Genomic and phenotypic characterisation of invasive neonatal and colonising group B Streptococcus isolates from Slovenia, 2001–2018

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Abstract

**Background:** Group B Streptococcus (GBS) is the leading cause of invasive neonatal disease in the industrialized world. We aimed to genomically and phenotypically characterise invasive GBS isolates in Slovenia from 2001 to 2018 and contemporary colonising GBS isolates from screening cultures in 2018.

**Methods:** GBS isolates from 101 patients (invasive isolates) and 70 pregnant women (colonising isolates) were analysed. Basic clinical characteristics of the patients were collected from medical records. Antimicrobial susceptibility and phenotypic capsular serotype were determined. Whole-genome sequencing was performed to assign multilocus sequence types (STs), clonal complexes (CCs), pathogenicity/virulence factors, including capsular genotypes, and genome-based phylogeny.

**Results:** Among invasive neonatal disease patients, 42.6% (n = 43) were females, 41.5% (n = 39/94) were from preterm deliveries (< 37 weeks gestation), and 41.6% (n = 42) had early-onset disease (EOD). All isolates were susceptible to benzylpenicillin with low minimum inhibitory concentrations (MICs; ≤0.125 mg/L). Overall, 7 serotypes were identified (Ia, Ib, II-V and VIII); serotype III being the most prevalent (59.6%). Twenty-eight MLST STs were detected that clustered into 6 CCs. CC-17 was the most common CC overall (53.2%), as well as among invasive (67.3%) and non-invasive (32.9%) isolates (p < 0.001). CC-17 was more common among patients with late-onset disease (LOD) (81.4%) compared to EOD (47.6%) (p < 0.001). The prevalence of other CCs was 12.9% (CC-23), 11.1% (CC-12), 10.5% (CC-1), 8.2% (CC-19), and 1.8% (CC-498). Of all isolates, 2.3% were singletons.

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Conclusions: A high prevalence of hypervirulent CC-17 isolates, with low genomic diversity and characteristic profile of pathogenicity/virulence factors, was detected among invasive neonatal and colonising GBS isolates from pregnant women in Slovenia. This is the first genomic characterisation of GBS isolates in Slovenia and provides valuable microbiological and genomic baseline data regarding the invasive and colonising GBS population nationally. Continuous genomic surveillance of GBS infections is crucial to analyse the impact of IND prevention strategies on the population structure of GBS locally, nationally, and internationally.

Keywords: Group B Streptococcus, GBS, Capsular type, Hypervirulent CC-17, Neonatal infection, Molecular epidemiology, Pathogenicity/virulence factors, Slovenia
or recto-vaginal ($n = 19$) screening swabs of pregnant women in 2018. All isolates were microbiologically characterized, however, only one isolate per patient was included in the analysis. If a patient had phenotypically identical GBS isolates cultured concomitantly from blood and CSF, the CSF isolate was included. Accordingly, blood isolates from 13 patients were excluded from the analysis, which resulted in the final number of 101 invasive GBS isolates. In the case of duplicate isolates from a woman in the colonization group, only the first isolate was included in the analysis (one isolate was excluded, which resulted in the final number of 70 colonising isolates). Finally, invasive isolates were divided into 2 subgroups based on the year of isolation: the early isolates (isolated 2001–2011; isolates from the laboratory in Ljubljana lacking) and the late isolates (isolated 2012–2018) (Supplementary Fig. 1). This was mainly performed to examine changes in the Slovenian GBS population and especially if the number and proportion of serotype III and GBS CC-17 isolates increased over time. However, it was also performed because national coverage of GBS isolates was only available from 2012 and onwards. The study was approved by the National Medical Ethics Committee in Slovenia (KME 54/07/15).

Phenotypic characterisation
Phenotypic characterisation was performed at the Institute of Microbiology and Immunology, Ljubljana, Slovenia. Species identification was performed by MALDI-TOF mass spectrometry (Bruker Daltonics, Bremen, Germany). Antibiotic susceptibility testing was performed and interpreted according to the EUCAST Clinical Breakpoint Tables v10.0 (www.eucast.org), using the disc diffusion method for vancomycin, levofloxacin, trimethoprim-sulfamethoxazole, erythromycin, clindamycin, and tetracycline on Mueller-Hinton fastidious agar. Minimum inhibitory concentrations (MICs) of benzylpenicillin and ampicillin were determined using the Etest (bioMérieux, Marcy l’Etoile, France) on Mueller-Hinton fastidious agar. Serotyping was conducted with ImmuLex Strep-B-Latex test (SSI Diagnostica, Hillerod, Denmark), as previously described [15]. After WGS-based ‘serotyping’ was available, all discrepant isolates were retested for the final result.

Genomic characterisation
Genomic characterisation was performed at the WHO Collaborating Centre for Gonorrhoea and other STIs, Örebro University Hospital, Örebro, Sweden. Briefly, all isolates were grown from frozen stocks on blood agar media at 36°C and bacterial suspensions were subjected to 60 min of lysis at 37°C after adding an enzyme cocktail [16] containing lysozyme (20 mg/mL), mutanolysin (250 U/mL), and lysostaphin (20 U/mL) (Sigma-Aldrich, Saint Louis, Missouri, USA). Extraction of genomic DNA was performed using QIAsymphony DSP Virus/Pathogen MIDI Kit (Qiagen, Hilden, Germany). Libraries were prepared using Nextera XT library preparation kit and WGS was performed on the Illumina MiSeq System (Illumina, San Diego, CA, USA) using Miseq Reagent kit V3 (600-cycle) producing 300 bp paired-end reads for each isolate with an average coverage of 126× per base (range: 82–180×). Reads were aligned to the chromosome of the S. agalactiae reference strain NEM316 (Genbank: NC_004368.1) using Burrows Wheeler Aligner (BWA) [17] with GATK indel realignment. Variant sites were identified from each isolate using bcftools (version 0.19) included in SAMtools (version 0.19) with default parameters [18] and filtered as described previously [19] to produce a multiple-sequence alignment.

De novo assembly was performed using CLC Genomics Workbench 12.0.1 and Velvet 1.2.10 assembler (https://github.com/dzerbino/velvet/tree/master) for confirmation [20]. MLST was performed from draft genomes and using the MLST tool (https://github.com/tseemann/mlst) as well as PubMLST (https://pubmlst.org). Clonal complexes were assigned using eBURST (http://eburst.mlst.net) [21]. Other genes of interest were extracted and characterised from the genome sequences using BLAST (https://blast.ncbi.nlm.nih.gov) and an in silico “PCR” method (https://github.com/egonoz/in_silico_pcr). WGS-based ‘serotyping’ was performed by analysing the variable region of the cps region [22].

Characterisations of surface and pathogenicity/virulence genes were performed in silico from draft genomes. Pili, ALP family (alp1, rib, R28, alpha), C5a peptidase (scpB), laminin/fibrinogen-binding proteins (lmb, fbsA, fbsB) and other adhesins (bibA, hvgA, srr-1, srr-2) genes were analysed. Previously described Pili, ALP, srr and hvgA genotypes [22] were determined using BLAST. For the genotypic characterisation of scpB, lmb, fbsA and fbsB, gene sequences were extracted from draft genomes, aligned with MUSCLE algorithm [23], and arbitrarily named using consecutive allele numbers. Neighbor-joining (N) trees were then constructed using SeaView 4.7 [24] and major clades were classified into allele numbers.

Phylogeny was achieved by mapping the reads to the reference genome of S. agalactiae NEM316 (NC_004368.1) using the bwa tool (http://bio-bwa.sourceforge.net) and constructing maximum-likelihood (ML) phylogenomic tree from the alignment using the generalized time reversible (GTR) substitution model and gamma distribution in the RAxML tool with 100 bootstraps (https://github.com/stamatak/standard-RAxML) [25]. Additionally, alignments were generated masked for recombination using the Gubbins tool (https://github.com/sanger-pathogens/gubbins) [26] and a second ML phylogenetic tree excluding regions of recombination was constructed. Phylogenetic trees were visualized with metadata using Microreact (https://microreact.org).
org) and Phandango (https://jameshadfield.github.io/phandango/#/). [27, 28].

Raw sequence data were deposited at the European Nucleotide Archive (ENA); project accession number PRJEB35421.

Statistical analysis
Descriptive statistics were used for sample characterisation. Chi-squared test was used for category proportion comparison between groups and subgroups. Significance was defined as \( p \)-values < 0.05.

Results
Patients and bacterial isolates
Basic patient characteristics are shown in Table 1. Briefly, 42.6% (\( n = 43 \)) of patients were females, 41.5% (\( n = 39/94 \)) were from preterm deliveries (< 37 weeks gestation), and 41.6% (\( n = 42 \)) had EOD. Altogether, 171 patients/isolates were included in the analysis, 101 from neonates/infants with IND (invasive) and 70 from consecutive pregnant women (colonising).

Antimicrobial susceptibility testing
All isolates were susceptible to benzylpenicillin, ampicillin, vancomycin, levofloxacin, and trimethoprim-sulfamethoxazole. The susceptibility to both erythromycin and clindamycin was > 80% (Supplementary Table 2). Most (87.2%, \( n = 149 \)) isolates were resistant to tetracycline; invasive isolates (\( n = 93, 92.1% \)) resistant at higher frequency than colonising isolates (\( n = 56, 80% \) \( p = 0.02 \)). None had elevated MICs (> 0.125 mg/L) of benzylpenicillin or ampicillin.

Phenotypic and molecular ‘serotyping’
A pairwise comparison of conventional phenotypic serotyping and molecular ‘serotyping’ is summarised in Supplementary Table 3. A serotype could be phenotypically determined for all isolates (\( n = 171 \)), while 4 isolates (2.3%) were non-typeable (NT) using the molecular method. Excluding the NT isolates, 87.4% (\( n = 146 \)) of serotype results were concordant between the two methods. Nine, 5 and 7 isolates assigned the phenotypic serotypes Ia, Ib and III, respectively, gave discordant results in the molecular typing. Molecular serotype combined with phenotypic serotype for the 4 NT isolates was used as a final result. Overall, 7 capsular serotypes (Ia, Ib, II, III, IV, V, and VIII) were identified (1a, Ib, II, III, IV, V, and VIII). Serotype III was the most common serotype overall (59.6% of isolates), as well as among invasive isolates (74.3%) and colonising isolates (38.6%). However, the proportion of serotype III isolates was significantly higher among the invasive isolates compared to the colonising isolates (\( p < 0.001 \)). The distribution of serotypes and CCs is depicted in Table 2.

Multilocus sequence typing
Twenty-eight STs were detected, of which 10 had previously not been described. Thirteen and 24 unique STs were detected among the invasive and colonising isolates, respectively, showing higher variability within the latter (\( p < 0.001 \)). Altogether, the STs were grouped into 6 CCs and 4 singletons based on eBURST analysis. CC-17, CC-23, CC-12, CC-1, and CC-19 included more than 10 isolates each. Overall, CC-17 was the most common CC, including 53.2% (\( n = 91 \)) of isolates. CC-17 was more common among invasive versus colonising isolates (67.3 vs. 32.9%; \( p < 0.001 \)), and LOD versus EOD isolates (81.4% vs. 47.6%; \( p < 0.001 \)) (Supplementary Tables 4, 5 and 6). However, the proportion of CC-17 isolates was not significantly different (\( p = 0.187 \)) in the early period (58.1%)

Table 1 Basic patient information

| Groups and categories | Number (%) |
|-----------------------|------------|
| PATIENTS              |            |
| Invasive disease       | 101 (100)  |
| Gender                |            |
| Female                | 43 (42.6)  |
| Male                  | 58 (57.4)  |
| Gestation (\( n = 94 \)) |            |
| Preterm (< 37 weeks)  | 39 (41.5)  |
| Term (\( \geq 37 \) weeks) | 55 (58.5)  |
| Disease type*         |            |
| Early-onset (EOD)     | 42 (41.6)  |
| Late-onset (LOD)      | 55 (54.5)  |
| Very late-onset (vLOD)| 4 (4)      |
| Geographical location |            |
| Ljubljana             | 42 (41.6)  |
| Celje                 | 25 (24.8)  |
| Maribor               | 30 (29.7)  |
| Koper                 | 4 (4)      |
| Year of disease       |            |
| 2001–2011 (early)     | 31 (30.7)  |
| 2012–2018 (late)      | 70 (69.3)  |
| Colonisation          | 70 (100)   |
| Age group             |            |
| Age \( \leq 30 \) years| 35 (50)    |
| Age > 30 years        | 35 (50)    |
| Geographical location |            |
| Ljubljana             | 70 (100)   |
| Year sample collection|            |
| 2018                  | 70 (100)   |
| Total                 | 171 (100)  |

*Disease type definition: EOD (1–7 days), LOD (8–90 days), vLOD (91–365 days)
Phylogeny and characterisation of pathogenicity/virulence genes

A SNP-based ML phylogenetic tree including metadata is shown in Fig. 1. Six clades with ≥5 isolates could be distinguished within the 5 major CCs. CC-19 was represented by 2 clades characterised by different serotypes, i.e. II and III. The majority (4/5, 80%) of these serotype II isolates were colonising and the serotype III isolates were predominantly invasive (n = 7/9, 78%). Overall, CC-17 isolates were almost exclusively assigned serotype III and they were predominantly invasive. However, two colonising CC-17 isolates were of serotype IV. A high homogeneity of surface and pathogenicity/virulence factors was observed within the CCs. As almost one third (32.9%) of non-invasive colonising isolates belonged to CC-17, it was difficult to compare the presence/absence of different pathogenicity/virulence factors between the invasive and colonising isolates. Typical profiles of pathogenicity/virulence factors of the 5 most common CCs are depicted in Table 3.

SNP-based ML phylogenetic tree was also constructed after excluding regions of abundant recombination using Gubbins [26] (Fig. 2). A striking difference in frequency of recombination within CC-17 isolates compared to isolates of other CCs was observed (Fig. 2). This highlights the importance of horizontal gene transfer and recombination in GBS, especially among non-CC-17 strains. In contrast, the CC-17 hypervirulent clade had few regions prone to recombination.

Discussion

In this first molecular epidemiology and genomic study of GBS in Slovenia, we show a high prevalence of hyper-virulent MLST CC-17 among invasive isolates (67.3%), but also among contemporary colonising (32.9%) isolates. The CC-17 isolates were relatively conserved genomically and mostly belonged to serotype III. Slovenian GBS isolates were uniformly susceptible to benzylpenicillin (MICs ≤0.125 mg/L), whereas the resistance to erythromycin (17%) and clindamycin (16%) was comparable to that of other European countries [29, 30].

The concordance between phenotypic and molecular “serotyping” methods was 87%, suggesting imperfect but mainly sufficient typing using also sequencing methods, particularly in view of the increasing availability of WGS and other molecular methods [22]. This concordance is also in line with two recent studies, that is, describing 87–94% concordance [31, 32]. Nevertheless, this sub-optimal concordance is important to take into account when performing, for example, surveillance studies informing vaccine design. Overall, 7 serotypes were identified, with serotype III accounting for the majority (60%). Serotype III isolates mostly belonged to CC-17 (52%), but some were assigned CC-19 (4.7%) and CC-23 (2.3%). Serotype III was predominantly associated with invasive disease (74% of invasive isolates). Serotypes among colonising isolates were more evenly distributed, consistent with data from a recent meta-analysis [33].
Fig. 1 Single nucleotide polymorphism (SNP)-based maximum-likelihood phylogenomic tree with bootstrap values for the major branches including metadata: consisting of isolate group (invasive/colonising), disease type (early-onset/late-onset), serotype, MLST sequence type, MLST clonal complex, and surface/pathogenicity/virulence factors genotype (pili, alpha-like protein family, hvgA, srr, scpB, lmb, fbsA, fbsB and bibA). Colour of the bar depicts the genotype or lack of any named genotype or MLST sequence type or clonal complex (white bars). Pili, ALP, srr and hvgA genotypes were named in accordance with Metcalf et al. [22]. Alleles of scpB, lmb, fbsA and fbsB were arbitrarily assigned consecutive numbers.

Table 3 Pathogenicity/virulence factors in group B Streptococcus isolates, belonging to the 5 major multilocus sequence typing (MLST) clonal complexes (CCs), cultured in Slovenia from 2001 to 2018. The most prevalent genotype within each CC and its proportion are shown.

| Clonal complex | Number (%) | Capsule (cps) [22] | Pili [22] | ALP family (alp1, alpha, rib, R28) [22] | CSa peptidase (scpB) | Laminin-binding protein (lmb) | Fibrinogen-binding protein A (fbsA) | Fibrinogen-binding protein B (fbsB) | Serine-rich proteins (srr) [22] | GBS immunogenic adhesin A (bibA) | Hypervirulent GBS adhesin (hvgA allele) |
|----------------|------------|---------------------|-----------|----------------------------------------|---------------------|-----------------------------|----------------------------------|----------------------------------|---------------------------------|---------------------------------|-------------------------------------|
| CC-1           | 18 (10.5)  | V (83.3)            | 1, 2A R28 (83.3) | allele-5 (72.2)                       | allele-1 (94.4)      | allele-5 (44.4)              | allele-2 (88.9)                  | srr-1 (94.4)                      | allele-2 (94.4)                  | neg                             | pos                                |
| CC-12          | 19 (11.1)  | Ib (63.2)           | 1, 2A alpha (89.5) | allele-2 (42.1)                       | allele-1 (100)       | allele-2 (36.8)              | allele-2 (68.4)                  | srr-1 (89.5)                      | allele-2 (94.7)                  | neg                             | neg                                |
| CC-17          | 91 (53.2)  | III (97.8)          | 1, 2B rib (84.6) | allele-1 (96.7)                       | allele-3 (74.7)      | allele-4 (100)               | allele-3 (85.7)                  | srr-2 (100)                       | allele-1 (100)                   | pos                             | neg                                |
| CC-19          | 14 (8.2)   | III (57.1)          | 1, 2A rib (92.9) | allele-3 (57.1)                       | allele-1 null       | allele-2 (78.6)              | allele-2 (78.6)                  | srr-1 (78.6)                      | allele-3 (78.6)                  | neg                             | neg                                |
| CC-23          | 22 (12.9)  | Ia (81.8)           | 2A alp1 (86.4) | allele-4 (90.9)                       | allele-1 (100)       | allele-1 (90.9)              | allele-1 (100)                  | srr-1 (100)                       | allele-4 (100)                   | neg                             | neg                                |

ALP alpha-like proteins, neg negative, pos positive
Pili, ALP, srr and hvgA genotypes were named in accordance with Metcalf et al. [22]. Alleles of scpB, lmb, fbsA and fbsB were arbitrarily assigned consecutive numbers.
GBS isolates in our study displayed a high level of genomic diversity with 28 MLST STs detected, 9 of which had not been described previously. The diversity was larger among the colonising isolates. Nevertheless, CC-17 comprised more than half of all isolates and was more common among the invasive and LOD isolates. This hypervirulent clone also showed a trend towards higher prevalence among the late subgroup of isolates (2012–2018) (58% vs. 71%), similar to a study from the Netherlands [13]. CC-17 had a characteristic profile of pathogenicity/virulence factors that included serotype III, pili 1-2B, ALP family rib, scpB allele-1, fbsA allele-4, fbsB allele-3, srr-2, bibA allele-1 and hvgA positive. These results are in-line with several previous studies [7–9, 13, 27].

The genome organisation of the frequently invasive CC-17 isolates was highly conserved with few recombination prone regions. This may indicate that CC-17 has already experienced an evolutionary selection to increase fitness for survival and pathogenicity/virulence. In contrast, non-CC-17 isolates were recombination prone, highlighting the importance of recombination and horizontal gene transfer in GBS evolution [12]. Interestingly, CC-1, CC-12 and CC-19, which are predominantly colonising CCs, belonged to the same clade after the regions of recombination were removed (Fig. 2).

The limitations of the present study included that we were not able to include isolates from all cases of IND due to the unavailability of GBS isolates from 2001 to 2010 in the largest Slovenian laboratory (in Ljubljana). Furthermore, colonising isolates were available only from 2018 and the laboratory in Ljubljana. Finally, we had limited clinical data from the IND cases. However, despite these limitations, a relatively large number of IND cases, isolates and standard genomic analysis tools provided us with detailed and reliable baseline information about the GBS population structure in Slovenia.

**Conclusions**

A high prevalence of hypervirulent CC-17 isolates, with low genomic diversities and characteristic profile of pathogenicity/virulence factors, was detected among invasive neonatal and colonising GBS isolates from pregnant women in Slovenia. This is the first genomic characterisation of GBS isolates in Slovenia and provides valuable microbiological and genomic baseline data regarding the invasive and colonising GBS population in Slovenia. Continuous genomic surveillance of GBS infections is crucial to analyse the impact of IND prevention strategies on the population structure of GBS locally, nationally and internationally.
Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12879-020-05599-y.

Additional file 1: Supplementary Table 1. Number of births in Slovenia during the years 2002–2018 and calculated representativeness of the sample based on the estimated incidence of invasive neonatal disease (IND) of 0.7/1000 births from reference 3. Supplementary Table 2. Antimicrobial susceptibility of Slovenian invasive neonatal and colonising pregnant women isolates of group B Streptococcus from early (2001–2011) and late (2012–2018) period (n = 171). Supplementary Table 3. Pairwise comparison of conventional phenotypic serotyping and molecular ‘serotyping’ results among invasive and non-invasive isolates of group B Streptococcus from early (2001–2011) and late (2012–2018) period (n = 171). Supplementary Table 4. Distribution of multilocus sequence typing (MLST) sequence types (STs) and clonal complexes (CCs) among Slovenian group B Streptococcus isolates overall and among isolates from infants with invasive neonatal disease (2001–2018) and pregnant woman with colonisation (2018). Supplementary Table 5. Distribution of multilocus sequence typing (MLST) and clonal complexes (CCs) among invasive neonatal group B Streptococcus isolates from Slovenia within two groups based on disease onset: Supplementary Table 6. Distribution of multilocus sequence typing (MLST) and clonal complexes (CCs) among invasive neonatal group B Streptococcus isolates from Slovenia within two groups based on time period of disease: Supplementary Fig. 1. Distribution of invasive group B Streptococcus isolates (n = 114) from different laboratories in Slovenia from 2001 to 2018. Unfortunately, during 2001–2010, isolates from the largest laboratory (in Ljubljana) were not preserved so whole genome sequencing of those isolates could not be performed. LJ Ljubljana; MB: Maribor; CE: Celje; KP: Koper. Supplementary Fig. 2. eBURST analysis of 28 multilocus sequence typing (MLST) sequence types (STs) identifying six clonal complexes (CCs) and four singletons, among Slovenian group B Streptococcus isolates from infants with invasive neonatal disease (2001–2018) and pregnant woman with colonisation (2018).

Abbreviations

ALP: Alpha like proteins; Bib: GBS immunogenic adhesins; CC: Clonal complex; Abbreviations

ALP: Alpha like proteins; Bib: GBS immunogenic adhesins; CC: Clonal complex; CSF: Cerebrospinal fluid; EOC: Early onset disease; Fbs: Fibrinogen binding proteins; GBS: Group B Streptococcus; HvgA: Hypervirulent GBS adhesin; IND: Invasive neonatal disease; Lmb: Laminin binding protein; LOD: Late onset disease; MALDI-TOF: Matrix assisted laser desorption/ionization-time of flight; MIC: Minimum inhibitory concentration; MLST: Multilocus sequence typing; NT: Non-typeable; ScpB: Csa peptidease; SNP: Single nucleotide polymorphism; Src: Serine rich protein; ST: Sequence type; vLOD: Very late onset disease; WGS: Whole genome sequencing

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Authors’ contributions

TP, DG, AI, SJ. MU designed the study. TP, ML, ŠG, LKC, MBI, AR, PF and ILK collected the patients and samples. TP, DG, MU, SJ analysed the data. TP and SJ wrote the first draft of manuscript. All authors were involved in finalising the manuscript and approved the final version of the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study was approved by the National Medical Ethics Committee (KME 54/07/15).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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