Molecular characteristics of *Streptococcus agalactiae* strains deficient in alpha-like protein encoding genes

Christina Gabrielsen,† Johan A. Mæland,† Randi Valsø Lyng, Andreas Radtke,‡ and Jan Egil Afset,†,*

**Abstract**

*Streptococcus agalactiae* (group B streptococci, GBS) are important human and animal pathogens, which can be subdivided based on different capsular polysaccharides and surface-anchored alpha-like proteins (Alps), as well as other proteins. Nearly all GBS strains possess an Alp (Alp GBS), although Alp-negative GBS (non-Alp GBS) do occur. In this study, 10 (1.1%) of 932 clinical human GBS tested lacked an Alp encoding gene. All 10 strains were from patients with bloodstream infection, confirming that non-Alp GBS can be highly virulent. All non-Alp GBS expressed one or more of the surface-anchored proteins R3, Z1 and Z2, while less than 10% of unselected clinical strains express any of these proteins. In contrast to Alp GBS, all non-Alp strains tested were PCR negative for the upstream sequence of the insertion site of the Alp encoding gene of Alp GBS. Genome sequencing showed that all but one of the 10 clinical non-Alp strains and the non-Alp reference strain CNCTC 10/84 lacked a region surrounding the Alp gene commonly present in Alp GBS strains. These strains instead harboured an 849 bp region not present in the Alp prototype strain A909. We have shown that non-Alp GBS differ from Alp GBS in the region surrounding the insertion site of Alp genes of Alp GBS as well as in their content of other surface proteins and that PCR for the upstream flanking region of the Alp gene may be useful for differentiation between Alp and non-Alp GBS.

**INTRODUCTION**

*Streptococcus agalactiae* (group B streptococci, GBS) are part of the normal flora on mucous membranes in a large proportion of humans, usually without causing disease. When they occasionally cause severe disease, this most commonly affects neonates or the elderly, although all ages may be affected. Bacterial components such as the capsular polysaccharide (CPS) and certain proteins, notably proteins located at the bacterial cell surface, are probably important in the generation of disease, but such components can also induce an immune response that may provide protective immunity [1, 2]. Presently, 10 structurally and immunologically distinct CPS variants (serotypes) of GBS are known, named Ia, Ib and II to IX. A large number of surface-localized GBS proteins have also been identified. These include the alpha-like proteins (Alps), members of which (and encoding genes) are *Ca* (*bca*), Alp1 (*alp1*), Alp2 (*alp2*), Alp3 (*alp3*), Alp4 (*alp4*) and Rib (*rib*) [1, 2]. *alp3* also occurs in some *Streptococcus pyogenes* (group A streptococci, GAS) strains and encodes the R28 protein of this species [3, 4]. The Alps share structural features which have mostly been revealed by sequence analysis of Alp genes. Major domains in Alps are the signal peptide, the N-terminal region, the repeat area with a variable number of large and tandemly arranged repeats and the C-terminal region [1, 2, 5]. Domains of different Alps often display high sequence similarity, which provides a structural basis for immunological cross-reactivity between different Alps, and may also provide cross-protective immunity, the latter of importance in the context of vaccine development [4]. Alp genes have been proposed to belong to horizontally transferred genomic islands [3, 6]. Inter-strain transfer of Alp genes or gene domains in combination with homologous recombination most likely explains the broad distribution of these genes among GBS strains and also structural characteristics of the...
Alps with their mosaicism and immunological properties [1, 2, 5]. Alps probably contribute to the virulence of GBS, although the proteins are not indispensable for virulence [7]. The function of Alps as adhesins in the invasion process may be particularly important for the ability of GBS to cause systemic infection [3, 8, 9]. GBS also possess a large number of additional surface-anchored proteins including the R3 protein [10] and the recently described proteins Z1 and Z2 [11].

Lachenauer et al. [5] provided evidence that the flanking regions of Alp genes in GBS were highly conserved and structurally identical or nearly identical irrespective of the Alp gene present, meaning Alp genes are allelic. Basic metabolic functions were identified in the upstream and downstream of the Rib protein, consistent with a silent Alp gene in this strain. It is therefore not surprising that strain 18RS21 did not diverge noticeably from other Alp gene-containing isolates with respect to the Alp gene flanking regions [5]. Clinical GBS isolates nearly always possess one of the Alp genes and nearly always express the corresponding Alp, and such isolates have most often been studied. In this work, we have used a combination of immunofluorescence (IF), molecular biology and bioinformatics methods to investigate phenotypic and genotypic traits of GBS lacking an Alp gene (non-Alp GBS) compared to Alp GBS.

METHODS

Bacterial strains and culture conditions

The strains included in this study were as follows: 932 GBS strains from invasive infections, mostly with bacteraemia, forwarded for extended characterization at the National Reference Laboratory for GBS at St Olavs Hospital, Trondheim, Norway, during the period 2011–2014; 28 reference and prototype strains from our strain collection; and four GAS strains (Table 1, details in Table 3). The strains were cultured overnight on blood agar plates or in Todd–Hewitt broth at 35 °C. The study was approved by the regional committee for medical and health research ethics (REC central no. 2016/1164).

<table>
<thead>
<tr>
<th>Categories of strains</th>
<th>No. (%) of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Alp</td>
</tr>
<tr>
<td>GBS</td>
<td></td>
</tr>
<tr>
<td>Clinical</td>
<td>922 (98.9)</td>
</tr>
<tr>
<td>Reference</td>
<td>26 (92.9)</td>
</tr>
<tr>
<td>GAS</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1. GBS and GAS strains included in the study

IF testing

Expression of the R3, Z1 and Z2 antigens was examined by whole-cell-based IF testing using the antisera described below. All antisera were used in 1:50 dilution. The tests were performed, and the signalling was recorded as described previously [12]. For analysis of R3, a murine monoclonal antibody (immunoglobulin M) raised against the R3 protein of the serotype V/R3, Z1, Z2 strain CNCTC 10/84 (ATCC 49447) was used [13]. In addition, a polyclonal antibody against the R3 protein was obtained from rabbit anti-whole cell sera raised against the serotype NT/Alp4, R3 strain 9828 (NCTC 9828) by exhaustive cross-absorption of the antisera with selected GBS isolates as described elsewhere [11]. In a similar manner, anti-Z1 and -Z2 sera were prepared by exhaustive cross-absorption of anti-CNCTC 10/84 whole cell serum [11]. Testing of a variety of reference and prototype strains and a large number of clinical isolates provided evidence that these antisera were specific for the protein antigens R3, Z1 and Z2, respectively [11, 14].

DNA extraction and PCR analyses

Bacterial DNA for PCR analysis was prepared by adding one colony to 100 µl each of lysis buffer and TE buffer in a 1.5 ml tube, which was then vortexed and heated with shaking at 95 °C for 15 min. The mixture was centrifuged at 14 100 g for 2 min, and 2 µl supernatant was then used as PCR template. Molecular CPS typing and serosubtyping were performed by a combination of single target and multiplex PCR designed to detect the Alp- and the Cβ-encoding genes, as described previously [15, 16]. PCR for alp flanking regions was done using primers for a site named N-2 near the N-terminus of the Cα-encoding gene bca of the Ca reference strain A909 (NCTC 11078) and for C-2 located downstream of the C-terminus of bca as described by Lachenauer et al. [5]. The primer sets were as follows: for N-2, 5′-GGGTCAATAACATTGCTACGCCAG-3′ (forward) and 3′-CCGTTTGTTTCGTCTGTGAAGTATC-5′ (reverse), and for the site C-2, 5′-GGTACATTCTTCTCTGTAAGTGGAAG-3′ (forward) and 5′-GACACTGGC AAGCTGATCGATCAAC-3′ (reverse). The N-2 and C-2 PCRs were done following the same protocol as for molecular serotyping [16].

Genotyping

Multiple-locus variant-repeat analysis (MLVA) was performed as described previously [17, 18]. In silico multilocus sequence typing (MLST) was done by blasting the nucleotide sequences of ORFs for each clinical non-Alp strain (see below) against alleles registered for each of the MLST loci available in the PubMLST database (http://pubmlst.org/sagalactiae/) and assigning a sequence type (ST) to each strain based on its allele profile [19]. Selected alleles were verified by Sanger sequencing, as previously described [20]. Relatedness between STs was assessed by eBURST analysis [21].
DNA isolation, whole-genome sequencing and assembly

Bacterial cells were treated with proteinase K (1.5 mg ml\(^{-1}\)), lysozyme (0.5 mg ml\(^{-1}\)) and mutanolysin (250 U ml\(^{-1}\)) for 15 min with shaking at 37 °C, and 15 min at 65 °C. RNase (2 mg ml\(^{-1}\)) was added to the lysate before vortexing. Genomic DNA was then isolated using the Qiagen MagAttract DNA Mini M48 kit and the Qiagen BioRobot M48 workstation (Qiagen) as described by the manufacturer. Illumina sequencing libraries were prepared using the Nextera XT sample prep kit (Illumina), which were then sequenced on the Illumina MiSeq platform (Illumina) with 300 bp paired-end read configuration (MiSeq Reagent kit v3). Reads were trimmed on quality using Trimmomatic [22]. Trimmed reads were assembled using the SPAdes Genome Assembler version 3.5.0 [23] with the BayesHammer read error correction and Mismatch Corrector modules. QUAST version 2.3 [24] was used for assessing the quality of assemblies. Contigs were filtered based on coverage (>5\(\times\)) and size (500 bp). The draft genomes were subsequently annotated using the RAST server [25]. Geneious (version R7.1.8) and MAUVE [26] were used for whole genome comparison and visualization, and AlienHunter [27] was used for prediction of horizontal gene transfer events.

RESULTS
Prevalence of non-Alp GBS

All 932 GBS isolates referred for analysis in our laboratory during the period 2011–2014 were invasive strains which had given rise to a variety of clinical illnesses, mostly with bacteraemia (>90%). The isolates originated from different body sites, although blood culture isolates predominated and were from patients of all ages. Based on characterization by multiplex PCR for all known Alp types, only 10/932 (1.1%) of the referred strains were non-Alp GBS, confirming the widespread occurrence of Alp genes and the correspondingly rare occurrence of missing Alp genes in invasive GBS (Table 1). All 10 non-Alp strains were from patients with bloodstream infections, ranging in age from <1 to 97 years, and eight of them were from women (Table 2). Two (7.1%) of the 28 reference and prototype GBS strains included in this study, CNCTC 10/84 and 08-17, lacked an Alp gene. Of the four GAS strains included, two were Alp3 PCR positive, which showed that these strains contained a homologue of the GBS gene\(\text{alp}3\).

Identification of R3, Z1 and Z2 antigens in non-Alp GBS

All 10 clinical non-Alp isolates expressed one or more of the proteins R3, Z1 and Z2. Six of them expressed all three proteins, while three strains expressed only the Z1 protein (Table 2). The two non-Alp reference and prototype GBS strains expressed all three proteins (Table 3). Of the 26 Alp gene-positive reference and prototype strains, nine (34.6%) expressed one or more of the three proteins. Thus, 10/10 (100%) of the invasive non-Alp strains and 9/26 (34.6%) of the reference and prototype Alp strains expressed at least one of the three protein antigens R3, Z1 and Z2. None of the \(\text{alp}3\) positive or negative GAS strains expressed any of the Alp proteins.

Serotypes and genotypes of non-Alp GBS

Although the clinical non-Alp strains had been isolated over a 4 year period and from diverse geographical locations in Norway, both serotyping, MLST and MLVA genotyping results showed that these strains belonged to closely related genotypes. Eight of the 10 invasive non-Alp GBS strains belonged to CPS type V, while the remaining two non-Alps belonged to CPS types II and III. The two non-Alp reference strains, CNCT10/84 and 08-17, were also CPS type V (Table 3). CPS type V strains thus predominated among the non-Alp GBS, at least in this small collection of strains. All eight clinical CPS type V strains and the two type V reference strains belonged to MLST clonal

<table>
<thead>
<tr>
<th>Strain</th>
<th>Patient characteristics</th>
<th>IF for proteins</th>
<th>PCR for</th>
<th>Genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sex Age (y)</td>
<td>R3 Z1 Z2 CPS type</td>
<td>C-2 N-2</td>
<td>MLVA</td>
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<tr>
<td>14-179</td>
<td>F 0</td>
<td>– – –</td>
<td>V –</td>
<td>2-7-21-3-0</td>
</tr>
<tr>
<td>14-192</td>
<td>F 50</td>
<td>+ + +</td>
<td>II –</td>
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</tr>
<tr>
<td>13-6</td>
<td>F 33</td>
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<td>V –</td>
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<td>13-87</td>
<td>M 0</td>
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<td>V –</td>
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<tr>
<td>12-165</td>
<td>F 78</td>
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<td>V –</td>
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<tr>
<td>12-221</td>
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<tr>
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<td>– –</td>
<td>V –</td>
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<tr>
<td>11-11</td>
<td>F 36</td>
<td>+ + +</td>
<td>III –</td>
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<td>11-19</td>
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<tr>
<td>11-206</td>
<td>F 49</td>
<td>– + –</td>
<td>V –</td>
<td>2-0-0-3-0</td>
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</table>

CC, clonal complex.
complex (CC) 26; six of them belonged to ST26 and two to closely related STs (ST805 and ST806). The CPS type II and III strains belonged to ST2 (CC1) and ST19 (CC19), respectively. The 10 clinical non-Alps belonged to five different MLVA profiles, but six of the eight type V strains had identical profiles. The results suggest restricted clonality of non-Alps.

Distinguishing between Alp and non-Alp GBS by PCR

PCR for the flanking region sites N-2 and C-2 located upstream and downstream, respectively, of the Ca-encoding gene bca of strain A909 was used in an attempt to distinguish between Alp and non-Alp GBS. All of the clinical non-Alp strains failed to generate amplicons for any of the
two flanking sites (Table 2), which was also the case with the Alp-negative reference strains, CNCTC 10/84 and 08-17 (Table 3). This was in contrast to the Alp-positive reference or prototype strains, all of which produced N-2 amplicons of ~620 bp with negligible strain-to-strain variation in size. The C-2 amplicons, however, varied in size; of the 26 Alp gene-positive reference and prototype strains, eight strains generated ~1320 bp C-2 amplicons, 16 strains generated ~670 bp amplicons and two Alp strains were C-2 PCR negative. These results indicate that the N-2, but not the C-2 PCR, may have potential to discriminate between Alp and non-Alp GBS.

**Characterization of the Alp insertion site in Alp GBS**

A comparison of the Alp insertion site in the genome of whole genome-sequenced GBS strains encoding five different Alp proteins showed that all strains encode an AraC family transcriptional regulator, an alpha-like protein as well as an integrase gene (pseudogene in strain A909) adjacent to the tRNA-Thr gene which is believed to be the Alp genomic island insertion site (Fig. 1). Based on this, we tentatively define an Alp core region (ACR) that encompasses these genes as well as a number of additional ORFs, mostly with unknown function, located upstream from the integrase gene in strains SS1, COH1 and NEM316. The upstream and downstream flanking regions of the ACR appear to be highly conserved in these strains, thus indicating that Alp genomic islands of different Alp types are inserted in the same location in the genome of GBS.

**Characterization of the Alp insertion site in non-Alp GBS**

The genome sequences of the 10 clinical non-Alp GBS strains were compared to the genome sequences of the bca-positive reference strain A909 and the non-Alp reference strain CNCTC 10/84. Prediction of horizontal gene transfer events in the A909 reference strain using Alien-Hunter software identified a 7501 bp region that includes the Alp gene, an Ara-C family transcriptional regulator (SAK_0516), an integrase pseudogene (SAK_0518), a tRNA-Thr encoding gene (SAK_0519) and three conserved hypothetical proteins (SAK_0520-SAK_0522) as a potential mobile genomic island (Fig. 1). The non-Alp reference strain CNCTC 10/84 was missing the majority of this region, corresponding to the 5705 bp ACR of strain A909, but retained the tRNA and conserved hypothetical protein encoding genes. Nine of the 10 clinical non-Alp strains similarly lacked the ACR of strain A909. The ACR includes the primer binding sites for both of the N-2 primers and for the forward C-2 primer, which confirms the ability of these PCRs to discriminate between Alp and non-Alp GBS. CNCTC10/84, as well as the 9/10 clinical non-Alps, contains an 849 region (tentatively named the non-Alp region (NAR)) encoding three short putative hypothetical proteins with unknown function that is not found in strain A909 (Fig. 2) nor in the GBS strains of other Alp types (Fig. 1). One of the clinical non-Alp strains, the CPS type II strain 14-192, differed by displaying significant sequence similarity to the A909 genome (Fig. 1). This strain contained the part of the ACR encoding the AraC family transcriptional regulator and lacked the NAR. In place of the Alp gene, however, was found a region homologous to part of the transposase of the IS element Issa4. The regions flanking NAR in the clinical non-Alp and CNCTC 10/84 strains appeared to be highly conserved between strains, with few polymorphisms and insertions or deletions, and to be homologous to the flanking regions found in Alp GBS (Fig. 1). A BLAST search against the NCBI non-redundant nucleotide database using the CNCTC 10/84 strain as query revealed eight additional sequenced GBS genomes that lack an ACR, including a single human strain and seven strains of animal origin (Camelus dromedarius and Nile tilapia). Although these strains display greater strain-to-strain sequence variation than the clinical non-Alps, which in some places affects the reading frames of flanking genes, these strains also appear to be missing an ACR and contain a homologue of

![Alignment of the Alp genomic region of reference strains with different Alp types. The N-2 and C-2 PCR products are displayed above the A909 sequence. High-to-low sequence conservation is indicated by a colour panel above the sequences (green to red accordingly). Grey blocks indicate homologous sequences, whereas black horizontal lines denote gaps in the sequence and vertical black lines display single-nucleotide polymorphisms. Genes are marked by arrows, for which the putative functions are indicated by the coloured key.](Image)
among the non-Alp GBS could be due to a specific genetic background in such strains making them less prone to acquisition of Alp genes or more prone to deletion of such genes than strains of other CPS types [31], but could also be because the type V strains in this study belong to the same clonal lineage. The latter is supported by genotyping, which showed that the eight CPS type V non-Alps belonged to STs of the same CC (CC26) and 6/8 strains had identical MLVA profiles (Table 2). However, the MLST results showed that the non-Alps of other CPS types belonged to different phylogenetic lineages of GBS (CC1 and CC19, respectively). This confirms that the non-Alp trait is not exclusively linked to one phylogenetic lineage. The deviation in gene content of the ARC/NAR region of one of the clinical non-Alp GBS strains compared to the nine other non-Alp strains may indicate that this has previously been an Alp GBS, because the type V strains in this study belong to the same clonal lineage. The latter is supported by genotyping, which showed that the eight CPS type V non-Alps belonged to STs of the same CC (CC26) and 6/8 strains had identical MLVA profiles (Table 2). However, the MLST results showed that the non-Alps of other CPS types belonged to different phylogenetic lineages of GBS (CC1 and CC19, respectively). This confirms that the non-Alp trait is not exclusively linked to one phylogenetic lineage. The deviation in gene content of the ARC/NAR region of one of the clinical non-Alp GBS strains compared to the nine other non-Alp strains may indicate that this has previously been an Alp GBS, where a deletion event has occurred to remove most of the ACR at a later stage.

Alps are complex and usually high-molecular-mass proteins, depending on the number of repeat units in the molecules [1, 5, 32]. Synthesis of these proteins is likely to impose considerable expenditure on the bacteria. This may support the notion that these proteins provide important benefits to GBS, for instance in the ability to survive in hostile environments or to adhere to and invade human or animal tissues. The reference strain CNCTC 10/84 has been considered highly virulent in spite of lacking an Alp. This may at least in part be due to hyperproduction of the cyl operon product β-haemolysin/cytolysin [7]. The 10 non-Alp GBS strains detected in this study had all caused bloodstream infection, supporting the notion that non-Alps can stream infection, supporting the notion that non-Alps can

In previous studies, non-Alp strains of CPS types Ia, Ib, III and V have been described [14, 16]. In this study, strains of CPS type V predominated, although CPS type II and III strains were also detected. This dominance of CPS type V
may be candidates to compensate for the lack of Alp, since these proteins seem to be expressed by most or all non-Alps. One or more of these proteins were expressed by all 10 invasive non-Alp strains analysed in this study as well as the two non-Alp reference strains. This constitutes a far higher expression frequency of these proteins by non-Alp GBS than the frequency of 8% found in a collection of 173 invasive human Alp GBS [14] and of 34.6% of the reference Alp GBS strains tested in this study (Table 3). However, the potential function as well as the sequences and other characteristics of R3, Z1 and Z2 are unknown at present but ought to be a challenge to investigators. The pathogenicity of GBS is certainly multifactorial [33], and a variety of other microbial factors may contribute to the pathogenicity of the non-Alp GBS strains.

The results from the N-2 PCR of the reference and prototype GBS strains in this study as well as genomic comparison of strains with different Alp types show that the upstream flanking region of the Alp gene is highly conserved, in accordance with what has previously been concluded by Lachenaux et al. [5]. All Alp-positive strains produced nearly identical amplicons with the N-2 PCR for a site in the upstream flanking region of the Alp gene. In contrast, the C-2 PCR, designed for a site on the downstream flanking region, produced less uniform results, consistent with a lower sequence similarity in the downstream flanking region in genomes of Alp GBS strains. Interestingly, the two alp3 PCR-positive GAS strains were also N-2 PCR positive, and the alp3 PCR-negative GAS strains were N-2 PCR negative. This indicates that at least a part of the region flanking the N terminus of alp3 in R28 protein-positive GAS is structurally similar to the corresponding region in Alp-positive GBS. This, however, appears not to be the case with the downstream flanking region in N-2 PCR-positive GAS, since the C-2 PCR was negative for all four GAS strains tested. These results are consistent with horizontal transfer of genetic elements (islands) between alp3-positive GBS and GAS strains, where the transferred elements seem to include both the alp3 gene and upstream stretches, in accordance with earlier notions [3, 5, 34]. The PCR results support the idea that the N-2 PCR may provide discrimination between Alp and non-Alp GBS and, consequently, may be useful in characterization of GBS and potentially also of GAS strains. For instance, the N-2 PCR may be an alternative to other methods in identification of type T28 (R28 positive) GAS strains, which are important human pathogens [4, 35].

The results of genomic sequencing of the 10 clinical non-Alp GBS strains from this study confirmed that there are differences between Alp and non-Alp GBS in the regions adjacent to the Alp insertion site (Fig. 1). The genomes of the non-Alp strains lacked a 5705 bp ACR when compared to the genome of A909, including the Alp gene and stretches both upstream and downstream of the gene, and the sites for the N-2 primers and the forward C-2 primer. The predicted horizontally transferred genomic island including the Alp gene of strain A909 is substantially shorter than what has been suggested previously for strain NEM316 [6], which suggests that Alp genomic islands differ in size and gene content. Furthermore, the flanking regions which in that publication were suggested to be part of this genomic island were in this study found to be highly conserved both in Alp- and non-Alp GBS strain (Figs 1 and 2). Together, these data indicate that the ACR, as defined by us, represents the horizontally transferred genomic island and, furthermore, that the Alp-encoding genomic islands are inserted into the same location in the genome of GBS of different Alp types.

In 9 of the 10 clinical non-Alp strains, the site corresponding to the ACR of Alp GBS was occupied by the 849 bp NAR region, whose function is unknown. Interestingly, a BLAST search of the NAR revealed homologous regions both for the NAR and immediate upstream and downstream flanking regions in non-Alp GBS isolated from animals, although the sequence similarity was lower than when compared to the clinical non-Alps in this study. The finding that one of the clinical non-Alp strains (14-192) deviated from the other non-Alps by having a sequence with significant homology to the upstream flanking region of the Alp gene of GBS shows that the non-Alp trait has appeared in different clonal lineages of GBS through independent genetic events.

In conclusion, we have found that some phenotypic as well as genotypic traits of non-Alp GBS differ from Alp-containing GBS and that at least some of these traits result from differences in the genomic region which harbours an Alp gene in Alp GBS. We have also shown that a PCR for the upstream flanking region of the Alp gene may be useful for differentiation between Alp and non-Alp GBS.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References
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