

Original Article

## Antimicrobial resistance and molecular characterization of *Streptococcus agalactiae* from pregnant women in southern China

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### Abstract

**Introduction:** This study aimed to characterize antimicrobial resistance (AMR), molecular determinants of AMR and virulence, as well as clonal relationship of *Streptococcus agalactiae* isolates from women at 35-37 weeks of gestation in the Chaoshan metropolitan area of southern China. **Methodology:** Bacterial strains isolated from vaginal swabs were identified and antimicrobial susceptibility tests were performed by using a Vitek 2 Compact system (BioMérieux, France). Resistance and virulence genes were detected by polymerase chain reaction (PCR) and the clonal relationship was analysed by multiple locus variable number tandem repeat analysis (MLVA). Statistical analysis was carried out by using SPSS software, version 19.0.

**Results:** All GBS were susceptible to benzylpenicillin, ampicillin, quinupristin/dalfopristin, tigecycline, linezolid and vancomycin, but a considerable proportion was resistant to clindamycin (29.67%), erythromycin (46.15%), azithromycin (63.74%), tetracycline (84.62%) and quinolones (25.27%). The carrier rates of *ermB* (69.04%) and *mefA/E* (64.28%) were detected in these GBS strains resistant to erythromycin. In terms of MLVA detection, 91 GBS strains were categorized into 43 genotypes and 6 clusters. All GBS harboured *hylB* and *cylE* genes, most of which carried a combination of *PI-1* and *PI-2a* genes as a common virulence gene profile.

**Conclusions:** The high level of resistance conferred by some corresponding resistance genes to macrolides, lincosamides and quinolones of GBS isolates from pregnant women in southern China, has reinforced the necessity for monitoring GBS strain resistance to the above agents. Comparative genetic studies of GBS isolates, especially efforts to understand the relationship between pilus islands and genotype, were essential for conducting infection control and epidemiological comparisons between countries.

**Key words:** *Streptococcus agalactiae*; antimicrobial resistance; resistance gene; virulence gene; genotype; MLVA.

*J Infect Dev Ctries* 2019; 13(9):802-809. doi:10.3855/jidc.11395

(Received 25 February 2019 – Accepted 17 July 2019)

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### Introduction

*Streptococcus agalactiae* (group B streptococcus, GBS), a common commensal of the female genital tract, has been considered the main cause of neonatal sepsis and meningitis and as the most common agent of invasive infections in pregnant women [1]. According to the previous studies in different countries, approximately 10.00%-31.50% of pregnant women are vaginally and/or rectally colonized with GBS [2-6]. The universal screening for maternal GBS colonization at 35 to 37 weeks' gestation and the use of intrapartum antibiotic prophylaxis have resulted in a nearly 80% reduction in the rate of neonatal GBS infection over the past 15 years, from 1.7 cases per 1,000 live births in the early 1990s to 0.34–0.37 cases per 1,000 live births in recent years [7]. Prenatal GBS screening is recommended by the Center for Disease Control and

Prevention of United States in all the pregnant women between 35 and 37 weeks of gestation [7]. In China, prenatal GBS screening is also a reference project in pregnant women.

GBS has been continuously susceptible to penicillin and other  $\beta$ -lactams. However, resistance to antimicrobials used as alternative therapy, especially macrolides, lincosamides and quinolones has been documented in different countries [4,8]. Erythromycin and clindamycin are given in cases in which there is a high risk of anaphylaxis to penicillin [9]. However, increasing resistance of GBS to erythromycin and clindamycin has been reported worldwide [10]. In GBS isolates, macrolide resistance is mediated mainly by two classes of resistance genes: the *mef* genes and the *erm* genes [11]. The *erm(B)*, *erm(A)* and *mef(A)* genes

have been detected in 70.51%, 46.15% and 6.41% of 156 GBS strains in Spain, respectively [12].

Quinolones are generally not used to treat GBS infections in pregnant women and newborns in China, but they are widely used for clinical and agricultural applications [13]. The continued widespread use of quinolones may increase the resistance of various pathogens, including *S. agalactiae*, to these drugs [14]. However, specific mutations found in a region of the *parC* and *gyrA/B* genes, called the quinolone resistance-determining region (QRDR), shows result in decreased binding to and activity of quinolones and represents the main mechanism of resistance to quinolones in GBS [15].

Pilus islands, haemolysins, and hyaluronidases played important roles in GBS adaptation and host specificity, and disease progression [16,17]. The hyaluronidase encoded by the *hylB* gene promotes the spread of bacteria in host cells [16]. The product of the gene *cylE* is a pore-forming toxin, referred to as extracellular  $\beta$ -haemolysin/cytolysin ( $\beta$ -H/C), which is toxic to a broad range of eukaryotic cells and results in cell invasion and evasion of phagocytosis [16]. Three pilus islands (PI-1, PI-2a, and PI-2b) had been identified, which encoded different pilus structures that mediated interactions with host cell [18,19]. Few studies, however, have characterized the distribution and genetic diversity of each PI in GBS strains from pregnant women in southern China.

Maternal and neonatal populations were those most commonly infected by GBS, and previous reports had mostly focused on the epidemiology of these special populations in other counties; however, little was known about the molecular characterization of the GBS isolates colonized in pregnant women in southern China. Therefore, this study was conducted to investigate and characterize the antimicrobial resistance, resistance genes, virulence determinants and genotypes of 91 GBS isolates collected from pregnant women in Chaoshan metropolitan area of southern China in 2015.

## Methodology

### Bacterial isolates

This study was conducted at the obstetrical department of a tertiary-level teaching hospital affiliated to the Shantou University Medical College (SUMC) in Shantou city in Guangdong, a populous province in southern China. The hospital (a tertiary-level teaching hospital with 1816 inpatient beds) serves the Chaoshan metropolitan area area in eastern Guangdong. A total of 91 non-duplicated GBS isolates

were systematically collected from vaginal swabs of pregnant women at 35-37 weeks of gestation during the period of January 1<sup>st</sup> to December 31<sup>st</sup>, 2015. These strains had previously been identified on the basis of Gram staining, colony morphology,  $\beta$ -haemolysis, and a positive CAMP test [20] on blood agar and were further identified by using Vitek 2 Compact system (bioMérieux, Lyon, France). *Enterococcus casseliflavus* (ATCC 700327) was used as the quality control strain for the GP card. Strains were stored at -80°C in the brain heart infusion broth containing 20% glycerol and 5% sheep blood.

### Antimicrobial susceptibility tests

Antimicrobial susceptibility tests containing benzylpenicillin, ampicillin, clindamycin, erythromycin, levofloxacin, quinupristin/dalfopristin, tetracycline, tigecycline, linezolid, vancomycin, ciprofloxacin and moxifloxacin were obtained by using the Vitek 2 compact system with the AST-GP67 card assembly kits (BioMérieux, Lyon, France), according to the manufacturer's instructions. Antimicrobial susceptibility test (AST) results for MICs (minimum inhibitory concentrations) were interpreted according to the criteria recommended by the Clinical and Laboratory Standards Institute [21]. *Staphylococcus aureus* (ATCC 29213) was used as a quality control strain.

### DNA extraction

Whole genomic DNA was extracted by using TIANamp Bacterial DNA Kit according to the manufacturer's instructions (Tiangen Biotech, Beijing, China).

### Detection of antimicrobial resistance genes and virulence determinant genes

Detection of antimicrobial resistance genes by PCR amplification was carried out with specific primers to screen for the following genes of interest (shown in the supplementary table): macrolide resistance genes (*ermB*, *ermTR* and *mefA/E*), fluoroquinolone resistance genes (*gyrA*, *gyrB* and *parC*), and virulence determinant genes (*cylE*, *hylB*, CAMP, *PI-1*, *PI-2a* and *PI-2b*), as had been described in the literatures [16,22]. Gene sequences were analysed by the Huada Genomics Company (Shen Zhen, China). The nucleotide sequences obtained were aligned with DNAMAN software. *Streptococcus agalactiae* strain 2603 V/R (ATCC BAA-611; GenBank accession number NC004116) was used as a reference strain for comparative analysis [22].

**Table 1.** Correlation analysis for phenotypes and genotypes in macrolide antibiotics.

Resistance genes	Erythromycin			cMLS <sub>B</sub>			iMLS <sub>B</sub>		
	Resistance rate (%)	B	P	Resistance rate (%)	B	P	Resistance rate (%)	B	P
<i>ermB</i>	69.04	-14.116	0.000	90.00	-14.73	0.001	45.45	-13.366	0.054
<i>mef(A/E)</i>	64.28	-29.394	0.000	55.00	-14.206	0.022	77.72	-17.173	0.000
<i>erm(TR)</i>	4.76	-0.769	0.054	0.00	0.000	0.997	9.09	-14.975	0.026
<i>ermB+mef(A/E)</i>	35.71	26.542	0.027	45.00	13.226	0.112	22.72	15.381	0.004
<i>mef(A/E)+erm(TR)</i>	2.38	0.821	1.000	0.00	14.206	0.996	4.54	0.138	1.000

B: Partial regression coefficient by logistic correlation.

**Multiple-locus VNTR analysis (MLVA)**

The genetic relationship between the strains was analysed by MLVA genotyping of the following six variable number tandem repeat (VNTR) alleles: SAG2, SAG3, SAG4, SAG7, SAG21, SAG22 [23]. Amplification and electrophoresis were performed as described in the literatures [23-25]. Determination of the Hamming’s distance (also called the categorical coefficient) and the unweighted pair-group method with arithmetic mean (UPGMA) for cluster category were run with NTSYSpc2.10e software, which made out the minimum spanning tree (MST) [26].

**Statistical analysis**

Statistical analysis on the relationship between AST and genotypes was performed by the logistic correlation method, while Spearman correlation was used to determine the relevance for antimicrobials, pilus islands and amino acid substitutions carried out with SPSS 19.0 software. Values of  $P \leq 0.05$  was considered to be statistically significant.

**Results**

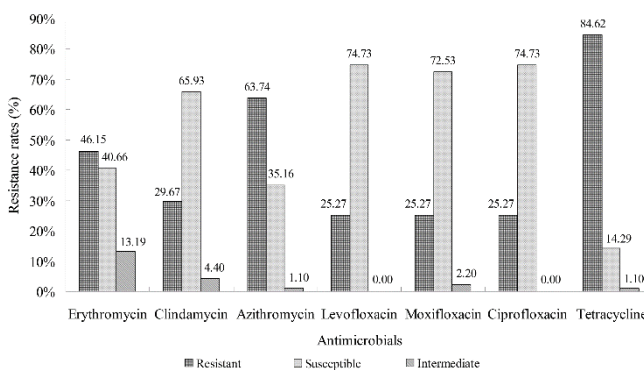
**Antimicrobial susceptibility**

For antimicrobial susceptibility profiles, all GBS strains were susceptible to benzylpenicillin, ampicillin, quinupristin/dalfopristin, tigecycline, linezolid and vancomycin. Resistance to clindamycin, erythromycin, azithromycin and tetracycline was detected in 27, 42, 58 and 77 strains, which accounted for 29.67%, 46.15%, 63.74% and 84.62%, respectively. In addition, resistance to levofloxacin, ciprofloxacin and moxifloxacin was the same as 25.27% (Figure 1).

**Resistance genes**

As shown in Table 1, among the 42 isolates resistant to erythromycin, 69.04% of them (29/42,  $B = -14.116$ ,  $P \leq 0.001$ ) carried the *ermB* gene while 64.28% (27/42,  $B = -29.394$ ,  $P \leq 0.001$ ) carried the *mef(A/E)* gene. A total of 35.71% of the strains (15/42,  $B = 26.542$ ,  $P \leq 0.05$ ) harboured the combination of *ermB* and *mef(A/E)* genes. For the 22 strains displaying the inducible macrolide-lincosamide-streptogramin B (iMLS<sub>B</sub>) phenotype, 16 strains (77.73%,  $B = -17.173$ ,  $P \leq 0.001$ ) were detected the *mef(A/E)* gene and 5 strains (22.73%,  $B = 15.381$ ,  $P \leq 0.005$ ) were found carriage for both *mef(A/E)* and *ermB* genes. However, ten strains (45.45%,  $B = -13.366$ ,  $P > 0.05$ ) only carried the *ermB* while two strains (9.09%,  $B = -14.975$ ,  $P \leq 0.05$ ) carried the *erm(TR)* gene.

**Figure 1.** Antimicrobial susceptibility profiles of 91 GBS isolates.

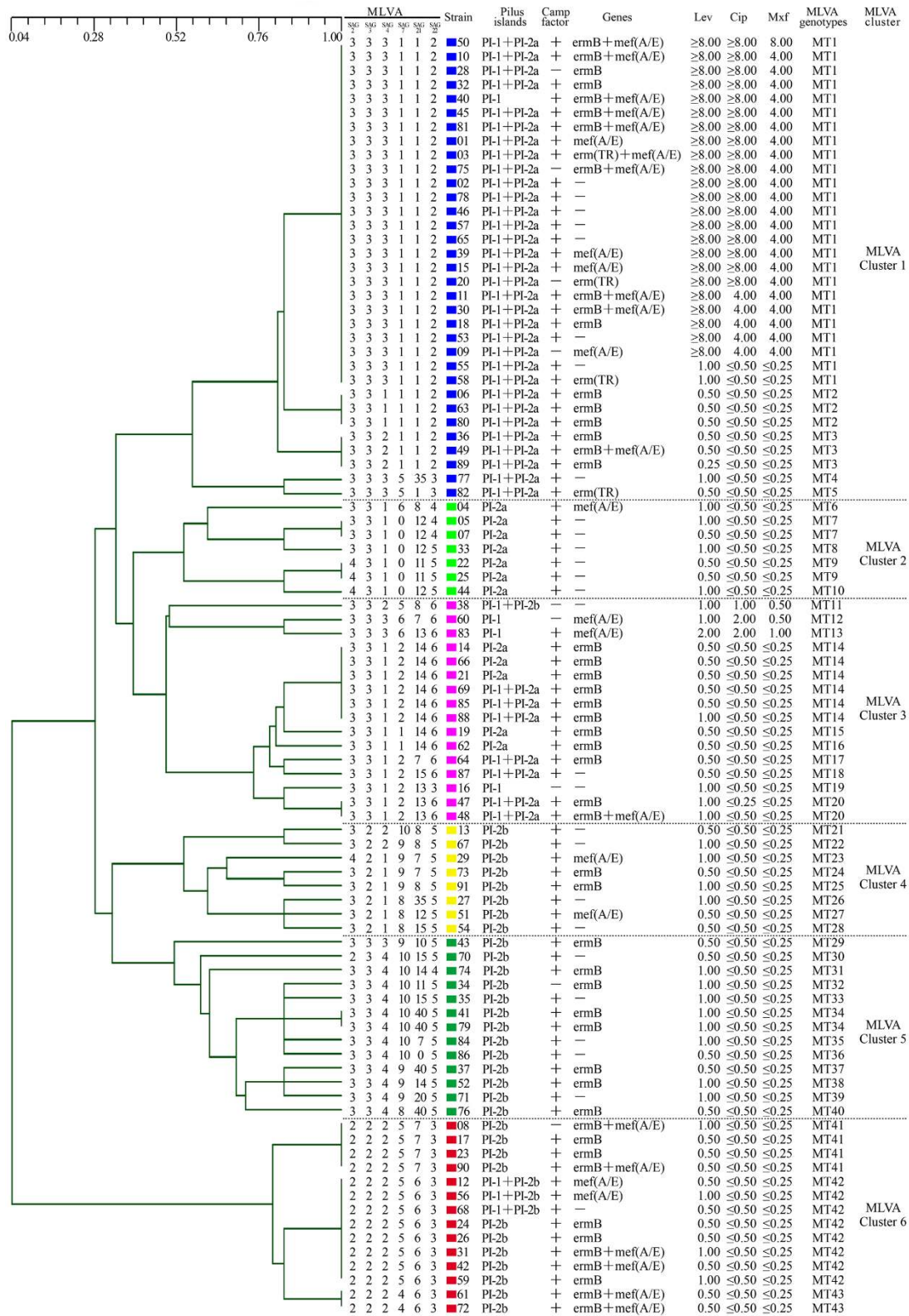


**Table 2.** Mutation types of amino acid substitutions based on phenotypic and genotypic characterizations of quinolone antimicrobials.

Mutation type	Proportion (%)	MIC (µg/mL)		Amino acid substitution			
		Lev	Cip	Mxf	GyrA	GyrB	ParC
I	4.35	≥ 8	≥ 8	8	S81L	—	S79Y
II	65.22	≥ 8	≥ 8	4	S81L	—	S79Y
III	8.70	≥ 8	≥ 8	4	—	—	S79Y
IV	17.39	≥ 8	4	4	S81L	—	S79Y
V	4.35	≥ 8	4	4	—	—	S79Y

Lev: Levofloxacin; Cip: Ciprofloxacin; Mxf: Moxifloxacin; S: serine; L: leucine; Y: tyrosine.

**Figure 2.** Clonal relationship analysis was performed by using the UPGMA algorithm of the NTSYSpc2.10e software.



91 GBS isolates were grouped into 6 clusters by the following characterizations: distribution of hemolysis pattern, pilus islands, CAMP factor, resistance genes, MICs of quinolone-resistance and MLVA types. Numbers of repeats were shown in each MLVA marker. The different clusters were marked with different colors: Cluster 1—blue; Cluster 2—light green; Cluster 3—pink; Cluster 4—yellow; Cluster 5—dark green; Cluster 6—red.

In terms of detection of the constitutive MLS<sub>B</sub> (cMLS<sub>B</sub>) phenotype, 90.00% (18/20, B = -14.73,  $P \leq 0.001$ ) and 55.00% (11/20, B = -14.206,  $P \leq 0.05$ ) harboured the *ermB* and *mef(A/E)* genes, respectively.

According to the mutation patterns of amino acid substitutions for quinolone antimicrobials, 23 GBS isolates which were insensitive to levofloxacin, ciprofloxacin and moxifloxacin, could be categorized into five patterns (Table 2). Pattern II was the predominant mutation form (65.22%, 15/23), in which strains showed resistance to levofloxacin and ciprofloxacin but intermediate to moxifloxacin, with amino acid substitutions such as S81L in *gyrA* and S79Y in *parC*, respectively. Meanwhile, these two amino acid substitutions were also detected in the strains belonging to pattern I, II and IV of 20 GBS isolates (86.96%, 20/23), while the rest ones (pattern III and V) conducted the only one substitution as S79Y. Synthetically, among the 23 levofloxacin-resistant strains, all had mutations in *gyrA* (86.95%, 20/23) and/or *parC* (100%, 23/23), without any mutation in *gyrB* gene. The majority of isolates with mutations displayed the same types of substitutions, such as S79Y ( $P \leq 0.001$ ) and S81L ( $P \leq 0.001$ ) (Table 3). However, three strains (No. 15, 18, and 40) showed resistance to levofloxacin, but no mutations were found in *gyrA* gene.

#### Detection of virulence determinants

PCR results showed that all strains carried at least one pilus island and both of *hylB* and *cylE* genes (Figure 2). Nine (9.9%) of the 91 GBS strains were CAMP factor-negative. Most of strains harboured *PI-2a* alone (13.19%, 12/91,  $P \leq 0.01$ ), or in combination with *PI-1* (42.86%, 39/91,  $P \leq 0.001$ ) (Table 3). The combination of *PI-1* and *PI-2a* was present in both cluster 1 and 3. The presence of *PI-2b* was uniquely observed in 32 strains (35.16%, 32/91,  $P \leq 0.01$ ) which belonged to cluster 4 to 6. Additionally, *PI-1* was found in combination with *PI-2b* (4.40%, 4/91,  $P > 0.05$ ).

#### GBS strain origins and genotypes detected by MLVA

The clonal relationship of 91 GBS isolates was assessed by MLVA (Figure 2). A total of 43 different MLVA types (MTs) were identified with a diversity index of 0.91. Six MLVA clusters were identified. The two major groups of similar MLVA profiles consisted of 25 (MT1, 27.5%) and 8 (MT42, 8.8%) isolates. In cluster 1, all isolates resistant to levofloxacin (25.27%, 23/91) belonged to the MT1 profile, and the majority of those isolates (95.56%, 22/23) harboured both *PI-1* and *PI-2a* genes.

#### Discussion

The increasing emergence of resistance to macrolides among GBS isolates is a therapeutic problem among patients who are allergic to  $\beta$ -lactams [13]. In our study, all strains were uniformly susceptible to penicillin, consistent with a previous report [13], indicating that penicillin remains an appropriate first-line agent option for treating GBS in genital infection for intrapartum. However, the resistance rates of erythromycin and clindamycin were 46.15% (42/91) and 29.67% (27/91), respectively. In comparison, the resistance rates of erythromycin and clindamycin in Chaoshan metropolitan area were lower than those in Beijing (85.7% and 73.2%, respectively) [14] and Shanghai (69.0% and 50.6%) [27], similar to France (46.0% and 37.8%) [28], but much higher than those in India (14.3% and 0.0%) [29] and Brazil (4.1% and 3.0%) [30]. Based on the observation in the high resistance to erythromycin in our study, we proposed that erythromycin should not be a priority antibiotic choice for intrapartum GBS chemoprophylaxis. Notably, The American CDC guidelines no longer recommend erythromycin as a routine antimicrobial for the treatment of GBS infection [9]. Generally, antimicrobial therapy should be guided by the antimicrobial susceptibility test and necessity for the continuous monitoring of antimicrobial susceptibility profiles should also be emphasized, as well.

**Table 3.** Correlation analysis for antimicrobials, pilus islands and amino acid substitutions.

Items	Erythromycin		Levofloxacin		Ciprofloxacin		Moxifloxacin	
	Rho	P	Rho	P	Rho	P	Rho	P
PI-1	0.204	0.052	0.460	0.000	0.574	0.000	0.607	0.000
PI-2a	-0.027	0.797	0.273	0.009	0.368	0.000	0.397	0.000
PI-2b	-0.003	0.977	-0.341	0.001	0.445	0.000	-0.472	0.000
PI-1+ PI-2a	0.181	0.086	0.436	0.000	0.527	0.000	0.566	0.000
PI-1+ PI-2b	-0.013	0.904	-0.082	0.437	-0.044	0.675	-0.048	0.652
S81L	0.263	0.012	0.731	0.000	0.861	0.000	0.873	0.000
S79Y	0.334	0.001	0.800	0.000	0.938	0.000	0.955	0.000
S81L+S79Y	0.263	0.012	0.731	0.000	0.861	0.000	0.873	0.000

Rho: the correlation coefficient by Spearman.

One of the main goals of this study was to determine the genetic basis of antimicrobial resistance. Among the 91 erythromycin-resistant strains, 42 carried one or two of three resistance genes (*ermB*, *mefA/E*, and *ermTR*). The high carrier rates of *ermB* and *mefA/E* implied that these two resistance genes might be the main mechanisms of GBS resistance to erythromycin. That the iMLS<sub>B</sub> isolates carried *ermB* and *mefA/E* at the same time showed consistency with the published data [16]. In our study, *ermTR* and iMLS<sub>B</sub> were found by statistical analysis to be correlated ( $P = 0.026$ ). To our knowledge, there are no relevant previous reports similar to this finding. The high level of resistance conferred by these resistance genes reinforces the necessity for monitoring GBS strain resistance to macrolides and lincosamides.

The incidence of levofloxacin and ciprofloxacin resistance in this study was both 25.27%, which was lower than that reported in China (37.7%), but higher than that in Japan (18.4%) and the United States (4.4%) [31]. One of the mechanisms of GBS resistance to quinolones is mutations in subunits A and B of the DNA topoisomerases (*gyrA*, *gyrB*, and *parC*) [13, 31]. In our study, the patterns of mutations detected in the QRDR of *GyrA* and *ParC* were S81L and S79Y, respectively, without mutations in *GyrB*. Such finding was similar to other studies [13], indicating that the quinolone-resistance mutations in GBS isolates were mainly due to substitutions in the *parC* and *gyrA* genes. Although, to the best of our knowledge, amino acid substitutions in *GyrB* have not been reported in GBS, the E476K substitution (corresponding to the E474K substitution in *S. pneumoniae*) may contribute to fluoroquinolone resistance [22].

In our study, at least one type of pilus island could be detected in each GBS isolate, and most GBS isolates carried the combination of *PI-1* and *PI-2a* genes, consistent with previous reports [32]. All GBS harboured *hylB* and *cylE* genes, most of which carried a combination of *PI-1* and *PI-2a* genes as a common virulence gene profile. Springman [18] reported that the presence of *PI-1* might increase the adaptability and colonization potential of certain strains in the human body. This pilus island allowed them to establish a niche in pregnant mothers and increase the likelihood of opportunistic infections and their subsequent spread to susceptible newborns. However, further studies and larger sample sizes are required to identify the continued colonization, invasion, and disease-related relationships of different pilus islands in GBS. Therefore, strengthening our understanding of *PI* distribution patterns and the genetic diversity of strains

from different sources and geographic locations is crucial for future efforts to develop GBS vaccines based on pilus islands [33].

In this study, we applied MLVA to characterize the genetic diversity of GBS isolates. This method converted our experimental results into digital alleles, which could be used to build a database for communication between laboratories. The diversity index obtained from our MLVA analysis for the bacterial population in this experiment was 0.91, which was higher than what had been reported in Brazil [16], but lower than that in France [23]. However, despite the close relatedness of several isolates, as judged by their capsular type and presence of pilus islands, this genotyping scheme could discriminate GBS isolates [18]. In fact, a total of 43 different genetic groups had been identified, which were further divided into 6 gene clusters. Because there was a clear correlation between gene cluster and *PI* profile, indicating that the *PI* genetic background in each gene cluster might be well conserved. All of the quinolone-resistant strains were confined to MT1 in cluster 1, and the vast majority (22 out of 23 strains) carried *PI-1* and *PI-2a* (only 1 strain carried *PI-1* alone). However, the correlation between pilus islands and quinolone resistance had not been studied, and the reason why this combination was only distributed in MT1 had not been found yet. Further research on these topics is needed in the future.

## Conclusions

This study revealed high resistance to macrolides, lincosamides and quinolones of GBS isolates from pregnant women in southern China, compared to some previous studies in other countries. The high level of resistance was conferred by resistance genes to macrolides, lincosamides and quinolones, which reinforced the necessity for monitoring GBS strain resistance to those agents. The genotypic characteristics showed MT1 to be a predominant MT in GBS strains from Chaoshan, which contributed to a better understanding of the epidemiology of GBS isolates. Comparative genetic studies of GBS isolates, especially efforts to understand the relationship between pilus islands and genotype, were essential for conducting infection control and epidemiological comparisons between countries.

## Limitation

The low number of *S. agalactiae* strains obtained in this study might lead to a slight deviation in the analysis. The clinical impact of GBS infections among affected patients had not been discussed. Some data

such as treatment outcomes (e.g. mortality versus discharge; birth outcomes), and hospitalization duration had not been collected and analysed to provide a measure on this regard.

### Acknowledgements

This study was supported by grants from the Medical Scientific Research Foundation of Guangdong Province, China (A2019263), Guangdong Provincial High-level University Key Discipline Construction Fund for “Research and Control of Infectious Diseases”, China (No.2015048), the Science and Technology Projects of Shantou City, China (2017119).

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**Conflict of interests:** No conflict of interests is declared.



**Annex – Supplementary Items****Supplementary Table 1.** Primer sequence descriptions for resistance and virulence genes.

Genes	Sequence (5'→3')	Length	Product length	Accession number
<i>ermB</i>	F: ATTGGAACAGGTAAAGGGC	19	442	NG047798.1/
	R: GAACATCTGTGGTATGGCG	19		EF422365.1
<i>ermTR</i>	F: GAAGTTTAGCTTTCCTAA	18	395	KP898896.1/
	R: GCTTCAGCACCTGTCTTAATTGAT	24		CP007631.2
<i>mefA/E</i>	F: GCGATGGTCTTGTCTATGGCTTCA	24	225	DQ445273.1/
	R: AGCTGTTCCAATGCTACGGAT	21		DQ445269.1
<i>gyrA</i>	F: GCCATGAGTGTCATTGTTGC	20	599	CP010875.1/
	R: ATCACCAAGGCACCAGTAGG	20		CP012480.1
<i>gyrB</i>	F: TTTCGTA CTGCCTTGACACG	20	650	CP012503.1/
	R: TCAACATCGGCATCAGTCAT	20		CP016391.1
<i>parC</i>	F: CGTTTTGGGCGCTATTCTAA	20	607	CP016391.1/
	R: TAGCGCCAGTTGGAAAATCT	20		CP015976.1
<i>hylB</i>	F: TGTCTCCGAGGTGACACTTGA ACT	24	124	U15050.1/
	R: TTGTGTTGTGACGGGTTGTGGATG	24		Y15903.1
<i>cylE</i>	F: TCGGAACAAGTAAAGAGGGTTCGG	24	130	AF093787.2/
	R: GGGTTTCCACAGTTGCTTGAATGT	24		AF157015.2
<i>CAMP</i>	F: CCAGGATAGGCGCCAAGAAT	20	363	X72754,.1
	R: TTTTGAGCCATTTGCTGGGC	20		
<i>PI-1</i>	F: AACCACTAGCAGGCGTTGTCTTTG	24	147	EU929540.1/
	R: TGAGCCCGGAAATTCTGATATGCC	24		EU929469.1
<i>PI-2a</i>	F: GCCGTTAGATGTTGTCTTCGTA CT	24	117	EU929374.1/
	R: TTTACTGCGGTCCCAAGAGCTTC	23		EU929330.1
<i>PI-2b</i>	F: AAGTCTTGACCAAGGATACGACGC	24	150	EU929426.1/
	R: ATCGTGTTACTTGCCTGCGTA	22		EU929391.1