Original Article

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Molecular Identification of Streptococcus agalactiae Using gbs1805 Gene and Determination of the Antibiotic Susceptibility Pattern of Isolates

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ABSTRACT

BACKGROUND AND OBJECTIVE: Streptococcus agalactiae is the major factor for sepsis and meningitis in newborns and pneumonia and bacteremia in the elderly. Despite the importance of this pathogen, no accurate statistics are available regarding the prevalence of associated infections in adults. The purpose of this study was to analyse the efficacy of gbs1805 gene in the identification of isolates using laboratory and bioinformatic analyses and determine the prevalence of infections caused by Streptococcus agalactiae.

METHODS: In this cross-sectional study, 522 urine samples and genital swabs were collected from patients with suspected urinary tract infections and analyzed using culture and biochemical tests. The Streptococcus agalactiae isolates were examined for the presence of gbs1805 gene and antibiotic susceptibility was determined using Clinical and Laboratory Standards Institute (CLSI) guidelines.

FINDINGS: In this study, 54 isolates of Streptococcus agalactiae (10.3%) were obtained from the infections. All the isolates had preserved gbs1805 genes. The highest and lowest sensitivities were related to ceftriaxone (76%) and erythromycin antibiotics (33%).

CONCLUSION: Identification of preserved gbs1805 gene in clinical samples can determine infections caused by Streptococcus agalactiae. Due to the growing resistance of isolates to penicillin, alternative antibiotics should be used for the treatment of infections caused by these bacteria.

KEY WORDS: Streptococcus Agalactiae, Molecular Diagnosis, gbs1805 Gene, Antibiotic Susceptibility.

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Introduction

Streptococcus agalactiae (S. agalactiae) is colonized in the urogenital tract, as well as respiratory and gastrointestinal systems. This bacterium can cause bacteremia, pneumonia, and meningitis in newborns (1) and is often developed by capsular serotypes Ia, III, and V. S. agalactiae causes bacteremia, pneumonia, and skin infections in non-pregnant women and older men. It also leads to wound infection, urinary tract

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infection, asymptomatic bacteriuria and cystitis, acute inflammation of the bladder and kidney, and acquired pelvic inflammation in pregnant women. Most adult diseases are caused by serotypes Ia and V (2, 3). Over the past decade, the incidence of infections caused by S. agalactiae, especially among the elderly with immunodeficiency, has increased. In fact, in the United States, the majority of deaths associated with S. agalactiae occurs among the elderly (4, 5). S. agalactiae colonization in pregnant women in the United States and other industrialized countries ranges between 16% and 24% (6).

Also, in 32-34% of men and non-pregnant women in USA, S. agalactiae is colonized in the urogenital tract (7). Vaginal colonization of S. agalactiae in pregnant women has been reported to be 5.2-26.7% in Iran (8-10). However, no studies are available regarding the colonization of urogenital systems in non-pregnant adults in Iran. These types of infection are transmitted through sexual intercourse or ingestion (7) and transmission to infants occurs through the birth canal or the amniotic fluid (11). Asymptomatic colonization of S. agalactiae in healthy people can cause the bacteria to be transmitted to high-risk people including pregnant women, the elderly, and people under treatment. Thus, in order to prevent transmission to high-risk individuals and infants, determination of the prevalence of infections, caused by S. agalactiae, is necessary. Antibiotic penicillin is the first-line treatment for infections caused by S. agalactiae. Although this bacterium is sensitive to penicillin, ampicillin, and vancomycin, it has become resistant to second-line antibiotics such as erythromycin and clindamycin (10-20%) (12). In recent years, cases of bacterial resistance to penicillin have been also reported (13). In several studies, several genes were used for the molecular detection of S. agalactiae in clinical samples. Some of these genes such as scpB and cfb were confirmed as diagnostic markers by Polymerase Chain Reaction (PCR) method (14, 15). Other genes such as lmb, bca, bac, and rib have been also examined, although their presence in all isolates was not confirmed (16). In bioinformatic genome study of S. agalactiae, a set of proteins have been identified which appear to be similar to glucan-binding proteins in other bacteria. Polysaccharide glucan is composed of D-glucose units. Glucan-binding proteins include enzymes that catalyze the synthesis of glucan and have the ability to hydrolize glucan to starch and cellulose, which are required for bacterial growth (17). Gbs1805 gene, which encodes glucan-binding proteins, has not been examined so far. Previous bioinformatic studies have shown that preserved gbs1805 gene only exists in S. agalactiae with a significant sequence. Therefore, it seems that a new index that is fully preserved and exists solely in the isolates of S. agalactiae can be useful for the clinical identification of such bacterial species. This study aimed to evaluate infections caused by S. agalactiae and determine the antibiotic susceptibility of isolates. We also addressed the efficiency of gbs1805 gene for molecular diagnosis of such infections

Methods

Sampling method: In this cross-sectional study, 522 urinary and genital swab samples from patients with suspected urogenital tract infections, referring to Noor and Markazi Pathobiology Laboratories, were studied.

Culture and antibiogram: After culturing the bacteria on blood agar and overnight incubation, Gram-positive beta-hemolytic bacteria were studied via catalase test, CAMP, and bile esculin test. CAMP-positive, catalase-negative bacteria which lacked the ability to grow in Bile esculin agar, were confirmed as S. agalactiae. Sensitivity of S. agalactiae isolates to penicillin (10 units), ampicillin (10 µg), vancomycin (30 µg), erythromycin (15 µg), clindamycin (2 µg) and ceftriaxone (30 µg) antibiotics was asssessed on Mueller Hinton blood agar; the isolates were incubated overnight at 37 °C and investigated according to Clinical and Laboratory Standards Institute (CLSI) guidelines, using Himedia discs (India).

Genome extraction: S. agalactiae isolates were inoculated in 5 ml lysogeny broth and incubated for 18-24 hours at 37 °C. After this period, the culture medium was centrifuged at 5000 rpm for 3 min. Then, 300 microliters of STET solution (Tris-Hcl, EDTA,

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Triton X100, NaCl) was added to the precipitation and boiled at 100 °C for 15 min. Afterwards, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to another sterile microtube and was placed at -20 °C for 20 min after adding isopropanol (three times the volume of supernatant).

After this period, the microtube was centrifuged at 14,000 rpm for 10 min; the supernatant was removed and incubated at 37 °C until the alcohol completely dried. Then, 50 µl of distilled water was added to the microtubes and stored at -20 °C. To assess the quality of the extracted DNA, we electrophoresed it on 1% agarose gel.

PCR technique: The primers in this study were designed based on gbs1805 gene information in JCVI sequence database. The sequences of forward and reverse primers in this study were determined as follows:

Forward primer: AAT ACA TAT AAC TAT GCA GTA GAT GTA

Reverse primer: ATT CGG ATA AAT GTA GCT AAC TAC

The preserved Gbs1805 gene in the evaluated isolates was amplified by PCR using 12 microliters of mixed mastermix (Sinaclon, Iran), two microliters of DNA (57 ng/ul), one microliter of each primer (20 pmol/µl), and 9 microliters of distilled water, according to the stages presented in table 1. The standard strain of S. agalactiae NEM316 was examined regarding the presence of gbs1805 gene, using the mentioned procedures; the resulting product was sent to Takapouzist Company for sequencing. To analyze the results, McNemar's test was applied and $p<0.05$ was considered statistically significant.

Table 1. PCR steps for the identification of gbs1805 gene

Steps	Temp/time	Number of Sequences
Initial denaturation	95°C/5 min	1
Secondary denaturation	95°C/1 min	30
Connection	59°C/45 sec	
Elongation	72°C/45 sec	
Final elongation	72°C/10 sec	

Results

Among 522 samples, 54 isolates of S. agalactiae (10.3%), including 6 genital swabs and 48 urinary samples, were obtained. In total, 55% and 45% of samples were from female and male subjects, respectively. As the results indicated, 85% of isolates were obtained from female samples. Antibiotic susceptibility of isolates was studied and the lowest and highest sensitivities were related to ceftriaxone and vancomycin (76%) and erythromycin (48%), respectively (table 2). Penicillin and erythromycin antibiotics were significantly different from ceftriaxone and erythromycin ($p<0.05$).

Table 2. Antibiotic susceptibility of S. agalactiae isolates

Antibiotics	Sensitive isolates N(%)	Isolates with intermediate sensitivity N(%)	Resistant isolates N(%)
Penicillin (10 units)	32(64)	-	18(36)
Ampicillin (10 µg)	29(58)	-	21(42)
Vancomycin (30 µg)	38(76)	-	12(24)
Ceftriaxone (30 µg)	38(76)	-	12(24)
Erythromycin (15 µg)	24(48)	18(36)	8(16)
Clindamycin (2 µg)	35(70)	6(12)	9(18)

All isolates of S. agalactiae, obtained by culturing, were studied with regard to preserved gbs1805 gene. Isolates including 432-bp bands in the electrophoresis of PCR products, were confirmed as carriers of gbs1805 gene (fig 1). The strain of S. agalactiae NEM316 was used as a positive control. Accordingly, all isolates of S. agalactiae contained gbs1805 gene. Gene sequencing results were recorded in the Gene Bank and no mutations were observed in the studied gene (Accession number: KM39696). The multiple alignment results showed that this gene was 100% similar in different strains of S. agalactiae and was

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completely preserved. In addition, gbs1805 gene was specific to S. agalactiae and could not be found in other bacteria.

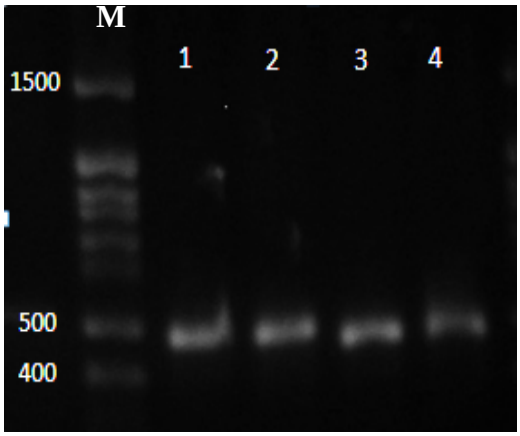


Figure 1: Electrophoresis of PCR product with an approximately 432-bp band for evaluating the presence of gbs1805 gene on 1% agarose gel. (M) Marker 100bp (Gene On, USA), (1) positive control, strain of S. agalactiae NEM316, 2-4 isolates of gbs1805 gene

Discussion

Among 522 samples examined in this study, 54 isolates of S. agalactiae were obtained, among which 88% were from positive urinary samples. Also, 85% of isolates were obtained from women, which indicates the higher rate of infections among women, compared to men. All S. agalactiae isolates in this study were positive for gbs1805 gene. In addition, 64% of isolates were sensitive to penicillin, and 48% were sensitive to erythromycin. According to previous studies on colonization and infection in pregnant women in Iran, the prevalence of colonization ranged from 5.2% to 26.7% (10, 11). In another study in Tehran, the prevalence of colonization in pregnant women was 9.6% (14). In our study, the number of pregnant women was 4 (3.8%), which is not comparable to the previously mentioned study due to the small sample size. In a twenty-year study in England and Wales during 1999 and 2010, the annual report showed that S. agalactiae infections had doubled in number (from 1.4 to 2.9 per 100,000 populations) and had significantly increased among adults (18). Other studies have also found that the prevalence of infections caused by S. agalactiae among adults has increased by 32% (19). In the present study, the prevalence of S. agalactiae infections was found to be 10.3%; also, 94% of cases were observed in adults. Although infection rates in newborns reduced due to antibiotic prophylaxis, an increasing rate of infections caused by S. agalactiae could be seen in adults. Antibiotic susceptibility testing of isolates showed that penicillin with 64% sensitivity is still a good option for the treatment of S. agalactiae infections. However, it should be noted that the reduced sensitivity of clinical isolates to penicillin indicates the need for suitable alternative antibiotics. Several studies have demonstrated that S. agalactiae isolates do not show any resistance to beta-lactam antibiotics; erythromycin resistance rate was reported to be 4.7-12% (20-22). Studies conducted in 2008 and 2011 in Japan showed that the sensitivity of clinical isolates of S. agalactiae to penicillin is declining due to changes in PBP2X protein in walls of bacteria. In fact, use of these antibiotics as first-line treatment for infections caused by S. agalactiae is being questioned (23, 24). In another study conducted in Norway, antibiotic resistance of 426 isolates to erythromycin, clindamycin, and tetracycline was 10.2%, 9.6%, and 76.6%, respectively. Also, sensitivity to penicillin in this study was 100% (25). In a study in Swiss, all 364 examined isolates were susceptible to penicillin. Erythromycin and clindamycin resistance rates (including inductive resistance) were 14.5% and 14%, respectively (26). In our study, the sensitivity of isolates to beta-lactams varied from 53% to 76%, and sensitivity to erythromycin was 33%.

It seems that increased resistance to beta-lactam antibiotics in isolates is due to the uncontrolled use of antibiotics, European and American guidelines for antibiotic use, and laboratory errors in determining the antibiotic susceptibility of S. agalactiae isolates in our country. Therefore, due to the increasing resistance to commonly used antibiotics, ceftriaxone (76% sensitivity) can be a good option for treating infections caused by S. agalactiae, especially in cases of allergy to penicillin or isolate resistance to antibiotics. In recent years, cfb and scpB genes (14, 15), as well as hylB, scpB, cylE, lmb, rib, bca and bac have been used

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for the identification of S. agalactiae (16). In a study by Dmitriev et al. on the detection of S. agalactiae isolates by scpB gene, it was found that this gene can be used as a diagnostic marker for S. agalactiae (27). In another study, the sensitivity and specificity of PCR were 99.6% and 100% for scpB gene, and 100% and 98% for cfb gene, respectively (10, 28). In a study in Malaysia, the presence of hylB, scpB, cylE and lmb genes in almost all S. agalactiae isolates (103 isolates) was confirmed, while rib, bca and bac were present in 29.1%, 14.6%, and 9.7% of isolates, respectively (16). In the present study, the gbs1805 gene was used to confirm the presence of S. agalactiae isolates, obtained by culture. It was determined that the gene was preserved in all isolates with no exception. Bioinformatic studies have indicated that this gene is specific to S. agalactiae and is not found in other bacteria. Based on laboratorial study, this gene can be used for the detection of S. agalactiae. In previous bioinformatic studies, several genes have been considered in gram-positive bacterial species; these studies help determine the efficacy of these genes for the diagnosis of bacterial species (29-33). Identification of preserved gbs1805 gene in clinical samples can be effective in the diagnosis of infections caused by S. agalactiae. Due to the growing resistance of isolates to penicillin and their high sensitivity to ceftriaxone, it appears that ceftriaxone can be a good alternative antibiotic for the treatment of infections caused by S. agalactiae.

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