











Comparative Analysis of the Molecular Characteristics of Group B *Streptococcus* Isolates Collected from Pregnant Korean Women Using Whole-genome Sequencing

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Background: The incidence of early- and late-onset sepsis and meningitis in neonates due to maternal rectovaginal group B *Streptococcus* (GBS) colonization may differ with serotype distribution and clonal complex (CC). CC17 strains are associated with hypervirulence and poor disease outcomes. GBS serotypes are distinguished based on the polysaccharide capsule, the most important virulence factor. We determined the sequence type distribution of GBS isolates from pregnant women in Korea and validated whole-genome sequencing (WGS)-based prediction of antimicrobial susceptibility and capsular serotypes in GBS isolates.

Methods: Seventy-five GBS isolates collected from pregnant Korean women visiting Wonju Severance Christian Hospital, Wonju, Korea between 2017 and 2019 were subjected to WGS using the NovaSeq 6000 system (Illumina, San Diego, CA, USA). Multilocus sequence types, serotypes, antimicrobial resistance genes, and hemolysin operon mutations were determined by WGS, and the latter three were compared with the results of conventional phenotypic methods.

Results: The predominant lineage was CC1 (37.3%), followed by CC19 (32.0%), CC12 (17.3%), and CC17 (4.0%). All isolates were *cps* typeable (100%, (75/75), and 89.3% of *cps* genotypes (67/75) were concordant with serotypes obtained using latex agglutination. The *cps* genotypes of the 75 isolates were serotypes III (24.0%), V (22.7%), and VIII (17.3%). All isolates harboring intact *ermB* and *tet* were non-susceptible to erythromycin and tetracycline, respectively. Three non-hemolytic strains had 1-bp frameshift insertions in *cyfE*.

Conclusions: The low prevalence of CC17 GBS colonization may explain the low frequency of neonatal GBS infections. WGS is a useful tool for simultaneous genotyping and antimicrobial resistance determination.

Key Words: Group B *Streptococcus*, Whole-genome sequencing, Serotype, Sepsis, Neonate

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INTRODUCTION

Group B *Streptococcus* (GBS) is a critical pathogen that causes meningitis and sepsis in neonates. Neonatal GBS isolates are mainly acquired from the maternal genitourinary tract. The most important virulence factor of GBS is its polysaccharide capsule encoded by *cps*. Based on the capsule, 10 serotypes (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX) with different disease-causing abilities are distinguished [1]. Serotypes and antimicrobial resistance profiles are important parameters for the characterization and treatment of invasive GBS infections [2]. Serotype III isolates belonging to clonal complex (CC)17 are associated with hypervirulence and poor disease outcomes [3-5]. In a study in the Netherlands, most isolates obtained from neonates showing invasive infections were clustered into one of five major lineages: CC17 (39%), CC19 (25%), CC23 (18%), CC10 (9%), and CC1 (7%) [3]. The number of neonatal GBS infections caused by CC17 isolates has significantly increased [3, 4, 6]. In contrast, CC1, CC12, and CC23 are more common in pregnant women [6]. In Korea, the prevalence of GBS lineages has not yet been reported.

With the rapid advances in bioinformatics that have allowed analyzing and storing large amounts of whole-genome sequencing (WGS) data, it has become possible and feasible to obtain genetic information of bacteria in clinical microbiology laboratories. Recent studies have used WGS for molecular capsular typing and antimicrobial resistance gene typing of GBS [7-9]. As comprehensive data, including multilocus sequence types (MLSTs), serotypes, resistomes, and virulence factors, can be extracted from a single WGS dataset, WGS is a practical and economical method as compared with conventional phenotypic methods [9, 10].

We for the first time investigated the sequence type (ST) distribution of GBS isolates collected from pregnant women in Korea and validated WGS-based antimicrobial susceptibility and capsular serotypes of the GBS isolates.

MATERIALS AND METHODS

Bacterial collection and WGS

In total, 75 GBS isolates were collected from pregnant Korean women visiting Wonju Severance Christian Hospital, Korea between May 2017 and May 2019, as previously reported [11]. The study was approved by the Yonsei University Wonju Severance Christian Hospital Institutional Review Board (IRB No. CR319119). Sequencing libraries for all isolates were prepared using the Twist

Library Preparation EF Kit (Twist Bioscience, San Francisco, CA, USA) according to the manufacturer's instructions. Briefly, extracted DNA was assessed using the Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Fifty nanograms of high-quality DNA in 40 μ L of 10 mM Tris-HCl (pH 8.0) was enzymatically fragmented, and DNA ends were repaired by dA-tailing in a C1000 Touch Thermal Cycler (Bio-Rad, Hercules, CA, USA). The dA-tailed DNA fragments were ligated with Twist Universal Adapters compatible with the NovaSeq 6000 system (Illumina, San Diego, CA, USA) at 20°C for 30 minutes. The ligated libraries were PCR-amplified in six cycles using Twist UDI primers in a thermal cycler. The quantities and size ranges of the final libraries were validated using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific) and Agilent 4200 TapeStation System (Agilent Technologies, Santa Clara, CA, USA). All libraries were equally pooled, and the pooled library was diluted to 2 nM for sequencing, denatured in 0.2 N NaOH, and diluted with 400 mM Tris-HCl (pH 8.0) to 400 pmol/L. The diluted library was sequenced using paired-end (2 \times 150 bp) sequencing on the NovaSeq 6000 system (Illumina). *De novo* assembly from the FASTAQ files generated after sequencing was performed using Unicycler (v0.4.0, <https://github.com/rwwick/Unicycler>). The median depth of coverage was 1,050 \times , with maximum depths of 2,264 \times to 529 \times .

Whole-genome analysis

The FASTA files generated after assembly were analyzed using tools provided on the Center for Genomic Epidemiology website (<http://www.genomicepidemiology.org/>) and the PubMLST database (<http://pubmlst.org>) to determine MLSTs, *cps* genotypes, antimicrobial resistance genes, and the hemolysin operon regions of the GBS isolates. The *cps* genotypes were analyzed using the *cpsG-K* region, as previously suggested [7, 8]. We compared the WGS-based serotypes, antimicrobial resistance genes, and phenotypic characteristics with the findings of a previous study that conducted antimicrobial susceptibility testing and serotyping of GBS isolates using the Strep-B-Latex kit (SSI Diagnostica, Hillerød, Denmark) [9].

Antimicrobial susceptibility test

Antimicrobial susceptibility was tested using the MicroScan MicroSTREP Plus Panel (Beckman Coulter, Brea, CA, USA), which covers ampicillin, penicillin, cefotaxime, ceftriaxone, cefepime, meropenem, levofloxacin, clindamycin, erythromycin, tetracycline, chloramphenicol, and vancomycin. GBS isolates showing inconsistent results between WGS and previous methods were

re-examined using WBS and the previously used method.

RESULTS

MLSTs of GBS isolates

The distribution of GBS isolates according to MLST CCs is shown in Table 1. The predominant CC was CC1 (N=28, 37.3%), followed by CC19 (N=24, 32.0%) and CC12 (N=13, 17.3%). CC1 was composed of ST1, ST2, ST667, and ST676, whereas CC19 was composed of ST19, ST27, ST335, and ST1911. Three isolates (4.0%) belonged to ST17—a hypervirulent strain. Two new STs (ST1911 and ST1912) were identified (Table 1).

Latex serotypes and *cps* genotypes

All isolates (100%, 75/75) were *cps* typeable, and 89.3% (67/75) of the *cps* genotypes were in agreement with serotypes determined using latex agglutination. Of the 16 isolates with discordant results, eight were confirmed by retesting using latex agglutination and WGS (Table 2). The *cps* genotypes of the 75 isolates mainly included serotypes III (N=18, 24.0%), V (N=17, 22.7%), and VIII (N=13, 17.3%). CC19 isolates were of sero-

Table 2. Retyping of isolates with discordant results between latex agglutination serotyping and *cpsG-K* genotyping

Isolate	Latex agglutination serotype		<i>cpsG-K</i> genotype
	Initial	Repeat	
WJ1	VII	VI	VI
WJ4	VIII	III	III
WJ10	IX	IX	III
WJ11	Ia	V	V
WJ12	VII	VII	V
WJ14	VIII	III	III
WJ21	IX	IX	Ib
WJ36	V	V	II
WJ37	V	V	II
WJ41	V	V	II
WJ47	IX	Ib	Ib
WJ50	VII	VII	V
WJ59	IX	Ib	Ib
WJ60	Ib, II	II	II
WJ64	Ib	Ib	II
WJ68	II	Ib	Ib

Abbreviation: *cps*, capsular polysaccharide gene.

Table 1. Distribution of MLST CCs and STs and *cps* genotypes among 75 GBS isolates collected from pregnant Korean women

CC	ST	<i>cps</i> genotype								Total
		Ia	Ib	II	III	V	VI	VII	VIII	
1	2								13	13
	1					6	3	1		10
	676			4						4
	667						1			1
19	19				5	8				13
	335				5					5
	27				3	2				5
	1911				1					1
12	10		5							5
	12			3						3
	654		3							3
	8		1							1
	1912		1							1
23	23	4								4
17	17				3					3
22	22			1						1
NA	498					1				1
NA	529				1					1
Total (%)		4 (5.3)	10 (13.3)	8 (10.7)	18 (24.0)	17 (22.7)	4 (5.3)	1 (1.3)	13 (17.3)	75

Abbreviations: CC, clonal complex; ST, sequence type; MLST, multilocus ST; *cps*, capsular polysaccharide gene; GBS, group B *Streptococcus*; NA, not assigned.

Table 3. Antimicrobial resistance genes in erythromycin- and/or tetracycline-resistant isolates according to *cps* genotypes and STs

<i>cps</i> genotype (N isolates)	CC	ST	Erythromycin resistance			Tetracycline resistance					
			MIC (µg/mL)	Susceptibility	<i>erm</i> mutation	MIC (µg/mL)	Susceptibility	<i>tet</i> mutation			
V (17)	1	1	256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
	19	19	256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			16	R	<i>ermA</i>	≥8	R	<i>tetM</i>			
			0.12	S	<i>ermA</i>	≥8	R	<i>tetM</i>			
			0.06	S	<i>ermA</i>	≥8	R	<i>tetM</i>			
			0.25	S	-	≥8	R	<i>tetM</i>			
			0.12	S	-	≥8	R	<i>tetM</i>			
			0.06	S	-	≥8	R	<i>tetM</i>			
			0.06	S	-	≥8	R	<i>tetM</i>			
			27	27	2	R	<i>ermB</i>	≥8	R	<i>tetO</i>	
					2	R	<i>ermB</i>	≥8	R	<i>tetO</i>	
	NA	498	0.06	S	-	≥8	R	<i>tetM</i>			
III (18)	19	19	256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			256	R	<i>ermB</i>	≥8	R	<i>tetM</i>			
			0.50	I	<i>ermB</i>	≥8	R	<i>tetM</i>			
			0.06	S	-	≥8	R	<i>tetM</i>			
			0.06	S	-	≥8	R	<i>tetM</i>			
			27	27	256	R	<i>ermB</i>	≥8	R	<i>tetO</i>	
					256	R	<i>ermB</i>	≥8	R	<i>tetO</i>	
					0.06	S	<i>ermB</i> (E128*)	≥8	R	<i>tetO</i>	
			33	33	2	R	<i>ermA</i>	≥8	R	<i>tetM</i>	
					5	1	R	<i>ermA</i>	≥8	R	<i>tetM</i>
						1	R	<i>ermA</i>	≥8	R	<i>tetM</i>
			1911	1911	1	R	<i>ermA</i>	≥8	R	<i>tetM</i>	
					0.06	S	<i>ermA</i>	≥8	R	<i>tetM</i>	
	0.06	S			-	≥8	R	<i>tetO</i>			
	0.06	S			-	≥8	R	<i>tetO</i>			
	17	17	4	R	<i>ermB</i>	≥8	R	<i>tetO</i>			
			4	R	<i>ermB</i>	≥8	R	<i>tetO</i>			
			0.06	S	-	≥8	R	<i>tetM</i>			
	Ia (4)	23	23	0.06	S	-	≥8	R	<i>tetM</i>		
0.25				S	-	≥8	R	<i>tetM</i>			
0.06				S	-	≥8	R	<i>tetM</i>			
0.06				S	-	≥8	R	<i>tetM</i>			
II (4)	12	12	0.06	S	-	≥8	R	<i>tetO</i>			
			0.06	S	-	≥8	R	<i>tetO</i>			
			0.06	S	-	≥8	R	<i>tetO</i>			
			22	22	256	R	<i>ermB</i>	≥8	R	<i>tetM</i>	

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Table 3. Continued

<i>cps</i> genotype (N isolates)	CC	ST	Erythromycin resistance			Tetracycline resistance		
			MIC (µg/mL)	Susceptibility	<i>erm</i> mutation	MIC (µg/mL)	Susceptibility	<i>tet</i> mutation
Ib (2)	12	8	0.06	S	-	≥8	R	<i>tetM</i>
		654	0.06	S	-	≥8	R	<i>tetO</i>
VI (2)	1	667	128	R	<i>ermB</i>	0.5	S	<i>tetO</i> (E203*)
		1	0.06	S	<i>ermB</i> [†]	0.5	S	-
VII (1)	1	1	0.06	S	-	≥8	R	<i>tetM</i>

*Stop codon; [†]A 15-bp fragment inserted at position 238.

Abbreviations: ST, sequence type; *cps*, capsular polysaccharide gene; CC, clonal complex; S, susceptible; I, intermediate; R, resistant; MIC, minimum inhibitory concentration; NA, not assigned.

types III and V, whereas CC12 were of serotypes Ib and II. CC1 isolates were of diverse serotypes, including II, V, VI, VII, and VIII.

Antimicrobial susceptibility and antimicrobial resistance genes

Erythromycin resistance was predicted by the presence of *erm*, which encodes a methylase (Table 3). Twenty-nine isolates (38.7%) harbored *erm*, including 20 harboring *ermB* and nine harboring *ermA*. Eighteen isolates carrying intact *ermB* were non-susceptible to erythromycin, with 13 isolates showing high-level resistance (minimum inhibitory concentration [MIC] ≥128 µg/mL). One isolate with a premature termination codon (E128stop) in *ermB* and one isolate with a 15-bp insertion in *ermB* were susceptible to erythromycin. Among the nine isolates harboring *ermA*, six were resistant to erythromycin, whereas the remaining three were susceptible. Forty-seven isolates (62.7%) harbored *tet*, including 34 harboring *tetM* and 13 harboring *tetO*. Although the 46 isolates having intact *tetM* or *tetO* genes were resistant to tetracycline, one isolate was susceptible to tetracycline because of a premature termination codon in *tetO* (E203stop). No β-lactam-resistant isolate was identified.

Hemolysin operon analysis

Among the 75 isolates, six were non-hemolytic. We analyzed the *cyl* operon (12 kb) and *abx1* regulatory gene reported previously [12]. A 1-bp insertion in *cylE* at position 903/2,004 was detected in three non-hemolytic strains belonging to ST27. *Abx1* mutations were not found in any of the isolates.

DISCUSSION

CC1 and CC19 are among the major GBS CCs associated with invasive disease and colonization in humans [6, 13, 14]. CC1 is phylogenetically close to CC19 [6, 15]. We found that 69.3% of

Korean pregnant women carried CC1 or CC19 GBS. CC17 GBS strains belong to a hypervirulent lineage of homogeneous serotype III clones and are associated with a disproportionately high frequency of invasive neonatal diseases, particularly, meningitis [3, 16]. In Korea, the prevalence of neonatal GBS cases is low [17, 18]. A multicenter study showed that 157 neonatal GBS cases were identified in 14 university hospitals of Korea between 1996 and 2005 [17]. Another study reported 10 GBS cases (0.3%) among 3,862 infants during 2010–2017 [18]. In this study, only three (4.0%) isolates were of CC17, and neonatal infection with maternal CC17 GBS was not observed. The low prevalence of CC17 GBS colonization may explain the low frequency of neonatal GBS infections.

Serotype classification of GBS is based on the capsular polysaccharide, of which 10 variants are known to exist. The capsular polysaccharide is encoded on the *cps* locus, which comprises 16–18 genes. Kapatai, *et al.* [7] have suggested that molecular serotypes based on the variable *cpsG-K* region demonstrated the best performance in terms of typeability and concordance with latex agglutination. Therefore, we compared phenotypic serotyping using latex agglutination with molecular genotyping using the *cpsG-K* region. All isolates (100%) were typeable by WGS-based *cpsG-K* genotyping, and 89.3% of the genotypes were concordant with latex agglutination results; isolates showing discordant results were retested. Similarly, Kapatai, *et al.* [7] reported a concordance rate of 86.7% in initial testing and of 98.2% in retesting between *cps* genotyping and latex agglutination results. However, one study has indicated that the determination of GBS serotypes is often hampered by poor capsule expression [19].

Serotypes Ia, Ib, II, III, and V account for 98% of the colonizing GBS isolates identified worldwide [5]. However, serotype distribution varies with geography and ethnicity. In the United States, Europe, and Australasia, serotypes Ia, II, III, and V account for

80%–90% of clinical isolates, whereas serotypes IV, VI, VII, VIII, and IX are relatively less frequent [5, 19]. Serotype III, which is associated with invasive disease, accounts for 25% worldwide; however, it is less frequent in South American (11%) and South-Eastern Asian (12%) populations [5]. Serotypes VI, VII, VIII, and IX are common in Asia [5]. Our results demonstrated that serotypes III and V are predominant in Korean pregnant women; they were found in 24.0% and 22.7% of the women, respectively, followed by serotype VIII (17.3%). These results suggest that continuous monitoring of serotype distribution is important in epidemiological and vaccine-related studies [19, 20]. Associations between STs and serotypes have been reported in the literature, with some studies reporting strong correlation and others very weak correlation [21, 22]. Ramaswamy, *et al.* [21] observed correlations between serotype III and ST17 and between Ib and ST12; serotype V was found to be present in all STs, except for ST17. Similarly, we found correlations between serotype III and ST17 and between serotype VIII and ST2.

We compared antimicrobial susceptibility results with antimicrobial resistance genes detected using WGS. Tetracycline resistance was predominantly caused by *tetM* and *tetO*, whereas macrolide resistance was predominantly due to the presence of *erm*, with *ermB* being more prevalent. In a previous study, resistant and intermediate-resistant GBS isolates showed high frequencies of *tetM* (97.6%) and low frequencies of *tetO* (2.4%), *ermB* (34.5%), and *ermTR* (10.3%) [23]. In this study, 45.3% harbored *tetM*, 17.3% harbored *tetO*, 26.7% harbored *ermB*, and 12.0% harbored *ermA*. Isolates carrying intact *tetM* or *tetO* were predicted to be resistant to tetracycline. Regarding erythromycin resistance, our results are similar to those reported by Mingoia, *et al.* [24], in which *ermB* and *ermA* were associated with high-level and variable-level erythromycin resistance, respectively. Multidrug resistance to erythromycin, clindamycin, and tetracycline, coupled with the recovery of non-susceptible isolates resistant to antimicrobial agents such as cefazolin, penicillin G, and ampicillins indicates the importance of GBS surveillance and antimicrobial susceptibility testing [23].

Mutations are localized predominantly in the *cyl* operon, encoding the β -hemolytic pigment biosynthetic pathway, and in *abxI*, encoding a CovSR regulatory partner [12]. In the present study, three non-hemolytic strains (WJ16, WJ29, and WJ46) had 1-bp frameshift insertions at nucleotide 903 of *cylE*. However, *abxI* mutations were not found.

This study had some limitations. First, our results are not representative of all pregnant Korean women, as we used single-center data. Second, we collected the isolates from pregnant

women, not from neonates, and the strains involved in GBS infection in these two groups are not necessarily the same.

In conclusion, CC1 and CC19 GBS are prevalent in pregnant Korean women. The low prevalence of CC17 GBS, which mainly causes neonatal invasive infection, explains the low frequency of neonatal GBS infections in Korea. WGS data can predict the serotypes of GBS isolates based on *cps* genotypes. Detection of *tetM* and *tetO* and *ermB* is predictive of resistance to tetracycline and erythromycin, respectively. Therefore, WGS is a useful tool for simultaneous genotyping and antimicrobial resistance determination.

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AUTHOR CONTRIBUTIONS

Lee K and Uh Y designed the study; Uh Y, Bae HG, Won D, Yun W, Choi JK, and Lee H collected and identified clinical isolates and performed molecular studies; Lee Y analyzed the data; Lee Y, Lee K, and Uh Y wrote, edited, and reviewed the manuscript. All authors revised and accepted the final version of the manuscript.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article are reported.

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