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Original article

Genotyping of Clinical *Streptococcus agalactiae* Strains Based on Molecular Serotype of Capsular (*cps*) Gene Cluster Sequences Using PCR Assay in Hamadan during 2013-2014

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Background: Group B streptococcus (GBS) is the major cause of serious life threatening infections in neonates, pregnant women, and other adults with underlying diseases. Capsular polysaccharide typing is a significant way for epidemiological studies of GBS, the pathogenesis, and other studies associated with GBS infections including surveillance programs and vaccine development in future. Molecular serotyping (MS) methods offer more accurate and reliable typing of bacteria. The aim of current study was to differentiate genotypes of clinical GBS isolates based on PCR assay to acquire information about the distribution of GBS types in Hamadan, Iran.
Materials and Methods: A total of 62 clinical GBS strains including vaginal swabs, urine cultures, and blood culture isolates were examined for genotyping using multiplex PCR assay.
Results: Among the 62 GBS isolates examined, all capsular types, except VI, VII, and VIII, were found. Type III was the predominant type with 35 isolates (56.5%), followed by Type V with 11 isolates (17.7%), Type II with 7 isolates (11.3%), Type Ia with 5 isolates (8.1%), and Types Ib and IV with similar prevalence of 2 isolates (3.2%) for each type.
Conclusion: The results of the current study demonstrated that Type III is the predominant type in Hamadan, followed by Types V, II, Ia, Ib, and IV, respectively. Using MS method leads to accurate, sensitive, specific, and fast typing of GBS isolates. The advantages of MS method allow it to analyze various populations and to examine invasive and colonizing isolates in extensive epidemiological studies and surveillance activities. In fact, MS will facilitate the proper formulation of candidate GBS vaccines.

Keywords: *Streptococcus agalactiae*, Multiplex PCR, Genotyping techniques, Molecular epidemiology, Vaccines

1. Background

Streptococcus agalactiae, also known as Group B streptococcus (GBS) is the major cause of serious life threatening infections including sepsis, pneumonia, and meningitis in neonates and young infants, as well as other serious infections in women at gestational and postpartum period, individuals with diabetes, immunocompromised patients, adults, and the elderly. Mortality rate due to GBS infection in newborns is 4 to 6% and higher in premature infants (1-3).

Based on recognized capsular polysaccharide (CPS) antigens (capsular polysaccharide synthesis *cps* gene cluster), GBS is classified into 13 variants (serotypes), from which 9 serotypes, for example, Ia, Ib, and II-VIII, are considered important clinically. Capsular polysaccharides are regarded as significant virulence factors and epidemiological markers; thus, they can be used as the major components of conjugate vaccines. Distribution of these serotypes is different among geographical regions and studied populations (e. g. between pediatric and adult individuals) and changes over time. Based on reported studies in USA and Europe, it has been shown that Serotypes Ia, II, III, and V are the main causes of human GBS disease, as these serotypes have been found in 80-90% of clinical isolates. In addition, high prevalence of Serotype III in neonatal disease and also the emergence of Serotype V in adult disease have been shown (4-9).

In facta fundamental and crucial way for epidemiological studies of GBS, the pathogenesis, and other studies related to GBS infections including surveillance programs and vaccine invention in future is Capsular polysaccharide typing. In fact, many attempts have been focused on using CPS as immunoprophylactic antigens (5, 9)..

Typing of bacteria is often required when two or several samples (strains) are suspected to have epidemiological relationship, for instance, in nosocomial or foodborne outbreaks. Another situation in which typing is required can be for epidemiological surveillance of one infectious disease after certain period of time in order to follow disease development and design possible infection control approaches. As a part of surveillance method, recording and storing of outbreaks-related typing results are required for comparison with possible future outbreaks or with other related investigations. Another application of typing methods can be for making comparison among strains of bacterial species isolated from one patient in order to differentiate pathogenic strains from nonpathogenic ones or endogenic strains from exogenic ones (10-12).

Conventionally, GBS capsular typing is performed by serotyping (immunological) methods including coagglutination (13), latex agglutination (14),capillary precipitin (15), double immunodiffusion (16), and enzymeimmunoassay (17); however, complicated interpretation, being expensive, and commercially availability of their reagents for only a subset of serotypes are often limitation of these methods. Moreover, due to possible capsule operon mutations or rearrangements and the fact that encapsulation levels of GBS is different among strains, particularly those under experiment condition(i.e. some strains

maybe low encapsulated and/or none-encapsulated, thereby either have poor capsule expression or lack of it, so these strains are considered as non-typeable strains lacking detectable capsular polysaccharide), therefore, typing of such strains using immunological methods may fail or be difficult. Additionally, subjects such as strains not expressing related capsular polysaccharide genes (non-encapsulated) or expressing polysaccharide variants that fail to react with used antiserums, are non-distinguishable using this methodologies (18, 19).

Although appropriate phenotypic methods can be used for outbreak isolates in short time, but such methods are not adequate for evolutionary studies generally, and it has been known that these methods lack adequate discriminatory power (12).

PCR-based typing has demonstrated that non-typeable strains resulted from immunological methods usually harborthe genetic information to synthesis these polysaccharides and consequently are typeable by PCR assay; in fact, molecular methods such as PCR assay offer more accurate and reliable typing of bacteria than phenotypic methods (20). On the other hand, in case of occurring mutations in the capsule synthesis genes, the molecular typing methods result in non-typeable strains. Currently, the sequences of CPS type-specific gene regions from GBS have been available (8, 21, 22); thereby CPS genotyping can be designed using both single PCR and/or multiplex PCRs (23, 24).

2. Objectives

The aim of the current study was to determine capsular polysaccharide serotypes of clinical GBS isolates using multiplex PCR assay in Hamadan to obtain accurate information about the distribution of GBS serotypes in this region, which can provide some knowledge to facilitate the proper formulation of candidate GBS vaccines in future.

3. Materials and Methods

3.1. Collection and identification of isolates

A total of 62 clinical GBS strains (56 and 6 samples from female and male individuals, respectively) including vaginal swabs (*n*=16, one sample from non-pregnant woman and fifteen samples from pregnant women isolated from 203 collected specimens), urine cultures (*n*=45), and blood culture (*n*=1) isolates were collected from educational hospitals and private clinic centers during nine months from June 2013 through February 2014 in Hamadan, Iran. Total samples from male individuals were urine cultures. Information about the age of each collected sample was recorded. Based on Centers for Disease Control and Prevention (CDC) guidelines(1), processing of the vaginal swabs was performed as follows: specimen swabs were inoculated into Limbroth (Pronadisa Co, Spain) as a selective and enrichment medium and incubated at 35–37°C in 5% CO_2 (in candle jar) for 18–24 hours, then subcultured on Trypticase Soy agar(TSA) (Merck Co, Germany) with 5% blood and incubated at 35–37°C in 5% CO_2 for 18–24 hours. Conventional phenotypic methods including gram staining, catalase test, sodium hippurate hydrolysis, bile esculin agar test, and CAMP reaction were used for microbiological presumptive identification of the isolates. The presumptive identified isolates as GBS were subcultured on Trypticase Soy agarwith 5% blood and incubated at 35–37°C in 5% CO_2 for 18–24 hours to obtain single pure GBS colonies. Then these isolates were inoculated in Brain Heart Infusion (BHI) broth (Merck Co, Germany) containing glycerol and blood and preserved in deep freeze (-70°C) condition for further use.

3.2. DNA extraction from isolates

DNA was extracted from isolates using alkaline lysis method as following procedure: Three or four colonies from overnight cultured isolate were suspended in a sterile microtube containing 60 µL of lysis buffer (0.05 NNaOH, 0.25% sodium dodecyl sulfate-SDS) then vortexed and heated at 95°C for 15 min. Afterwards the microtube was added 540 µL of TE buffer (50 mM Tris HCl, 1 mM EDTA, pH 8) for diluting the obtained cell lysate. Subsequently, the microtube was centrifuged at 10,000 rpm for 5 min to sediment cell debris. The supernatant was transferred to a new sterile microtube and used for PCR assay or frozen at -20°C for further use (25).

3.3. Confirmation of identified isolates as GBS using PCR assay

PCR assay targeting the 780-bp*atr*gene (GenBank accession number: AF15135) which is specific for *S. agalactiae* species as internal positive control, was performed for confirmation or definitive identification of the isolates. The forward and reverse primer sequences were CAACGATTCTCTCAGCTTTGTTAA and TAAGAAATCTCTTGTGCGGATTTC, respectively (26). The PCR reaction volume was 20 µL including 2 µL of bacterial DNA, 1 µL of forward primer, 1 µL of reverse primer, 10 µL of 2x Taq Premix-Master mix (Parstous Biotech Co, Iran), and 6 µL of sterile double distilled water. Amplification of thermal cycles were as follows: an initial denaturation step for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 55°C for 55 s, and 72°C for 1 min, and a final extension cycle of 72°C for 10 min using Bio-Rad Thermal Cycler. For positive and negative controls, the *S. agalactiae* ATCC 12386 and *Enterococcus faecalis* ATCC 29212 were used, respectively. PCR products and 50-bp DNA size marker (Fermentase Co, USA) were run simultaneously on 1.5% agarose gel stained with DNA safe stain (SinaClon Co, Iran) at 80 V for 1 hour. Finally, the agarose gel was visualized and photographed using UV transilluminator (Vilbert Lourmat Co, Japan).

3.4. Molecular serotyping of GBS isolates using multiplex PCR assay

Each confirmed isolate as GBS was examined for genotyping (molecular serotyping) using multiplex PCR assays targeting nine *cps* genes introduced by Poyart et al, 2007 (24). For this purpose, two reaction mixes were prepared. The mix (i) was contained the primers for Ia, Ib, II, IV, and V and the mix (ii) contained the primers for III, VI, VII, and VIII. The first reaction mix contained 2.5 µL of bacterial DNA, 1 µL of each forward primer, 1 µL of each reverse primer, and 12.5 µL of 2x Taq Premix-Master mix (Parstous Biotech Co, Iran). The second reaction mix contained 2.5 µL of bacterial DNA, 1 µL of each forward primer, 1 µL of each reverse primer, 12.5 µL of 2x Taq Premix-Master mix, and 2 µL of sterile double distilled water. Final volume of each reaction mix was 25 µL. Amplification of thermal cycles were as follows: an initial denaturation step for 3 min at 94°C, followed by 30 cycles of 94°C for 30 s, 58°C for 1min, and 72°C for 1 min, and a final extension cycle of 72°C for 5 min. As mentioned confirmatory PCR assay methodology, PCR products were run on agarose gel and then visualized and photographed using UV transilluminator. The 50-bp and 1-kb DNA size marker (Fermentas Co, USA) were used in these assays.

3.5. Statistical analysis

Correlation between genotypes and samples type as well as between genotypes and age groups (≤30 and >30) were analyzed by Chi-Square Test. The test of significance was two-tailed and a *P* value <0.05 was considered statistically significant. All analyses were performed using SPSS software version 20.

4. Results

In case of confirmatory PCR assay, the samples (PCR product) presenting an amplicon size of 780-bp were considered positive for GBS (Figure 1). All the 62 presumptive identified isolates produced amplicon size of 780-bp and thus were confirmed as

GBS strains. All 62 GBS strains produced amplicons from CPS related primer pairs with genotypes of Ia, Ib, and II-V (Figure 2). In none of the examined GBS strains, amplicon size indicating genotypes of VI-VIII was observed. Results of genotyping for collected samples using multiplex PCR assay as well as correlation between samples type and genotypes are presented in Table 1. Among the 62 GBS isolates examined, all capsular types, except VI, VII, and VIII, were found. Type III was the predominant type with 35 isolates (56.5%), followed by Type V with 11 isolates (17.7%), Type II with 7 isolates (11.3%), Type Ia with 5 isolates (8.1%), and Type Ib and IV with similar prevalence of 2 isolates (3.2%) for each type. Statistical result of correlation between age groups and genotypes was $P = 0.963$ (data not shown).

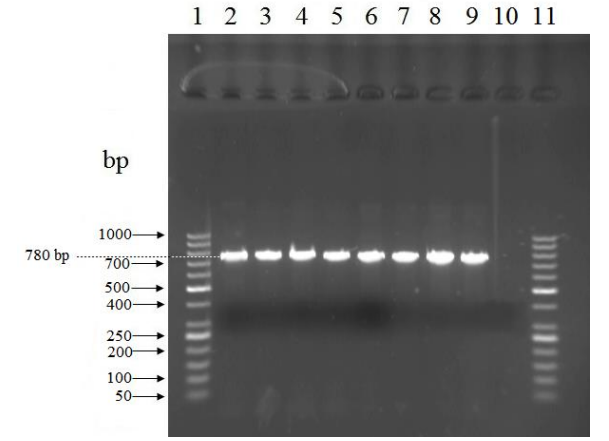

Figure 1. Results of gel electrophoresis from confirmatory PCR assay targeting 780-bp *atr* gene. Lanes 1 and 11: 50-bp DNA size marker; lane 2: *S. agalactiae* ATCC 12386 (as positive control); lanes 3 to 9: clinical GBS samples; and lane 10: *E. faecalis* ATCC 29212 (as negative control).

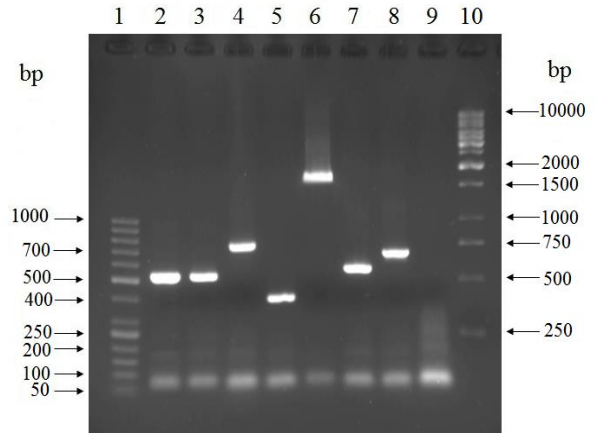

Figure 2. Results of gel electrophoresis from Multiplex PCR assay for genotyping of GBS. Lanes 1: 50-bp DNA size marker; lane 2 and 3: *S. agalactiae* ATCC 12386 and clinical GBS sample, respectively, with amplicon size of 521-bp denoting Type Ia; lane 4: clinical GBS sample with amplicon size of 770-bp denoting Type Ib; lane 5: clinical GBS sample with amplicon size of 397-bp denoting Type II; lane 6: clinical GBS sample with amplicon size of 1826-bp denoting Type III; lane 7: clinical GBS sample with amplicon size of 578-bp denoting Type IV; lane 8: clinical GBS sample with amplicon size of 701-bp denoting Type V; lane 9: sample without DNA template (as negative control); and lane 10: 1-kb DNA size marker.

Table 1. Results of genotyping using multiplex PCR assay and correlation between genotypes and samples type.

		Samples Type			Frequency	Percent (%)	*p* value*
		Vaginal swab	Urine	Blood			
Genotypes	Ia	4	1	0	5	8.1	**0.005**
	Ib	0	2	0	2	3.2	0.462
	II	3	4	0	7	11.3	0.266
	III	4	30	1	35	56.5	**0.003**
	IV	1	1	0	2	3.2	0.433
	V	4	7	0	11	17.7	0.346
	VI	0	0	0	0	0.0	-
	VII	0	0	0	0	0.0	-
	VIII	0	0	0	0	0.0	-
Total		16	45	1	62	100	

* *P* value for determining correlation between genotypes and samples type based on Chi-Square Test.

5. Discussion

The GBS capsule is a significant virulence factor and antigenic determinant; therefore, it is considered as one of the main targets in investigations for developing vaccine against GBS infections in future. The future promising GBS capsular polysaccharide-protein conjugate vaccines must be consisted of more frequent and virulent serotypes which are involved in circulation associated with the disease in different populations; thereby correct capsular typing of clinical isolates is essential for predicting vaccine coverage; consequently, applying sensitive and specific methods are required for achieving this purpose (27-29).

Considering these fact, the aim of current study was to differentiate genotypes of clinical GBS isolates based on PCR assay to acquire information about the distribution of GBS types in Hamadan, Iran.

Among the 62 GBS isolates examined, all capsular types, except VI, VII, and VIII, were found. Types III and V were the most prevalent types with the sum of 46 isolates (74.2%). Type III was the predominant type with 35 isolates (56.5%), followed by Type V with 11 isolates (17.7%), Type II with 7 isolates (11.3%), Type Ia with 5 isolates (8.1%), and Types Ib and IV with similar prevalence of 2 isolates (3.2%) for each type.

Reviewing prevalence of GBS types in pregnant women universally, the results were as follows: in USA, although a majority of reported studies in 1990s showed Type V as the predominant type among colonizing isolates, but Types Ia and III have been more frequent in recent years. In Canada, Types Ia, III, and V were common similarly, and in Europe, except for Greece, Type III was predominant in many countries (30); additionally, Type Ib is emerging in Germany (31). Based on a recent study performed in three African countries, similar prevalence of Types III and V has been reported. In Asian countries, after Type III as the predominant type, Types Ib and V are common similarly (32); however, an unusual case has been reported in Japan in which Types VI and VIII were predominant in Japanese pregnant women (33). In the Middle-east, Types Ia, II, III, and V are more frequent (32), but in the United Arab Emirates, Type IV is the most common (34).

Considering above findings, the obtained results of the current study is consistent with the universal prevalence of GBS types, in which Types III and V have been reported to be the most frequent types in many countries. Similarly, our findings are also consistent with the study performed by Ippolito et al. (32), in which they reported Types Ia, II, III, and V as the most frequent types in the Middle-east.

Following results have been reported in other studies performed in Iran. In arecent study performed in Ardabil, in

2012, Jannati et al. used capsular antiserum for serotyping of isolates and showed that Serotypes V (19.6%), II (12.5%), and IV (12.5%) were the most frequent serotypes, followed by Serotypes III (10.7%), VI (10.7%), Ib (8.9%), Ia (7/1%), VII (5/3%), and VIII (5/3%); in addition, 7.1% of the strains were non-typeable (35). Another recent study performed by Yasini et al. in Kashan, in 2012, PCR assay was used and genotyping results were as follows: the most common types were III (32.14%), V (21.43%), and IV (14.3%), respectively, followed by Ia (10.7%), VI (10.7%), Ib (7.13%), and VII (3.6%). Types II and VIII were not identified (36). According to the study by Nahaei et al. in Tabriz, in 2007, serotypes of *S. agalactiae* strains isolated were as follows: Ia (17.6%), Ib (13.4%), II (14.2%), III (9.5%), IV (8.2%), V (19.5%), and nontypable (17.6%) using capsular antiserum (37). In another study by Rahnama et al. in Tehran, in 2010, it has been shown that the prevalence of GBS types among 50 GBS strains using PCR assay were Serotype III with 25 isolates (50%) and Serotype V with 8 isolates (16%) as the predominant serotypes, followed by Serotypes Ia and II with similar prevalence of 7 isolates (14%). In this study, Serotypes Ib, IV, VI, VII, and VIII were not found, and 3 strains were classified as non-typeable (38).

Considering above studies, it is revealed that the studies using PCR assay for genotyping have partly consistent results with each other and universal cases. It seems that dispersal distribution of GBS types in other studies using conventional serotyping may be associated with limitation of this method for serotyping.

Statistically some associations between some types (i.e. Types Ia and III) and the source of isolations were observed in current study. It is thought to be correlation between Type Ia and vaginal colonization (P = 0.005), and also between Type III and urine samples (P = 0.003); however, due to partly small number of samples examined in current study, these associations may not be considered as significant and accurate. On the other hand, being no statistically significant correlation between age groups and genotypes (P = 0.963), regardless of other similar related study (10), can also be attributed to small number of examined samples and/or can be due to being no approximate equivalence between male and female samples number.

Intrapartum antibiotic prophylaxis (IAP) can only prevent from neonatal early-onset disease (EOD), while the vaccine is expected to prevent from both late-onset disease (LOD) and EOD. A vaccine can be administered to adult or pregnant women as well as other at risk individuals including immunocompromised adults, individuals with underlying disease, and the elderly. According to investigations, it has been found that Type III often appears in neonatal disease, followed by Types Ia, Ib, II, and V. In addition, these antigens are the cause of 96 -88% of the neonatal and adult diseases cases, respectively; therefore, a possible CPS vaccine must be consisted of these mentioned capsular antigens (39).

To ensure the efficient development of such vaccine as GBS, surveying worldwide distribution pattern of frequent serotypes will be important in order to ensure that one global GBS vaccine involves the most appropriate bacterial components. It is important to restate that serotypes vary geographically in different populations and over time; therefore, this may affect vaccine formulation in future seriously. Moreover, prevalence of GBS serotypes is age dependent and different between colonizing and invasive strains (10, 11).

Bacterial epidemiologists use typing methods for studying distribution and dynamics of population of human bacterial pathogens (continuous variation of different GBS clones in human population) in environmental and clinical situation, which include transmission patterns of bacteria and identification of risk factors, to control infectious disease in human population(10, 12).

The most frequent method used for GBS typing is conventional serotyping (CS) or immunological method such as latex agglutination based on specific polyclonal antibodies. However, the accuracy of the obtained results is highly dependent on several agents. In fact, serological methods due to some limitations are only moderately reliable; for example, due to the fact that some GBS strains either lack (non-encapsulated strains) of capsular polysaccharides or have low expression of detectable capsular polysaccharides (different encapsulation level of GBS among strains), thus, the CS method is not efficient for typing of these strains. Also, these methods can cause misidentification of certain serotypes due to the problem associated with immunological cross reaction. Moreover, CS requires high-titre serotype-specific antisera and is highly dependent on the quality of the typing of sera used and the technical experience of the operator. Being expensive and not cost-effective (particularly, commercial serotyping kits) and laborious procedure to perform and less sensitive than molecular methods, are other limitations of CS method. With regard to the mentioned limitations, significant proportion of the isolates is non-typeable (NT) by this method. In contrast, PCR-based typing (genotyping) methods such as molecular serotyping (MS) provide several applications in bacterial typing systems and show a simple modifiable level of differentiation. MS identification methods are attractive due to several advantages including being specific, their high discriminatory power, producing clearer results, high reproducibility, relatively simple and fast to perform, and great availability of equipments and required materials. Advanced molecular methods have the ability to differentiate strains collections into many variable types (5, 8, 9, 18, 24, 40, 41).

6. Conclusion

Based on The results of this research, current study demonstrated that Type III is the predominant most prevalent type in Hamadan, followed by Types V, II, Ia, Ib, and IV, respectively. Using MS method such as PCR assay as an alternative way to CS method leads to obtain an accurate, sensitive, specific, and fast typing of GBS isolates is MS methods such as PCRR assay; in addition, MS method results in reduced misclassification of non-typeable isolates associated with CS. Therefore, MS can be used to confirm serotyping results obtained by CS method such as latex agglutination. The advantages of MS method help allow it to analyze different various populations and to study examine invasive and colonizing isolates in extensive a wide range of epidemiological studies and surveillance activities. In fact, MS will facilitate make the proper formulation of candidate GBS vaccines much easier.The obtained data from current study can be useful for designing future coming GBS vaccine for this geographic region (i.e. Hamadan city). So it is recommended that such similar studies about GBS typing, particularly molecular genotyping, being done periodic in all over the Iran cities (in order to get general information about GBS strains in a certain geographical regions and also to investigate about prevalence of this microorganism in society and hospitals) for obtaining complementary information about the distribution of GBS types in Iran.

Conflict of Interest

There isn't any conflict of interest in this study.

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Authors' Contributions

Seyed Masoud Mousavi, Mona Nassaj, and Mohammad Reza Arabestani contributed in all steps of the study designing, practical, data analyzing and writing manuscript; Haleh Nazeri contributed in practical step; Manijeh Rahmanian contributed in vaginal sample collection and Hasan Hoseinzadeh reviewed manuscript and indicated some comments. All of the authors read and revised the manuscript and approved its final version.

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