MAJOR ARTICLE

# Multilocus Sequence Typing of Serotype III Group B Streptococcus and Correlation with Pathogenic Potential

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**Serotype III group B streptococcus (GBS) causes more invasive disease in infants than do other serotypes in North America. We used multilocus sequence typing to identify clones within 28 invasive serotype III GBS isolates identified from a population-based study and 55 serotype III GBS colonizing isolates from a cohort of women from the same population. Ten allelic sequence types (STs) were identified and primarily involved 2 profiles: ST-19 (57.1% of invasive isolates and 58.2% of colonizing isolates) and ST-17 (32.1% of invasive isolates and 29.1% of colonizing isolates). On concatenation, the 10 allelic profiles converged into 3 groups. Group 1 consisted of ST-19 complex, ST-36, and ST-1, and was closely related to reference genome 2603V/R (serotype V). Group 2 consisted of ST-17 complex. Group 3 consisted of ST-23 complex and was closely related to the serotype III genome strain NEM 316. Neither of the major sequence types or groups was more commonly associated with invasion ($P = .61$) or with lower levels of maternal capsular polysaccharide–specific IgG (0.89 $\mu$g/mL and 0.39 $\mu$g/mL, respectively) for ST-19 and ST-17 ($P = .86$). The close association of genomic strain 2603V/R (serotype V) with ST-19 suggests that the phenomenon of capsule switching may have occurred.**

Group B streptococcus (GBS) has been the major cause of bacterial sepsis and meningitis in newborns for the past 3 decades [1–7]. Antibodies to type-specific capsular polysaccharides (CPSs) of GBS in the serum of animals used in experiments and of human neonates correlate with protection from GBS disease [8, 9].

In a recent cohort study of 1207 pregnant women in Alberta, Canada [10], we compared the GBS serotype distribution of vaginal-rectal colonizing isolates recovered from these women with the GBS serotype distribution of all isolates recovered from neonates with early-onset disease (onset occurring at <7 days of age) identified by population-based surveillance. We also determined serum concentrations of GBS serotypes Ia, Ib, II, III, and V CPS-specific immunoglobulin G (IgG), according to the serotype of the vaginal-rectal colonizing strain. Serotype III accounted for 20.6% of the colonizing strains available for typing, but it accounted for 37% of the invasive isolates from neonates ($P <$ .01). Maternal colonization with serotype III was least likely to be associated with moderate concentrations of serotype III GBS CPS-specific IgG, compared with other serotypes. We concluded that, in our study population, serotype III GBS was more invasive in neonates born to mothers colonized with GBS serotype III, compared with other serotypes. This may, at least in part, be due to a poor maternal serotype III CPS-specific IgG re-

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sponse to vaginal or rectal colonization. However, it was apparent that colonization with some serotype III isolates was associated with robust maternal serotype III CPS-specific serum IgG responses, whereas colonization with some other serotype III isolates was not.

Previous investigators have postulated the existence of high-virulence clones of serotype III [11–14]. We thus used multilocus sequence typing (MLST) to distinguish genotypes, to determine whether distinct clones among the serotype III GBS isolates assessed are associated with differential maternal serotype III CPS-specific IgG responses, and to infer whether one clone is more virulent than others, on the basis of the MLST patterns comparing neonatal invasive and maternal colonizing strains. We also compared, by use of MLST, the genetic profile of isolates in the present study with known genomic strains of GBS and group A streptococcus (GAS).

MLST has provided much information about the population biology of several bacterial pathogens [15–20]. The technique is based on the same approach used in multilocus enzyme electrophoresis (MLEE), in that genetic variation from multiple chromosomal locations is indexed. However, MLST has major advantages over MLEE, in that the sequence data that are produced are stable, unambiguous, and easily repeatable within and between laboratories. MLST involves direct characterization of gene alleles through the sequencing of nucleotides from gene fragments, whereas MLEE is an indirect characterization of gene alleles through electrophoresis of gene products. MLST has recently been developed for GBS, and an initial database of the allelic profiles of 152 isolates obtained from neonates and adults with serious invasive disease or asymptomatic genitourinary carriage has been established [21] (available at: http://sagalactiae.mlst.net).

## PATIENTS AND METHODS

***Population.*** The population from which the GBS isolates in the present study were recovered has been described elsewhere [10]. In brief, all invasive isolates were identified from prospective, population-based active surveillance for early-onset GBS disease in neonates in Alberta, Canada, from 1 January 1995 through 31 December 1999. Informed consent was obtained from all patients and/or their parents. The study received ethical approval from the Conjoint Medical Ethics Committee of the University of Calgary (Calgary, Alberta, Canada). "Invasive disease" was defined by the presence of clinical signs of infection in neonates <7 days of age, in association with isolation of GBS from a sterile site (e.g., blood, cerebrospinal fluid, joint, pleural or peritoneal fluid, surgical tissue, etc.). The colonizing GBS isolates were vaginal-rectal GBS strains from a cohort of pregnant women in Calgary [10]. Serum samples were obtained from a subset of these women and were tested for concentrations of GBS serotype III CPS-specific IgG, by use of ELISA, as described elsewhere [8]. CPS-specific capsular serotyping of all the GBS isolates was performed at the National Center for Streptococcus (Edmonton, Alberta, Canada).

***Methods.*** Twenty-eight invasive serotype III GBS isolates recovered from neonates with early-onset disease and 55 colonizing serotype III isolates recovered from pregnant women (gestation, 35–37 weeks) from a previous study [10] were subcultured twice onto Columbia agar (we presume) with 5% horse blood. DNA was extracted from each strain by use of the DNeasy kit (Qiagen). Internal fragments (size, ~400–500 bp) of 7 housekeeping genes, which were chosen for GBS characterization by use of MLST, were amplified from DNA extract by polymerase chain reaction (PCR). The 7 loci that were chosen were *pheS* (phenylalanyl transfer RNA synthetase), *atr* (amino-acid transporter protein), *tkt* (transketolase), *glcK* (glucose kinase), *sdhA* (L-serine dehydratase), *glnA* (glutamine synthetase), and *adhP* (alcohol dehydrogenase). MLST was performed on these isolates by use of methods published elsewhere [21].

Results were analyzed using sequence navigator software (Perkin-Elmer Applied Biosciences). For each locus, every different sequence was assigned a distinct allele number, and each isolate was defined by a series of 7 integers that corresponded to the alleles at the 7 loci. Complete genome sequences of serotype III GBS and serotype V GBS have been previously published elsewhere [22, 23]. In a second analysis, sequences for the 7 loci were concatenated (i.e., combined end to end) into a single "supergene" for each sequence type (ST). The nucleotide differences between the supergene sequences were used to infer phylogenetic relationships among STs. The phylogenetic trees were calculated using the MEGA program with the neighbor-joining algorithm and the Kimura 2-parameter mutation model of genetic distance [24]. We used the genome information from GenBank as a source of sequence data for the 7 homologous genes in the MLST system, so that the reference strains could be included in the analysis for comparative purposes. For the present analysis, to allow for better comparison, sequences for the 2 reference genomes of *Streptococcus agalactiae* (GBS) (2603V/R [22] [available at: http://www.tigr.org] and NEM 316 [23]) and from the 4 genomes of *Streptococcus pyogenes* [25–27] were added to root the phylogeny.

All data were entered into an Access database (version 7.0; Microsoft) and were analyzed using SPSS (version 11; SPSS). Median levels of GBS CPS-specific IgG in serum samples were calculated. Comparisons of antibodies in these different groups of women were calculated using the $\chi^2$ test and Fisher's exact test, for categorical variables, and the Kruskal-Wallis test, for comparison of the distribution of antibodies between the 2 major groups. For calculation of antibody logarithmic means, we used the following formula: logarithim of antibody =

**Table 1. Allelic profiles of 83 serotype III group B streptococcal strains.**

| Sequence type | Allelic profile[a] | No. (%) of invasive strains[b] ($n$ = 28) | Source of invasive strains | No. (%) of colonizing strains[c] ($n$ = 55) | No. (%) of all strains ($n$ = 83) |
|---|---|---|---|---|---|
| 1 | 1,1,2,1,1,2,2 | 0 | Not applicable | 2 (3.6) | 2 (2.4) |
| 17 | 2,1,1,2,1,1,1 | 9 (32.1) | Blood (6 isolates); blood and CSF (3 isolates) | 16 (29.1) | 25 (30.1) |
| 19 | 1,1,3,2,2,2,2 | 16 (57.1) | Blood (15 isolates); blood and CSF (1 isolate) | 32 (58.2) | 48 (57.8) |
| 23 | 5,4,6,3,2,1,3 | 0 | Not applicable | 1 (1.8) | 1 (1.2) |
| 31 | 2,1,1,6,1,1,1 | 1 (3.6) | Blood | 0 | 1 (1.2) |
| 32 | 2,1,1,2,7,1,1 | 0 | Not applicable | 1 (1.8) | 1 (1.2) |
| 33 | 5,6,4,3,2,1,3 | 1 (3.6) | Blood | 0 | 1 (1.2) |
| 34 | 5,7,6,3,2,1,3 | 1 (3.6) | Blood | 0 | 1 (1.2) |
| 35 | 1,1,3,2,2,5,2 | 0 | Not applicable | 1 (1.8) | 1 (1.2) |
| 36 | 1,1,3,2,2,2,7 | 0 | Not applicable | 2 (3.6) | 2 (2.4) |

**NOTE.** Sequence type (ST)–17 complex, ST-17, ST-31, and ST-32; ST-19 complex, ST-19, ST-35, and ST-36; ST-23 complex, ST-23, ST-33, and ST-34. CSF, cerebrospinal fluid.

[a] Allelic profiles for each gene are presented in the following order: *adhP, pheS, atr, glnA, sdhA, glcK,* and *tkt.*

[b] Isolated from neonates <7 days of age.

[c] Isolated from pregnant women.

logarithm(1 + antibody). $P < .05$ was considered to be statistically significant.

## RESULTS

Among the 28 serotype III GBS invasive isolates recovered from neonates and the 55 serotype III colonizing isolates recovered from pregnant women, 10 allelic STs were identified. On the basis of these allelic profiles, most of the isolates (invasive and colonizing) belong to 1 of 2 major sequence types: ST-19 (57.1% of invasive isolates and 58.2% of colonizing isolates) or ST-17 (32.1% of invasive isolates and 29.1% of colonizing isolates) (table 1). These 10 sequence types could be grouped into clonal complexes that include single- and double-locus variants: ST-17 complex (includes ST-17, ST-32, and ST-31; $n = 27$); ST-19 complex (includes ST-19, ST-35, and ST-36; $n = 51$); and ST-23 complex (includes ST-23, ST-33, and ST-34; $n = 3$). ST-1 ($n = 2$) was not a part of any complex. There was no statistical significance in the relative frequency of the major allelic ST complexes, in a comparison of invasive isolates with serotype III colonizing isolates: (number of invasive isolates in the ST-17 complex, 10 [of 27]; number of colonizing isolates in the ST-19 complex, 16 [of 51]; $P = .61$).

***Concatenation.*** The sequences of 7 coding regions were

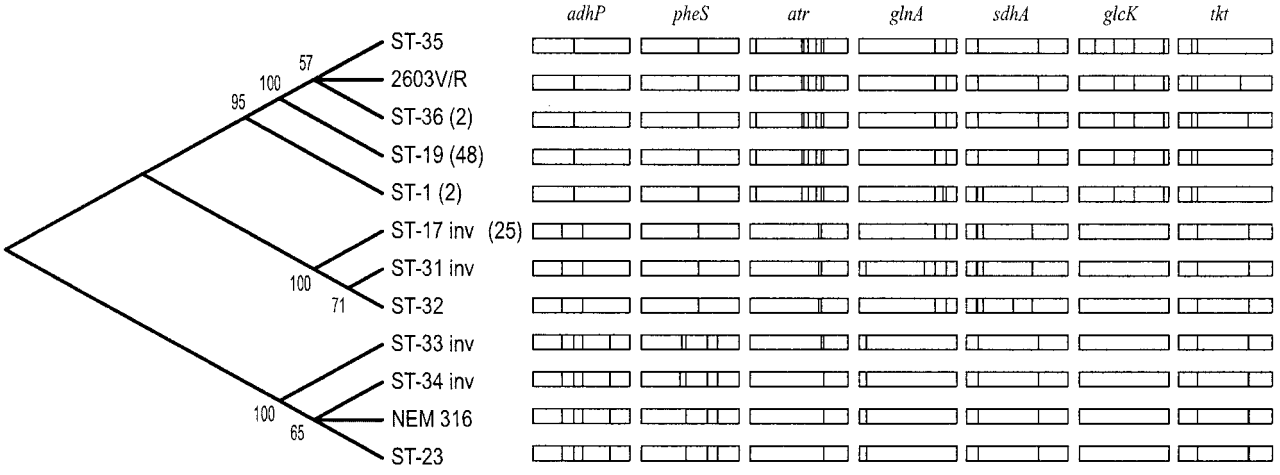


**Figure 1.** *Left,* Branching pattern (topology) and percentage of bootstrap trees that support particular clusters. For example, 100% of the trees produced by random sampling of the positions in the sequences cluster ST-33, ST-34, and ST-23, together with the sequence type of genome strain NEM 316. The trees were rooted with the *Streptococcus pyogenes* sequences. *Right,* Diagram of the nucleotide differences defining the alleles for each of the 7 genes. The vertical lines denote positions in the sequence where mutations have occurred. inv, Invasive isolate; ST, sequence type.

**Table 2. Sequence divergence between serotype III group B streptococcus (GBS) strains, according to pairwise comparison of 3 groups of sequence types.**

| Group[a] comparison | Divergence, % | $d_S \times 100$, mean ± SE | $d_N \times 100$, mean ± SE |
|---|---|---|---|
| GBS group 1 vs. group 2 | 0.7 | 1.85 ± 0.40 | 0.22 ± 0.09 |
| GBS group 1 vs. group 3 | 0.8 | 2.06 ± 0.42 | 0.32 ± 0.11 |
| GBS group 2 vs. group 3 | 0.6 | 1.57 ± 0.38 | 0.20 ± 0.08 |
| GBS vs. GAS[b] | 20.9 | 87.46 ± 5.33 | 9.18 ± 0.71 |

**NOTE.** $d_S \times 100$, No. of synonymous differences calculated per 100 sites; $d_N \times 100$, no. of nonsynonymous differences calculated per 100 sites; GAS, group A streptococcus; SE, standard error.

[a] Group 1, sequence type (ST)–19 complex and ST-1; group 2, ST-17 complex; group 3, ST-23 complex.

[b] The mean divergence between GBS and *Streptococcus pyogenes* (GAS) is provided for comparison.

concatenated and analyzed at the sequence level, on the basis of the nucleotide differences between STs. Among the 3456 total nucleotide sites, there were 42 variable positions and 36 informative sites (i.e., variable sites where the rare point mutation occurs in ⩾2 strains). Nucleotide differences were tabulated for 2 different types of sites in the protein-coding genes. Synonymous (or silent) sites include the first and third positions in codons where nucleotide mutations do not change the amino acid in the protein because of the redundancy in the genetic code. Nonsynonymous (or replacement) sites include the second position and the first and third positions in some codons where a nucleotide change results in an amino-acid substitution in the predicted protein. The average difference between strains, per 100 synonymous sites, was 0.92, so that, on average, a pair of GBS strains from the present study differed at ~1% of the synonymous sites. Nonsynonymous (amino acid–changing) sites were more conserved and differed at ~0.11% of those sites. A phylogenetic analysis that used the Kimura 2-parameter model of mutation and the neighbor-joining algorithm was used to generate 1000 bootstrap trees. The consensus tree and bootstrap confidence values support 3 groups of sequences (figure 1): group 1, which consisted of 53 isolates, including the ST-19 complex (51 isolates) and ST-1 (2 isolates); group 2, which consisted of ST-17 complex (27 isolates); and group 3, which consisted of ST-23 complex (3 isolates). Group 1 converged with reference genome 2603V/R (serotype V), whereas group 3 converged with the serotype III genome strain NEM 316.

We estimated the divergence between the GBS groups for both synonymous and nonsynonymous sites (table 2). The divergence ranged from 1.5 to 2 synonymous differences per 100 synonymous sites and from 0.2 to 0.3 nonsynonymous differences per 100 nonsynonymous sites (table 2). This degree of divergence between GBS groups is approximately one-fiftieth of the distance between the GBS and the GAS.

There also was no significant difference in the frequency of invasive isolates between the groups of sequence types, by use of this analysis method. In clonal group 1, 16 (30%) of 53 isolates were invasive (excluding reference genome 2603V/R), and, in group 2, 10 (37%) of 27 isolates were invasive ($P = .54$). It was noteworthy that clonal group 3 (ST-23 complex), which includes genomic strain NEM 316 (serotype III), was rare in the present study. Furthermore, genome strain 2603V/R (serotype V) was phylogenetically closely related to the group that includes the major type III subclone in the present study (group 1, primarily ST-19). Group 2, which includes the second most common ST in the present study (ST-17), was divergent from both genome strains NEM 316 and 2603V/R.

***Association of the allelic type of the colonizing strain with maternal serotype III CPS–specific IgG serum level.*** Forty-seven women who were colonized with serotype III GBS also had a serum concentration of serotype III CPS-specific IgG determined during the same visit when the vaginal-rectal swab specimens were obtained, to assess GBS colonization status. The serotype III CPS-specific IgG serum levels ranged from 0.012 to 49.5 μg/mL (median, 0.43 μg/mL) (table 3). The 2 most common allelic types and median antibody levels were compared. There was no difference between the median serotype III CPS-specific IgG concentrations in serum samples obtained from women colonized with the 2 major allelic types ST-19 and ST-17 (0.89 μg/mL and 0.39 μg/mL, respectively; $P = .86$) or between the logarithmically transformed mean values (0.9 μg/mL vs. 0.86 μg/mL, respectively; $P = .54$). Similarly, there were no differences in the proportion of women with serotype III GBS CPS-specific IgG levels <0.5 μg between the 2 major ST complexes (ST-19 complex [in 14 of 30 women]

**Table 3. Allelic profiles and serotype III capsular polysaccharide–specific immunoglobulin G (IgG) concentrations in serum samples obtained from pregnant women (gestation, 35–37 weeks).**

| ST | No. of valid samples[a] ($n = 47$) | IgG concentration,[b] μg/mL | | |
|---|---|---|---|---|
| | | Median | Minimum | Maximum |
| 1 | 1 | … | 0.124 | 0.124 |
| 19 | 27 | 0.89 | 0.025 | 16.84 |
| 36 | 2 | 0.069 | 0.025 | 0.113 |
| 35 | 1 | … | 0.223 | 0.223 |
| 17 | 14 | 0.39 | 0.012 | 49.51 |
| 32 | 1 | … | 7.28 | 7.28 |
| 23 | 1 | … | 0.074 | 0.074 |

**NOTE.** ST, sequence type.

[a] Not all women had serum samples obtained for serotype III capsular polysaccharide–specific IgG determination.

[b] For the group of 47 samples, the median concentration was 0.43 μg/mL, and the range of concentrations (minimum and maximum values) was 0.012–49.51 μg/mL.

vs. ST-17 complex [in 8 of 15 women]; $P = .67$). Finally, the analysis showed no difference between the 2 major groups, as determined by concatenation ($P = .63$).

## DISCUSSION

Previous authors have demonstrated, using primarily pooled isolates, the existence of highly virulent clones of GBS [11–14]. However, there have been few studies in which epidemiologically well-characterized isolates that were carefully collected, over time, from the same geographical location, have been analyzed with molecular methods. Using MLST, we identified 10 allelic profiles among the serotype III GBS invasive isolates recovered from neonates in a population-based study and among colonizing isolates recovered from pregnant women, with the majority clustering into 3 groups on concatenation. The present analysis supports the observation, already made by others, that there are distinct phylogenetic lineages of serotype III GBS [11–14]. In contrast to the findings of a previous study [11], there was an equal distribution of these groups among colonizing isolates and invasive isolates recovered from neonates with early-onset disease. This suggests that, in the Canadian population involved in the present study, the invasiveness of serotype III GBS in neonates <7 days of age, as determined by MLST, is proportional to maternal colonization rates and is independent of the clonal group. Additional studies are needed to better understand the difference in virulence between the clones identified in the present study and other previously identified clones. In particular, it would be important to examine other GBS serotypes to see whether there are MLST patterns associated with invasive disease, regardless of serotype.

We have previously shown that serotype III GBS colonization among pregnant Canadian women is associated with lower serotype III CPS-specific IgG serum levels than is colonization with other GBS serotypes [10]. In the present study, we could not identify a specific clone more likely to be associated with a low specific IgG level. This may be related to an inadequate sample size and, thus, inadequate power, or it may suggest that production of serotype III CPS-specific IgG is dominated more by possession of the serotype III CPS than by the overall genetic profile of the GBS. The findings of the present study corroborate the findings from previous studies in which it has been shown that type-specific immunity is needed for protection [8, 28, 29]. These data, along with the proportional distribution of colonizing and invasive strains, do not support the presence of a "virulent clone" of serotype III GBS in the population in the present study. More studies are needed to better define whether virulent clones do exist in other populations.

Hauge et al. [14] used restriction fragment–length polymorphism analysis on *Eco*RI- and *Msp*I-digested whole-cell DNA, to examine the genetic diversity and associations in a collection of 85 Danish strains of GBS. They showed a population separating into 6 major lineages that correlated with individual GBS serotypes. Similar to the data that we obtained with MLST, the data from their analysis showed that the GBS population examined was predominantly clonal. In particular, serotype III strains divided into 2 distant evolutionary lineages, 1 of which lacked expression of hyaluronidase activity.

An interesting finding of the present study was the discovery that the genome strain sequenced by 2603V/R (serotype V) was phylogenetically closely related to the clonal group that includes our major serotype III subclone (group 1), on the basis of sequencing of regions from 7 housekeeping genes. This serotype-independent clustering supports the possibility of capsule switching, even though such events have not yet seen demonstrated to occur spontaneously in GBS. Similar observations have been made by Tettelin et al. [21] and Jones et al. [22]. Serotype III CPS is produced under the direction of the *cps*III locus and comprises 16 genes within 15.5 kb of contiguous chromosomal DNA, with an operon that is transcribed as a single polycistronic message [30]. It previously has been shown that suppression of the gene $cps_{III}H$ in a type Ia strain resulted in suppression of CPS Ia synthesis and in production of CPS that reacted with serotype III–specific polyclonal antibody [30]. Similarly, expression of the putative Ia polymerase gene in a serotype III strain reduced synthesis of III CPS with production of a type Ia immunoreactive capsule. These findings suggest that a single gene can confer serotype specificity in organisms producing complex polysaccharides and that genetically diverse strains possessing the appropriate genome can share the same serotype.

We have confirmed that MLST is a very useful method for the classification of serotype III GBS isolates. We were unable to demonstrate, in the Canadian population evaluated in the present study, the existence of a highly virulent clone among serotype III GBS isolates, because rates of invasion in neonates <7 days of age mirrored rates of colonization during pregnancy, and because no clonal type was associated with a lower maternal serotype III CPS-specific IgG concentration in maternal serum samples. The observation of distinct clonal groups within capsular serotype III GBS is consistent with the findings obtained, by MLEE and other methods, in previous studies. Our data support the hypothesis that closely and divergently related clones of GBS may share the genes coding for a particular CPS and that the phenomenon of capsule switching may have occurred. If this is the case, it has significant implications for vaccine programs that are targeted against the CPS. Intensive surveillance would be needed to ensure that there is no emergence of nonvaccine CPS serotypes.

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