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Unveiling the genetic landscape of *Streptococcus agalactiae* bacteremia: emergence of hypervirulent CC1 strains and new CC283 strains in Tehran, Iran

Anis Mohammadi[1,2], Chakameh Amini[3], Parmida Bagheri[4], Zahra Salehi[5] and Mehdi Goudarzi[1,6*]

Abstract

Background The emergence of *Streptococcus agalactiae* infections in patients with bacteremia is increasing. It is crucial to investigate the epidemiology, molecular characteristics, biofilm status, and virulence analysis of *Streptococcus agalactiae* in these patients.

Methods In this cross-sectional study, 61 *S. agalactiae* isolated from blood infection were subjected to characterization through antimicrobial susceptibility tests, biofilm formation, multilocus sequence typing (MLST), and PCR analysis for detecting resistance (*tet* and *erm* family) and virulence genes *(alp2/3*, *alp4*, *bca*, *bac*, *eps*, *rib*, *lmb*, *cylE*, and pilus island genes).

Results Overall, 32.7% of the isolates demonstrated an inducible clindamycin resistance phenotype. The results showed that 49.2, 24.6, and 8.2% of confirmed *Streptococcus agalactiae* strains were classified as strong, intermediate, and weak biofilm-forming strains, respectively. *tet*(M) (57.1%) was recovered most, followed by *tet*(M) + *tet*(L) (14.3%), *tet*(S) + *tet*(K) (10.7%), *tet*(M) + *tet*(K) (8.9%), *tet*(M) + *tet*(K) + *tet*(O) (5.4%), and *tet*(S) + *tet*(L) + *tet*(O) (3.6%). Three virulence gene profiles of *cylE*, *lmb*, *bca*, *rib* (24.6%; 15/61), *cylE*, *lmb*, *rib*, *alp3* (19.7%; 12/61), and *cylE*, *lmb*, *bac*, *rib* (16.4%; 10/61) were detected in approximately two-thirds of the isolates. MLST revealed that the 61 isolates belonged to six clonal complexes, including CC1 (49.2%), followed by CC12 (18%), CC19 (13.1%), CC22 (9.8%), CC17 (6.6%), and CC283 (3.3%), and 11 different sequence types (STs), including ST1 (24.6%), ST7 (14.8%), ST918 (13.1%), ST2118 (9.8%), ST19 (9.8%), ST48 (6.6%), ST1372 (4.9%), ST22 (4.9%), ST40 (4.9%), ST734 (3.3%), and ST283 (3.3%). Remarkably, all CC1 and CC12 isolates, three-fourths of CC19, and half of CC22 were confirmed as biofilm producers. Conversely, CC17 and CC28 isolates were found to be nonproducers. The occurrence of strong biofilm formation was limited to specific CCs, namely CC1 (34.4%), CC12 (8.2%), CC19 (3.3%), and CC22 (3.3%).

Conclusion The high prevalence of CC1 and CC12 clones among *S. agalactiae* strains reflects the emergence of these lineages as successful clones in Iran, which is a serious concern and poses a potential threat to patients.

*Correspondence:
Mehdi Goudarzi
gudarzim@yahoo.com

Full list of author information is available at the end of the article

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Background

Streptococcus agalactiae strains are common residents of the gastrointestinal and genitourinary tracts of humans and cause various infections, such as skin and soft tissue infections, urinary tract infections, endocarditis, pneumonia, meningitis, and bacteremia [1]. Evidence has recently designated *S. agalactiae* isolated from blood infections as a high-priority pathogen with the potential to increase fatality rates globally [2]. Bloodstream infections (BSIs) caused by *S. agalactiae* strains are relatively rare, accounting for only 1–2% of all such infections. Previous studies in European countries have reported an incidence rate of 1.2–4.1 per 100,000 for *S. agalactiae* BSIs, depending on the year and population studied. However, according to a 2016 analysis, the United States seems to have a higher incidence rate, with an estimated 11 cases per 100,000 [3]. In the United States, nonpregnant adults now account for over 75% of invasive *S. agalactiae* cases and approximately 90% of associated deaths. A population-based surveillance conducted in Canada, from 2011 to 2018 revealed an incidence rate of 4.7 per 100,000 for *S. agalactiae* BSIs with an increasing annual incidence of β-hemolytic streptococcal bloodstream infections from 2011 to a peak in 2016, followed by a decrease [4–7].

Reports have also indicated that numerous *S. agalactiae* virulence factors, including surface proteins, adhesion factors, capsules, immune evasion factors, and toxins, are related to the pathogenesis of this bacterium [2, 8–11]. Among these virulence factors, it seems that surface antigens belonging to the alpha-like protein (*Alp*) family and the pilus islands (PIs) significantly contribute to the pathogenesis of this bacterium [12–17]. Alp proteins play a crucial role in the invasion of human epithelial cells by *S. agalactiae* through their interaction with glycosaminoglycans present on the surface of host cells, allowing them to traverse through the cell layer. Pili in *S. agalactiae* have been extensively studied, indicating their significant involvement in adhesion and invasion mechanisms. They have been suggested as promising candidates for vaccines due to their crucial roles in these processes. Evidence commonly regards pili as significant factors in colonization. The pili of *S. agalactiae* are appendages anchored to the bacterial cell wall, extending outward from the surface. Within each gene, there are three structural subunit proteins encoded, which could be considered promising candidates for vaccines [2, 13, 16].

Information on molecular characteristics of *S. agalactiae* related to bacteremia is very important. The epidemiological studies are imperative to monitor the possible dissemination of these strains and to adopt rational infection control strategies for the prevention of sequential outbreaks [10, 12, 14–16]. Various *S. agalactiae* population epidemiology-based studies have identified five main genetically unique lineages, namely CC1, CC10, CC17, CC19, and CC23. These lineages are commonly associated with both invasive disease and asymptomatic colonization in individuals of different age groups. Earlier evidence indicated that ST17 and ST19 strains were more frequently associated with invasive disease. However, in adult individuals with invasive infections caused by *S. agalactiae*, CC17 strains are less commonly identified compared to CC19 [7, 18–20]. Molecular epidemiology studies have indicated various clonal distributions of invasive *S. agalactiae* strains. For example, CC1 was the most frequently identified invasive type in Taiwan [18], CC23 in East African [19], CC19 in China [8], CC17 in Japan [7], CC10 in Ethiopia [20], and CC17 in Poland [21]. According to the earlier data, the major invasive *S. agalactiae* clone found in Iranian hospitals was the CC/ST19 clone [22]. Although several studies have recently focused on understanding the serotypes and antibiotic resistance pattern of *S. agalactiae* isolated from clinical samples in Iran, there is very little information about genetic diversity and biofilm formability of *S. agalactiae* isolated from BSIs. Therefore, it is crucial to gain insight into the molecular characteristics of this bacterium. This study attempted to identify the biofilm production, virulence determinants, antibiotic resistance genes, and genetic variability of *S. agalactiae* strains isolated from blood infections.

Results

Identification and antibacterial susceptibility pattern

In the current study, 61 clinical isolates of *S. agalactiae* obtained from BSIs were used. The samples were collected from patients with an age range of 18–65 years. The mean age of the patients was 39.2 years. The results revealed that all isolates displayed sensitivity toward vancomycin and penicillin. Overall, resistance to all of the antibacterial agents was not detected among the tested isolates. The majority of isolates (91.8%) were resistant to tetracycline. This was followed by erythromycin (86.9%), chloramphenicol (67.2%), clindamycin (42.6%), levofloxacin (19.7%), and linezolid (13.1%). In total, 77% (47/61) of isolates were found to be multidrug resistance (MDR). We found eleven resistance patterns in the examined isolates, wherein erythromycin, tetracycline, clindamycin, chloramphenicol (19.7%; 12/61), erythromycin, tetracycline, chloramphenicol (19.7%; 12/61),

Table 1 Antibiotic resistance patterns of 61 *S. agalactiae* isolated from blood infection

Number of antibiotics	Antibiotic resistance patterns†	*N* (%)
6	ERY, TET, CHL, LIN, LEV, CLI	3 (4.9)
5	ERY, LEV, LIN, TET, CHL	3 (4.9)
	ERY, TET, CHL, CLI, LIN	1(1.6)
4	ERY, TET, CLI, CHL	12 (19.7)
3	ERY, TET, CHL	12 (19.7)
	ERY, TET, CLI	10 (16.4)
	ERY, TET, LEV	5 (8.2)
	CHL, LIN, LEV	1 (1.6)
2	ERY, TET	4 (6.6)
	ERY, CHL	3 (4.9)
	TET, CHL	6 (9.9)
-	Without resistance	1 (1.6)

†ERY, erythromycin; TET, tetracycline; CLI, clindamycin; LEV, levofloxacin; LIN, linezolid; CHL, chloramphenicol

Table 2 Categorization of biofilm production of *S. agalactiae* strains isolated from blood

Average OD range†	Biofilm degree	Number (%)
>0.932	Strong producer	30 (49.2)
0.520< OD ≤ 0.932	Moderate producer	15 (24.6)
0.342 < OD ≤ 0.520	Weak producer	5 (8.2)
≤ 0.342	Non-producers	11 (18)

†Optical density

Table 3 Distribution of resistance pattern among non-biofilm and biofilm producer *S. Agalactiae* isolates

Biofilm status	Phenotypic resistance† (No; %)	Number (%)
Strong producer	ERY, TET, CHL, LIN, LEV, CLI (3; 10)	30 (49.2)
	ERY, LEV, LIN, TET, CHL (3; 10)	
	ERY, TET, CHL, CLI, LIN (1; 3.3)	
	ERY, TET, CLI, CHL (2; 6.7)	
	ERY, TET, CLI (5; 16.7)	
	ERY, TET, LEV (3; 10)	
	ERY, TET, CHL (7; 23.3)	
	ERY, CHL (2; 6.7)	
	ERY, TET (3; 10)	
	TET, CHL (1; 3.3)	
Moderate producer	ERY, TET, CLI, CHL (5; 33.3)	15 (24.6)
	ERY, TET, LEV (2; 13.3)	
	ERY, TET, CLI (1; 6.7)	
	ERY, TET, CHL (5; 33.3)	
	ERY, CHL (1; 6.7)	
	ERY, TET (1; 6.7)	
Weak producer	ERY, TET, CLI, CHL (4; 80)	5 (8.2)
	TET, CHL (1; 207)	
Non-producer	ERY, TET, CLI, CHL (1; 9.1)	11 (18)
	ERY, TET, CLI (4; 36.4)	
	CHL, LIN, LEV (1; 9.1)	
	TET, CHL (4; 36.4)	
	Without resistance (1; 9.1)	

†ERY, erythromycin; TET, tetracycline; CLI, clindamycin; LEV, levofloxacin; LIN, linezolid; CHL, chloramphenicol

erythromycin, tetracycline, clindamycin (16.4%; 10/61), tetracycline, chloramphenicol (9.9%; 6/61), and erythromycin, tetracycline, levofloxacin (8.2%; 5/61) were the top five frequently detected profiles (Table 1). In the current research, 20 (32.7%) of the isolates were confirmed as clindamycin resistance (ICR). Constitutive clindamycin-resistant (CCR) isolates were detected at a prevalence rate of 42.6%.

Biofilm formation

By the microtiter plate (MtP) assay, 50 isolates (82%) were found to be able to produce biofilms to different degrees. Eleven isolates (18%) did not have the ability to form biofilms and were classified as non-biofilm producers. Table 2 gives information about different degrees of biofilm production in the *S. agalactiae* isolates tested.

In Table 3, the highest resistance rate among strong biofilm producers belonged to erythromycin (96.7%; 29/30) and tetracycline (93.3%; 28/30), while the lowest resistance rate was recorded for levofloxacin (30%; 9/30) and linezolid (23.3%; 7/30). One isolate, which showed susceptibility to all tested antibiotics, did not produce biofilm. However, all of the weak producer isolates were resistant to tetracycline and chloramphenicol.

Resistance gene detection

Table 4 depicts the prevalence of resistance genes in clinical isolates of *S. agalactiae*. In this investigation, the findings indicated that all erythromycin-resistant *S. agalactiae* carried at least one macrolide resistance gene. Resistance to macrolides was mainly due to *erm*(B), which was contained in 39 isolates (63.9%), followed by *erm*(A) in 20 isolates (32.8%) and *mef*(A/E) in 14 isolates (22.9%). Among the 20 ICR *S. agalactiae* strains, 11 isolates (55%) carried both *erm*(B)+*erm*(A), 5 isolates (25%) carried *erm*(A) alone, and 4 isolates (20%) carried *erm*(B) alone, while in 26 *S. agalactiae* isolates with simultaneous resistance to clindamycin and erythromycin, 15 isolates (57.7%) carried *erm*(B) alone, one isolate (3.8%) carried *mef*(A/E) alone, one isolate (3.8%) carried *erm*(A) alone, 7 isolates (27%) carried *erm*(B)+*mef*(A/E), and two isolates (7.7%) carried *erm*(A)+*erm*(B). Regarding 56 tetracycline-resistant isolates, the results indicated that *tet*(M) (57.1%) was recovered the most, followed by *tet*(M)+*tet*(L) (14.3%), *tet*(S)+*tet*(K) (10.7%), *tet*(M)+*tet*(K) (8.9%), *tet*(M)+*tet*(K)+*tet*(O) (5.4%), and *tet*(S)+*tet*(L)+*tet*(O) (3.6%). At least one *tet* gene was identified in each tetracycline-resistant isolate.

Pilus island and virulence gene detection

Our analysis indicated that among the 61 *S. agalactiae* strains, 40 isolates (65.6%) carried the *rib* gene, 29 isolates (47.5%) carried *bca*, 24 isolates (39.3%) carried *apl2*, 22 isolates (36.1%) carried *alp3*, 14 isolates (23%) carried

Table 4 Distribution of PI-encoding genes in different molecular types of *S. agalactiae* strains isolated from blood infection

CC	ST	Pilus island genes (*N*; %)	Total *N* (%)
CC1 (30; 49.2)	ST1	PI-1 + PI-2a (10; 66.6), PI-1 + PI-2b (3; 20), PI-2a (1; 6.7), PI-2b (1; 6.7)	15 (24.6)
	ST7	PI-1 + PI-2a (5; 55.6), PI-1 + PI-2b (2; 22.2), PI-2a (1; 11.1), PI-2b (1; 11.1)	9 (14.8)
	ST2118	PI-1 + PI-2a (6; 100)	6 (9.8)
CC12 (11; 18)	ST918	PI-1 + PI-2a (5; 62.5), PI-1 + PI-2b (2; 25), PI-2b (1; 12.5)	8 (13.1)
	ST1372	PI-1 + PI-2a (2; 66.7), PI-2b (1; 33.3)	3 (4.9)
CC19 (8; 13.1)	ST19	PI-1 + PI-2a (6; 100)	6 (9.8)
	ST734	PI-1 + PI-2a (2; 100)	2 (3.3)
CC22 (6; 9.8)	ST22	PI-1 + PI-2b (2; 66.7), PI-2a (1; 33.3)	3 (4.9)
	ST40	PI-1 + PI-2b (2; 66.7), PI-2b (1; 33.3)	3 (4.9)
CC17 (4; 6.6)	ST48	PI-1 + PI-2b (3; 75), PI-2a (1; 25)	4 (6.6)
CC283 (2; 3.3)	ST283	PI-1 + PI-2b (1; 75), PI-2b (1; 25)	2 (3.3)

Table 5 Distribution of antibiotic resistance genes in molecular types of *S. agalactiae*

ST	Resistance genes *N* (%)								Total *N* (%)
	erm(A)	erm(B)	tet(M)	tet(K)	tet(S)	tet(L)	tet(O)	mef(A/E)	
ST1	6 (40)	8 (53.3)	15 (100)	1 (6.7)	-	5 (33.3)	1 (6.7)	6 (40)	15 (24.6)
ST7	8 (88.9)	4 (44.4)	9 (100)	1 (11.1)	-	2 (22.2)	1 (11.1)	-	9 (14.8)
ST2118	5 (83.3)	4 (66.7)	2 (33.3)	-	-	2 (33.3)	1 (16.7)	1 (16.7)	6 (9.8)
ST918	-	8 (100)	8 (100)	1 (12.5)	-	-	-	7 (87.5)	8 (13.1)
ST1372	-	3 (100)	3 (100)	1 (33.3)	-	-	-	-	3 (4.9)
ST19	-	6 (100)	-	6 (100)	6 (100)	-	-	-	6 (9.8)
ST734	-	-	2 (100)	2 (100)	-	-	-	-	2 (3.3)
ST22	1 (33.3)	3 (100)	3 (100)	1 (33.3)	-	-	-	-	3 (4.9)
ST40		3 (100)	2 (66.7)	-	1 (33.3)	1 (33.3)	1 (33.3)	-	3 (4.9)
ST48	-	-	3 (75)	1 (25)	-	-	1 (25)	-	4 (6.6)
ST283	-	-	1 (50)	-	-	-	-	-	2 (3.3)

bac, and 3 isolates (4.9%) carried *alp4*. Simultaneous carriage of the *cylE* and *lmb* genes was confirmed in all tested isolates. We identified seven different patterns of virulence gene combinations, wherein *cylE*, *lmb*, *bca*, *rib* (24.6%; 15/61), *cylE*, *lmb*, *rib*, *alp3* (19.7%; 12/61), *cylE*, *lmb*, *bac*, *rib* (16.4%; 10/61), *cylE*, *lmb*, *alp2/3* (16.4%; 10/61), and *cylE*, *lmb*, *bca*, *alp2* (16.4%; 10/61) were the top five identified patterns. Screening of PI-encoding genes showed that the combination pattern PI-1+PI-2a (59%; 36/61) was detected most frequently, followed by PI-1+PI-2a (24.6%; 15/61), whereas the carriage rate of PI-2b or PI-2a alone was found in 6 (9.8%) and 4 (6.6%) isolates, respectively.

MLST

In the current research, all *S. agalactiae* strains were successfully typed using the MLST technique. A total of eleven particular sequence types (STs), including ST1 (24.6%; 15/61), ST7 (14.8%; 9/61), ST918 (13.1%; 8/61), ST2118 (9.8%; 6/61), ST19 (9.8%; 6/61), ST48 (6.6%; 4/61), ST1372 (4.9%; 3/61), ST22 (4.9%; 3/61), ST40 (4.9%; 3/61), ST734 (3.3%; 2/61), and ST283 (3.3%; 2/61), were identified and categorized into six clonal complexes (CCs). As presented in Table 4, the most prevalent CC was CC1 (49.2%; 30/61), followed by CC12 (18%; 11/61), CC19 (13.1%; 8/61), CC22 (9.8%; 6/61), CC17 (6.6%; 4/61), and CC283 (3.3%; 2/61). CC1, CC12, CC19, and CC22 represented different STs. Table 4 shows that ICR *S. agalactiae* strains were distributed to three particular STs, including ST1 (55; 11/20), ST7 (30; 6/20), and ST2118 (15; 3/20), all of which belonged to CC1. Overall, the most common STs in CCR *S. agalactiae* isolates included ST2118 (11.5%; 3/26), ST918 (30.8%; 8/26), ST1372 (11.5%; 3/26), ST19 (23.1%; 6/26), ST734 (7.7%; 2/26), ST22 (11.5%; 3/26), and ST40 (3.9%; 1/26).

Isolates simultaneously harboring *tet*(M), *tet*(K), and *tet*(O) belonged to ST1, ST7, and ST48 (one isolate each). The findings also showed that the isolates carrying *tet*(S)+*tet*(L)+*tet*(O) belonged to ST2118 and ST40 (one isolate each). Isolates harboring *tet*(M)+*tet*(L) genes were mainly found in ST1 (5 isolates), ST7 (2 isolates), and ST2118 (one isolate). Isolates harboring *tet*(M)+*tet*(K) simultaneously belonged to ST734 ($n=2$), ST918 ($n=1$), ST1372 ($n=1$), and ST22 ($n=1$). Overall, macrolide resistance genes were strongly linked with specific STs. *erm*(B)+*erm*(A) were present in ST1 ($n=5$), ST2118 ($n=4$), ST7 ($n=3$), and ST22 ($n=1$). All 7 isolates carrying *erm*(B)+*mef*(A/E) belonged to ST918. The results are presented in Table 5.

Variability in PI types was observed within the majority of the STs, but some STs were also strongly linked with specific STs, such as ST2118, ST19, and ST734 with PI-1+PI-2. The distribution of biofilm formation in different CCs of *S. agalactiae* strains is presented in Fig. 1. Notably, all CC1 and CC12, three-fourths of CC19, and half of CC22 isolates were confirmed as biofilm producers, while all CC17 and CC28 isolates were found to be nonproducers. Strong biofilm formation was exclusive to ST1 (30%; 9/30), ST7 (23.3%; 7/30), ST2118 (16.6%; 5/30), ST918 (10%; 3/30), ST19 (6.7%; 2/30), ST40 (6.7%; 2/30), and ST1372 (6.7%; 2/30). As stated earlier, among 61 *S. agalactiae* strains isolated from blood infection, seven virulence patterns were identified.

Phylogenetic analysis

The consensus tree was constructed by the Maximum Likelihood Analysis from the combined seven loci *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt* of 11 STs of *S. agalactiae* isolated from blood infection. We chose one representative strain from each ST for phylogenetic analysis. All STs in this study were placed in separate clades. The ST2118 clade takes a basal position relative to the other clades. Bootstrap branch supports above 75% are shown (Fig. 1). Also, Fig. 1 indicates that *cylE*, *lmb*, *rib*, *alp3* was the most prevalent profile among ST1 and ST7 isolates; *cylE*, *lmb*, *bac*, *rib* in ST918 isolates; *cylE*, *lmb*, *alp2/3* in ST1372, ST40, and ST22 isolates; *cylE*, *lmb*, *bca*, *rib* in ST19 isolates; and *cylE*, *lmb*, *bca*, *alp2* in ST48 isolates.

Discussion

To our knowledge, the molecular characteristics of *S. agalactiae* associated with blood infection, as well as its biofilm production, virulence, and adhesion factors, remain unexplored in Iran. Based on our current knowledge, this is the first document to reveal the genetic variability, biofilm production, and virulence detection of *S. agalactiae* strain isolates from blood infections in Iran.

The *S. agalactiae* isolates with the CCR phenotype (42.6%) were higher than isolates with the ICR phenotype (32.7%). Our findings, supported by evidence from other studies [13], indicate an increase in resistance to clindamycin in *S. agalactiae* worldwide. Possible explanations for this increased prevalence are the genetic diversity of bacteria, excessive consumption of this antibiotic, abuse of antibiotics, self-treatment, and the transmission of resistant strains from hospitals and communities, and vice versa.

In line with some previous studies that indicated PI-1 and PI-2a as the most prevalent PI family genes in *S. agalactiae* strains, we noticed a high percentage of PI-1 (83.6%), followed by PI-2a (65.6%), and PI-2b (36.1%) among our strains [9, 16]. We showed that the combination pattern of PI genes was greater than that of PI genes

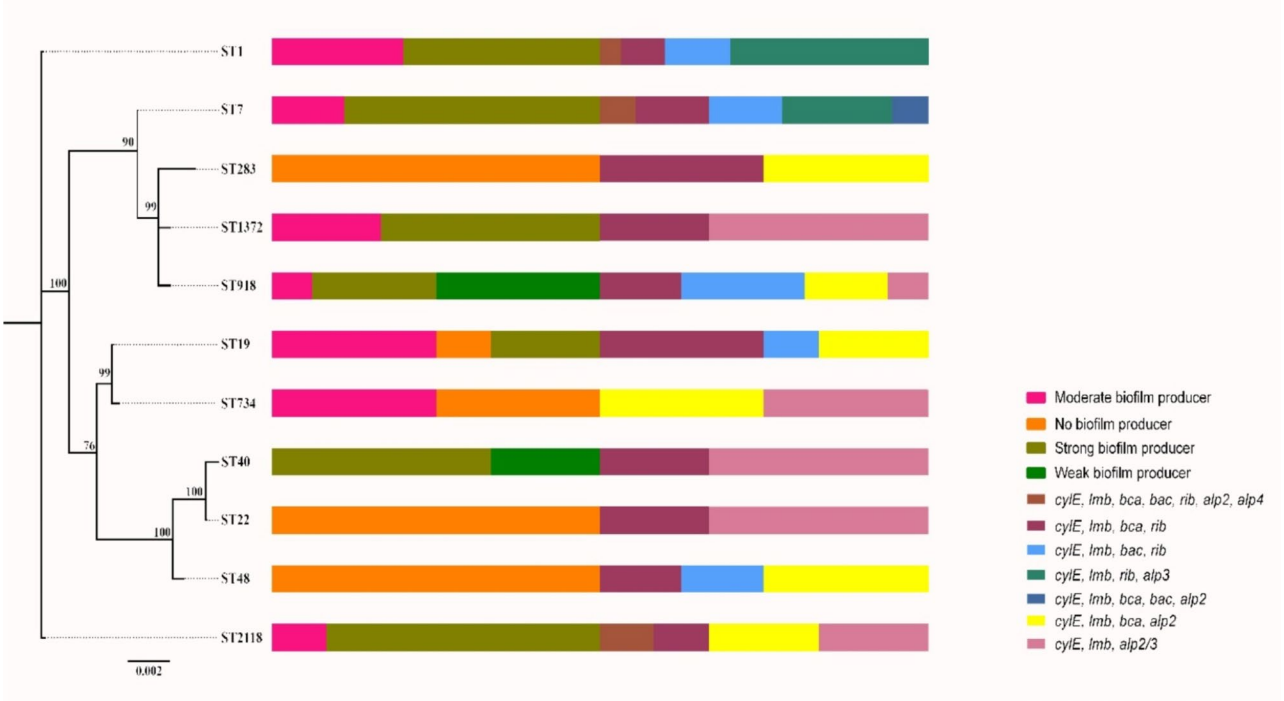

Fig. 1 The phylogenetic analysis of 11 STs of *S. agalactiae* strains. The consensus trees were constructed by the RAxML analysis of the combined seven loci *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*. The phylogeny was constructed with 1,000 bootstrap replications. Bootstrap branch supports above 75% are shown. Also, the distribution of biofilm formation and the distribution of different virulence genes have been shown in STs of *S. agalactiae* isolated from blood infection

alone. In similar surveys conducted in China [9] and Ireland [23], a high prevalence of PI genes in combination compared to PI genes alone was also reported. In a study conducted in Africa, Madzivhandila et al. found similar results, reported that PI1+PI2b, PI2a, PI1+PI2a, and PI2b were observed in 45.1%, 29.8%, 24.8%, and 0.2% of isolates, respectively. Our research indicated that all isolates harbored at least one of the PI genes. Based on our findings, all the CC19 isolates were found to carry PI-1+PI-2a. Additionally, the majority of CC22 (66.7%), CC17 (75%), and CC283 (50%) isolates harbored PI-1+PI-2b. This result is in agreement with Khan et al.'s study, which reported the presence of at least one of the PI genes in *S. agalactiae*, with the majority of them being PI-1+PI-2a. This study also reported that the PI-1+PI-2b combination was exclusively found in CC17 isolates [3].

The main finding of the present research, based on the phylogenetic analysis, was the genetic variability of the tested strains with a high prevalence of CC1 (49.2%), which corresponded to ST1, ST7, and ST2118. All the strains of this clone were biofilm producers, had ICR phenotypes, and were positive for PI-1+PI-2a (70%), PI-1+PI-2b (16.6%), PI-2a (6.7%), and PI-2b (6.7%). According to our data, the *tet*(M) gene, as the most widespread *tet* family gene among CC1 isolates, was detected in 86.7% of isolates, followed by *tet*(L) (30%), *tet*(O) (10%), and *tet*(K) (6.79%). This clone was also reported in *S. agalactiae* strains obtained from Iran (5.3%) [24], China (13%) [17], and Sweden (10.2%) [11]. According to the evidence, there is an expected correlation between the virulence gene and specific STs. Our observations indicated that *alp3* was only detected in CC1 strains. All of the CC1 isolates were found to carry *cylE* and *lmb* genes. This finding is in line with research conducted in the UK by Khan et al. that reported the predominance of *alp3* in CC1 isolates [3]. In the research conducted by Rojo-Bezares et al. on 375 *S. agalactiae* strains isolated from pregnant women, *alp2*/3 and *lmb* genes were detected in all of the CC1 isolates [25]. In a study conducted in Spain, 16.8% of *S. agalactiae* from postmenopausal women exhibited CC1/ST1 and were all resistant to erythromycin and clindamycin [12]. Recently, a study conducted in the USA reported the presence of CC1 in 12% of the examined isolates, which was identified to have PI-1+PI-2a as the most frequent PI type, followed by PI-1+PI-2b, PI-2a, and PI-2b [16]. A similar survey conducted by Khan et al. in the UK on 192 *S. agalactiae* isolates (179 invasive and 13 non-invasive) showed that 91.4% of CC1 isolates were tetracycline resistant, and 82.9% carried *tet*(M), and 3 isolates carried *tet*(O), *tet*(S) and *tet*(W) each [3]. Overall, we found that 63.3% and 53.3% of CC1 *S. agalactiae* isolates harbored *erm*(A) and *erm*(B), respectively. In similar surveys conducted in UK, the carriage rates of *erm*(A) and *erm*(B) among *S. agalactiae* isolated from various clinical specimens were found to be 45.4% and 54.5%, respectively [3]. This controversy about the carriage of *erm* family genes may be due to the irrational prescribing or consumption of these antibiotics, public health policy, different sources of recovery of resistant *S. agalactiae*, or the possible exchange of *erm* and *mef*(A/E) genes among different *S. agalactiae* strains through the transfer of mobile genetic elements. Further investigation is required to validate the claims that the high prevalence of PIs, biofilm formation, and adhesion genes in the CC1 clone may play a significant role in its ability to colonize human cells. These assertions are currently speculative and necessitate verification. Therefore, additional research is necessary to comprehend the underlying factors that contribute to the adherence and colonization of human cells exhibited by the CC1 clone. However, it was hypothesized that the CC1 clone would have a higher likelihood of being identified in BSIs related to S. *agalactiae* in Iranian hospitals.

In the present study, CC12, which corresponded to ST918 and ST1372, was the second most frequently detected clone with simultaneous resistance to erythromycin and clindamycin and biofilm production. This confirms the previous results of Emaneini et al. in Iran, who showed CC12 in 10.5% of *S. agalactiae* isolated from newborns in intensive care units with full resistance to erythromycin, tetracycline, and clindamycin and carriage of *scpB*, *hlyB*, *bca*, and *bac* virulence genes [24]. A recent report by Moltó-García from Spain showed the emergence of ST12 in *S. agalactiae* from postmenopausal women at a low level (1.9%) [12]. These results coincide with those produced by Jiang et al., who reported ST12 as the third most predominant type of *S. agalactiae* causing community-onset and nosocomial infections in non-pregnant adults. They indicated that all the isolates were resistant to both erythromycin and clindamycin and showed the CCR phenotype [8]. Additionally, in a study conducted by Liu and colleagues in China in 2021, it was reported that CC12 was the most common clone of *S. agalactiae* isolated from blood infections, accounting for 86.7% of cases. They found that CC12 exhibited similarities to CC10 and CC1 in terms of virulence gene profiles. Liu et al. demonstrated that CC12 is an emerging clone of *S. agalactiae* that is both highly virulent and resistant to multiple drugs. This clone was found to be responsible for causing invasive and often fatal infections in pediatric patients [26]. In the current study, the high prevalence of *bac* in CC12 strains is in line with a study from Spain that indicated the presence of *alphaC*+*bac* in 88.9% of ST12 strains [25]. Consistent with earlier research, we found that CC12 isolates were linked to a high percentage of adhesion, PIs, and antibiotic resistance-related genes. This characteristic can enhance its ability to survive in

the hospital setting and potentially lead to more severe illnesses in patients.

The current research highlights the emergence of CC19, which corresponded to two STs, including ST19 and ST734. This CC has been the predominant clone in many areas of the world. For example, a report on 60 *S. agalactiae* from Ethiopia highlighted CC19 (18.8%) as the second predominant type among tested isolates [20]. Similarly, Emaneini et al. in Iran reported CC19 as the most prevalent CC among *S. agalactiae* isolated from neonates (68.4%) [24]. Current findings in relation to the detection of *rib* in CC19 strains (50%) are in line with the study of Khan et al. in the UK, which indicated the *rib* gene was detected in the majority of CC19 (79.3%) isolates [3]. This result is similar to that of the study by Rojo-Bezares et al., who indicated that the *rib* gene was present in all CC19 strains (ST19, ST28, ST110, ST335, and ST601) [25]. This CC with a high frequency of the CCR phenotype and full resistance to tetracycline with a different carriage rate of the *tet*(K) (100%), *tet*(M) (25%), *tet*(S) (75%), and *erm*(B) (75%) genes was also reported in studies from different countries, including Poland (17%), Sweden (18.7%), Malaysia (8%), China (30.6%), and Taiwan (7.1%) [11, 18, 21, 27]. A study conducted by Khan et al. from the UK reported a prevalence rate of 78.6% of *tet*(M) among tetracycline-resistant *S. agalactiae* [3]. The differences in *tet* prevalence might be due to differences in the expression or transmissibility of these genes, but further investigation is needed to elucidate the underlying reasons for these differences. In similar surveys conducted in Portugal, an association between macrolide resistance and the CC19 lineages was noted [28]. These discrepancies among CC19 may reflect differences in the horizontal transfer of macrolide- and tetracycline resistance genes. Since CC19 might be highly pathogenic, careful attention is required to avoid possible problems and the spread of this strain.

The fourth known predominant genotype in the present research was CC22 (ST22 and ST0). Approximately 70% of these strains had PI-1+PI-2b and CCR phenotypes; half of the strains produced biofilms, and all isolates carried tetracycline and erythromycin resistance genes. In concordance with our analysis, the presence of this clone, along with tetracycline resistance genes and CCR phenotypes, has been described by several investigators [29, 30]. To date, there have been few reports on the detection of CC22, and this is the first report to present clinically relevant data. This clone is suspected to be prevalent in a limited area.

According to published data, CC17 is a successful invasive hypervirulent clone that is distributed in different parts of the world. In the present survey, this clone was detected in bloodstream infections at a low level (6.6%), which is in agreement with Strakova et al.'s study that reported ST17 in CSF (85%) and blood (32%) isolates [31]. Likewise, a study by Bohnsack et al., conducted in the USA, displayed a strong correlation between CC17 and invasive infections, while this correlation was not detected in CC23 strains [32]. According to the evidence, ST17 and ST19 strains were most frequently detected in invasive infections, which confirms our results in the present research [33, 34]. We detected *rib* gene in 50% of CC17 isolates. This result is similar to that of the UK study by Khan et al., who indicated that the *rib* gene was the predominant virulence gene in *S. agalactiae* strains (100%) [3]. According to the study results of Rojo-Bezares et al., in Spain, the *rib* gene was detected in all of CC17 *S. agalactiae* isolates [25]. In line with findings reported from China, Poland, Kenya, Ethiopia, and Taiwan [10, 18–20], we noticed a high antibiotic resistance rate and no biofilm formation ability in CC17 strains. For instance, out of 4 CC17 isolates, none were biofilm producers; approximately 75% of isolates carried *tet*(M), 25% carried *tet*(O), and *tet*(K), while ICR and CCR phenotypes and carriage of erm family genes were not detected in any of the tested isolates. Khan and colleagues conducted an epidemiological study on 193 *S. agalactiae* strains obtained from clinical sources. They discovered that all CC17 isolates showed resistance to tetracycline. Among these isolates, 90.6% carried the *tet*(M) gene, while 9.3% carried the *tet*(O) gene [3]. The findings from the aforementioned studies align with our research, which confirms the diversity of these strains.

For the first time in Iran, we explored the emergence of CC/ST283 *S. agalactiae*-related bacteremia (3.3%). This study also found that CC/ST283 isolates did not have any ability to form biofilms, while PI-1+PI-2b (50%) and aPI-2b (50%) were detected among the isolates. Although the first report of the emergence of this clone dates from 1985, the human outbreak of fish-borne *S. agalactiae* was first reported in 2015 in Singapore, with the CC/ST283 clone accounting for 70% of the outbreak [35]. Subsequent reports from China, France, and Thailand confirmed an increasing prevalence of this CC in Asia due to the consumption of undercooked aquaculture foods. According to the evidence, CC283 has been widespread in Southeast Asia for over two decades in humans [35, 36]. Therefore, it is predicted that this clone would be more likely to be frequently detected in Asia since the consumption of raw seafood, especially tilapia, is common in this region. Syuhada and colleagues conducted an epidemiological study on 256 *S. agalactiae* strains obtained from tilapia in Malaysia. The researchers examined the virulence gene profiles of these isolates. Their findings revealed that ST7 and ST283 were the most prevalent clones. They indicated that ST283 had higher virulence compared to ST7, as it carried a greater

number of virulence genes, including *cspA*, *bac*, *fbsB*, *lmb*, *bca*, *pavA*, *cyl*, *scpB*, and others [37].

Contrary to expectations, the detection of the CC283 clone in Iran was rare; however, the results of this study suggest that further studies should be performed to complete the epidemiological picture of this clone.

The complex and diverse carriage of adhesion and alpha-protein-like genes in different CCs of *S. agalactiae* strains may be linked to the variability in the expression of the aforementioned factors, the possibility of transfer through mobile genetic elements, and their exclusive presence in different clones. Regarding the presence of *alp* genes in *S. agalactiae* strains, both the present and prior studies suggest the vital role of these genes in the pathogenesis of bacteria and their potential to be promising vaccine candidates. To our knowledge, this study is the first report showing the genetic diversity of *S. agalactiae* isolated from bloodstream infections. Overall, our findings indicate that there is genetic diversity with a strong ability for biofilm formation and the simultaneous carriage of resistance genes in clonal lineages. The observed genetic variability accentuates the potentially significant health risk posed by *S. agalactiae* in patients with bacteremia. Since CC1 and CC12, as successful clones in patients with bacteremia, are highly pathogenic and could cause a worse prognosis, careful attention to the spread of these clones is needed.

Materials and methods

Study design and identification of bacterial isolates

This cross-sectional study was conducted at the Department of Microbiology, Shahid Beheshti University of Medical Sciences (SBMU) over a duration of two years from February 2021 to January 2023. This study was performed at the Affiliated Hospitals of Shahid Beheshti University of Medical Sciences, Tehran, Iran. The Medical Ethics Committee of Shahid Beheshti University of Medical Sciences approved the study. All methods were performed in accordance with the relevant guidelines and regulations. BSI was defined as the presence of at least one positive blood culture for *S. agalactiae* in patients exhibiting signs of probable infection. For patients with persistent BSIs caused by the same organism, only the initial episode was included. If patients experienced multiple episodes of BSIs during the same admission period, only the first infection episode was counted for prevalence analysis, but subsequent episodes were included for analysis of microbiological characteristics and clinical outcomes. A total of 61 *S. agalactiae strains* causing blood infection were included in the present study. These samples were streaked on blood agar containing 5% sheep blood (BA, Oxoid, UK) for *S. agalactiae* isolation. Briefly, all suspected isolates from blood agar were gram-stained and tested for standard biochemical assays (sodium hippurate hydrolysis, catalase, and CAMP test) [8, 9]. All phenotypically confirmed *S. agalactiae* isolates underwent polymerase chain reaction (PCR) assay for *atr* gene detection and final confirmation. The sequences (5′ to 3′) of the oligonucleotide primers were: Forward-CA ACGATTCTCTCAGCTTTGTTAA and reverse-TAA GAAATCTCTTGTGCGGATTTC [38]. The protocol of this study received approval from the Ethics Committee of the Shahid Beheshti University of Medical Sciences in Tehran, Iran (IR.SBMU.MSP.REC.1399.623). Informed consent was obtained from all of the participants in the study.

Determination of minimum inhibitory concentrations (MICs) and macrolide resistance phenotypes

For antimicrobial susceptibility testing of the isolated *S. agalactiae*, the microbroth dilution method was applied using commercially available antibiotic powders, including erythromycin, vancomycin, linezolid, penicillin, clindamycin, tetracycline, chloramphenicol, and levofloxacin (Sigma–Aldrich, St. Louis, Mo), according to the Clinical and Laboratory Standards Institute (CLSI) methodology. The results were interpreted as recommended by the CLSI guidelines (CLSI 2022). The identification of ICR in *S. agalactiae* isolates was performed using a broth microdilution test (0.5 µg/mL clindamycin and 1 µg/mL erythromycin). Isolates with resistance to both clindamycin and erythromycin were considered CCR strains. Any growth in wells containing erythromycin and clindamycin was considered a positive test. MDR of *S. agalactiae* strains was specified as resistance to ≥3 classes of antibacterial agents, as previously explained [13, 39, 40]. The experiment was controlled using the *Streptococcus pneumoniae* ATCC 49,619 reference strain.

Evaluation of biofilm formation

Biofilm formation of *S. agalactiae* was performed by MtP assay, according to Goudarzi et al.'s previous research [39]. First, the *S. agalactiae* isolates were cultured in trypticase soy broth (TSB, Merck, Darmstadt, Germany) with 1% glucose and subsequently incubated at 37 °C overnight. The broth dilution was then performed at a ratio of 1:100. Next, two hundred microliters of the diluted cultures were inoculated in a sterile 96-well flat bottom polystyrene microtiter plate (Greiner Bio-One International, Kremsmunster, Austria) and subsequently incubated for 24 h at 37 °C without shaking. Then, the plate was air dried, and the wells were washed three times with 200 µl of phosphate-buffered saline (PBS; pH 7.2) to discard all nonadherent bacteria. Bacterial cells adhering to the slides were stained with 0.1% safranin after being fixed with 99% methanol. To remove the excess stain, the plate was rinsed under running tap water. After drying, 1 mL of 95% ethanol was added per well to solubilize the

adherent stain. TSB containing 1% glucose was used as a negative control. An ELISA plate reader (plate reader, model–A4, serial no.-1910, Das, Italy) was used to measure the optical density (OD) at 490 nm. A cutoff value (ODc) was determined for each microtiter plate according to the following formula: ODc=average OD of negative control + (3× standard deviation (SD) of negative control). The resulting OD value of a tested strain was calculated by subtracting the ODc value from the average OD value of the strain (OD=average OD of a strain - ODc) [41]. In the present study, negative- and positive-biofilm controls were the wells that initially contained medium without any bacteria (non-inoculated) and wells with a biofilm-producing bacterium (*S. agalactiae* ATCC 13813), respectively.

Extraction of genomic DNA and detection of resistance determinants

According to a prior description, the phenol–chloroform assay was used to extract the total genomic DNA of all the isolates. All isolates underwent a PCR assay for the presence of resistance determinants, *erm*(A), *mef*(A/E), and *erm*(B), as macrolide-mediated resistance genes and *tet*(S), *tet*(K), *tet*(M), *tet*(L), and *tet*(O) as tetracycline resistance genes, using specific oligonucleotides described by Palmeiro et al. [24, 42, 43]. The amplification products were electrophoresed on a 1.2–1.8% agarose gel.

Identification of alpha-protein-like and pilus island genes

All isolates were screened for the presence of *alp2/3* (C alpha-like protein 2/3), *alp4*, *bca* (alpha C protein), *eps* (epsilon protein-encoding gene), and *rib* (cell surface rib protein) genes by multiplex PCR to detect alpha-protein-like and pilus island genes using specific oligonucleotide primer sequences described by Creti et al. [44]. To evaluate the presence of adhesion factors, a PCR assay was performed to detect *lmb* (human laminin-binding protein), *cylE* (beta-hemolysin), and *bac* genes (cell surface beta C protein), as previously described [8]. PI-1-, PI-2a-, and PI-2b-mediated virulence was detected by using PCR as described elsewhere [13].

MLST

To determine the clonal lineage and genetic diversity of the isolates, MLST was performed as described earlier. In this technique, seven *S. agalactiae* housekeeping genes (*adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*) were amplified by PCR assay. Afterwards, amplified fragments were subjected to bidirectional DNA sequencing. In the next step, STs were determined by comparing the aforementioned sequences of all seven housekeeping genes with standard alleles listed on the online MLST website (http://pubmlst.org/sagalactiae/). STs were further grouped into different CCs based on similar allelic profiles.

Phylogenetic analysis

The bioinformatics data were analyzed for 11 STs (a combination of seven genes) in this study. The sequence dataset was aligned using the MEGA7.0.21 software. The program RAxML version 8.2 (Miller et al., 2010; Stamatakis, 2014) was run on the CIPRES Science Gateway. Optimization in RAxML was carried out using the GTRCAT option. Bootstrap values for maximum likelihood were 1,000 replicates, with one search replicate per bootstrap replicate.

Abbreviations

MLST	Multilocus sequence typing
CC	Clonal Complex
SD	Standard deviation
MtP	Microtiter plate
CCR	Constitutive clindamycin-resistant
CLSI	Clinical and laboratory standards institute
MIC	Minimum inhibitory concentration
PCR	Polymerase chain reaction
ICR	Inducible clindamycin resistance
MDR	Multidrug-resistant
PI	Pilus Island
OD	Optical density

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Author contributions

MG conceived and designed the study. MG, AM, CHA, and PB contributed to comprehensive research. BP, MG, and AM wrote the paper. ZS analyzed data and prepared figures. MG, AM, CHA, ZS and PB participated in manuscript editing.

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Data availability

All data are presented in manuscript.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Shahid Beheshti University of Medical Sciences in Tehran, Iran (IR.SBMU.MSP.REC.1399.623). Informed consent was obtained from all of the participants in the study. All methods were performed in accordance with the relevant guidelines and regulations.

Consent for publication

Not applicable.

Conflict of interest

The authors declare no conflicts of interest directly relevant to this work.

Author details

[1]Department of Microbiology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]Department of Microbiology, Faculty of Advanced Science and Technology, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran
[3]Department of Microbiology, Faculty of Biological Sciences, North Tehran Branch, Islamic Azad University, Tehran, Iran

[4]Department of Biotechnology, Faculty of Life Sciences and Biotechnology, Shahid Beheshti University, Tehran, Iran
[5]Department of Mycology, Pasteur Institute of Iran, Tehran, Iran
[6]Faculty of Medicine, Shahid Behesthi University of Medical Sciences, Koodak-yar St., Daneshjoo Blvd, Velenjak, Chamran HWY, Tehran, Iran

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