Original Article

Determination of Capsular Serotypes, Antibiotic Susceptibility Pattern, and Molecular Mechanism of Erythromycin Resistance among Clinical Isolates of Group B *Streptococcus* in Isfahan, Iran

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Abstract

Background: Documented streptococcal resistance to erythromycin has recently been raised. The aim of this study is to identify the molecular mechanism of erythromycin resistance among group B *Streptococcus* (GBS) strains and to correlate with the clinical origin of strains. **Materials and Methods:** A total number of 134 colonizing (n = 36), invasive (n = 36), noninvasive (n = 46), and asymptomatic (n = 16) GBS isolates were characterized by the detection of *dltS* gene, capsular serotyping, antibiotic susceptibility profiles using disc diffusion method, and screening of the *ermB*, *ermTR*, and *mefA* resistance genes. **Results:** The distribution of capsular serotypes was as follow: serotype III (24.6%), Ia (21.6%), V (17.9%), Ib (14.9%), II (8.9%), IV (8.9%), VI (1.5%), and VII (1.5%). From 134 GBS isolates, 51 (38%) isolates were resistant to erythromycin. The constitutive macrolide lincosamide streptogrmin B (MLSB) was the most common resistance phenotype (62.7%), followed by inducible MLSB (27.4%) and M phenotype (9.8%). Erythromycin resistance rate was higher among asymptomatic GBS strains (13/16, 81.2%). Serotype III was the most prevalent type among resistant isolates (41.1%). The *ermB* gene highly distributed among resistant strains (64.7%), followed by *ermTR* (21.5%) and *mefA* (9.8%). The *ermB* gene was related to constitutive MLSB phenotype (84.3%, $P < 0.05$) and serotypes III (61.9%), Ib (87.5%), and V (83.3%). All M phenotype strains harbored *mefA* gene and were in association with serotype Ia (90%). **Conclusion:** The current study suggests that ribosomal modification with *erm* genes is the main mechanism of erythromycin resistance. Because of relatively high prevalence of erythromycin resistance, double disc test highly recommended for GBS disease treatment and intrapartum prophylaxis among penicillin intolerant patients in our region.

Keywords: *Antibiotic resistance, erythromycin, microbial, Streptococcus agalactiae*

Introduction

Streptococcus agalactiae or group B *Streptococcus* (GBS) based on Lancefield classification is a leading cause of serious neonatal and adults infections.[1] GBS is often a part of normal flora of gastrointestinal and genitourinary tracts of healthy women.[2] Transmission during labor from colonized mothers to their babies can cause life-threatening infections and development of early onset disease (EOD) and late onset disease (LOD). Since colonized mothers can act as reservoir of GBS, Centers for Disease Control and Prevention, and the American Academy of Pediatrics recommended intrapartum antibiotic prophylaxis (IAP) to prevent perinatal GBS disease.[3] According to these recommendations, antenatal screening of pregnant women at 35–37 weeks of pregnancy with swab culture of rectovaginal secretions is done.[4] Pregnant mothers who have a positive urine or rectovaginal swab culture should receive IAP during delivery.[5] Penicillin is drug of choice for the treatment of GBS infections and IAP. However, reduced susceptibility to penicillin has been reported.[6] Erythromycin and clindamycin are used as alternative therapeutic agents for β-lactam allergic patients. Resistance to the erythromycin and clindamycin is not uncommon and increasingly has been observed worldwide.[6] Erythromycin resistance is mediated by two mechanisms that were first elucidated in Group A *Streptococcus* and *Streptococcus pneumoniae*. These mechanisms are ribosomal methylation of 23S rRNA by *erm* (erythromycin ribosome methylation)

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gene and efflux pump. Methylase enzymes encoded by over the forty variants with *ermA*, *ermB*, and *ermTR* identified in *Streptococcus*.[7] They block binding of macrolide (including erythromycin), lincosamides (including clindamycin), and streptogramin B (MLSB) to the 50S ribosomal subunit, leading to cross-resistance. Based on the constitutive or inducible expression of *erm* gene, two MLSB phenotypes have been described. Inducible MLSB phenotype occurs when resistance to erythromycin induces resistance to clindamycin.[7] This phenotype can detect with double-disc diffusion test (D-zone test) that make a D-shape zone of inhibition around the clindamycin disc.[8] With a single disc diffusion test, these strains appear susceptible to the clindamycin. In the clinical laboratories, D-zone test should be considered as a routine test for the detection of the inducible MLSB (iMLSB) phenotype to prevent treatment failure with clindamycin and emergence of constitutive MLSB phenotype.[8] Another resistance mechanism is mediated by an efflux pump encoded by the mef*A*/*E* gene. This pump confers the resistance to macrolides but not lincosamides, a characterization called M phenotype.[7]

Capsular typing is a traditional method for the classification of GBS. According to the capsular polysaccharide (CPS), GBS classified to the ten serotypes (Ia, Ib, and II-IX). The CPS is one of the most important virulence factors and a target helping vaccine development.[1]

Despite the clinical impact of GBS infections and increasing resistance rates to some antimicrobial agents, there are a limited number of studies reporting antimicrobial susceptibility profiles among GBS strains circulating in Iran. We undertook this study to document antimicrobial susceptibility testing, capsular genotyping and the presence of three macrolide resistance genes among GBS strains isolated from colonizing, invasive, noninvasive, and asymptomatic infections.

Materials and Methods

Bacterial isolates

In this cross-sectional study from July 2016 to September 2018, a total number of 134 nonduplicate GBS were collected from Alzahra and Shahid Beheshti hospitals in Isfahan, Iran. These isolates included colonizing GBS (n = 36) collected from swab culture of rectovaginal secretions of pregnant women at 35–37 weeks of gestations, invasive GBS collected from sterile body fluids of nonpregnant women such as blood and joint infections (n = 36), noninvasive GBS obtained from urinary tract infections, vaginal discharges, abscess, and tracheal tube secretions (n = 46, male = 9, female = 37), as well as asymptomatic women with positive urine culture with colony count $<10^5$ CFU/mL (n = 16). All isolates identified with morphology of colony, beta hemolysis on the sheep blood agar, Gram stain, catalase reaction, CAMP test, and polymerase chain reaction (PCR) amplification and detection of 952 bp *dltS* gene specific for GBS species after DNA extraction.[9]

Antibiotic susceptibility profile

The disc diffusion method based on the criteria of the Clinical and Laboratory Standards Institute (CLSI) 2018 edition was utilized to determine the antimicrobial susceptibility patterns to the following nine antimicrobial discs (Mast, Merseyside, UK) and concentrations: penicillin (10units), vancomycin (30 μg), ceftriaxone (30 μg), tetracycline (30 μg), clindamycin (2 μg) and erythromycin (15 μg), cefepime (30 μg), cefotaxime (30 μg), and levofloxacin (5 μg). For clindamycin, the detection of inducible resistance was performed by double disc diffusion testing (DD-test) in order to categorize the isolates as iMLSB, constitutive MLSB (cMLSB) (constitutive MLSB), M and L phenotypes.[8]

DNA extraction

The genomic DNAs of GBS isolates were extracted using a simple boiling method. Briefly, a loopful of bacterial biomass was suspended in 300 μl of TSE buffer (50 mM Tris hydrochloride [pH 7.5], 1% SDS, 25 mM EDTA), and the suspension was heated at 95°C for 20 min and centrifuged at 10,000 g for 10 min. The supernatant was taken as DNA lysate and was kept at −20°C for the molecular assay.[10]

Capsular genotyping

Capsular genotyping was performed using nine pairs of primers and multiplex PCR assay for the detection of Ia, Ib, and II-VIII serotypes as previously described.[9] A primer pairs (*dltS*-F and *dltS*-R) targeting the GBS-specific *dltS* gene were also included as an internal positive control.[9]

Detection of macrolide resistance genes

All erythromycin-resistant GBS surveyed for the presence of the *ermTR*, *ermB*, and *mefA* genes using PCR method with previously published primers.[11]

Statistical analysis

The SPSS Statistics V. 20.0 (IBM SPSS Statistics for Windows, Armonk, NY, United States: IBM Corp) were used for statistical analysis. Association between capsular serotype with the distribution of erythromycin-resistance genes was assessed by applying Fisher's exact test. Differences were considered statistically significant at $P < 0.05$.

Results

Of 134 GBS isolates studied in this study, 51 isolates were resistant to erythromycin. A number of 15/51 colonizing isolates (29.4%), 15/51 (29.4%) invasive isolates, 8/51 (15.6%) noninvasive GBS, and 13/51 (25.4%) asymptomatic isolates were erythromycin resistant.

According to the source of GBS isolates, 41.6% of colonizing GBS (15/36), 41.6% of invasive GBS (15/36), 17.3% of noninvasive GBS (8/46), and 81.2% of asymptomatic GBS isolates (13/16) were resistant. Therefore, we found high presence of erythromycin resistance among asymptomatic adults.

Capsular genotyping of 134 GBS isolates showed that all isolates were typeable and except for serotype VIII and IX, all types were detected. The distribution of capsular serotypes was as follow: serotype III (33, 24.6%), Ia (29, 21.6%), V (24, 17.9%), Ib (20, 14.9%), II (12, 8.9%), IV (12, 8.9%), VI (2, 1.5%), and VII (2, 1.5%). For colonizing GBS, serotype Ia was frequently detected (11, 30.5%), followed by III (8, 22.2%) and Ib and V (each 6, 16.6%). For invasive isolates, serotype III (11, 30.5%) followed by Ib and V (each 5, 13.8%), IV and Ia (each 4, 11.1%). For noninvasive GBS strains, serotypes III and V (each 10, 21.7%) were the most common capsular types. Asymptomatic GBS isolates had serotype Ia as most common type (31.2%). Distribution of serotypes across source was demonstrated in Figure 1.

All isolates were susceptible to penicillin, cefotaxime, and vancomycin. Based on CLSI criteria, two isolates were nonsusceptible to cefepime and ceftriaxone. Resistance to the levofloxacin was detected in 15 isolates (11.1%). The overall frequency of tetracycline resistance was 91%. Out of the 134 isolates studied, 51 (38%) isolates were resistant to erythromycin and 42 (31.3%) of them to clindamycin [Table 1]. Results of double-disc diffusion test revealed that the cMLSB phenotype was the most frequent (62.7%), followed by iMLSB (27.4%) and M phenotype (9.8%) [Table 2]. In respect to the prevalence of serotypes among erythromycin-resistant GBS, we found that 41.1% of resistant isolates were belonged to serotype III ($P < 0.05$). Among the cMLSB-GBS strains, CPS-III and V (18/32, 56.2%) and iMLSB-GBS strains CPS-III and Ib (10/14, 71.4%) were the most frequent serotypes. In contrast, M phenotype strains were predominately associated with serotype Ia isolates (4/5, 90%). Details are summarized in Table 3. The *ermB* gene and to a lesser extent *ermTR* gene were widely distributed (64.7% and 21.5%, respectively). However, only five isolates were positive for *mefA* gene (9.8%). Resistance genes *ermB* and *ermTR* were combined in one invasive strain with CPS-V and cMLSB phenotype. In this study, modification of 23S rRNA of 50S subunit of ribosome due to methylases encoded by *ermB* and *ermTR* genes was the most common reason of resistance to erythromycin. The cMLSB phenotype was in association with *ermB* gene (84.3%) ($P < 0.05$). Besides, two genes *ermB* and *ermTR* were found in approximately equal amounts in iMLSB phenotype strains. The M phenotype was detected only in isolates harboring the *mefA* gene (100%) [Table 2]. According to source of GBS isolates, cMLSB phenotype/erm*B* gene was most common phenotype and genotype among colonizing, invasive, noninvasive, and asymptomatic strains [Figure 2].

Discussion

The increasing trend in the rates of resistance to erythromycin and clindamycin among GBS isolates has raised concerns about the use of these antibiotics as alternative agents for the prophylaxis or treatment of GBS infections in beta-lactam allergic patients.[7] In current study, a rate of 38% resistance to erythromycin was detected. In

Table 1: Antibiotic susceptibility profile of 134 Group B *Streptococcus* isolates

Antibiotic	Susceptible, *n* (%)	Intermediate, *n* (%)	Resistant, *n* (%)
Penicillin	134 (100)	-	-
Cefepime	132 (98.5)	-	2 (1.5)
Ceftriaxone	132 (98.5)	-	2 (1.5)
Cefotaxime	134 (100)	-	-
Vancomycin	134 (100)	-	-
Tetracycline	12 (8.9)		122 (91.1)
Levofloxacin	106 (79.1)	13 (9.7)	15 (11.1)
Clindamycin	61 (45.5)	31 (23.2)	42 (31.3)
Erythromycin	53 (39.6)	30 (22.4)	51 (38)

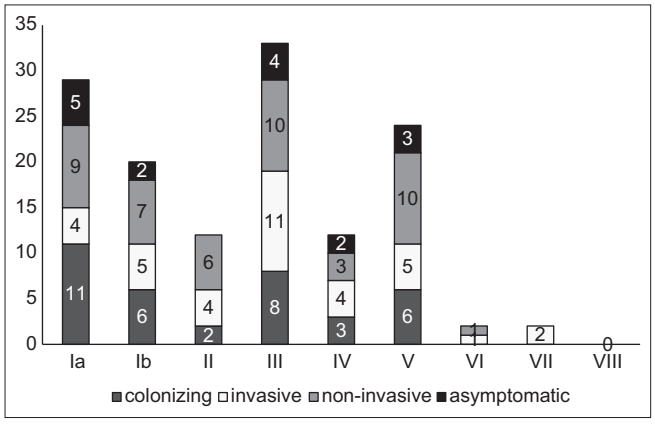

Figure 1: Distribution of serotypes according to the source of 134 Group B *Streptococcus* isolates

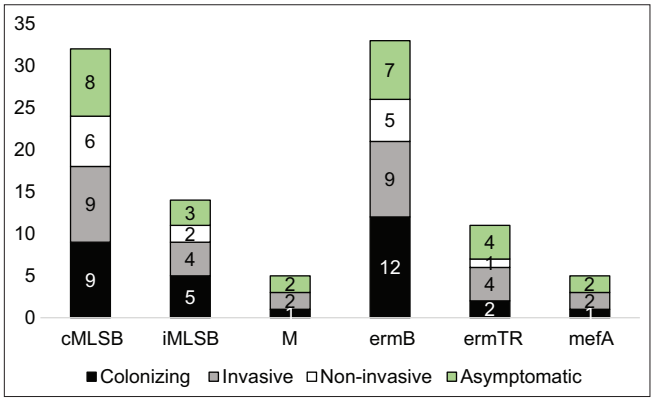

Figure 2: Distribution of erythromycin resistance phenotypes/genotypes according to source of 51 resistant isolates

Table 2: Distribution of resistance genes across erythromycin resistance phenotypes

Erythromycin resistance genes	Erythromycin resistance phenotype			Total, *n* (%)
	cMLSB, *n* (%)	iMLSB, *n* (%)	M phenotype, *n* (%)	
ermB	27	6	-	33 (64.7)
ermTR	4	7	-	11 (21.5)
mefA	-	-	5	5 (9.8)
Negative PCR	1	1	-	2 (3.9)
Total	32 (62.7)	14 (27.4)	5 (9.8)	51 (100)

MLSB: Macrolides, lincosamides, and streptogramin B, cMLSB: Constitutive MLSB, iMLSB; Inducible resistance to MLSB, PCR: Polymerase chain reaction

Table 3: Distribution of resistance phenotypes and genotypes across serotypes among 51 erythromycin-resistant Group B *Streptococcus*

Serotypes	Ia, *n* (%)	Ib, *n* (%)	II, *n* (%)	III, *n* (%)	IV, *n* (%)	V, *n* (%)	VI, *n* (%)	VII, *n* (%)	Total, *n* (%)
Resistant strain	8 (15.6)	8 (15.6)	2 (3.9)	21 (41.1)	3 (5.8)	6 (11.7)	2 (3.9)	1 (1.9)	51 (100)
ermB	4 (12.1)	7 (21.8)	1 (2.8)	13 (39.3)	2 (5.7)	5 (14.2)	-	1 (2.8)	33 (64.7)
ermTR	-	1 (8.3)	1 (8.3)	5 (41.6)	1 (8.3)	1 (8.3)	2 (16.6)	-	11 (21.5)
mefA	4 (80)	-	-	1 (20)	-	-	-	-	5 (9.8)
cMLSB	4	5	2	13	2	5	-	1	32 (62.7)
iMLSB	-	3	-	7	1	1	2	-	14 (27.4)
M	4	-	-	1	-	-	-	-	5 (9.8)

Two serotypes III had negative results in PCR amplification of all three genes. MLSB: Macrolides, lincosamides, and streptogramin B, cMLSB: Constitutive MLSB, iMLSB; Inducible resistance to MLSB, PCR: Polymerase chain reaction

addition, we found most of the erythromycin-resistant isolates were obtained from the rectovaginal samples of pregnant women and sterile body fluids of women with invasive infections (30/51, 58.8%). This issue leads to an increase in using vancomycin and cefazolin for the prophylaxis and treatment, which could enhance the emergence of vancomycin-resistant strains in the future.[12] A meta-analysis by Khademi and Sahebkar found that the overall prevalence of resistance to erythromycin among pregnant women was 21%.[13] However, Jalalifar *et al*. found a higher presence of erythromycin resistance among adults with urinary tract infections.[14] Results of a study showed lower rate of 25% resistance to the erythromycin among colonizing and invasive GBS in adults.[15] Besides, the erythromycin resistance was particularly high in the USA (54%), France (41.7%), and China (74.1%), but in Spain, only 20.7% of GBS strains were resistant.[7,16,17] Comparison of distribution of resistant isolates in each group showed this rate was higher among asymptomatic GBS (13/16, 81.2%). Borchardt *et al*. reported a high prevalence of resistance to erythromycin among asymptomatic adults in comparison to invasive ones.[18] Other study from Poland showed the proportion of erythromycin resistance among invasive versus carriage and noninvasive isolates was similar.[19]

Capsular serotyping of GBS is crucial in determining the pathogenicity of the isolates and vaccine development.[20] Placental transfer of anti-CPS-specific GBS antibodies from the mother to the fetus reduces the risk of invasive GBS disease with the evidence of protection against both EOD and LOD. Five serotypes Ia, Ib, II, III, and V are the predominant types of GBS that cause infections in neonates and adults.[1] Literature of review showed that a hexavalent polysaccharide–protein conjugate vaccine (Ia, Ib, II, III, IV, and V) has the potential to prevent up to 93% of worldwide maternal colonizing isolates, 95% of maternal invasive GBS disease, 99% of GBS-associated stillbirth, and 99% of infant invasive GBS disease. Although evidence is still limited, a vaccine targeting maternal colonization could provide additional protection against neonatal disease.[5,20,21] Our results showed serotypes Ia, III, and V accounted for 64.1% of GBS isolates. With regard to the source of isolates, the most prevalent serotype in colonizing and asymptomatic GBS isolates was Ia and in invasive and noninvasive GBS were serotypes III and V. Maternal colonization by serotype Ia is seen globally, but prevalence and geographical distribution of serotypes is different.[5] Regarding maternal colonization, previous studies in our country showed despite our results, CPS III was the most common serotype.[22-24] Serotype Ia was the most prevalent type in the United States, United kingdom, and South Korea contributing to maternal colonization and EOD.[5] CPS III is well known for its association with infections in neonates and adults, and it has been reported throughout the world.[1,5] Several investigations revealed the importance of serotype V for its association with infections in nonpregnant adults.[2] In this study, most of serotype V stains were belonged to patients with noninvasive infections. Another studies in the United Kingdom, South Africa, and France found serotype V as dominant CPS among maternal disease and colonization.[2,25] In this study, a high prevalence of serotype III among erythromycin-resistant

GBS isolates was observed. Previous studies found an association between erythromycin resistance with serotypes II, III, or V.[8,14]

Results of double-disc diffusion test revealed that cMLSB phenotype was the most common among erythromycin resistant strains followed by iMLSB and M phenotype. Many studies have confirmed the high prevalence of the cMLSB phenotype over the other two phenotypes.[15,16,26] Based on our finding, 64.7% of the erythromycin-resistant strains carried the resistance gene *ermB* and cMLSB phenotype was strongly related to this gene. Other investigations in our country, France, and China confirmed our results and *ermB* was the most frequent gene related to erythromycin resistance.[16,24,27] However, all M phenotype strains in this study were associated with the *mefA* gene. The efflux pump encoded by *mefA* gene unable to pump out clindamycin and other lincosamides even in the presence of erythromycin. Hence, strains harboring *mefA* gene cannot develop MLSB or L phenotype. This issue confirmed with other previous studies.[28]

Previous studies have suggested a possible association between serotype V or III strains and the presence of the *ermB* gene and between serotype Ia and the *mefA* gene.[8,24,29] We also found such associations: the *mefA* gene was found in 50% of the resistant serotype Ia strains, and the *ermB* gene in 61.9% of the resistant serotype III strains, 87.5% of the resistant serotype Ib strains, and 83.3% of the resistant serotype V strains.

Erythromycin resistance in two of our strains was not associated with either the *mefA* or the *ermB* and *ermTR* genes. Such resistance in beta-hemolytic streptococci may be related to mutations in ribosomal proteins, as previously reported for *S. pneumonia*.[30]

Conclusion

All GBS isolates included in this study were susceptible to penicillin, cefotaxime, and vancomycin. These results confirm that the use of penicillin as a drug of choice for prophylaxis and treatment of GBS infections in Iran is appropriate. A high rate of erythromycin resistance (38%) was found among the GBS isolates studied. These results agree with data from the other parts of our country, which showed a resistance rate of 21%[13] but differ from the 52% erythromycin resistance rate reported by others.[14] This discrepancy may be related to the different isolate collection periods and source of GBS. Erythromycin and clindamycin should no longer be relied upon as an alternative agent for prophylaxis and treatment of GBS infections in Iran without susceptibility testing. Most of resistant isolates were serotype III/cMLSB$^+$/*ermB*$^+$. Dominant distribution of cMLSB phenotype showed ribosomal modification with *ermB* gene is the main mechanism of erythromycin resistance. Since iMLSB-GBS isolates appear susceptible to clindamycin but may be inappropriate for the treatment of infection, the use of the double disc diffusion test is strongly recommended.

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Conflicts of interest

There are no conflicts of interest.

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