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Original Article

Determination of Genotypes of *Streptococcus Agalactiae* Isolated from both Urine and Vagina of Pregnant Women Referred to Gynecology Clinics of Yazd, Iran -2015

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ABSTRACT

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Background and Aims: Group B *streptococcus* (GBS) may cause neonatal infection during and or after the delivery, and is the leading cause of sepsis, bacteremia, pneumonia and meningitis. The virulence factors are carried by both capsule and surface proteins by which serotypes and genotypes are determined. However, some genotypes are believed to be related in severity of neonatal diseases, therefor this investigation was aimed to determine the surface proteins genotype of detected GBS from both vagina and urine of pregnant women.

Materials and Methods: In the present study, a total of 346 vaginal and urine samples were obtained from the same pregnant women. Following culturing the samples on sheep blood agar medium and related tests (CAMP, Hiporate hydrolysis) the suspected colonies were further confirmed by polymerase chain reaction (PCR) technique. The detected GBS species were then genotyped using multiplex PCR assay.

Results: Out of 346 specimens, 57 (16.47%), and 33 isolates (9.5%) were identified as vaginal and urine GBS, respectively. Genotype *rib* was predominant in both vaginal discharge and urine specimens, by frequencies of 56.1% and 54.5%, respectively. Other genotypes of vaginal GBS were as *epsilon* (36.8%), *anlpha-C* (3.5%), and *alp2/3* (3.5%); while GBS in urine were as *epsilon* (36.4%), *alp2/3* (6.1%), and *anlpha-C* (3%).

Conclusions: The prevalence of GBS in both vaginal and urine samples were reliable in comparison to other societies. These results showed that genotype *rib* of GBS, which seems to be associated to neonatal diseases, is dominant genotype in both urine and vaginal samples.

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Introduction

Streptococcus agalactiae or Group B Streptococcus (GBS) presents in the 10-40 percent of healthy women [1]. This bacterium colonizes in rectum and vagina of women. In these women, GBS may causes infections of endometrium, obstetric surgical wound and also causes bacteremia, obstetric sepsis, and meningitis [2-4]. GBS causes two kinds of newborns infections: early-onset diseases (EODs); and late-onset diseases (LODs). EODs affect newborns in the first week of their life, but LODs infections manifest during the 2nd week to 3rd month. Fatality rate of EOD infections is about 4-6 per cent, while those of LOD are 2-6 percent. Though, EOD infections are more dangerous, prophylaxis could prevent much of these infections during delivery, but prophylaxis does not affect LOD infections. For this reason, LODs are more prevalent that EODs, nowadays [5]. Most of the EOD infections occur before delivery. Because of optimal PH and conditions of uterus during pregnancy, GBS growths and colonizes in genital tracts of pregnant women, then spreads toward amniotic membrane and penetrates into amniotic fluid, infects pulmonary tracts of fetus. Afterward, GBS spread through the circulation systems causes septicemia, meningitis, and osteomyelitis. The source of infections in the case of LODs is not clear. In some cases, the bacterium transfer from hospital staff to newborn child, and in some cases infected mothers are the source of infections [6].

Nine serotypes have been defined for GBS based on *cps* gene clusters, which varies in distribution in different geographical areas, type of diseases and age of patients [7, 8]. In order to epidemiological and pathogenetical studies, phenotypic and molecular markers are very important. Besides capsular polysaccharide antigens, cell surface protein antigens, which contributes in pathogenesis and immune response inductions are very important [9]. Among protein surface, the *rib* is believed to be associated with neonatal diseases and remaining protein surface are found mostly in women infection [10]. Information regarding the GBS surface proteins genotyping in Iran is limited, therefore determination of GBS genotype is very important in epidemiological studies and designing of efficient vaccine [1]. The aim of this study was to investigate the GBS molecular genotyping isolated from urine and vaginal samples of pregnant women in Yazd, Iran. In addition, the prevalence of GBS from urine and vagina samples of these women was determined.

Materials and Methods

Sample obtaining and culture

In this cross-sectional study, from June 2015 to December 2016, a total of 346 vaginal swap and urine samples were obtained from 15-40 years old pregnant women, who have not taken any antibiotic within last two weeks referring to Mojibian Hospital, Seyyedoshohada Clinic, and Baghayipour Clinic of Yazd, Iran. The research was approved by the Ethics Committee of Shahid Sadoughi university of medical sciences, Yazd, Iran. Following swapping samples from vaginal by physician, preparation of urine sample was asked consequently. Samples were

transferred to Microbiology Laboratory of Shahid Sadoughi University of Medical Sciences, immediately, and cultured on blood agar base medium (Liofilchem, Italy) enriched with 5-10% sheep blood. Blood agar plates were incubated at 35°C for 18-24 h.

Bacterium identification

β-hemolysis colonies were selected and identified using Gram staining and specific tests, such as catalase, CAMP (Christie-Atkins-Munch-Petersen), and hyporate hydrolysis tests. Identified colonies were cultured in broth media (BHI, Liofilchem, Italy) and incubated for 16 h.

Genomic DNA extraction

Genomic DNA was extracted using following protocol: the selected bacteria were grown in a 5 ml culture until saturated, then 1.5 ml of the culture was microcentrifuged until a compact pellet was formed. The pellet was resuspended in 567 µl Tris EDTA (TE) buffer (10 mM Tris-Cl, 1mM Ethylenediaminetetraacetic acid [PH 8]). Followed by, 30 µl of 10% sodium dodecyl sulfate and 3µl of 20 mg/ml proteinase K (Promega,) were added, mixed thoroughly, and incubated 1 hr at 37°C. Next, 100µl of 5M NaCL were added and mixed thoroughly. After that, 80 µl CTAB/NaCL (4.1 g NaCl, 10 g CTAB in 100 ml H_2O) solutions were added, mixed thoroughly, and incubated at 65°C for 10 min. One volume (0.7 to 0.8 ml) of 24:1 chloroform/isoamyl alcohol was added, mixed thoroughly, and microcentrifuged 4 to 5 min. The supernatant was transferred to a fresh tube and 1 volume of 25:24:1 phenol/ chloroform/ isoamyl alcohol was added with thorough extraction and microcentrifuged for 5 minutes. The supernatant was transferred to a fresh tube and 0.6 volume of isopropanol was added and mixed gently. After brief centrifugation, supernatant was discarded and 70% ethanol was added to the pellet. After further centrifugation, pellet was dried and resuspended in 100µl TE buffer [11]. The quality and quantity of isolated DNA were measured by 0.7% agarose gel electrophoresis (Life Technology) and nanodraop (Eppendorf, Germany), respectively. DNA was stored in -20 °C for further studies.

Polymerase Chain Reaction (PCR)

Final confirmation of screened colonies was carried out by PCR using group B specific antigens primers [12] as follows: F: 5'-AGGAATACCAGGCGATGAACCGAT-3', R:5'-TGCTCTAATTCTCCCCTTATGGC-3' as reverse primer (amplicon size: 952 bp); the final volume for PCR reaction was 20 µl (4.5 µl of water, 10 µl of 2X PCR Master Mix (Amplicon, Denmark), 2.5 µl of working primers with final concentration of 10 pmol, and template DNA (3 µl). The thermal profile was as follows performed in thermocycler (Eppendorf, Germany): 94°C for 300 seconds as initial denaturation for one cycle; 94°C for 60 seconds, 55°C for 60 seconds, and 72°C for 60 seconds, for 30 cycles; with a final extension at 72°C for 300 seconds. Reference strains used as PCR positive control were kindly received from Professor Kong: center of Infectious Disease, New South Wales, and Australia.

Multiplex-Polymerase Chain Reaction

In order to genotyping the different isolates, Multiplex PCR was carried out using specific primers [13] as follows: 5'-

TGATACTTCACAGACGAAACAACG-3' and 5'- TACATGTGGTAGTCCATCTTCACC-3' as forward and reverse primers, for alpha-C genotype, respectively (amplicon size: 398bp); 5'-TGATACTTCACAGACGAAACAACG-3' and 5'- CATACTAGCTTTTAAATCAGGTGA-3' as forward and reverse primers, for *rib* genotype, respectively (amplicon size: 295 bp); 5'-TGATACTTCACAGACGAAACAACG-3' and 5'-CCAGATACATTTTTTACTAAAGCGG-3' as forward and reverse primers, for *epsilon* genotype, respectively (amplicon size: 200 bp); 5'-TGATACTTCACAGACGAAACAACG-3' and 5'-CACTCGGATTACTATAATATTTAGCAC-3' as forward and reverse primers, for alp2/3 genotype, respectively (amplicon size: 334 bp); 5'-TGATACTTCACAGACGAAACAACG-3' and 5'-TTAATTTGCACCGGATTAACACCAC-3' as forward and reverse primers, for alp4 genotypes, respectively (amplicon size: 110 bp).

The volume of reaction was as follows: the final volume for PCR reaction was 20 µl (2 µl of water, 10 µl of 2X PCR Master Mix (Amplicon, Denmark), 5 µl of working primers with final concentration of 10 pmol, and template DNA (3 µl). The thermal profile was as follows performed in thermocycler (Eppendorf, Germany): 94°C for 300 seconds as initial denaturation for one cycle; 94°C for 45 seconds, 56°C for 30 seconds, and 72°C for 35 seconds, for 25 cycles; with a final extension at 72°C for 300 seconds. 1.5% agarose gel electrophoresis was performed on PCR products, and sequencing (MacroGen, Korea) was performed for confirmation.

Statistical analysis

Chi-square test using SPSS software (Ver. 16) was used for statistical analysis ($p<0.05$ considered as significant). Histograms were drawn by Graph Pad Prism 6 software (GraphPad Company, California and USA).

Results

GBS identification

Selection of GBS colonies was based on *β*-hemolysis reaction. Consequently, Gram staining test was performed and selected; colonies were used for further specific test such as catalase, CAMP and hyporate hydrolysis test (Fig. 1).

Frequency of GBS among pregnant women

Results of phenotypic tests and confirmation by PCR method using group B specific primers (Fig. 2) showed that out of 346 specimens, 57 isolates (16.47%), and 33 isolates (9.5%) were identified as GBS of vaginal discharge, and urine, respectively.

GBS genotyping

Multiplex PCR results showed that among vaginal isolates, 56.1% of GBS were genotype *rib* (n=18); and frequencies of genotype *epsilon*, *alpha-C*, and *alp2/3* were as 36.8%, 3.5%, and 3.5%, respectively (Fig. 3). However, these frequencies among urine isolates were a little different. In urine isolates, the frequency of genotype *rib* was 54.5%, and for other mentioned genotypes were 36.4%, 3%, and 6.1%. The genotype *apl4* did not found here.

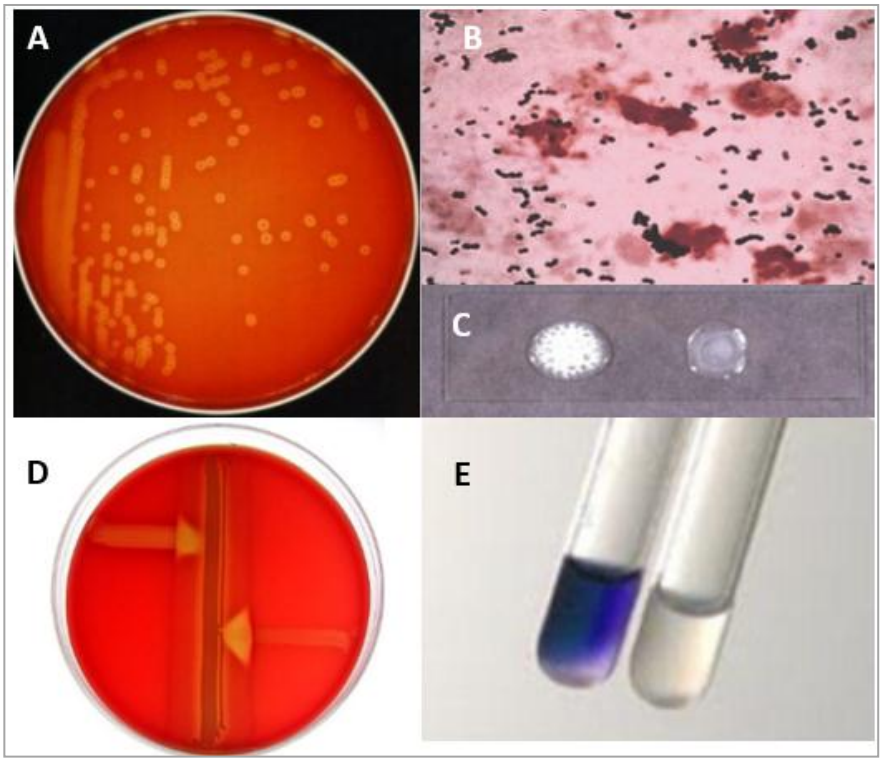

Fig. 1. GBS identification tests. **A:** Beta-hemolysis test. **B:** Gram staining test. **C:** Catalase test. **D:** CAMP test. **E:** Hyporate hydrolysis test

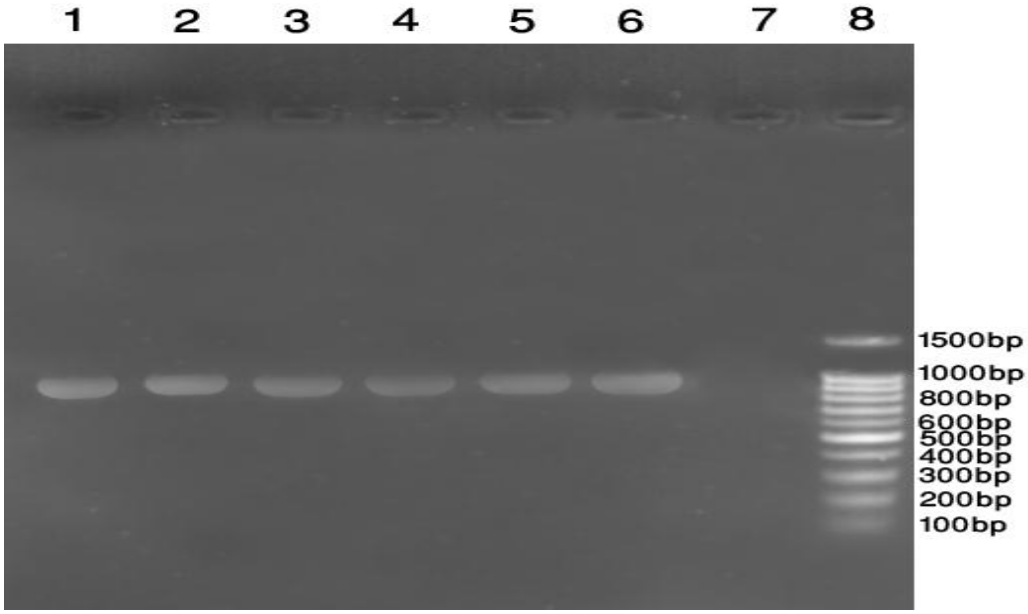

Fig. 2. PCR with specific primers of group B streptococcus. Lanes 1-5 show GBS (952bp). Lane 6 shows the positive control, and lane 8 shows the 100bp DNA ladder

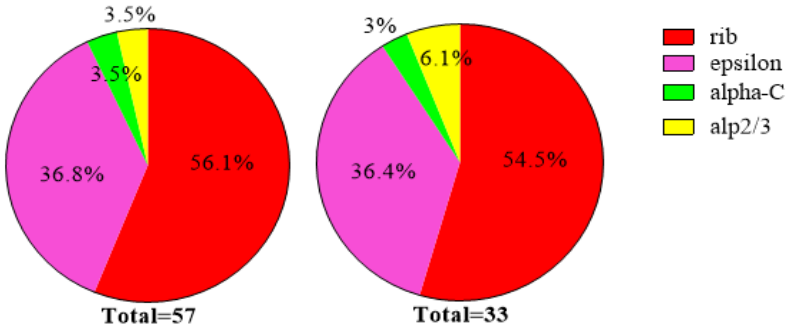

Fig. 3. Frequency of GBS genotypes in vaginal (left) and urine (right) isolates

The correlation between genotypes and age, number, and month of pregnancy

Accordingly, we grouped the persons in terms of age (<30 years and≥30 years),the number of pregnancy (first pregnancy, second pregnancy and more than second pregnancy), and months of pregnancy (first 4.5 month and second 4.5 month) and then the correlations of genotypes among themwere analyzed. The results showed that there is no significant correlation between age of pregnancy (p=0.41 for urine isolates, and p=0.13 for vaginal isolates), number of pregnancy (p=0.51 for urine isolates, and P=0.95 for vaginal isolates), and month of pregnancy (p=0.86 for urine isolates, and p=0.37 for vaginal isolates) with genotypes.

Correlation between GBS genotypes of vaginal discharge and urine isolates

The association between different vaginal and urine serotypes showed that out of 346 pregnant women, 26 persons were GBS positive for both vaginal and urine samples, and in all cases, genotypes of the urine isolates were same as the vaginal genotypes (Fig. 4) (p=0.000).

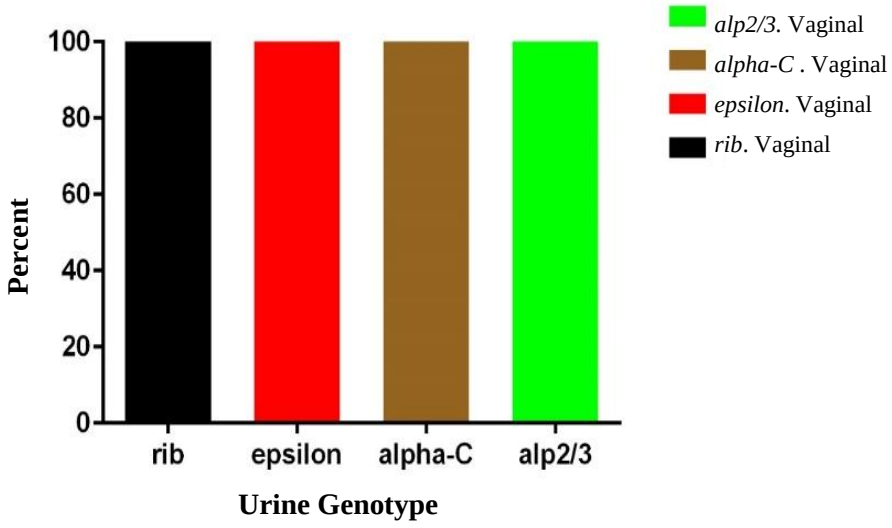

Fig. 4. The frequency of urine isolates genotypes among vaginal isolates genotypes

Discussion

GBS is one of the most important causing death in newborn babies. Colonization in vagina of pregnant women is one of the most important risk factor in newborn children infections [6]. The prevalence of GBS among pregnant women varies in different countries. In one study that carried out in 2010, prevalence of GBS in Turkey, Lebanon, Israel, Germany, Sweden, Philippine, and France was 9.2%, 17.7%, 12.3%, 23%, 25.4%, 7.5%, and 8%, respectively [12]. The results of the present study showed that the frequency of GBS among vaginal and urine samples collected from the same case women was 16.47%, and 9.53%, respective. This result almost concur with two other reports conducted by Sadeh et al. 15.4% [14] and Absalan et al. 19.6% [15] in the city of Yazd, Iran. In a study carried out by Fatemi et al. in Tehran, it was revealed that 20.6% of pregnant women were vaginal GBS positive. They used agar-plate method for identification [16]. The differences between this finding and our results besides geographical location may be because of the method of identification they used. In the present study, molecular methods were used, then, the results seem more reliable. Bidgani et al. (2016) showed that 27.6% of vaginal sample of pregnant women in Ahvaz were GBS positive [17]. This result is different from our finding and other studies which carried out in Yazd and Tehran, and however, shows that GBS is more prevalent in Ahvaz populations. The present study showed that out of 33 urine sample, 54.5% are genotype rib which followed by genotype epsilon, alp2/3, and alpha-C, by frequency of 36.4%, 6.1%, and 3%. In addition, vaginal genotyping showed that 56.1% of 57 samples are genotype rib, followed by genotype epsilon, alpha-C, and alp2/3 by frequency of 36.8%, 3.5%, and 3.5%, respectively. Note that genotype *alp4* was not detected in the present study (Fig. 3). The genotyping data of GBS in Iranian pregnant women is very limited. In a very recent study, which performed by Sadeh et al. (2016), showed that genotype rib is more prevalence in pregnant and non-pregnant adults in Yazd (by frequency of 53%); prevalence of other genotypes, including epsilon, alp2/3, alpha-C, and alp4 were as followed: 38%, 6%, 3%, and 0% [14]. These results are completely consistent with our investigation. The location and the method of assessment used in Sadeh et al. and our study were both the same. Therefore, the closeness of results in these two studies could be explained by these two factors. In a study conducted by Persson et al. (2008), revealed that six genotypes, including rib, epsilon, alp3, alp2, alpha-C, and Beta-C were surveyed in newborns and adults, separately. They showed that genotype rib is more prevalent (42%), and followed by alp3, epsilon, alpha-C, and alp2, by frequency of 26%, 14%, 3% and 1%, respectively. Genotype beta-C was not found solely, and in all cases was found by genotype alpha-C (11%), alp2 (0.3%), alp3 (0.3), rib (0.3%) [18]. Genotype rib in Persson study and in the present study was the most

prevalent genotype. In another study, which carried out in Poland on pregnant women, resulted that the prevalence of GBS was found 30%, and the frequency of genotypes epsilon, rib, alp2, alpha-C, and alp3 were 26%, 22%, 17%, and 14%, respectively [1]. These results showed that in Polish pregnant women, the frequency of different genotypes are relatively homogenous. In conclusion, the present study revealed that the prevalence of GBS from vagina and urine of pregnant women were reliable when compared to the results from other studies reported from most countries around the world (average of 40%). In addition, it was found that the surface protein rib was dominant among both vaginal and urine GBS isolates. Although, move survey seems necessary with higher cases population, their report may be enough significant for formulation of vaccine based on the protein surface rib. As mentioned before, rib surface protein is directly associated with the neonatal disease, so paying attention to prevent treat the GBS carrier pregnant women is highly suggested.

Conflict of Interest

Authors declare that there is no conflict of interest.

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