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Assessment of conventional PCR and real-time PCR compared to the gold standard method for screening *Streptococcus agalactiae* in pregnant women

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ABSTRACT

Group B Streptococcus is a causative agent of invasive neonatal infections. Maternal colonization by *Streptococcus agalactiae* is a necessary condition for vertical transmission, with efficient screening of pregnant women playing an essential role in the prevention of neonatal infections. In this study, we aimed to compare the performance of conventional polymerase chain reaction and real-time PCR assays as screening methods for *S. agalactiae* in pregnant women against the microbiological culture method considered as the gold-standard. A total of 130 samples from pregnant women were analyzed for sensitivity, specificity, positive predictive value, and negative predictive value. Statistical analysis was performed using the SPSS software, version 20.0. The verified colonization rate was 3.8% with the gold-standard, 17.7% with conventional PCR assay, and 29.2% with the real-time PCR test. The trials with conventional PCR and real-time PCR had a sensitivity of 100% and a specificity of 85.6% and 73.6%, respectively. The real-time PCR assay had a better performance compared to the gold-standard and a greater detection rate of colonization by *S. agalactiae* compared to conventional PCR assay. With its quick results, it would be suitable for using in routine screenings, contributing to the optimization of preventive approaches to neonatal *S. agalactiae* infection.

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Introduction

Streptococcus agalactiae or group B streptococcus (GBS) is a commensal microorganism that colonizes the lower gastrointestinal and genitourinary tracts. However, it is also a major causative agent of invasive infections in newborns, pregnant women, and elderly and immunocompromised patients.[1–3]

GBS is the leading infectious cause of neonatal diseases such as pneumonia, meningitis, and sepsis, with high morbidity and mortality rates in the United States.[4–6] The incidence of neonatal GBS infections ranges from 0.80 to 3.06 per 1000 live births in developing countries.[7] In Brazil, some studies have indicated an incidence between 0.39 and 1.0 per 1000 live births.[8–11]

Maternal colonization with GBS is a necessary condition for vertical transmission at delivery, as well as for the occurrence of early-onset neonatal infection. In 1996, the Centers for Disease Control and Prevention (CDC) released guidelines for the prevention of neonatal GBS infections. The guidelines were updated in 2002 and 2010. Current guidelines recommend universal screening of pregnant women between 35 and 37 weeks of gestation using combined vaginal and anal clinical specimens. In positive cases, the mother should receive intrapartum antibiotic prophylaxis.[12] In Brazil, however, there is currently no specific prevention strategies for neonatal infections by GBS, and universal screening routine in pregnant women is not standardized for the prenatal period.[13]

Microbiological culture is considered the gold-standard method for GBS screening, but the turnaround time for results is between 48 and 72 h. Furthermore, it has limitations in detecting low number of bacteria, which may lead to false negative results.[14,15]

The study of sensitive, specific, and rapid techniques for detecting GBS in pregnant women is extremely important to optimize a preventive approach for neonatal infections. The aim of this study was to assess the performance of standard polymerase chain reaction (PCR) and real-time PCR (qPCR) as screening methods for GBS in pregnant women when compared to the gold-standard.

Materials and methods

Clinical specimens

One hundred and thirty combined rectal/vaginal specimens were collected to conduct this study, as per CDC recommendations.[12] Clinical specimens were collected from pregnant women at 35 weeks or more of gestation who had received care at the Hospital de Clínicas de Porto Alegre (HCPA) between August 2014 and November 2014.

Microbiology tests

The swabs were inoculated onto a Todd Hewitt selective medium (Himedia Laboratories, India) supplemented with gentamicin (8 μg/mL) and nalidixic acid (15 μg/mL). The selective medium was incubated at 36° C, 5% CO_2 for 18 h. It was then subcultured onto blood agar plates (BioMerieux®, Marcy-l'toile, France), which were incubated at 35–37 °C, 5% CO_2 for 18–24 h. After incubation, the plates were inspected for β-hemolytic colonies. If no β-hemolytic colonies were observed after 24 h, plates were reincubated for another 24 h and inspected again. The β-hemolytic colonies whose morphology was consistent with group B *Streptococcus* were subcultured in broth and submitted to the CAMP (Christie, Atkins, Munch, Petersen) test. The colonies that tested positive were presumptively considered GBS.

Molecular analysis

Bacterial isolates preparation and DNA extraction

The swabs were incubated for 15 to 18 h onto a Todd Hewitt selective medium. After centrifugating the broth, the precipitate was washed with a 1× PBS solution and resuspended it in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 7.5). This solution was submitted to thermal lysis. Thermal lysis was performed using the TE solution for 15 min at 100 °C to lyse bacterial cell walls. The concentration of the extracted DNA was assessed under a spectrophotometer. All DNAs were diluted to 5 ng/μL and stored at −20 °C until use in PCR assays.

Conventional polymerase chain reaction (PCR)

For the conventional PCR reaction, we used primers 5′-CAA CGA TTC TCT CAG CTT TGT TAA-3′ and 5′-TAA GAA ATC TCT TGT GCG GAT TTC-3′, producing a 779-bp fragment that is specific to the *atr* gene.[16] This target gene encodes an amino acid transporter protein, gs0538, which is specific to GBS. A positive control confirmed by DNA sequencing was used in the assay (http://www.ncbi.nlm.nih.gov/BLAST).

A positive control confirmed by DNA sequencing was used in the assay.

Amplification conditions were described in a previous study.[17] The amplicons were detected by electrophoresis using 10 μL of the amplified reaction mixture in a 2% agarose gel containing SYBR® Safe DNA gel stain (Invitrogen®, Calbad, USA) as dye. A 100-bp molecular weight marker (Invitrogen®, Calbad, USA) and a positive control for *S. agalactiae* were used to evaluate the PCR products. The fragments presenting a 779-bp amplicon were considered positive for GBS.

Real-time PCR (qPCR)

The real-time PCR (qPCR) reaction targeted the *cfb* gene, which codifies a diffusible extracellular protein produced by GBS (CAMP factor). We used primers Sag59 5′-TTTCACCAGCTGTATTAGAAGTA-3′ and Sag190 5′-GTTCCCTGAACATTATCTTTGAT-3′. As described by Ke et al., these primers were specific to *S. agalactiae* and tested against many other species which also have genes similar to *cfb*.[18] For the internal control (IC) reaction, we used a synthetic IC, with primers TBIC90F 5′-ATCGCTGATCCGGCCACA-3′ and TBIC90R 5′-TCGGTGACAAAGGCCACGTA-3′ for detection. The positive control was the same as in conventional PCR.

The qPCR reaction was based on the SYBR® Detection System (Invitrogen™, USA). The reaction was carried out

using the Platinum SYBR® Green amplification kit – qPCR SuperMix-UDG, with 10 μM of the reverse and forward primers and a DNA concentration adjusted to 5 ng/μL. Amplification was performed in the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA) under the following conditions of temperature and cycling: one initial cycle at 50 °C for 2 min; one cycle at 95 °C for 10 min; 40 cycles at 95 °C for 15 s and at 60 °C for 1 min to amplify DNA; and 2 cycles at 95 °C for 15 s and at 60 °C for 15 s to measure melting temperature (T_m) and detect fluorescence.

The results were analyzed using the cycle threshold (C_t) and T_m. Positive results for the GBS target are a T_m between 76 and 78 °C, and for the IC, 82–84 °C.

Limit of detection (LoD)

The LoD of the qPCR assay was determined using four serial dilutions (5, 10, 50 and 100 copies/μL) of a commercial strain of GBS (AmpliRun, Vircell, Granada, Spain). The dilutions were submitted to the qPCR protocol in 46 trials. The LoD was defined as the lowest dilution tested with positive results (DNA amplification product) in at least 95% of the replicates.

Statistical analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the PCR technique were calculated using culture as the gold standard. Concordance between assays was determined using the *kappa* coefficient. Statistical analysis was performed using the SPSS® software, version 20.0 (IBM, Inc., Chicago, USA).

Results

Among the 130 clinical specimens used in the study, all five (3.8%) samples positive in the CAMP test were positive by conventional PCR for the *atr* gene. In comparison, 23 (17.7%) of the clinical specimens tested positive for GBS colonization with conventional PCR, and 38 (29.2%) tested positive with qPCR. Tables 1 and 2 show the results for conventional PCR and qPCR assays, respectively, compared to the microbiological culture.

All of the culture positive clinical samples tested positive with both PCR techniques, indicating a sensitivity of 100% of the assays. Among the 125 clinical specimens with culture negative results, 18 turned out positive in the conventional PCR assay, and 107 were negative in both, pointing to a specificity of 85% for the test. The qPCR assay presented a specificity of 73.6% when compared to the, given that 92 clinical specimens were negative in both methods.

Both conventional PCR and qPCR techniques presented a negative predictive value of 100% and a positive predictive value of 21.7% and 13.1%, respectively. Concordance between microbiological culture results and both conventional PCR and qPCR were weak (*kappa* = 0.314 and 0.177, respectively).

The LoD found in the qPCR assay was 10 copies/μL of *S. agalactiae*.

Discussion

Maternal rectovaginal colonization with SGB is the main risk factor for vertical transmission during labor and for the occurrence of early-onset neonatal infection. The CDC recommends screening programs for pregnant women between 35 and 37 weeks of gestation to detect *S. agalactiae* colonization, as an intrapartum antimicrobial prophylaxis is required for colonized women.[12] Since the introduction of SGB screenings in the USA, the early neonatal infection rate by SGB decreased from 1.8 cases per 1000 live births to 0.26 cases per 1000 live births.[19] However, in Brazil there are no public health policies in place for the standardization of universal routine screenings of pregnant women during the prenatal period.[13]

In our study, the maternal colonization rate was 3.8% with microbiological culture, 17.7% with conventional PCR, and 29.2% with qPCR. Other Brazilian studies using both microbiological and PCR methods for SGB detection found a colonization rate between 14.6% and 35.9%.[20–23] The colonization rates in pregnant women can vary widely due to factors related to the characteristics of the studied population and the detection methods used.[24,25]

With the gold standard method, the percentage of pregnant women who tested positive in our study was low in comparison to the PCR assays. Similarly, Castellano-Filho et al.[26] have described a colonization rate of 32.6% detected by conventional PCR compared to 9.5% with the gold standard. These data are consistent with other studies assessing qPCR assays, which showed considerable increase in the identification of pregnant women colonized by SGB.[27–29]

In our study, the sensitivity of both PCR methods was 100%. De-Paris et al.[17] also evaluated the use of conventional PCR as a method for SGB detection in pregnant women and found a high sensitivity rate (100%) for the test. In studies where the qPCR technique was used, we found variable and lower sensitivity values, between 89.1% and 95.4%.[28,30,31] Despite presenting high sensitivity (100%), the qPCR assay assessed in the present study also detected a higher number of positive clinical specimens compared to the conventional PCR.

The use of a selective medium before PCR is recommended by the CDC and is essential to ensure high sensitivity in the screening test, such as the one observed in our study.[12] Submitting clinical specimens without previous enrichment to the PCR assay reduces the time until results, but affects performance. Mashouf et al.[32] performed a direct assay using conventional PCR with the 16S rRNA gene as a target. The test sensitivity was 88.23% compared to the microbiological culture method. The direct use of clinical specimens in the qPCR also shows lower sensitivity (<75%) compared to the gold-standard.[33]

The discrepancy between the rates of positive results identified in the culture (3.8%) and those obtained with conventional PCR (17.7%) and real-time PCR (29.2%) may e due to non-viable bacteria or a low bacterial load in the collected clinical specimens. In these situations, the gold-standard method is usually insufficient for detection.[34] For that reason, although microbiological culture is considered the gold-standard for SGB detection, it is important to highlight

Table 1 – Comparison between the conventional PCR technique and the microbiological culture (gold standard) in detection of *S. agalactiae*.

Techniques	Microbiological Culture (gold standard)		Total	Kappa
	Positive	Negative		
Conventional PCR				
Positive	5 (100%)	18 (14.4%)	23 (17.7%)	0.314
Negative	0 (0%)	107 (85.6%)	107 (82.3%)	
Total	5 (100%)	125 (100%)	130 (100%)	

Table 2 – Comparison between the qPCR technique and the microbiological culture (gold standard) in detection of *S. agalactiae*.

Techniques	Microbiological Culture (gold standard)		Total	Kappa
	Positive	Negative		
qPCR				
Positive	5 (100%)	33 (26.4%)	38 (29.2%)	0.177
Negative	0 (0%)	92 (73.6%)	92 (70.8%)	
Total	5 (100%)	125 (100%)	130 (100%)	

situations in which term infants with neonatal infections are born to women who tested negative for colonization.[35,36]

It is therefore relevant that the chosen methodology for routine screening programs has a high analytical sensitivity, such as the one verified in the qPCR assay in this study, which was capable of detecting as low as 10 copies/μL of *S. agalactiae*. The LoD indicated in our assay is superior to the one described by El Aila et al.[26] who performed a qPCR assay with the same target gene (*cfb*), but using a probe system. They found a minimum LoD of 20 copies/μL.

The PPV of the conventional PCR and qPCR techniques were 21.7% and 13.1%, respectively. The predictive values relate to the specificity and prevalence of colonization, which means that the low PPV found in the conventional PCR and qPCR assays must be analyzed comparatively to the gold-standard, in which there were five (3.8%) positive clinical samples.[37]

In our study, the specificity of the conventional PCR technique was 85.6%, a slight increase compared to the 82.6% found by Munari et al.[22] for the *atr* gene. The qPCR reaction showed a specificity of 73.6% when compared to the culture. This result is consistent with that reported by Yeung et al.[38] (73.1%), who targeted the *cpsG* gene locus of the SGB capsular polysaccharide. Both PCR assays presented an NPV of 100%. This percentage is clinically important as it ensures that negative cases are indeed true, thus preventing unnecessary exposure of pregnant women to antimicrobial prophylaxis during labor.

The specificity of PCR assays must be analyzed against the results of the microbiological culture. Although the use of a selective broth and chromogenic media facilitates the β-hemolytic identification of *S. agalactiae*, there is a non-hemolytic fraction of SGB strains that hinder detection by the traditional method.[12] In practice, this means that the results considered as false-positives in the PCR techniques are in fact true positives, bearing in mind the greater sensitivity of the molecular method. Indeed, in the Microbiology Laboratory they investigate only β-hemolytic colonies in the CAMP test, since this characteristic appears in most *S. agalactiae*. The low positivity rate in the culture is basically due to the growth of the anal-vaginal microbiota, which hinders the isolation of *S. agalactiae*, and not because we do not investigate the non-hemolytic colonies of *S. agalactiae*, which have a low percentage in the species.

In a study performed by Feuerschuette et al.,[27] the samples with discrepant results, negative by the culture and positive by real-time PCR, were submitted to conventional PCR, which provided mainly positive results. The results obtained with qPCR were thus considered true positives, while the negative results with culture were considered false negative.

In the case of our study, the low specificity of the PCR techniques may result from its high performance when detecting SGB in samples where the culture method fails.

Over the past few years, many commercial kits to detect SGB by qPCR were released. Among them are the IDI-Strep B kit (IDI, Sainte-Foy, QC, Canada) and the GeneXpert kit (Cepheid, Sunnyvale, CA, EUA), both of which target the *cfb* gene. Studies have assessed the performance of these tests as an option to detect maternal intrapartum colonization, with sensitivity results between 85%-98.5% and specificity results between 97.6% and 99.6%.[39–42] However, both the operating platforms and the kits have an elevated cost, making them economically unfeasible as a screening strategy in the prevention of neonatal diseases by SGB in developing countries like Brazil.

Following the CDC recommendations is effective in preventing neonatal infections by SGB, reducing around 80% of their incidence.[18] However, to achieve such rates, it is essential to use sensitive, specific and fast screening methods that allow for the correct detection and adequate approach to colonized pregnant women.

The high sensitivity of a test is an essential parameter when screening is the focus, also considering that the failure in identification of individual positive results is a risk for the development of diseases.[37] The PCR techniques assessed in our study could be suitable for screening routines due to their higher sensitivity and faster turnaround time of results compared to traditional cultures. The NPV (100%) of the tests is also relevant as it indicates reliability in correctly identifying negative clinical samples and ensures the rational use

of antimicrobial prophylaxis. The qPCR technique had a better performance in identifying positive SGB clinical specimens compared to conventional PCR. These results may be related to a better sensitivity of the real-time modality, which uses a fluorophore and allows for a more specific amplification system. Besides, the amplification can be monitored and the number of molecules in each cycle can be quantified as the reaction occurs, providing results in a considerably shorter time compared to the gold-standard. Our study confirms the high sensitivity of the qPCR method and its potential for use in screening routines, providing reliable and safe results for the rational use of antimicrobial prophylaxis in the prevention of SGB vertical transmissions and in the occurrence of neonatal infections.

Conflicts of interest

The authors declare no conflicts of interest.

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