Characterisation of invasive group B streptococci based on investigation of surface proteins and genes encoding surface proteins

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ABSTRACT

The joint distributions of the six genes *bca*, *bac*, *ε/alp1*, *alp2*, *alp3* and *rib* (encoding α-C-protein, β-C-protein, ε/Alp1, Alp2, Alp3, and Rib, respectively) and the proteins α-C-protein, β-C-protein and Rib were investigated in invasive isolates of group B streptococcus (GBS). In total, 297 invasive isolates (123 from neonates, 174 from adults) from south-west Sweden were collected during a 13-year period. Genes were detected using multiplex and specific PCRs, and expression of the surface proteins was demonstrated using monoclonal antibodies. The genes studied were found alone or in combinations in 294 (99%) of the invasive isolates. The most common genes were *rib* ($n = 127$ isolates, 43%), *alp3* ($n = 78$, 26%) and *ε/alp1* ($n = 42$, 14%). The *bac* gene was never found alone, but was found in combination with one other gene in 36 isolates. The surface proteins studied were detected alone or in combinations in 152 (51%) isolates, with the most common being Rib ($n = 80$, 27%), α-C-protein ($n = 68$, 23%) and β-C-protein ($n = 24$, 8%). Several genes were associated significantly with particular serotypes (e.g., *ε/alp1* with serotype Ia; *bca* and *bac* with serotypes Ib and II; *rib* with serotype III; *alp3* with serotype V). Overall, it was concluded that demonstration of different genes and surface proteins of GBS strains can be useful in epidemiological studies and in formulation of vaccines, but disappointingly, no single gene or surface protein included in the study was sufficiently common for it to be considered as the basis for a successful GBS vaccine.

Keywords Genotype, group B streptococcus, serotype, *Streptococcus agalactiae*, surface protein, vaccine candidates

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INTRODUCTION

The importance of *Streptococcus agalactiae* (group B streptococcus; GBS) as a major pathogen in invasive neonatal infections and infections in pregnant women is well-documented [1–7]. GBS is also increasingly common as a pathogen causing severe infections in adults with underlying medical conditions [8,9]. Since recommendations for intra-partum antibiotics for mothers in labour at risk of GBS infection have been widely implemented, the incidence of early-onset GBS infection has declined to <1/1000 live births [1–7]. However, the incidence of late-onset disease has not declined. An attractive alternative to intra-partum antibiotics would be vaccination of young women to protect their neonates against GBS infection. An effective vaccine might protect a large proportion of neonates against early- and late-onset GBS sepsis, and might also prevent GBS-related still-births and premature births.

Based on the capsular polysaccharide (CPS) antigens, nine GBS serotypes (Ia, Ib, II–VIII) have been recognised to date. The major serotypes that cause invasive infections in both neonates and adults are serotypes III, V and Ia [2–6,8,9]. Antibodies against CPS provide type-specific protection [10], and a multivalent conjugate vaccine, containing serotypes Ia, Ib, II, III and V

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conjugated with a protein, has been a major focus of interest. Conjugate vaccines composed of CPS and tetanus toxoid have been evaluated in healthy adults [11,12], and a type III CPS–tetanus toxoid conjugate vaccine has been tested in women at 31–32 weeks of gestation [13].

GBS can also be classified on the basis of surface protein antigens. The first surface protein identified in GBS was the C antigen [14]. The C antigen is composed of the α and β proteins [15]. A GBS strain can express α-C-protein or β-C-protein, or both. The major surface-localised proteins include α-C-protein and β-C-protein, the R-proteins R1, R3 and R4 (the last of these has been shown to be identical to Rib [16,17]), and the α-like proteins Alp2 and Alp3 [18,19], which may be variants of R1 protein [20]. The ϵ protein has also been called α-like protein Alp1. Several of the surface protein antigens induce protective immunity in animal models [15,21,22], and surface proteins common for many strains would have a potential role in vaccine development.

The *bca*, ϵ/*alp1*, *bac*, *rib*, *alp2* and *alp3* genes encode α-C-protein, ϵ/Alp1, β-C-protein, Rib, Alp2 and Alp3, respectively. Studies of surface proteins, and of the genes encoding these proteins, are important for epidemiological analysis of GBS infections, and may also be of use in the development of a GBS vaccine that is suitable for use in widespread geographical areas. Accordingly, the aims of the present study were to analyse the gene content and surface protein distributions among invasive GBS isolates from a defined geographical area, to monitor the population dynamics over a 13-year period, and to compare the distribution of the different genes and proteins among age groups and capsular serotypes. An additional aim was to identify surface protein antigens that might be of use in the development of GBS vaccines.

MATERIALS AND METHODS

Invasive GBS isolates were collected as part of two previous studies conducted in south-west Sweden. In total, 136 invasive GBS isolates from the first study, collected between 1988 and 1997 at the Department of Clinical Bacteriology, Sahlgrenska Hospital, Göteborg, or between 1995 and 1997 from four other bacteriological laboratories in south-west Sweden [23], were available for the present investigation. In the second study, 161 invasive GBS isolates were collected prospectively between 1998 and 2001 from the six bacteriological laboratories in the counties of Västra Götaland and Halland in south-west

Sweden [5]. These laboratories serve all 13 hospitals in the two counties. The mean population of the surveillance area was 1 767 215, and the total number of live births during the entire study period was 298 639 (http://www.scb.se). Of the 297 isolates, 143 were from female and 154 were from male patients, with 123 isolates from neonates and infants aged 0–180 days, and 174 from adults aged 19–96 years.

GBS isolates were collected from normally sterile samples (blood, cerebrospinal fluid and synovial fluid). Isolates were identified as GBS according to colony morphology, microscopy following Gram's stain of smears, and coagglutination with group-specific reagents (Streptest; Murex Biotec, Dartford, UK). The isolates were initially stored in broth at −70°C, and then freeze-dried and transported to St Olav's University Hospital, Trondheim, where surface protein typing and genotyping were performed. Only one isolate from each infectious episode was included in the study. Clinical data (age, gender, gestational age, underlying medical conditions, clinical manifestations and outcome) were obtained from the individual hospital notes of the patients. All hospital notes and relevant data were available for all patients. At the time of the second study (1998–2001), the policy at most of the hospitals for intrapartum antimicrobial prophylaxis was that recommended by the American College of Obstetricians and Gynecologists [24].

Antibody-based surface protein typing was performed using murine monoclonal antibodies against GBS α-C-protein, β-C-protein and Rib in an indirect whole-cell-based fluorescent antibody test, using reagents and methods described previously [25]. The resulting fluorescence, detected using a Nikon epifluorescence microscope, was graded from 0 to 3+, with scores of 2+ and 3+ being indicative of a positive test.

A multiplex PCR [26] was used to detect *bca*, ϵ/*alp1*, *rib* and *alp2*/*alp3*, encoding α-C-protein, ϵ/Alp1, Rib and Alp2/Alp3, respectively. Primers were synthesised by Eurogentech SA (Liege, Belgium). All isolates positive by PCR for *alp2*/*alp3* were further tested by *alp2*- and *alp3*-specific PCRs. All isolates were examined for *bac*, encoding β-C-protein, using primer pairs as specified previously [27]. Bacterial lysates were prepared as described previously [28]. PCR products were detected using an Agilent 2100 Bioanalyzer (Agilent Technologies, St Clara, CA, USA) as recommended by the manufacturer.

The capsular serotypes of the isolates included in this study have been described previously [5,23].

Statistical analysis

Proportions were compared using two-tailed Fisher's exact tests with the Bonferroni correction for multiple comparisons.

Ethical approval

The Central Ethics Review Board of Göteborg University and Lund University approved the two studies.

RESULTS

Surface proteins

The surface α-C-protein, β-C-protein and Rib were detected alone or in combinations in 152 (51%) of the 297 isolates. The most commonly

Table 1. Surface proteins detected using fluorescent antibody tests in invasive group B streptococci isolated from neonates and adults

	No. of isolates (%)		
Surface protein	Neonates	Adults	Total
Rib	43 (35)	37 (21)	80 (27)
α-C-protein	22 (18)	25 (14)	47 (16)
β-C-protein	1 (1)	3 (2)	4 (1)
α-C-protein and β-C-protein	2 (2)	19 (11)	21 (7)
None detected	55 (45)	90 (52)	145 (49)
Total	123	174	297

Table 2. Genes encoding surface proteins found among invasive group B streptococci isolated from neonates and adults

	No. of isolates (%)		
Gene	Neonates	Adults	Total
rib	74 (60)	52 (30)	126 (42)
alp3	19 (15)	58 (33)	77 (26)
ε/alp1	22 (18)	20 (11)	42 (14)
bca and *bac*	6 (5)	27 (16)	33 (11)
bca		10 (6)	10 (3)
alp2	1 (1)	2 (1)	3 (1)
alp2 and *bac*		1 (1)	1 (0.3)
alp3 and *bac*		1 (1)	1 (0.3)
Rib and *bac*		1 (1)	1 (0.3)
None detected	1 (1)	2 (1)	3 (1)
Total	123	174	297

detected protein was Rib ($n = 80$, 27%), followed by α-C-protein ($n = 68$, 23%). The most common combination was α- and β-C-protein ($n = 21$, 7%) (Table 1).

Gene content

The six genes investigated (*bca*, *bac*, *rib*, *ε/alp1*, *alp2* and *alp3*) were identified alone or in combinations in 294 (99%) of the 297 GBS isolates. The most common gene identified alone was *rib* ($n = 126$, 42%), followed by *alp3* ($n = 77$, 26%) and *ε/alp1* ($n = 42$, 14%). The most common combination was *bca* and *bac* ($n = 33$, 11%) (Table 2). The *alp2* gene was only detected in four

isolates. All combinations of genes included *bac*; indeed, *bac* was never found alone, but only in combination with other genes.

Correlation between gene content and surface proteins

The correlation between genes and surface proteins is summarised in Table 3. The *ε/alp1* and *bca* genes were more common in isolates in which α-C-protein was detected than in the sum of all other isolates (for *ε/alp1*, 31/68 vs. 11/229, p <0.0001; for *bca*, 36/68 vs. 7/229, p <0.0001). The *ε/alp1* or *bca* genes were found in 67/68 (98.5%) of isolates positive for α-C-protein. Surprisingly, *bca* was also more common in isolates in which β-C-protein was detected (24/25 vs. 19/272, p <0.0001), even though this gene does not encode β-C-protein. The *bac* gene was more common in isolates in which β-C-protein was detected (21/25 vs. 15/272, p <0.0001), and *rib* was more common in isolates in which Rib was detected (72/80 vs. 55/217, p <0.0001). All of the eight Rib-positive, *rib*-negative isolates contained *alp3*.

The α-C-protein was detected in 31 (74%) of 42 *ε/alp1*-positive isolates, and also in 36 (84%) of 43 *bca*-positive isolates. β-C-protein was detected in 21 (58%) of 36 *bac*-positive isolates. Rib surface protein was detected in 72 (57%) of 127 *rib*-positive isolates, and also in eight of 78 *alp3*-positive isolates.

Correlation among serotype, gene content and surface proteins

The distributions of genes and surface proteins among the different capsular serotypes are shown in Tables 4 and 5, respectively. Most genes and surface proteins were detected in isolates of all capsular serotypes. However, certain types were associated significantly with specific serotypes

Table 3. Correlation between gene content and surface proteins

	Genes										
Surface proteins	*rib*	*alp3*	*ε/alp1*	*bca* and *bac*	*bca*	*alp2*	*alp2* and *bac*	*alp3* and *bac*	*rib* and *bac*	None detected	Total
Rib	71	8							1		80
α-C-protein			31	12	4					1	48
β-C-protein				3			1				4
α- and β-C-proteins				17	3						20
None detected	55	69	11	1	3	3		1		2	145
Total	126	77	42	33	10	3	1	1	1	3	297

Table 4. Correlation between capsular serotype and gene content in invasive isolates of group B streptococci

Capsular serotype	Genes										
	rib	*alp3*	*ε/alp1*	*bca* and *bac*	*bca*	*alp2*	*alp2* and *bac*	*alp3* and *bac*	*rib* and *bac*	None detected	Total
Ia	2	2	22	2	2	1				1	32
Ib		3	1	17	5			1			27
II	1		8	10	1	1	1				22
III	116	3	1		1						122
IV	4	2	5	1							12
V		67	3		1	1	1			2	77
Not typeable	1		2	1							5
Total	126	77	42	33	10	3	1	1	1	3	297

Table 5. Correlation between capsular serotype and surface protein in invasive isolates of group B streptococci

Capsular serotype	Surface protein					
	Rib	α-C-protein	β-C-protein	α-C-protein and β-C-protein	None detected	Total
Ia		19		2	11	32
Ib	2	11	1	8	5	27
II	1	8	2	7	4	22
III	65	2			54	121
IV	4	4		1	5	14
V	7	2	1		66	76
Not typeable	1	2		1		4
Total	80	48	4	20	145	297

Table 6. Significant associations between gene content and serotype, and between surface protein and serotype

	Gene/specific serotype	Gene/other serotypes	p
ε/alp1 in Ia	22/32 (69%)	20/265 (8%)	<0.0001
bac in Ib	18/27 (67%)	18/270 (7%)	<0.0001
bca in Ib	22/27 (81%)	21/270 (8%)	<0.0001
bac in II	11/22 (50%)	25/275 (9%)	0.0001
bca in II	11/22 (50%)	32/275 (12%)	0.0008
rib in III	116/122 (95%)	11/175 (6%)	<0.0001
alp3 in V	67/77 (87%)	10/220 (5%)	<0.0001
rib in V	1/77 (1%)	126/220 (57%)	<0.0001
	Protein/serotype	Protein/other serotypes	
α-C-protein in Ia	21/32 (66%)	47/265 (18%)	<0.0001
Rib in III	65/122 (53%)	15/176 (8%)	<0.0001
α-C-protein in Ib	19/27 (70%)	49/270 (18%)	<0.0001
α-C-protein in II	15/22 (68%)	53/275 (19%)	<0.0001
β-C-protein in Ib	9/27 (33%)	15/270 (6%)	0.0004
β-C-protein in II	9/22 (41%)	15/275 (5%)	0.0001

(e.g., *ε/alp1* with serotype Ia; *bca* and *bac* with serotypes Ib and II; *rib* with serotype III; and *alp3* with serotype V) (Table 6). α-C-protein was associated significantly with serotypes Ia, Ib and II, β-C-protein was associated with serotypes Ib and II, and Rib protein was associated with serotype III (Table 6).

Differences between neonates and adults

The differences in the distributions of surface proteins and their corresponding genes according to serotype among neonates and adults are summarised in Table 7. The *rib* gene was more common among neonates than among adults (74/123 vs. 53/174, p <0.0001). The *alp3* gene was more common among adults than among neonates (59/174 vs. 19/123, p <0.0005). There were no differences in protein expression between the age groups. The differences found could be explained by differences in serotypes between the two age groups; serotype III dominated among neonates (p <0.0001), while serotype V dominated among adults (p 0.0002). There were no significant changes in gene content or surface proteins during the 13-year period in which the study isolates were collected (results not shown).

DISCUSSION

To date, epidemiological studies of GBS infections have used a classification according to CPS and surface proteins, and several methods have been devised for serotyping [29–31]. Variations in serotype/surface proteins have provided an important tool for following GBS infections in a population [1–6]. However, a weakness of antibody-based surface protein typing is that several strains can react with two or more antisera, indicating a high degree of cross-reactivity [32]. GBS strains show considerable variations at the genetic level, and genes under strong environmental selection may undergo mutations that are not detected by PCR. Genotyping methods, including pulsed-field gel electrophoresis, multilocus enzyme electrophoresis and multilocus sequence typing, have been used to characterise and distinguish specific clones among GBS isolates [33–36]. Molecular serotyping methods for CPS have also been established [37,38]. In the present study, fluorescent antibody tests and PCRs were used to characterise invasive GBS

Table 7. Correlation of capsular serotypes of group B streptococci with surface proteins and gene content, stratified according to age group (neonates or adults)

Age	Capsular type	Cα				Cα, Cβ		Cβ			Rib											Total
		ε	bca, bac	bca	n	bca, bac	bca	bca, bac	bac, alp2	bac	rib	alp3	rib, bac	rib	ε	bca	bca, bac	alp2	alp3	alp3, bac	n	Total
Neonate	Ia	12	1											1	2			1	1			18
	Ib		2			2																4
	II	3						1							1							5
	III	1									42			30					1			74
	IV	2												1								3
	V											1							16		1	18
	NT	1																				1
	Total	19	3			2		1			42	1		32	3			1	18		1	123
Adult	Ia	5			1	1	1							1	3	1			1			14
	Ib	1	6	2		6	1	1				1	1			2		1	2			24
	II	3	2			6	1	1			1				1		1			1		17
	III			1							23			22					1			47
	IV	1	1			1					4				2				2			11
	V	1		1					1			6			2			1	45		1	58
	NT	1				1					1											3
	Total	12	9	4	1	15	3	2	1		29	7	1	23	8	3	1	2	51	1	1	174

Cα, α-C-protein; Cβ, β-C-protein; R4, Rib protein; ε, ε/alp1 gene; n, not found.

isolates on the basis of surface proteins and their corresponding genes.

The genes encoding surface proteins were found to constitute a heterogeneous group, with no particular gene dominating, either alone or in combination with others. The genes studied were found in 99% of the invasive isolates, either alone or in nine different combinations. The overall distribution of these genes was similar to that reported previously [18,27,37]. The three proteins studied were found at high frequencies (57–84%) in isolates harbouring the corresponding genes, and vice versa (84–98.5%), but the correlations were not complete.

The surface proteins of GBS are likely to play an important role during different stages of GBS infection. They confer immunity in animal models [15,21,22] and are therefore considered to be of potential importance in vaccine development. α-C-protein and ε/Alp1, Rib, Alp2 and Alp3 all belong to the group of ladder-forming proteins. Individual proteins exhibit extended regions composed of long identical repetitions [39]. Sections of the ladder-forming proteins show sequence homology. The proteins are highly complex immunologically, and have both protein-specific sites and sites that are similar [18]. The surface proteins included in the present study, i.e., α-C-protein, β-C-protein and Rib, were detected in only 51% of the invasive GBS isolates studied (neonates 55%, adults 48%), which is in contrast with results from other studies in Europe

and the USA, in which the detection rates were higher (70–90%) [4,40]. In the present study, there was a higher proportion of serotype V strains [4,40,41]. Serotype V often carries *alp3*, against which no antiserum exists. This might have contributed to the lower expression rate of surface proteins found in the present study. Alternatively, the proteins might have been expressed in insufficient quantities to be detected by the antibody-based test used. Furthermore, it is not known whether the expression rate under in-vitro growth conditions is the same as that *in vivo*.

Rib was found less commonly than in a study from the USA [40]. This difference could be caused by geographical variation, a lower expression rate, or the use of a Rib monoclonal antibody in the present study. In contrast, *rib* was found at a higher frequency (43%) in the present study than in another study from the USA (28%) [41]. This second US study found *bca* in 29% of isolates, compared with only 14% of isolates in the present study. The difference in the proportion of *bac* was less pronounced (12% in the present study vs. 20% in the US study).

It has been shown previously that Alp3 possesses an antigenic determinant that is also present in Rib. This region is called the Rib/Alp3 common site, and explains the cross-reactivity between these proteins [20, 42]. In serotype V, the dominant surface protein gene is *alp3* (p <0.0001) encoding Alp3 [19]. In the present study, eight of 78 isolates with *alp3* also expressed Rib, and all 80

Rib-positive isolates harboured either *rib* or *alp3*. This could be a result of the Rib/Alp3 common site. Antibodies targeting the Rib/Alp3 common determinant could hamper the reliability of Rib detection by antibody-based methods. Alp3 possesses several sites for antibody binding, but only contains antigenic determinants that are shared with other streptococcal proteins. It may therefore be difficult to produce Alp3-specific antibodies [42].

GBS can traverse placental membranes and weaken their tensile strength, gain access to the foetus within the amniotic cavity, induce placental membrane rupture and/or trigger premature delivery. The lung of the neonate is the initial focus for GBS infection, leading to free access to the bloodstream. Invasive strains enter epithelial cells more efficiently than strains from the vaginal mucosa of asymptomatic women [43]. The surface proteins play an important role in the pathogenesis of GBS infection. It has been shown that strains passing from mother to neonate may undergo mutations in *bca*, encoding α-C-protein, and that these mutations coincide with a loss of susceptibility to antibody-mediated killing by polymorphonuclear leukocytes [44,45].

The CPS antibodies confer protective immunity [10], and the capsule protects the bacteria from phagocytosis. The capsular serotype distribution varies over time, among different countries and among populations. The most important serotypes among invasive GBS strains infecting neonates are currently serotypes III, V and Ia. Serotype III is the serotype found most frequently among neonates in both Europe and North America [1–7]. The present study confirmed relationships reported previously among serotypes and genes encoding surface proteins [27,29,32,33]. Significant associations between serotype Ia and ε/*alp1*, between serotype III and *rib*, between serotype V and *alp3*, and between serotypes Ia, Ib and II and *bca* and *bac*, were found, but the associations were not absolute, and many different combinations of genes were seen in most serotypes.

Vaccine development for GBS has focused on CPS antigens, with studies showing that conjugated polysaccharide vaccines are well-tolerated [11–14]. Unconjugated polysaccharides are poorly immunogenic, but a covalent coupling with proteins stimulates a T-cell-dependent antigenic recognition that profoundly enhances immunogenicity. There are several immunogenic GBS surface proteins that could be carriers for the CPS antigens, and/or act as protective antigens themselves. In a vaccine study in mice [46], α-C-protein was covalently coupled with serotype III CPS and was shown to be an effective carrier. Rib also protected mice, without coupling to polysaccharides [47]. Several studies have attempted to identify a single protective protein that would be present in all strains. Sip, present in all strains, generates an immune response and protection against invasive GBS infection in animal models [48], and has shown promise as a vaccine candidate, but further studies have shown that the surface accessibility of Sip depend on the presence of the CPS [49].

Protein antigens resulting in protection have been shown to be effective only against those strains in which the antigens are sufficiently exposed on the bacterial surface, even though the strain carries the corresponding encoding genes [49]. A CPS conjugate vaccine for this population would need a combination of several CPSs and/or protein antigens. The variability in surface antigen expression makes it very important to select strains with high expression and accessibility of the antigen when developing a vaccine formula. Development of effective vaccines and implementation of vaccine strategies will be one of the key future challenges for prevention of neonatal GBS infections.

The isolates in the present study were obtained from both children and adults. Elderly individuals and patients with underlying disease have an increased risk of invasive GBS infection. In this group of patients, almost nothing is known concerning potentially protective CPS or protein antibodies. The *alp3* gene dominated in adults and the *rib* gene in neonates, but there were no differences in protein expression between neonates and adults. The differences in gene distributions could be explained by differences in the capsular serotype distribution of GBS strains between adults and neonates (with serotype III being most common in neonates and serotype V in adults). There were no changes in the gene distribution or protein expression over time, which contrasted with time-related changes in the capsular serotype distribution of the same strains (most importantly, an increase in serotype V) [5].

In conclusion, characterisation of invasive GBS strains by investigating genes coding for surface proteins can be used as a complement to capsular serotyping of GBS. Based on the investigation of

six genes encoding surface protein, 99% of the invasive strains in the present study could be characterised. Certain genes were significantly more common in some capsular serotypes than in others. The detection rate of the proteins studied was rather low (51%), perhaps because of a high proportion of serotype V isolates, but the results of immunological typing were difficult to interpret, and further investigations are needed to identify reliable markers of protein expression. Epidemiological studies of GBS are important for the development of GBS vaccines suitable for use in a range of geographical areas. Genes encoding immunogenic surface proteins should therefore be studied in different parts of the world and over time. The present study identified many serovariants for possible use in epidemiological studies and the formulation of vaccines, but no single surface protein included in this study appears to be adequate for use in a successful GBS vaccine on its own.

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