

Molecular Typing of *Streptococcus agalactiae*- cMLSB Phenotype Isolates by Enterobacterial Repetitive Intergenic Consensus-PCR (ERIC-PCR) in Isfahan, Iran

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ABSTRACT

Backgrounds: Group B *Streptococcus* (GBS) is an important opportunistic bacterial pathogen that could cause serious infections, especially in neonates, adults, and the elderly. In GBS isolates, a macrolide resistance phenotype that confers constitutive resistance to macrolide-lincosamide-streptogramin B antibiotics (cMLSB phenotype) has become a global concern. On the other hand, little is known about the genetic relatedness and diversity of GBS isolates isolated from various patients in Iran. Hence, this study aimed to determine the genetic relatedness and molecular typing of cMLSB-GBS isolates using enterobacterial repetitive intergenic consensus-PCR (ERIC- PCR) technique.

Materials & Methods: A total of 100 GBS isolates were collected from patients with urinary tract infections (UTIs). Among them, 52 erythromycin-resistant GBS isolates were selected, and double-disc diffusion (D-zone) technique was applied to determine the MLSB phenotype among the isolates based on CLSI criteria. Then the genetic relatedness of MLSB-GBS isolates was assessed using ERIC-PCR fingerprinting method.

Findings: Among 52 erythromycin-resistant GBS isolates, 38 isolates were identified with cMLSB phenotype, nine isolates with M phenotype, and five isolates with iMLSB phenotype. The analysis of ERIC-PCR patterns revealed eight different ERIC types that were divided into seven clusters (A-G) and one single type. Also, four isolates were non-typeable. ERIC type A/ serotype Ib was the most prevalent clone among the isolates.

Conclusion: The current study findings showed a high level of diversity and multiclinal spread of the cMLSB phenotype in Isfahan. ERIC type A/ serotype Ib is the predominant clone circulating among erythromycin-resistant GBS strains.

Keywords: Molecular typing, Group B *Streptococcus*, Antibiotic resistance, Erythromycin-resistant GBS, ERIC-PCR, Iran.

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Introduction

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a member of the gastrointestinal and genitourinary tracts normal flora in women [1, 2]. The colonization rate of healthy women with GBS has been reported to be about 10–40% [3]. Colonized pregnant women could act as a reservoir and transfer GBS to their babies during labor. Neonatal sepsis and meningitis are two life-threatening clinical manifestations that could lead to death [3]. Besides, GBS causes a wide range of localized and systemic infections in immunocompromised patients and the elderly, such as pneumonia, joint and soft tissue infections, and urinary tract infections (UTIs) [4, 5]. Although GBS is less prevalent than Enterobacteriaceae in UTIs (2-3% of cases), it could cause serious infections [6]. These infections include asymptomatic bacteriuria, cystitis, urethritis, and pyelonephritis [7].

Penicillin is the antibiotic of choice for intrapartum antibiotic prophylaxis (IAP) and treatment of GBS infections, but erythromycin, levofloxacin, and clindamycin are alternative choices for β -lactam allergic patients. Fortunately, resistance to penicillin remains very low. However, various investigations have reported high resistance of GBS to erythromycin and clindamycin [6, 8]. There are two macrolide resistance mechanisms in streptococci, including ribosomal modification via methylases that confer inducible or constitutive resistance to lincosamides and streptogramin B, characterizing macrolide-lincosamide-streptogramin B (MLSB) phenotypes, as well as drug efflux via a membrane-bound protein, characterizing M phenotype [9]. Double-disc diffusion (D-zone) test could be applied as a simple and reliable method to define inducible and constitutive clindamycin resistance [10].

There are various methods used for GBS

genotyping [4]. A capsular polysaccharide is considered as a virulence factor that could help escape the immune system. Latex agglutination using anti-capsular antibodies is one of the serotyping methods, but the reliability of molecular detection is higher than conventional methods like serotyping. Polymerase chain reaction (PCR)-based capsular gene (cps) typing methods are molecular techniques used for this purpose [11]. Multiplex PCR is one of the molecular typing methods that could detect capsular polysaccharide serotypes [12]. Moreover, multilocus sequence typing (MLST), pulse-field gel electrophoresis (PFGE), random amplification of polymorphic DNA (RAPD), and ribotyping are also used for genotyping [13–17]. However, due to the limitations of these methods in terms of convenience, time spent performing methods, and special equipment, other methods need to be considered [18]. Studies have revealed that unique sequences, such as enterobacterial repetitive intergenic consensus (ERIC) and repetitive extragenic palindromic (REP) sequences, located in the genome of prokaryotes could be used for genotyping [13]. ERIC is a 127 bp palindromic sequence that is characterized as an intergenic inverted-repeat element. Discrimination of bacterial strains due to the significant diversity in copy number of these elements is possible [19]; for example, copy numbers in *Escherichia coli* and *Salmonella Typhimurium* strains have been extrapolated to be about 30 and 150, respectively [20]. Amplification of ERIC sequence is done using PCR [19]EndNote>.

Objectives: Despite the clinical burden of GBS infections and their high resistance to antibiotics [21–23], there are limited studies reporting specific clones circulating among macrolide-resistant GBS isolates in Iran. In the current study, ERIC-PCR was performed to characterize and distinguish specific clones among GBS isolates with cMLSB

resistance phenotype in Isfahan, Iran.

Materials and Methods

Bacterial isolates: In our previous study, 100 GBS isolates collected from patients with UTIs were analyzed to determine antibiotic susceptibility patterns, capsular genotyping, and surface proteins profile [24]. These isolates were used in the present research.

Antimicrobial susceptibility testing to determine GBS isolates with MLSB phenotype: Double-disc diffusion (D-zone) technique was used to determine the MLSB phenotype based on the Clinical and Laboratory Standards Institute (CLSI) criteria. The clindamycin disc (2 µg) was placed 12 mm (edge-to-edge) far from the erythromycin disc (15 µg) (MAST, Merseyside, UK) on a Mueller-Hinton agar plate supplemented with 5% sheep blood previously inoculated with bacterial suspension equivalent to 0.5 McFarland. *Staphylococcus aureus* ATCC 25923 was applied as a quality control strain. Inducible MLSB resistance (iMLSB) was defined as showing resistance to erythromycin but susceptibility to clindamycin and the formation of a D-shaped zone of inhibition around clindamycin, which flattens towards erythromycin. Resistance to both clindamycin and erythromycin was considered as constitutive MLSB resistance (cMLSB). Susceptibility to clindamycin but resistance to erythromycin without blunting (without a D-shaped zone) was regarded as an efflux mechanism (M phenotype) [25]. GBS isolates with cMLSB phenotype were subjected to ERIC-PCR.

ERIC-PCR: DNA of GBS isolates was extracted using phenol-chloroform method as previously described [26]. ERIC PCR was done with ERIC primers consisting of ERIC 1 (5'-ATG TAAGCTCCTGGGGATTAC-3') and ERIC 2 (5'-AAG TAA GTG ACT GGG GTG AGCG-3') [27]. PCR program was carried out

with a pre-denaturation step at 95°C for 5 min, followed by 30 cycles of denaturation (at 95°C for 60 s, 59°C for 50 s, and 72°C for 60 s) and a final extension at 72°C for 10 min [28]. Finally, PCR products were run on 1.5% agarose gel in Tris/Borate/EDTA buffer for 60 min, and the gel was visualized by the documentation system after staining with safe stain loading dye (CinnaGen Co., Tehran, Iran).

Statistical analysis: In the current study, SPSS software (IBM SPSS statistics for windows, V. 20) was applied for statistical analysis. In this regard, Chi-square test was performed to characterize associations between variables. A *p* value of less than 0.05 was regarded as statistically significant (*p* < .050). ERIC patterns were surveyed by GelJ software Version 2.0 [29]. GBS isolates with a similarity coefficient of ≥80% were clustered as the same genotypes.

Findings

In this study, out of 52 erythromycin-resistant GBS isolates, 38 (73%) isolates were identified with cMLSB phenotype, nine (17.3%) isolates with M phenotype, and five (9.6%) isolates with iMLSB phenotype. The distribution of Alp family genes, capsular genotypes, and resistance patterns among GBS isolates with iMLSB and M phenotypes is presented in Table 1.

Capsular type Ib was the most prevalent serotype among cMLSB-GBS isolates (15 of 38, 39.4%), followed by serotype V (seven isolates, 18.4%), II and III (each in four isolates, 10.5%), Ia (three isolates, 7.9%), and IV (two isolates, 5.2%). Also, three (7.9%) isolates were non-typeable. Among Alp family genes, *alpha-c* gene was the most prevalent virulence gene detected in cMLSB-GBS isolates (16 of 38, 42.1%). Also, 16 (42.1%) isolates harbored *rib* and *epsilon* genes (each in eight isolates), and only six (15.7%) isolates were positive for *alp2/3* gene. The

distribution of capsular genotypes, antibiotic resistance patterns, and Alp family virulence genes among ERIC types is presented in Table 2.

ERIC-PCR fingerprinting of 38 cMLSB-GBS isolates is presented in Figure 1. Accordingly, the banding pattern showed two to seven bands. Also, four out of 38 isolates did not show any pattern and were non-typeable. The analysis of ERIC PCR patterns revealed eight different ERIC types based on a cut-off point of 80%. Accordingly, 34 cMLSB-GBS isolates were classified into seven clusters (ERIC type A-G) and one single type (type H). The most prevalent ERIC type was type A detected in eight isolates (21%), followed by type B (six isolates, 15.7%), C (five isolates, 13.5%), D and E (each in four isolates, each 10.5%), F and G (each in three isolates, each 7.9%), and H (one isolate) (Figure 1). ERIC type A/ serotype Ib was the most prevalent clone circulating among cMLSB-GBS isolates (six isolates, 15.7%).

Discussion

The increasing prevalence of clindamycin and erythromycin-resistant GBS isolates in recent years has caused concerns regarding the administration of these antibiotics to prevent or treat GBS infections [30]. Erythromycin resistance has been reported to be high particularly in China (74.1%), the USA (54%), and Italy (43.7%) [31]. In this study, among 52 (52%) erythromycin-resistant GBS strains, 73% were identified with cMLSB phenotype, but the frequency of iMLSB and M phenotypes was low. Several studies have also reported the high prevalence rate of the cMLSB phenotype compared to the other phenotypes [32-34]. The present study results confirmed that the main mechanism of erythromycin resistance is ribosomal modification via 23S rRNA-methylases that confer cMLSB and iMLSB phenotypes. Interestingly, the current study results

Table 1) Distribution of Alp family virulence genes, capsular genotypes, and resistance patterns among GBS isolates with iMLSB and M phenotypes

MLSB Phenotype	No. of Isolate	Resistance Pattern	Alp Family Gene	Capsular Type
iMLSB	39	EM, TET	Alp 2/3	III
	40	EM, TET	Rib	III
	41	EM, TET, PG, CPM, CRO, CTX	Alp 2/3	Ib
	42	EM, TET	Alpha-c	V
	43	EM, TET	Rib	III
M phenotype	44	EM, TET	Epsilon	II
	45	EM, TET, PG, CPM, CRO, CTX	Rib	III
	46	EM, TET	Epsilon	III
	47	EM, TET, LEV	Epsilon	V
	48	EM, TET	Epsilon	V
	49	EM, TET	Epsilon	III
	50	EM, TET, LEV	Epsilon	II
	51	EM, TET	Epsilon	V
	52	EM, TET	Alp 2/3	III

TET: tetracycline, EM: erythromycin, LEV: levofloxacin, PG: penicillin, CPM: cefepime CTX: cefotaxime, CRO: ceftriaxone

Table 2) Molecular characterization of 38 cMLSB-GBS isolates

No. of Isolate	Resistance Pattern	Alp Family Gene	Capsular Type	ERIC Type
1	TET, EM, CD	Rib	Ib	A
2	TET, EM, CD	Alpha-c	Ib	A
3	TET, EM, CD, LEV	Epsilon	V	A
4	TET, EM, CD	Alp 2/3	III	A
5	TET, EM, CD	Alp 2/3	Ib	A
6	TET, EM, CD	Alpha-c	Ib	A
7	TET, EM, CD	Alpha-c	Ib	A
8	TET, EM, CD	Alpha-c	Ib	A
9	TET, EM, CD	Epsilon	II	B
10	TET, EM, CD	Alpha-c	II	B
11	TET, EM, CD	Alpha-c	V	B
12	EM, CD	Epsilon	IV	B
13	TET, EM, CD, PG, CPM, CRO, CTX, LEV	Rib	Ib	B
14	TET, EM, CD	Alpha-c	Ia	B
15	EM, CD	Rib	NT	D
16	TET, EM, CD	Alpha-c	Ib	D
17	TET, EM, CD, PG, CPM, CRO, CTX, LEV, VA	Rib	NT	D
18	EM, CD, PG, CPM, CRO, CTX	Epsilon	NT	D
19	TET, EM, CD, LEV	Epsilon	V	Single
20	TET, EM, CD, LEV	Epsilon	II	G
21	TET, EM,CD	Rib	III	G
22	TET, EM, CD	Alpha-c	Ia	G
23	TET, EM, CD	Alpha-c	II	F
24	TET, EM, CD	Alpha-c	Ib	F
25	TET, EM, CD	Alp 2/3	V	F
26	TET, EM, CD	Rib	Ib	C
27	TET, EM, CD	Alpha-c	Ib	C
28	TET, EM, CD	Alpha-c	Ib	C
29	TET, EM, CD	Alp 2/3	V	C
30	TET, EM, CD	Alpha-c	Ib	C
31	TET, EM, CD	Alp 2/3	V	E
32	TET, EM, CD	Alp 2/3	IV	E
33	TET, EM, CD, LEV	Epsilon	V	E
34	TET, EM, CD	Epsilon	Ia	E
35	TET, EM, CD	Alpha-c	Ib	NT
36	TET, EM, CD	Rib	III	NT
37	EM, CD	Rib	III	NT
38	TET, EM, CD	Alpha-c	Ib	NT

CD: clindamycin, VA: vancomycin, TET: tetracycline, EM: erythromycin, LEV: levofloxacin, PG: penicillin, CPM: cefepime, CTX: cefotaxime, CRO: ceftriaxone, NT: non-typeable

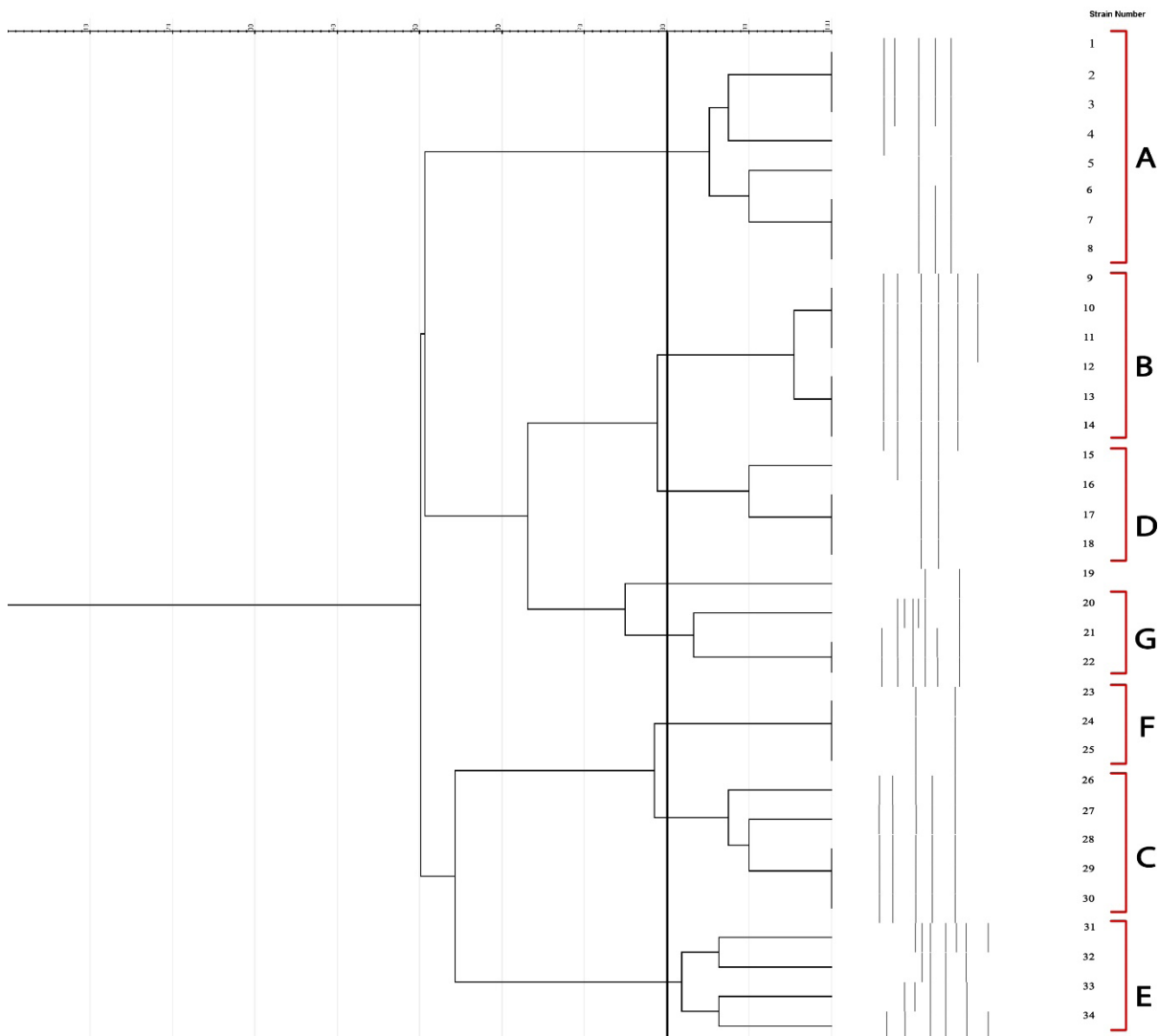


Figure 1) Dendrogram showing the genetic relatedness between ERIC-PCR patterns of 34 group B streptococci strains with cMLSB resistance phenotype. Four isolates were non-typeable.

showed a high prevalence of serotype Ib in cMLSB-GBS isolates. Previous studies have shown that, unlike serotypes III and V, serotype Ib is not a remarkable capsular type among macrolide-resistant GBS isolates in adults. A study in China showed that serotype Ib was in association with levofloxacin resistance [35-38]. However, among our GBS strains with iMLSB and M phenotypes, serotypes III and V were dominant (78.5%). Besides, this study results showed that all of the cMLSB-GBS isolates harbored at least one Alp family virulence gene. Alp family antigens increase the potential of GBS strains to invade host cells and cause severe

infections [4]. Most of the cMLSB-GBS isolates were positive for the presence of *alpha-c* gene. However, among iMLSB-GBS and M-GBS isolates, *epsilon* gene was frequently detected. Regarding the distribution of Alp family genes among different serotypes, this study results revealed that out of 16 erythromycin-resistant GBS isolates with serotype Ib, 12 (75%) isolates harbored *alpha-c* gene. In addition, out of 11 isolates with serotype III, six (54.5%) isolates were positive for *rib* gene. Due to the very small number of GBS isolates examined in this study, no association was found between virulence genes and capsular serotypes or

ERIC types, but a previous study showed an association between serotype Ib and *alpha-c* gene as well as serotype III and *rib* gene [38]. The current study findings showed that capsular type Ib was the most common serotype. Asian countries have the most prevalence of non-pregnant adult diseases attributable to serotype Ib; for example, in a study in South Korea, 22% of GBS isolates causing infection in adults were identified with serotype Ib [39]. However, in several studies in France, the USA, and the United Kingdom, serotype Ia has been reported to be associated with infections in adults [40]. Such differences in distribution could also be observed due to differences in GBS sources like pregnant, neonatal, and adult populations [39].

ERIC-PCR method has a reliable discriminatory power and could be used to identify significant GBS clones causing outbreaks in humans [27]. ERIC profiles of cMLSB-GBS isolates generated a dendrogram with two major clusters with 50% similarity. These two clusters were subdivided into several clusters with different levels of similarity in each. This observed variability suggests the multiclonal spread of the cMLSB phenotype among our GBS isolates. This finding is consistent with the finding of a study in India, in which all GBS isolates were disseminated from one ERIC type E [27]. By reviewing the literature, no information was found about the genetic population of erythromycin or clindamycin-resistant GBS isolates using ERIC-PCR fingerprinting. In this study, ERIC type A was the most prevalent type detected in cMLSB-GBS isolates. Statistical analysis results showed a significant association between serotype Ib and ERIC type A.

Conclusion

The current study, as the first report about the molecular characterization of cMLSB-

GBS isolates using ERIC-PCR, showed that GBS species could be easily fingerprinted by ERIC-PCR technique. The isolates were well-characterized by seven clusters (ERIC type A-G), indicating the multiclonal spread of erythromycin-clindamycin resistant GBS strains. ERIC type A/ serotype Ib is the predominant clone circulating among erythromycin-resistant GBS strains in this region. The predominant distribution of cMLSB and iMLSB phenotypes demonstrates that ribosomal modification via methylases is the major mechanism of erythromycin resistance. It would be helpful to determine genes involved in resistance. Due to the high prevalence of clindamycin and erythromycin resistance among GBS populations, there is a need for molecular characterization and continuous monitoring of antibiotic resistance patterns of GBS isolates to better manage the prophylaxis and treatment of infections and find antibacterial targets unique for resistant GBS isolates.

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Ethical permission: The current study had no human participants, and bacterial strains isolated from clinical samples collected from the microbiology laboratory were used.

Conflict of interest: None.

Authors' Contribution: Conceptualization, methodology supervision, writing, original draft preparation: JS, MT, DS; investigation and software analysis: JS, MR writing, reviewing, and editing: MR, KM; visualization, funding acquisition, and project administration: NEB.

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