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ORIGINAL ARTICLE

Genetically diverse serotypes III and VI substitute major clonal disseminated serotypes Ib and V as prevalent serotypes of *Streptococcus agalactiae* from 2007 to 2012

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Abstract *Background: Streptococcus agalactiae* [group B *Streptococcus* (GBS)] has become more prevalent in nonpregnant women, the elderly, and people who are immunocompromised. We investigated the serotype and genomic changes of GBS human isolates from different hospitals from 2007 to 2012.
Methods: The serotype and genotype of 658 GBS human isolates were determined with multiplex polymerase chain reaction and pulsed field gel electrophoresis analysis. Multilocus sequence typing analysis determined the sequence type (ST) of the major clones of serotypes Ib, V, and VI.
Results: Most of the isolates were collected from urine samples (60.5%) with a reduction in the rate from 74.6% in 2007 to 34.5% in 2012 and from infected patients older than 30 years (72.6%). The female/male ratio differed depending on the source: 3.52 in the urine group, 0.48 in the wound group, and 0.43 in pus. Serotypes Ib (16.5%), III (16.9%), V (27.2%), and VI (17.6%) were the most predominant among the nine serotypes identified and were separated into two prevalence patterns: a decrease in serotypes Ib and V and an increase in serotypes III and VI from 2007 to 2012. The prevalence change was associated with the urine group.

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Additionally, serotype VI become more prevalent in blood samples in four hospitals. The pulsed field gel electrophoresis analysis demonstrated three genetic patterns: limited pulsotypes and a major clonal dissemination for serotypes Ib and V, diverse pulsotypes for serotypes III, and diverse pulsotypes with a major clonal dissemination for serotype VI. Multilocus sequence typing analysis of the major clones identified ST12 for serotype Ib and ST1 for serotypes V and VI.

Conclusion: Rapid genomic variations with different evolutionary patterns have led to the establishment of serotypes III and VI as the predominant GBS serotypes.

Introduction

Gram-positive *Streptococcus agalactiae* (group B *Streptococcus*; GBS) is a normal human gastrointestinal and genitourinary flora. Therefore, GBS commonly infects the vagina, is more prevalent in pregnant women than nonpregnant women,[1] and causes early-onset or late-onset sepsis in newborns. Vaccination has been used to prevent GBS infection in neonates.[2–4] However, GBS infections have increased in immunocompromised and elderly persons and frequently occur in the summer months.[5,6]

The prevalence of the GBS infection varies depending on serotypes, countries, years, and patient age. In Taiwan, GBS infection was higher in early-onset cases than in late-onset cases in neonates with a rate of 0.11–1.30/per 1000 newborn children.[7,8] Serotype III was the major serotype that caused bacteremia and meningitis in patients younger than 18 years, whereas serotype II was the major serotype causing these diseases in nonpregnant adults.[9] In Japan, the most prevalent serotype was serotype VIII (35.8%), followed by serotype VI (24.7%), which are more frequently identified in pregnant women.[10] In Korea, the most prevalent serotypes were serotypes Ib and III in 1979–2000 and then serotypes V and III in 2006–2007.[11,12] In England and Wales, GBS caused the most common disease, meningitis, in neonates, and serotype III was the most prevalent serotype from 1991 to 2010.[5] Multilocus sequence typing (MLST) has been used for the phylogenetic analysis of strains in outbreaks or recent infections.[13,14]

Earlier we reported that serotype V was the most prevalent serovar with high resistance to erythromycin (91.0%) and clindamycin (82.1%) in 2007–2008.[6] In this study, we extended the previous study to characterize the GBS isolates collected from Chiayi Chang Gung Memorial Hospital (CGMH) during 2007–2012 and from four hospitals in 2012. The aim of this study was to investigate the genetic change of the four major serotypes.

Materials and methods

Bacterial isolates and identification

This study was approved by the research ethics committee of CGMH. Including the isolates from early reports,[6,15] a total of 562 GBS isolates were collected from Chiayi CGMH in 2007, 2008, 2011, and 2012. The other GBS isolates were from Linkou (34 isolates) and Kaoshiung (23 isolates) CGMH and Chi-Mei Hospital (CMH, 39 isolates) in 2012. The GBS genomic DNA was purified using a DNA extraction kit (Quality Systems, Taipei, Taiwan). Then, 50–100 ng genomic DNA was used as the template for polymerase chain reaction (PCR) identification of GBS and the serotypes. Serotypes were determined by various PCR products and size using 19 primers and conditions as previously described.[16] The PCR products were separated on a 1.5% agarose gel. After staining with ethidium bromide, the images were recorded and analyzed.

Genotyping by pulsed field gel electrophoresis

Briefly, 250 μL of an overnight bacterial culture was centrifuged and resuspended with 250 μL of ddH_2O. The solution was mixed with an equal amount of 1.6% agarose to make plugs. The plugs were treated with 1 mL of lysis solution (2mM Tris–HCl, 0.1M EDTA, and 1M NaCl, 1 mg/mL lysozyme) at 37°C for 12–14 hours and were then digested with 1 mg/mL of proteinase K at 50°C for 12–14 hours. The plugs were then washed with TE buffer (10mM Tris, 1mM EDTA, pH 8.0) at least 10 times at 37°C with shaking and were then digested with *Sma*I (New England Biolabs, Ipswich, MA, USA) at 25°C for 12–16 hours. *Xba*I-digested *Salmonella* Braenderup (H9812) was used as the size marker. The chromosomal DNA was separated using the following conditions: an initial switch time of 4 seconds, a final switch time of 70 seconds, a runtime of 14 hours, an angle of 120°, and 6.5 V at 14°C followed by an initial switch time of 4 seconds, a final switch time of 70 seconds, a runtime of 14 hours, and an angle of 120° at 4 V at 14°C in a Bio-Rad CHEF MAPPER. The gel was stained with ethidium bromide and was then recorded and analyzed.

MLST analysis

MLST analysis was performed by sequencing the genes for alcohol dehydrogenase (*adhP*), phenylalanyl tRNA synthetase (*pheS*), aminoacid transporter (*atr*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA)*, glucose kinase (*glcK*), and transketolase (*tkt)*.[14] The PCR products were purified with a DNA purification kit (ProTECH, Taipei, Taiwan) and were then sequenced. The sequence was confirmed with the National Center for Biological

Information BlastX program. MLST analysis was performed on representative isolates from serotypes Ib, V, and VI using the protocols and software provided at http://pubmlst.org/sagalactiae/.

Results

GBS infections associated with the sampling sources

Among the 562 GBS isolates, the predominant isolates were mainly collected from urine with a prevalence of 60.5% (340/562); the remaining isolates were collected from other sources, including bone, cerebrospinal fluid, dialysis fluid, and sputum (58, 10.4%), wounds (50, 8.9%), blood (45, 8%), and pus (30, 5.3%) (Table 1). However, the prevalence of urine isolates has changed annually with a decrease from 74.6% in 2007 to 34.5% in 2012 and an increase from 2% in 2007 to 25.1% in 2012 for the other sources with a fluctuation for blood and pus.

Serotype distribution associated with age

Multiplex PCR identified nine serotypes—Ia, Ib, and II—VIII—for 555 isolates; however, the serotypes could not be identified for seven isolates (Table 2). Four major serotypes accounted for 78.2% isolates with serotype V (27.2%) being the most prevalent followed by serotype VI (17.6%), serotype III (16.9%), and serotype Ib (16.5%). The remaining serotypes each accounted for less than 9% of the total isolates. By separating the patient ages into five groups, we found that most of the GBS infections occurred in patients in the over 30-year-old age range for all of the serotypes (Table 2). The most prevalent serotypes were serotypes V, VI, III, and I in all age groups; however, a decrease in serotypes Ib and V prevalence and an increase in serotypes III and VI prevalence was observed from 2007 to 2012 (Figure 1). Next, we investigated whether there was a sex preference among the source groups.

Serotypes associated with sex, major isolation sources, and years

The average female/male (F/M) ratio was 2.08, and the F/M ratio differed among the four major source groups with a higher rate of female infection in the urine group (3.52), a higher rate of male infection in the pus and wound groups, and a nearly even ratio in the blood source group (Table 3). The F/M ratio differed among serotypes with higher than average F/M ratios for serotypes Ib, II, V, VI, and VII, a near-average F/M ratio for serotype III, an equal F/M ratio for serotype Ia, and a lower F/M ratio for serotype IV.

The change in prevalence of the four major serotypes was associated with isolation sources (Table 4). For the major sampling group from 2007 to 2012, the serotype change in urine determined the trends of all of GBS isolates (Table 4). However, these four serotypes differed in prevalence among the four major isolation sources: serotype V was the most prevalent in the blood (28.9%) and urine (28.5%) samples, serotype V (36.7%) in the pus samples, and serotype VI (24.3%) in wound samples. Additionally, serotype III and serotype VI increased the prevalence in the wound and blood samples, respectively, in 2012.

Pulsotype change of the four major serotypes

The pulsed field gel electrophoresis analysis classified these four major serotypes into nine pulsotypes for serotype Ib, 27 pulsotypes for serotype III, 11 pulsotypes and one nontypable for serotype V, and 22 pulsotypes for serotype VI (Figures 2 and 3, Table 5). Serotypes Ib, V, and VI appeared to have a dominant clone dissemination of pulsotype I with a prevalence of 79.4%, 66.5% and 64.8% through 4 years, respectively, whereas serotype III showed genetic diversity without a dominant clone. Compared with the limited pulsotypes in serotypes Ib and V, new pulsotypes emerged annually in serotype VI: four pulsotypes in 2008, six pulsotypes in 2011, and five pulsotypes in 2012, which indicates that constant chromosomal variations occur in serotype VI to increase the genetic diversity and fitness of this serotype. MLST analysis of two isolates from the major pulsotype of serotypes Ib, V, and VI determined that the ST types were ST12 for serotype Ib and ST1 for serotypes V and VI.

Next, we investigated the genetic variations of GBS isolates from 2012 among the four hospitals although the GBS number was limited. The most prevalent serotypes differed among these four hospitals: serotypes III (21.8%) and VI (28.6%) for CGMH-Chiayi, serotypes V (17.6%) and VI (38.2%) for CGMH-Linkou, serotype Ib and VI for CGMH-Kaoshiung, and serotype IV (28.2%) and VI (25.6%) for (CMH, Supplementary Table S1). Major pulsotype I was the most prevalent for serotype Ib in CGMH-Chiayi (60%,6/10), Kaoshiung (71.4%,5/7), and CMH (100%, 3/3) hospitals and for serotype VI in CGMH-Chiayi (65.6%, 21/32), Kaoshiung (100%,7/7), Linkou (50%,6/12), and CMH (70%, 7/10).

Table 1 Source of *Streptococcus agalactiae* isolates from Chiayi Gung Memorial Hospital

Year	AB	B	CX	GTS	PUS	TS	U	WD	OTH	N	Total
2007		20 (10.4)	1 (0.5)		11 (5.7)		144 (74.6)	10 (5.2)	4 (2)	3 (1.6)	193 (100)
2008	2 (1.2)	9 (5.3)		1 (0.6)	8 (4.7)	1 (0.6)	121 (71.6)	16 (9.5)	10 (5.9)	1 (0.6)	169 (100)
2011		4 (4.9)	2 (2.5)	4 (4.9)	5 (6.2)	1 (1.2)	34 (42)	15 (18.5)	15 (18.5)	1 (1.2)	81 (100)
2012	1 (0.8)	12 (10.1)	3 (2.5)	16 (13.4)	6 (5)	1 (0.8)	41 (34.5)	9 (7.6)	30 (25.1)		119 (100)
Total	3 (0.5)	45 (8)	6 (1.1)	21 (3.7)	30 (5.3)	3 (0.5)	340 (60.5)	50 (8.9)	58 (10.4)	5 (0.9)	562 (100)

AB = abscess; B = blood; CX = endocervix discharge; GTS = genital tract swab and vaginal discharge; OTH = others, bone (1), cerebrospinal fluid (1), dialysate (1) and sputum (1); PUS = pus; TS = tissue; U = urine; WD = wound; N = no data.

Table 2 Association of ages with serotypes for *Streptococcus agalactiae* isolates from Chiayi Gung Memorial Hospital

Age	Serotype[N, %]										Total
	Ia	Ib	II	III	IV	V	VI	VII	VIII	ND[a]	
<1	3 (15)	3 (3.2)	1 (3.1)	4 (4.2)	4 (8.2)	3 (14.3)	3 (3.0)				21 (3.7)
1～30	5 (25)	13 (14.0)	9 (28.1)	11 (11.6)	7 (14.3)	13 (16.9)	17 (17.2)	1 (8.3)		1 (14.3)	77 (13.7)
31～50	5 (25)	30 (32.3)	5 (15.6)	27 (28.4)	12 (24.5)	41 (27.2)	23 (23.2)	4 (33.3)		4 (57.1)	151 (26.9)
51～70	3 (15)	29 (31.2)	8 (25.0)	30 (31.6)	15 (30.6)	47 (27)	35 (35.3)	5 (41.7)	1 (50)	1 (14.3)	174 (31.0)
>70	4 (20)	18 (19.4)	9 (28.1)	23 (24.2)	11 (22.4)	46 (33.8)	21 (21.2)	2 (16.7)	1 (50)	1 (14.3)	136 (24.2)
Total[b]	20 (3.6)	93 (16.5)	32 (5.7)	95 (16.9)	49 (8.7)	153 (27.2)	99 (17.6)	12 (2.1)	2 (0.4)	7 (1.2)	562 (100)

[a] Not determined.
[b] There were three strains belonged to serotype V without age data.

Discussion

The GBS serotypes differed genetically, and GBS infection is associated with patient age and sources. Therefore, serotypes evolve differently to become the prevalent serotypes among countries, such as serotype III in Portugal[17]; serotypes Ib, III, and VI in Norway[18]; serotypes Ia, Ib, and V from neonates in the United States[19]; serotypes Ia, II, III, and V in the United States and Europe[20,21]; serotypes Ib and III in adults and children in Japan[22]; serotypes III and V in pregnant women, neonates, and adults in Korea[11]; and serotypes Ia and VI in pregnant women[23] and in neonates and adults in Malaysia.[24] These results demonstrated that serotypes III, V, and VI may be the major serotypes worldwide.

In the present study, we investigated the genetic differences and the change in the prevalence of the

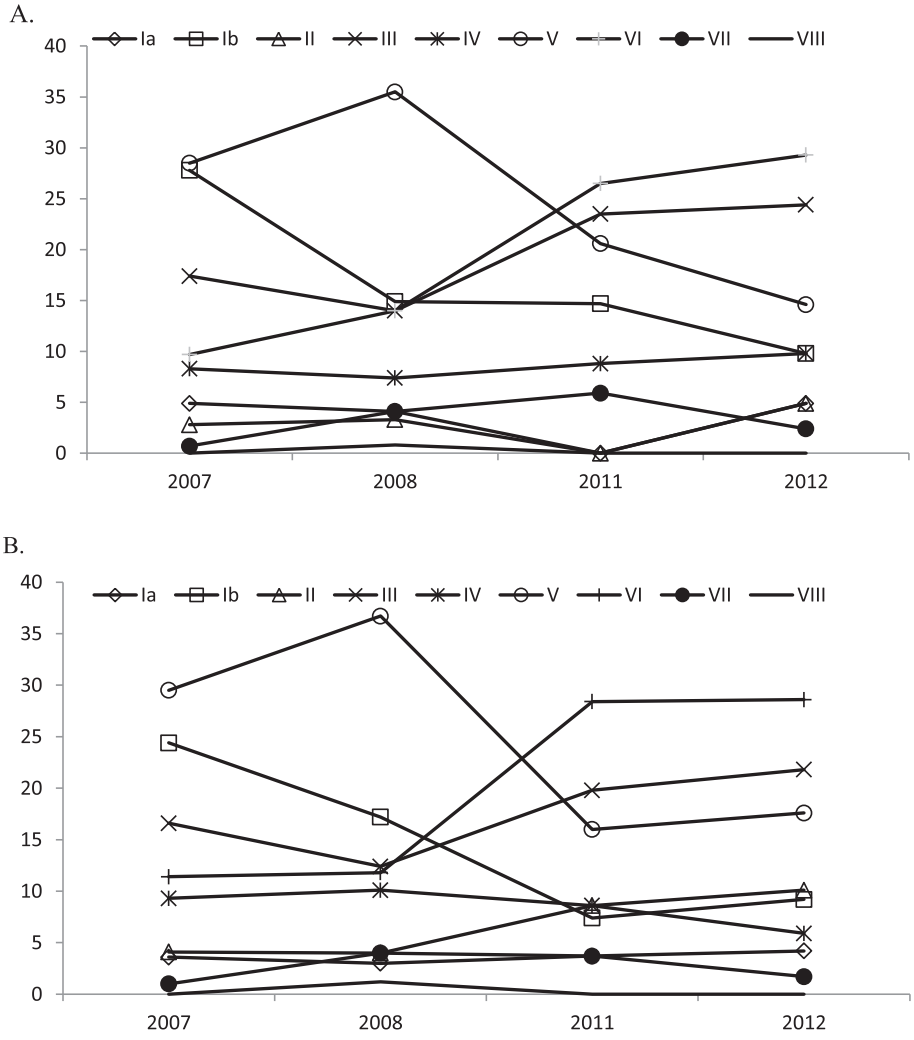

Figure 1. Pulsotypes of (A) serotype Ib, (B) V and VI isolates determined by pulsed field gel electrophoresis (PFGE) analysis.

Table 3 Association of sources with serotypes for *Streptococcus agalactiae* isolates from Chiayi Gung Memorial Hospital

Source	Sex	Serotype, *n* (%)										Total	F/M ratio
		Ia	Ib	II	III	IV	V	VI	VII	VIII	ND		
B	F		2 (8.3)	2 (8.3)	3 (12.5)		9 (37.5)	7 (29.2)			1 (4.2)	24 (100)	1.14
	M	1 (4.8)	5 (23.8)	2 (9.5)	3 (14.3)	1 (4.8)	5 (23.8)	4 (19)				21 (100)	
PUS	F		3 (33.3)	1 (11.1)	1 (11.1)		3 (33.3)	1 (11.1)				9 (100)	0.43
	M		2 (9.5)	2 (9.5)	3 (14.3)	3 (14.3)	8 (38.1)	2 (9.5)	1 (4.8)			21 (100)	
U	F	7 (2.6)	50 (18.9)	10 (3.8)	43 (16.2)	17 (6.4)	79 (29.8)	48 (18.1)	8 (3)	1 (0.4)	2 (0.8)	265 (100)	3.52
	M	6 (8)	17 (22.7)	1 (1.3)	17 (22.7)	11 (14.7)	18 (24)	4 (5.3)	1 (1.3)			75 (100)	
WD	F		2 (12.5)	2 (12.5)	4 (25)		4 (25)	2 (12.5)		1 (6.3)	1 (6.3)	16 (100)	0.48
	M		1 (2.9)	2 (5.9)	5 (14.7)	9 (26.5)	6 (17.6)	10 (29.4)	1 (2.9)			34 (100)	
Total	F	7 (2.2)	57 (18.2)	15 (4.8)	51 (16.2)	17 (5.4)	95 (30.3)	58 (18.5)	8 (2.5)	2 (0.6)	4 (1.3)	314 (100)	2.08
	M	7 (4.6)	25 (16.6)	7 (4.6)	28 (18.5)	24 (15.9)	37 (24.5)	20 (13.20)	3 (2.0)			151 (100)	
	F/M	1.00	2.28	2.14	1.82	0.71	2.58	2.9	2.67			2.08	

B = blood; ND = not determined; OTH = other sources; PUS = pus; U = urine; WD = wound.

Table 4 The prevalence of major serotypes in four isolation sources

Serotype	Sources	2007	2008	2011	2012	Total
Ib	Blood	25.0 (5/20)	11.1 (1/9)	0 (0/4)	8.3 (1/12)	15.6 (7/45)
	Pus	9.1 (1/11)	37.5 (3/8)	0 (0/5)	16.6 (1/6)	16.7 (5/30)
	Wound	0 (0/10)	18.8 (3/16)	0 (0/15)	0 (0/9)	6.0 (3/50)
	Urea	27.8 (40/144)	14.9 (18/121)	14.7 (5/34)	9.5 (4/42)	19.7 (67/340)
	Total	24.9 (46/185)	16.2 (25/154)	10.3 (6/58)	8.7 (6/69)	17.6 (82/465)
III	Blood	15.0 (3/20)	11.1 (1/9)	0 (0/4)	16.7 (2/12)	13.3 (6/45)
	Pus	18.2 (2/11)	0 (0/8)	20.0 (1/5)	16.7 (1/6)	13.3 (4/30)
	Wound	20.0 (2/10)	6.3 (1/16)	13.3 (2/15)	44.4 (4/9)	18.0 (9/50)
	Urine	17.4 (25/144)	14.0 (17/121)	23.5 (8/34)	47.6 (20/42)	20.6 (70/340)
	Total	17.3 (32/185)	12.3 (19/154)	19.0 (11/58)	39.1 (27/69)	19.1 (89/465)
V	Blood	25.0 (5/20)	55.6 (5/9)	25.0 (1/4)	16.7 (2/12)	28.9 (13/45)
	Pus	27.3 (3/11)	50.0 (4/8)	25.0 (2/5)	33.3 (2/6)	36.7 (11/30)
	Wound	40.0 (4/10)	31.3 (5/16)	0 (0/15)	11.1 (1/9)	20 (10/50)
	Urine	28.5 (41/144)	35.5 (43/121)	20.6 (7/34)	14.3 (6/42)	28.5 (97/340)
	Total	28.6 (53/185)	37.0 (57/154)	20.7 (12/58)	15.9 (11/69)	28.2 (131/465)
VI	Blood	20.0 (4/20)	11.1 (1/9)	25.0 (1/4)	41.7 (5/12)	24.4 (11/45)
	Pus	9.1 (1/11)	0 (0/8)	20.0 (1/5)	16.7 (1/6)	10.0 (3/30)
	Wound	10.0 (1/10)	6.3 (1/16)	43.8 (7/16)	33.3 (3/9)	24.0 (12/50)
	Urine	9.7 (14/144)	14.0 (17/121)	26.5 (9/34)	28.6 (12/42)	15.3 (52/340)
	Total	10.8 (20/185)	12.3 (19/154)	31.0 (18/58)	30.4 (21/69)	16.8 (78/465)

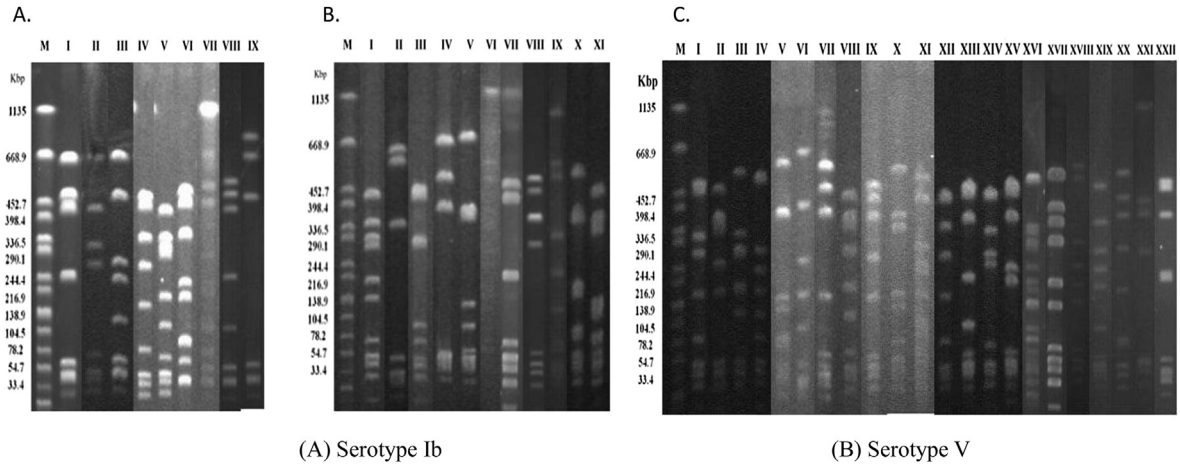

Figure 2. Pulsotypes of (A) serotype Ib, (B) V and (C) VI isolates determined by pulsed field gel electrophoresis (PFGE) analysis.

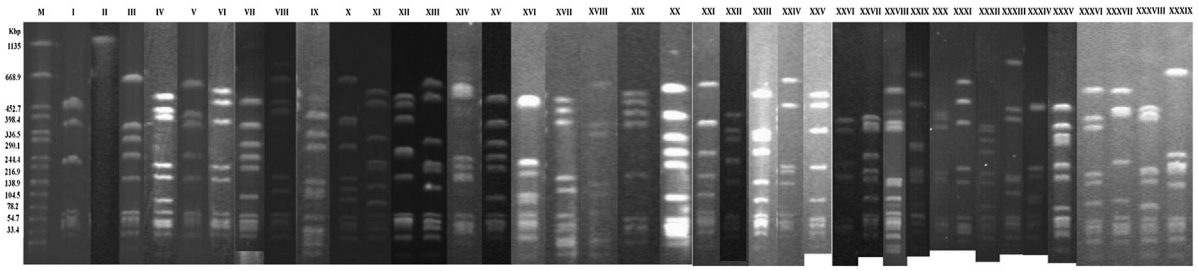

Figure 3. Pulsotypes of serotype III isolates determined by pulsed field gel electrophoresis (PFGE) analysis.

predominant serotypes Ib, III, V, and VI. However, there are three genetic patterns of the most prevalent serotypes: a major clonal dissemination with limited pulsotypes for serotype Ib and V, a major clonal dissemination with a large number of a single pulsotype for serotype VI, and no major clone with the most genetically diverse pulsotypes (Table 5). This genetic difference leads to changes in prevalence with a decrease in serotypes Ib and V and an increase in serotypes III and VI from 2007 to 2012, although this change is associated with the prevalence change in the urine group (Figure 2). Conversely, an increase in the genetic diversity may have caused serotypes III and VI to become the most prevalent serotypes, especially serotype VI in the other three hospitals in 2012.

Table 5 Number of PFGE patterns in serotype Ib, III, V, and VI isolates

Pulsotypes	Serotype, *n* (%)			
	Ib	III	V	VI
I	77 (79.4)	4 (3.9)	109 (66.5)	81 (64.8)
II	1 (1)	3 (2.9)	14 (8.5)	6 (4.8)
III	1 (1)	6 (5.8)	1 (0.6)	2 (1.6)
IV	2 (2.1)	3 (2.9)	9 (5.5)	3 (2.4)
V	2 (2.1)	4 (3.9)	6 (3.7)	1 (0.8)
VI	2 (2.1)	12 (11.7)	4 (2.4)	4 (3.2)
VII	10 (10.3)	2 (1.9)	5 (3)	3 (2.4)
VIII	1 (1)	1 (1)	1 (0.6)	2 (1.6)
IX	1 (1)	5 (4.9)	1 (0.6)	3 (2.4)
X		2 (1.9)	1 (0.6)	2 (1.6)
XI		1 (1)	1 (0.6)	1 (0.8)
XII		4 (3.9)		4 (3.2)
XIII		1 (1)		2 (1.6)
XIV		2 (1.9)		2 (1.6)
XV		6 (5.8)		1 (0.8)
XVI		2 (1.9)		1 (0.8)
XVII		6 (5.8)		1 (0.8)
XVIII		1 (1)		1 (0.8)
XIX		2 (1.9)		1 (0.8)
XX		1 (1)		1 (0.8)
XXI		7 (6.8)		1 (0.8)
XXII		1 (1)		2 (1.6)
XXIII		3 (2.9)		
XXIV		1 (1)		
XXV		4 (3.9)		
XXVI		5 (4.9)		
XXVII		2 (1.9)		
ND			9 (5.5)	
Total	97 (100)	103 (100)	164 (100)	125 (100)

ND = not determined.

It has been reported that there is clonal dissemination of clonal complex 1, mostly ST1, of serotype VI in the blood isolates in Taiwan,[25] and the major clones of serotype V and VI belong to ST1.[26] In this study, we observed that serotype VI increased in prevalence in blood samples, and serotypes V and VI were the two most prevalent serotypes (Tables 2 and 4). Although these two serotypes belong to ST1, serotype VI has substituted serotype V to become the most prevalent serotype in 2011 and 2012. Despite a major clonal dissemination of both of these serotypes, serotype VI evolved a large number of genotypes in the four sampled hospitals throughout the study years (Supplementary Table S2), whereas the prevalence of serotype V gradually decreased and was limited in the numbers of pulsotypes. The change in the prevalence by year is related to the rapid evolution of a new clone from a major clone in serotype VI, which has led to increased adaptation compared to the conserved serotypes in serotype V.

Vaccination has been used to reduce prevalent GBS infection in neonates. In this study, GBS infection occurs frequently in nonpregnant female and elderly patients and less frequently in young adults and neonates (Table 2). The reduced infection rate in neonates is not associated with vaccinations as vaccines have not been administered at the study hospitals. As a normal flora component in the human gastrointestinal and genitourinary tracts, more GBS infections were observed in females than in males, and the most dominant serotypes were Ib, III, V, and VI in urine samples. A female prevalence was not observed in other sources. In contrast, GBS infection was more frequently found in the pus and wound samples from males with serotype V as the most prevalent in pus and an almost equal predominance of serotypes IV and VI in wounds (Table 3). In conclusion, GBS serotype change was associated with genomic variations, and the sample size affected the prevalence and sex preference.

Conflicts of interest

All of the contributing authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jmii.2015.05.022.