiol. Immunol. 93:121–124.

Blumberg, H.M., Stephens, D.S., Licitra, C. Pigott, N., Facklam, R., Swaminathan, B., Wachsmuth, I.K., 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), J. Infect. Dis. 166:574-579.

Bohnsack, J.F., Widjaja, K., Ghazizadeh, S., Rubens, C.E., Hillyard, D.R., Parker, C.J., Albertine, K.H., Hill, H.R., 1997. A role for C5 and C5a-ase in the acute neutrophil response to group B Streptococcal infections. J. Infect. Dis. 175:847–855.

Bohnsack, J.F., Mollison, K.W., Buko, A.M., Ashworth, J.C., Hill, H.R., 1991. Group B Streptococci inactivate complement component C5a by enzymic cleavage at the C-terminus. Biochem. J. 273: 635-640.

Bolduc, G.R., Baron, M.J., Gravekamp, C., Lachenauer, C.S., Madoff, L.C., 2002. The alpha C protein mediates internalization of group B Streptococcus within human cervical epithelial cells. Cell. Microbiol. 4:751-758.

Bolduc, G.R., Baron, M.J., Gravekamp, C., Lachenauer, C.S., Madoff, L.C., 2002. The alpha C protein mediates internalization of group B Streptococcus within human cervical epithelial cells. Cell. Microbiol. 4(11):751 – 758.

Boyer, K.M., Gadzala, C.A., Kelly, P.D., Burd, L.I., Gotoff, S.P., 1983. Selective intrapartum hemoprophylaxis of neonatal group B Streptococcal early-onset disease. II. Predictive value of prenatal cultures. J. Infect. Dis. 148:802-809.

Brigtse, A.K., Kasper, D.L., Baker, C.J., Jennings, H.J., Guttormsen, H.K., 2002 Induction of cross-reactive antibodies by immunization of healthy adults with type Ia

and Ib group B streptococcal polysaccharidetetanus toxoid conjugate vaccines. J. Infect. Dis.185:1277-84.

Brigtsen, A.K., Kasper, D.L., Baker, C.J., Jennings, H.J., Guttormsen, H.K., 2002. Induction of cross-reactive antibodies by immunization of healthy adults with type Ia and Ib group B Streptococcal polysaccharide tetanus toxoid conjugate vaccines. J. Infect. Dis. 185; 1277-84.

Brimila, B., Barthellc, E., Heindrichsd, U., Kuhne, M., Luttickena, R., Spellerberge, B., 2006. Epidemiology of *Streptococcus agalactiae* colonization in Germany. Int. J. Med. Microbiol. 296(1):1473-1483.

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

Brodeur, B.R., Boyer, M., Charlebois, I., Hamel, J., Couture, F., Rioux, R.C., Martin, D., 2000. Identification of group B Streptococcal Sip protein, which elicits cross-protective immunity. Infect. Immun. 68:5610–5618.

Brodeur, B.R., Boyer, M., Charlebois, I., Hamel, J., Couture, F., Rioux, C.R., Martin, D.R., 2000. Identification of group B Streptococcal Sip protein, which elicits cross-protective immunity. Infect. Immun. 68(10):5610–5618.

Bulgakova, T.N., Grabovskaya, K.B., Ryc, M., Jelinkova, J., 1986. The adhesin structures involved in the adherence of group B streptococci to human vaginal cells. Folia Microbiol. (Praha). 31:394-401.

Butler, K.M., Baker, C.J., Edwards, M.S., 1987. Interaction of soluble fibronectin with group B Streptococci. Infect. Immun. 55(10):2404-2408.

Campbell,J.R., Hillier, S.L., Krohn, M.A., Ferrieri, P., Zaleznik, D.F., Baker, C.J., 2000. Group B streptococcal colonization and serotype-specific immunity in pregnant women at delivery.Obstet. Gynecol. 96(4):498-503.

Carlemalm, S.M., Areschoug, T., Larsson, C., Lindahl, G., 1999. The R28 protein of *Streptococcus pyogenes* is related to several group B Streptococcal surface proteins, confers protective immunity and promotes binding to human epithelial cells. Mol. Microbiol. 33:208– 219.

Carlemalm, S.M., Stenberg, L., Lindahl, G., 1993. Protein rib: a novel group B Streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. J. Exp. Med. 177:1593–1603.

Centers for Disease Control and Prevention. 2002. Prevention of perinatal group B Streptococcal disease: revised guidelines from CDC. Morb. Mortal. Wkly. Rep. 51:1-22.

Centers for Disease Control and Prevention. 2005. Early-onset and lateonset neonatal group B Streptococcal disease—United States, 1996–2004. Morb. Mortal. Wkly. Rep. 54:1205–1208.

Chaffin, O.C., Beres, B.S., Harry, H. Yim, H.H., Rubens, C. E., 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.

Chalifour, R.J., Michon, F., Jennings, H.J., Lacroix, M., 1991. Use of defined oligosaccharide epitopes in an ELISA for group B streptococcus. J. Immunol. Methods. 154(1):69-76.

Chaudhary, U., Sabherwal, U., Chugh, T.D., 1981. Prevalence of group B Streptococci in obstetrical cases. Indian J. Med. Res. 73:710-714.

Cheng, Q., Carlson, B., Pillai, S., Eby, R., Edwards, L., Olmsted, S.B., Cleary, P., 2001. Antibody against surface-bound C5a peptidase Is opsonic and initiates macrophage killing of group B Streptococci. Infect. Immun. 69(4):2302-2308.

Cheng, Q., Debol, S., Lam, H., Eby, R., Edward, L., Matsuka, Y., Olmsted, S.B., Cleary, P.P., 2002. Immunization with C5a peptidase or peptidase type III polysaccharide conjugate vaccines enhance clearance of Group B Streptococci from lings of infected mice. Infect. Immun. 70:2408-2413.

Chmouryguina, I.I., Suvorov, A.N., Carlson, B., Cleary, P., 1997. Structural and functional similarity of C5a-ase enzymes from group A and B Streptococci. Adv. Exp. Med Biol. 418:757–759.

Christie, R., Atkins, N. E., Munch-Petersen E., 1944. A note on the lytic phenomenon shown by group B Streptococci. Aust. J. Exp. Biol. Med. Sci. 22:197-200.

Cieslewicz, M.J., Kasper, D.L., Wang, Y., Wessel, M.R., 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(5):139-146.

Cotter, P.A., DiRita, V.J., 2000. Bacterial virulence gene regulation: an evolutionary perspective. Annu. Rev. Microbiol. 54:519–565.

Dalal, S., Lahiri, A., Parel, C.C., 1998. Carriage rate of group B Streptococci in pregnant women and evaluation of different isolation media. J. Indian Med. Assoc. 96:360-1366.

Daly, J.A., Seskin, K.C., 1988. Evaluation of rapid, commercial latex techniques for serogrouping beta-hemolytic streptococci. J. Clin. Microbiol. 26:2429–2431.

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

Deng, L., Kasper, D.L., Krick, P.T., Wessels, R.M., 2002. Characterization of the linkage between the type III capsular polysaccharide and the bacterial cell wall of group B Streptococcus. J. Biol. Chem. 275:7497–7504.

Dermer, P., Lee, C., Eggert, J., Few, B., 2004. A history of neonatal group B Streptococcus with its related morbidity and mortality rates in the United States. J. Pediatr. Nurs. 19:357–363.

Deuerling, E., Schulze-Specking, A., Tomoyasu, T., Mogk, A., Bukau, B. 1999. Trigger factor and DnaK cooperate in folding of newly synthesized proteins. Nature. 400:693–696.

Dillon, H.C., Khare S.J., Gray B.M., 1987. Group B Streptococcal carriage and disease: a 6-year prospective study. J. Pediatr. 110:31-36.

Donnenberg, M.S., Calderwood, S.B., Donohue, R.A., Keusch, G.T., Kaper, J.B., 1990. Construction and analysis of TnphoA mutants of enteropathogenic Escherichia coli unable to invade HEp-2 cells. Infect. Immun. 58:1565-1571.

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

Edward, M.S., Baker, C.J., 2005. Group B Streptococcal infections in elderly adults. Clin. Infect. Dis. 41:839–847.

Edward, M.S., Nizet, V., Baker, C.J., 2006. Group B Streptococcal infections. In: Infectious Diseases of the Fetus and Newborn Infant, 6th Edition. Eds: Remington, J., Klein, J., Baker, C.J., Wilson, C.B., Elselvier Saunders, Philadelphia. pp. 404-464.

Eickhoff, T.C., Klein, J.O., Daly, A.K., Ingall, D., Finland, M., 1964. Neonatal sepsis and other infections due to group B beta-hemolytic Streptococci. N. Engl. J. Med. 271:1221–1228.

Ekin, I.H., Gurturk, K., 2006. Characterization of bovine and human group B Streptococci isolated in Turkey. J. Med. Microbiol. 55:517–521.

Enright, M.C., Spratt, B.G., 1998. A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease. Microbiol. 144:3049–3060.

Eren, A., Kucukercan, M., Oguzoglu, N., Unal, N., Karateke, A., 2005. The carriage of group B Streptococci in Turkish pregnant women and its transmission rate in newborns and serotype distribution. Turk. J. Pediatr. 47:28–33.

Falkow, S., 1991. Bacterial entry into eukaryotic cells. Cell 65:1099-1102.

Federle, M.J., McIver, K.S., Scott, J.R., 1999. A response regulator that represses transcription of several virulence operons in the group A Streptococcus. J. Bacteriol. 181:3649–3657.

Ferretti,J.J., Ajdic, D., McShan, W.M., 2004. Comparative genomics of streptococcal species Indian. J. Med. Res.119:1-6.

Ferrieri, P., Cleary, P.P., Seeds, A.E., 1977. Epidemiology of group B Streptococcal carriage in pregnant women and newborn infants. J. Med. Microbiol. 10:103-114.

Ferrieri, P., Flores, A.E., 1997. Surface protein expression in group B Streptococcal invasive isolates, In: T. Horaud, A. Bouvet, R. Leclercq, H. de Montclos, and M. Sicard (ed.), Streptococci and the host. Proceedings of the 13th Lancefield International Symposium on Streptococci and Streptococcal Diseases. Plenum Press, New York, N.Y. pp. 635–637.

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

Franciosi, R.A., Larsen, J.W., Zimmerman, R.A., 1973. Group B Streptococcal neonatal and infant infections. J. Pediatr. 82:707–718.

Gardam, M.A., Low, D.E., Saginur, R., 1998. Group B Streptococcal necrotizing fasciitis and streptococcal toxic shock-like syndrome in adults. Arch. Intern. Med. 158:1704-1708.

Gase, K., Ferretti, J.J., Primeaux, C., McShan, W.M., 1999. Identification, cloning, and expression of the CAMP factor gene (*cfa*) of group A Streptococci. Infect Immun. 67:4725-4731.

Gautier, L., Cope, L. Bolstad, B.M. Irizarry, R.A., 2004. affy—analysis of Affymetrix GeneChip data at the probe level. Bioinform. 20:307–315.

Gibson,R.L., Lee, M.K., Soderland, C., Chi, E.Y., Rubens, C.E., 1993. Group B streptococci invade endothelial cells: type III capsular polysaccharide attenuates invasion.Infect. Immun. 61(2):478–485.

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

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

Graham, M.R., Smoot, L.M. Migliaccio, C.A. Virtaneva, K., Sturdevant, D.E. Porcella, S.F., Federle, M.J., Adams, G.J., Scott, J.R., Musser, J.M., 2002. Virulence control in group A Streptococcus by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. Proc. Natl. Acad. Sci. (USA). 99:13855–13860.

Gravekamp, C., Kasper, D.L.,Paoletti,L.C., Madoff, L.C., Alpha C Protein as a Carrier for Type III Capsular Polysaccharide and as a Protective Protein in Group B Streptococcal Vaccines. Infect. Immune. 67(5):2191-2496.

Gryllos, I., Levin, J.C. Wessels, M.R., 2003. The CsrR/CsrS two component system of group A Streptococcus responds to environmental. Mg^{2+}. Proc. Natl. Acad. Sci. (USA) 100:4227–4232.

Gutekunst, H., Eikmanns, B.J., Reinscheid, D.J., 2003. Analysis of RogB-controlled virulence mechanisms and gene repression in *Streptococcus agalactiae*. Infect. Immun. 71:5056–5064.

Guttormsen, H.K., Baker, C.J., Edwards, M.S., Paoletti, L.C., Kasper, D.L., 1996 Quantitative determination of antibodies to type III group B Streptococcal polysaccharide. J. Infect. Dis.173:142–150.

Haft, R.F., Wessels, M.R., Mebane, M.F., Conaty, N., Rubens, C.E., 1996. Characterization of *cpsF* and its product CMP-N-acetylneuraminic acid synthetase, a group B Streptococcal enzyme that can function in K1 capsular polysaccharide biosynthesis in *Escherichia coli*. Mol. Microbiol. 19:555-563.

Hansan, S. M., Uldbjerg, N., Kilian, M., Sorensen, U.B., 2004. Dynamics of *Streptococcus agalactiae* colonization in women during and after pregnancy and in their infants. J. Clin. Microbiol. 42:83–89.

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

Hauge, M., Jespersgaard, C., Poulsen, K., Kilian, M., 1996. Population structure of *Streptococcus agalactiae* reveals an association between specific evolutionary lineages and putative virulence factors but not disease. Infect. Immun. 64: 919-925.

Heard, S.R., Mawn, J.A., 1993. New phenotypic scheme for group B Streptococci. J. Clin. Pathol. 46:145-148.

Heath, A., DiRita, V.J., Barg, N.L., Engleberg, N.C., 1999. A two component regulatory system, CsrR-CsrS, represses expression of three *Streptococcus pyogenes* virulence factors, hyaluronic acid capsule, streptolysin S, and pyrogenic exotoxin B. Infect. Immun. 67:5298–5305.

Heath, P.T., Feldman, R.G., 2005. Vaccination against group B Streptococcus. 4(2):207-218.

Hensler, M.E., Miyamoto, S., Nizet, V., 2008. Group B Streptococcal b-hemolysin/cytolysin directly impairs cardiomyocyte viability and function. PLoS One. 3(6):e2466

Henslera, M.E., Quacha, D., Hsieha, C., Dorana, K.S., Nizeta, V., 2008. CAMP Factor is not essential for systemic virulence of group B Streptococcus. Microb. Pathog. 44(1):84–88.

Hood, M., Janney, A., Dameron, G., 1961. Beta hemolytic streptococcus group B associated with problems of the perinatal period. Am. J. Obstet. Gynecol. 1961. 82:809–818.

Hoogkamp-Korstanje, J.A., Gerards, L.J., Cats, B.P., 1982. Maternal carriage and neonatal acquisition of group B Streptococci. J. Infect. Dis. 145:800-803.

Hoshina, K., 1997. Minimization of the number of pregnant women to be treated with preventive procedure against GBS infection by means of antibody measure. Acta. Paediatr. Jpn. 39:546–549.

Hughes, M. J.G., Moore, J.C., Lane, J.D., Wilson, R., Pribul, P.K., Younes, Z.N., Dobson, R.J., Everest, P., Reason, A.J., Redfern, J.M., Greer, F.M., Paxton, T., Panico, M., Morris, H.R., Feldman, R.G., Santangelo, J.D., 2002. Identification of major outer surface proteins of *Streptococcus agalactiae.* Infect. Immun.70:1254–1259.

Jackson, R. J., Dao, M.L., Lim, D.V., 1994. Cell-associated collagenolytic activity by group B Streptococci. Infect. Immun. 62:5647-5651.

Jarva, H., Hellwage, J., Jokiranta, T.S., Lehtinen, M.J., Zipfel, P.F., Meri, S., 2004. The group B streptococcal beta and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion. J. Immunol.172:3111-3118.

Jennings,H.J., Johnson,K.G., Kenne,L., 1983. The structure of an R-type oligosaccharide core obtained from some lipopolysaccharides of *Neisseria meningitidis*. Carbohydr. Res. 121:233–241.

Jerlstrm, P.G., Chhatwal, G.S., Timmis, K.N., 1991. The IgA-binding beta antigen of the c protein complex of group B Streptococci: sequence determination of its gene and detection of two binding regions. Mol. Microbiol. 5:843.

Jerlstrom, P.G., Talay, S.R.,Weigand P.V., Timmis K. N, Chhatwal G. S.,1996. Identification of an immunoglobulin A binding motif located in the β-antigen of the c protein complex of group B streptococci. Infect. Immune. 64(7):2787-2793.

Ji, Y., Carlson, B., Kondagunta, A., Cleary, P.P., 1997. Intranasal immunization with C5a peptidase prevents nasopharyngeal colonization of mice by the group A Streptococcus. Infect. Immun. 65:2080–2087.

Ji, Y., Schnitzler, N., DeMaster, D., Cleary, P.P., 1998. Impact of M49, Mrp, Enn, and C5a peptidase proteins on colonization of the mouse oral mucosa by *Streptococcus pyogenes*. Infect. Immun. 66:5399–5405.

Jiang, S.M., Cieslewicz, M. J., Kasper, D.L., Wessels, M.R., 2005. Regulation of virulence by a two-component system in group B Streptococcus. J. Bact. 187(3):1105-1113.

Johnson, D.R., Ferrieri, P., 1984. Group B Streptococcal Ibc protein antigen: distribution of two determinants in wild-type strains of common serotypes. J. Clin. Micro. 19:506–510.

Johri A.K., Margarit, I., Broenstrup, M., Brettoni, C., Hua,L., Gygi, S.P., Telford, J.L., Grandi, G.,Paoletti, L.C., 2007. Transcriptional and proteomic profiles of group B Streptococcus type V reveal potential adherence proteins associated with high-level Invasion. Infect. Immun. 75 (3):1473–1483.

Johri, A.K., Margarit, I., Broenstrup, M., Brettoni, C., Hua, L., Gygi, S.P., Telford, J.L., Grandi, G., Paoletti, L.C., 2007 Transcriptional and proteomic profiles of group B Streptococcus type V reveal potential adherence proteins associated with high-level invasion. Infect. Immun.75:1473– 1483.

Johri, A.K., Padilla, J., Malin, G., and Paoletti, L.C., 2003. Oxygen regulates invasiveness and virulence of group B Streptococcus. Infect. Immune. 71:6707–6711

Johri, A.K., Paoletti, L.C., Glaser, P., Dua, M., Sharma, P.K., Grandi, G., Rappuoli, R., 2006. Group B Streptococcus: global incidence and vaccine development. Nat. Rev. Microbiol. 4:932-942.

Jolley, K.A., Feil, E.J., Chan, M.S., Maiden M.C., 2001. Sequence type analysis and recombinational tests (START). Bioinform. 17:1230–1231.

Jones, A.L., Knoll, K.M., Ruben, C.E., 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, J.F., Takahashi, S., Oliver, K.A., Chan, M.S., Kunst, F., Glaser, P., Rusniok, C., Crook, D.W., Harding, R.M., Bisharat, N., Spratt, B.G., 2003. Multilocus sequence typing system for group B Streptococcus. J. Clin. Microbiol. 41:2530-2536.

Jurgens, D., Sterzik, B., Fehrenbach, F.J., 1987. Unspecific binding of group B Streptococcal cocytolysin (CAMP factor) to immunoglobulins and its possible role in pathogenicity. J. Exp. Med. 165:720-732.

Kaushik, D.K., Sehgal, D., 2008. Developing antibacterial vaccines in genomics and proteomics Era. Scand. J. Immunol. 67:544-552.

Kjems, E., Perch, B., Henrichsen, J., 1980. Serotypes of group B Streptococci and their relation to hyaluronidase production and hydrolysis of salicin. J. Clin. Microbiol. 11:111–113.

Kling, D.E., Gravekamp, C., Madoff, L.C., Michel, J.L., 1997. Characterization of two distinct opsonic and protective epitopes within the alpha C protein of the group B Streptococcus. Infect. Immun. 65(4):1462–1467.

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

Kong, F., Gidding, H.F., Berner, R., Gilbert, G.L., 2006. *Streptococcus agalactiae* Cb protein gene (bac) sequence types, based on the repeated region of the cell-wall-spanning domain: relationship to virulence and a proposed standardized nomenclature. J. Med. Microbiol. 55:829–837.

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

Kratovac, Z., Manoharan, A., Luo, F., Lizano, S., Bessen, D.E., 2007. Population genetics and linkage analysis of loci within the FCT region of *Streptococcus pyogenes*. J. Bacteriol. 189:1299 –310.

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

Kvam, A.I., Efstratiou, A., Bevanger, L., Cookson, B.D., Marticorena, I.F., George, R.C., Maeland, J.A., 1995. Distribution of serovariants of group B Streptococci in isolates from England and Norway. J. Med. Microbiol. 42:246-250

Lachenauer, C.S., Creti, R., Michel, J.L., Madoff, L.C., 2000. Mosaicism in the alpha-like protein genes of group B Streptococci. Proc. Natl. Acad. Sci. (USA) 97(19): 9630–9635.

Lachenauer, C.S., Kasper, D.L., Shimada, J., Ichiman, Y., Ohtsuka, H., Kaku, M., Paoletti, L.C., Ferrieri, P., Madoff, L.C., 1999. Serotypes VI and VIII Predominate among group B Streptococci isolated from pregnant Japanese women. J. Infect. Dis. 179:1030–1033.

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

Lakshmi, V., Das, S., Shivanand, P.G., Savithri, P., Rao, K., 1988. Incidence of group-B beta haemolytic streptococci in the vaginal flora of pregnant women. Indian J. Pathol. Microbiol. 31:240-244.

Lancefield, R.C., 1934. Serological differentiation of specific types of bovine haemolytic streptococci (group B). J. Exp Med. 59:441–458.

Lancefield, R.C., Dole, V.P., 1946. The properties of T antigen extracted from group A hemolytic streptococci. J. Exp. Med. 84:449 –471.

Lancefield, R.C., McCarty, M., Everly, W.N., 1975. Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. J. Exit Med. 142:165.

Lang, S., Palmer, M., 2003. Characterization of *Streptococcus agalactiae* CAMP factor as a pore-forming toxin. J. Biol. Chem. 278:38167–38173.

Larsson, C., Holmgren, J., Lindahl, G., Bergquist, C., 2004. Intranasal immunization of mice with group B Streptococcal protein Rib and cholera toxin B subunit confers protection against lethal infection. Infect. Immun. 72(2):1184–1187.

Larsson, C., Stalhammar-Carlemalm, M., Lindahl, G., 1996. Experimental vaccination against group B Streptococcus, an encapsulated bacterium, with highly purified preparations of cell surface proteins Rib and α. Infect. Immun. 64:3518–3523.

Larsson, C., Stalhammar-Carlemalm, M., Lindahl, G., 1999. Protection against experimental infection with group B Streptococcus by immunization with a bivalent protein vaccine. Vaccine. 17(5):454-458.

Lauer, P., Rinaudo, C.D., Soriani, M., Margarit, I., Maione, D., Rosini, R., Taddei, A.R., Mora, M., Rappuoli, R., Grandi, G., Telford, J.L., 2005. Genome analysis reveals pili in group B Streptococcus. Science. 309:105.

Lawrence, M.C., Pilling, P.A., Epa, V.C., Berry, A.M., Ogunniyi, A.D., Paton, J.C., 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. Structure. 6:1553–1561.

Levin, J.C., Wessels, M.R., 1998. Identification of *csrR/csrS*, a genetic locus that regulates hyaluronic acid capsule synthesis in group A Streptococcus. Mol. Microbiol. 30:209–219.

Limansky, A.S., Sutich, E.G., Guardati, M.C., Toresani, I.E., Viale, A.M., 1998. Genomic diversity among *Streptococcus agalactiae* isolates detected by a degenerate oligonucleotide-primed amplification assay, J. Infect. Dis. 177:1308-1313.

Lin B., Averett, W.F., Novak, J., Chatham, W.W., Hollingshead, S.K., Coligan, J.E., Egan, M.L., Pritchard, D.G., 1996. Characterization of PepB, a group B Streptococcal. oligopeptidase. Infect. Immun. 64:3401-3406.

Lin, F.Y.C., Clemens, J.D., Azimi, P.H., 1998. Capsular polysaccharide types of group B Streptococcal isolates from neonates with early-onset systemic infection. J. Infect. Dis. 177:790-2.

Lindahl, G., Stalhammar-Carlemalm, M., Areschoug, T., 2005. Surface proteins of *Streptococcus agalactiae* and related proteins in other bacterial pathogens. Clin. Microbiol. Rev. 18(1):102-127.

Liu, G.Y., Sobczak, S., Benirschke, K., Nizet, V., Heldt, G.P. 2005. Virulence role of group B Streptococcus beta-hemolysis/cytolysin in a neonatal rabbit model of early-onset pulmonary infection. J. Infect. Dis. 191(8):1287-1291.

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

Madoff, L.C., Michel, J.L., Gong, E.W., Rodewald, A.K., Kasper, D.L., 1992. Protection of neonatal mice from group B Streptococcal infection by maternal immunization with beta C protein. Infect. Immun. 60(12):4989-4994.

Madoff, L.C., Paoletti, L.C., Kasper, D.L., 2006. Surface structures of group B Streptococci important in human immunity. In: Gram positive pathogens. (ed. Fischetti, V) ASM Press, 2006, pp 169.

Madureira, P., Baptista, M., Vieira, M., Magalha, V., Camelo, A., Oliveira, L., Ribeiro,A., Tavares, D., Cuot, P.T., Vilanova, M., Ferreira, P., 2007. *Streptococcus agalactiae* GAPDH is a virulence-associated immunomodulatory protein. J. Immunol. 178:1379–1387.

Maeland, J.A., Bevanger, L., Lyng, R.V., 2004. Antigenic determinants of alpha-like proteins of *Streptococcus agalactiae.* Clin. Diagnos. Laborat. Immunol. 11(6):1035–1039.

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

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

Maisey, H.C., Doran, K.S. Nizet, V., 2008. Recent advances in understanding the molecular basis of group B Streptococcus virulence. Expert Rev. Mol. Med.10:27.

Mancuso, G., Cusumano, V., Genovese, F., Gambuzza, M., Beninati, C., Teti, G., 1997. Role of interleukin 12 in experimental neonatal sepsis caused by group B Streptococci. Infect. Immun 65:3731-3735.

Mani, V., Jadhav, M., Sivadasan, K., Thangavelu, C.P., Rachel, M., Prabha, J., 1984. Maternal and neonatal colonization with group B Streptococcus and neonatal outcome. Indian Pediatr. 21:357-363.

Manning, S.D., Ki, M., Marrs, C.F., Kugeler, K.J., Borchardt, S.M., Baker, C.J., Foxman, B., 2006. The frequency of genes encoding three putative group B Streptococcal virulence factors among invasive and colonizing isolates. BMC Infect. Dis. 6:116–123.

Marchaim, D., Hallak, M., Uzan, L.G., Peled, N., Riesenberg, K., Schlaeffer, F., 2003. Cell wall proteins of group B Streptococcus and low incidence of neonatal disease in southern Israel. J. Reprod. Med. 48:697–702.

Marchlewicz, B.A., Duncan, J.L., 1981. Lysis of erythrocytes by a hemolysin produced by a group B Streptococcus sp. Infect. Immun 34:787–794.

Carlemalm,M.S., M., Stenberg, L, Lindahl, G., 1993. Protein Rib: a novel group B Streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections J. Exp. Med. 177:1593-1603.

Maternal and Reproductive Health: National Family Health Survey (NFHS-2), 1998-1999. Vol. 2003. Mumbai, India: International Institute for Population Sciences (IIPS), 2000:http://www.nfhsindia.org/india.html.

.

Matsubara, K., Sugiyama, M., Hoshina, K., Mikamo, H., Baba, K., 2000. Early onset neonatal sepsis caused by serotype VIII group B Streptococci. Pediatr. Infect. Dis. J. 19:359–360.

Mello, L.V., De-Groot, B.L., Li, S., Jedrzejas, M.J. 2002. Structure and flexibility of *Streptococcus agalactiae* hyaluronate lyase complex with its substrate. Insights into the mechanism of processive degradation of hyaluronan. J. Biol. Chem. 277:36678–36688.

Mereghetti, L., Sitkiewicz, I., Green, N.M., Musser, J.M., 2008a. Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (group B Streptococcus) in response to incubation with human blood. Plos ONE. 3(9):3143.

Mereghetti, L., Sitkiewicz, I., Green, N.M., Musser, J.M., 2008b. Remodeling of the *Streptococcus agalactiae* transcriptome in response to growth temperature. PLoS ONE. 3:2785.

Mereghetti, L., Sitkiewicz, I., Green, N.M., Musser, J.M., 2009. Extensive adaptive changes occur in the transcriptome of *Streptococcus agalactiae* (group B Streptococcus) in response to incubation with human blood. Plos ONE. 3(9):3143

Michd, J.L., Madoff, L.C., Kling, D.E., Kasper, D.L., Ausubel, F.M., 1991. Cloned alpha and beta c-protein antigens of group B Streptococci elicit protective immunity. Infect. lmmun.59:2023.

Michel, J. L., Beseth, B. D., Madoff, L.C., Olken, S.K., Kasper, D.L., Ausubel, F.M., 1994. Genotypic diversity and evidence for two distinct classes of the C protein alpha antigen of group B Streptococcus. In: Totolian A, editor. Pathogenic streptococci: present and future. St. Petersburg, Russia: Lancer Publications. pp. 331–332

Michel, J.L., Madoff, L.C., Kling, D.E., Kasper, D.L., Ausubel, F.M., 1991. C proteins of group B streptococci. In: Genetics and Molecular Biology of Streptococci, Lactococci, and Enterococci. Dunny, G.M., Cleary, P.P., McKay, L.L., editors. American Society for Microbiology, Washington, DC. 214-218.

Michel, J.L., Madoff, L.C., Olson, K., Kling, D.E., Kasper, D.L, 1992. Ausubel FM. Large, identical, tandem repeating units in the C protein alpha antigen gene, *bca*, of group B Streptococci. Proc. Natl. Acad. Sci. (USA) 89:10060–10064.

Mikamo, H., Johri, A.K., Paoletti, L.C., Madoff, L.C., Onderdonk, A.B., 2004. Adherence to, invasion by, and cytokine production in response to serotype VIII group B Streptococci. Infect. Immun. 72:4716–4722.

Miller, A.A., Engleberg, N.C., Dirita, V.J., 2001. Repression of virulence genes by phosphorylation-dependent oligomerization of CsrR at target promoters in *S. pyogenes*. Mol. Microbiol. 40:976–990.

Musser, J.M., Mattingly, S.J., Quentin, R., Goudeau, A., Selander, R.K., 1989. Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease, Proc. Natl. Acad. Sci. (USA). 86:4731-4735.

Musser, J.M., Mattingly, S.J., Quentin,R., Goudeau,R., Selander, R.K.,1989 Identification of a high-virulence clone of type III Streptococcus agalactiae (group B Streptococcus) causing invasive neonatal disease. Proc. Natl. Acad. Sci. (USA). 86(12): 4731-4735.

Nair, S., Poyart, C., Beretti, J.L., Veiga-Fernandes, H., Berche, P., Trieu-Cuot, P., 2003. Role of the *Streptococcus agalactiae* ClpP serine protease in heat-induced stress defence and growth arrest. Microbiol. 149:407–417.

Nizet, V., Ferrieri, P., Rubens, C.E., 2000. Molecular pathogenesis of group B streptococcal disease in newborns. In: Streptococcal Infections: Clinical Aspects, Microbiology,and Molecular Pathogenesis. Stevens, D.L., and Kaplan, E.L. (eds). New York: Oxford University Press, pp. 180–221.

Osrin, D., Vergnano, S., Costello, A., 2004. Serious bacterial infections in newborn infants in developing countries. Curr. Opin. Infect. Dis. 17:217–224.

Palacios, G.C., E.K. Eskew, F. Solorzano, S.J. Mattingly. 1997. Decreased capacity for type-specific-antigen synthesis accounts for high prevalence of nontypeable strains of group B Streptococci in Mexico. J. Clin. Microbiol. 35:2923-2926.

Pancholi, V., Fischetti V.A., 1998. Alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. J. Biol.Chem. 273:14503–14515.

Pancholi,V., Fischetti, V.A., 1998. Alpha-enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. J. Biol. Chem. 273:14503-14515.

Paoletti, L.C., Ferrieri, P., Madoff, L.C., 1999. Serotypes VI and VIII predominate among group B Streptococci isolated from pregnant Japanese women. J. Infect. Dis. 179:1030–1033.

Paoletti, L.C., Kasper, D.L., 2002. Conjugate vaccines against group B Streptococcus types IV and VII. J. Infect. Dis. 186:123–126.

Paoletti, L.C., Kasper, D.L., 2003. Glycoconjugate vaccines to prevent group B Streptococcal infections. Expert. Opin. Biol. Ther. 3:975–984.

Paoletti, L.C., Madoff, L.C., 2002.Vaccines to prevent neonatal GBS infection. Semi. Neonatal. 7:315–323.

Paoletti, L.C., Madoff, L.C., Kasper, D.L., 2000. In: Gram- Positive Pathogens. (2nd eds) Fischetti, V.A., Novick, R.P.,Ferretti, J.J., Portony, D.A.& Rood, J.I.), ASM Press, Washington DC. pp. 137–153.

Paoletti, L.C., Pinel, J., Johnson, K.D., Reinap, B., Ross, R.A., Kasper, D.L., 1999. Synthesis and preclinical evaluation of glycoconjugate vaccines against group B Streptococcus types VI and VIII. J. Infect. Dis. 180:892-895.

Paoletti, L.C., Wessels, M.R., Michon, F., DiFabio, J., Jennings, H.J., Kasper, D.L., 1992. Group B Streptococcus type II polysaccharide-tetanus toxoid conjugate. Vaccine. 60(10):4009-4014.

Paoletti, L.C., Wessels, M.R., Rodewald, A.K., Shroff, A.A., Jennings, H.J., Kasper, D.L., 1994. Neonatal mouse protection against infection with multiple group B Streptococcal (GBS) serotypes by maternal immunization with a tetravalent GBS polysaccharide-tetanus toxoid conjugate vaccine. Infect. Immun. 62(8):3236-3243.

Pass, M.A., Gray, B.M., Khare, S., Dillon, H.C., 1979. Prospective studies of group B Streptococcal infections in infants. J. Pediatr. 95:437-443.

Phares, C.R., Lynfield, R., Farley, M.M., Mohle-Boetani, J., Harrison, L.H., Petit, S., Craig, A.S., Schaffner, W., Zansky, S.M., Gershman, K., Stefonek, K.R., Albanese, B.A., Zell, E.R., Schuchat, A., Schrag, S.J., 2008. 1999-2005 Epidemiology of invasive group B Streptococcal disease in the United States. JAMA. 299(17):2056-65.

Podbielski A., Blankenstein, O., Lutticken, R., 1994. Molecular characterization of the cfb gene encoding group B streptococcal CAMP-factor. Med. Microbiol. Immunol. 183:239-256.

Prakash, K., Ravindran, P.C., Sharma, K.B., 1976. Group B beta haemolytic streptococci and their serological types in human infections. Indian J. Med. Res. 64:1001-1007.

Prakobphol, A., Xu, F., Hoang, V.M., Larsson, T., Bergstrom, J., Johansson, I., Frangsmyr, L., Holmskov, U., Leffler, H., Nilsson,V., Boren, T., Wright, J.R., Stromberg, N., Fishera, S.J., 2000. Salivary agglutinin, which binds *Streptococcus mutans* and *Helicobacter pylori*, is the lung scavenger receptor cysteine-rich protein gp-340. J. Biol. Chemist. 275(52): 39860–39866.

Pritchard, D.G., Gray, B.M., Egan, M.L., 1992. Murine monoclonal antibodies to type Ib polysaccharide of group B Streptococci bind to human milk oligosaccharides. Infect. Immun.60:1598.

Pritchard, D.G., Lin, B., Willingham, T.R., Baker, J.R., 1994. Characterization of the group B Streptococcal hyaluronate lyase. Arch. Biochem. Biophys. 315:431–437.

Pritzlaff, C.A., Chang, J.C.W., Kuo, S.P., Tamura, G.S., Rubens, C.E., Nizet, V., 2001. Genetic basis for the β-haemolytic/cytolytic activity of group B *Streptococcus*.Mol. Microbiol. 39(2):236-248.

Puopolo, K.M., Madoff, L.C., Eichenwald, E.C., 2005. Early-onset group B Streptococcal disease in the era of maternal screening. Pediatrics. 115:1240–1246.

Ragunathan, P., Spellerberg, B., Ponnuraj, K., 2009. Expression, purification, crystallization and preliminary crystallographic analysis of laminin-binding protein (Lmb) from Streptococcus agalactiae. Acta. Crystallogr. Sect. F Struct. Biol. Cryst. Commun. 65(Pt 5):492-494.

Rainardl, P., Boulard, C., 1992 .Opsonization of *Streptococcus agalactiae* of bovine origin by complement and antibodies against group B polysaccharide .Infect. Immun. 60(11):4801-4808.

Rappuoli, R., 2000. Reverse vaccinology. Curr. Opin. Microbiol. 35:445–450.

Ratner, H.B; Weeks, L.S., Stratton, C.W., 1986. Evaluation of spot CAMP test for identification of group B Streptococci. J. Clin. Microbiol. 24(2):296-297.

Regan, J.A., Klebanoff, M.A., Nugent, R.P., 1991. The epidemiology of group B Streptococcal colonization in pregnancy. Vaginal infections and prematurity study group. Obstet Gynecol. 77(4):604-610.

Renata, O., Graner, M., Dani, A.P., Duncan, M.J., 2006. Functional analysis of glucan binding protein B from *Streptococcus mutans*. J. Bact. 188(11):3813–3825.
Rench, M.A., Baker, C.J., 1993. Neonatal sepsis caused by a new group B Streptococcal serotype. J. Pediatr. 122:638–640.

Rioux, S., Martin, D., Ackermann, H.W., Dumont, J., Hamel, J., Brodeur, B.R., 2001. Localization of surface immunogenic protein on group B Streptococcus. Infect. Immun. 69(8):5162-5165.

Rioux, S., Martin, D., Ackermann, H.W., Dumont, J., Hamel, J., Brodeur, B.R., 2001. Localization of surface immunogenic protein on group B Streptococcus. Infect. Immun. 69(8):5162–5165.

Roberto, R., Daniela, C.R., Marco, S., Peter, L., Marirosa, M., Domenico, M., Annarita, T., Isabella, S., Claudia, G., Cecilia, B., Scilla, B., Immaculada, M., Guido, G., John L. T., 2006. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol. Microbiol. 61:126-141.

Rojas, J., Larsson, L.E., Hellerqvist, C.G., Brigham, K.L., Gray, M.E., Stahlman, M.T., 1983 Pulmonary hemodynamic and ultrastructural changes associated with group B Streptococcal toxemia in adult sheep and newborn lambs. Pediatr. Res. 17:1002-1008.

Rolland, K., C. Marois, V. Siquier, B. Cattier, and R. Quentin. 1999. Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and *hylB* gene analysis. J. Clin. Microbiol. 37:1892-1898.

Rolland, K., Marois, C., Siquier, V., Cattier, B., Quentin, R., 1999. Genetic features of *Streptococcus agalactiae* strains causing severe neonatal infections, as revealed by pulsed-field gel electrophoresis and hylB gene analysis, J. Clin. Microbiol. 37: 1892-1898.

Rosini, R., Rinaudo, C.D., Soriani, M., Lauer, P., Mora, M., Maione, D., Taddei, A., Santi, I., Ghezzo, C., Brettoni, C., Buccato, S., Margarit, I., Grandi, G., TelforD, J.L., 2006. Identification of novel genomic islands coding for antigenic pilus-like structures in *Streptococcus agalactiae*. Mol. Microbiol. 61(1):126-141.

Rouillard, J.M., Zuker, M., Gulari, E., 2003. OligoArray 2.0: design of oligonucleotide probes for DNA microarrays using a thermodynamic approach. Nucleic Acids Res. 31:3057-3062.

Rubens, C.E., Wessels, M.R., Heggen, L.M., Kasper, D. L., 1987. Transposon mutagenesis of type III group B Streptococcus: correlation of capsule expression with virulence. Proc. Natl Acad. Sci. (USA). 84:7208–7212.

Rubens, C.E., Smith, S., Hulse, M., Chi, E.Y., Belle, G.V., 1992. Respiratory epithelial cell invasion by group B streptococci. Infect Immun. 60(12):5157-5163.

Rubens, C.E., Raff, H.V., Jackson, J.C., Chi, E.Y., Bielitzki, J.T., Hillier, S.L. 1991. Pathophysiology and histopathology of group B Streptococcal sepsis in *Macaca nemestrina* primates induced after intraamniotic inoculation: evidence for bacterial cellular invasion. J. Infect. Dis. 164:320-330.

Ruhlmann, J., Liebold, B.W., Jurgens, D., Fehrenbach, F. 1988. Complete amino acid sequence of protein B. FEBS microbial. Lett. 235:262-266.

Schneewind O., Friedrich, K., Lutticken, R., 1988. Cloning and expression of the CAMP factor of group B Streptococci in Escherichia coli. Infect. Immun. 56:2174-2179.

Schubert, A., Zakikhany, K., Schreiner, M., Frank, R., Spellerberg, B., Eikmanns, B.J., Reinscheid, D.J., 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., 1998. Epidemiology of group B Streptococcal disease in the United States: shifting paradigms. Clin. Microbiol. Rev. 11:497–513.

Schuchat, A., Wenger, J. D. 1994. Epidemiology of group B Streptococcal disease. Risk factors, prevention strategies, and vaccine development. Epidemiol. Rev. 16(2):374-402.

Schwartz-Linek, S.U., Hook, M., Potts, J.R., 2004. The molecular basis of fibronectin-mediated bacterial adherence to host cells. Mol. Microbiol. 52:631–641.

Seepersaud, R., Hanniffy, S.B., Mayne, P., Sizer, P., Page, R.L., Wells, J.M., 2005. Characterization of a novel leucine-rich repeat protein antigen from group B Streptococci that elicits protective immunity. Infect. Immun 73 (3):1671-1683.

Seepersaud,R., Hanniffy, S.B., Mayne,P., Sizer,P.,Page, R.L .,Wells, J.M., 2003. Characterization of a novel leucin rich repeat protein antigen from Group B streptococci that elicit protective immunity. Infect. Immun. 73:1671–1683.

Segura,M., Gottschalk,M., Olivier, M., 2004. .Encapsulated *Streptococcus suis* Inhibits Activation of Signaling Pathways Involved in Phagocytosis. Infect. Immune. 72(9):5322-5330.

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(2):100-111.

Shottmuller.H., 1903. Die artunterscheidung der fur den menschen pathogen streptokokken durch blutagar. Munch. Med. Whchenschr. 50:849-853.

Sitkiewicz, I., Musser, J.M., 2009. Analysis of growth-phase regulated genes in *Streptococcus agalactiae* by global transcript profiling. BMC Microbiol. 9:32.

Skalka B., Smola, J., 1981. Lethal effect of CAMP-factor and UBERIS-factor--a new finding about diffusible exosubstances of *Streptococcus agalactiae* and *Streptococcus uberis*. Zentralbl. Bakteriol. 249:190-194.

Slotved, H.C., Elliott, J., Thompson, T., Konradsen, H.B., 2003. Latex assay for serotyping of group B Streptococcus Isolates. J. Clin. Microbiol. 41(9):4445–4447.

Spellerberg, B., Rozdzinski, E., Martin, S., Weber-Heynemann, J., Schnitzler, N., Lütticken, R., Podbielski, A., 1999. Lmb, a Protein with Similarities to the LraI

Adhesin Family, Mediates Attachment of *Streptococcus agalactiae* to Human Laminin. Infect. Immun. 67(2):871-878.

Stalhammar-Carlemalm, M., Stenberg, L., Lindahl, G., 1993. Protein rib: a novel group B Streptococcal cell surface protein that confers protective immunity and is expressed by most strains causing invasive infections. J. Exp. Med. 177:1593-1603.

Stock, A.M., Robinson,V.L., Goudreau, P.N., 2000. Two-component signal transduction. Annu. Rev. Biochem. 69:183–215.

Slotved, H.C., Kong, F., Lambertsen, L., Sauer, S., Gilbert, G.L., 2007. Serotype IX, a proposed new *Streptococcus agalactiae* serotype. J. Clin. Microbiol. 45:2929–2936.

Stoll, B.J., Schuchat, A., 1998. Maternal carriage of group B streptococci in developing countries. Pediatr Infect Dis J. 17:499- 503.

Sukhnanand, S., Dogan, B., Ayodele, M.O., Zadoks, R.N., Craver, M.P.J., Dumas, N.B., Schukken, Y.H., Boor, K.J., Wiedmann. M., 2005. Molecular subtyping and characterization of bovine and human *Streptococcus agalactiae* isolates. J. Clin. Microbiol. 43(3):1177–1186.

Suvorov, A.N., Cleary, P.P., Ferrieri, P., 1991. C5a peptidase gene from group B streptococci. In: Dunny G, Cleary P, McKay L, editors. Genetics and molecular biology of streptococci, lactococci, and enterococci. Washington, D.C.: American Society for Microbiology; pp. 230–232.

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

Takahashi, S., Aoyagi, Y., Adderson, E.E., Okuwaki, Y., Bohnsack, J.F.,1999. Capsular sialic acid limits C5a production on type III group B streptococci. Infect Immun.67:1866–1870.

Tamura, G.S., Kuypers, J.M., Smith, S., Raff, H., Rubens, C.E., 1994. Adherence of group B Streptococci to cultured epithelial cells: roles of environmental factors and bacterial surface components. Infect. Immun. 62:2450– 2458.

Tamura, G.S., Nittayajarn, A., Schoentag, D.L., 2002. A glutamine transport gene, glnQ, is required for fibronectin adherence and virulence of group B Streptococci. Infect. Immun.70:2877–2885.

Teti, G., Mancuso, G., Tomasello. F., 1993. Cytokine appearance and effects of anti-tumor necrosis factor antibodies in a neonatal rat model of group B Streptococcal infection. Infect. Immun. 61:227–235.

Teti, G., Tomasello, F., Chiofalo, M.S., Orefici, G., Mastroeni, P., 1987. Adherence of group B Streptococci to adult and neonatal epithelial cells mediated by lipoteichoic acid. Infect. Immun. 55:3057-3064.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Donati, C., Medini, D., Ward, N.L., Angiuoli, S.V., Crabtree, J., Jones, A.L., Durkin, A.S., Deboy, R.T., Davidsen, T.M., Mora, M., Scarselli, M., Ros, M.I., Peterson, J.D., Hauser, C.R., Sundaram, J.P., Nelson, W.C., Madupu, R,, Brinkac, L.M., Dodson, R.J., Rosovitz, M.J., Sullivan, S.A., Daugherty, S.C., Haft, D.H., Selengut, J., Gwinn, M.L., Zhou, L., Zafar, N., Khouri, H., Radune, D., Dimitrov, G., Watkins, K., O'Connor, K.J., Smith, S., Utterback, T.R., White, O., Rubens, C.E., Grandi, G., Madoff, L.C., Kasper, D.L., Telford, J.L., Wessels, M,R., Rappuoli, R., Fraser, C.M., 2005. Genomic analysis of multiple pathogenic isolates of *Streptococcus agalactiae*: Implications for the microbial 'pan-genome'. Proc. Natl Acad. Sci. (USA). 102, 13950–13955.

Tettelin, H., Masignani, V., Cieslewicz, M.J., Eisen, J.A., Peterson, S., Wessels, M.R., Paulsen, I.T., Nelson, K.E., Margarit, I., Read, T.D., Madoff, L.C., Wolf, A.M., Beanan M.J., Brinkac, L.M., Daugherty, S.C., DeBoy, R.T., Durkin, A.S., Kolonay, J.F., Madupu, R., Lewis, MR., Radune, D., Fedorova, N.B., Scanlan, D., Khouri, H., Mulligan, S., Carty, H.A., Cline, R.T., Aken, V.S.E., Gill, J., Scarselli, M., Mora, M., Iacobini, E.T., Brettoni, C., Galli, G., Mariani, M., Vegni, F., Maione, D., Rinaudo, D., Rappuoli, R., Telford, J.L., Kasper, D.L., Grandi, G., Fraser, C.M., 2002. Complete genome sequence and comparative genomic analysis of an emerging human pathogen, serotype V *Streptococcus agalactiae*. Proc. Natl Acad. Sci. (USA). 99:12391– 12396.

Tettelin, H., Medini, D., Donati, C., Masignani, V., 2006. Towards a universal group B Streptococcus vaccine. Expert Rev. Vaccines. 5(5):687-694.

Tusher, V., Tibshirani, G.R., Chu. G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. Proc. Natl. Acad. Sci. (USA). 98:5116–5121.

Tyrrell, G.J., Kennedy, A., Shokoples, S.E., Sherburne, R.K. 2002. Binding and invasion of HeLa and MRC-5 cells by *Streptococcus agalactiae*. Microbiol. 148:3921–3931.

UNAIDS, World Health Organization. 2007. AIDS epidemic update. Available from: http://data.unaids.org/pub/EPISlides/2007/2007 epiupdate en.pdf.

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

Vallette, J.D., Goldenberg, R.N., Suguihara, C., Delmoral, T.,Martinez, O., Lin, J., Thompson, R.C., Bancalari, E., 1995. Effect of an interleukin-1 receptor antagonist on the hemodynamic manifestations of group B Streptococcal sepsis. Pediatr. Res. 38:704-708.

Valentin-Weigand, P., Chhatwal, G.S., 1995. .Correlation of epithelial cell invasiveness of group B streptococci with clinical source of isolation. Microbial pathogen. 19(2):83-91.

Vanberg, C., Lutnaes, B.F., Langsrud, T., Nes, I.F., Holo, H., 2007. Propionibacterium jensenii produces the polyene pigment granadaene and has hemolytic properties similar to those of Streptococcus agalactiae. Appl. Environ. Microbiol. 73:5501– 5506.

Wagner, W.B., Kubinv, M., Ryc, M., 1980. Immunoelectron microscopic study of the location of group-specific and protein type-specific antigens of group B Streptococci. J Gen. Microbiol 118:95-105.

Walsh, J.A., S. Hutchins. 1989. Group B Streptococcal disease: its importance in the developing world and prospect for prevention with vaccines. Pediatr. Infect. Dis. J. 8:271–277.

Weimer, B., Xie, Y., Chou, L., Cutler, A., 2004. Gene expression arrays in food. In: J. L. Barredo (ed.), Microbial products and biotransformation. Humana Press, Totowa, N.J. p. 333–343.

Wenger, J.D., Hightower, A.W., Facklam, R.R., Gaventa, S., Broome, C.V. 1990. Bacterial meningitis in the United States, 1986: report of a multistate surveillance study. The Bacterial Meningitis Study Group. J. Infect. Dis. 162:1316–1323.

Wessels, M.R., Muñoz, A., Kasper, D.L., 1987. A model of high-affinity antibody binding to type III group B Streptococcus capsular polysaccharide. Proc. Natl. Acad. Sci. (USA). 84(24):9170–9174.

Wessels, M.R., Paoletti, L.C., Kasper, D.L., DiFabio, J.L., Michon, F., Holme, K., Jennings, H.J., 1990. Immunogenicity in animals of a polysaccharide-protein conjugate vaccine against type III group B Streptococcus. J. Clin Invest. 86(5):1428-1433.

Wessels, M.R., Paoletti, L.C., Pinel, J., Kasper, D.L., 1995. Immunogenicity and protective activity in animals of a type V group B Streptococcal polysaccharide-tetanus toxoid conjugate vaccine. J. Infect. Dis. 171:879–884.

Wessels, M.R., Paoletti, L.C., Rodewald, A.K., Michon, F., Difabio, J., Jennings, H.J., Kasper, D.L., 1993. Stimulation of protective antibodies against type Ia and Ib group B Streptococci by a type Ia polysaccharide-tetanus toxoid conjugate vaccine. Infect. Immun. 61:4760–4766.

Wexler, D., Chenoweth, E.D., Cleary, P., 1985. A streptococcal inactivator of chemotaxis: a new virulence factor specific to group A Streptococci. Proc. Natl. Acad. Sci. (USA). 82:179–180.

WHO Report, April 2003. State of the art of new vaccines Research & Development: Initiative for Vaccine Research, World Health Organization, Geneva.

WHO, global burden of disease (GBD), 2002 estimates (revised). Available from: http://www.who.int/healthinfo/bodestimates/en/.

Wibawan, I.T., Lammler, C., Pasaribu, F.H., 1992. Role of hydrophobic surface proteins in mediating adherence of group B Streptococci to epithelial cells. J. Gen. Microbiol. 138:1237–1242.

Winram, S.B., Jonas, M., Chi, E., Rubens, C.E., 1998. Characterization of group B Streptococcal invasion of human chorion and amnion epithelial cells *in vitro*. Infect. Immun. 66:4932-4941.

Wessels, M.R., Benedí, W.J. Jennings, H.J., Michon, F., DiFabio, J.L., Kasper, D.L., 1989. Isolation and characterization of type IV group B Streptococcus capsular polysaccharide. Infect. Immun. 57(4): 089-1094.

Wessels, M,R,, Pozsgay, V., Kasper, D.L., Jennings, H.J., 1987. Structure and immunochemistry of an oligosaccharide repeating unit of the capsular polysaccharide of type III group B Streptococcus. J. Biol. Chem. 262:8262–8267.

Woods, C.J., Levy, C.S., 2009. Group B Streptococal infection. *e medicine*

Xie, Y., 2003. Determining the influence of extracellular proteinase from *Brevibacterium linens* on the metabolism of *Lactococcus lactis* spp. *lactis* using functional genomics. Ph.D. dissertation. Utah State University, Logan.

Yang, Y.H., Dudoit, S., Luu, P., Speed, T. P., 2001. Normalization for cDNA microarray data. Proc. SPIE. 4266:141–152.

Zaleznik, D.F., Rench, M.A., Hillier, S., Krohn, M.A., Platt, R., Lee, M.L., Flores, A.E., Ferrieri, P., Baker, C.J., 2000. Invasive disease due to group B Streptococcus in pregnant women and neonates from diverse population groups. Clin. Infect. Dis. 30:276-281.

Zangwill, K.M., Schuchat, A., Wenger, J.D., 1992. Group B Streptococcal disease in the United States, 1990: report from a multistate active surveillance system. MMWR CDC Surveill Summ.

Appendices

1. Todd Hewitt Broth (THB)

Constituents	Composition
Beef heart infusion from peptic digest of animal tissue	20 gm/L
Dextrose	2 gm/L
Sodium chloride	2 gm/L
Sodium phosphate	0.40 gm/L
Sodium carbonate	2.50 gm/L

1. Blood Agar Base 2 (BAP)

Constituents	Composition
Proteose peptone	15 gm/L
Liver extract	2.50 gm/L
Yeast Extract	5 gm/L
Sodium chloride	5 gm/L
Agar	15 gm/L

(Note: add 7% of sheep blood after autoclaving the media when temperature goes down to 37-40^{0}C)

2. **Morpholinopropane sulphonic acid (MOPS buffer)**

Constituents	Composition
MOPS	200 mM
Sodium acetate	50 mM
EDTA	10mM

3. **Phosphate Buffer Saline (PBS)**

Constituents	Composition
Sodium chloride	8.0 gm/L
Potassium chloride	0.2 gm/L
Di-sodium hydrogen phosphate	1.44 gm/L
Potassium di-hydrogen phosphate	0.24 gm/L

(Note: Adjust the pH to 7.4 with HCl and store the buffer at room temperature)

4. **RPMI 1640 Culture Media**

Constituents	Composition
Inorganic Salts	
Calcium nitrate x $4H_2O$	100.00 mg/L
Potassium chloride	400 mg/L
Magnesium sulfate dried	48.44 mg/L
Sodiumchloride	5959 mg/ml
di-Sodium hydrogen phosphate	800 mg/ml
Amino acids	
L-Arginine x HCl	241.86 mg/ml

L-Asparagine x H_2O	50.00 mg/ml
L-Aspartic acid	20.00 mg/ml
L-Cystine	50.00 mg/ml
L-Glutamine	300 mg/ml
L-Glutamic acid	20 mg/ml
Glycine	10 mg/ml
L-Histidine Base	15 mg/ml
L-Hydroxyproline	20.00 mg/ml
L-Isoleucine	50 mg/ml
L-Leucine	50 mg/ml
L-Lysine x HCl	40 mg/ml
L-Methionine	15 mg/ml
L-Phenylalanine	15 mg/ml
L-Proline	20 mg/ml