Prevalence and Serotype Determination of *Streptococcus agalactiae* Isolated From Non-Pregnant Women in Tehran, Iran

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Abstract- Consequence of *Streptococcus agalactiae*, Group B Streptococcus (GBS) relating infant's diseases are well documented. Although many women carry this bacterium in their vagina, they may transfer to their infant during delivery and may result in different neonatal invasive diseases. The aim of this study was to determine the prevalence of GBS and serotyping the isolated species among un-selective non-pregnant women who attended two gynecology clinics in Tehran. In this cross-sectional study, a total of 560 vaginal samples collected from non-pregnant women. Following inoculation of the specimen on Blood Agar, the standard technology was applied for the final identification of GBS. Detected GBS species were further confirmed using specific PCR directed on *dlts* gene. Capsular serotyping was done by using the multiplex polymerase chain reaction (PCR) method. The chi-square method was used for statistical analysis. Fifty (8.9%) out of 560 non-pregnant women were carriers of GBS. The most common types were III (36%), followed by type II (32%), Ia (26%), and Ib (6%), respectively. Results represent that the prevalence rate of GBS in non-pregnant women was reliable and similar to what obtained from pregnant women. In addition, the serotype III was found the most dominant types, as well as other investigations in the Tehran area. Therefore, vaccine designation based on type III is recommended.

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Keywords: Group B streptococcus; Multiplex polymerase chain reaction (PCR); Non-pregnant women; Capsular serotyping

Introduction

Streptococcus agalactiae or Streptococcus group B (GBS) is a facultative gram-positive bacterium and is recently known as an important cause of neonatal diseases in both adult and children. Reports from different sources represent that 15-45% of women harbor the GBS in their vagina and may transfer to their newborn during delivery (1). Meningitis, sepsis, and pneumonia are those important infectious diseases that new-born babies may suffer from contaminated mothers (2).

Infants diseases caused by GBS may be classified into two groups; early-onset diseases (EOD, from birth to day 6th) and late-onset diseases (LOD, from day 7 to 89). The EOD is usually manifested in the first week of birth, whereas LOD appears between the second two and third month (3,4).

Although different virulence factors have been recognized in GBS, polysaccharide capsule seems the most important factor. Based on antigenic properties, capsular polysaccharide (CPS), the GBS has been classified into ten serotypes; Ia, Ib, and II- IX (5,6). However, different investigations in Iran revealed that serotype III is the most common GBS detected from both pregnant and non-pregnant women (7).

Investigations revealed that type III is the main causative of meningitis in LOD, while the GBS serotype Ia is usually common in EOD. Since the GBS serotypes distribution is geographic differences, the determination of serotype is necessary for vaccine designation in a different population (8).

The aim of this study was to determine the prevalence of GBS among non- pregnant women who attended the gynecology clinic at Javaheri and Shohada hospitals in Tehran, Iran. Following isolation of GBS

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from vaginal specimens, all have further processed for capsular type using multiplex PCR.

Materials and Methods

Sample collection and culture

A total of 560 swab samples from the vagina of non-pregnant married women aged 15-45-year-old referred to Javaheri and Shohada gynecology department (2015-2016) were collected and subjected to culture using sheep blood agar medium (Merck, Germany). All selected cases were not used any antibiotic 2 weeks prior to sampling. Note that sampling was done by an expert obstetrician. Following overnight incubation, at 37˚ C, the suspected beta-hemolytic colonies were further tested for exact recognition of GBS, including CAMP and hippurate hydrolysis. All isolated species were then frozen at -20˚ C for later type determination.

Genomic DNA extraction and molecular detection of GBS

The extraction of genomic DNA from *S. agalactiae* was performed using a bacterial extraction kit (Gene All, South Korea).

In order to corroborate the isolated GBS, the PCR method was programed using primers dlts- F, dlts- R (Takapouzist, Iran) (Table 1) targeted dlts gene. The reaction mixture was prepared in a final volume of 20 µl containing 2.5 µM of each primer with the final concentration of 10 Pmol, 10 µl of 2X PCR master mix (Amplicon, Denmark) and 3 µl of template DNA. Amplification was carried out in an automated PCR machine, as follows:

The reaction mixture was first raised to 94˚ C for 5 min, followed by 30 cycles for 1 min at 94˚ C, annealing for 1 min at 55˚ C and 1 min at 72˚ C for elongation. The whole process was completed with a final elongation cycle of 5 min at 72˚ C.

Table 1. CPS Type-Specific Primers and Amplicon Size of PCR Products (5)

Primer name	Sequence (5′ to 3′)	Gene target(s)	Amplicon size(s) (bp)
Ia-F	GGTCAGACTGGATTAATGGTATGC	*cps1aH*	
Ia-R	GTAGAAATAGCCTATATACGTTGAATGC	*cps1aH*	521 and 1,826
Ib-F	TAAACGAGAATGGAATATCACAAACC	*cps1bJ*	
Ib-R	GAATTAACTTCAATCCCTAAACAATATCG	*cpsIbK*	770
II-F	GCTTCAGTAAGTATTGTAAGACGATAG	*cps2K*	
II-R	TTCTCTAGGAAATCAAATAATTCTATAGGG	*cps2K*	397
III-F	TCCGTACTACAACAGACTCATCC	*cps1a/2/3I*	
III-R	AGTAACCGTCCATACATTCTATAAGC	*cps1a/2/3J*	1,826
IV-F	GGTGGTAATCCTAAGAGTGAACTGT	*cps4N*	
IV-R	CCTCCCCAATTTCGTCCATAATGGT	*cps4N*	578
V-F	GAGGCCAATCAGTTGCACGTAA	*cps5O*	
V-R	AACCTTCTCCTTCACACTAATCCT	*cps5O*	701
VI-F	GGACTTGAGATGGCAGAAGGTGAA	*cps6I*	
VI-R	CTGTCGGACTATCCTGATGAATCTC	*cps6I*	487
VII-F	CCTGGAGAGAACAATGTCCAGAT	*cps7M*	
VII-R	GCTGGTCGTGATTTCTACACA	*cps7M*	371
VIII-F	AGGTCAACCACTATATAGCGA	*cps8J*	
VIII-R	TCTTCAAATTCCGCTGACTT	*cps8J*	282
dltS-F	AGGAATACCAGGCGATGAACCGAT	*dltS*	
dltS-R	TGCTCTAATTCTCCCCTTATGGC	*dltS*	952

Molecular serotyping

In order to perform molecular serotyping, two sets of the multiplex PCR reaction were taken (Table 1). Amplification of both multiplex reactions with the final volume of 20 µl for PCR reaction (2 µl of water, 10 µl of 2X PCR Master Mix) (Amplicon, Denmark), 5 µl of working primers with the final concentration of 10 pmol, and template DNA (3 µl).

The PCR processing was achieved by 94˚ C denaturation for 5 min, followed by 1 min at 94˚ C for 35 cycles as denaturation. Then, the mixture was subjected to the annealing process at 49.5˚ C and 60˚ C for the first and second set, 1 min, followed by 1 min at 72˚ C for the extension.

The whole process was completed with a final extension at 72˚ C for 5 min. analyzing of amplicons was achieved using 1% agarose gel and then visualized with gel imagers (Life Technologies, USA).

Statistical analysis

In this survey, the chi-square test was applied to evaluate the prevalence of isolates, and $P \leq 0.05$ was considered significant by SPSS 16.

Results

Among 560 swab samples collected from recto-vaginal of non-pregnant women, 50 (8.9%) samples were identified as GBS positive. Figure 1 disclosed the specific PCR for the exact detection of GBS using dlts.

Results obtained from multiplex PCR indicate that 18 (36%) of GBS positive samples were capsular type III, 16 (32%) type II, 13 (26%) type Ia and 3 (6%) Ib. Note that the types IV, V, VI, VII, and VIII were not isolated (Table 2).

As table 2 indicates, the range of ages among participated cases was 25-39; When the data was analyzed, it was found that the serotype III was absent in women under 25 years old, but, However, it is higher in aged 30-34-year-old (Table 2).

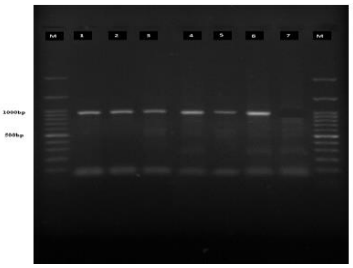

Figure 1. Gel electrophoresis of the PCR reaction with specific primers of group B streptococcus. Lane M shows the 100bp DNA ladder, lane 1 shows the positive control, Lanes 2-6 show GBS (952bp) and lane 7 shows the negative control

Table 2. Distribution of capsular polysaccharide serotypes based on age among non- pregnant women

		Serotypes					
		Ia (%)	**Ib (%)**	**II (%)**	**III (%)**	**Negative (%)**	**Total (%)**
Age	>25	3 (4.2)	1 (1.4)	3 (4.2)	0	65 (90.3)	72 (100)
	25-29	3 (2.4)	0	3 (2.4)	4 (3.2)	115 (92)	125 (100)
	30-34	2 (1.6)	0	3 (2.4)	7 (5.6)	113 (90.4)	125 (100)
	35-39	3 (2.3)	1 (0.8)	4 (3)	5 (3.8)	119 (90.2)	132 (100)
	≤40	2 (1.9)	1 (0.9)	3 (2.8)	2 (1.9)	98 (92.5)	106 (100)
Total (%)	50 (8.9)	13 (26)	3 (6)	16 (32)	18 (36)	510 (91.1)	560 (100)

P=0.860

Discussion

In the present study, 560 recto-vaginal swab specimens were collected for the detection of GBS. Overall, 50 samples were found positive for GBS, reflecting the prevalence of 8.9% for this unselected population referred to two gynecology clinics in Tehran. In general, data reported from WHO revealed that about 15-45% of women carry the GBS in their genital-urinary system (1).

When our findings were compared to other studies reported from several populations in Tehran, it was found that different study has revealed a variable rate of colonization. For example, in an investigation conducted by Hadavand *et al*., in Tehran showed that among 210 vaginal samples only 3.3% were positive for GBS (9), whereas Fatemi *et al*., (10), Aali *et al.*, (11), Jahed *et al.*, (12) reported 20,6%, 9.2% and 5.3% respectively (Table 3).

Table 3. Prevalence of GBS from some selected population

Authors	**Years of isolation**	**Place**	**Sample size**	**Colonization rate (%)**
Hadavand et al (9)	2010-2011	Tehran (Iran)	210	7 (3.3%)
Fatemi et al(10)	2008	Tehran (Iran)	330	68 (20.6)
Aali et al (11)	2005-2006	Kerman (Iran)	206	19 (9.2)
Jahed et al(12)	2008	Tehran (Iran)	246	13 (5.3)
Absalan et al (13)	2012	Yazd (Iran)	250	49 (19.6)
Sadeh et al(8)	2013-2014	Yazd (Iran)	650	100 (15.4)
Najarian et al(3)	2015-2016	Yazd (Iran)	346	57 (16.47)
Lu B et al(14)	2011-2013	China	2850	201 (7.1%)
Sharmila et al(15)	2006-2008	India	300	7 (2.33)
Busetti et al(16)	2002-2005	Italy	5020	901 (17.9)
Melo et al(17)	2011-2014	Brazil	496	141 (28.4)
Kawatra et al(18)	2016	Americas	23163	4360 (19.7)

Different similar investigations concerning the prevalence and type distribution of GBS performed by our microbiology team in Yazd showed the frequency of GBS as follows:

Absalan *et al*., (13) tested 250 vaginal samples and found 19.6% of cases were GBS positive. Later, Sadeh *et al*., (8) reported that among 650 swab samples, 15.4% and Najarian *et al*., (3) indicated that among 346 specimens, 16.7% were positive for GBS. Our finding (8.9% rate) was consistent with the experience of Aali *et al.,* (11) but not in concur with others (8,13).

When we reviewed the prevalence of GBS in some countries, it was found controversy in the rate of GBS colonization among their reports. In Chaina (14) 7.1%, Italy (16) 17.9%, Americas (18) 19.7%, Brazil (17) 28.4% and India (15) 2.3% of their unselected women population were GBS vaginal carrier (Table 3).

In general, there is controversy in regard to the prevalence rate, and there is no absolute reason to explain the subject. However, different geographical location, cultural technique, the experience of technical stuff, the concentration of specimen, transfer medium, amount of time elapsed before inoculation and using antibiotic by the participant before sampling may all be important factors influence the isolation of GBS (3,7,13).

In the present study, the isolated GBS were subjected to serotyping using multiplex PCR and found that 36 (18%) were type III, followed by 32 (16%) II, 6 (3%) Ib, and 13 samples (26%) type Ia. As table 2 indicates, there was no type III under 25 years old women, but this serotype was the most common between ages 30-35 years old. This result corresponds with our previous finding reported by Sadeh *et al.,* (8) in Yazd population, who reported figure of GBS serotypes III (50%), followed by II (25%), Ia (12%), V (11%) and Ib (2%). In another similar study conducted by Najarian *et al.,* (3) in Yazd revealed that 54.4% of GBS isolated from pregnant women were type III, followed by II (26.3%), Ia (12.3%), Ib (3.5%) and V (3.5%).

In another survey carried out by Beigverdi *et al.,* (19) in Tehran presented that type III with 65.8% was predominant, followed by Type II (14,6%), Ib (7.3%) and V (4.9%). In a similar study conducted by Nahaei (20) *et al*., in Tabriz revealed that serotype V was the most common. This, however, may reflect the necessity of specific clones in different geographical areas or sources of bacterial detection. Although the percentage of serotypes isolated in the present study almost correspond with other findings reported from Tehran (19), Hamadan (21), and our previous works in Yazd (3,8), the serotype V was not detected among our isolated GBS. This, however, may be due to the limited number of cases we investigated; another possibility is that the specimen might have been collected from a group of women in a specific geographical area.

When our findings were compared with some other studies published from different countries, we found that the serotype distribution in Australia (22), serotype III, Ia, and V were the most common serotypes, and in Turkey (23) serotypes Ia, Ib, II, III, and IV were common, but in Japan (24) serotypes VI and VIII were predominant among Japanese population.

In conclusion, results obtained from this study, together with many other publications, revealed that serotype III is predominant among pregnant and non-pregnant women. Hence this serotype is directly related to LOD in infants. Therefore establishment of an adequate screening program seems critical to identify and treat the infected pregnant women before delivery (8).

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