



Isolation and Characterization of *Streptococcus agalactiae* and its Capsular Antigen, Along with *Mycoplasma hominis* and *Listeria monocytogenes*, as Abundant Infections in Women with Abortion in Iran

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Abstract

Background: The accurate diagnosis of infections can significantly enhance preventative measures against abortion.

Objectives: The rates of *Streptococcus agalactiae*, *Mycoplasma hominis*, and *Listeria monocytogenes* were investigated in the vaginal secretions of women with abortion. Furthermore, this study aimed to detect *S. agalactiae* capsular types by multiplex Polymerase Chain Reaction (PCR) and sequencing.

Methods: The study collected vaginal samples obtained from a cohort of women with abortions of unknown cause from various healthcare facilities across Iran, as well as their counterparts who did not experience any reproductive issues. Molecular identification was performed by a multiplex PCR protocol for the amplification of specific regions within the bacterial 16S rRNA gene. The capsular polysaccharide of *S. agalactiae* was identified by multiplex PCR of the caps gene. Then, the sequences of the amplified gene were analyzed by Mega X. Finally, some factors that exerted a noteworthy influence on the likelihood of miscarriage were determined.

Results: The study found *S. agalactiae*, *M. hominis*, and *L. monocytogenes* in the vaginal secretions of women with abortion, with respective frequencies of 9.09%, 4.54%, and 2.7%, which were more than the frequencies in the pregnant healthy women. The caps III (4%) and caps V (6%) genes were identified in the *S. agalactiae* isolates. The sequences of caps III or caps V did not show any differences among the isolates containing each gene.

Conclusions: The findings demonstrated the significance of preemptively managing prevalent infections and investigating risk factors in women prior to pregnancy. No difference in the sequences of the caps genes is promising for the adoption of vaccination and therapeutic strategies.

Keywords: Abortion, *Streptococcus agalactiae*, *Mycoplasma hominis*, *Listeria monocytogenes*, Multiplex Polymerase Chain Reaction, Capsular Antigen

1. Background

Abortion is the unplanned and natural ending of a pregnancy occurring before the 20th week, usually involving a fetus weighing approximately 500 g. In the context of abortion, infectious agents have been shown to exert noteworthy efficacy (1, 2). Fatal pregnancy termination has a high prevalence in both developed and impoverished countries. This prevalence is reported as 3.9%, involving Group B *Streptococcus* (GBS) in women of

childbearing age globally (3). Different statistics have been reported for abortion in Iran, but most of them are near global statistics (4). Congenital infections pose a significant threat to fetal health in humans, with bacterial agents emerging as a crucial contributor (5). Several bacterial agents have been identified as common causes of abortion. These include *Mycoplasma hominis*, *M. genitalium*, *Ureaplasma urealyticum*, *Streptococcus agalactiae*, *Chlamydia trachomatis*, *Listeria monocytogenes*, *Brucella*, and *Helicobacter pylori* (6-8).

A wide range of noteworthy events and advancements have emerged during the calendar year of 2021. Among the common bacterial factors involved in abortion, *S. agalactiae* (as a GBS member) is an important agent. *Streptococcus agalactiae* is aerobic, capsule-forming, Gram-positive cocci among the normal flora of the urinary-genital and lower digestive tract of many adults, so it has been isolated from the urinary-genital and digestive tracts of about 25% of healthy adult women (9). A meta-analysis determined that approximately 19.7 million pregnant women were affected by rectovaginal colonization with GBS in 2020 (5). The colonization of the bacterium in pregnant women generally remains without noticeable symptoms; however, it has the potential to lead to urinary tract infections, septicemia, cystitis, pyelonephritis, wound infections, chorioamnionitis, endometritis, miscarriages, and labor fever (10, 11). Recent research has demonstrated a gradual escalation of antibiotic resistance in numerous nations with respect to GBS strains (12-14).

The identification of bacterial infections in women who have experienced miscarriages and those within the childbearing age could potentially mitigate the financial burden on patients and the healthcare system stemming from the administration of antibiotic treatment for these infections (2, 7). Therefore, this study was designed to investigate the prevalence of three abundant infections in the vaginal secretions of women with abortion. On the other hand, GBS serotypes have a high presence in many geographic regions, and some serotypes are most prevalent in specific areas. Therefore, continuous monitoring studies, such as this study, are essential for determining the distribution of capsule serotypes in order to design specific antigen vaccination and treatment protocols optimally (15). In relation to the geographical distribution of *S. agalactiae* serotypes, it has been reported that serotype III is the most common type in Europe, Central Asia, Africa, and Australia. Other serotypes that have been reported in low-income countries were serotypes I, II, and V, in sequence (16, 17).

2. Objectives

This study was conducted in Isfahan, Iran, to determine the prevalence of *M. hominis*, *L. monocytogenes*, and *S. agalactiae* in the vaginal secretions of women who have had a history of miscarriage by multiplex PCR. Furthermore, the types of capsular antigen were detected in *S. agalactiae* isolates by multiplex PCR and sequencing. Additionally, it was sought to assess the impact of certain risk factors on miscarriage in these women, utilizing demographic information obtained from the patients.

3. Methods

3.1. Isolation of the Bacteria from Vaginal Samples

The bacterial samples studied in this study included 110 samples of vaginal secretions from women who had 2 - 3 previous abortions and were referred to gynecological departments because of a sudden abortion. A proven cause of abortions in these women had not been detected. Also, 110 samples were taken from pregnant women without reproductive problems or genital infections. The Cochran formula was used for the determination of the sample size, and a cluster sampling method was performed. The studied women were all married, aged between 21 and 35 years, with no assisted reproductive methods for pregnancy. These women had been referred to different medical centers with a gynecology department in Isfahan, Iran. The samples were taken immediately after abortion from the middle third area of the vagina with sterile swabs by gynecologists. They were transferred to the microbiology laboratory in 0.9% normal saline on ice for use in culturing and Todd Hewitt Broth medium (Merck, Germany) as a transfer medium (18). At the time of sampling, a questionnaire was completed to collect the demographic information of the patients, including education and economic status, age, blood groups, weight during pregnancy, the number of abortions, and the patient's stress status and vitamin D level.

3.2. Designing the Multiplex Polymerase Chain Reaction Protocol for the Identification of the Isolates

All chemicals were obtained from Merck, Germany. All isolates were kept at - 70°C. The DNA was extracted from each isolated bacterium by phenol-chloroform method (19, 20). The multiplex Polymerase Chain Reaction (PCR) was used for the identification of the isolates based on the sequence of the 16S rRNA gene in the extracted DNA from each isolate by using specific primers. The sequences of the primers and their relevant reference strains are shown in Appendix 1. The PCRs were done by using the reaction mixture (CinaClon, Iran) containing 1X PCR buffer, 1.5 mM MgCl₂, 200 μM dNTPs, 0.4 μM of each primer, 1-unit Taq DNA polymerase, and 0.5 μg/mL of the template DNA in a total volume of 25 μL in a T100 thermal cycler (Bio-Rad, USA).

The reaction protocols included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 35 s, primers' annealing at the temperatures and times obtained in gradient reactions for each primer, and extension at 72°C for 35 s. Each reaction was ended by a final extension at 72°C for 5 min. The PCR products were finally visualized by 1% agarose gel electrophoresis (NOGEN, Iran) and confirmed

in polyacrylamide gel electrophoresis (NOGEN, Iran). The PCR products were sequenced (BIONEER, South Korea), and the obtained sequences were analyzed using the BLAST server to find the homologies in GenBank. The confirmed sequences were deposited in the NCBI genomic database. According to the Tm of the primers and the length of PCR products, a multiplex PCR protocol was designed for the simultaneous diagnosis of three infections by *M. hominis*, *L. monocytogenes*, and *S. agalactiae*. For this purpose, different concentrations of DNAs from reference strains were used as templates in multiple PCR to obtain the best amplification results.

3.3. Capsular Antigen Detection

The capsular polysaccharide type in *S. agalactiae* isolates was detected by a multiplex PCR method on the bacterial purified DNA using specific oligonucleotide primers presented in Appendix 2. The PCR temperature protocol included an initial step at 94°C for 3 min, followed by 40 repetitive cycles of 94°C for 45 s, 54°C for 40 s, and 72°C for 30 s each, and a final step at 72°C for 10 min. The evolutionary history was inferred by using the maximum likelihood method and JTT matrix-based model. All positions with less than 95% site coverage were eliminated. Evolutionary analyses were conducted in MEGA X.

3.4. Statistical Analysis

Descriptive statistics, including numbers, percentages, means, and standard deviation, and inferential (analytical) statistics, including chi-square, independent *t*, and Fisher's exact tests, were employed. Also, SPSS 22 software was used for statistical analysis of data. The graphs were drawn using Excel software.

4. Results

4.1. Abortion Risk Factors in the Studied Women

The highest frequency of blood groups in the examined samples was related to blood group O⁺ in both groups, and the lowest frequency was related to blood group AB⁻ in both groups, with no significant differences between blood groups (*P* = 0.05) (Appendix 3). The results of examining some other abortion risk factors in the studied women are shown in Appendix 4. Between healthy women who have had an abortion and even their husbands, the frequency of high school graduates and academic education was higher than the high school undergraduated women, while there was no significant difference between them. As the results showed, moderate economic status was most common

in both aborted and healthy pregnant groups. There was no significant difference in the frequency distribution of women based on economic status between the healthy pregnant group and the group with abortions.

The results showed no significant difference between the two groups in terms of having a history of cesarean section. Also, no significant difference was seen between the two groups in terms of overweight. The stress during pregnancy was significantly greater in women with abortions than in healthy pregnant women. Concerning vitamin D levels, the results showed that the chance of miscarriage in women with normal vitamin D levels was significantly lower than that in women with vitamin D deficiency. The results of urinary infection, vaginal infection, and co-infection examination in the 2 groups of studied women are presented in Table 1. The women with abortions were significantly more infected than the healthy pregnant women. The women who had infections in both groups were sampled for isolation and identification of bacteria.

4.2. Bacterial Isolates

The results are shown in Table 2. Based on the result of Fisher's exact test, significant differences were seen between the two groups of abortion and healthy pregnant women in terms of the presence of *L. monocytogenes* and *M. hominis*. In comparison, there was no significant difference between the two groups in terms of the presence of *S. agalactiae*.

4.3. Designed Multiplex PCR Protocol

A multiplex PCR was designed for the simultaneous amplification of the 16S rRNA gene in *M. hominis* (a 213 bp fragment), *L. monocytogenes* (a 417 bp fragment), and *S. agalactiae* (a 331 bp fragment). The results for the detection of the strains in some samples with different concentrations of template DNA are shown in Appendix 5.

4.4. Capsular Antigen Detection in *Streptococcus agalactiae* Isolates

In a total of 10 isolates of *S. agalactiae*, the caps III and caps V genes were identified with frequencies of 40% (4 isolates) and 60% (6 isolates), respectively. The phylogenetic trees are shown in Figure 1. As shown, no difference was seen in the sequences of each of the caps III or caps V genes.

5. Discussion

The presence of genital infections has been associated with impaired fertility (21). Diagnosing bacterial infection

Table 1. Frequency Distribution of Various Types of Infections in Two Groups of Patients and Healthy Pregnant Women

Variables	No. (%)		Statistics	P-Value	Odds Ratio (95% Confidence Level)
	Healthy Pregnant	With Abortion			
Urinary infection					29.66 (6.94 - 126.73)
No	108 (98.2)	71 (64.5)			
Yes	2 (1.8)	39 (35.5)	41.038	0.001	
Vaginal infection					22.029 (6.57 - 73.88)
No	107 (97.3)	68 (61.8)			
Yes	3 (2.7)	42 (38.2)	42.491	0.009	
Double infection					21.33 (2.79 - 163.82)
No	110 (100)	92 (83.6)			
Yes	0.0 (0.0)	18 (16.4)	16.648	0.008	
Total	110	110	100	100	

Table 2. Frequency Distribution of Abortion-causing Bacteria in Two Groups of Patients and Controls

Bacterial Isolate	No. (%)		Statistics	P-Value	Odds Ratio (95% Confidence Level)
	Healthy Pregnant	With Abortion			
<i>Mycoplasma hominis</i>			—	0.489	—
Negative	110 (100)	105 (95.46)			
Positive	0 (0.00)	5 (4.54)			
<i>Listeria monocytogenes</i>			—	0.622	—
Negative	110 (100)	107 (97.3)			
Positive	0 (0.00)	3 (2.7)			
<i>Streptococcus agalactiae</i>			7.135	0.008	3.270 (1.8 - 321.091)
Negative	109 (99.09)	90 (81.81)			
Positive	1 (0.90)	10 (9.09)			

in women can prevent more miscarriages and decrease its economic and health impacts on women in society (22). Several risk factors may predispose a pregnant woman to early pregnancy loss, among which infection is one of the most important factors (23). In this study, a novel multiplex PCR method was used for the simultaneous detection of 3 bacteria: *M. hominis*, *L. monocytogenes*, and *S. agalactiae*, which can be used as a complementary method that has been proposed previously for the simultaneous detection of other vaginal infections related to abortion (24-26). In the present study, *S. agalactiae*, *M. hominis*, and *L. monocytogenes* were detected in the vaginal secretions of women with abortion in the frequencies of 4.54%, 2.7%, and 9.09%, respectively. *Mycoplasma hominis* and *L. monocytogenes* were not found in the vaginal secretions of healthy pregnant women. The frequency of *S. agalactiae* in the vaginal secretions of those women was significantly lower than that in the women with abortion. In another study in Iran, the frequency of GBS was 7% in the samples

taken from the vaginal secretions of 100 pregnant women (27).

It has been suggested that infection with *M. hominis* may be a common cause of spontaneous abortion (28). It has been reported that the colonization rate of these two bacteria in patients with miscarriage was higher than their frequencies in normal pregnancy groups. These bacteria are opportunistic pathogens of the urinary tract that are able to change the microenvironment of the uterine cavity against the normal growth of the fetus by causing multiple infections (29). In a study, the incidence of *L. monocytogenes* infection was found to be 3.66% among women with spontaneous abortion, 1.83% among women with normal delivery, 3% among fertile women, and 0% among infertile women (30). In another study, the prevalence of *L. monocytogenes* contributing to human spontaneous abortions was determined to be 14.8% (31). In another study, the correlation between *M. hominis* and spontaneous abortion in gravid women was

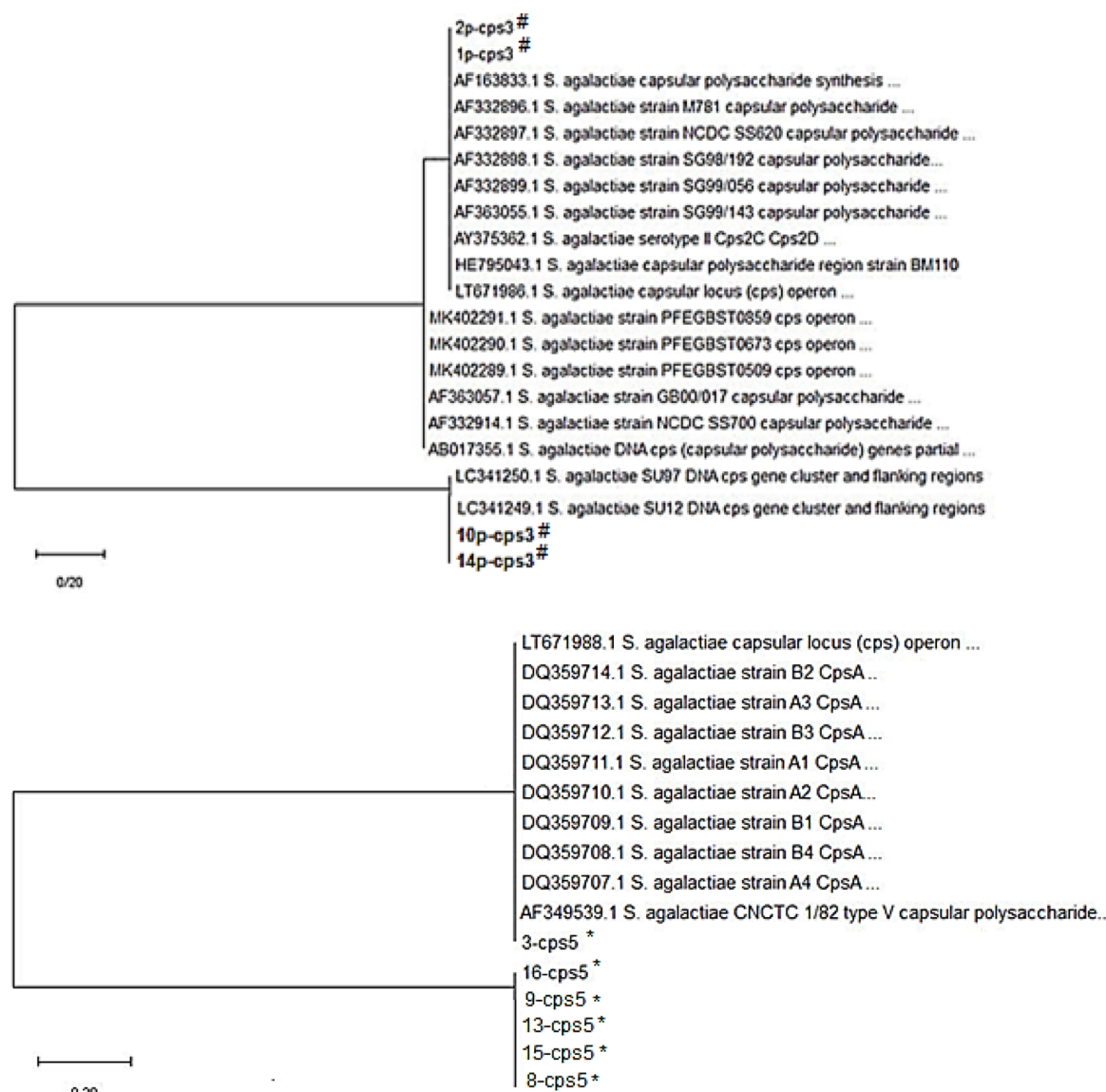


Figure 1. The phylogenetic trees of caps III and caps V genes in 10 isolates of *Streptococcus agalactiae*. The isolates containing caps III are shown by #, and the isolates containing caps V are shown by *.

assessed. The *M. hominis* infection (spontaneous abortion) was found in 2 individuals in the case group, constituting 1.83% of the group's population.

In both the case and control groups, there was no observed correlation between *M. hominis* infection and spontaneous abortion (32). In the present study, the serotypes III (4%) and V (6%) were prevalent in the *S. agalactiae* isolates. This prevalence was similar to the prevalence that has been reported in Asia, although in some regions, serotypes I and II also have been reported

(16, 17). The fact that the sequences of each caps III or caps V gene did not have any differences among the *S. agalactiae* isolates can be of concern for the health care system and vaccine designers for controlling the abortions caused by this bacterial infection.

5.1. Conclusions

The application of a previously developed novel multiplex PCR method is proposed for the concurrent identification of *L. monocytogenes*, *M. hominis*, and *S.*

agalactiae in women with a history of abortion. In this study, the sequences of the caps genes had no difference, which is promising for adopting vaccination and therapeutic strategies.

Supplementary Material

Supplementary material(s) is available [here](#) [To read supplementary materials, please refer to the journal website and open PDF/HTML].

Footnotes

Authors' Contribution: A. B., M. D., and A. M. A. designed the study and wrote the paper; A. B. performed the study; A. B., M. D., and H. G. S. analyzed the data.

Conflict of Interests: The authors declare no conflict of interest.

Data Reproducibility: The data presented in this study were uploaded during submission as a supplementary file (Appendices) and will be openly available for readers upon request.

Ethical Approval: This study has been approved by the Ethics Committee of the Falavarjan Branch, Islamic Azad University, with the ethics code [IR.IAU.FALA.REC.1398.025](#).

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