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Population-level transition of capsular polysaccharide types among sequence type 1 group B *Streptococcus* isolates with reduced penicillin susceptibility during a long-term hospital epidemic

Noriyuki Nagano[a,1,*], Shota Koide[a], Wataru Hayashi[a], Yui Taniguchi[a], Hayato Tanaka[a], Yoshihiko Maeyama[a], Masahiro Suzuki[b], Kouji Kimura[c], Yoshichika Arakawa[c], Yukiko Nagano[c,1]

[a] Department of Health and Medical Sciences, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto, Nagano 390-8621, Japan
[b] Department of Microbiology, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
[c] Department of Bacteriology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan

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ABSTRACT

Over a 35-month period, group B *Streptococcus* isolates with reduced penicillin susceptibility (PRGBS) were detected from elderly patients at a regional hospital in Japan, accompanying population-level transition of PRGBS serotypes. The genetic relatedness of 77 non-duplicate PRGBS from 73 patients was analysed. Serotype III PRGBS predominated (16 serotype III/1 serotype Ib) in the first 9 months (period I), then 3 serotype Ib isolates appeared transiently for the next 3 months (period II), which was replaced predominantly by serotype Ia (20 serotype Ia/1 serotype III/1 non-typeable) for 9 months (period III). In the last 14 months (period IV), besides 25 serotype Ia isolates, 10 serotype III were also identified. Serotypes III and Ia isolates, belonging to ST1, shared G329V, G398A, V405A and G429D substitutions in penicillin-binding protein 2X. Of three strains subjected to whole-genome sequencing, serotype III strain SU12 (period I) had a higher degree of genomic similarity with serotype Ia strain SU97 (period III) than serotype Ib strain SU67 (period II) based on average nucleotide identity and single nucleotide polymorphisms. Analysis of the *cps* gene clusters and the upstream and downstream flanking sequences revealed that disruption of the hyaluronidase gene located upstream of *cpsY* by insertion of IS*1548* was found in strain SU12, whereas ΔIS*Sag8* was inserted between tRNA-Arg and *rpsA* genes located downstream of *cpsL* in strain SU97. Interestingly, most serotype III PRGBS re-emerging in period IV had this tRNA-Arg–ΔIS*Sag8*–*rpsA* region. Capsular switching and nosocomial transmission may possibly contribute to population-level serotype replacement among ST1 PRGBS isolates.

1. Introduction

Group B *Streptococcus* (GBS; *Streptococcus agalactiae*) is one of the most important causes of life-threatening infections such as sepsis and meningitis in neonates [1]. GBS is also associated with invasive infections in non-pregnant adults, particularly in elderly and immunocompromised individuals [2].

The GBS capsular polysaccharide is a major virulence determinant for invasive infection and is a target of protective immunity. GBS are serologically classified based on the capsular polysaccharide synthesis (*cps*) gene cluster into ten serotypes (Ia, Ib and II–IX) as well as non-typeable. Most invasive GBS diseases in non-pregnant adults in the USA, Canada, France and Taiwan are caused by serotype V strains, especially those belonging to sequence type (ST) 1 [3–5]. A recent increase of serotype IV among adult invasive disease strains has been reported in North America and European countries [6–8]. A high diversity of genetic lineages has been observed among serotype IV isolates, including ST452 in clonal complex (CC) 23, ST459 and ST196 in CC1, and *hvgA*-positive ST291, which is a single locus variant (SLV) of ST17 and has originated from a capsular switching event from serotype III to IV within CC17 [9,10]. Although only a few studies are available in Japan, the serotypes most frequently associated

* Corresponding author. Tel.: +81 263 37 2381; fax: +81 263 37 2370.
E-mail address: naganon@shinshu-u.ac.jp (N. Nagano).
[1] These two authors contributed equally to this work.

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with invasive infections, mainly sepsis in adults, are serotype Ib, followed by serotype V [11]. Very recent studies have revealed that serotype Ib, ST10 is predominant among GBS strains from invasive infections, including streptococcal toxic shock-like syndrome cases in adults [12,13].

Our study group has accumulated epidemiological and molecular analysis findings on GBS isolates with reduced susceptibility to penicillin (PRGBS), which is the first-line antibiotic for GBS disease therapy and for intrapartum chemoprophylaxis. At least two key amino acid substitutions in penicillin-binding protein 2X (PBP2X) have been demonstrated to contribute to a considerable reduction in β-lactam susceptibility [14]. Besides these key substitutions, other amino acid substitutions have also been found in PBP2X, PBP2B and PBP1A among PRGBS isolates, depending on their minimum inhibitory concentrations (MICs) for β-lactams [14–16]. These PRGBS from Japan have most commonly been isolated from respiratory specimens from elderly patients, and serotype VI and ST1 or ST458 (a SLV of ST1 within CC1) are frequently associated with them [16,17]. PRGBS is capable of surviving persistently at the site of infection for >3 weeks, and nosocomial spread of multidrug-resistant PRGBS with serotype VI and ST458 has also been reported [15,18].

During a 35-month period at a small-scale regional hospital in Japan, PRGBS isolates were frequently detected from elderly patients, accompanying transition of their serotypes, mainly from serotype III in the previous period to serotype Ia in the latter period. The present study examined such a unique serotype transition phenomenon among these PRGBS isolates.

2. Materials and methods

2.1. Bacterial isolates

A total of 77 non-duplicate PRGBS clinical isolates (penicillin MIC $\geq$ 0.25 mg/L) recovered from 66 transtracheal aspirates (TTAs), 4 blood cultures, 3 catheter urine cultures, 2 intravenous catheter tip cultures and 2 nasal cavity cultures were collected from 73 adult patients in a 299-bed regional general hospital in Japan from April 2011 to February 2014. The 77 isolates included 2 isolates each obtained from different specimens of two patients, 2 isolates showing different antimicrobial susceptibility profiles from the same patient, and 2 isolates showing different serotypes recovered from the same specimens obtained on different days from the same patient. The 73 adult patients across eight wards (including 30 and 27 patients, respectively, from two main internal medicine wards) comprised 47 males (64.4%) and 26 females (35.6%) with a mean $\pm$ standard deviation age of 83.0 $\pm$ 9.0 years. During the study period, a total of seven non-duplicate penicillin-susceptible GBS with reduced cephalosporin susceptibility (CTB^{r}PSGBS) clinical isolates recovered from five TTAs and one each from a urine culture and pus culture were also collected from seven adult patients (six males and one female with a mean age of 77.9 $\pm$ 11.7 years) [19]. Identification of the GBS isolates was performed by the latex agglutination method using a Seroiden Strepto Kit Eiken® (Eiken Chemical Co., Tokyo, Japan). The GBS isolates were grown overnight in Todd–Hewitt broth and were then stored in glycerol at −80 °C until use.

2.2. Serotyping assays

Capsular serotyping was performed using antisera (Denka Seiken, Tokyo, Japan) for the type-specific capsular polysaccharides Ia, Ib and II–VIII. Molecular capsular typing by multiplex PCR was also performed as described previously [20].

Based on the shifts in predominant serotypes, the 35-month isolation period (April 2011 to February 2014) was divided into four periods: periods I (April–December 2011); period II (January–March 2012); period III (April–December 2012); and period IV (January 2013–February 2014).

2.3. Antimicrobial susceptibility testing

MICs were determined by the broth microdilution method using a MicroScan® MICroFAST panel type 5J (Beckman Coulter) and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines [21,22]. MIC determinations were repeated independently three times for each strain to ensure the reproducibility of the MIC results using the quality control strain *Streptococcus pneumoniae* ATCC 49619. Susceptibility testing with a ceftibuten disk was also performed by the Kirby–Bauer disk diffusion method [23].

2.4. Amplification and sequencing of pbp genes

Analysis of *pbp* genes was performed as previously described [16,18]. In brief, the full-length *pbp* genes were amplified from genomic DNA using PCR primers specific for *pbp2x, pbp2b* and *pbp1a* as well as PrimeSTAR® HS DNA Polymerase (Takara Bio Inc., Shiga, Japan). Amplified DNA fragments were purified using a Wizard® SV Gel and PCR Clean-up System (Promega, Madison, WI). Sequencing analysis on both strands of purified DNA fragments was performed using consecutive primers, a BigDye® Terminator v.1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and an ABI 3730x/DNA Analyzer (Applied Biosystems).

The nucleotide sequences obtained were assembled into a consensus sequence, which was aligned with the published sequence of the corresponding genes of strain *S. agalactiae* 2603V/R (ATCC BAA-611; GenBank accession no. **NC_004116.1**) using BioEdit v.5.0.9 software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

2.5. Multilocus sequence typing (MLST)

MLST was performed for sequencing seven housekeeping genes (*adhP, pheS, atr, glnA, sdhA, glcK* and *tkt*) amplified by PCR as described previously [18,24]. Allelic profile assignment and ST determination were made using the GBS MLST databases (http://pubmlst.org/sagalactiae).

2.6. Whole-genome sequencing (WGS)

WGS was performed on serotype III PRGBS strain SU12 from a TTA (period I) and serotype Ia PRGBS strain SU97 from blood (period III) that were isolated 1 year apart from two different patients, as well as serotype Ib CTB^{r}PSGBS strain SU66 from a TTA and serotype Ib PRGBS strain SU67 from TTA (period II) that were isolated 4 days apart from the same patient (Fig. 1A). Genomic DNA was purified using a Wizard® Genomic DNA Purification Kit (Promega) and was subjected to sequencing on an Illumina HiSeq 2500 system (Illumina Inc., San Diego, CA) generating 100-bp paired-end reads. Approximately 12 million Illumina reads were processed and merged into contigs using the de novo assembly pipeline A5-miseq [25]. The obtained contigs were mapped to the *S. agalactiae* NEM316 reference genome (ATCC 12403; GenBank accession no. **NC_004368**) using CONTIGuator2 (http://contiguator.sourceforge.net/) to create a pseudocontig sequence, which was annotated by RAST (Rapid Annotation using Subsystem Technology) v.2.0 [26]. EasyFig v.2.2.2 [27] was used for comparative analysis of *cps* gene clusters and their flanking regions.

Average nucleotide identity based on MUMmer calculation (ANIm) of paired genomes was calculated among isolates SU12, SU66, SU67, SU97 and serotype V ST1 *S. agalactiae* strain SS1 (GenBank accession no. **CP010867**, reference 7), serotype III

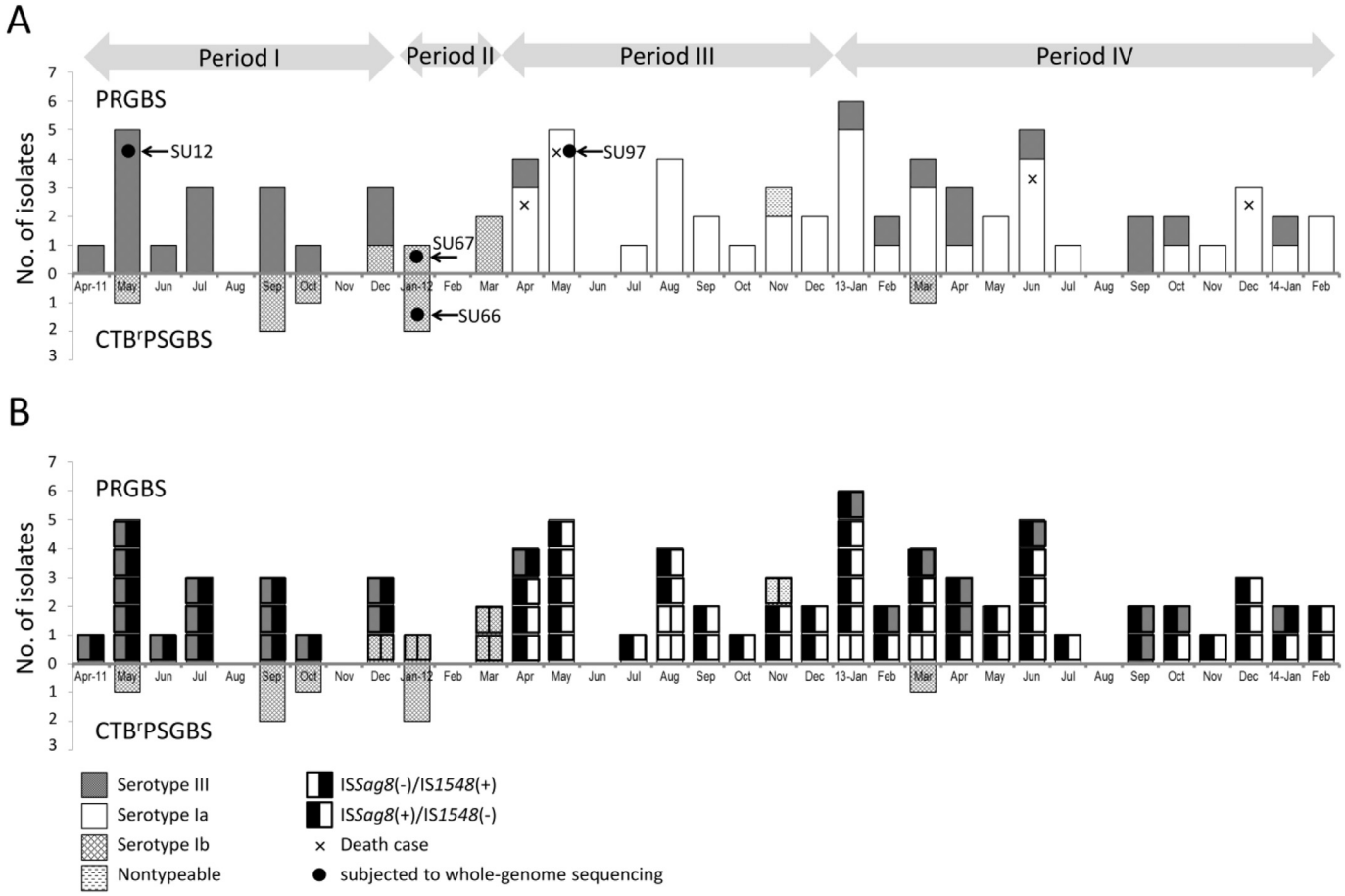


Fig. 1. Group B *Streptococcus* isolates with reduced susceptibility to penicillin (PRGBS) ($n = 77$) detected in a regional general hospital in Japan: (a) transition of serotypes during periods I to IV; and (b) presence or absence of ΔIS*Sag8* between tRNA-Arg and *rpsA* genes or of IS*1548* in the hyaluronidase gene *hylB* for each isolate. CTB^{r}PSGBS, penicillin-susceptible group B *Streptococcus* with reduced cephalosporin susceptibility.

ST23 *S. agalactiae* NEM316 and serotype Ia ST7 *S. agalactiae* A909 (GenBank accession no. **NC_007432**) using JSpeciesWS with the default parameters (http://jspecies.ribohost.com/jspeciesws/) to assess nucleotide-level genomic similarity [28]. A phylogenetic heatmap based on pairwise comparison matrices was constructed using R v.3.5.0. Single nucleotide polymorphism (SNP)-based phylogeny among the abovementioned seven strains was analysed using CSI Phylogeny 1.4 with default parameters (https://cge.cbs.dtu.dk/services/CSIPhylogeny/) [29]. The genome of strain SU12 was used as reference sequence and the phylogenetic tree was visualised using FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

2.7. Analysis of insertion sequence (IS) elements

For PRGBS and CTB^{r}PSGBS clinical isolates, forward (ISSag8F, 5′-TCGAACCCCTATCCCAAGAAC-3′) and reverse (ISSag8R, 5′-CGCAAGCAGAAGGTGAAAAAG-3′) primers were used to determine whether or not ΔIS*Sag8* was inserted between the tRNA-Arg and *rpsA* genes (1827 bp vs. 269 bp). To determine whether or not IS*1548* was inserted in the hyaluronidase gene *hylB*, forward (IS1548F, 5′-ACCGCTACTTATCGTCGTTTGG-3′) and reverse (IS1548R, 5′-AGCCAAGCCAGACTCTCTTT-3′) primers were used (1439 bp vs. 113 bp). Insertion of ΔIS*Sag8* or IS*1548* was confirmed by sequencing all PCR products.

2.8. Nucleotide sequence accession numbers

The GenBank accession numbers for the *cps* gene clusters and their flanking regions of strains SU12 and SU97 in this study are **LC341249** and **LC341250**, respectively.

3. Results

3.1. Antimicrobial susceptibility of PRGBS isolates

The MIC range, the MICs at which 50% (MIC_{50}) and 90% (MIC_{90}) of the isolates were inhibited, and the percentage susceptibility of the 77 PRGBS isolates to the antimicrobial agents tested are listed in Table 1. The isolates had a penicillin MIC_{50} of 0.25 mg/L, an MIC_{90} of 0.5 mg/L and an MIC range of 0.25–0.5 mg/L, which is above the CLSI's susceptible MIC of ≤0.12 mg/L. The rate of non-susceptibility of PRGBS to cefotaxime and cefepime was 97% and 100%, respectively, whereas 49% of the isolates were non-susceptible to ampicillin. Although they were susceptible to meropenem, an increasing trend of MICs (0.25–0.5 mg/L) within the susceptible range was observed. The rates of erythromycin, clindamycin and levofloxacin resistance were 74%, 23% and 45%, respectively. All PRGBS and CTB^{r}PSGBS isolates exhibited no growth inhibition zones around the ceftibuten disks. The CTB^{r}PSGBS isolates were susceptible to penicillin (MICs of 0.06–0.12 mg/L) [19].

Table 1
Minimum inhibitory concentration (MIC) distribution of antimicrobial agents for 77 clinical isolates of group B streptococci with reduced penicillin susceptibility.

Antimicrobial agent	MIC (mg/L)			%S/%R or NS
	Range	MIC_{50}	MIC_{90}	
Penicillin	0.25–0.5	0.25	0.5	0/100
Ampicillin	0.25–0.5	0.25	0.5	51/49
Cefotiam	4 to >4	4	>4	–[a]
Cefotaxime	0.5–1	1	1	3/97
Ceftriaxone	0.25–1	0.5	1	58/42
Cefditoren	0.25–0.5	0.5	0.5	–[a]
Cefepime	1 to >2	2	2	0/100
Cefixime	1 to >1	>1	>1	–[a]
Meropenem	0.25–0.5	0.25	0.5	100/0
Erythromycin	≤0.12 to >1	>1	>1	26/74
Clarithromycin	≤0.12 to >1	1	>1	26/74
Clindamycin	≤0.12 to >1	≤0.12	>1	77/23
Levofloxacin	1 to >8	2	>8	55/45
Vancomycin	0.5–1	0.5	0.5	100/0

$MIC_{50/90}$, MICs at which 50% and 90% of the isolates are inhibited, respectively; S, susceptible; R, resistant; NS, non-susceptible.
[a] – Indicates no breakpoint available.

3.2. Capsular type shift among PRGBS isolates

In 74 of 77 isolates, serotyping results were concordant with molecular serotyping results; 26 serotype III, 3 serotype Ib and 45 serotype Ia. Of the remaining three non-typeable isolates, two were assigned to serotypes III and Ia each by genotyping but one remained non-typeable. A shift in serotypes was observed within the isolates collected over the 35-month period as shown in Fig. 1A. Namely, the serotype III PRGBS population predominated (16 serotype III/1 serotype Ib) in the first 9 months (period I), then 3 serotype Ib isolates appeared transiently for the next 3 months (period II), which was replaced predominantly by a serotype Ia population (20 serotype Ia/1 serotype III/1 non-typeable) for 9 months (period III). In the last 14 months, in addition to 25 serotype Ia isolates, 10 serotype III PRGBS isolates were also identified (period IV). Among these PRGBS, two isolates recovered from TTAs from the same patient in May 2011 and May 2012 showed different serotypes (III and Ia). All four PRGBS isolates derived from blood samples belonged to serotype Ia and a consequent poor prognosis was observed. Serotype Ib CTB^{r}PSGBS was also recognised in six isolates in periods I and II, as reported previously by us, and in one isolate in period IV (Fig. 1A) [19].

3.3. Amino acid substitutions in PBP2X, PBP2B and PBP1A, and multilocus sequence typing

For analysis of amino acid substitutions in PBP2X, PBP2B and PBP1A as well as MLST, 25 PRGBS isolates were arbitrarily chosen depending upon their serotypes and dates of isolation: 12 (11 serotype III and 1 serotype Ib) from period I; 1 (serotype Ib) from period II; 5 (serotype Ia) from period III; and 7 (4 serotype III and 3 serotype Ia) from period IV. Irrespective of the period, PRGBS of serotypes Ia and III shared several amino acid substitutions in PBPs (Table 2). The PBP2X amino acid substitutions included a key substitution (V405A) and three additional substitutions (G329V, G398A and G429D). One amino acid substitution in PBP1A (T587I) was shared by serotype Ia PRGBS in periods III and IV and serotype III PRGBS in period I. However, these amino acid substitutions were not detected in either serotype Ib PRGBS or serotype Ib CTB^{r}PSGBS, except for V405A in PBP2X of Ib PRGBS [19].

MLST analysis revealed that all 25 PRGBS isolates were assigned to ST1, a founder of CC1, irrespective of their capsular types (Table 2). Serotype Ib CTB^{r}PSGBS isolates also belonged to ST1 [19].

3.4. Analyies of genetic relatedness and cps gene cluster regions among PRGBS isolates

The assembled pseudocontig sequences of four strains (serotype III PRGBS strain SU12, serotype Ia PRGBS strain SU97, serotype Ib PRGBS strain SU67 and serotype Ib CTB^{r}PSGBS strain SU66) were ca. 2.0–2.2 Mb in length. Sequence similarity searching using BLAST revealed that SU12 shared 99% sequence identity with SU97 (93% query coverage) and with SU66, SU67 and SS1 (90% query coverage). SU12 showed the highest ANIm values of 99.84% with SU97, followed by 99.68%, 99.67% and 99.63% with SU66, SS1 and SU67, respectively. ANIm values showed 99.96% nucleotide similarity between strains SU66 and SU67. The phylogenetic tree constructed based on ANIm analysis indicated that serotype III strain SU12 and serotype Ia strain SU97 clustered together were more closely related to serotype V strain SS1 than to serotype Ib strains SU66 and SU67 clustered together (Fig. 2). The SNP-based phylogenetic approach showed that strains SU12 and SU97, differing from each other by 957 SNPs, were clustered together, whereas there were 46 SNP differences between strains SU66 and SU67 forming another cluster (Fig. 3). Strain SS1 belonging to ST1 and A909 (ST7) clustered together.

In-depth analysis of the *cps* gene clusters and the upstream and downstream flanking sequences (ca. 55-kb region) is shown in Fig. 4. The 12.7-kb *cps* gene clusters of strains SU12 and SU97 exhibited overall 99.3% nucleotide identity, except that serotype-specific *cpsG* and *cpsH* genes had 70.5% and 28.9% nucleotide identities, respectively. The insertion sequence Δ*ISSag8*, which was bracketed by a 20-bp repeat sequence (AGACTTGCTTTAG-CAAGTCT) was located 9.7 kb downstream of the *neuA* gene (between tRNA-Arg and *rpsA*) for strain SU97, whereas disruption of the hyaluronidase gene *hylB* located 21 kb upstream of *cpsY* caused by insertion of *IS1548* was found in strain SU12. Except for these differences, strains SU12 and SU97 shared 99.9% and 93.6% sequence identities at the region 16.0 kb upstream and 26.1 kb downstream from the *cps* gene clusters, respectively (Fig. 4A). The 55-kb *cps* gene clusters and their flanking region of strains SU66 and SU67 exhibited overall 99.8% sequence identity, where insertion of either Δ*ISSag8* between tRNA-Arg and *rpsA* genes or *IS1548* into *hylB* structural genes were not detected (Fig. 4B). Strains SU12, SU97, SU66 and SU67 shared major virulence factors including fibrinogen-binding protein A (*fbsA*), fibrinogen-binding protein B (*fbsB*), fibronectin-binding protein (*pavA*), C5a peptidase (*scpB*), laminin-binding protein (*lmb*), cAMP factor (*cfb*), haemolysin III (*spb1*) and serine protease CspA (*cspA*), but did not have C-β protein (*bac*). For genes of the alpha-like protein (Alp) family, they had C-α protein (*bca*) but did not have Rib (*rib*), alpha-like protein 1 (also called epsilon; *alp1*), alpha-like protein 2 (*alp2*), alpha-like protein 3 (*alp3*) or alpha-like protein 4 (*alp4*).

3.5. Prevalence of ΔISSag8 or IS1548 insertion

As shown in Fig. 1B, PCR and sequencing revealed that no Δ*ISSag8* insertion combined with *IS1548* insertion was confirmed in all 16 serotype III isolates in period I. Whereas Δ*ISSag8* insertion combined with no *IS1548* insertion was confirmed in 18 of 20 serotype Ia isolates in period III. The Δ*ISSag8* insertion combined with no *IS1548* insertion was also confirmed among 23 of 25 serotype Ia isolates, and notably, among 9 of 10 serotype III isolates in period IV. Four serotype Ib PRGBS and seven CTB^{r}PSGBS isolates resulted in no insertion of both Δ*ISSag8* and *IS1548*.

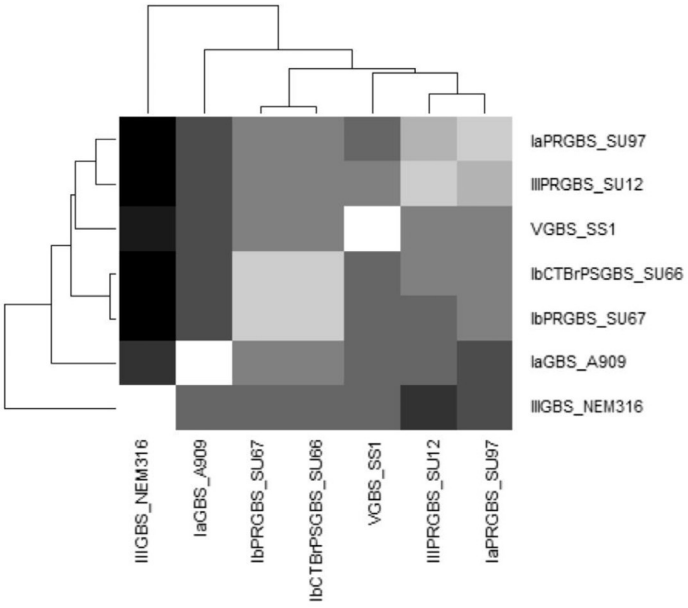
4. Discussion

Mortality rates vary from 3% to >30% for adult GBS invasive infection, where relative higher rates are noted in elderly patients

Table 2
Penicillin-binding protein (PBP) amino acid substitutions in PRGBS and CTB^{r}PSGBS clinical isolates.

Category	Capsular type	*n*	MLST	Amino acid substitutions in PBPs								
				PBP2X							PBP2B	PBP1A
PRGBS	Ia	8 (periods III & IV)	ST1	G329V		G398A		V405A	G429D		ND	T587I
	III	11 (period I)	ST1	G329V		G398A		V405A	G429D		H633Y (7) or ND (4)	T587I
		4 (period IV)	ST1	G329V		G398A		V405A	G429D		ND	P385L
	Ib[a]	2 (periods I & II)	ST1				A400V	V405A		Q557E	T567I	ND
CTB^{r}PSGBS	Ib[a]	3 (periods I & II)	ST1		T394A				G429S		T567I	ND
		3 (periods I & II)	ST1		T394A						T567I	T145A

PRGBS, group B streptococci with reduced penicillin susceptibility; CTB^{r}PSGBS, penicillin-susceptible group B streptococci with reduced cephalosporin susceptibility; MLST, multilocus sequence typing.
[a] Previously described by Nagano et al. [19].



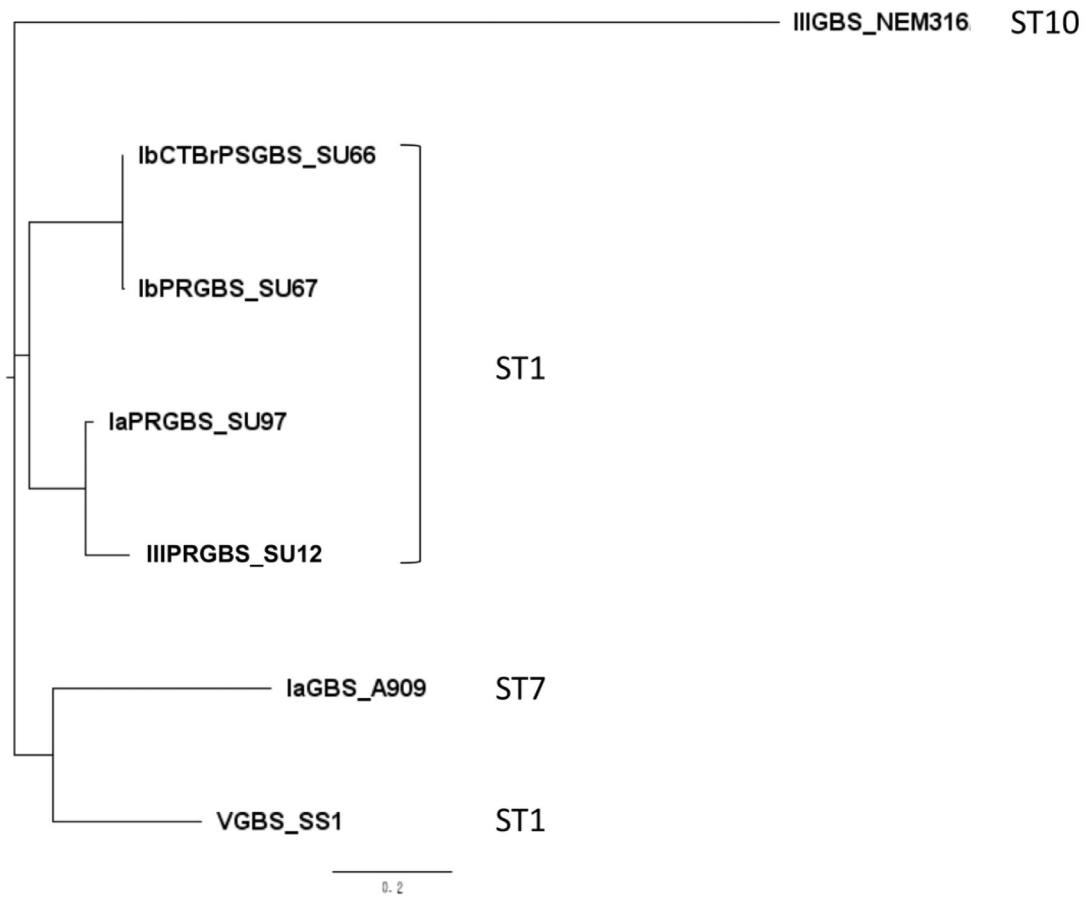
ANIm matrix table

	IIIPRGBS_SU12	IaPRGBS_SU97	IbCTB^{r}PSGBS_SU66	IbPRGBS_SU67	VGBS_SS1	IIIGBS_NEM316	IaGBS_A909
IIIPRGBS_SU12	100	99.84	99.68	99.63	99.67	99.04	99.46
IaPRGBS_SU97	99.84	100	99.69	99.71	99.62	99.2	99.44
IbCTB^{r}PSGBS_SU66	99.68	99.68	100	99.96	99.62	99.31	99.53
IbPRGBS_SU67	99.63	99.71	99.95	100	99.61	99.3	99.53
VGBS_SS1	99.67	99.62	99.62	99.61	100	99.34	99.46
IIIGBS_NEM316	99.04	99.19	99.3	99.3	99.34	100	99.33
IaGBS_A909	99.46	99.44	99.53	99.53	99.46	99.33	100

Fig. 2. Genetic relatedness of group B *Streptococcus* with reduced susceptibility to penicillin (PRGBS) strains established by average nucleotide identity based on MUMmer calculation (ANIm). A heatmap and a dendrogram was generated using the distance matrix of ANIm between each pair of genomes among serotype III PRGBS strain SU12 (IIIPRGBS_SU12), serotype Ia PRGBS strain SU97 (IaPRGBS_SU97), serotype Ib penicillin-susceptible GBS with reduced cephalosporin susceptibility (CTB^{r}PSGBS) strain SU66 (IbCTB^{r}PSGBS_SU66), serotype Ib PRGBS strain SU67 (IbPRGBS_SU67), serotype V GBS strain SS1 (VGBS_SS1), serotype III GBS strain NEM316 (IIIGBS_NEM316) and serotype Ia GBS strain A909 (IaGBS_A909).

with underlying diseases [30]. In particular, a fatal outcome is significantly associated with adult patients with bacteraemia [31]. Thus, the development of multidrug resistance, including to β-lactams, in GBS isolates is of clinical concern. The Japan Nosocomial Infections Surveillance (JANIS) data of the Ministry of Health, Labour and Welfare (http://www.nih-janis.jp/) show a clear trend towards the increasing occurrence of penicillin non-susceptibility among GBS isolates from various clinical sources, from 4.8% (472/9738 isolates) in 2008 to 5.8% (2022/34 988 isolates) in 2016, which is extremely higher than the rate of 0.7% (13/1975 isolates) in 2015 from US Centers for Disease Control and Prevention (CDC) Active Bacterial Core surveillance (ABCs) [32]. Furthermore, PRGBS isolates in Japan have been characterised to be commonly associated with serotype VI and CC1 (ST1 or ST458), although serotype V is predominant among ST1 GBS isolates in many other countries. Of note, serotypes of PRGBS isolates in this study were serotypes III, Ia and Ib, which have been very rarely reported among ST1 GBS [24].

In the present study, a dynamic change of capsular polysaccharide types mainly from serotype III to Ia was confirmed in ST1 PRGBS populations that shared amino acid substitutions in PBPs in a medium-sized hospital in Japan. Serotype III strain SU12 showed the highest ANIm value of 99.84% with serotype Ia strain SU97, and SU12 and SU97 formed a distinct cluster in the SNP-based phylogenetic analysis, confirming the close genetic relatedness of these two strains sharing virulence factors. Strains SU12 and SU97 were



SNP difference matrix

	IIIPRGBS _SU12	IaPRGBS _SU97	IbCTB^{r}PSGBS _SU66	IbPRGBS _SU67	VGBS _SS1	IIIGBS _NEM316	IaGBS _A909
IIIPRGBS_SU12	0	957	3022	3027	3434	7369	4920
IaPRGBS_SU97	957	0	2436	2440	3716	7575	4315
IbCTBrPSGBS_SU66	3022	2436	0	46	4131	7270	4535
IbPRGBS_SU67	3027	2440	46	0	4138	7277	4542
VGBS_SS1	3434	3716	4131	4138	0	7260	4478
IIIGBS_NEM316	7369	7575	7270	7277	7260	0	7749
IaGBS_A909	4920	4315	4535	4542	4478	7749	0

Percentage of strain SU12 reference genome covered by all isolates: 83.4709976798144
1780812 positions was found in all analyzed genomes.
Size of strain SU12 reference genome: 2133450 bp

Fig. 3. Genetic relatedness among group B *Streptococcus* with reduced susceptibility to penicillin (PRGBS) strains based on single nucleotide polymorphism (SNP) differences. A phylogenetic tree was constructed using the distance matrix of SNP differences between each pair of genomes among serotype III PRGBS strain SU12 (IIIPRGBS_SU12), serotype Ia PRGBS strain SU97 (IaPRGBS_SU97), serotype Ib penicillin-susceptible GBS with reduced cephalosporin susceptibility (CTB^{r}PSGBS) strain SU66 (IbCTB^{r}PSGBS_SU66), serotype Ib PRGBS strain SU67 (IbPRGBS_SU67), serotype V GBS strain SS1 (VGBS_SS1), serotype III GBS strain NEM316 (IIIGBS_NEM316) and serotype Ia GBS strain A909 (IaGBS_A909).

characterised by insertion of IS*1548* within the *hylB* structural gene upstream of the *cpsY* gene, and ΔIS*Sag8* downstream of the *neuA* gene, respectively. Disruption of *hylB* by IS*1548* insertion identified in SU12 was also found among other 15 serotype III PRGBS isolates detected in period I. On the other hand, the tRNA-Arg–ΔIS*Sag8*–*rpsA* region identified in SU97 was also found among other 17 serotype Ia PRGBS detected in period III. Thus, nosocomial transmission may likely contribute to the spread of serotype III PRGBS in period I and serotype Ia PRGBS in period III.

The *hylB* gene disrupted by IS*1548* insertion has been described in hyaluronidase-negative serotype III GBS strains derived from endocarditis and septicaemia cases in adults [33]. Since hyaluronidase, as one of the virulence factors produced by GBS, plays an important role in evading the host immune system by degrading host-generated immunostimulatory hyaluronic acid fragments [34], restoring the original intact *hylB* gene could become beneficial for the pathogenicity of serotype Ia PRGBS in period III. Indeed, PRGBS recovered from all four patients with poor

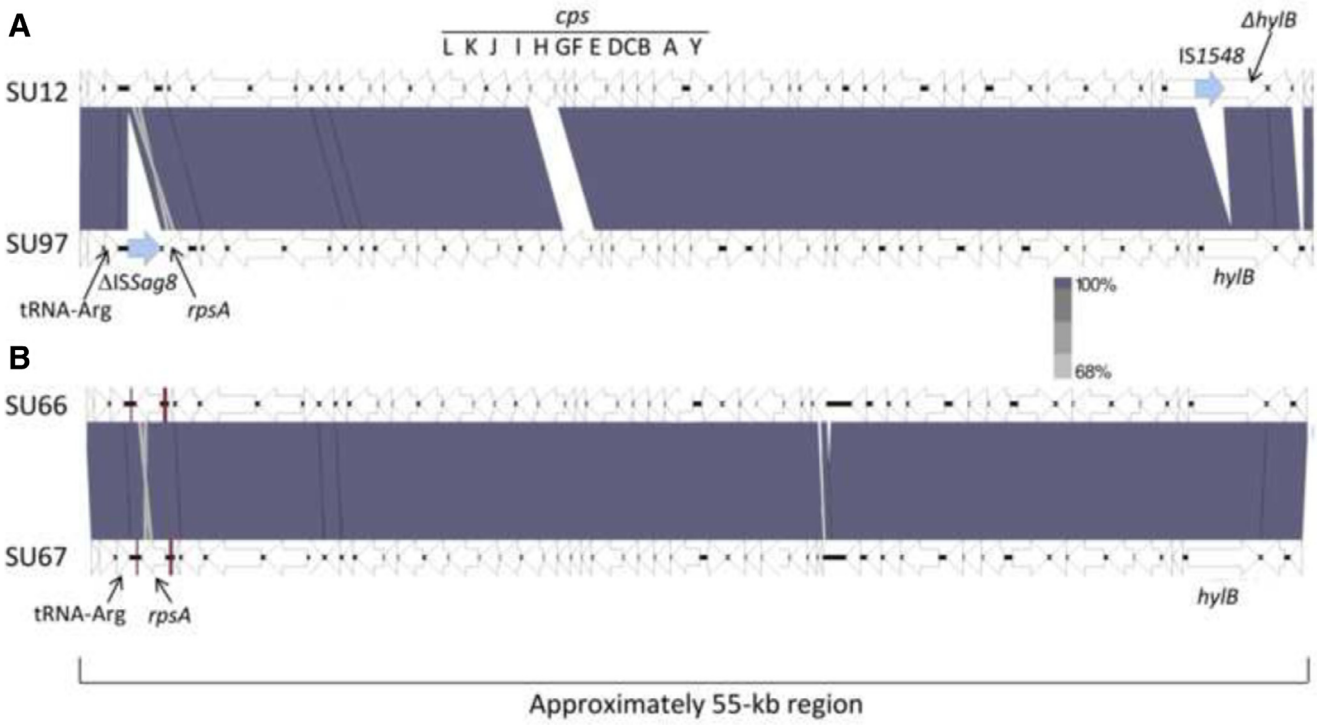


Fig. 4. Alignment of an ca. 55-kp region including *cps* gene clusters and the upstream and downstream flanking sequences using EasyFig v.2.2.2: (a) comparison between serotype III PRGBS strain SU12 and serotype Ia PRGBS strain SU97; and (b) comparison between serotype Ib CTB^{r}PSGBS strain SU66 and serotype Ib PRGBS strain SU67. Genes are indicated by arrows and the level of sequence similarity is shown in the gradient scale. PRGBS, group B *Streptococcus* with reduced susceptibility to penicillin; CTB^{r}PSGBS, penicillin-susceptible group B *Streptococcus* with reduced cephalosporin susceptibility.

prognosis in periods III and IV were serotype Ia isolates with intact *hylB* genes. IS*Sag8*, which is peculiar to serotype Ia, is found in five copies in the published genome sequence of serotype Ia *S. agalactiae* A909 but is not found in serotype III *S. agalactiae* NEM316. None of the five copies of IS*Sag8* in *S. agalactiae* A909 are located between tRNA-Arg and *rpsA* genes. This tRNA-Arg–ΔIS*Sag8*–*rpsA* region has 99% (4750/4776 bp) sequence identity to only a bovine milk isolate SA111 in France (GenBank accession no. **LT545678**), so it may be a unique region within serotype Ia PRGBS populations in periods III and IV detected in this hospital. Interestingly, most (nine of ten) serotype III PRGBS that re-emerged in phase IV had this tRNA-Arg–ΔIS*Sag8*–*rpsA* region.

Population-level changes in the serotype prevalence among ST1 PRGBS isolates may suggest the contribution of a capsular switch event, which has so far been reported in ST1 GBS [35], and nosocomial transmission of such capsule-switched isolates. The possibility of replacement of one nosocomial PRGBS by another PRGBS of different serotype cannot be excluded. However, at the whole-genome level, the relationship between serotype III strain SU12 and serotype Ia strain SU97 showed greater genetic similarity than the relationship between serotype III strain NEM316 and serotype Ia strain A909, so capsular switching is more likely to be involved in this phenomenon. The driving force for capsular switching may be derived from bacterial stress generated by antibiotics and/or from immune pressure by anti-capsule antibodies [36,37]. Consequently, escape from immunity to serotype III as well as advantageous characteristics such as reversion back to a normal *hylB* gene and reduced susceptibility to β-lactams may allow serotype Ia PRGBS to achieve successful survival and long-term spread. PRGBS has commonly been associated with multidrug resistance [18,38]. Therefore, capsular switching in PRGBS is a potential medical concern because it allows it to evade naturally occurring immunity and to resist antimicrobial therapy, thus it may cause more serious and life-threatening infections in the elderly and immunocompromised populations.

CTB^{r}PSGBS isolates including strain SU66, a unique serotype Ib PSGBS lineage in that it showed reduced susceptibility to several cephalosporins such as ceftibuten, were detected in periods I, II and IV, and serotype Ib PRGBS including strain SU67 was detected in period II. Genome sequencing analysis revealed a high degree of genetic similarity between strains SU66 and SU67.

This study highlights the long-term epidemic of ST1 PRGBS among elderly patient populations. Continuous monitoring and genetic characterisation of these isolates enabled us to find the population-level serotype transition that may have been associated with capsular switching events and nosocomial transmission. A limitation of the present study is the impossibility of determining the concentration of anti-capsule antibodies in the serum of patients. None the less, this study, including a fatal outcome in four elderly patients with bacteraemia, provides important findings for vaccine development strategies.

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Competing interests

None declared.

Ethical approval

Not required.

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