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Risk factors and drug resistance in early-onset neonatal group B streptococcal disease*#

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In recent years, group B streptococcus (GBS) has become an important pathogen that causes infections in many neonatal organs, including the brain, lung, and eye (Ballard et al., 2016). A series of studies performed on GBS infections in western countries have revealed that GBS is one of the primary pathogens implicated in perinatal infection, and GBS infections are a major cause of neonatal morbidity and mortality in the United States (Decheva et al., 2013). In China, GBS is mainly found by screens for adult urogenital tract and perinatal infections, and neonatal GBS infections have been rarely reported. The incidence rate of early-onset neonatal GBS disease is thought to be lower in China than in western countries; however, this data is controversial since it also reflects the clinical interest in GBS (Dabrowska-Szponar and Galinski, 2001).

The clinical manifestations of neonatal GBS infections can be divided into two subtypes based on the time of onset of postnatal symptoms: early and late onset. Early-onset GBS infections occur by vertical (maternal-infant) transmission under normal conditions (Barbadoro et al., 2011). Crago et al. (2012) have identified the risk factors associated with different fetus prognoses in mothers with GBS infections. However, few researchers have examined the diverse risk factors associated with neonatal GBS disease or the effect of interactions.

In this study, factors associated with neonatal GBS disease were divided into three categories: factors of gravidas, delivery, and neonates. We collected samples that included 135 cases (mother-neonate pairs) of early-onset neonatal GBS disease and 234 controls in accordance with clinical diagnostic criteria between January 2007 and December 2015 in Women's Hospital of Zhejiang University School of Medicine, the Eye Hospital of Wenzhou Medical University, and Wenzhou People's Hospital, China. There was an increasing trend in the overall incidence of early-onset neonatal GBS disease (Fig. 1a). Twenty-two parameters associated with disease were selected for univariate analysis (Table 1). Five factors (gravida urinary tract

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infection, premature rupture of membranes, gestational age, bilirubin of neonates, and phototherapy of neonates) were statistically significant (P<0.05).

A binary multivariate logistic regression model (IBM SPSS 21.0) was performed, revealing that the gravida urinary tract infection, premature rupture of membranes, and gestational age were statistically significant (P<0.05, Table 2). Thus, the probability prediction model of early-onset neonatal GBS disease was as follows:

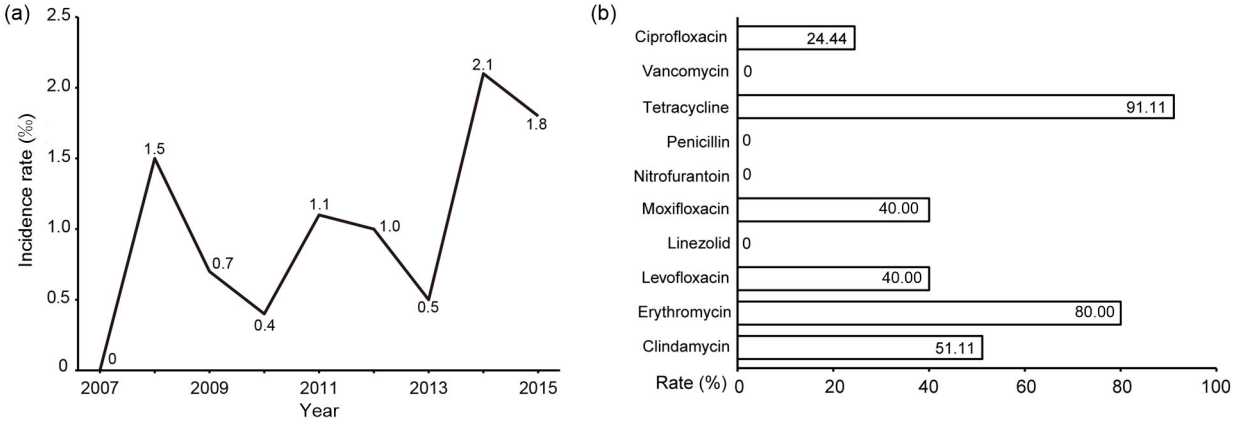

Fig. 1 Incidence and drug-resistance rate in group B streptococcus

Table 1 Univariate analysis in group B streptococcus

Factor	Case (n=135)	Control (n=234)	χ^2 or t	P-value
Factors of gravidas				
Puerpera age (year)	30.4±6.1	29.5±4.0	−1.242	0.215
Parity (each time)	1.6±0.9	1.9±1.2	1.186	0.237
Delivery time (each time)	1.2±0.5	1.2±0.4	−1.043	0.298
Pregnancy hypertension syndrome (no/yes)	132/3	221/13	1.560	0.212
Gestational diabetes mellitus (no/yes)	123/12	210/24	0.182	0.670
Endocrine disease (no/yes)	132/3	227/7	0.011	0.916
Urinary tract infection (no/yes)	129/6	207/27	5.291	0.021*
Factors of delivery				
Embryo number (1/(2 or 3))	132/3	222/12	1.184	0.277
Premature rupture of membrane (<18 h/≥18 h)	78/57	222/12	77.484	1.34×10^{-18}*
Character of amniotic fluid (normal/abnormal)	114/21	195/39	0.078	0.781
Volume of amniotic fluid (normal/abnormal)	132/3	221/13	1.560	0.212
Fetal distress (no/yes)	93/42	178/56	2.262	0.133
Mother's fever (no/yes)	126/9	223/11	0.645	0.422
Way of delivery (normal/abnormal)	87/48	128/106	3.342	0.068
Factors of neonates				
Gestational age (mature/premature)	134/1	186/48	27.371	1.68×10^{-7}*
Gender (male/female)	75/60	132/102	0.025	0.873
Weight (g)	3101±602	3078±729	−0.204	0.838
Neonatal Apgar5 score (point)	9.7±1.4	9.8±1.2	0.312	0.755
Neonatal respiratory disease (normal/abnormal)	120/15	201/33	0.677	0.411
Neonatal survival (survival/death)	134/1	232/2	0.014	0.907
High bilirubin (no/yes)	63/72	165/69	20.619	5.61×10^{-6}*
Phototherapy (no/yes)	81/54	193/41	22.628	1.97×10^{-6}*

Values for case and control are expressed as mean±standard deviation or number/number. Apgar5: 5-min Apgar score. * P<0.05

$$P=e^{y}/(1+e^{y}),$$
$$y=-3.8A+4.1B-3.2C-0.7,$$

where P is probability, A represents gravida urinary tract infection (no, A=0; yes, A=1), B represents premature rupture of membrane (<18 h, B=0; ≥18 h, B=1), and C represents gestational age (mature, C=0; premature, C=1).

A study of drug susceptibility in 135 cases was performed by the disk diffusion (K–B) method. We found that the strains were not resistant to vancomycin, penicillin, nitrofurantoin, or linezolid. However, 51.11%, 80.00%, and 91.11% of the strains were resistant to clindamycin, erythromycin, and tetracycline, respectively (Fig. 1b).

Table 2 Logistic regression model in group B streptococcus

Factor	*B*	SE	OR	*P*-value
Gravida urinary tract infection	−3.8	0.86	19.62	9.4×10^{-6}
Premature rupture of membranes	4.1	0.73	30.81	2.8×10^{-8}
Gestational age	−3.2	1.02	9.58	2.0×10^{-3}
Constant value	−0.7	0.14	27.04	2.0×10^{-7}

B: regression coefficient; SE: standard error; OR: odds ratio

Twelve strains of GBS were screened for subsequent experiments, revealing that two were sensitive to clindamycin, erythromycin, tetracycline, and levofloxacin, whereas the other ten were drug-resistant. We then amplified 25 candidate drug-resistance genes (polymerase chain reaction (PCR) primers are shown in Table S1), revealing the *aac6*, *ant6-I*, *aph3-III*, and *TEM* genes in the GBS samples (Fig. 2). We obtained *aac6* amplicons of about 250 bp, and this gene showed low expression in the two drug-sensitive strains. The *ant6-I* amplicons of about 600-bp products in all strains, as well as that of *aph3-III*, were not expressed in drug-sensitive strains, but a 300-bp product in some (6/10) drug-resistant strains was found. Finally, a *TEM* amplicon of about 500 bp in some drug-sensitive strains (1/2) and drug-resistant strains (9/10) was found. Therefore, GBS drug resistance may be closely related to the presence of *aph3-III* (Fig. 2c).

PCR products of drug-resistant genes were sequenced by Sanger sequencing (Invitrogen, Shanghai, China), and sequences were aligned and visualized using the BioEdit 7.2.2. We found that *aac6*, *ant6-I*, and *TEM* were expressed in both drug-sensitive and drug-resistant strains; hence, we sequenced the PCR products of these three genes in the 12 GBS strains.

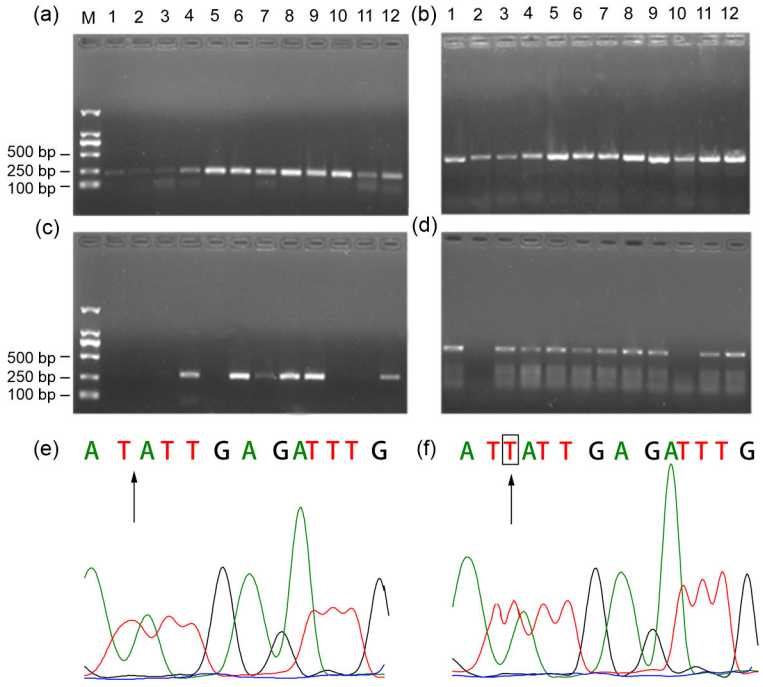

Fig. 2 Agarose gel electrophoresis and key mutation point in group B streptococcus

(a) *aac6* (220 bp); (b) *ant6-I* (597 bp); (c) *aph3-III* (292 bp); (d) *TEM* (535 bp); (e) Drug-sensitive strains in *acc6*; (f) Drug-resistant strains in *acc6*. M: marker

The *aac6* sequences had a deletion mutation (T) in the drug-sensitive strains but not in the drug-resistant strains (Figs. 2e and 2f), suggesting that the mutation attenuated the GBS drug resistance.

As we obtained samples over the years, the rate of early-onset neonatal GBS disease detected in blood cultures has increased, suggesting that more attention should be paid to GBS prevention. Notably, our logistic regression analysis revealed three factors that were associated with variations in GBS: premature rupture of membranes was identified as a risk factor, and the other two factors were found to be protective factors. Perhaps the bacteria involved in gravida urinary tract infections have a competitive relationship with GBS and other bacteria (Yu et al., 2016). To our knowledge, the low GBS infection rate of premature neonates has not been previously reported.

The US Center for Disease Control and Prevention (CDC) recommended penicillin as the first-choice drug for early-onset neonatal GBS disease treatment in China (Li et al., 2009). Erythromycin and clindamycin were recommended for penicillin-resistant strains or for patients who were allergic to penicillin (Juncosa-Morros et al., 2014). The high level of resistance to clindamycin, erythromycin, and tetracycline may reflect overuse of this drugs in the general population. The Clinical and Laboratory Standards Institute (CLSI) recommends that clinical trials can be conducted without the need for antimicrobial susceptibility testing and experience with penicillin-based antimicrobial therapy (Schrag et al., 2000). Persson et al. (2008) reported that the prevalence of penicillin-resistant infections had increased year-over-year in Sweden. Moreover, the erythromycin resistance rate had reached 14%–20%, and the clindamycin resistance rate had reached 10%–16%. However, in our study, the results of early-onset GBS infection in in vitro susceptibility tests revealed that the sensitivity of penicillin was 100%, and the erythromycin and clindamycin resistance rates were 80.00% and 51.11%, respectively. Therefore, the applicability of the US CDC guidelines for treatment is questionable. When GBS-positive cases are identified to avoid the possibility of serious consequences for neonates, we suggest the application of early and adequate doses of penicillin or ampicillin. These coupled with other forms of neonatal prophylaxis and treatment can effectively reduce neonatal mortality (Dabrowska-Szponar and Galinski, 2001). For penicillin-resistant or allergic cases, GBS should be isolated for susceptibility testing as soon as possible, and an effective drug should be identified from drug susceptibility test results.

Strains carrying drug-resistant genes are common causes of drug resistance (Yang et al., 2015). In this study, PCR analyses revealed that 4 of 25 well-known drug-resistance genes were present in different GBS samples. Among these, the aminoglycoside antibiotic resistance genes *aac6* and *ant6-I* were expressed both in drug-sensitive and drug-resistant strains, while *aph3-III* was expressed only in drug-resistant strains (6/10). Sequencing results revealed that the PCR products of the *aac6* gene contained a deletion mutation that may lead to decreased expression of this drug-resistance gene and thus decreased resistance to relevant antibiotics. The β-lactamase gene *TEM*, which confers penicillin resistance in many strains (Pimenta et al., 2014), was also detectable in the GBS strains (10/12). However, no penicillin resistance was found in this study, suggesting that the *TEM* gene may not be the major cause of the GBS resistance to penicillin. Finally, the *ermB* and *mefA* genes (Harimaya et al., 2007), which are closely related to the *TetM* gene for tetracycline resistance (Fischer et al., 2013), are worthy of further studies and may be associated with tumors (Hao et al., 2017; Zhang et al., 2017).

Our study systematically analyzed the influence of various factors on GBS infection. Using the chi-square test and *t*-tests, 5 out of 22 factors showed a significant relationship with infection. We examined three major factors, gravida urinary tract infection, premature rupture of membranes, and gestational age, further using logistic regression to build a model of the GBS infection incidence rate, in the hope that this would contribute to the development of methods to prevent neonatal GBS infection. Moreover, drug resistance analyses of GBS patients offer a useful reference dataset that can be used to treat GBS more effectively. Finally, our PCR and sequencing results from drug-resistant strains explore the mechanisms of drug resistance in GBS.

Contributors

Ying-wei WANG and Yao-qiang DU performed the whole experimental research and data analysis, wrote and

revised the manuscript. Xiao-lin MIAO and Guang-yong YE collected the samples and filtered the significant risk factors. Yi-yun WANG used logistic regression to build a risk model. Ai-bo XU and Yun-zhong JING performed the PCR and Sanger sequencing. Yu TONG and Kai XU collected the samples and analyzed the drug-resistance results. Mei-qin ZHENG checked the statistical methods. Dong CHEN and Zhen WANG contributed to the study design, data analysis, and manuscript writing.

Compliance with ethics guidelines

Ying-wei WANG, Yao-qiang DU, Xiao-lin MIAO, Guang-yong YE, Yi-yun WANG, Ai-bo XU, Yun-zhong JING, Yu TONG, Kai XU, Mei-qin ZHENG, Dong CHEN, and Zhen WANG declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008 (5). Informed consent was obtained from all patients for being included in the study. Additional informed consent was obtained from all patients for whom identifying information is included in this article.

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List of electronic supplementary materials

Table S1 PCR primers in this study

中文概要

题　目：新生儿早发型无乳链球菌感染的风险因素及耐药分析

目　的：新生儿早发型无乳链球菌（GBS）是一种可致婴儿脑、肺和眼部发生感染甚至死亡的细菌。筛查其感染风险因素并建立概率预测模型有助于疾病的预防和控制，进一步分析 GBS 菌株的耐药类型和耐药基因，为疾病的临床诊断和治疗提供指导意见。

创新点：首次归类了新生儿感染 GBS 的众多因素，按照孕妇、分娩过程、胎儿因素分为三类，统计筛选出重要风险因素并建立 Logistic 回归的概率预测模型，并通过聚合酶链式反应（PCR）筛查到了相关耐药基因。

方　法：收集 2007 年 1 月至 2015 年 12 月，浙江大学医学院附属妇产科医院、温州医科大学附属眼视光医院和温州市人民医院的实验组 135 例早发型 GBS 感染和对照组 234 例无感染的新生儿及其母亲的临床资料和标本，进行数据统计分析并构建概率预测模型，采用传统的药敏实验对所有 GBS 阳性标本进行耐药分析，同时 PCR 筛查耐药基因并测定序列。

结　论：这 9 年间新生儿早发型 GBS 感染率呈上升趋势。孕妇泌尿系统感染、胎膜早破、胎龄这三项因素与感染 GBS 的结局存在显著关联，由此得出的 Logistic 回归模型可用于预测感染 GBS 的概率。135 株 GBS 对克林霉素、红霉素、四环素的耐药菌株比例分别为 51.11%、80.00%、91.11%，同时未发现对万古霉素、青霉素、呋喃妥因、力奈唑胺的耐药性。PCR 筛查发现 *aac6*、*ant6-I*、*aph3-III* 和 *TEM* 四个耐药基因，其中 *aph3-III* 仅存在于耐药株中，而测序发现 *aac6* 基因在非耐药株中相比耐药株存在一个缺失突变。临床上尽早对患者分离的 GBS 药敏试验，有助于合理用药和有效治疗。

关键词：新生儿；无乳链球菌；风险因素；耐药性