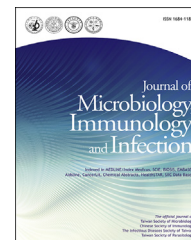




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ORIGINAL ARTICLE

Clonal dissemination of invasive and colonizing clonal complex 1 of serotype VI group B *Streptococcus* in central Taiwan



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Received 18 July 2014; received in revised form 14 October 2014; accepted 5 November 2014

Available online 11 November 2014

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KEYWORDS

group B
Streptococcus;
multilocus sequence
typing (MLST);
pulse-field gel
electrophoresis
(PFGE);
sequence type

Background/Purpose: The aim of this study was to investigate clinical presentation, serotype distribution and genetic correlation of group B streptococcus (GBS) diseases. Since serotype VI prevalence far exceeded that reported in prior studies, genetic relationship of isolates was further analyzed.

Methods: GBS isolates obtaining from patients with invasive diseases and pregnant women with colonization between June 2007 and December 2010 were analyzed. All isolates were tested for serotypes by multiplex PCR assay and pulsed-field gel electrophoresis (PFGE). Serotype VI isolates were further analyzed by multilocus sequence typing (MLST).

Results: A total of 134 GBS isolates were recovered from blood of 126 patients with invasive disease (94.0%) and anogenital swabs of 8 pregnant women (6.0%). Most common serotype was Ib (21.6%), followed by V (20.1%), VI (18.7%), III (15.7%), II (11.9%), Ia (11.2%), and IX (0.7%). Serotype VI was also the leading type in infants with early onset disease (EOD; 3/8, 37.5%) and colonizing pregnant women (3/8, 37.5%). PFGE distinguished 33 pulsotypes, reflecting genetic diversity among GBS isolates. Among 25 serotype VI isolates tested, 14 were ST-1, seven were ST-679, three were ST-678, one was ST-681, and distributed into four PFGE pulsotypes. ST-678, ST-679, and ST-681 were novel sequence types; ST-678 and ST-679 are single-locus variants of ST-1 that belongs to clonal complex (CC) 1.

Conclusion: CC1 dissemination of serotype VI GBS thus emerges as an important invasive pathogen in infants and nonpregnant adults in central Taiwan. Serotype prevalence of GBS must be continuously monitored geographically to guide prevention strategy of GBS vaccines.

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Introduction

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is a major cause of invasive infection in neonates, pregnant women, and nonpregnant adults older than 65 years or those immunocompromised by underlying medical conditions.^{1–3} GBS, an encapsulated gram-positive bacterium, colonizes human gastrointestinal, and genital tracts. Neonates acquire colonization and infection with GBS vertically, either *in utero* by the ascending route or at delivery. Since initiation of maternal intrapartum chemoprophylaxis (IAP) in 1996 in the United States, the incidence of early-onset disease (EOD) has declined by 65% compared with the preprevention era baseline rate in 1993.⁴ However, maternal IAP does not reduce the incidence of late-onset disease (LOD).^{1,4} In addition to neonates, the growing prevalence of invasive GBS disease in nonpregnant adults has been reported, with common clinical syndromes of bacteremia without focus, skin and/or soft-tissue infection, and pneumonia.^{1–3} Most (88.0%) adult GBS cases had underlying conditions, diabetes the most common (41.0–44.4%).^{1,2} Vaccinating pregnant women with glycoconjugate vaccine could potentially prevent invasive GBS diseases of newborns.^{5,6}

By structure of surface capsular polysaccharide (CPS), isolates were serotyped as Ia, Ib, II–VIII, and provisional IX.⁷ Serotypes Ia, Ib, II, III, and V accounted for 96% of neonatal cases^{1,8} and 88–93% of adult cases,^{1–3} targets of protective immunity, and candidates of GBS vaccines. By contrast to these, serotype VI has been rarely cited in Europe and America: 1% in infants younger than 90 days⁸ and 0.1% in nonpregnant adults,² yet predominant in Japan among GBS strains isolated from pregnant women with colonization (5.6–24.7%)^{9–11} and neonates with EOD

(8.5%).¹² In Taiwan, the past 20 years have seen serotype VI reported sporadically: 2.6% in patients with invasive disease during 1994–2004,¹³ and 4% between 2001 and 2004.¹⁴ Still, prevalence rose to 10.5% in nonpregnant patients between 2006 and 2008¹⁵ and 12% in cases of invasive disease from 1998 to 2009.¹⁶ To understand the epidemiology of GBS diseases in central Taiwan, we collected GBS isolates from invasive disease patients or pregnant women with colonization, analyzing serotype distribution and genetic correlation by pulse-field gel electrophoresis (PFGE). Since serotype VI prevalence far exceeded that reported in prior studies, genetic relationship of isolates was further analyzed by multilocus sequence typing (MLST).

Methods

GBS isolates

From June 2007 to December 2010, GBS isolates obtaining from blood of patients with invasive diseases along with anogenital swabs of colonizing pregnant women at China Medical University Hospital (CMUH) in central Taiwan, were analyzed. One isolate per patient was included, GBS strains grouped by clinical outcome and anatomical isolation sites: invasive strains from blood ($n = 126$) and colonizing strains from anogenital specimens ($n = 8$). Case details were gleaned from medical records. Primary bacteremia was defined as the presence of viable bacteria in blood without an obvious site of active infection. GBS disease in infants was categorized as early onset disease (EOD; from birth to 6 day old), and late onset disease (LOD; aged 7–89 days). This project was approved by the Institutional Review Board of our hospital (DMR100-IRB-237-1).

Identification and serotyping

Isolates cultured onto 5% sheep blood plate for 24 hours at 37°C in 5% CO₂ atmosphere were identified according to colony morphology, β-hemolysis, Gram staining, and Lancefield grouping with type B antisera. Serotyping was performed via multiplex PCR assay previously described by Imperi et al.¹⁷

Antimicrobial susceptibility testing

Isolates were rated for susceptibility to penicillin, clindamycin, and erythromycin, as per Clinical and Laboratory Standards Institute guidelines by microdilution MIC method.¹⁸ The double-disk diffusion test was applied to uncover inducible clindamycin resistance.

Pulsed-field gel electrophoresis

Genomic DNA of all isolates was prepared and subsequently digested by restriction endonuclease *Sma*I (New England BioLabs, Beverly, MA, USA) as previously described,¹⁹ with modification. After digestion, PFGE was performed on the CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA), with these parameters: switch time of 1–5 seconds for 8 hours, 3.5–45 seconds for 12 hours; 6 V/cm; 14°C; 120° angle. PFGE patterns were then examined with Bio-numerics software (Applied Maths, Kortrijk, Belgium) and a dendrogram was generated using the unweighted pair group

method with arithmetic mean (UPGMA) algorithm. The Dice similarity coefficient was employed, with optimization position tolerance settings of 1.0% and 1.5%, respectively. Isolates with similarities of ≥80% clustered as highly genetically related and grouped as one pulsotype (PT).

Multilocus sequence typing

MLST evaluated 25 serotype VI isolates, sequencing seven housekeeping genes, as originally described,²⁰ sequence type (ST) determined via *Streptococcus agalactiae* MLST database (<http://pubmlst.org/sagalactiae>). Sequence types not previously described were submitted and assigned at the *S. agalactiae* MLST database.

Statistical analysis

Data were analyzed using SAS version 9.3 (SAS Institute, Cary, NC). Trend over time was analyzed using logistic regression analysis. Chi-square test or Fisher's exact test were used for the comparison of proportions; 2-tailed $p < .05$ was considered to be statistically significant.

Results

A total of 134 GBS isolates were recovered from the blood of 126 (94.0%) patients with invasive disease and anogenital swabs of eight pregnant women (6%; Table 1). Among invasive cases, infants had higher prevalence of serotype III

Table 1 Serotype distribution of group B streptococcus disease in children, pregnant women and non-pregnant adults, by age, clinical manifestations, and resistance of erythromycin and clindamycin, 2007–2010

Clinical manifestation or site	Total (n = 134)	Isolate type							p-value ^c
		Ia (n = 15)	Ib (n = 29)	II (n = 16)	III (n = 21)	V (n = 27)	VI (n = 25)	IX (n = 1)	
Isolates									0.608
Colonizing isolates	8 (6.0)	0 (0.0)	1 (3.5)	0 (0.0)	2 (9.5)	2 (7.4)	3 (12.0)	0 (0.0)	
Invasive isolates	126 (94.0)	15 (100.0)	28 (96.6)	16 (100.0)	19 (90.5)	25 (92.6)	22 (88.0)	1 (100.0)	0.001
Invasive isolates according to age									
Infant	17 (13.5)	3 (20.0)	1 (3.6)	0 (0.0)	9 (47.4)	1 (4.0)	3 (13.6)	0 (0.0)	
EOD ^a	8	2	1	0	2	0	3	0	
LOD ^b	9	1	0	0	7	1	0	0	
Adolescent	1 (0.8)	0 (0.0)	1 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	
Non-pregnant adults	108 (85.7)	12 (80.0)	26 (92.9)	16 (100.0)	10 (52.6)	24 (96.0)	19 (86.4)	1 (100.0)	
Primary bacteremia	8	0	0	1	2	3	1	1	
Sepsis	73	8	20	12	5	16	12	0	
Sepsis & cellulitis	24	4	6	3	3	3	5	0	
Sepsis & septic arthritis/osteomyelitis	3	0	0	0	0	2	1	0	
Death in invasive isolates	12 (9.5)	0 (0.0)	2 (7.1)	2 (12.5)	0 (0.0)	4 (16.0)	4 (18.2)	0 (0.0)	0.265
Isolates not susceptible to									
Erythromycin	59 (44.0)	3 (20.0)	26 (89.7)	1 (6.3)	11 (52.4)	12 (44.4)	6 (24.0)	0 (0.0)	<.001
Clindamycin	55 (41.0)	2 (13.3)	25 (86.2)	0 (0.0)	10 (47.6)	12 (44.4)	6 (24.0)	0 (0.0)	<.001

Data were presented as n (%).

^a EOD, early-onset disease.

^b LOD, late-onset disease.

^c p-value from Chi-square test or Fisher's exact test.

isolate whereas non-pregnant adults had higher prevalence of serotypes Ib, II, V, and VI. Among patients with invasive diseases, the overall mortality was 9.5% (12/126), and mortality did not differ with regard to serotypes ($p = 0.265$). Clinical presentations of 17 infants emerged as 8 EOD and 9 LOD. One adolescent with acute lymphocytic leukemia had sepsis. Of 108 nonpregnant adults (57% female), the most common manifestation was sepsis ($n = 73$, 68%), followed by sepsis with cellulitis ($n = 24$, 22%), primary bacteremia ($n = 8$, 7%), and sepsis with arthritis/osteomyelitis ($n = 3$, 3%).

Overall, types Ib ($n = 29$, 21.6%) and V ($n = 27$, 19.1%) were predominant, followed by VI ($n = 25$, 18.7%), III ($n = 21$, 15.7%), II ($n = 16$, 11.9%), Ia ($n = 15$, 11.2%), and IX ($n = 1$, 0.7%) (Table 1). Type III was most common in infants and type VI (37.5%) in infants with EOD. Of 108 nonpregnant adults, Type Ib (24.1%) was the most frequent and Type VI (17.5%) was third. Type VI (37.5%) was also the most common in colonizing isolates. Every isolate proved susceptible to penicillin; still, 44% (59/134) and 41% (55/134) of strains were resistant to erythromycin and clindamycin, respectively. Inducible clindamycin resistance was not detected in these strains. Serotype Ib showed highest resistance to erythromycin (86%) ($p < .001$) and clindamycin (90%) ($p < .001$). (Table 1) Among 25 serotype VI isolates, resistance rates to erythromycin and clindamycin both tallied 24% (Tables 1 and 2). The proportion of invasive serotype VI increased from 12.5% in 2007 to 15.9% (unpublished data, CMUH) in 2012, but the increasing trend was not statistically significant (P for trend = 0.9236).

Isolates were assessed with PFGE, 33 pulsotypes distinguished by Dendrogram (Fig. 1). We defined a major pulsotype as comprising at least five strains: i.e., six major PFGE pulsotypes (3, 4, 18, 25, 26, 27) containing 96 (72%) of 134 isolates (Table 3, Fig. 1), with pulsotype 27 (25%, 34/134) the most common. Serotypes Ib and V were the most homogenous; Ia, II, III, and VI were heterogenous, each contained two or three pulsotypes (Table 3). Pulsotypes 3, 4, and 25 contained solely isolates of serotypes Ia, III, and VI, respectively. Pulsotypes 18, 26, and 27 showed more diversity: each comprised two or three serotypes (Table 3).

Table 2 depicts clinical data of 25 patients infected or colonized with serotype VI isolates, distributed into four pulsotypes. Pulsotype 26 was the most common, accounting for 64% ($n = 16$) of 25 isolates; six (24%) pulsotype 25, two (8%) pulsotype 28, and one (4%) pulsotype 29. The 25 serotype VI isolates studied by MLST contained four sequence types, including ST-1 ($n = 14$), and three novel STs, ST-678 (allelic profile 1,37,2,1,1,2,2) ($n = 3$), ST-679 (allelic profile 1,9,2,1,1,2,2) ($n = 7$), and ST-681 (allelic profile 35,2,2,1,1,2,2) ($n = 1$). ST-678, ST-679, and ST-681 had never been defined before this study. All but one serotype VI isolate (Patient 12, ST-681) displayed ST-1 or single-locus variant (SLV) of it (ST-678 and ST-679), belonging to clonal complex (CC) 1. Isolates from three neonates with EOD and three pregnant women with colonization were ST-1 ($n = 4$) or ST-679 ($n = 2$). Four nonpregnant adults with sepsis and underlying conditions died. No correlation surfaced between pulsotypes, STs, and isolation dates.

Discussion

In this study, serotype VI was predominant in EOD infants (3/8, 37.5%) and colonized pregnant women (3/8, 37.5%), ranking third (17.5%) among nonpregnant adults with invasive infection. Overall, 25 isolates (18.7%) belonged to serotype VI in 134 GBS isolates; 96% of them ST-1, ST-678, or ST-679 (the latter 2 SLVs of ST-1), based on MLST analysis and belonging to clonal complex (CC) 1, divided into four pulsotypes. Previous studies disclosed 9.4%²¹³ to 12%¹⁶ prevalence of serotype VI in southern and northern Taiwan. Therefore, prevalence of 18.7% in this study points to clonal dissemination of serotype VI GBS, CC1 as an important pathogen in central Taiwan.

Earlier (1994–2004) study in southern Taiwan identified only 2.6% invasive isolates as serotype VI.¹³ A 1998–2009 study in northern Taiwan disclosed its emergence in nonpregnant adults with invasive GBS disease, 12% prevalence.¹⁶ In addition, 10.5% of GBS isolates from nonpregnant patients were serotype VI in southern Taiwan (2006–2008).¹⁵ A recent study in southern Taiwan (2008–2010) unveiled 9.4% isolates obtaining from hospitalized non-pregnant adults as serotype VI.²¹ Based on upward trends of serotype VI GBS in Taiwan,^{13,15,16,21} Japan,^{10,11,22} Malaysia,²³ Thai–Myanmar border,²⁴ and our current study (18.7%), it is possible that type VI has emerged as a prominent pathogen in Taiwan since the late 1990s and in Asia since the mid 1980s.

Invasive GBS serotypes vary among geographic regions and age groups.^{2,8} Globally, in infants younger than 90 days, the main serotypes were III (49%) and Ia (23%).⁸ This study showed similar results, but type Ib (21.6%) was most common in nonpregnant adults, sharply differing from Skoff et al² where serotypes V (29.2%), Ia (24.3%), II (13.5%), and III (11.4%) were predominant. In our study, 17.5% of invasive and 37.5% of colonizing strains were serotype VI, far higher than previous studies: 1% in infants <90 days old⁸ and 0.1% in nonpregnant adults.² In addition, our study showed that serotype VI strains obtained from term infants with EOD and colonized pregnant women tallied 37.5%. Further evidence arose from our 2011–2012 surveillance: 14% (19/136) invasive and 13.6% (9/66) colonizing isolates from pregnant women were serotype VI (unpublished CMUH data). Serotype VI was first detected in 1977 at the Centers for Disease Control, Atlanta.²⁵ A majority of serotype VI distributed in Japan since the mid 1980s accounted for 5.6–26.6% in pregnant women with colonization^{10,11,22} and EOD (8.5%).¹² Pregnant women colonized with it were also common in Malaysia (17%),²³ Thai–Myanmar border (16.7%),²⁴ and Iran (12.5%).²⁶ Nonetheless, serotype VI has been reported sporadically in America,²⁷ Korea,²⁸ Greece,²⁹ The Netherlands,³⁰ Kuwait,³¹ and Australia.³²

Besides ST1, novel sequence types 678 and 679, SLV of ST1 never reported, belonging to CC1, were exhibited by serotype VI in this study. In addition, an earlier study (2001–2004) revealed two strains of serotype VI GBS with CC1 (ST1).¹⁴ Noninvasive serotype VI GBS strains with reduced penicillin susceptibility belonging to ST1 and ST-458 (a SLV of ST-1) were reported in Japan.^{33,34} Wong et al¹⁶ noted emergence of serotype VI GBS as an invasive

Table 2 Clinical data and general characteristics of 25 serotype VI GBS isolates from patients with invasive GBS disease or colonization during 2007–2010

Patient	Specimen ID	Date (mo/yr)	Age (yr)/Sex	Clinical syndrome	Underlying Diseases ^b	Outcome	Pulsotypes	MLST ^c	Clindamycin susceptibility ^a	Erythromycin susceptibility ^a
1	012	11/2007	31/F	Colonization		Survived	25	ST-1	R	R
2	034	11/2007	67/F	Sepsis	L	Survived	25	ST-1	R	R
3	067	05/2009	54/F	Sepsis & cellulitis	DM, M	Survived	25	ST-679	S	S
4	096	10/2009	57/M	Sepsis & osteomyelitis	DM, L	Survived	25	ST-1	S	S
5	135	07/2010	35/F	Sepsis & cellulitis	M	Survived	25	ST-679	S	S
6	137	07/2010	60/M	sepsis	M, L	Survived	25	ST-1	S	S
7	002	06/2007	71/M	Sepsis & cellulitis	DM, M, L	Survived	26	ST-678	S	S
8	014	11/2007	33/F	Colonization		Survived	26	ST-1	S	S
9	020	11/2007	26/F	Colonization		Survived	26	ST-679	S	S
10	040	04/2008	1d/F	EOD & sepsis		Survived	26	ST-679	S	S
11	060	03/2009	77/F	Sepsis	DM, M	Survived	26	ST-1	S	S
12	065	04/2009	69/F	Sepsis	DM	Survived	26	ST-1	S	S
13	079	07/2009	59/F	Sepsis		Survived	26	ST-681	S	S
14	089	09/2009	1d/M	EOD & sepsis		Survived	26	ST-1	S	S
15	102	12/2009	62/M	Sepsis	r	Survived	26	ST-1	S	S
16	107	01/2010	83/M	Sepsis	DM, L	Died	26	ST-679	R	R
17	112	03/2010	63/M	Sepsis	L, r	Died	26	ST-1	S	S
18	117	03/2010	54/M	Sepsis	M, L	Died	26	ST-1	R	R
19	119	03/2010	1d/M	EOD & sepsis		Survived	26	ST-1	R	R
20	124	04/2010	84/M	Primary bacteremia	DM	Survived	26	ST-679	S	S
21	134	07/2010	83/M	Sepsis	L	Survived	26	ST-678	S	S
22	136	07/2010	86/M	Sepsis	M	Survived	26	ST-679	S	S
23	055	12/2008	41/M	Sepsis	DM, L	Survived	28	ST-1	S	S
24	083	07/2009	59/M	Sepsis & cellulitis	M	Survived	28	ST-678	S	S
25	024	09/2007	54/M	Sepsis	M	Died	29	ST-1	R	R

^a S, susceptible, MIC <0.25 µg/mL; R, resistant, MIC ≥1µg/mL.

^b DM, diabetes mellitus; L, liver diseases; M, malignancy; r: renal diseases.

^c Multilocus sequence type of 7 housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, *tkt*). Allelic profiles of ST-1 (1, 1, 2, 1, 1, 2, 2); ST-678 (1, 37, 2, 1, 1, 2, 2); ST-679 (1, 9, 2, 1, 1, 2, 2); ST-681 (35, 2, 2, 1, 1, 2, 2).

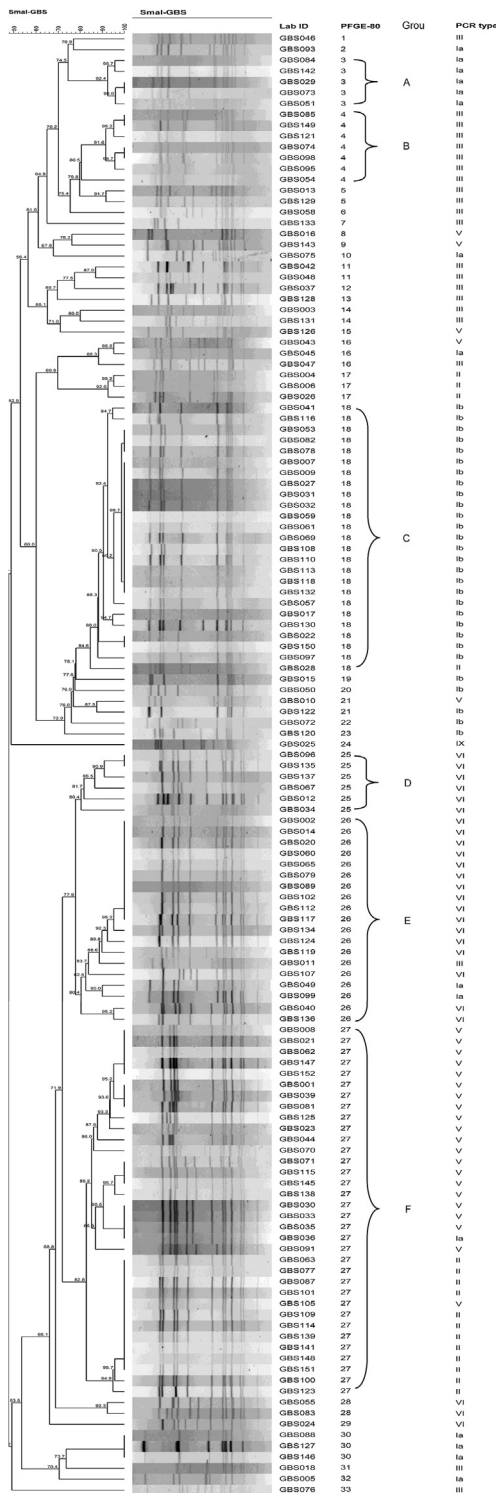


Figure 1. Phylogenetic analysis of the pulsed field gel electrophoresis (PFGE) profiles of 134 GBS strains obtained during 2007–2010. The dendrogram was concocted by the unweighted pair group method with arithmetic mean (UPGMA) algorithm. Pulsotypes, shown to the right of the dendrogram with PFGE-80, were termed as strains clustering on the dendrogram at an 80% similarity cutoff point. Additionally denoted are isolates designation and serotype by multiplex-PCR identified in each isolate.

Table 3 Major pulsotypes correlating with serotype among 96 of 134 GBS isolates during 2007-2010

PFGE Pulsotypes	No. of isolates in each serotype						
	Total	Ia	Ib	II	III	V	VI
3	5	5					
4	7				7		
18	25		24	1			
25	6						6
26	19	2			1		16
27	34	1		12		21	
Total	96	8	24	13	8	21	22

pathogen since 2006 in northern Taiwan, later in southern Taiwan by Wang et al. after 2008.²¹ Specific clone CC1 serotype VI might play a role in vertical transmission in cases with EOD from colonized pregnant women and invasive disease in immunocompromised nonpregnant adults in central Taiwan. Serotype VI should be considered for developing glycoconjugated GBS vaccine in Asia.

Our research found pulsotypes 3, 4, and 25 shared by isolates with specific serotypes, whereas pulsotypes 18, 21, 26, and 27 consisted of isolates belonging to two or three serotypes (Table 3, Fig. 1). Serotypes Ib and V were most homogenous, with unique pulsotypes; Ia, II, III, and VI heterogenous, including two or three pulsotypes and indicating greater genetic diversity. Similar heterogeneity between serotypes and PFGE pulsotypes has been reported in southern Brazil,³⁵ Italy,³⁶ and America.³⁷ Cieslewicz et al.³⁸ viewed GBS capsular polysaccharide structural diversity as driven by horizontal gene transfer, through the introduction of novel DNA sequence and genetic recombination. Martins et al.³⁷ later unearthed clear evidence for *in vivo* capsular switching in *Streptococcus agalactiae*. Classical and convenient typing by bacteria capsular polysaccharide composition fails to mirror genetic diversity of species.³⁹

The main limitation of this study is the small sample size, especially in infants and colonized pregnant women. Still, to our knowledge, this is the largest case series of invasive serotype VI GBS infection by strains belonging to genetic lineage CC1, regarding detailed clinical manifestations, PFGE, and MLST studies, particularly in infants, pregnant women, and nonpregnant adults. Immunizing pregnant women with capsular (CPS)-based conjugate vaccines (Ia, Ib, II, III, V), prevalent serotypes found in North America and Europe, could potentially prevent invasive GBS disease of newborns.³⁷ These provide no essential protection for individuals in areas where predominant capsular types such as serotype VI have arisen. The resistance to clindamycin and erythromycin in serotype VI GBS isolates from pregnant cases with colonization and newborns with EOD is also alarming. Neonatal GBS infection was still a common threat in Taiwan, with incidence of 1 per 1000 live births of infants during 2001–2005.⁴⁰ Considering geographical location, the emergence of type VI, CC1 GBS strains in central Taiwan mandates inclusion in the GBS vaccine in Asia for pregnant women. GBS serotype distribution should be continuously monitored to guide prevention strategies, such as effective vaccines.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The study was partly funded by grants from China Medical University Hospital (DMR-100-049) at Taichung, Taiwan, Chang Gung Memorial Hospital (CMRPG3B0642) at Taoyuan, Taiwan, and the National Science Council (NSC-101-2320-B-182A-002-MY3). We thank Hui-Chen Chen for performing PFGE, and Prof. Philippe Glaser (Institut Pasteur, Paris, France) for critical reading of the manuscript.

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