Serotype Distribution and Resistance Genes Associated with Macrolide and Fluoroquinolone Resistance in *Streptococcus agalactiae* Isolates from a Hospital in Southern Taiwan

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Background: Antimicrobial resistance of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) has been emerging worldwide. We aimed to examine the correlation of drug-resistant genes with serotypes and with the mutations of the quinolone resistance-determining region (QRDR) in GBS isolates.

Methods: A total of 323 human GBS isolates were collected from a hospital in southern Taiwan. Laboratory investigation included serotyping by a multiplex polymerase chain reaction (PCR) method, antimicrobial susceptibility testing by a disc diffusion method, and mechanism analysis of the resistance to macrolides and fluoroquinolones by PCR and sequencing methods.

Results: Multiplex PCR showed that the most prevalent serotypes were Ib, III, V, and VI, mostly isolated from urine. The *ermB* gene was highly prevalent in serotypes Ib and V and was associated with clindamycin and macrolide resistance. GBS with a serine-to-leucine mutation at codon 81 in GyrA and with a serine-to-phenylalanine or -tyrosine mutation at codon 79 in ParC had a higher minimum inhibitory concentration of levofloxacin than isolates with only an aspartic acid-to-tyrosine mutation at codon 83 (>32 μg/ml vs. 16 μg/ml) in GyrA.

Conclusions: The most prevalent GBS serotypes were Ib, III, V, and VI. The *ermB* and *mefE* genes carried in serotypes Ib and V were highly associated with the resistance to macrolides and clindamycin. Mutations at codon 79 and codon 83 of ParC were the major determining factors for high-level fluoroquinolone resistance. (*Biomed J 2015;38:215-220*)

Key words: antimicrobial resistance, fluoroquinolone, macrolide, serotype, *Streptococcus agalactiae*

At a Glance Commentary

Scientific background of the subject

GBS is one of the most common pathogens causing meningitis, bacteremia, and pneumonia with high mortality in neonates, non-pregnant woman, and the elderly. Drug-resistant GBS have been emerging worldwide.

What this study adds to the field

The most prevalent GBS serotypes in southern Taiwan were Ib, III, V, and VI in this study. The *ermB* and *mefE* genes carried in serotypes Ib and V were highly associated with the resistance to clindamycin and macrolides. Mutations within the QRDR region of ParC were determined as genetic factors for high-level fluoroquinolone resistance.

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Streptococcus agalactiae (Group B *Streptococcus*, GBS) is one of the most common pathogens causing meningitis, bacteremia, and pneumonia, with high mortality rates in neonates,[1-3] non-pregnant women, the elderly, and immuno-compromised patients.[4,5] To treat GBS infection, penicillin, ampicillin, and cefazolin are the drugs of choice; for patients allergic to penicillin or cephalosporins, vancomycin, macrolides (such as erythromycin, azithromycin, and clarithromycin), and lincosamides (clindamycin) may be used as the alternative drugs.[6-9] Vancomycin resistance has not been reported in GBS. However, in the USA, 12% of pregnant women are allergic to penicillin, and 25% and 7% of GBS isolates from these women are resistant to erythromycin and clindamycin, respectively.[8] Resistance to these drugs is higher in isolates from Taiwan; resistance to erythromycin increased from 19 to 46% and to clindamycin from 18 to 37% in isolates between 1994 and 1997.[10]

The mechanism of macrolide resistance is primarily through acquisition of the resistance genes *ermA*, *ermB*, *ermC*, *ermF*, *ermQ*, or *ermT*, which encode 23S rRNA methylases,[11,12] or *mef*, which encodes an efflux pump that excludes 14-and 15-membered macrolides from the cell.[6,12,13] In addition, mutations in the quinolone resistance-determining region (QRDR) of GyrA or ParC result in the emergence of fluoroquinolone-resistant GBS.[14,15]

Multiplex polymerase chain reaction (PCR) can be used to differentiate GBS isolates into 10 serotypes based on genetic variations in the capsular polysaccharide (CPS) operon and 6 serotypes based on blood agglutination.[13,16-18] Recently, we reported highly erythromycin/clindamycin-resistant phenotypes in different serotypes, with constitutive macrolide–lincosamide–streptogramin B ($cMLS_B$) as the most prevalent resistance type, followed by inducible macrolide–lincosamide–streptogramin B ($iMLS_B$), macrolide (M), and lincosamide–streptogramin A (LSA).[19]

In this study, we investigated the prevalence of *ermB* and *mefE* and the association of these genes with different serotypes and antimicrobial resistance. Mutations in the QRDR region of GyrA and ParC in fluoroquinolone-resistant GBS isolates were also determined.

METHODS

Bacterial sources

Between 2007 and 2008, 322 *S. agalactiae* isolates were collected from blood, cerebrospinal fluid (CSF), pus, wounds, urine, vaginal discharge (VA), and other samples from patients treated in Chang Gung Memorial Hospital in Chiayi. After identification, the isolates were maintained on blood agar at 37°C and 5% CO_2 for use in future experiments.

Antimicrobial susceptibility test

The disk diffusion method was used to measure resistance to penicillin (PEN), ceftriaxone (CRO), azithromycin (AZM), erythromycin (ERY), clindamycin (CLI), levofloxacin (LEV), and moxifloxacin (MOX) according to the Clinical and Laboratory Standards Institute (CLSI) standards and guidelines for *Streptococcus pneumoniae* ATCC49619.[20] The disks (Bacto™) were purchased from Becton, Dickinson and Company (Sparks, MD, USA). The minimum inhibitory concentration (MIC) of LEV and ciprofloxacin (CIP) was determined for each fluoroquinolone-resistant GBS isolate using the Etest® (BioMérieux, Marcy-l'Etoile, France). To ensure accuracy, all isolates tested for PCR and antimicrobial susceptibility were confirmed at least twice for reproducibility.

PCR serotyping and identification of the *ermB* and *mefE* resistance genes

GBS isolates were identified by PCR amplification of the *dltS* gene and differentiated into 10 serotypes using multiplex PCR, as previously described.[16,17] The primers used to amplify the *erm* and *mefE* genes are listed in Table 1. A protocol for detection of *ermB* and *mefE* using multiplex PCR was developed in which 366-and 268-bp PCR products were simultaneously amplified and then separated on a 1.5% agarose gel. After staining with ethidium bromide (EtBr), images were recorded under UV illumination.

Sequencing of the QRDR region of *gyrA* and *parC*

The QRDR region of the *gyrA* and *parC* genes from five fluoroquinolone-resistant isolates and two fluoroquino-

Table 1: Primers used to amplify *ermB, mefE*, and the QRDR regions of the *gyrA* and *parC* genes

Gene	Primer	Sequence (5'→3')	T (ºC)	Product size (bp)
ermB	ermB-F	GCATTTAACGACGAAACTGGCT	54	366
	ermB-R	GAAAGCATTCCGCTGGCAGCT		
ermC	ermC-F	TGAAATCGGCTCAGGAAAAGG	54.4	80
	ermC-R	GTCTATTTCAATGGCAGTTACG		
ermF	ermF-F	GCATACCTTTGTTCCTCGGT	54.4	196
	ermF-R	GAGGTGAATACTTCTTGAGTGC		
ermQ	ermQT-F	ATTGGTCCAGGAAAAGGTCAT		
	ermQ-R	CTAGCCACATATCAGTTGGT	53	274
ermT	ermT-R	GATGCAGTTTATGCACCCCT	54.4	651
mefE	mefE-F	AAACAGGATCTGCGATGGTCT	54	268
	mefE-R	CGGAGTATAAGAGTGCTGCA		
parC	parC-F	CCTTGAATGATAGCGCCAGT	45	575
	parC-R	GTTGCCGGATATTCGTGATG		
gyrA	gyrA-F	CGCCATGAGTGTCATTGTTG'	52	452
	gyrA-R	CAATACCAGTTGCACCATTGAC		

Abbreviation: QRDR: Quinolone resistance-determining region

lone-susceptible isolates was amplified using the primers listed in Table 1. The PCR products were purified using the Wizard PCR Prep DNA Purification System (Promega, Madison, WI, USA). The purified PCR products were sequenced using an ABI3730 autosequencer. The sequences were analyzed using the SeqMan and Megalign programs in the Lasergene software (DNASTAR, Inc., Madison, WI, USA).

RESULTS

Clinical isolates and serotype analysis

Majority (91.9%) of the 322 GBS isolates were associated with non-invasive infection, with urine (73.9%) being the most frequent. A substantial amount (8.1%) of invasive infections was found, including 25 bacteremia cases. Overall, eight serotypes (Ia, Ib, and II-VII) were identified. The prevalence ranged from 1.9% for serotype Ia to 35.7% for serotype V [Table 2]. Serotype distribution did not differ significantly between invasive and non-invasive infections. However, serotypes Ia and VII were not found in our invasive isolates.

Antimicrobial susceptibility

All isolates were susceptible to the β-lactams PEN and CRO, and only five isolates were resistant to fluoroquinolone. The prevalence of resistance to AZM, ERY, and CLI was 56.2%, 53.1%, and 52.5%, respectively [Table 3].

Prevalence of the *ermB* and *mefE* genes

Although five *erm* genes (*ermB*, *ermC*, *ermF*, *ermQ*, and *ermT*) were examined in this study, only *ermB* was identified in detail. We developed a multiplex PCR protocol using primers for both *ermB* and *mefE* that could amplify simultaneously 366-bp and 268-bp PCR products from *ermB* and *mefE*, respectively. The prevalence of the GBS isolates carrying *ermB* and *mefE* was 68.0% and 1.9%, respectively, while 2.8% of the isolates possessed both *ermB* and *mefE*; however, 27.3% of the isolates lacked both *ermB* and *mefE* (*ermB*$^-$ *mefE*$^-$) [Table 3].

Regarding the association of resistance genes with antimicrobial resistance, we found that 75.8% of the *ermB*$^+$ isolates were resistant to each of the three drugs, indicating that the *ermB* gene may be the major factor associated with the resistance to the three drugs in GBS isolates. However, the *ermB* and *mefE* genes were not the only determinants responsible for the resistance to AZM, ERY, and CLI in GBS isolates. We observed that 24.2% of the *ermB*$^+$ isolates and 33.3% of the *ermB*$^+$ *mefE*$^+$ isolates were susceptible to all three drugs. In contrast, 85.2% of the *ermB*$^-$ *mefE*$^-$ isolates were susceptible to all three drugs, while 14.8% of the *ermB*$^-$ *mefE*$^-$ isolates were resistant to at least one of the three drugs [Table 3].

Table 2: Prevalence of each serotype of GBS isolates associated with sources of isolation

Serotype	Total, *n* (%)		Source, *n* (%)			
			Invasive		Non-invasive	
Ia	6	(1.9)	0	(0.0)	6	(100.0)
Ib	65	(20.2)	6	(9.2)	59	(90.8)
II	11	(3.4)	1	(9.1)	10	(90.9)
III	43	(13.4)	3	(7.0)	40	(93.0)
IV	32	(9.9)	2	(6.3)	30	(93.8)
V	115	(35.7)	10	(8.7)	105	(91.3)
VI	45	(14.0)	4	(8.9)	41	(91.1)
VII	5	(1.6)	0	(0.0)	5	(100.0)
Total	32 2	(100.0)	26	(8.1)	296	(91.9)

Abbreviations: Invasive: Blood, cerebrospinal fluid, urine; Non-invasive: Pus, wound, vaginal discharge; GBS: Group B *Streptococcus*

Table 3: Association of the azithromycin, erythromycin, and clindamycin resistance-associated genes *ermB* and *mefE* with resistance phenotypes in GBS isolates

Resistance gene	Azithromycin	Erythromycin	Clindamycin		Total (%)
ermB+	S	S	R	4	219 (68.0)
	S	R	S	3	
	R	S	R	4	
	R	R	S	7	
	R	R	R	148	
	S	S	S	53	
mefE+	R	S	S	1	6 (1.9)
	R	R	S	4	
	S	R	R	1	
ermB+*mefE*+	S	R	S	1	9 (2.8)
	R	R	S	2	
	R	R	R	3	
	S	S	S	3	
ermB mefE	R	S	S	2	88 (27.3)
	S	S	R	1	
	R	S	R	2	
	R	R	S	2	
	R	R	R	6	
	S	S	S	75	
Total *n* (%)	181 (56.2)	1715 (3.1)	169 (52.5)		322 (100)

Abbreviations: S: Susceptible; R: Resistant; GBS: Group B *Streptococcus*

Association of resistance genes with serotypes

The ratio of the *ermB*$^+$ number to the *ermB*$^-$ *mefE*$^-$ number was then used to reflect the susceptibility to the three drugs in GBS isolates. The ratio was 2.49 (219/88) in total. However, the ratio differed among serotypes; a ratio of 2 (4/2) was shown in serotype Ia, 20.3 (62/3) in serotype Ib, 1.74 (7/4) in serotype II, 1.5 (22/15) in serotype III, 0.87 (13/15) in serotype IV, 3.1 (84/27) in serotype V, 1.5 (26/18) in serotype VI, and a ratio of 0.25 (1/4) in serotype VII [Table 4]. The results showed that all serotypes (with the *ermB*$^+$/*ermB*$^-$ *mefE*$^-$ ratio of > 1), except

serotypes IV and VII, had higher rates of resistance to the three drugs.

Fluoroquinolone resistance-associated mutations in the QRDR regions of GyrA and ParC

Sequence analysis revealed that all LEV- and MOX-resistant isolates carried an identical serine-to-leucine mutation (S81L) at codon 81 of GyrA and one of two separate mutations in ParC: A serine-to-tyrosine mutation (S79Y) at codon 79 or an aspartic acid-to-phenylalanine (D83F) or aspartic acid-to-tyrosine mutation (D83Y) at codon 83 [Table 5]. The MICs of LEV were 16 μg/ml for the isolates with a mutation at codon 79 of ParC and >32 μg/ml for the isolates with a mutation at codon 83 of ParC. The mutations in LEV- and MOX-resistant isolates did not affect the MIC of CIP at the tested concentrations (greater than 32 μg/ml), but they did affect the MIC of LEV.

Table 4: Prevalence of *ermB* and *mefE* in different serotypes of GBS isolates

Serotype	Number	Prevalence, %	Resistance gene, *n* (%)			
			ermB$^+$	*mefE*$^+$	*ermB*$^+$ *mefE*$^+$	*ermB*$^-$ *mefE*$^-$
Ia	6	1.9	4 (66.7)	0 (0)	0 (0)	2 (33.3)
Ib	65	20.2	62 (95.3)	0 (0)	0 (0)	3 (4.7)
II	11	3.4	7 (63.6)	0 (0)	0 (0)	4 (36.4)
III	43	13.4	22 (51.2)	3 (7.0)	3 (7.0)	15 (34.9)
IV	9.9	32	13 (40.6)	9.4 (3)	1 (3.1)	15 (46.9)
V	115	35.7	84 (73.0)	0 (0)	4 (3.5)	27 (23.5)
VI	45	14.0	26 (57.8)	0 (0)	1 (2.2)	18 (40.0)
VII	5	1.6	1 (20.0)	0 (0)	0 (0)	4 (80.0)
Total	322	100	219 (68.0)	6 (1.9)	9 (2.8)	88 (27.3)

Abbreviation: GBS: Group B *Streptococcus*

Table 5: Minimum inhibitory concentrations of levofloxacin and ciprofloxacin and mutations in the QRDR of *GyrA* and *ParC* in fluoroquinolone-resistant GBS isolates

Isolates	Resistance to		MIC (μg/ml)		Mutation in QRDR region of		
	LEV	MOX	LEV	CIP	GyrA	ParC	
G25*	S	S	0.5	0.5	S_{81}	S_{79}	D_{83}
G39*	S	S	0.5	0.5	S_{81}	S_{79}	D_{83}
G15	R	R	16	>32	S81L	S_{79}	D83Y
G22	R	R	16	>32	S81L	S_{79}	D83Y
G57	R	R	>32	>32	S81L	S79F	D_{83}
G233	R	R	16	>32	S81L	S_{79}	D83Y
G309	R	R	>32	>32	S81L	S79Y	D_{83}

*The G25 and G39 strains are fluoroquinolone-sensitive GBS isolates and used as a control. Abbreviations: CIP: Ciprofloxacin; LEV: Levofloxacin; MOX: Moxifloxacin; S: Susceptible; R: Resistant; D: Aspartic acid; F: Phenylalanine; L: Leucine; S: Serine; Y: Tyrosine, GBS: Group B *Streptococcus,* QRDR: Quinolone resistance-determining region

DISCUSSION

S. agalactiae is a zoonotic pathogen of fish, bovines, and humans.[19,21,22] Most GBS isolates were collected from female patients.[19] The predominant human serotype differs between countries, with serotype V being the primary serotype in the USA,[21,23] serotype III in France and Zimbabwe, and serotypes III and V being the primary serotypes in Korea.[16,24,25] In this study, although the total number of GBS isolates decreased from 2007 to 2008, the predominant serotypes varied. Serotype V, serotype Ib, and serotype III were predominant in 2007; however, serotype V, serotype VI, and serotype Ib were prevalent in 2008. Although the present study was performed in only one hospital, the data appeared to suggest that serotype V is gradually becoming the dominant serotype, as it is in the USA and Korea.

In a previous study, we determined that the predominant ERY/CLI resistance phenotype was $iMLS_B$ in serotype Ib and $cMLS_B$ in serotypes III and V.[19] The prevalent resistance genes vary between countries, with *ermB*, *ermTR*, and *mefA* being prevalent in Spain and the USA[26] and *ermB* and *lnuB* being prevalent in Korea.[25] The association of the *erm* and *mef* drug resistance genes with drug resistance phenotype and serotype was seldom reported. In Korea, *ermB* was frequently identified in serotypes III and IV.[27] Nevertheless, in this study, *ermB* was primarily identified in serotypes Ib and V, and *mefE* in serotypes III and IV [Table 4], implicating that these two drug resistance genes are frequently transferred between serotypes through lateral gene transfer.[28,29] Further investigation is required to determine whether serotype switching is involved in the observed differences in *ermB*-associated serotypes.[30]

The prevalence of CLI and ERY resistance in the tested isolates differed,[25,27] and *ermB* was present in 91.9% of ERY-resistant isolates and in 84.0% of CLI-resistant isolates in Korea.[25] However, in this study, *ermB* was present in those isolates with a higher population resistant to AZM (90.6%), ERY (95.9%), or CLI (94.1%). In addition, 14.8% *ermB*$^-$ *mefE*$^-$ isolates were still resistant to one of these drugs. These results indicate that not only *ermB* is the major gene responsible for resistance to these three drugs, but also other genes, such as *ermTR, mefA*, and *lnuB,* may be involved, as is the case in Korea.[25,26] We also determined that 57 isolates that possessed *ermB* or *mefE* were susceptible to all three drugs, indicating the possibility that mutations in these genes inhibit the function of the encoded proteins.

Since the discovery of fluoroquinolone-resistant GBS isolates carrying a serine-to-leucine mutation at codon 81 of GyrA and a serine-to-phenylalanine mutation at codon 79 of ParC in 2003,[14,15,31] an aspartic acid-to-tyrosine mutation at codon 83 of ParC was also identified in fluoroquinolone-resistant GBS isolates.[32] Isolates with double mutations at

codon 81 of GyrA and codon 79 and/or codon 83 of ParC had been reported to have the same MIC (>32 μg/ml) of LEV.[14,15,31,32] However, in this study, differences in the MICs of the tested drugs for fluoroquinolone-resistant GBS isolates with mutations in ParC were observed. With the identical mutation at codon 81 of GyrA, the fluoroquinolone-resistant isolates with the second mutation of a serine-to-phenylalanine or -tyrosine at codon 79 of ParC had an at least twofold higher MIC than those with the second mutation of a serine-to-tyrosine at codon 83 of ParC. Because these mutations are similar, with a change to tyrosine at codon 79 or codon 83, the variations in MIC for LEV may be due to positional conformation change.

Conclusions

The most prevalent GBS serotypes Ib, III, V, and VI were primarily isolated from urine samples. CLI and macrolide (AZM and ERY) resistance were highly associated with the presence of the *ermB* gene. The resistance genes *ermB* and *mefE* were associated with specific serotypes Ib and V. At least a twofold increase was observed in the MIC of LEV between isolates carrying mutations at codon 79 and codon 83 of ParC.

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