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Molecular detection of *Streptococcus agalactiae* and its abundant capsular serotypes in vaginal secretions of women with abortion in Isfahan, Iran

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ABSTRACT

Streptococcus agalactiae which is naturally found in the women intestine and vagina, can cause complications leading to the abortion. The aim of this study was molecular detection of *Streptococcus agalactiae* and its abundant capsular serotypes in vaginal secretions of women with abortion in 2020 in Isfahan. Samples were taken from vaginal secretions of 110 women with abortions referred to different hospitals in Isfahan from April 2019 to March 2020. Patients were asked to fill out a questionnaire containing demographic characteristics. Following isolation, the bacteria were identified by using biochemical tests and PCR. Bacterial capsular polysaccharides were identified by multiplex PCR using specific primers. The results of biochemical and molecular tests showed that among the isolated bacteria from aborted women participating in this study, 20 isolates (18.1%) were *Streptococcus agalactiae*. The type III capsular serotype had the highest frequency (42.13%). Other serotypes were Ia (18.27%), II (16.73%), V (13.9%), Ib (8.7%), and IX (0.9%). The serotypes VII, VIII, and VIIV were absent in the isolates. The results of this study showed a significant presence of *Streptococcus agalactiae* in women with abortion with the highest frequent capsular serotype related to the type III. Although this bacterium is a member in the normal flora of women's vagina, it is one of the most important causes of miscarriage. To better understand the role of this bacterium in the abortion, more investigations, with more efficient methods are needed to be done on the mechanisms of this effect.

1. Introduction

Abortion is one of the most common reproductive problems (Fathalla, 2020; Renner et al., 2014), which usually occurs in the early second trimester of pregnancy (Skjeldestad et al., 1998). About 15% of pregnancies are commonly associated with abortion (Soper, 2020). In the study of pregnancies leading to abortion, infections have been one of the most common causes of abortion (Fujii, 2002).

Streptococcus agalactiae is found as a natural flora in the intestines and vagina of women but can cause preterm labor and abortion; as well as sepsis, pneumonia, meningitis, endometritis, and chorioamniotitis in infants (Török and Day, 2005; Zimmermann et al., 1996). This bacterium, which is classified among Lancefield group B streptococci, is an immobile and catalase negative bacterium. These types of streptococci produce various pathogenesis factors such as beta-hemolysin and C5a peptidase (Spellerberg, 2000). Beta-hemolysin damages endothelial and epithelial

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cell membranes, and the enzyme c5a peptidase breaks down the complement protein c5a, delaying the defense of macrophages and neutrophils (Cole, 1990). The capsule of *Streptococcus agalactiae* also lets the microorganism to escape from the immune system and disrupts phagocytosis (Edwards and Baker, 2015). Capsule antigens play an important role in the classification of group B streptococci (GBS) and are therefore classified into 9 serotypes Ia, Ib, II to VIII (Yang et al., 2021).

One of the most important methods to prevent *Streptococcus agalactiae* infection is vaccination. Many attempts have been made to produce effective vaccines against this infection (Tizard, 2021). Due to the high presence of group B streptococci serotypes in many geographic regions, and the presence of some serotypes in specific areas; continuous monitoring and extensive epidemiological studies are required on the distribution of capsule serotypes in order to optimally design specific antigen-containing vaccines (Melin, 2011).

Several methods have been used to determine the capsular serotypes in group B streptococci. Conventional methods are generally costly and time consuming, and require a high titer of serotype-specific antigen. Another problem is the existence of isolates that have recently increased dramatically but cannot be categorized by routine methods (Rodrigues Pereira et al., 2020). The use of molecular techniques is very practical and effective due to their high speed and specificity for the detection of *Streptococcus agalactiae* isolates and its capsular antigens (O'Higgins et al., 2014). The aim of this study was to molecularly investigate the frequency of *Streptococcus agalactiae* and to determine its most abundant capsular serotypes in vaginal secretions of aborted women in Isfahan, Iran.

2. Materials and Methods

2.1. Sampling

This descriptive-analytical study was performed in a period of 12 months, from April 2019 to March 2020. After obtaining ethics permits, 110 samples of vaginal secretions were collected from women with abortion, referred to medical centers in Isfahan and 110 samples were taken from healthy pregnant women without reproductive problems were collected. Demographic information of each patient including history of abortion, history of underlying diseases, abnormal weight gain, vitamin D level, and the history of stress was recorded at the time of sampling. Vaginal specimens were taken with sterile swabs from the middle third of vagina (Ghazvini and Keikha, 2021) and transferred to the microbiology laboratory in Islamic Azad University, Falavarjan branch, in a tube containing sterile normal saline.

2.2. Isolation and biochemical identification of bacteria

Samples were first cultured on Todd Hewitt broth (Merck, Germany) and incubated for 24 h at 37 °C (Melin, 2011). Then the bacterial suspension was inoculated into blood agar medium (Merck, Germany) containing 5% sheep blood and incubated at 37 °C for 24 h, in an anaerobic jar with 5% CO_2 (Shetty et al., 2016). To identify *Streptococcus* spp., colonies were analyzed based on microscopic morphology, Gram staining, capsule formation, type of hemolysis, catalase reaction, Coagulase reaction, Voges–Proskauer reaction, Christie Atkins Munch Petersen (CAMP) reaction, hippurate hydrolysis, , and resistance to bacitracin disk (Lin et al., 2006; Ghazvini and, Keikha, 2021). After microbiological and biochemical analusis, molecular identification test was performed.

2.3. DNA extraction

After purification of bacteria on blood agar medium, single colonies were inoculated into Mueller Hinton broth (Merck, Germany) and incubated at 37 °C for 18 h. In the next step using DNA extraction kit (Arongene Pars, Iran, catalog No. AGNB102), according to the manufacturer protocol.

2.4. Molecular identification of Streptococcus agalactiae among the isolates

Specific primers were designed to amplify the 16S rRNA gene in the studied bacteria (Table 1). To do this, the gene sequence was first homologically evaluated using the ClustalW server and the primers were designed by Gene runner V. 6.5.52. beta 64 software. In the next step, 1 µl of the extracted DNA (containing 0.5

µg of the purified DNA) was added to a PCR mixture (CinnaClone, Iran) containing 1X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTP mix, 0.4 µm of each forward and reverse primers and 1 unit of Taq DNA polymerase enzyme. The reaction was performed at a thermal condition including one step at 95 °C for 5 min followed by 35 cycles including 94 °C for 35 s, 58 °C for 35 s, and 72 °C for 30 s, ended with one final step at 72 °C for 5 min. The reaction was done in a thermocycler (Boecco, Germany, model TC SQ). PCR products were detected by 1% agarose gel electrophoresis using green viewer fluorescent dye, then visualized on a UV light at the wavelength of 254 nm in a gel documentation system (Fard Azma, Iran, model FG 2). To confirm the size of PCR products, the experiment was repeated in SDS polyacrylamide gel electrophoresis (SDS PAGE) by staining with $AgNO_3$ (Merril, 1990).

2.5. Molecular identification of capsular polysaccharide

To identify the capsular polysaccharide type in Streptococcus agalactiae isolates, multiplex PCR method was performed on the bacterial purified DNA using specific oligonucleotide primers presented in Table 2, and PCR temperature program shown in Table 3.

Table 1. The characteristics of the designed primer pairs for molecular identification of Streptococcus agalactiae

Primer	Sequence	Sequence length (bp)	Target Gene	Design
F	GGAGCAGAAGTGACAGGTGG	332	16S *rRNA*	This study
R	GTGCTGATCCGCGATTACTAG			

Table 2. Primer pairs used for Streptococcus agalactiae serotyping based on capsular antigen coding genes amplification by multiplex PCR(Poyart et al., 2008).

Primer name	Sequence	Target gene	Sequence length (bp)	references
Ia-F	GGTCAGACTGGATTAATGGTATGC	*cps1ан*	521	(Zhang et al., 2018)
Ia-R	GTAGAAATAGCCTATATACGTTGAATGC	*cps1ан*		
Ib-F	TAAACGAGAATGGAATATCACAAACC	*cps 1bj*	770	(Zhang et al., 2018)
Ib-R	GAATTAACTTCAATCCCTAAACAATATCG	*cps 1bj*		
II-F	GCTTCAGTAAGTATTGTAAGACGATAG	*cps 2k*	397	(Boonyayatra et al., 2020)
II-R	TTCTCTAGGAAATCAAATAATTCTATAGGG	*cps 2k*		
III-F	TCCGTACTACAACAGACTCATCC	*cps 1a/2/3I*	1826	(Zhang et al., 2018)
III-R	AGTAACCGTCCATACATTCTATAAGC	*cps 1a/2/3j*		
IV-F	GGTGGTAATCCTAAGAGTGAACTGT	*cps 4N*	578	(Poyart et al., 2008)
IV-R	CCTCCCCAATTTCGTCCATAATGGT	*cps 4N*		
V-F	GAGGCCAATCAGTTGCACGTAA	*cps 5O*	701	(Zhang et al., 2018)
V-R	AACCTICTCCTCACACTAATCCT	*cps 5O*		
VI-F	GGACTTGAGATGGCAGAAGGTGAA	*cps 6 I*	487	(Poyart et al., 2008)
VI-R	CTGTCGGACTATCCTGATGAATCTC	*cps 6 I*		
VII-F	CCTGGAGAGAACAATGTCCAGAT	*cps 7M*	371	(Emaneini et al., 2016)
VII-R	GCTGGTCGTGATTTCTACACA	*cps 7M*		
VIII -F	AGGTCAACCACTATATAGCGA	*cps 8J*	282	(Zhang et al., 2018)
VIII-R	TCTTCAAATTCCGCTGACTT	*cps 8J*		
dltS-F	AGGAATACCAGGCGATGAACCGAT	*dlts*	952	(Poyart et al., 2008)
dltS-R	TGCTCTAATTCTCCCCTTATGGC	*dlts*		

Table 3. Multiplex PCR temperature program used for *Streptococcus agalactiae* serotyping based on capsular antigen coding genes (Poyart et al., 2008).

Number of cycles	Time	Temperature	phase
1	3	94 °C	Hot start
40	45	94 °C	Denaturation
	40	54 °C	Annealing
	30	72 °C	Extension
1	10	72 °C	Final extension

2.6. Statistical analysis of data

Statistical analysis of data was performed by analysis of variance (ANOVA) and t-test in SPSS 22 software.

3. Results

3.1. Presence of Streptococcus agalactiae in the experimental groups

In the present study, a total of 220 bacterial isolates were obtained from the samples. The results from the identification of bacteria based on biochemical testing are shown in Table 4. A number of 6 *Streptococcus agalactiae* isolates were detected in this study which their PCR products on 1% agarose and 10% acrylamide gels are shown in Figure 1. The results from identification methods showed that 18.1% of the isolated bacteria from the patient group and 3.6% of the isolated bacteria from the control group were *Streptococcus agalactiae* strains (Tables 5).

3.2. The results obtained from the statistical comparisons of demographic data in the experimental groups

Statistical comparison of the results obtained from the demographic information of the control and patient groups is given in Table 6. Findings indicate that there is a statistically significant relationship between the history of abortion, history of underlying diseases, patient stress and low levels of vitamin D with increasing abortion rates between the two groups ($P < 0.05$). Also, the rate of infection associated with abortion in the group of patients infected with *Streptococcus agalactiae* was 1.0 ± 5.08 ($P \leq 0.05$). Statistical analysis of the data also showed that there was a significant relationship between *Streptococcus agalactiae* infection and the abortion ($P < 0.05$).

Table 4. The results from morphological and biochemical tests for identification of *Streptococcus agalactiae*

Test	Result
Morphology	Rod
Gram staining	+
Hemolysis	Beta
Capsule	+
Catalase	-
Coagulase	-
VP	-
CAMP	+
Hippurate hydrolysis	+
Resistance to bacitracin disk	+

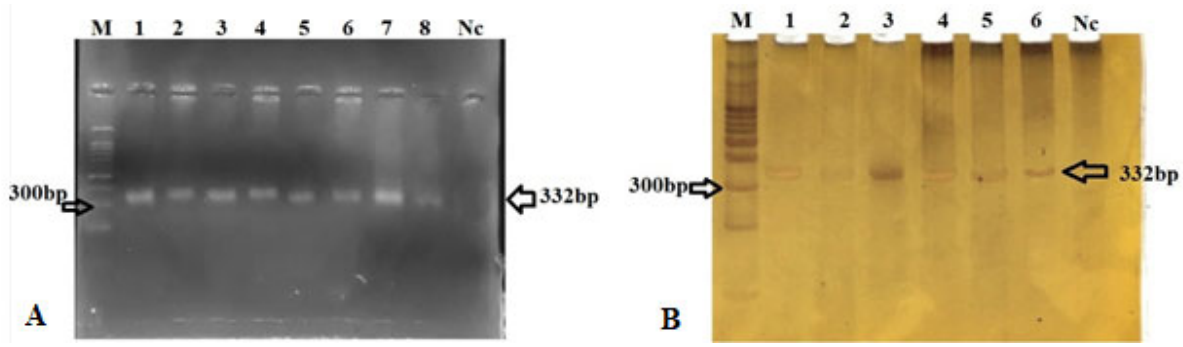

Figure 1. PCR products of 16SrRNA gene amplification in 1% agarose gel (A) and 10% polyacrylamide gel (B). Lane M: 100 bp weight marker; Lanes 1-6: PCR products in 6 isolated Streptococcus agalactiae; Lanes 7 and 8: positive control (Streptococcus agalactiae ATCC12386; Lane Nc: negative control. PCR products with a 332 bp size represent the bacterium.

3.3. *Capsular polysaccharide serotypes distribution among the isolates*

The results obtained from molecular analysis for detection of capsule serotype distribution showed that type III had the highest frequency (42.13%) among the isolates. The most other frequent serotypes were Ia, II, V, and IX. The serotypes VIIV, VIII, VII were not observed. The serotypes frequencies are illustrated in Figure 2.

Table 5. Frequency distribution of research units based on the presence of Streptococcus agalactiae in the 2 studied groups.

Variable	Result	Group				Statistics	P value	Odds ratio (95% confidence interval)
		Aborted		Control				
		Number	Percent	Number	Percent			
Streptococcus agalactiae	-	90	81.9	103	93.6	7.135	0.008	3.270 (8.091-1.321)
	+	20	18.1	7	6.4			

Table 6. The average of the results from some demographic characteristics of patients in this study.

Indicators		*Streptococcus agalactiae* infection			Abortion		
		P value	Control	Sample	P value	Control	Sample
Abnormal weight gain	-	0.081	1	13	0.180	57	47
	+		3	7		53	63
Employment	Employed	0.056	2	6	0.091	45	43
	Housewife		2	14		65	67
Underlying diseases	+	0.030	2	9	0.067	85	80
	-		2	11		25	30
History of abortion	+	0.009	0	7	0.032	8	9
	-		4	13		102	96
History of stress	-	0.005	2	7	0.021	65	68
	+		2	13		45	42
Vitamin D level	Normal	0.037	1	6	0.067	56	57
	Low		3	14		54	53

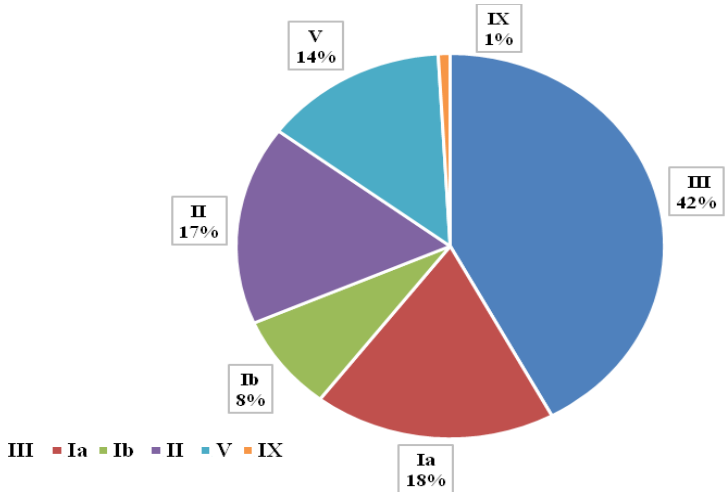

Figure 2. The frequencies of *Streptococcus agalactiae* serotypes among the studied patients.

4. Discussion

Streptococcus agalactiae is among the normal gut flora in some humans, but can also be colonized in secondary organs such as the vagina in women (Liu et al., 2018). This bacterium can cause urinary tract infections in men and urinary tract, uterine, and pelvic infections which may lead to infertility or miscarriage in women (Billick and Gold, 2019; Njum et al., 2019). For these reasons, the studies on the presence of *Streptococcus agalactiae* and finding the risk factors affecting this infection can help to prevent maternal and neonatal infections.

Streptococcus agalactiae has different presence in the female reproductive system in different geographical areas which varies from 1% to 40% (Yoshida et al., 2021). These great differences may because of the factors affecting the infection including race, climate, age, sexual activity rate, and finally the methods used for the isolation and identification of this bacterium (Phuoc et al., 2021; Xie et al., 2021).

The present study examined 220 isolates in one year in Isfahan, of which 18.1% in the patient group and 3.6% in the control group were identified as *Streptococcus agalactiae*. The results obtained according to the filled-out questionnaire about the demographic characteristics of the patient and control groups showed that there was a statistically significant relationship between the history of abortion, weight gain, high stress and low levels of vitamin D with increasing abortion rates between the two groups. A history of miscarriage, underlying disease, high stress, and low levels of vitamin D have also increased the risk of vaginal infection by *Streptococcus agalactiae*.

The studies by Boeh et al. (2019), Brooks et al. (2019), and Bearak et al. (2020) have found an association between an increased risk of miscarriage and a history of miscarriage. Research by Jacob et al. (2019), and Akdag Topal and Terzioglu (2019) have also shown that women with anxiety and stress are associated with higher rates of abortion. There are also several studies that have shown the relationship between vitamin D deficiency in the blood with an increase in abortion rates, including the studies of Tayeb et al. (2019) and Ibrahim (2020). Also, the existence of a history of underlying diseases such as hypertension, and diabetes has been shown in the studies of Somers (2020), Wu et al. (2018) as well as Ghodrati et al. (2019), all of the results are similar to the results of the present study.

In a study conducted by Liu et al. (2018) on 300 pregnant women, during childbirth, and on their infants, the rate of mothers carrying *Streptococcus agalactiae* was 17% (Liu et al., 2018). Silvestre et al. (2020) showed that 12% of pregnant women examined during childbirth carried *Streptococcus agalactiae*, and that the factors including the age, weight gain, and high stress were contributed to the colonization of the bacterium. The results of this study are partially similar to the findings of the present study.

Regan et al. (1996) in a large four-year study examined the association between the establishment of *Streptococcus agalactiae* during pregnancy and its consequences. They tested vaginal secretions for *Streptococcus agalactiae* at 7 medical centers in the United States at 23 to 26 weeks of gestation, and during childbirth for comparison of the number of abortions and premature births among the infected women with *Streptococcus agalactiae* and uninfected women. They found that the presence of *Streptococcus agalactiae* at 23 to 26 weeks' gestation was associated with an increased risk of miscarriage.

Various studies have shown that *Streptococcus agalactiae* is an important cause of miscarriage and stillbirth (Edwards and Baker, 2015). The results of molecular epidemiology in the present study also showed that 18.1% of women with abortion were infected with *Streptococcus agalactiae*, which was consistent with the mentioned previous studies. In the study of Cai et al. (2020) which used multiplex PCR to identify the serotypes of group B Streptococci, the results showed a high agreement of group B streptococci identification by microbiological methods in culture media and PCR. Similarly, in the present study, the results of biochemical tests and PCR were highly consistent. The fetus is protected against many infectious agents. The fetal immune system does not develop until a few months after birth. Antibodies that cross the placental barrier, including immunoglobulin G (IgG), protect the fetus against many infections, however, during

pregnancy, the fetus can be exposed to some of the mother's infectious agents. Pregnancy infection is transmitted from mother to fetus through the blood or through ascending pathways (Takahashi et al., 2021). Ascending infections are usually the result of pathogens that travel up the vagina or cervix due to premature or prolonged rupture of membranes or during pregnancy. Amniotic fluid infection is a major cause of miscarriage and may occur in the presence of a healthy membrane without clinical symptoms (Regan et al., 1996). *Streptococcus agalactiae* has been isolated from amniotic fluid and has been frequently found in inflammation of fetal membranes (Yoshida et al., 2021). There have been several reports of the *Streptococcus agalactiae* invasion into amniotic fluid without rupture of embryonic membranes (Naeye and Peters, 1980). Takahashi et al. (2021) pointed to the relation between amniotic fluid infection and abortion, and also cited invisible intrauterine infection as the leading cause of abortion. They have identified *Streptococcus agalactiae* as a key pathogen in this field. Other study has identified inter-amniotic infections as major causes of miscarriage in asymptomatic women with healthy amniotic membranes (Regan et al., 1996). The study by Piedimonte et al. (2018) have also shown that some pathogenic bacteria, such as GBS, primarily attack the amniotic fluid and chorioamnionic fluid, then enter the fetus, leading to miscarriage.

The global distribution of GBS serotypes varies based on geographical location, racial differences, and study time (Njum et al., 2019). In a study conducted by Haimbodi et al. (2021), out of 1129 patients, 3.18% were infected with *Streptococcus agalactiae* in Namibia among which 29% had the capsule type Ia, 27% had the capsule type III, and 17% had the capsule type V. Types VII and VIII were not found in their study. In a study conducted in Korea in 2010, serotypes III (8.29%), V (27.27%) and Ia (17%) had the highest frequency (Seo et al., 2010). In a study in Tabriz in 2010, the presence of GBS was 2.5% and the presence of serotypes were V (5.19%), Ia (9.17%), II (2.16%), Ib (6.13), III (5.9%) and IV (2.8%) (Nahaei et al., 2007). Contrary to the results of this study, serotypes III and IV had a low prevalence.

In this study, the serotype III had the most frequency (42.13%) and the frequencies of the other serotypes were 18.27% for Ia, 8.07% for Ib, 16.73% for II, 13.9% for V, and 0.9% for IX. Two types of carbohydrate antigens have been defined in the cell wall of *Streptococcus agalactiae*. The group B antigen is common in all strains, and the type of capsular polysaccharide antigen has led to classify this bacterium in 10 serotypes (Jackson et al., 1995). In various studies, it has been determined that the distribution of serotypes of *Streptococcus agalactiae* is different in various geographical areas. A systematic review and meta-analysis on the prevalence of *Streptococcus agalactiae* infection in low-income countries in 2012 showed that the dominant serotype which was identified in all these areas with existing data, was the serotype III (48.9%); followed by serotypes Ia (22.9%), Ib (7.0%), II (6.2%) and V (1.9%). In general, five serotypes Ia, Ib, II, III and V included 85% of all serotypes in these countries (Edmond et al., 2012). In A recent study on the geographical distribution of *Streptococcus agalactiae* has shown that the serotype III has been the most common type in Europe, Central Asia, Africa, and Australia (Asadollahi et al., 2021). The two above studies show that the serotypes III have more been observed in most areas of Central Asia since 2012 than other genotypes. Also, the results of the two studies are consistent with the results of this study. Determination of type of the serotypes in each region helps to plan vaccination and treatment protocols (Bobadilla et al., 2021) and determining them in Isfahan in this study can be helpful for future decisions in this area.

Conclusion

According to the results of previous studies as well as the result of the present study, it seems that *Streptococcus agalactiae* has a role in the deficiencies in female reproductive system such as the abortion. In order to better understand the mechanisms that this bacterium applies for induction of these deficiencies, further researches are required.

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Refereces

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