Identification of Group B Streptococci Using *16S rRNA, cfb, scpB,* and *atr* Genes in Pregnant Women by PCR

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Received: 15 Feb. 2016; Accepted: 22 Aug. 2016

Abstract- *Streptococcus agalactiae* is acommensalorganism, but it may cause infection in susceptible hosts. The aim of this study was to evaluate PCR assay compared with conventional culture method for direct detection of *Streptococcus agalactiae*. Total of 203 paired low vaginal swabs were collected from women at 35-37 weeks of pregnancy from June 2013 through February 2014 for detection of *Streptococcus agalactiae* using PCR assay targeting 16S rRNA, *cfb*, *scpB*, and *atr* genes and culture method following broth enrichment. The results were recorded and evaluated for determining of sensitivity, specificity, positive and negative predictive values of PCR assaycompared with culture method. Prevalence of *Streptococcus agalactiae* was determined as 7.39% (n=15) using culture method; 19.70% (n=40) by PCR targeting 16S rRNA gene; 18.23% (n=37) by targeting *atr* gene; 17.24% (n=35) by *cfb* gene; and 8.87% (n=18) by *scp*B gene. Generally, a total of 49 specimens were considered true positive (27 samples by PCR assay using the four genes in sum, 4 samples only by *atr* gene PCR, 3 samples only by *cfb* gene PCR, 2 samples only by culture method, and 13 samples by PCR assay and culture method in common) and prevalence of *Streptococcus agalactiae* determined 24.14% in Hamadan. The current data demonstrated that performing only culture method for detecting GBS from pregnant women leads to missed false negative carrier individuals. Thus, it is recommended that both the PCR assay and conventional culture method to be performed in order to detect *Streptococcus agalactiae*.

Acta Med Iran, 2016;54(12):765-770.

Keywords: *Streptococcus agalactiae*; Pregnancy; Polymerase chain reaction

Introduction

Group B streptococcus (GBS; *Streptococcus agalactiae*) is a normal part of women's vaginal and gastrointestinal tract flora (commensal organisms); however, it can give rise to life-threatening infections in some vulnerable hosts including infants, pregnant women and nonpregnant adults with chronic diseases (such as underlying diseases like diabetes and cancer). Moreover, GBS is the main cause of invasive bacterial disease in infants (1,2).

Also, there is a higher possibility for pregnant women to develop GBS related diseases both before and after childbirth; urinary tract infection or more serious diseases like chorioamnionitis or bacteremia are such diseases. A number of studies have been demonstrated that such diseases constitute 6.3 percent (3) and 11 percent (4) of all diseases caused by invasive GBS. It is also worth noting that maternal infections can lead to premature delivery, low birthweight babies or abortion. In a significant portion of pregnant women, GBS colonization may transiently, chronically, or periodically happen thereby GBS infection can be detected in aspecific period of pregnancy time (5). Using rapid screening test available for detecting GBS-related infections, carrier mothers can be identified before or during childbirth. Consequently, unnecessary administration of prophylactic antibiotics for non-infected mothers during labor is avoided, and this kind of screening is usually carried out at 35-37 weeks of pregnancy (6).

It is believed that cultures or tests which are carried out during the last five weeks before delivery are fairly accurate in predicting the presence of GBS in themother during childbirth (7). It is well known that vertical transmission of this organism in babies can be prevented

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by administration of prophylactic antibiotics to mothers during labor. Therefore, prevention of EOD in infants is possible only if the mother has received antibiotics during labor (8). Nowadays, the golden standard recommended by Center for Disease Control (CDC) for detecting rectovaginal between 35 to 37 weeks of pregnancy is the culture method so that rectovaginal swabs are cultivated in a selective medium at first, and then the cultures are passaged on the agar medium.

However, the method requires a cultivation period of about 24-72 hours in order for achieving the desired results, which is too time-consuming to be useful during labor (9,10). Furthermore, according to review studies conducted in this area, the probability of a GBScolonization to be predicted by this method is just about 87 percent, and because of transient nature of GBS colonization, a negative result of culture at 35-37 weeks of pregnancy should not be considered as a definite proof of the absence of infection.

On the other hand, several studies indicate insufficient sensitivity of cultures to detect GBS colonization in labor before or close to birth, and low sensitivity and specificity of other rapid tests introduced in this area (such as antigen-based tests, and procedure-based hybridization or hybridization-based methods) for direct identification of GBS from clinical samples of pregnant women are demonstrated. Several specific culture medium have been introduced which are able to rapid identification of GBS colonization based on their unique ability to produce pigment; but some GBS strains are not able to produce pigment (strains without hemolysis) which is why the specific culture medium is not a good choice to be used as a tool for their rapid identification (11). Rapid and reliable detection of GBS colonization in pregnant mothers, especially those who have had a poor obstetric care, would be useful and will allow us to more effectively prevent GBS infection. According to the description above, the need for a rapid, specific, and sensitive test for direct detection of GBS from clinical samples is an imperative for planning a simpler and more effective prevention program. Accordingly, this study was designed to investigate *16S rRNA*, *scpB* (*S. agalactiae* C5a peptidase), *atr* and *cfb* (CAMP factor) genes as targets in *Streptococcus agalactiae* for rapid and direct detection of GBS.

Materials and Methods

Study design

The study was performed from June 2013 to February 2014. The study was approved by the ethical committee of Hamadan university medical sciences, and the written consent was obtained from all the patients. The included individuals were women at 35-37 weeks of pregnancy with no clinical problem, referred to prenatal care of Fatemieh Hospital and/or private clinic centers in Hamadan. The criterion for exclusion was a pregnant woman at <35 weeks of gestation. The information about age and history of antibiotic usage until two weeks prior to the study was questioned and recorded. A total of 203 double-vaginal specimens were collected using sterile cotton tip swabs from thelower vagina for each pregnant woman. One of the swabs was placed into an examination tube containing Stuart transport medium (for microbiological culture), and the another one was placed into an examination tube containing 1 mL phosphate buffered saline (1x PBS) with pH=7.2 (for direct DNA extraction and performing PCR).

Isolation and detection by culture method

Upon the collected samples, the swab specimens in Stuart transport medium were removed and inoculated into Todd-Hewitt broth (Pronadisa Co., Spain) supplemented with 1% yeast extract, 10 µg/mL of colistin and 15 µg/mL of nalidixic acid (Lim broth). This inoculated selective broth was incubated at 35-37°C in 5% CO_2 (in candle jar) for 18-24 hours. The incubated broth was subcultured to a plate containing tryptic soy agar with 5% defibrinated sheep blood and was incubated at 35-37°C in 5% CO2 for 18-24 hours. Then, the plates were inspected and identified for GBS organisms by the following criteria: colonies with narrow zone of beta hemolysis, Gram-positive cocci, catalase negative, resistance to bacitracin and trimethoprim/ sulfamethoxazole (SXT) diagnostic antibiotic discs, sodium hippurate hydrolysis-positive, and CAMP [Christie, Atkinson, Munch, Peterson] positive.

DNA extraction and polymerase chain reaction assay

DNA was extracted by thermal lysis (boiling method) (12) with some modifications. The 405-bp 16S rRNA gene was chosen as the GBS primer (GenBank accession number: 2353759) for PCR. The forward and reverse sequences of the primers were CGCT-GAGGTTTGGTGTTTACA (40-61) and CACTCCTACCAACGTTCTTC (445-465), respectively (13). Homology checking was performed using the BLAST database. And also the three other atr (GI No.AF15135), cfb (GI No.X72754) and scpB (GI No. AF189002) genes, as previously described (14,15), were used too. These primers were synthesized by BioneerOligoNucleotide Synthesis Co. (Korea).

The PCR reaction was 20 µL which was prepared as follows: 10 µL of 2x premix master mix (Parstous Biotech Co., Iran), 5 µL sterile double-distilled water, 1 µL of the forward primer, 1 µL of the reverse primer, and 3 µL of the DNA sample. The DNA samples as well as a positive control (a sample including DNA from S. agalactiae ATCC 12386) and a negative control (a sample without template) were amplified by an initial denaturation step for five minutes at 94° C, followed by 35 cycles of 94° C for 45 seconds, 60° C for 60 seconds, and 72° C for one minute, and a final cycle of 72° C for seven minutes in a Bio-Rad thermal cycler. After amplification, 5 µL of each amplification product was combined with 1 µL of 6x DNA loading dye buffer (Parstous Biotech Co., Iran) and analyzed by electrophoresis on a 1.5% (w/v) agarose gel, stained with DNA safe stain (Cinna Clone Co., Iran).

Determining specificity and limit of detection (sensitivity) in polymerase chain reaction (16S rRNA primers)

To assess the specificity of the PCR assay, a battery of 10 non-GBS microorganisms (Table 1) was tested for the gene. The lower limit of detection (sensitivity) of the PCR was determined by performing a 10-fold serial dilution of 10^8 CFU/mL (standard 0.5 McFarland) GBS from 10^8 to 10^0 CFU/mL.

Table 1. The list of bacterial species used for assessing specificity of PCR method in detecting GBS with target of 16s rRNA, cfb, atr, scpB

Bacterial species
Streptococcus mutans *
*Streptococcus pneumoniae**
Neisseria sica *
Escherichia coli ATCC 25922
*Klebsiellapneumoniae**
Proteus mirabilis *
Citrobacterfreundii *
*Staphylococcus aureus*ATCC 25423
*Enterococcus faecalis*ATCC 29212
*Enterococcus faecium**

* Clinical isolates

Statistical analysis

The PCR results for four genes were compared with the results of the golden standard and using the related equation sensitivity, specificity, positive predict value (PPV), negative predict value (NPV), and efficiency of the PCR results was determined. Pearson correlation analysis and Chi-Square test were utilized to assess the correlation between two methods. All statistical analyses were performed with SPSS software (version 21).

Results

Identification of *GBS* by culture and PCR

The prevalence of GBS colonization among pregnant women (at 35-37 weeks) was 7.39 percent. However, using PCR method (*16s rRNA*), it was found that 40 of 203 (19.7 percent) samples had a positive GBS result. To ensure the true positive results, all positive results were repeated (Table 2).

Table 2. Comparison of PCR results to culture results (as golden standard)

Test		PCR results											
		16 s rRNA			*cfb*			*atr*			*scpB*		
		Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total	Pos	Neg	Total
Culture (goldstandard)	**Positive**	13	2	15	11	4	15	12	3	15	9	6	15
	Negative	27	161	188	24	164	188	25	163	188	9	179	188
	Total	40 (19.70%)	163	203	35 (17.24%)	168	203	37 (18.23%)	166	203	18 (8.87%)	185	203

The results obtained for *16s rRNA* gene were compared to the results obtained for other genes, i.e. *atr*, *cfb*, *scpB*. Using *cfb*, *atr*, and *scpB* genes, 35,37, and 18 samples have been determined to have a positive result, respectively; It was interesting that all these samples that had positive results were among the 40 samples that already their positive results had been determined using *16s rRNA* PCR method. So, it can be concluded that all

these 40 samples were true positive results (Table 2).

Specificity of *scpB, atr, cfb,* and *16s rRNA* genes targeting PCR method

For assessing the specificity of primers (genes) used in PCR test, ten bacterial species other than GBS were examined. For seven of these ten species, there was no specific band, whereas the weak band (amplicon) of 405 bp was found for *S.aureus (ATCC 25423), E.faecalis* (ATCC 29212), and *E. faecium* (clinical isolates); suggesting that *16s rRNA* is the probable region of homology between GBS and bacterial species. Moreover, no specific band was found in *scpB*, *atr*, and *cfb* genes targeting PCR for all ten bacterial species.

Limit of detection (sensitivity) of *scpB, atr, cfb,* and *16s rRNA* genes targeting PCR method

The results of the study demonstrated that the PCR method by using *16s rRNA,cfb* and *atr* genes targeting is capable of detecting concentration as low as 100 cells per mililiter (10^2 CFU/ml); However, the results showed that the limit of detection for *scpB* gene targeting PCR is 10^3 CFU/ml. Furthermore, the results obtained by PCR method, as a direct diagnostic test, including specificity, sensitivity, PPV, NPV, and efficiency, were compared with the results of culture method, as the golden standard and presented in Table 3.

Table 3. Specificity, sensitivity, PPV, NPV, and efficiency of PCR results

PCR test	Sensitivity (%)	Specificity (%)	PPV%	NPV%	Efficiency
16S rRNA PCR	86.67	85.64	32.50	98.77	85.71
***cfb* PCR**	73.33	87.23	31.43	97.62	86.21
***atr* PCR**	80	86.70	32.43	98.19	86.21
***scpB* PCR**	60	95.21	50	96.76	92.61

Discussion

The present study has been carried out in order to make a comparison between two methods used for direct diagnosis of GBS colonization in vaginal samples of pregnant women between 35 and 37 weeks of pregnancy; gene targeting PCR and the conventional culture method. In addition to this primary goal, the study also attempted to assess the effects of age and previous use of antibiotics on GBS colonization. Regarding these objectives, pregnant women were divided into two age categoriesunder age 30 and over age 30, similarly, and based on the previous use of antibiotics, divided into women with theprevious use of antibiotics and women without previous of antibiotics. Using the Pearson correlation coefficient and Chi Square analysis, it was revealed that there is no significant difference between age and BBS colonization (*P.value*=0.622). Of the 203 participants in the study, 41 had a history of taking antibiotics within two weeks before the examination; among them, five women had the positive result of GBS colonization (three of them were only determined by PCR method, and two of them were determined by both methods). Of the 162 participants who had not a history of taking antibiotics 37 women had positive test result; 24 of them were only diagnosed by PCR method, two of them were only diagnosed by the culture method, and 11 samples were diagnosed by both methods. However, no significant relationship was observed between the two groups for GBS colonization (*P*.value=0.178). The positive test result for *scpB*, *cfb*, *atr*, and *16s rRNA* gene targeting PCR were 19.7, 18.23, 17.24, and 8.87 percent, respectively. These proportions were much higher than what was obtained by the culture method, i.e. 7.39 percent. These findings are in good agreement with the finding of other studies from other countries (16-20). Furthermore, it was stressed by the CDC in their last report on appropriate methods for preventing GBS disease in newborns that PCR-based methods have a good sensitivity and also it was recommended to carry out screening test using both methods - PCR-based and the culture methods (21).

In the present study, it was demonstrated that the prevalence of vaginal GBS colonization among Hamadan's pregnant women is 24.14 percent that is compatible with the findings of other studies, which reported a range from 10 to 30 percent (19). Moreover, epidemiological studies conducted in Iran have been indicated that GBS colonization rate is in a range from 5.3 to 26.7 percent (22-29). The study conducted by Fatemi*et al.*, in Tehran, Iran, found that the prevalence of GBS colonization was 19.7 and 20.6 percent using 16s rRNA gene targeting PCR and the culture methods, respectively. The sensitivity and specificity of these methods were 82.3 and 96.5 percent, respectively. The

recent study carried out by Bakhteyari *et al*., showed that the prevalence of GBS was determined 9.3 percent by culture method and 11.2 percent by cfb gene targeting PCR. In this study, the sensitivity, specificity, NPV, and PPV of the method were 100, 98, 100, and 100 percent, respectively (30).

In the study conducted by Fabien Rallu *et al*., the GBS prevalence was 16 percent by culture method while the prevalence of GBS by using *scpB* gene targeting was 22 percent (31). Another study carried out by Fernanda de-Paris, Brazil; the GBS prevalence was 26.99 percent by using *atr* gene targeting while the prevalence of GBS by the culture was 15.96 percent (12).

The different amount of prevalence reported by various study can be due to several factors related to the procedure used for sampling, preparing or analyzing samples including; the age of pregnant women involved in the study, sampling from different parts of the body, use of different culture techniques, use of various gene targeting PCR methods. Moreover, these differences can stem from the racial or geographical diversity. It is difficult to compare the positive test result of PCR for bacterial nucleic acids with a negative test result obtained by the culture method. One reason for this contradiction can be due to the high sensitivity of PCR method for detecting circulating nucleic acids, non-proliferating GBS, and/or dead GBS which can be observed in people who received an antibiotic treatment (30,31).

Errors and mistakes in collecting, storing, and shipping the samples, especially for those with a low concentration, are other factors that can influence the bacterial growth by the culture method. On the other hand, as already mentioned, about 1% to 5% of all GBS strains are Non-hemolytic, and in such cases, the GBS colonies may be invisible, or it may be difficult to differentiate them from other similar colonies from other Non-hemolytic Streptococcus and Enterococcus. The last possible answer for a positive test in PCR but negative in culture method is the possible differences in the two samples taken from an individual.

The development of rapid tests for detecting GBS colonization would help us to screen pregnant women during delivery in a more efficient manner. It is recommended that both the culture and PCR methods should be routinely performed in order to achieve more reliable results.

Acknowledgement

The authors would like to thank the vice chancellor of Hamadan University of Medical Sciences for their financial supports.

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