

Molecular Characterization of Group B Streptococcus Serotypes By Multiplex Polymerase Chain Reaction

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OBJECTIVE: Group B Streptococcus (GBS) serotypes (Ia, Ib and II to IX) are classified based on variations in their capsular polysaccharide; their prevalence differs between different geographic areas. We examined the prevalence of all GBS serotypes in rectal and vaginal swab samples obtained from 363 pregnant women followed at a Brazilian referral center (Hospital da Mulher Professor Doutor José Aristodemo Pinotti); bacterial susceptibility to antibiotics was further determined.

METHOD: Prevalence of positive GBS was evaluated by latex agglutination and by multiplex PCR analysis; bacterial susceptibility to antibiotics, such as clindamycin, erythromycin, levofloxacin, linezolid, penicillin and tetracycline was determined by the disk diffusion method.

RESULTS: (a) standard GBS culture and the multiplex PCR analysis tested positive for 83 swabs, collected from 72 women (prevalence of GBS colonization: 72/363; 20%); the most prevalent Serotype was Ia (n=43/83; 52%), followed by serotype V (n=14/83; 17%); according to anatomical origin, serotype Ia accounted for 27/59 (46%) and 16/24 (67%) of the vaginal and rectal samples, respectively; PCR also identified serotypes Ib, II, III and VI. Serotype VI is rarely described and had not been previously reported in Brazil or in Latin America. (b) The latex agglutination test only identified 44 positive samples, all of which were serotyped: 34 of these samples (77%) had serotypes matching those identified by multiplex PCR. (c) Only one sample (serotype Ia) showed resistance to erythromycin and clindamycin.

CONCLUSION: Regional studies on GBS serotypes prevalence are essential to guide immunoprophylactic interventions (vaccines) and the implementation of adequate antibiotic prophylaxis or treatment. In this study, the incidence of the serotype VI, a new and rare serotype of GBS was described for the first time in a Brazilian population.

KEYWORDS: Capsular polysaccharides; Group B Streptococcus; Molecular serotyping; Multiplex PCR; Streptococcus agalactiae; serotype VI.

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INTRODUCTION

Group B Streptococcus (GBS) or Streptococcus agalactiae is one of the major causes of neonatal infection, with a great impact on perinatal morbidity and

mortality.¹⁻⁴ Maternal colonization at delivery predisposes the newborn to early-onset invasive GBS-mediated disease.⁵

There are currently ten recognized GBS serotypes (Ia, Ib and II to IX), classified on the basis of variations in their capsular polysaccharide composition;^{3,6-8} serotype specificity is closely related to bacterial virulence.⁹ Understanding the prevalence of different serotypes

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in a specific population may allow the implementation of more adequate prophylactic measures, appropriate antibiotic prescription and even future development of specific vaccines.^{3,10,11} Such characterization is still at a very preliminary stage in most of the developing countries.¹²

GBS serotyping is conventionally performed by the latex agglutination method, with a high rate of untypable strains or erroneous classifications.^{3,11,13,14} In this context, the use of more sensitive and specific methods of GBS identification, such as molecular approaches based on the amplification of the nucleotide sequences of genes responsible for the expression of GBS capsular components, allow the recognition of strains not detected by conventional methods.^{10,11} Among these molecular methods, multiplex PCR, which permits the detection of the ten GBS serotypes in a single reaction, is particularly amenable to routine laboratory use because of its ease of implementation, low cost and increased reliability compared to conventional serotyping.

The aim of this study was to use multiplex PCR, recently adopted by the pan-European program DEVANI (Design of a Vaccine against Neonatal Infections) as the standard molecular method for GBS capsular gene analysis,^{11,15,16} to screen vaginal-rectal samples from pregnant women for GBS serotypes.

METHODS

Sample Collection. This project was approved by the institutional Review Board (CONEP, Brazilian Ministry of Health: # 724/2009) and samples were collected after agreement and signature of a written informed consent. This study included women in the 35th to 37th week of pregnancy, with preterm premature rupture of membranes or preterm labor at the the Medical referral center of the State University of Campinas, in southeastern Brazil.

Samples of vaginal and rectal swabs were collected and inoculated into separate tubes containing Todd-Hewitt broth medium. After 24 hours incubation, the broth was subcultured onto blood agar culture medium. The blood agar plates were incubated at 37°C in a CO₂ atmosphere for 24-72 hours. After culture and scoring for the presence of beta hemolysis, the colonies were transferred to another blood agar plate along with *Staphylococcus aureus* isolates for the CAMP (Christie, Atkins, Munch-Peterson) test. The final identification was performed by automation in Vitek 2 (bioMérieux, Jacarepaguá, Rio de Janeiro, Brazil). After this test, the colonies were transferred to a tube containing brain heart infusion culture medium and refrigerated at 4-8 °C for 48 hours until DNA extraction or were refrigerated with glycerol 5% and conserved at -80 °C.

DNA extraction was performed as described by Thomasini,¹⁷ with minor modifications, using lysis (10 mM Tris-HCl, pH 8.3, 150 mM NaCl, 1 mM EDTA, 0.4% sodium

dodecyl sulfate) and proteinase K (1% proteinase K, 50% glycerol, 10 mM Tris-HCl, pH 7.5, 20 mM CaCl₂) solution. The mixture was then incubated in water bath for 2 h at 55 °C, mixed with 100 µL of phenol and 100 µL of chloroform, and incubated for 10 min on ice. The mixture was then centrifuged at 15,000 rpm for 10 min, at room temperature, and the supernatant containing DNA transferred to another tube and mixed with 25 µL of 3 M sodium acetate, pH 5.2 and 900 µL of cold ethanol for DNA precipitation. After gentle mixing, the material was centrifuged (15,000 rpm, 10 min, at room temperature) and the DNA was resuspended with 25 µL of sterile water and stored at -20 °C.

Multiplex PCR was performed according to Imperi et al.¹⁵, with minor adaptations. The reaction mixture consisted of 5 µL of 10x buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl), 80 µM of each dNTP (dATP, dCTP, dGTP and dTTP), 1.5 mM MgCl₂, 0.2 µM of each oligonucleotide, 1 U of "Platinum Taq" enzyme (Life Technologies), 1 µL of DNA and sufficient sterile water to give a final reaction volume of 50 µL.

Amplifications were generated in an Eppendorf Mastercycler gradient thermocycler using the following conditions: initial denaturation at 94°C for 1 min and final extension at 72°C for 10 min, for a total of 35 cycles. Each cycle consisted of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension of 72°C for 1 min. An aliquot (5 µL) of the amplification product was subjected to electrophoresis in a 2% agarose gel in TAE buffer (40 mM Trizma base, 20 mM glacial acetic acid and 1 mM EDTA, pH 8.0). The size of the amplicons varied among serotypes (**Figure 1**).

Serotyping with a commercial latex agglutination test (containing reagents to serotypes Ia, Ib and II to IX; Strep-B-Latex Kit; Statens Serum Institute, Copenhagen, Denmark) was performed according to the manufacturer's instructions.¹⁶

Antimicrobial susceptibility was assessed by the disk diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines¹⁸ for clindamycin, erythromycin, levofloxacin, linezolid, penicillin and tetracycline.

RESULTS

The study was performed on 83 clinical isolates from vaginal and rectal swabs that tested positive by standard GBS culture; the swabs were obtained from 72 women tested GBS positive out of 363 pregnant women enrolled in this study. Figure 2 shows the flowchart of included samples and GBS testing. The included women were 13-50 years old (mean age: 27.9 years); the GBS prevalence was 20% (n= 72/363). The vaginal and rectal swabs were obtained from women in three conditions: between the 35th and 37th week of pregnancy (n= 271), with preterm rupture of

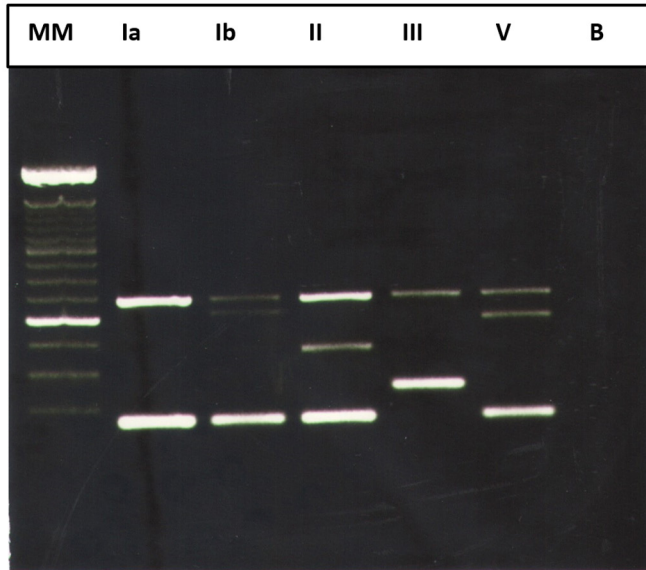


Figure 1. Gel electrophoresis of the multiplex PCR amplification products. (Five serotypes of six detected) In the sequence: MM –Molecular Marker of a 100 bp DNA ladder (Invitrogen®), Lanes Ia, Ib, II, III and V – GBS serotypes, B – blank.

membranes (n= 25) or preterm labor (n= 67). Forty-four of the 83 positive culture samples (53%) were serotyped by latex agglutination. Of these, 34 (77%) presented matching results by latex agglutination and multiplex PCR. Divergent results between the two methods were observed in ten samples.

All the 83 positive swab samples were serotyped by multiplex PCR: 59 were of vaginal origin and 24 of rectal origin; serotype Ia was the most prevalent, followed by serotype V. When the two anatomical sites were considered separately, the most prevalent GBS serotype was also Ia, as shown in Table 1. Among the eleven pregnant women who were simultaneously positive for samples collected at both anatomical sites, four had serotype Ia. The multiplex PCR test identified the serotypes Ib, II, III and VI as shown in Table 1. Serotype VI is rarely described.

In terms of susceptibility to antibiotics, the 44 samples were tested but only one of the samples (positive for serotype Ia) showed antimicrobial resistance to two antibiotics (clindamycin and erythromycin). The

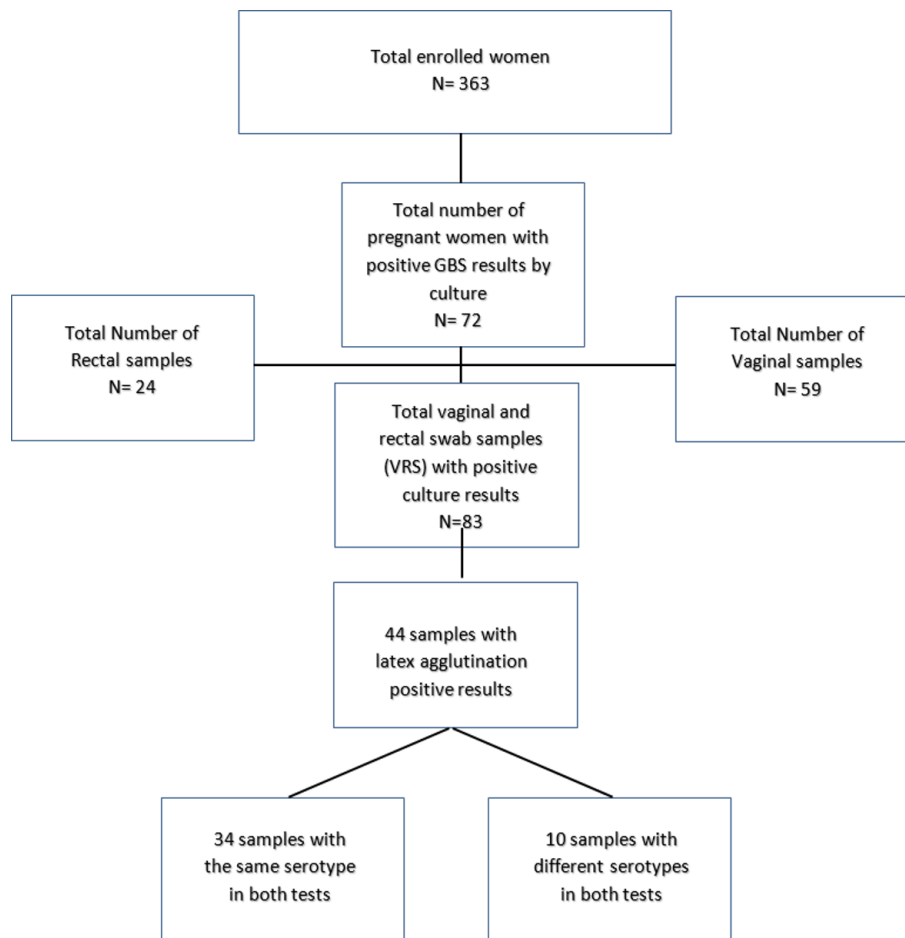


Figure 2. Flowchart of included samples and GBS testing.

Table 1 - Prevalence of GBS serotypes identified by multiplex PCR in positive standard culture samples (rectal and vaginal samples).

SEROTYPES	*VAGINAL SAMPLES (%)	*RECTAL SAMPLES (%)	TOTAL OF SAMPLES (%)
Ia	27 (46)	16 (67)	43 (52)
V	12 (20)	2 (8)	14 (17)
II	10 (17)	2 (8)	12 (15)
Ib	4 (7)	3 (13)	7 (8)
III	6 (10)	0 (0)	6 (7)
VI	0 (0)	1 (4)	1 (1)
TOTAL	59	24	83 (100)

* Of 11 pregnant women in whom positivity was observed in samples from both anatomical sites, four (36%) had the serotype Ia.

other samples (n= 43) were sensitive to clindamycin, erythromycin, levofloxacin, linezolid, penicillin and tetracycline.

DISCUSSION

The aim of this study was to determine the utility of the multiplex PCR technique to detect Group B Streptococcus serotypes and to determine the prevalence of different serotypes in order to characterize the local population and possibly predict the impact of future prophylactic vaccination. An appropriate understanding of the serological type of GBS is very useful in epidemiological studies and for the development of vaccines¹⁹ since each serotype is responsible for a different immunological response. In addition, there is a close relationship between serologic type and virulence in these microorganisms.^{9,15,20-22} In our study, multiplex PCR identified the serotypes Ia, V, II, Ib, III, VI. Serotype VI is rarely described: it had never been reported in Brazil or in other Latin American countries until this day.^{3,21,23}

The tests currently available for bacterial serotyping, such as the capillary precipitation test developed by Lancefield²⁰ and latex agglutination, are based on antiserum reactivity against the capsular polysaccharides of the ten known serotypes. However, both tests yield a large proportion of untypable strains, in addition to problems related to their high cost and reproducibility during routine laboratory use. Untypable strains result from the absence of capsular polysaccharides, the occurrence of non-specific reactions with antiserum for specific serotypes, or the absence of the minimum number of detectable antigens required by the test.^{10,24-26}

The development of molecular techniques such as multiplex PCR to identify specific sequences of group B Streptococcus DNA responsible for the expression of bacterial serotypes has allowed the characterization of several strains previously classified as untypable by conventional methods.^{11,27} In a study by Kong et al.²⁷ 207 GBS

strains were serotyped using the latex agglutination test, the capillary precipitation test and multiplex PCR. Of these, only 71 (34.3%) strains were successfully typed by conventional methods, while 204 (98.5%) strains were typed by multiplex PCR. In another study, Poyart et al.¹³ serotyped 426 streptococcal strains by conventional methods and multiplex PCR; 397 (93%) strains were successfully serotyped by conventional methods, whereas 425 (99.7%) were characterized by multiplex PCR. These studies confirmed the high sensitivity and specificity of multiplex PCR compared to conventional serotyping methods and its high discriminatory power in epidemiological studies. In agreement with these findings, in the present study none of the samples was untypable by multiplex PCR. In addition, compared to other techniques, multiplex PCR is cheaper and faster to run.²⁸

Serotypes vary in their distribution and prevalence between different geographic areas. Such geographic differences have been demonstrated for Brazil and other countries.^{3,8,19,29-31} In Brazil, a country of continental proportions, a recent study revealed that in 434 isolates obtained from symptomatic adults and from colonized patients, serotypes Ia (27,6%), II (19,1%), Ib (18,7%) and V (13,6%) were the most predominant; when only the colonized patients were considered, serotype II was the most prevalent in the southeastern region, followed by serotype Ia, while in the northeastern region serotype Ib is the most prevalent, followed by serotype V. Serotype Ia is most prevalent in the mid-western and south regions of the country, followed by serotypes II and V. Another study involving GBS isolates from women of reproductive age identified in most cases of GBS the capsular serotypes Ia (42,2%), II (10,8%), III (14,5%) and V (30,1%).⁸

Focused on pregnant women, this study found that serotype Ia was the most prevalent serotype, in agreement with previous literature reports indicating that despite geographic differences in prevalence, this serotype is the most frequently isolated from pregnant women in Latin America;²³ with regard to the prevalence of GBS serotypes in relation to anatomical sites (vaginal and rectal) of sample collection, serotype I prevalence at the two sites was consistent with previous findings.^{21,23} Surprisingly, serotype V was also frequent at both sites. One possible reason for this discrepancy with other studies may be that most of the previous investigations used conventional serotyping techniques that yielded high rates of untypable strains, possibly including serotype V; this bias in detection probably affected the conclusions regarding the serotypic profile of the study population.^{3,21,22,29,30}

A trivalent GBS polysaccharide-protein conjugate vaccine composed of capsular epitopes from serotypes Ia, Ib and III is currently in phase-II evaluation among pregnant women in Europe, North America and Africa. The vaccine used serotypes Ia, Ib and III, and seemed to be successful

in eliciting an adequate maternal and neonatal immune response, with a favorable safety profile. More studies are needed, but the path to decrease the incidence of early and late neonatal disease with a maternal vaccine is open. These serotypes cause 70-80% of all invasive GBS disease in early infancy.^{32,33} The possibility of shifts in serotype prevalence, such as the emergence of type IV,³⁴ the presence of new serotypes and the occurrence of untypable strains^{6,11} support the need for ongoing surveillance of serotypes.³⁴

In our samples, GBS was still uniformly sensitive to penicillin, the first-choice antibiotic. Clindamycin or erythromycin is recommended for intrapartum prophylaxis against GBS in penicillin-allergic women with a high risk of anaphylaxis or when therapeutic failure is suspected.^{35,36} As shown here, only one isolate (serotype Ia) was resistant to clindamycin and erythromycin.

■ CONCLUSION

Multiplex PCR allowed 100% GBS serotyping of the 83 positive samples of vaginal and rectal origin, with a major prevalence of serotype Ia. In this study, the incidence of the serotype VI, a new and rare serotype of GBS, was described for the first time in the Brazilian population.

Our results confirm the importance of implementing a highly sensitive, specific serotyping technique for successful serotypic profiling of a population. Determination of the correct serotypic profile should improve the efficiency of vaccination programs and antibiotic therapy.

■ CONFLICT OF INTEREST

The authors declare that they have no conflict of interest relating to this study.

■ AUTHOR CONTRIBUTION

PDA, CRCC, SCBC, MLN and RPJ designed the study, PDA, EBVA, SCBC and TBSP did the work and interpreted the molecular and genomic data, JF, MG, JSR and KGO did the molecular assays, JF, JBG, MG, and TBSP collected the samples, CEL did the microbiological assays, and PDA, MLC and SCBC analyzed the epidemiological data and drafted the manuscript. All authors read and approved the final manuscript.

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CARACTERIZAÇÃO MOLECULAR DOS SOROTIPOS DE ESTREPTOCOCOS DO GRUPO B POR REAÇÃO MULTIPLEX PCR

OBJETIVO: Os sorotipos (Ia, Ib e II ao IX) do estreptococo do grupo B (GBS) são classificados baseado nas variações em seus polissacarídeos capsulares; sua prevalência difere entre diferentes áreas geográficas. Nós examinamos a prevalência de todos os sorotipos do estreptococo do grupo B em amostras de swabs vaginal e retal obtidas de 363 mulheres seguidas em um centro de referência brasileiro, o Hospital da Mulher Professor Doutor José Aristodemo Pinotti; a susceptibilidade bacteriana a antibióticos foi também determinada.

MÉTODO A prevalência de estreptococo do grupo B positivo foi avaliada por aglutinação em látex e através de análise por multiplex PCR; susceptibilidade bacteriana a antibióticos, tais como clindamicina, eritromicina, levofloxacina, linezolida, penicilina e tetraciclina foi determinada pelo método de disco difusão.

RESULTADOS: (a) Tanto a cultura padrão para estreptococo do grupo B quanto a análise por multiplex PCR testaram positivos para 83 swabs. A prevalência para colonização por GBS foi 20%. O sorotipo Ia foi o mais prevalente (n= 43/83; 52%), seguido pelo sorotipo V (n= 14/83; 17%); De acordo com a origem anatômica, o sorotipo Ia positivou 27/59 (46%) e 16/24 (67%) das amostras vaginais e retais, respectivamente; o teste de PCR também identificou os sorotipos Ib, II, III, VI. O sorotipo VI é raramente descrito e não reportado no Brasil ou na América Latina até esta data. (b) O teste de aglutinação em látex somente identificou 44 amostras positivas, todas das quais foram sorotipadas: 34 destas amostras (77%) tiveram os sorotipos coincidindo com aqueles identificados pela multiplex PCR. (c) Somente uma amostra (sorotipo Ia) mostrou resistência a eritromicina e clindamicina.

CONCLUSÃO: Estudos regionais sobre a prevalência dos sorotipos do estreptococo do grupo B são essenciais para guiar medidas imunoprolifáticas (vacinas) e a implementação de adequada antibiótico profilaxia. Neste estudo, a incidência do sorotipo VI foi descrita pela primeira vez na população Brasileira, um novo e raro sorotipo do estreptococo do grupo B.

PALAVRAS-CHAVE: Polissacarídeos capsulares; Estreptococos do grupo B; Sorotipagem Molecular; multiplex PCR; Streptococcus agalactiae; sorotipo VI

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