

Experimental Study on *Streptococcus agalactiae* Genotype and Erythromycin Resistance in Neonatal Sepsis

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ABSTRACT

This study aimed to evaluate *Streptococcus agalactiae* genotype and erythromycin resistance in neonatal sepsis. After obtaining the mothers' informed consent, trained nurses sampled 430 neonatal specimens of sepsis from the ear canal, oral cavity and umbilical cord immediately after childbirth and implemented a cross-sectional study. By Gram staining, morphology, hemolysis mode, catalase and CAMP tests, the isolate was identified as *S. agalactiae*. All 455 isolates were tested for antimicrobial susceptibility by the disc diffusion method. Multilocus sequence typing was used to serotype *S. agalactiae* involving sequencing of 7 housekeeping genes. The erythromycin resistance genes-erm (B), erm (A) and mef (A) were detected by PCR. Results showed that there were 286 cases (66.51%) of neonates delivered naturally, and 144 cases (33.49%) of neonates delivered by cesarean section. A total of 455 strains were tested, including 253 strains (55.60%) of Gram-positive bacteria with 100 strains (21.98%) of *S. agalactiae* and 52 strains (11.43%) of *Staphylococcus epidermidis*, 178 strains (39.12%) of Gram-negative bacteria with 45 strains of *Klebsiella pneumoniae* (9.89%), 36 strains of *Escherichia coli* (7.91%), 36 strains of *Pseudomonas aeruginosa* (7.91%), and 323 strains of *Citrobacter freundii* (7.03%). *S. agalactiae* had the highest resistance of 87 (87.00%) to erythromycin, followed by resistance to azithromycin 83 (83.00%) and clindamycin 78 (78.00%). In children with neonatal sepsis, *S. agalactiae* serotypes were mainly Ia, Ib, and III, accounting for 29.00%, 35.00%, and 19.00% respectively. The main genotypes were ST651, ST103 and ST176, which account for 19.00%, 17.00% and 15.00% respectively. The ST19 type 13.00%, ST27 type 8.00%, ST17 Type 11.00%, ST10 type 12.00%, ST485 type 5.00%. The ST103 and ST485 isolates were classified as serotype Ia, the ST10 and ST176 isolates were classified as serotype Ib, and ST17 and ST19 isolates were classified as serotype III. Among the strains of *S. agalactiae*, 40.23% (35/87) carry erm (A) gene, 35.63% (31/87) carry erm (B) gene, and 24.14% (21/87) carry mef (A) gene. erm (A) gene was the most common gene in ST19 strain (7/11, 63.64%), and erm (B) gene was the most common gene in ST176 and ST651 strains (6/12, 50.00%; 8/18, 44.44%), while mef (A) gene was the most common gene in ST17 strain (5/11, 45.45%). In general, *S. agalactiae* genotypes in neonatal sepsis were mainly ST651, ST103 and ST176, and the main serotypes are Ia, Ib, and III. There was good consistency between ST and serotype, and a significant difference was shown in erythromycin resistance and ST distribution, which highlights the value of new epidemiological trend detection by monitoring multiple characteristics and provides inspiration for the development of multivalent *S. agalactiae* vaccines.

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Introduction

Streptococcus agalactiae as an important human pathogen that causes neonatal pneumonia, sepsis and meningitis may appear in the form of early or late-onset in neonates and infants (1). *S. agalactiae* is still the main cause of neonatal mortality and morbidity in many parts of the world (2). *S. agalactiae* occupies gastrointestinal and reproductive tracts of approximately 25% of healthy pregnant women, which will be passed to neonates during delivery (3). Colonization of *S. agalactiae* has a relation with

increased risk of nosocomial infections and community-related infections of the organism (4). The capsular polysaccharide of *S. agalactiae* has chemical and antigenic differences, which can be subdivided into ten serotypes, namely Ia, Ib, and II-IX (5). Vaccines against *S. agalactiae* infection must include more common serotypes associated with the disease in different populations (6). The epidemiological distribution of these serotypes may vary in several aspects, including geographic area, the profile of the study population, and the source of bacterial isolates

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(7, 8). In the United States and certain European countries, the four serotypes (Ia, II, III and V) are usually the most common isolates (9). Several studies conducted in Brazil have described the production of serotypes Ia, Ib, II, III, IV and V (10). Penicillin or amoxicillin is used for chemoprevention during childbirth and has been used in pregnant women with risk factors for vaginal *S. agalactiae* in order to reduce the incidence of early-onset diseases in neonates (11). Erythromycin is recommended for prenatal prophylaxis of *S. agalactiae* in women who are at high risk of penicillin allergy or treatment failure (12). The main purpose of this study is to evaluate the *S. agalactiae* genotype and erythromycin resistance in neonatal sepsis.

Materials and methods

Study population and sample collection

This cross-sectional study was conducted on August 15th, 2018. The project was approved by the research ethics committee of the hospital. After obtaining the mothers' informed consent, trained nurses sampled neonatal specimens of sepsis from the ear canal, oral cavity and umbilical cord immediately after childbirth. The culture was carried out using conventional methods, and the isolate was identified as *S. agalactiae*.

Identification of *S. agalactiae*

During transportation, the swab was inoculated into a selective medium (Trans-Vag broth containing 8µg/mL gentamicin and 15µg/mL nalidixic acid) at 4°C, and further inoculated for 24 hours at 37°C under 5% CO₂. Then subculture a circle of broth onto 5% sheep blood agar and incubate at 37°C, 5% CO₂ for 24 hours. Through a combination of Gram staining, morphology, hemolysis mode, catalase test and Christie Atkins Munch Peterson (CAMP) test, the isolate was identified as *S. agalactiae*.

Antimicrobial susceptibility test

According to the CLSI guidelines, all 455 isolates were tested for sensitivity to erythromycin, azithromycin, clindamycin, amoxicillin, cefazolin, penicillin, levofloxacin, tetracycline and teicoplanin by disc diffusion method. The resistance phenotype was determined by the erythromycin-fosfomycin double-disc test.

Serotype analysis

Multi-locus sequence typing was used for serotyping of *S. agalactiae* involving sequencing of 7 housekeeping genes. Sequence types (STs) and alleles were confirmed by database inquiry (<http://eburst.mlst.net>). By using the eBURST software program (Department of Epidemiology of Infectious Diseases, Imperial College London, UK; London, UK; <http://eburst.mlst.net>), cluster ST into clonal complexes (CC). Serotype all the isolates by HCl extraction method and Double immunodiffusion test or capillary precipitation test. The capsular polysaccharides (Ia, Ib and II-V) prepared indoors by standard methods were subjected to antiserum typing. The isolates that cannot be typed are referred to as NT.

Determination of erythromycin resistance gene

Screen all strains for erythromycin resistance genes. erm (B), erm (A) and mef (A) genes were detected by PCR amplification using the aforementioned primers and subjected to minor modifications, and properties of PCR products were checked by sequencing. In short, PCR has a final volume of 25 mL, which contains PCR buffer, 0.2 mM of each deoxynucleoside triphosphate, 0.5 mM of each primer, 2 IU of Taq polymerase and 25 ng of template DNA. The cycling conditions are as follows: initial denaturation at 94°C for 2 minutes, then cycling at 94°C for 1 minute, 55°C for 1 minute and 72°C for 1 minute for 35 cycles (finally extended at 72°C for 10 minutes). The products were separated by electrophoresis in 1.6% agarose gel in TBE (8.9 mM Tris, 8.9 mM boric acid, 0.25 mM EDTA; pH 8.0) buffer. *S. pyogenes* CIP107003, *S. pyogenes* CIP107002 and *S. agalactiae* 03-226 were used as positive controls for erm (B), erm (A) and mef (A) genes, respectively.

Data analysis

All data were entered into EpiData 3.1 database in duplicate. The minimum spanning tree illustrates the relationship between STs and the serotype of *S. agalactiae* isolates. Categorical variables were compared by Fisher's exact test. These analyses were performed using STATA version 13.0. $P < 0.05$ indicates a statistically significant difference.

Results and discussion

General information statistics

This study involved 430 children with neonatal sepsis. The male to female ratio was 228:202, the average gestational age was 38.26 ± 1.57 , and the mother's gestational age was 27.16 ± 3.19 . There were 286 cases (66.51%) of neonates delivered naturally, and 144 cases (33.49%) of neonates delivered by cesarean section. All the children had symptoms within 3 weeks of birth. There were 126 cases of neonatal hyperbilirubinemia, 157 cases of fever, 134 cases of shortness of breath, and 13 cases of congenital heart disease. (Table 1)

Table 1. General information statistics of patients with neonatal sepsis

Item	n=430	
Gender (male: female)	228:202	
Gestational age (week)	38.26 ± 1.57	
Mother's gestational age (year)	27.16 ± 3.19	
Birth weight (g)	3005.17 ± 316.28	
Birth way	Normal delivery	286(66.51%)
	Cesarean section	144(33.49%)
	premature delivery factor	135(31.40%)
Maternal risk factors	Fever during pregnancy	76(17.67%)
	Premature rupture of membranes	58(13.49%)

Analysis of pathogenic bacteria in neonatal sepsis

430 cases of neonatal sepsis patients were analyzed for strain infection. A total of 455 strains were tested, including 253 strains (55.60%) of Gram-positive bacteria with 100 strains (21.98%) of *S. agalactiae* and 52 strains (11.43%) of *Staphylococcus epidermidis*, 178 strains (39.12%) of Gram-negative bacteria with 45 strains of *Klebsiella pneumoniae* (9.89%), 36 strains of *Escherichia coli* (7.91%), 36 strains of *Pseudomonas aeruginosa* (7.91%), and 323 strains of *Citrobacter freundii* (7.03%). (Table 2)

Analysis of strain drug resistance to antibiotics

A total of 455 strains were detected in children with neonatal sepsis. The strains were cultured and tested for resistance. Where, *S. agalactiae* has the highest resistance of 87 (87.00%) to erythromycin, followed by resistance to azithromycin 83 (83.00%) and clindamycin 78 (78.00%). Other strains have the highest drug resistance of 167 (47.04%) to tetracycline, followed by resistance to clindamycin 128 (36.06%). (Table 3)

Table 2. Analysis of pathogenic bacteria in neonatal sepsis

Pathogenic bacteria	Strain	Proportion
Gram-positive bacteria (n=253)	<i>S. agalactiae</i>	100 21.98%
	<i>Staphylococcus Epidermidis</i>	52 11.43%
	Coagulase-negative <i>Staphylococcus</i>	30 6.59%
	<i>Staphylococcus aureus</i>	24 5.27%
	<i>Listeria monocytogenes</i>	20 4.40%
	<i>Enterococcus faecalis</i>	15 3.30%
Gram-negative bacteria (n=178)	<i>S. hemolyticus</i>	12 2.64%
	<i>Klebsiella pneumoniae</i>	45 9.89%
	<i>Escherichia coli</i>	36 7.91%
	<i>Pseudomonas aeruginosa</i>	36 7.91%
	<i>Citrobacter freundii</i>	32 7.03%
	<i>Enterobacter cloacae</i>	24 5.27%
Fungi (n=24)	<i>Enterobacter aerogenes</i>	5 1.10%
	<i>Candida albicans</i>	24 5.27%

Table 3. Analysis of strain drug resistance to antibiotics

Antibiotics	<i>S. agalactiae</i> (n=100)	Other strains (n=355)
Erythromycin	87(87.00%)	54(15.21%)
Azithromycin	83(83.00%)	108(30.42%)
Clindamycin	78(78.00%)	128(36.06%)
Amoxicillin	45(45.00%)	69(19.44%)
Cefazolin	7(7.00%)	79(22.25%)
Penicillin	3(3.00%)	74(20.85%)
Levofloxacin	53(53.00%)	49(13.80%)
Tetracycline	22(22.00%)	167(47.04%)
Teicoplanin	19(19.00%)	74(20.85%)

Serotype distribution of *S. agalactiae* in sepsis

In children with neonatal sepsis, *S. agalactiae* serotypes were mainly Ia, Ib, and III, accounting for 29.00%, 35.00%, and 19.00% respectively. Type II, IV, V and non-classifiable types account for 8%, 5%, 2.00%, and 2.00% respectively. (Table 4)

Table 4. Serotypes of *S. agalactiae* in neonatal sepsis

Serotype	Number of cases	Proportion
Ia	29	29.00%
Ib	35	35.00%
II	8	8.00%
III	19	19.00%
IV	5	5.00%
V	2	2.00%
NT	2	2.00%

Genotype distribution in neonates with sepsis

Among the children with neonatal sepsis, the main genotypes were ST651, ST103 and ST176, which account for 19.00%, 17.00% and 15.00% respectively. In terms of proportion of other genotypes: ST19 type 13.00%, ST27 type 8.00%, ST17 Type 11.00%, ST10 type 12.00%, ST485 type 5.00%. (Figure 1, Table 5)

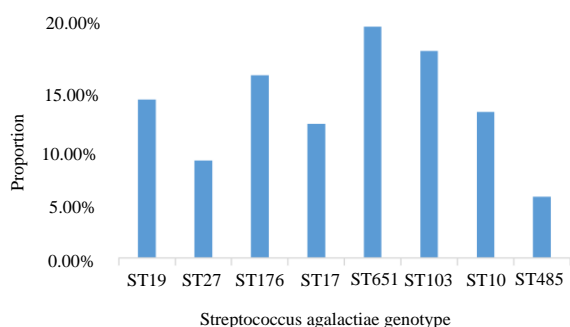


Figure 1. Genotype of *S. agalactiae* in neonatal sepsis

Table 5. Genotype of *S. agalactiae* in neonatal sepsis

Genotype	n	Proportion
ST19	13	13.00%
ST27	8	8.00%
ST176	15	15.00%
ST17	11	11.00%
ST651	19	19.00%
ST103	17	17.00%
ST10	12	12.00%
ST485	5	5.00%

Relationship between serotype and genotype of *S. agalactiae* in neonatal sepsis

The relationship between serotype and genotype of *S. agalactiae* in children with neonatal sepsis was studied. The results show good consistency between certain ST and serotypes. The ST103 and ST485 isolates are classified as serotype Ia, the ST10 and ST176 isolates are classified as serotype Ib, and ST17 and ST19 isolates are classified as serotype III. (Figure 2, Table 6)

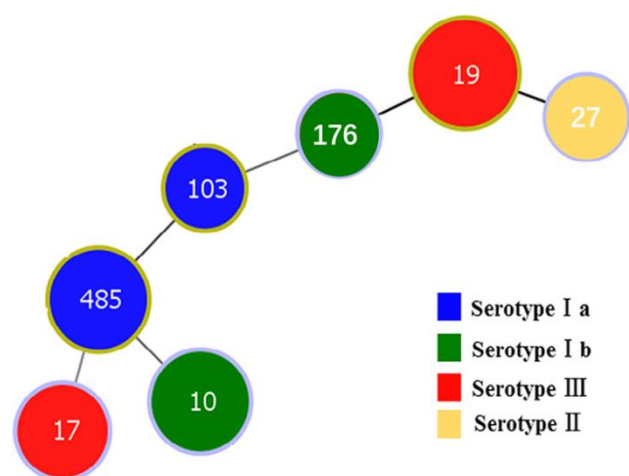


Figure 2. Correlation between genotype and serotype distribution of *S. agalactiae* isolates

Table 6. Correspondence between serotype and genotype of *S. agalactiae* in neonatal sepsis

Genotype	n	Ia	Ib	II	III	IV	V/NT
ST19	13	0	0	0	13	0	0
ST27	8	0	0	8	0	0	0
ST176	15	0	15	0	0	0	0
ST17	11	0	0	0	6	3	2
ST651	19	7	8	0	0	2	2
ST103	17	17	0	0	0	0	0
ST10	12	0	12	0	0	0	0
ST485	5	5	0	0	0	0	0

***S. agalactiae* genotype and erythromycin resistance in neonatal sepsis**

Among the strains of *S. agalactiae*, 40.23% (35/87) carry erm (A) gene, 35.63% (31/87) carry erm (B) gene, and 24.14% (21/87) carry mef (A) gene. There was a significant difference in erythromycin resistance and *S. agalactiae* genotype distribution. erm (A) gene is the most common gene in ST19 strain (7/11, 63.64%), and erm (B) gene is the most common gene in ST176 and ST651 strains (6/12, 50.00%; 8/18, 44.44%), while mef (A) gene is the most common gene in ST17 strain (5/11, 45.45%). (Figure 3, Table 7)

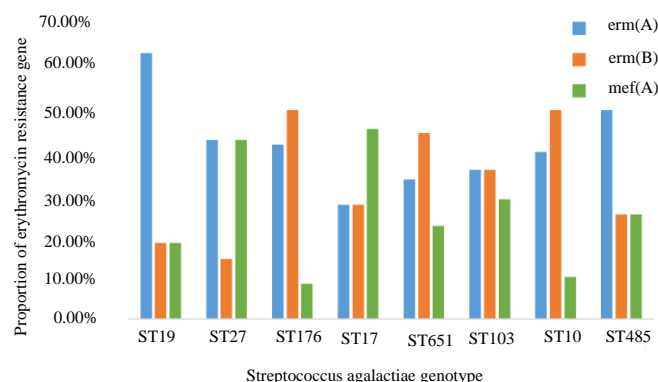


Figure 3. The relationship between *S. agalactiae* genotype and erythromycin resistance

Table 7. *S. agalactiae* genotype and erythromycin resistance

Genotype	n	erm(A)	erm(B)	mef(A)
ST19	11	7(63.64%)	2(18.18%)	2(18.18%)
ST27	7	3(42.86%)	1(14.29%)	3(42.86%)
ST176	12	5(41.67%)	6(50.00%)	1(8.33%)
ST17	11	3(27.27%)	3(27.27%)	5(45.45%)
ST651	18	6(33.33%)	8(44.44%)	4(22.22%)
ST103	14	5(35.71%)	5(35.71%)	4(28.57%)
ST10	10	4(40.00%)	5(50.00%)	1(10.00%)
ST485	4	2(50.00%)	1(25.00%)	1(25.00%)

S. agalactiae is an important cause of neonatal sepsis and meningitis. In the past decade, *S. agalactiae* has an increasingly higher correlation with invasive disease in non-pregnant adults (13).

Serotype determination has traditionally been used in epidemiological studies of *S. agalactiae*, which greatly helps the development of a broadly protected vaccine that contains capsular polysaccharides or polysaccharides bound to proteins (14, 15). The distribution of serotypes varies by region. Studies conducted in European countries, the United States and Latin America have shown that serotypes Ia, Ib, III or V are usually the most common (16). Type III and type V serum predominate in infection cases of neonates and non-pregnant adults, while in Portugal, type Ia serum predominates in *S. agalactiae*-induced invasive infections in non-pregnant adults (17). In Brazil, previous studies have shown that serotypes Ia, II, III and V are found in 68.2% isolates, mainly from vaginal specimens of asymptomatic pregnant women (18). In this study, serotypes Ia, Ib, and III were the most common, and serotypes VI to VIII were not found.

In our study, the prevalence of *S. agalactiae* colonies in infants was 1.3%, similar to that of Japan (1.3%), but lower than that in Bangladesh (6.3%), Turkey (17.3%) and Gambia (24.8%) of previous research. Colonization of *S. agalactiae* exhibits significant differences between different populations, which may be related to race, ethnicity, and genetic composition (19). It is worth noting that the prevalence is high in Italy (21.9%, sampling from ear, throat and rectum) and Gambia (24.8%, sampling from nasopharynx and rectum), but low in Pakistan (3.1%, sampling from abdominal skin and ears) and Greece (2.4%, sampling from the ear canal, throat and umbilical cord) (20). These findings indicate the importance of sampling points in determining the colonization of neonatal *S. agalactiae*. Only sampling from the skin and mucous membrane surfaces (such as the ear canal, oral cavity and umbilical cord) may underestimate the true prevalence of *S. agalactiae* in our study. It is worth noting that neonatal specimens of *S. agalactiae* were taken immediately after birth, indicating that the sampling was performed via vertical transmission of the mother.

Penicillin is a first-line drug recommended for the prevention and treatment of *S. agalactiae* disease.

Similar to the latest meta-analysis, 97% of all *S. agalactiae* isolates in this study are susceptible to penicillin infection, indicating that penicillin is still the first choice for prevention. It is recommended to use erythromycin and clindamycin as alternatives, especially in neonates allergic to penicillin. The resistance rates to erythromycin (87.00%) and clindamycin (78.00%) were similar to previous domestic reports (74.1%-92.5% and 64.2%-87.5%, respectively). In addition, the latest meta-analysis reveals that the resistance rate to clindamycin and erythromycin is significantly higher in Asia than in non-Asian countries. These findings suggest that alternative treatments for neonates intolerant to penicillin should be guided by the antibiotic resistance model. We observed that approximately 66.00% of isolates were multi-drug resistant, and the main multi-drug resistance model featured resistance to erythromycin, azithromycin and clindamycin. Consistent with our research, reports of invasive *S. agalactiae* isolates from neonates in China indicate that 90% are multi-drug resistant, and these isolates show drug resistance to erythromycin, clindamycin and tetracycline. According to research on *S. agalactiae*-induced community and hospital-acquired infections in non-pregnant women, Chinese adults have an overall drug resistance rate of 23%, but the drug resistance model remains unclear. These findings highlight the importance of continuous monitoring of antibiotic resistance.

As far as we know, for *S. agalactiae* isolates, there is a rare mechanism of drug resistance. Regarding the macrolide resistance genes, *erm* gene modifies the ribosome through methylation and inhibits erythromycin binding, while *mef* gene encodes a membrane protein that acts as a drug pump (21). In our study, the most common erythromycin resistance genes are *erm* (A), *erm* (B) and *mef* (A). Similarly, the high prevalence of *erm* (A) and *erm* (B) has also been reported in South Korea and Barcelona (22). *erm* (A) gene is the most common gene in ST19 strain (7/11, 63.64%), and *erm* (B) gene is the most common gene in ST176 and ST651 strains (6/12, 50.00%; 8/18, 44.44%), while *mef* (A) gene is the most common gene in ST17 strain (5/11, 45.45%). A Spanish study found that the correlation between these genes in *S. pneumoniae* may be caused by the presence of a conjugative transposon that encodes *erm*(B) and *tetM*,

but the underlying relationship and mechanism in *S. agalactiae* remain unclear (23). Future research must pay more attention to the genetic mechanism of resistance of *S. agalactiae* isolates worldwide to antibiotics.

Our study found that the most common ST is ST651, followed by ST103 and ST176, which is similar to the results of France, Italy and Poland (24, 25). However, there are various reports about other antibiotic-resistant bacteria such as *Escherichia coli*, *Staphylococcus aureus*, and *Acinetobacter baumannii* strains (26, 27), but there are little data on the differentiation of *S. agalactiae* clones based on phenotype and molecular characteristics (28-30). We observed good consistency between certain ST and serotypes, such as ST17/ST19 and III serotypes, ST103/ST485 and Ia serotype, as well as ST10/ST176 and Ib serotype. The latest research on invasive infection of *S. agalactiae* also reveals good consistency between certain ST and serotypes (for example, ST19 and III, ST23 and Ia, ST12 and Ib, ST1 and V).

In summary, we distinguished *S. agalactiae* clones in neonatal sepsis by using a variety of phenotypes and molecular characteristics and found a specific phenotype-genotype combination of the *S. agalactiae* clones. *S. agalactiae* genotypes in neonatal sepsis are mainly ST651, ST103 and ST176, and the serotypes are mainly Ia, Ib, III. There is good consistency between ST and serotype, and a significant difference is shown in erythromycin resistance and ST distribution, which highlights the value of new epidemiological trend detection by monitoring multiple characteristics and provides inspiration for the development of multivalent *S. agalactiae* vaccines. In addition, our findings indicate a high prevalence of multi-drug resistant *S. agalactiae*, which highlights the importance of continuous monitoring of antibiotic resistance.

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None.

Interest conflict

None.

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