

## Associations between capsular serotype, multilocus sequence type, and macrolide resistance in *Streptococcus agalactiae* isolates from Japanese infants with invasive infections

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### SUMMARY

*Streptococcus agalactiae* (group B streptococcus; GBS) isolates ( $n=150$ ) from infants with invasive infections between 2006 and 2011 were analysed for capsular serotype, multilocus sequence type, and antibiotic susceptibility. In cases with late-onset disease ( $n=115$ ), primary meningitis was predominant (62·6%), but represented only 39·1% in cases with early-onset disease ( $n=23$ ). The most common serotype was III (58·7%), followed by Ia (21·3%) and Ib (12·7%). Sequence types (STs) of serotype III strains included ST17 (50·0%), ST19 (26·1%), ST335 (18·2%), ST27 (4·5%), and ST1 (1·1%). Predominant STs of serotypes Ia and Ib were ST23 (81·3%) and ST10 (84·2%), respectively. No penicillin-resistant strains were detected, but 22·0% of strains had *mef(A/E)*, *erm(A)*, or *erm(B)* genes, which mediate macrolide resistance. A new ST335, possessing an *mef(A/E)* gene belonging to clonal complex 19 gradually increased in frequency. Improved prevention of invasive GBS infections in infants requires timely identification, and ultimately vaccine development.

**Key words:** Capsular type, invasive diseases, multilocus sequence typing, neonates, *Streptococcus agalactiae*.

### INTRODUCTION

*Streptococcus agalactiae*, or Lancefield group B streptococcus (GBS), is a leading cause of neonatal infections with high mortality and morbidity rates [1, 2]. GBS infections may be divided into early-onset disease (EOD), which occurs within the first 6 days

after birth, and late-onset disease (LOD), which occurs from days 7 to 89 after birth [3]. EOD is caused by vertical transmission from mothers to neonates during childbirth, and presents most frequently as sepsis, followed by meningitis [4–6]. LOD mostly presents as meningitis due either to transmission from the mother or contact with environmental sources [2].

The initial guidelines issued in 1996 [3] and the revised guidelines of 2002 [7] for the prevention of neonatal GBS infections in the USA proposed the use of a prophylactic intrapartum antimicrobial agent in pregnant carriers. These preventive measures were

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associated with a significant decline in the incidence of EOD over the past decade in the USA, while the incidence of LOD remained unchanged [8, 9]. The guidelines were again revised in 2010 [10]. Active Bacterial Core Surveillance (ABCs) recently estimated the incidence of invasive GBS as 0.25 EOD and 0.26 LOD cases/1000 live births in 2010 (<http://www.cdc.gov/abcs/reports-findings/survreports/gbs10>).

In 2008, the Japan Society of Obstetrics and Gynecology (JSOG) adopted similar guidelines, recommending the universal screening of pregnant women at 33–37 weeks gestation and the administration of a prophylactic intrapartum antibiotic to those who test positive for GBS. The incidence of neonatal GBS infections was estimated to be 0.4/1000 live births in the Hokkaido area in 2010 [11]. A nationwide surveillance study in Japan between 2004 and 2010 reported a decrease in mortality due to EOD and LOD, but the incidence of GBS infection remained unchanged [12].

In the 10 capsular polysaccharide serotypes, types Ia, Ib, and III most frequently cause neonatal disease [13–16]. In particular, about 80% of LOD is caused by type III GBS [13]. Current PCR methods can readily identify GBS serotypes [17, 18]. Multilocus sequence typing (MLST), developed by Jones *et al.* [19] is also used to distinguish genetic lineages to probe the associations between specific GBS genotypes and diseases. A lineage with enhanced invasive capacity expressing serotype III has been characterized as sequence type 17 (ST17) by most MLST studies [20–22]. Another report indicates that capsular type Ia of ST23 and ST24 demonstrates enhanced potential for invasive diseases, especially EOD [23].

In the present study we aimed to clarify the genetic diversity of GBS isolates responsible for invasive infections in neonates in Japan from 2006 to 2011. The GBS isolates sent to our laboratory by medical institutions participating in the surveillance group underwent serotype characterization by real-time PCR and MLST. Susceptibility to antimicrobials and the presence of macrolide (ML) resistance genes were also investigated.

## MATERIALS AND METHODS

### Strains

The study population was limited to patients aged <1 year with invasive GBS infections including sepsis, meningitis, pneumonia, and others such as purulent

arthritis. GBS strains isolated from normally sterile clinical specimens such as blood, cerebrospinal fluid, and joint fluid, were collected from the 351 medical institutions participating in the Active Surveillance of Invasive Pneumococcal and Streptococcal Infections in Japan. During 2006–2011, our laboratory at Kitasato Institute for Life Sciences received a total of 150 GBS strains accompanied by an anonymous survey form completed by the attending physician.

### Capsular serotyping

To identify the capsular serotypes of GBS by real-time PCR, we constructed nine sets of primers and molecular beacon (MB) probes, according to Poyart *et al.* [17] and Igarashi & Mitsuhashi [24]. One colony was picked from each agar plate and placed in 50  $\mu$ l lysis solution containing 2 U mutanolysin (Sigma Aldrich, Germany). The lytic reaction was performed for 10 min at 37 °C and 10 min at 60 °C, followed by 5 min at 94 °C. Lysate was added to each of six tubes containing the PCR mixtures for the individual capsular types. The combinations were as follows: types Ia and Ib in tube A, type II in tube B, types III and IV in tube C, types V and VI in tube D, types VII and VIII in tube E, and the *dltS* gene in tube F.

The PCR reaction mixture (total volume, 50  $\mu$ l) consisted of 20 pmol of each primer, 25 pmol of each probe, 2  $\times$  multiplex power mix (Bio-Rad, USA), and DNase- and RNase-free distilled water. DNA amplification was performed for 40 cycles at 95 °C for 10 s, 50 °C for 30 s, and 72 °C for 20 s.

### MLST analysis

Primer sets corresponding to seven housekeeping genes (*adhP*, *atr*, *glcK*, *glnA*, *pheS*, *sdhA*, *tkt*) used for MLST analysis were constructed in reference to the MLST website (<http://pubmlst.org/sagalactiae/>). MLST was applied to the sequence for these seven genes according to previously described methods [19], with alleles and sequence type (ST) assignments determined using the *S. agalactiae* MLST database. Alleles and sequence types not previously posted were entered into the *S. agalactiae* MLST database.

### Antibiotic susceptibility and resistance mechanism

Susceptibility testing of GBS strains was performed using an agar dilution method conforming to the

Table 1. Correlation between capsular serotypes and diseases according to age at onset

Onset of disease	Disease	Strains (%) <sup>*</sup>	Capsular serotype (%)										
			Ia	Ib	II	III	IV	VI	VIII				
Early-onset disease (<7 days)	Meningitis	9 (39.1)	3	1		4							
	Sepsis	14 (60.9)	5	3		1							
	Subtotal (%)	23	8 (34.8)	4 (17.4)	3 (13.0)	5 (21.7)							
Late-onset disease (7–89 days)	Meningitis	72 (62.6)	9	6		56							
	Sepsis	34 (29.6)	12	2	2	17							1
	Other	9 (7.8)	1	4		3	1						
Subtotal (%)	115	22 (19.1)	12 (10.4)	2 (1.7)	76 (66.1)	1 (0.9)				1 (0.9)			1 (0.9)
Older infants (90 days to <1 year)	Meningitis	6 (50.0)		1		5							
	Sepsis	5 (41.7)	2	1		2							
	Other	1 (8.3)		1									
	Subtotal (%)	12	2 (16.7)	3 (25.0)		7 (58.3)							
Total (%)		150	32 (21.3)	19 (12.7)	5 (3.3)	88 (58.7)	1 (0.7)			4 (2.7)			1 (0.7)

<sup>\*</sup> Percentage of each subtotal.

standards of the Clinical and Laboratory Standards Institute ([www.clsi.org](http://www.clsi.org)). Oral antimicrobial agents employed in this study were penicillin G (PEN), ampicillin (AMP), amoxicillin (AMX), and erythromycin (ERY). Parenteral agents were cefotaxime (CTX), panipenem (PAM), meropenem (MEM), and vancomycin (VAN).

ML resistance genes of *erm(A)*, *erm(B)*, and *mef(A/E)* were identified by real-time PCR. The primer and probe sets and PCR conditions required modification of previously described methods [25].

## RESULTS

### Relationship between serotype and GBS diseases according to age at onset

The 150 cases of invasive GBS infection we collected between 2006 and 2011 are summarized in Table 1 according to the serotype of the GBS strain and the age at onset as follows: EOD, within 6 days after birth ( $n=23$ , 15.3%); LOD, from 7 to 89 days after birth ( $n=115$ , 76.7%); and from 90 days to 1 year ( $n=12$ , 8.0%). GBS diseases are categorized as meningitis, sepsis (including a few bacteraemic cases), or other infections including purulent arthritis.

In EOD cases, sepsis (60.9%) was more frequent than meningitis (39.1%), but in LOD cases, meningitis (62.6%) exceeded sepsis (29.6%) as well as other infections (7.8%). This difference between EOD and LOD was statistically significant ( $P=0.011$ ), while the findings of LOD did not differ significantly from those in older infants ( $P=0.669$ ).

Infants with low birth weight or underlying congenital diseases were substantially represented in both EOD cases (26.1%) and LOD cases (19.1%). Patients with outcomes of death and neurological sequelae, respectively, were 13.0% and 8.7% for EOD, and 0.9% and 7.0% for LOD.

Of the EOD cases, serotype Ia was most frequent (34.8%), followed by serotype III (21.7%) and serotype Ib (17.4%). In contrast, serotype III was most prevalent in LOD cases (66.1%) and in older infants (58.3%), especially for meningitis and sepsis. Serotype distribution differed significantly depending on the disorders in LOD ( $P<0.001$ ), but not in EOD ( $P=0.213$ ) or older infants ( $P=0.206$ ).

Of all tested strains, the most frequent serotype was III (58.7%), which was followed by Ia (21.3%) and Ib (12.7%); other types had lower incidences.

Table 2. Relationships between clonal complex, sequence type, and serotype in 150 invasive GBS isolates

Clonal complex	Sequence type	Serotype							Total (%)
		Ia	Ib	II	III	IV	VI	VIII	
CC1	ST1	1	1	5	1	1	3	1	13 (8.7)
	ST3		1						1 (0.7)
CC10	ST10		16						16 (10.7)
	ST12		1						1 (0.7)
CC17	ST17				44				44 (29.3)
CC19	ST19	1			23				24 (16.0)
	ST27				4				4 (2.7)
	ST335*				16		1		17 (11.3)
CC23	ST23	26							26 (17.3)
	ST24	1							1 (0.7)
	ST88	1							1 (0.7)
	ST144*	1							1 (0.7)
	ST145*	1							1 (0.7)
	Total	32	19	5	88	1	4	1	150

\* New sequence types identified in this study.

### Association between serotype and MLST

Associations between capsular serotype and ST or clonal complex (CC) for all GBS strains were analysed by MLST using the GBS website. As shown in Table 2, strains were classified into 13 STs and five CCs. New STs identified in this study included ST144 and ST145, belonging to CC23; and ST335, belonging to CC19. Correlations between the serotype and ST were evident, such as serotype Ia with ST23, serotype Ib with ST10, and serotype III with ST17, ST19, and ST335.

### Phylogenetic relationship between ST and CC

Figure 1 is a dendrogram of STs based on the results of MLST using an unweighted pair-group method with average linkages (UPGMA). One half of serotype III isolates, identified as ST17 of CC17 ( $n=44$ ), represented known high-virulence strains; the remainder belonged to ST19, ST27, and ST335 of CC19 ( $n=43$ ), except for a single strain of ST1. Notably, CC17 was plotted near CC61 (ST61, ST76, ST91), a bovine-derived GBS strain.

Five STs, including the frequently occurring ST23, which belongs to CC23 in serotype Ia, clearly differed from other CCs. ST10 and ST12, accounting for most of serotype Ib, belonged to CC10. ST1, belonging to CC1, was distributed in seven serotypes.

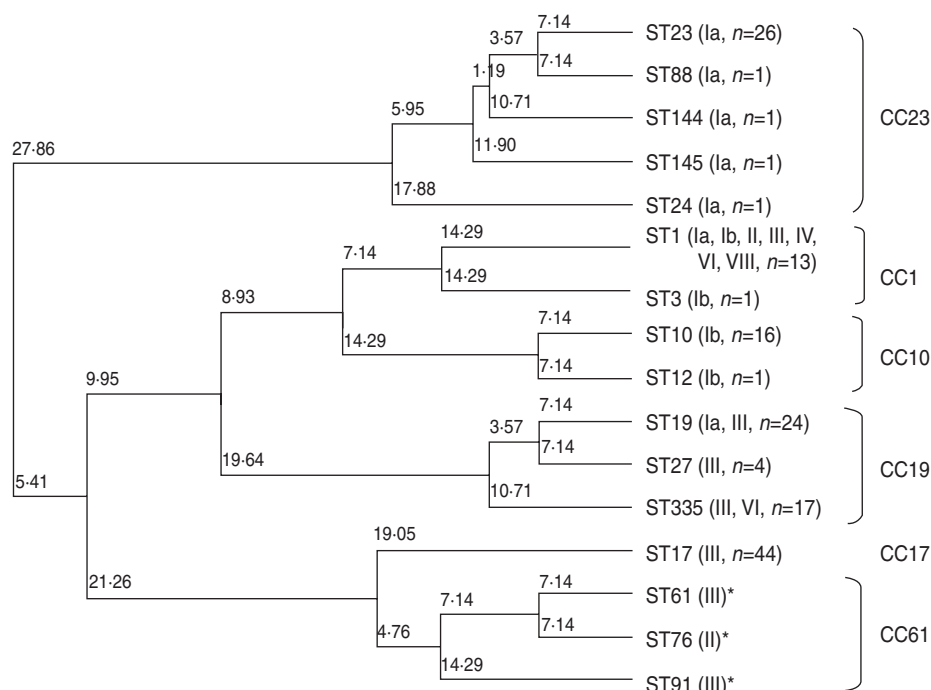
### Antimicrobial susceptibility and ML resistance

The minimum inhibitory concentration (MIC) range, MIC<sub>50</sub>–MIC<sub>90</sub>, for GBS of eight antimicrobial agents including three penicillins, cefotaxime, two carbapenems (MEM and PAM), VAN, and ERY are shown in Table 3. No  $\beta$ -lactam-resistant strains were identified. The MIC<sub>90</sub> for ML-resistant strains was  $\geq 64 \mu\text{g/ml}$ .

Table 4 shows the correlation between the ML resistance gene, serotype, and ST. Strains possessing the *erm(B)* gene mediating high ML resistance, the *erm(A)* gene mediating inducible high ML resistance, and the *mef(A/E)* gene mediating intermediate ML resistance (2–16  $\mu\text{g/ml}$ ), were 8.7%, 12.0%, and 1.3%, respectively, totalling 22.0% of all strains. ML resistance was particularly prevalent in serotype Ia belonging to ST23 and in serotype III belonging to ST335, ST17, and ST19.

### DISCUSSION

In 1996, guidelines for the prevention of neonatal GBS infections using antimicrobial prophylaxis first were issued in the USA [3]. The guidelines were subsequently revised to include universal screening for vaginal GBS colonization in pregnant women at 35–37 weeks gestation [7]. Epidemiological studies later found that the recommendations had



**Fig. 1.** Neighbour-joining analysis of allelic identities of 13 sequence types (STs) in clinical isolates of GBS and three reference bovine-derived strains. Numbers at nodes are the percentages of 1000 bootstrap replicates in which these nodes appeared. Only nodes with percentages exceeding 50% were included. \* Known bovine-derived strains. CC, Clonal complex

**Table 3.** Susceptibility of invasive GBS isolates to eight antimicrobial agents

Antimicrobial agent	MIC <sub>50</sub> (µg/ml)	MIC <sub>90</sub> (µg/ml)	MIC range (µg/ml)
Penicillin G	0.063	0.063	0.031–0.063
Ampicillin	0.125	0.125	0.063–0.125
Amoxicillin	0.063	0.063	0.063–0.125
Cefotaxime	0.063	0.063	0.031–0.063
Meropenem	0.063	0.063	0.031–0.125
Panipenem	0.031	0.031	0.016–0.031
Vancomycin	0.5	0.5	0.25–0.5
Erythromycin*	0.031	≥64	0.016–≥64

MIC, Minimum inhibitory concentration.

\* Strains possessing macrolide resistance genes are as follows: *erm(B)*, 8.7%; *erm(A)*, 12.0%; *mef(A/E)*, 1.3%.

contributed to a decrease in EOD, but not in LOD [10].

In 2008, JSOG recommended universal GBS screening of pregnant women at 33–37 weeks gestation as well as intrapartum prophylaxis with an antimicrobial agent for GBS-positive cases [26]. The results of continued study of meningitis indicate that prophylactic intrapartum administration of

antimicrobial agents after the introduction of the JSOG guidelines led to a decrease in the incidence of EOD, but the incidence of LOD remained unchanged [27, 28].

In response to this problem, revised US guidelines were issued in 2010. The application of a rapid diagnostic test for GBS identification was added, and the antimicrobials used for intrapartum prophylaxis were changed [10]. In Japan, similar revised guidelines were also published in 2011 [29].

As previously reported [20, 30], our results also show that meningitis is overwhelmingly caused by capsular type III. GBS colonization of pregnant Japanese women in an advanced gestational stage was reported as 15% according to bacterial culture, but increased to 22% with real-time PCR [24]. Capsular types V, Ia, and Ib were predominant; type III accounted for only 12% of colonization cases in Japan.

The ability of type III GBS to frequently cause meningitis has been attributed to neuraminidase activity [31], the presence of Spbl surface proteins involved in adhesion to and invasion of epithelial cells by GBS [32], fibrinogen receptor FbsA [33], and pili that are involved in the invasion of brain

Table 4. Relationships between macrolide resistance gene, serotype, and sequence type

Capsular serotype	Isolates ( <i>n</i> )	Macrolide resistance gene			Resistant isolates <i>n</i> (%)
		<i>erm</i> (A)	<i>erm</i> (B)	<i>mef</i> (A/E)	
Ia	32	ST23 (4)*	ST19 (1)	ST23 (2)	7 (21.9)
Ib	19	ST12 (1)			1 (5.3)
II	5				0
III	88	ST17 (1), ST335 (11)	ST17 (5), ST19 (6), ST27 (1)		24 (27.3)
IV	1	ST1 (1)			1
VI	4				0
VIII	1				0
Total (%)	150	18 (12.0)	13 (8.7)	2 (1.3)	33 (22.0)

\* Numbers of isolates with a given gene and sequence type are given in parentheses.

microvascular endothelial cells [34]. CspA serine protease-like surface protein has been reported to inactivate chemokines through its anti-phagocytotic action [35]. If strains of highly pathogenic capsular type can be rapidly identified, the prevention of neonatal GBS infections should be enhanced.

The use of multiplex PCR to rapidly detect capsular type has been reported by Poyart *et al.* [17]. We also developed a real-time PCR method to identify GBS and capsular types. A rapid PCR method with high sensitivity and specificity is most useful as a screening to prevent neonatal GBS infections.

Relationships between capsular type, virulence, and MLST have been studied by many investigators [19–23, 36, 37]. The capsular type III strain of ST17 belonging to CC17 has been linked to high pathogenicity [19–23, 36, 37] as a high-virulence clone [38, 39]. Our results showed that half of the capsular type III strains were ST17 and the remainder were ST19 or its new subtype, ST335, belonging to CC19. Based on MLST analysis, CC17 is placed near lineage CC61, a bovine pathogen [40]. Our results also placed CC61 near CC17 on the phylogenetic tree. However, analysis of 15 housekeeping genes did not favour such a conclusion [41]. Although *cps* gene recombination or capsular switching has been suggested [36], it remains to be investigated whether such gene recombination occurs between human and bovine-derived GBS strains.

$\beta$ -lactam-resistant GBS isolates have not explicitly been confirmed anywhere in the world. However, in 2008, Kimura *et al.* reported that penicillin-resistant GBS (PRGBS) strains possessing a few amino acid

substitutions adjacent to conserved motifs of Ser-Ser-Asn or Lys-Ser-Gly in PBP2x had been isolated from adult sputum [42]. The capsular types were Ib, III, VI, and VIII. The PRGBS of capsular type III, belonging to ST19, was also isolated from elderly patients in the USA [43].

CDC guidelines [10] have recently been revised, increasing the dose of PEN used for prophylaxis. From now on, it will be necessary to monitor the  $\beta$ -lactam susceptibility of GBS isolates – especially those derived from neonates. An additional important point is that 22.0% of our Japanese strains had genes mediating ML resistance. ML-resistant strains are increasing worldwide. When isolates from pregnant women who are allergic to penicillin are resistant to ML, VAN should be used for intrapartum antibiotic prophylaxis.

To control invasive neonatal GBS infection most effectively, the development of a GBS vaccine is an extremely important goal.

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#### DECLARATION OF INTEREST

None.

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