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Research Paper

Molecular Detection of Virulence Genes and Multi-drug Resistance Patterns in *Streptococcus Agalactiae* in Clinical Bovine Mastitis: Tehran and Alborz Provinces, Iran

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ABSTRACT

Introduction: *Streptococcus agalactiae* is one of the important causes of mastitis in cows. The ability of *S. agalactiae* to cause disease depends on the production of a large number of virulence factors encoded by different genes. The overuse of antibiotics to treat mastitis can lead to antibiotic resistance. This research was conducted to detect selected virulence genes and assess the antibiotic resistance of *S. agalactiae*.

Materials & Methods: A total of 30 bacteria that isolated from clinical cases of mastitis and characterized as *S. agalactiae* by conventional cultural methods was collected from veterinary diagnostic laboratories. Then, these isolates undergo further characterization by detecting virulence genes by molecular methods and antibiotic resistance by disc diffusion method.

Results: Of these, 24 samples were confirmed as *S. agalactiae* through the detection of the two *16S-23S rRNA* genes. The disk diffusion method, using a panel of 10 antimicrobial agents, showed a large number of strains resistant simultaneously to six antibiotics. Five virulence genes *bac*, *bca*, *cylE*, *hylB*, and *cfb* were screened by polymerase chain reaction (PCR). The *cfb* and *hylB* genes were found in 95.83 % of the isolates,while the *cylE* gene was detected in 29.16% of the isolates. The *bca* and *bac* genes were not detected in any of the isolates. The absence of *bac* and *bca* genes suggests that they likely have minimal impact on the pathogenesis of *S. agalactiae* mastitis in dairy cows, while the *hylB* and *cfb* genes play a crucial role in this condition.

Conclusion: The results presented here are one of the first molecular data concerning these five virulence genes in *S. agalactiae* isolates causing bovine mastitis in the Tehran and Alborz provinces, providing a foundation for the development of diagnostic, preventive, and therapeutic methods.

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1. Introduction

Streptococcus agalactiae, the only known member of group B streptococci, was initially differentiated from other streptococci by Rebecca Lancefield in the 1930s after being isolated from milk and cows with bovine mastitis [1]. This bacterium causes mastitis in cows and pneumonia and meningitis in human infants [2, 3].

In cases of mastitis, the genus *Streptococcus* accounts for 25-50% of the isolated pathogens worldwide [4]. Meanwhile, *S. agalactiae* is a significant cause of mastitis in cows. *S. agalactiae* can persist in the mammary gland for extended periods without causing symptoms, and the disease progresses slowly [5, 6]. *S. agalactiae* is transmitted through infected mammary glands and contaminated environmental sources, such as milking machines and bedding [2]. *S. agalactiae* infection in dairy cows is a major factor in reducing milk production and the quality of milk products. Milk from cows with mastitis reduces the quality of dairy products. Changes in milk composition not only decrease its nutritional value and cause processing issues but also shorten the shelf life of liquid milk products [5, 7, 8].

The pathogenicity of *S. agalactiae* depends on the production of a large number of virulence factors, each encoded by different genes. For instance, the virulence factors alpha protein C, beta protein C, hyaluronidase, CAMP factor, and B-hemolysin are encoded by *bca*, *bac*, *hylB*, *cfb*, and *cylE* genes, respectively. These virulence genes were reported in some *S. agalactiae* obtained from mastitis milk samples [9, 10]. Previously, Ahmadi et al. (2009) in Urmia, Iran and Momtaz et al (2012) in Isfahan, Iran detected *S. agalactiae* in milk samples using PCR method [11, 12].

The most common treatment for mastitis involves administering intramammary antibiotics in the infected parts of the udder and injection [13]. However, the overuse of antibiotics to treat mastitis over a long period can lead to antibiotic resistance. This can result in the need to increase antibiotic dosages, leading to the accumulation of high levels of antibiotics in milk and dairy products, which can then be transferred to humans [14]. Antibiotic resistance has been described as one of the most significant global threats of the 21st century for this reason [15]. Therefore, it is crucial to determine antibiotic resistance in bacteria isolated from mastitis cases for effective treatment of this disease [16].

Therefore, this study aimed to determine antibiotic resistance and describe the distribution of virulence genes in isolates to support in the prevention and control of bovine mastitis.

2. Materials and Methods

2.1. Collection of isolates of *S. agalactiae* and *16S rRNA* sequence analysis

30 isolates of *S. agalactiae* were isolated from 400 milk samples collected from mastitis-affected cows in 10 herds in industrial cattle farms in Alborz and Tehran provinces. The isolates were provided by the Mabna Laboratory, located in Mehrshahr, Karaj, Alborz, Iran. The samples were frozen in 30 microtubes with a size of 2 mL containing 1% glycerol and paraffin at -20 ˚C, then transferred to Karaj Branch of Islamic Azad University Research Laboratory. All *S. agalactiae* isolates were confirmed using *16S rRNA* polymerase chain reaction (PCR)

2.2. Analysis of antimicrobial susceptibility

All confirmed isolates underwent susceptibility testing for 10 commonly used antimicrobial agents in Tehran and Alborz provinces dairy farms The antibiotics tested included erythromycin (15 µg), ceftiofur (30 µg), penicillin (10 µg), ciprofloxacin (5 µg), streptomycin (10 µg), kanamycin (30 µg), tetracycline (30 µg), neomycin (30 µg), florfenicol (30 µg), and clindamycin (2 µg). Susceptibility was assessed using the disc diffusion method on Mueller-Hinton agar plates, supplemented with 5% sheep blood. The cultures were incubated overnight (16–18 h) at 37˚C in an atmosphere containing 5% CO_2.

2.3. Genomic DNA extraction

Template DNA was obtained by boiling bacterial colonies. Therefore, each bacterial isolate was cultured in 2 mL of Muller- Hinton broth, then transferred to 2 mL microtubes, and centrifuged (Hermle Z233MK-2) at 5000 rpm (2374×g) for 10 minutes. Then the supernatant was discarded, and 200 microliters of distilled water was added to the remaining sediment. Then the microtubes were placed in the hot block (Techne-DB.2D) for 10 minutes at 100 ˚C to disrupt the bacterial walls and release the bacterial genome. The microtubes were once again placed in a centrifuge at 5000 rpm (2374×g) for 10 minutes. Ultimately, the liquid supernatant was utilized as the genomic DNA.

Table 1. PCR primers of genes and cycling conditions used to identify and characterize *S. agalactiae*

Gene	Primer Sequence (5'---> 3')	Amplicon Size (bp)	Ref.
16S-23S rRNA1[1]	Fw: TGTTTAGTTTTGAGAGGTCTTG Rv: CGTGGAATTTGATATAGATATTC	150	[16]
16S-23S rRNA2[1]	Fw: GGAAACCTGCCATTTGCG Rv: TAACTTAACCTTATTAACCTAG	281	[16]
bac[2]	Fw: AAGCAACTAGAAGAGGAAGC Rv: TTCTGCTCTGGTGTTTTAGG	479	[16]
bca[3]	Fw: TGATACTTCACAGACGAAACAACG Rv: TACATGTGGTAGTCCATCTTCACC	398	[16]
cfb[4]	Fw: TTTCACCAGCTGTATTAGAAGTA Rv: GTTCCCTGAACATTATCTTTGAT	153	[16]
cylE[5]	Fw: CATTGCGTAGTCACCTCCC Rv: GGGTTTCCACAGTTGCTTGA	380	[17]
hylB[2]	Fw: CACCAATCCCCACTCTACTA Rv: TGTGTCAAACCATCTATCAG	444	[16]

[1]94 ˚C (600 s); 30 cycles of 94 ˚C (60 s), 55 ˚C (60 s), 72 ˚C (60 s); final extension 72 ˚C (420 s).

[2]94 ˚C (300 s); 30 cycles of 94 ˚C (30 s), 53°C (30 s), 72 ˚C (60 s); final extension 72 ˚C (240 s).

[3]96 ˚C (180 s); 30 cycles of 95 ˚C (60 s), 58°C (45 s), 72 ˚C (45 s); final extension 72 ˚C (600 s)

[4]94 ˚C (180 s); 40 cycles of 95 ˚C (20 s), 55 ˚C(30 s), 72 ˚C (120 s); final extension 72 ˚C (300 s)

[5]94 ˚C (180 s); 34 cycles of 94 ˚C (20 s), 56 ˚C (20 s), 72 ˚C (45 s); final extension 72 ˚C (300 s)

2.4. Detection of virulence genes

All confirmed isolates were screened for the presence of the following virulence genes: *bac* (C-β protein), *bca* (C- α protein), *cfb* (CAMP factor), *cylE* (β-hemolysins/ cytolysin) and *hylB* (hyaluronidase) [9, 17].

The concentrations of components in the reaction mixtures used for amplifying gene fragments were selected based on experimental results and references shown in Table 1. For each gene, 12.5 µL of 2x Taq DNA Polymerase Master Mix RED with 1.5 mM $MgCl_2$ (Ampliqon Co. Denmark), 0.5 µL of each primer (0.4 µM for bca), and 1 µL of template DNA were placed in each microtube. Then the total volume of each microtube reached 25 µL with distilled water. Each reaction included a positive control (DNA isolate containing the tested gene) and a negative control (nuclease-free water) in a thermocycler (Applied Biosystems- en61327). The primer sequences and conditions used for amplification of DNA fragments are presented in Table 1. Also, PCR temperatures and conditions are shown in footnotes of the Table 1.

3. Results

3.1. Antimicrobial susceptibility

Antimicrobial susceptibility testing of the isolates showed that 100% of the 24 confirmed isolates (Unconfirmed isolates are number 2, 8, 10, 25, 26, and 30) of *S. agalactia* were susceptible to penicillin, ciprofloxacin, and ceftiofur, and 75% were susceptible to florfenicol. All 24 isolates were resistant to the streptomycin, kanamycin, tetracycline, and neomycin. The resistance rates for clindamycin and erythromycin were 95.8% and 91.6%, respectively (Table 2).

3.2. Prevalence of virulence genes

The presence of five virulence genes of *S. agalactiae* (*bca*, *cylE*, *cfb*, *hylB*, and *bac*) were tested in all 24 confirmed isolates, shown in Figures 1, 2, 3, 4, 5, and 6. The results showed that *cfb* and *hylB* genes were detected in 95.8% of *S. agalactiae* isolates. Also, *cylE* gene was detected in 29.1% of these isolates, while the *bac* and *bca* genes were not detected in these isolates. Three distinct virulence gene profiles were identified and the virulence gene profile *cfb-hylB* was common among isolates, as shown in Table 2.

4. Discussion

S. agalactiae is considered one of the major mastitis pathogens. To the best of our knowledge, this is one of the first molecular studies that characterizes *S. agalactiae* isolates circulating among cattle with mastitis in Tehran and Alborz provinces, Iran. Of the 30 original strains identified as *S. agalactiae* by biochemical tests, only 24 were confirmed genetically, resulting in an isolation rate of 80.0%. The presence of virulence factors in a pathogen significantly influences disease progres-

Table 2. Multi-drug resistance patterns and virulence gene profiles in 24 *S. agalactiae* isolates

Antibiotic Resistance and Virulence Gene Profile	Patterns	No. (%) of Isolates
Antibiotics resistance patterns	N, CC, FF, TE, E, K, ST	3(12.5)
	N, CC, TE, E, K, ST	19(79.16)
	N, CC, TE, K, ST	1(4.16)
	N, TE, K, ST	1(4.16)
Virulence gene profile	*cylE*	1(4.16)
	cfb, hylB	23(95.83)
	cfb, hylB, cylE	6(25)

Abbreviations: N: Neomycin; CC: Clindamycin; FF: Florfenicol; TE: Tetracycline; E: Erythromycin; ST: Streptomycin.

sion [9]. Concerning the virulence genes screened in this study, five virulence genes were detected, including *bac*, *bca*, *cfb*, *hylB*, and *cylE*.

The *hylB* gene encodes the hyaluronidase protein [18], an enzyme that increases the spread of infection by hydrolyzing hyaluronic acid in the connective tissue [19, 20]. In previous studies, the frequency of hylB virulence gene has been reported in more than 95% of the investigated isolates [7, 18-24]. In this study, the virulence gene *hylB* was seen in 23 of the 24 confirmed isolates of *S. agalactiae* (95.83%), highlighting its importance in improving mastitis by *S. agalactiae*.

The *cfb* virulence gene encodes the CAMP factor, which induces pore formation in host cell membrane [9, 18]. Most studies have reported a frequency above 90% in most researches [7, 9, 18, 19, 21, 24-26]. The next reported frequency was 68.96% and the lowest frequency of this gene was 38.09% [27]. With these interpretations, we can conclude that this virulence gene is also one of the most abundant virulence genes of *S. agalactiae*. In this research, *cfb* virulence gene was founded in all isolates but one (95.83%).

The virulence gene *cylE*, by encoding the B-hemolysin protein, increases the invasion of this bacterium into host cells [9]. Different frequencies have been reported in different countries for this virulence gene. The high-

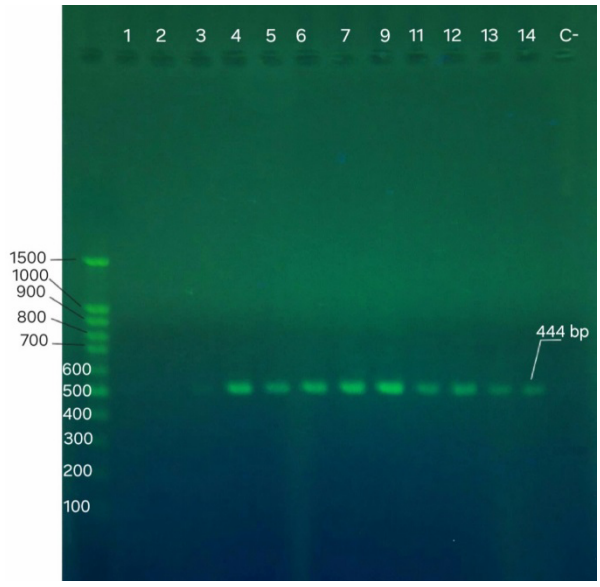

Figure 1. *hylB* gene specific PCR for *S. agalactiae* (444 bp)

Note: First lane: Ladder; The last lane: Nuclease-free water as negative control, lanes 1 to 14 relates to samples 1 to 14 that isolated from clinical bovine mastitis (Lane 2 relates to isolate that is Unconfirmed).

est frequency reported for the *cylE* virulence gene was 100% [19-21]. Also, in some researches, the frequency of this gene has been reported as 93% [18, 26]. Frequencies of 78% and 68.2% were reported [9, 22]. The lowest mentioned frequency for this gene was 23.80% [27]. In this research, *cylE* virulence gene was found in 7 isolates out of 24 confirmed isolates of *S. agalactiae* (29.16%). According to the clinical reports, the cows affected with these isolates showed sever clinical mastitis.

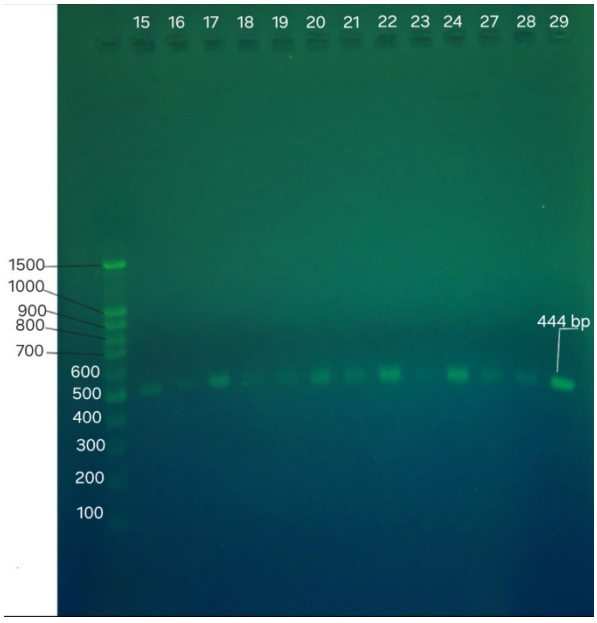

Figure 2. *hylB* gene specific PCR for *S. agalactiae* (444 bp)

Note: First lane: Ladder; Lane 15 to 29 relates to samples 15 to 29 that isolated from clinical bovine mastitis.

Among the reviewed articles from various countries, the *bca* and *bac* virulence genes have the lowest frequency of occurrence. The virulence gene, *bca*, encodes surface protein C alpha antigen. This protein mediates the adhesion of bacteria to the epithelial cells of the host. The *bac* virulence gene encodes surface protein C beta antigen, responsible for binding to immunoglobulin A [9]. In most studies, the frequency of the *bca* and *bac* virulence genes was less than 10% and, in some cases,

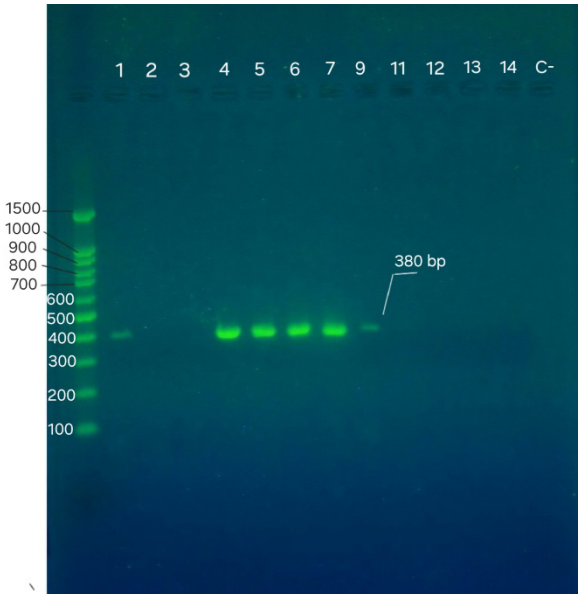

Figure 3. *cylE* gene specific PCR for *S. agalactiae* (380 bp)

Note: First lane: Ladder; The last lane: Nuclease-free water as negative control, lanes 1 to 14 relates to samples 1 to 14 that isolated from clinical bovine mastitis (lane 2 relates to isolate that is unconfirmed).

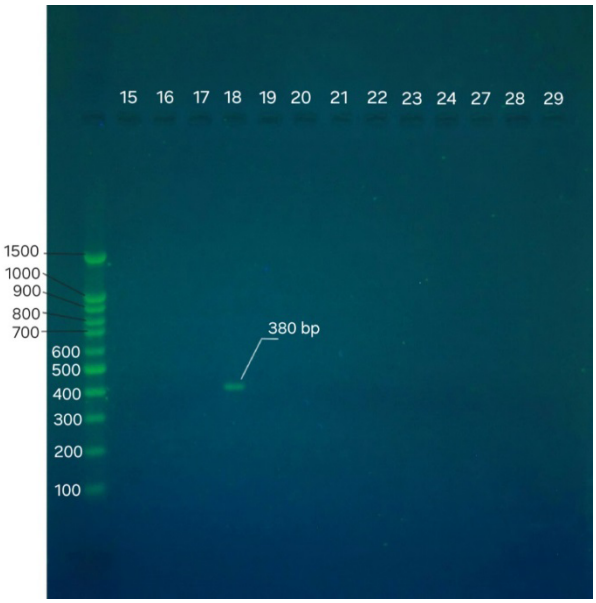

Figure 4. *cylE* gene specific PCR for *S. agalactiae* (380 bp)

First lane: Ladder; Lane 15 to 29 relates to samples 15 to 29 that isolated from clinical bovine mastitis.

even 0% [7, 9, 18-21, 25]. In this study, *bac* and *bac* virulence genes were not found in any of the 24 confirmed isolates of *S. agalactiae* (0%). This can be related to the relatively small samples size collected in this study.

These results indicated that the *bac* and *bca* virulence genes probably do not significantly contribute to the pathogenesis of mastitis caused by *S. agalactiae* in dairy cows, and these two genes are less important in the virulence of *S. agalactiae* than the virulence genes *hylB*, *cylE*, and *cfb*. It can be concluded that the *hylB* and *cfb* genes play a significant role in the pathogenesis of mastitis caused by *S. agalactiae* in dairy cows.

The most common treatment for mastitis involves administering antibiotics directly into the infected parts of udder and giving intramuscular injections [13]. In this study, we conducted susceptibility testing for 10 commonly used antibiotics to treat clinical mastitis in dairy cows in Tehran and Alborz Provinces. We found that all 24 isolates showed 100% resistance rate to streptomycin, neomycin, tetracycline, and kanamycin, while they ex-

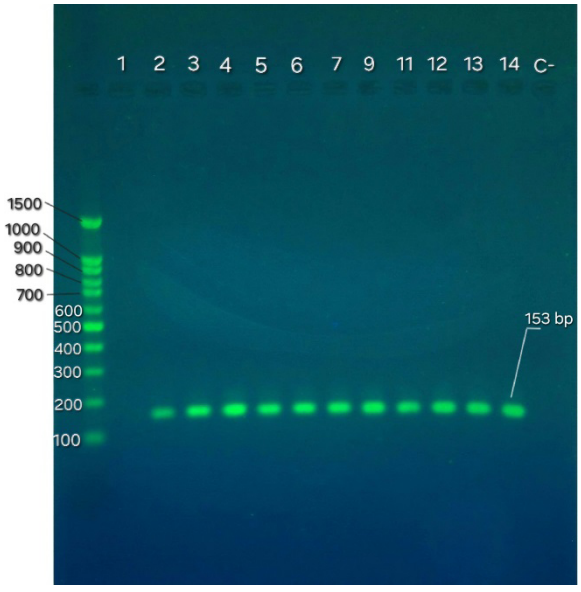

Figure 5. *cfb* gene specific PCR for *S. agalactiae* (153 bp)

Note: First lane: Ladder; The last lane: Nuclease-free water as negative control, lanes 1 to 14 relates to samples 1 to 14 that isolated from clinical bovine mastitis (lane 2 relates to isolate that is Unconfirmed).

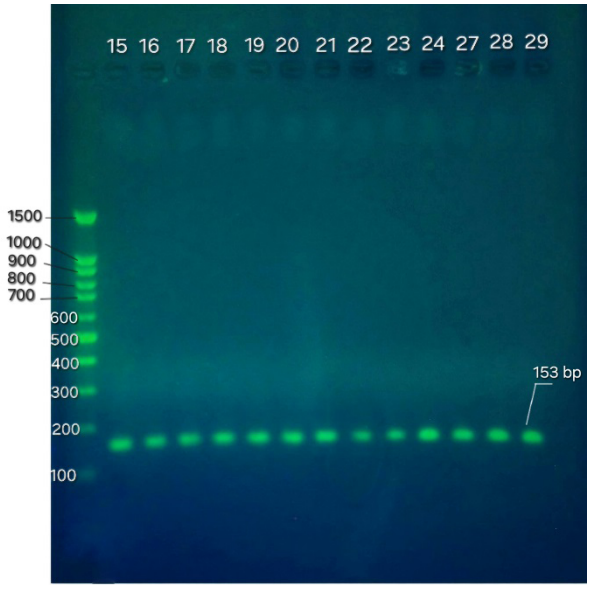

Figure 6. *cfb* gene specific PCR for *S. agalactiae* (153 bp)

Note: First lane: Ladder; Lane 15 to 29 relates to samples 15 to 29 that isolated from clinical bovine mastitis.

hibited high sensitivity to penicillin, ciprofloxacin, and ceftiofur. Also, the resistance rate in 24 isolates was over than 90% for clindamycin and erythromycin and it was 12.5% for florfenicol.

The results of this study indicate that penicillin, ciprofloxacin, and ceftiofur, may be suitable drug choices for treating *S. agalactiae* mastitis in the of Tehran and Alborz Provinces. However, *S. agalactiae* can eventually develop resistance to these antimicrobial agents. Therefore, these three antibiotics should not be considered a long-term solution. Also, the virulence genes investigated in this study can provide helpful data for the preparation of vaccines for use in livestock in the Tehran and Alborz provinces. We detected several virulence profiles associated with *S. agalactiae* intramammary infections.

5. Conclusion

Overall, the findings suggest that *bac* and *bca* virulence genes probably do not significantly contribute to the pathogenesis of mastitis caused by *S. agalactiae* in dairy cows, although this can be influenced by the relatively small size of the samples collected in this study. Also, the *hylB* and *cfb* genes play a significant role in the pathogenesis of mastitis caused by *S. agalactiae* in dairy cows. On the other hand, according to the results of the disk diffusion test, we have determined that penicillin, ciprofloxacin, and ceftiofur are the most effective antibiotics for treating mastitis caused by *S. agalactiae*. These data will assist us in closely monitoring *S. agalactiae* strains, improving diagnostic methods, and developing prevention, treatment strategies including the potential for vaccine production.

Ethical Considerations

Compliance with ethical guidelines

All experimental procedures were carried out in accordance with established the ethical research, ensuring the welfare and safety of the participants.

Data availability

The data supporting the findings of this study are available upon request from the corresponding author.

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Authors' contributions

Conceptualization and study design: Hadi Pourtaghi; Data acquisition: Fatemeh Hashemi Haghighi and Farhad Moosakhani; Experiments and data interpretation: Hadi Pourtaghi, Fatemeh Hashemi Haghighi, Naser Harzandi, and Farhad Moosakhani; Writing the original draft: Fatemeh Hashemi Haghighi; Review and editing: Hadi Pourtaghi and Naser Harzandi; Supervision, project administration, technical, and material support: Hadi Pourtaghi, Naser Harzandi, and Farhad Moosakhani.

Conflict of interest

The authors declared no conflict of interest.

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A collection of *S. agalactia* isolates investigated in this study was gathered by the Mabna laboratory in March and April 2024.

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