MOLECULAR CHARACTERISATION OF BACTERIA

***Streptococcus agalactiae* β gene and gene product variations**

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Streptococcus agalactiae (group B streptococci; GBS) are serotyped on the basis of the capsular polysaccharide antigens and subtyped on the basis of the strain-variable and surface-localised c proteins c^{α} , c^{β} , and R proteins. This study compared c^{β} protein detection and the polymerase chain reaction (PCR) for β gene detection, by examining 50 clinical GBS strains. The c^{β} protein was detected by antibody-based immunofluorescence in a GBS whole-cell assay and Western blotting by probing with the anti- c^{β} antibody or human IgA. Absorption experiments were performed to test for surface-anchoring of c^{β} ; and bacterial supernates were examined to test for c^{β} production. Primers for the PCR target regions resulted in a 620-bp product that included β gene-encoding IgA-binding domains. The results demonstrated four categories of GBS with respect to the β gene and the c^{β} protein: (1) strains (16 of 50) that harboured the β gene and regularly expressed normal surface-localised c^{β} with a M_r of 120 kDa; (2) strains (5 of 50) that harboured the gene but did not express the protein; (3) strains (2 of 50) that harboured the gene but expressed a c^{β} that was not surface-localised and had reduced M_r ; (4) strains (27 of 50) without β gene and c^{β} expression. One strain amongst the third group generated a PCR product of 1330 bp. These results demonstrate considerable strain variability of the β gene of GBS and of its product the c^{β} protein.

Introduction

Streptococcus agalactiae (group B streptococcus; GBS) is an important cause of invasive disease in neonates, of pregnancy-associated disease in women, and of non-pregnancy-related disease in adults [1]. Serological classification of GBS plays an important role in their epidemiological study. GBS can be serotyped on the basis of the capsular polysaccharide (CHO) antigens and subtyped on the basis of strain-variable proteins which are bound to the bacterial cell surface. The surface-anchored proteins include the c proteins c^{α} and c^{β} and the R proteins. In addition, two other protein antigens, γ and δ , have also been described within the c protein fraction [2]. Immunoprecipitation or fluorescent antibody testing (FAT) are commonly used to detect both the CHO antigens and the protein antigens with polyclonal or, increasingly, monoclonal antibodies (MAbs). MAbs have been generated for c^{α} [3, 4], c^{β} [5], the R4 protein [6], and for some of the capsular CHO antigens [7]. Recently, GBS isolates from England and Norway were serotyped and subtyped,

the latter being performed with anti- c^{α} , - c^{β} and -R4 MAbs in a FAT. This revealed a total of 29 GBS serovariants among the 334 isolates examined [8]. The protein c^{α} occurred in 45.5% of the isolates, c^{β} in 24.3%, and R4 in 32.3% [8]. In addition to their importance as sero-subtype markers, the surface-localised proteins may function as virulence factors [9, 10] and they appear to be targets for protective antibodies in experimental infections [4, 11, 12]. c^{β} also has the ability to bind human IgA by its Fc fragment [13].

The present study focused on c^{β} and the β gene, which has been sequenced [14, 15]. The gene encodes a protein with an N-terminal signal sequence (S), two IgA-binding domains (A and B), a cell wall-spanning domain (W), and a membrane-spanning domain (M) on the C-terminal side of the protein [15]. Domain A on the N-terminal side is responsible for most of the IgA-binding capacity of c^{β} [16]. Recent studies have demonstrated variability among GBS isolates in surface-anchoring of c^{β} and in its ability to bind the Fc region of human IgA [17].

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The present study examined 50 GBS isolates from

neonatal and adult infections by antibody-based techniques for c^{β} protein detection and by PCR-based methods for β gene detection.

Materials and methods

Bacterial strains examined

A total of 50 GBS strains was analysed. The isolates were from adult and neonatal infectious disease cases in different Norwegian hospitals and had been forwarded to this laboratory for serotyping. All isolates had been included in a previous study of GBS serotype distribution [8]. Strains of the CHO antigen types Ia ($n = 21$), Ib ($n = 13$) and II ($n = 16$) were arbitrarily chosen from the strain collection to provide a suitable number for the purpose of this study. Amongst this collection, strains FAT positive ($n = 16$) or negative ($n = 34$) for c^{β} protein production were included. The strains A909 (serotype Ia/ $c^{\alpha\beta}$) and 46/94 (II) were used as positive and negative control strains for c^{β} production. Bacteria were preserved in Greaves's medium at -80°C and were cultured on blood agar plates or in Todd-Hewitt broth as described previously [18].

MAb

Generation and characterisation of the anti- c^{β} MAb (IgG1/ κ isotype) have been described previously [5]. The antibody had a titre of 10^6 in a GBS whole-cell ELISA [5].

Polyclonal anti- c^{β} serum

Protein c^{β} from GBS strain 15626 (IV/ c^{β}) was prepared by HCl extraction [18] of the bacteria and purified by affinity chromatography with CNBr-activated Sepharose (Pharmacia, Uppsala, Sweden) with bound IgG isolated from rabbit antiserum against c^{β} protein [3]. Immunisation of rabbits was performed as described previously [3].

FAT

Slides with GBS were prepared and the tests were performed and interpreted as described previously [8, 19].

Indirect ELISA

Todd-Hewitt broth supernates of cultured GBS A909 were used for coating after precipitation with trichloroacetic acid, 5% w/v, solubilisation of the precipitate in phosphate-buffered saline (PBS) pH 7.2, in a volume that corresponded to a 100-fold concentration of the supernate, followed by dialysis against PBS. The optimal coating dilution was determined by titration of the anti- c^{β} MAb. ELISA was performed as described previously [5] with peroxidase-labelled rabbit anti-

bodies against murine immunoglobulins (Dakopatts, Glostrup, Denmark) and colour development with *o*-phenylenediamine- H_2O_2 . The reaction was stopped after 10 min and OD₄₉₅ was recorded. In probing with rabbit anti- c^{β} serum, antibody binding was detected with peroxidase-conjugated swine anti-rabbit IgG (1 in 1000; Dakopatts).

Sandwich ELISA

A sandwich ELISA was used to detect c^{β} in concentrated fluid culture supernates. Coats for c^{β} capture were prepared with human IgA (5 $\mu\text{g}/\text{ml}$; Sigma). After washing and blocking [5], concentrated supernates which were diluted 1 in 2 in PBS with Tween 20 0.05% v/v (PBS-T) were applied and incubated (20°C ; 1 h). After washing, captured c^{β} was detected with the anti- c^{β} MAb or the rabbit antiserum as described for the indirect ELISA. The OD recordings were matched against those recorded when concentrated supernates of the positive (strain A909) and negative control strain (strain 46/94) were tested.

Immunoblotting

Components of SDS-lysed bacteria were separated by SDS-PAGE with polyacrylamide 12% gels and transferred on to PVDF membranes (BioRad, Richmond, CA, USA), pore size 0.2 μm , as described previously [3]. The membranes were processed for probing with the anti- c^{β} MAb (1 in 1000), rabbit anti- c^{β} serum (1 in 1000) or human IgA (5 $\mu\text{g}/\text{ml}$). Immunoglobulin binding was detected with peroxidase-labelled antisera as in ELISA or peroxidase-labelled goat antibody to human IgA (1 in 1000; Sigma) and colour development with *o*-phenylenediamine- H_2O_2 .

Absorption

GBS cell deposits were pelleted, washed with PBS, and adjusted to $c. 10^{10}$ cfu/ml in PBS. Equal volumes of the suspension and the anti- c^{β} MAb (1 in 2000) or rabbit antiserum (1 in 1000) in PBS-T, were mixed and incubated at 20°C for 1 h. Bacteria were pelleted (2000 *g*, 10 min) and the supernate was tested in ELISA for anti- c^{β} activity, with coats prepared with concentrated culture supernate of strain A909.

Primers and probe

Oligonucleotides were synthesised (Med Probe, Oslo, Norway) on the basis of published sequences of the β gene [15]. The two 24-mer primers corresponded to the nucleotides 1337–1360 (5'-AAGGCTATGAGTGAGAGCTTGGAG-3') and 1917–1940 (5'-CTGCTCTGGT-GTTT TAGGA ACTTG-3'), respectively. These primer positions resulted in a deduced amplification product of 604 bp which included a part of each of the IgA-binding domains A and B [15]. For hybridisation, an internal 39-mer probe targeting the nucleotide position

1686–1724 (5'-CAGTCACAGCTAAAAGTGATTCG-AAGACGACGTTGGACT-3') of the β gene [15] was synthesised. The probe was biotin-labelled in the 5'-end. Probe and primers were used without further purification and were preserved as described previously [20].

PCR amplification

Overnight broth cultures of GBS were diluted 1 in 10 in saline and 100 μ l were centrifuged (11 000 *g*; 5 min). The pellet was washed three times in saline, suspended in 100 μ l of deionised water, and 10 μ l of the suspension were added to 90 μ l of Chelex-100 lysis reagent: Chelex-100 (Bio-Rad) 15% w/v, Tween 20 1% v/v and Nonidet P-40 1% v/v in autoclaved ultra-pure water. The mixture was heated (95°C; 15 min), diluted 1 in 10 in deionised water and 10 μ l were mixed with 88 μ l of the PCR mixture: 2 μ l (10 mM) of each of the nucleotides dATP, dGTP, dTTP and dCTP, 0.4 μ l (200 mM) of each of the primers, 10 $\times$ PCR amplification buffer (Boehringer Mannheim) and 69.2 μ l of deionised water in thin-walled reaction tubes and with 2 drops of sterile mineral oil overlaid. After heating at 94°C for 5 min, 2 μ l of *Taq* DNA polymerase (2.5 U; Boehringer Mannheim) were added and the mixture was run in a DNA thermal cycler (Perkin-Elmer Cetus Corp., Norwalk, CT, USA) which had been prewarmed to 94°C. A total of 37 PCR cycles were run at 94°C (1 min) for denaturation, 60°C (1 min) for primer annealing, and 72°C (1 min) for DNA extension. The reaction was terminated at 4°C and PCR products were detected by agarose gel electrophoresis with agarose 2% gels as described previously [20].

Southern blot analysis

Agarose gel-separated PCR products were transferred to a Zeta Probe blotting membrane (BioRad) as described previously [20]. The membranes were processed for

hybridisation as described [20], and were hybridised in a hybridisation oven at 50°C for 60 min in a hybridisation solution [20] which contained 1 pmol of the biotinylated DNA probe. The membrane was washed twice (5 min; 20°C) in a washing solution [20], incubated at 20°C for 60 min with a streptavidin-peroxidase conjugate (1 in 2000; Sigma), washed in PBS-Tween 20, and developed with *o*-phenylenediamine-H₂O₂.

Results

All 50 GBS strains included in the present study, previously tested by an anti-*c* β MAb-based FAT [8], were examined by PCR (β -PCR) for β gene detection (Table 1). All 16 strains FAT-positive for *c* β were also β -PCR positive. Of the 16 FAT⁺/ β -PCR⁺ isolates, strains 3–8 shown in Table 2 were selected for further investigation. The remaining FAT⁺/ β -PCR⁺ isolates were not examined further.

Fig. 1 shows β -PCR and Western blot results with the positive (no. 1) and negative (no. 2) control isolates and strain nos. 3–8. All strains except the negative control generated a PCR product of 620 bp, which is close to the theoretical estimate of 604 bp. In immunoblotting, these strains showed a major band calculated at *c.* 120 kDa and a variable number of more weakly stained bands when probed with the anti-*c* β MAb. Thus, the strains shown in Fig. 1 produced quite similar results in the FAT and Western blotting

Table 1. Correlation of *c* β protein production tested by the FAT and PCR for β gene detection

β -PCR	Number of strains	
	FAT-positive	FAT-negative
Positive	16	7
Negative	0	27

Table 2. Designation, serotype and results obtained in the FAT, β -PCR and Western blotting with GBS isolates

Strain no.	Strain designation	Serotype	FAT*	β -PCR	Western blotting with	
					MAb*	IgA
1 [†]	A909	Ia/ <i>c</i> $\alpha\beta$	+	+	+	+
2 [‡]	46/94	II	–	–	–	–
3	161616/92	Ia/ <i>c</i> $\alpha\beta$	+	+	+	+
4	114007/92	Ia/ <i>c</i> $\alpha\beta$	+	+	+	+
5	19/94	Ib/ <i>c</i> $\alpha\beta$	+	+	+	+
6	14/95	Ib/ <i>c</i> β	+	+	+	+
7	3/94	II/ <i>c</i> β	+	+	+	+
8	26/94	II/ <i>c</i> β	+	+	+	+
9	23/94	Ia/ <i>c</i> α	–	+	–	–
10	45/95	Ia	–	+	–	–
11	13/94	Ib/ <i>c</i> α	–	+	–	–
12	56/94	Ib/ <i>c</i> α	–	+	–	–
13	R90/1653	Ib	–	+	–	–
14	31/94	II	–	+	+	+
15	23/95	IIc α	–	+	+	+

*With an anti-*c* β MAb.

[†]Positive control strain.

[‡]Negative control strain.

Fig. 1. Gel electrophoresis of PCR products of the amplified β gene of GBS strains (a) and Western blots of SDS-lysates of whole cell of the same strains probed with an anti- c^β MAb (b). Strains analysed are indicated at the top of each lane (see Table 2).

for c^β detection and in the β -PCR for β gene detection. The PCR product of these strains hybridised to the internal DNA probe (not shown).

Of the 34 FAT-negative strains, seven produced an amplification product in the β -PCR (Table 1). These strains are listed as nos. 9–15 and included capsular type Ia, Ib, and II GBS (Table 2). Six of the seven strains generated a 620-bp PCR product and one type II strain (no. 14) a 1330-bp product (Fig. 2a). The tests were repeated twice at an interval of several weeks and the results were reproducible. The PCR products were tested in a Southern hybridisation assay. The internal DNA probe hybridised to the PCR product of the FAT⁻/ β -PCR⁺ strains with the exception of strain no. 14, which generated the 1330-bp product (Fig. 2b). Concordant results were obtained when the amplification mixtures were tested in a dot hybridisation assay (data not shown).

When strain nos. 9–15 were probed in immunoblotting against the anti- c^β MAb only two (nos. 14 and 15) showed antibody binding, resulting in major lines of *c.* 94 kDa and *c.* 84 kDa, respectively (Fig. 2c), well below the M_r of 'normal' c^β . Strains were tested for binding of human IgA by its Fc fragment by Western blotting. The positive control GBS strain A909 and the FAT⁺/ β -PCR⁺ strain nos. 3–8 showed IgA-binding as expected, resulting in banding patterns similar to those obtained with the MAb (not shown). Also, the FAT⁻/ β -PCR⁺ strain nos. 14 and 15 bound IgA resulting in banding patterns similar to those that

Fig. 2. GBS strains tested for PCR products of the amplified β gene (a), for hybridisation of the products to an internal DNA probe (b), for binding of an anti- c^β MAb (c) and for human IgA Fc fragment binding (d). Strains analysed are indicated at the top of each lane (see Table 2).

were generated with the MAb, whereas strain nos. 9–13 failed to bind IgA (Fig. 2d).

For further testing of surface anchoring of c^β , the anti- c^β MAb was absorbed by whole cells of all the isolates shown in Table 2 and then tested for antibody levels against the A909 c^β protein. Strains 1 and 3–8 removed the anti- c^β antibodies, corresponding to OD₄₉₅ reductions which ranged from 70 to 90%, whereas strains 2 and 9–15 gave antibody absorption results corresponding to OD₄₉₅ reductions of <5%. These results accord with the FAT results and strongly indicate that strains 9–15 did not possess surface-bound c^β .

As the c^β protein is secreted by GBS [21], supernates of fluid cultures of all of the strain nos. 1–15 were examined. Concentrated (50-fold) culture supernates were tested in a sandwich ELISA in which IgA was the capturing immunoglobulin. Strain nos. 1, 3–8 and 14–15 showed MAb-binding corresponding to OD₄₉₅ recordings in the range 0.800–1.000 above the background. MAb-binding was not recorded when supernates of strains 2 and 9–13 were tested. Thus, all the

c^β -producing strains secreted the protein. Strains 9–13, negative for c^β in Western blotting, showed no c^β secretion. This was confirmed in experiments in which the concentrated supernates were used for coating in an indirect ELISA.

Production of c^β defective in IgA-binding has been reported [16, 17]. The study tested if c^β with deficiency in both IgA and MAb-binding might exist. Strains 1–15 were examined with polyclonal anti- c^β serum in experiments similar to those performed with the anti- c^β MAb, the FAT, immunoblotting, absorption and testing of culture supernates. In all experiments the results correlated with those obtained by probing with MAb or IgA. These results substantiate the assumption that the β -PCR positive strains (9–13) did not express c^β protein and that the c^β expressed by strains 14 and 15 was not anchored to the bacterial cell surface.

Discussion

The serogroup B polysaccharide is specific for *S. agalactiae*. However, these bacteria include a large number of variants based on genotypic or phenotypic characters [8, 22, 23]. In classification based on phenotypic markers, identification of the capsular antigens which define serotype and of strain-variable and surface-localised proteins which define subtypes, have been used most widely. With regard to the strain-variable protein c^β , strains have been categorised as either c^β positive or c^β negative by immunological tests. However, the present study has provided evidence of the heterogeneity of c^β and the gene encoding this protein.

The present study found that all 16 strains which tested FAT-positive for c^β were also β -PCR positive, producing a PCR product close to the estimated size of 604 bp. The regularity by which FAT-positive strains generated the β -PCR product correlates with the findings by other investigators who used a different primer set, which resulted in a 592-bp fragment of the β gene [24]. In immunoblotting, the FAT⁺/ β -PCR⁺ strains showed a major c^β band of 120 kDa which is lower than the *c.* 130-kDa c^β band described by other investigators [13, 25] and the theoretical estimate of a M_r of 126.841 Da based on sequence analysis [15]. Minor diversity of the M_r estimate may be due to the methodology, as a comparatively high polyacrylamide concentration (12%) in SDS-PAGE and a low molecular-weight standard were used.

Surprisingly, seven of 34 GBS strains which were FAT-negative for c^β , generated a PCR product in the β -PCR. Six of these strains produced a product that was indistinguishable from the 620-bp product of the FAT⁺/ β -PCR⁺ strains; whereas one strain (no. 14)

generated a 1330-bp product. The β gene abnormality of strain 14 was substantiated by failure of its PCR product to hybridise to the DNA probe which targeted an internal stretch of the amplified gene fragment. Strain 14 also produced an IgA and c^β antibody-binding protein, supporting the notion that this strain harboured the β gene, although abnormal with a possible insertion segment. The amplification product of the other seven FAT[−]/ β -PCR⁺ strains hybridised to the internal DNA probe, confirming that these strains harboured the β gene.

The seven FAT[−]/ β -PCR⁺ strains subdivided into two categories, those that did not produce the c^β protein (strains 9–13) and those that produced antibody and IgA-binding proteins (14 and 15). As the former strains generated a normal PCR product, the silent β gene in those strains could be due to a defective gene regulatory function. To our knowledge, no data concerning factors that control β gene expression are available. Failure to express gene products occurs quite frequently in streptococci [26, 27]. The FAT[−]/ β -PCR⁺ strains 14 and 15 expressed gene products, but these products differed from normal c^β . Firstly, c^β of these strains showed a major band of *c.* 94 kDa and *c.* 84 kDa, respectively, well below the M_r estimate of 120 kDa for normal c^β . Secondly, none of these proteins was surface-anchored in the bacteria, as shown by the negative FAT and inability of these bacteria to absorb the anti- c^β antibody. However, the abnormal c^β proteins were secreted during bacterial growth. Thus, it is likely that the signal peptide on the N-terminus of the protein [15] behaved normally and secured transmembrane transportation of the proteins. The proteins also behaved normally with respect to IgA-binding, indicating the integrity of the IgA binding domains, which are located N-terminally to the cell wall-spanning region [14, 15]. The reduced M_r of these c^β s and failure to bind to the bacterial cell surface could be explained by the fact that these proteins were truncated at the C-terminal end, perhaps due to deletions in a proline-rich area [14, 15] with a three-residue periodicity in this half of the protein [15]. Such deletions would affect the cell wall and membrane-spanning domains of c^β and surface-anchoring of the protein.

β gene amplification has been described previously, but the results were not compared with antibody-based testing [24]. In a recent study, testing of IgA binding by GBS strains and DNA-DNA hybridisation with a β gene DNA probe, showed concordant results [28]. The discrepancy between the results of that study and those of the present study may be related to methodological differences or the fact that GBS from different geographical areas were tested, or both. The present study did not detect c^β -expressing GBS which failed to bind IgA, but only strains 1–15 were tested for IgA-binding. GBS expressing c^β which lack the ability to bind IgA have been reported [16, 17], as has

c^{β} which did not anchor to the bacterial cell surface [17]. Brady and Boyle [17] found that four of 18 c^{β} -producing strains secreted the protein without bacterial binding. They found that the unbound but secreted c^{β} s were low mol.wt proteins in the range 38–55 kDa, well below the M_r s of similar c^{β} described in this study; although even these had reduced M_r s. The present study substantiates the findings by other investigators [16, 17] that a number of variations may affect the GBS c^{β} protein. In addition, the present studies have demonstrated the existence of GBS with unexpressed β gene or with a β gene with major abnormality.

The c^{β} protein is important in the serological typing of GBS, which, in this laboratory, is based on FAT with whole cells of the bacteria. For c^{β} detection, this test requires surface-anchoring of the proteins. More GBS strains will test positive for c^{β} if methods which detect the protein irrespective of cell surface binding are used; still more strains will test positive if c^{β} protein detection is replaced by β gene detection. Of the 50 isolates examined, 16 of 50 (32%), 18 of 50 (36%) and 23 of 50 (46%), respectively, would show positive results in the three methodological approaches outlined above. This must be taken into consideration in the selection of test methods for typing of GBS. As the 50 strains included in the present study were selected from a diverse collection of clinical isolates on the basis of FAT results for c^{β} expression and capsular antigen type, the prevalence of the various c^{β} and β gene variants may differ from that of the whole strain collection.

As the GBS c^{β} protein is a target for protective antibodies [4, 11, 12], this protein has been proposed as a vaccine candidate, either unconjugated or as a carrier protein in a capsular polysaccharide- c^{β} conjugate vaccine [29]. Although the protective c^{β} epitopes have not been mapped they are probably located on the N-terminal side of the cell wall-spanning region which we believe are absent in the defective c^{β} proteins described in this study. In that case, the defective proteins should possess the protective epitopes. However, it remains to be examined if immunisation with defective c^{β} will induce protective immunity and if these proteins could have advantages over normal c^{β} in a protein or conjugate vaccine.

These findings have demonstrated the existence of GBS with the following categories of β gene and c^{β} protein variations: (1) strains (16 of 50) that harboured the gene and regularly expressed surface-anchored c^{β} ; (2) strains (5 of 50) that harboured the gene but failed to express c^{β} ; (3) strains (2 of 50) that harboured the gene but expressed an abnormal c^{β} ; (4) strains (27 of 50) without β gene and c^{β} expression. One strain within the third category possessed an abnormal β gene. Our explanations of some of the findings are

speculative. Further experiments are required to elucidate the β gene and c^{β} protein variations and if these variations influence virulence, GBS carriage or disease.

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