Development of Conventional and Real-Time PCR Assays for the Rapid Detection of Group B Streptococci

Danbing Ke, Christian Ménard, François J. Picard, Maurice Boissinot, Marc Ouellette, Paul H. Roy, and Michel G. Bergeron

Background: Group B streptococci (GBS), or *Streptococcus agalactiae*, are the leading bacterial cause of meningitis and bacterial sepsis in newborns. Currently available rapid methods to detect GBS from clinical specimens are unsuitable for replacement of culture methods, mainly because of their lack of sensitivity.

Methods: We have developed a PCR-based assay for the rapid detection of GBS. The cfb gene encoding the Christie-Atkins-Munch-Petersen (CAMP) factor was selected as the genetic target for the assay. The PCR primers were initially tested by a conventional PCR method followed by gel electrophoresis. The assay was then adapted for use with the LightCycler™. For this purpose, two fluorogenic adjacent hybridization probes complementary to the GBS-specific amplicon were designed and tested. In addition, a rapid sample-processing protocol was evaluated by colony-forming unit counting and PCR. A total of 15 vaginal samples were tested by both standard culture method and the two PCR assays.

Results: The conventional PCR assay was specific because it amplified only GBS DNA among 125 bacterial and fungal species tested, and was able to detect all 162 GBS isolates from various geographical areas. This PCR assay allowed detection of as few as one genome copy of GBS. The real-time PCR assay was comparable to conventional PCR assay in terms of sensitivity and specificity, but it was more rapid, requiring only ~30 min for amplification and computer-based data analysis. The presence of vaginal specimens had no detrimental effect on the sensitivity of the PCR with the sample preparation protocol used. All four GBS-positive samples identified by the standard culture method were detected by the two PCR assays.

Conclusion: These assays provide promising tools for the rapid detection and identification of GBS.

Nonstandard abbreviations: GBS, group B streptococci; CAMP, Christie-Atkins-Munch-Petersen; CFU, colony-forming unit; and GNS broth, Todd-Hewitt broth containing nalidixic acid (15 mg/L) and gentamicin (8 mg/L).
detection and identification of GBS directly from clinical specimens of colonized women (10, 11). GBS-specific PCR-based assays have demonstrated better sensitivity, but they require complicated procedures that are not applicable to clinical use (13–15).

A real-time amplification-detection apparatus with air thermal cycling and fluorescently monitored product analysis (LightCycler™) in a closed-tube assay format recently has been developed (16–18). This new technology is particularly attractive because it is able to avoid carryover and requires ~30 min for completion of a 45-cycle PCR.

GBS can be presumptively identified by the Christie-Akins-Munch-Petersen (CAMP) test, based on detection of a diffusible extracellular protein (CAMP factor) produced by the majority of GBS (19). The cfb gene encoding the CAMP factor is present in virtually every GBS isolate and is an obvious candidate for the development of a PCR assay for identification of GBS (20). In this study, a pair of GBS-specific PCR amplification primers were designed from the cfb gene and initially evaluated by conventional PCR using agarose gel electrophoresis. Subsequently, the assay was adapted for use with the LightCycler, which allowed for shorter running time and real-time detection of amplicons by using fluorescence measurements. These assays were shown to be specific and highly sensitive for the detection and identification of GBS.

Materials and Methods

MICROORGANISMS

A total of 162 GBS strains, including 5 reference strains obtained from the American Type Culture Collection (ATCC 13813, ATCC 12400, ATCC 12403, ATCC 12973, and ATCC 27591) were used in this study. Of the 157 clinical isolates of GBS, 117 were of human origin and 40 were of bovine origin. The 117 clinical isolates of human origin were from (a) the Microbiology Laboratory of the Centre Hospitalier Universitaire de Québec, Pavillon Centre Hospitalier de L’Université Laval, Ste-Foy, Québec, Canada (n = 25); (b) the National Centre for Streptococcus, University of Alberta Hospital, Edmonton, Alberta, Canada (n = 91); and (c) the CDC, Atlanta, GA (n = 1). The 40 strains isolated from cow milk samples were obtained from the Faculté de Médecine Vétérinaire, Université de Montréal, St-Hyacinthe, Québec, Canada. All strains were grown on sheep blood agar at 37 °C under an aerobic atmosphere. The identification of all GBS strains was confirmed by both the CAMP test and the Streptex latex agglutination test (Murex Diagnostics). Stock cultures were stored frozen (−80 °C) in brain-heart infusion medium containing 100 mL/L glycerol.

A wide variety of gram-positive and gram-negative bacterial strains as well as two fungal species obtained from the ATCC were used to test the specificity of the PCR assays (Table 1). All strains were grown on media under conditions that support optimal growth.

DNA ISOLATION

Genomic DNA from all strains tested was obtained using the G NOME kit (Bio101) with modification. A RNase pretreatment was added before quantification of genomic DNA. The concentrations of DNA preparations were calculated by measuring the absorbance at 260 nm or by comparison with DNA calibrators after agarose gel electrophoresis.

OLIGONUCLEOTIDES

Two pairs of fluorescently labeled adjacent hybridization probes (STB-F/STB-C hybridizing to GBS-specific amplicons and IC-F/IC-C hybridizing to the internal control amplicon) were synthesized and HPLC-purified by Operon Technologies (Table 2) and were designed to meet the recommendations of the manufacturer (Idaho Technology) (16). These adjacent probes, which are separated by one nucleotide, allow fluorescence resonance energy transfer to generate an increased fluorescence signal when hybridizing to their target sequences. The probes STB-F and IC-F were labeled with fluorescein, and STB-C and IC-C were labeled with Cy5™ (Amersham Pharmacia Biotech). The Cy5-labeled probes contained a 3′-blocking phosphate group to prevent extension of the probes during the PCR reactions.

CONSTRUCTION OF THE INTERNAL CONTROL

An internal control was constructed essentially as described previously by Rosenstraus et al. (21). A 252-bp DNA fragment consisting of a 206-bp sequence not found in GBS flanked by the sequences of each of the two GBS-specific primers was used as a template for the internal control. This fragment was cloned into the pCR2.1 vector (Invitrogen). The recombinant plasmid, named pSTB, was isolated from transformed Escherichia coli by the Qiagen plasmid mini kit (Qiagen). The purified plasmid was then linearized with EcoRI (New England Biolabs) and serially diluted. The concentration of the linearized plasmid was optimized to permit amplification of the 252-bp internal control product without significant detrimental effect on the GBS-specific amplification.

PCR AMPLIFICATION

Relevant characteristics and optimal PCR conditions for the GBS-specific conventional and real-time PCR assays are given in Table 3. Strict precautions to prevent car-
Table 1. Bacterial strains used to test the specificity of the GBS-specific PCR assay.

<table>
<thead>
<tr>
<th>Gram-positive aerobic bacteria</th>
<th>Gram-negative aerobic bacteria</th>
<th>Gram-negative anaerobic bacteria</th>
<th>Fungi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abiotrophia adiacens ATCC 49175(^a)</td>
<td>Actinobacillus suis ATCC 26784</td>
<td>Bacteroides fragilis ATCC 25285(^a)</td>
<td>C. albicans ATCC 10231(^a)</td>
</tr>
<tr>
<td>A. defectiva ATCC 49176(^a)</td>
<td>A. haemolyticus ATCC 27914</td>
<td>Bifidobacterium breve ATCC 15700(^a)</td>
<td>C. krusei ATCC 34135</td>
</tr>
<tr>
<td>Bacillus anthracis ATCC 4229</td>
<td>Bordetella pertussis ATCC 9797</td>
<td>Citrobacter diversus ATCC 27028</td>
<td>C. parapsilosis ATCC 26403</td>
</tr>
<tr>
<td>B. cereus ATCC 14579</td>
<td>Buikholderia cepacia ATCC 25416</td>
<td>Citrobacter freundii ATCC 8090</td>
<td>C. tropicalis ATCC 8103</td>
</tr>
<tr>
<td>Actinomyces pyogenes ATCC 19411</td>
<td>Citrobacter koseri ATCC 17733</td>
<td>Enterobacter aerogenes ATCC 13048</td>
<td>C. viscosus ATCC 52591</td>
</tr>
<tr>
<td>Corynebacterium urealyticum ATCC 43042</td>
<td>Citrobacter freundii ATCC 8090</td>
<td>Escherichia coli ATCC 10412</td>
<td>C. yeasts ATCC 70081</td>
</tr>
<tr>
<td>Enterococcus avium ATCC 14025</td>
<td>C. freundii ATCC 8090</td>
<td>Klebsiella pneumoniae ATCC 10030</td>
<td></td>
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</tbody>
</table>
ryover of amplified DNA were used (22). Pre- and post-PCR manipulations were conducted in separate areas. Aerosol-resistant pipette tips were used to handle all reagents and samples. Control reactions to which no DNA was added were performed routinely to verify the absence of DNA carryover.

Concomitant amplification of the internal control allowed verification of the efficiency of the PCR to ensure that there was no significant PCR inhibition by the test sample. For conventional PCR, the internal control was amplified simultaneously with the GBS genomic target. On the other hand, for real-time PCR, the control was amplified in a separate reaction vessel because only two fluorescent signals can be monitored in the same capillary with the LightCycler model used.

SPECIFICITY AND SENSITIVITY TESTS

The specificity of the conventional PCR assay was verified using purified genomic DNA (0.1 ng/reaction) from a battery of ATCC reference strains representing 105 aerobic and 18 anaerobic bacterial species as well as 2 fungal species (Table 1). These microbial species included 28 species of streptococci and many members of the typical vaginal and anal flora. The specificity of the real-time PCR assay was verified by testing genomic DNA from bacterial species that are phylogenetically close to GBS, including members of the genera *Streptococcus*, *Lactococcus*, *Enterococcus*, *Abiotrophia*, *Peptostreptococcus*, and *Listeria*. Some species encountered in the typical vaginal flora were also tested (Table 1). A total of 162 clinical isolates of GBS from various origins were also tested to further validate the GBS-specific conventional PCR assay by performing amplifications from standardized bacterial suspensions before adapting the assay to the LightCycler platform.

The detection limit (i.e., minimal number of genome copies that can be detected) of the PCR assays was determined by serial twofold dilutions of purified GBS genomic DNA from five GBS ATCC strains. To evaluate the efficiency of the IDI DNA extraction kit (Infectio Diagnostics Inc.) to prevent PCR inhibition, three GBS-specific conventional PCR assay by performing amplifications from standardized bacterial suspensions before adapting the assay to the LightCycler platform.

The efficacy of the IDI DNA extraction kit to lyse GBS was evaluated by comparing the minimal number of colony-forming units (CFUs) detected with the preparations without pretreatment to those prepared by using the IDI kit. The detection limits in CFUs were determined using cultures of three GBS strains (ATCC 13813, 12400, and 27591) in the logarithmic phase of growth (absorbance at 600 nm, ~0.6) diluted 10-fold in phosphate-buffered saline. Each 10-fold dilution in phosphate-buffered saline was either added directly to the PCR reaction mixture or processed using the IDI DNA extraction kit before PCR amplification. The number of CFUs was estimated by standard plating procedures.

CLINICAL SPECIMENS AND GBS-SELECTIVE CULTURE AND PCR

Vaginal specimens were collected from 15 consenting pregnant women admitted for delivery at the Centre Hospitalier Universitaire de Québec, Pavillon Saint-François d’Assise, using a polyurethane-tipped swab (Cultette™; Beckon Dickinson) after excessive discharge. 

Table 2. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Oligonucleotides</th>
<th>Nucleotide sequences</th>
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<tbody>
<tr>
<td>GBS-specific primers⁴</td>
<td></td>
</tr>
<tr>
<td>Sag59</td>
<td>5'-TTTCACCCAGCTGTATTAGAAAGTA-3'</td>
</tr>
<tr>
<td>Sag190</td>
<td>5'-GTTCCCTGAACATTATCTTTGAT-3'</td>
</tr>
<tr>
<td>STB-F</td>
<td>5'-TGCTGTGCTCAAGATAATCCACAGTTGA-phosphate-3'</td>
</tr>
<tr>
<td>STB-C</td>
<td>5'-TTATTGACGGTCTCCGACAGGA-fluorescein-3'</td>
</tr>
<tr>
<td>Adjacent hybridization probes specific for the GBS amplicon</td>
<td></td>
</tr>
<tr>
<td>IC-F</td>
<td>5'-TTATTGACGGTCTCCGACAGGA-fluorescein-3'</td>
</tr>
<tr>
<td>IC-C</td>
<td>5'-TTATTGACGGTCTCCGACAGGA-fluorescein-3'</td>
</tr>
</tbody>
</table>

⁴The GBS-specific primers amplify a fragment of 153 bp. The nucleotide positions based on the cfb sequence of GBS (X72754) are 369–391 for Sag59 and 500–522 for Sag190.
Results

SELECTION OF GBS-SPECIFIC PRIMERS AND FLUORESCENTLY LABELED PROBES

GBS-specific primers and probes (Table 2) were chosen from GBS unique regions selected from a multiple sequence alignment of the \textit{cfb} genes from GBS, \textit{S. uberis}, and \textit{S. pyogenes} (data not shown). Sequence comparison of streptococcal \textit{cfb} genes showed that these three genes were fairly divergent, with nucleotide identities ranging from 60.8\% to 66.7\%, hence facilitating the design of specific oligonucleotides.

EVALUATION OF THE GBS-SPECIFIC CONVENTIONAL PCR ASSAY

The specificity of the assay was assessed using purified genomic DNA from the panel of gram-positive and gram-negative bacterial species as well as fungal species listed in Table 1. This assay was specific because only DNAs from GBS strains could be amplified (Fig. 1). Many members of the vaginal or anal flora tested (23), including \textit{E. coli}, \textit{Candida albicans}, \textit{Gardnerella vaginalis}, enterococci, coagulase-negative staphylococci, \textit{Lactobacillus} spp., \textit{Peptostreptococcus} spp., and \textit{Bacteroides} spp. were not amplified by the GBS-specific PCR assay. Moreover, the PCR assay was able to efficiently detect all 162 GBS strains used in this study, including reference ATCC strains as well as clinical isolates of both human and bovine origins, thereby showing a perfect correlation with standard culture-based identification methods.

The detection limit of the assay was determined using purified genomic DNA from the five ATCC strains of GBS. The detection limit for all five ATCC strains was one genome copy of GBS. When we used the IDI extraction kit, as few as one genome copy of GBS was also detected from all three GBS-negative vaginal samples to which genomic DNA of GBS had been added. In terms of CFUs, the PCR assay was able to detect 1–3 CFUs from mid-log phase cultures when the IDI DNA extraction kit was used compared with 10–24 CFUs with diluted cultures added directly to the PCR mixture without pretreatment. These results confirmed the high sensitivity of our GBS-specific PCR assay as well as the efficacy of the IDI kit for lysis of GBS cells and prevention of significant PCR inhibition.

During PCR amplification, the internal control template (i.e., linearized pSTB) integrated into all PCR reactions allowed verification of the efficiency of all amplifications. The 252-bp PCR product for the internal control was amplified by the GBS-specific primers. Thus, the GBS-specific primer pair could amplify both the target genomic sequence in GBS and the internal control template. This strategy allows validation of the amplification primers, simplification of the assay, and prevention of

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### Table 3. Characteristics of the GBS-specific conventional and real-time PCR assays.

<table>
<thead>
<tr>
<th></th>
<th>Conventional PCR</th>
<th>Real-time PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thermocycler</td>
<td>PTC-200 DNA Engine (MJ research)</td>
<td>LC-32 LightCycler (Idaho Technology)</td>
</tr>
<tr>
<td>Heating/cooling rate</td>
<td>3 °C/s</td>
<td>20 °C/s</td>
</tr>
<tr>
<td>Reaction vessel</td>
<td>0.2 mL plastic tube</td>
<td>Glass capillary</td>
</tr>
<tr>
<td>Reaction volume</td>
<td>20 μL</td>
<td>7 μL</td>
</tr>
<tr>
<td>Use of internal probes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Sample volume</td>
<td>2 μL</td>
<td>0.7 μL</td>
</tr>
<tr>
<td>Internal control</td>
<td>Coamplified</td>
<td>Amplified separately</td>
</tr>
<tr>
<td>PCR Master Mix</td>
<td>0.4 μmol/L primers, 200 μmol/L dNTP, 10 mmol/L Tris-HCl, 2.5 mmol/L MgCl₂, 3.3 g/L BSA, 10 copies of pSTB, 0.5 U of Taq polymerase, 50 mmol/L KCl</td>
<td>0.4 μmol/L primers, 200 μmol/L dNTP, 10 mmol/L Tris-HCl, 3.5 mmol/L MgCl₂, 0.45 g/L BSA, 70 copies of pSTB, 0.5 U of KlenTaq, 16 mmol/L (NH₄)₂SO₄, 0.2 μmol/L fluorescent probes</td>
</tr>
<tr>
<td>PCR program</td>
<td>Hold: 3 min/94 °C, 40 cycles: 1 s/55 °C, 30 s/55 °C, Hold: 2 min/72 °C</td>
<td>Hold: 3 min/94 °C, 45 cycles: 0 s/55 °C, 14 s/55 °C, 5 s/72 °C</td>
</tr>
<tr>
<td>Maximum reactions/run</td>
<td>96</td>
<td>32</td>
</tr>
<tr>
<td>Duration of amplification</td>
<td>65 min</td>
<td>35 min</td>
</tr>
<tr>
<td>Detection</td>
<td>Gel electrophoresis (25 min)</td>
<td>Fluorescence monitoring (real-time)</td>
</tr>
</tbody>
</table>

\(\text{a BSA, bovine serum albumin.} \\
\text{b With the real-time PCR assay, the internal control was amplified separately. The 7 μL reaction mixture for the internal control also contained 70 copies of linearized plasmid pSTB per capillary and the two fluorescently labeled probes (IC-F and IC-C) specific for the 252-bp internal control amplification product rather than those specific for the GBS-specific amplification product.} \\
\text{c Both Taq polymerase (Promega) and KlenTaq1 (AB peptides) were coupled with TaqStart™ antibody (Clontech) at molar ratios of 1:14 and 1:56, respectively.)}
potential detrimental competition between different PCR primer pairs. As expected, when GBS DNA was absent, the internal control was always amplified efficiently (Fig. 1). When GBS DNA was present, amplification of the internal control was either lower or absent because of competitive inhibition by amplification of the GBS genomic target. It is critical that there be no significant competitive inhibition from the internal control template to minimize a decrease in the sensitivity of the assay.

EVALUATION OF THE GBS-SPECIFIC REAL-TIME PCR ASSAY
The specificity of the real-time PCR assay was also verified using a battery of bacterial species, including streptococci (28 species), enterococci, lactococci, and Peptostreptococcus spp., as well as members of typical vaginal and anal flora (Table 1). Only GBS could be detected by the production of an increased fluorescence signal that was interpreted as a positive PCR result. The fluorescence resonance energy transfer signal for the internal control was always amplified efficiently (Fig. 1). When GBS DNA was present, amplification of the GBS target DNA increased (data not shown). The real-time PCR assay showed the same sensitivity as the conventional PCR assay described above (Fig. 2).

IDENTIFICATION OF GBS COLONIZATION IN PREGNANT WOMEN
Among 15 vaginal samples obtained from pregnant women at delivery, 4 were positive for GBS, whereas the other 11 samples were negative for GBS as determined by both the standard culture method and the two PCR assays. For conventional PCR, the time required for sample processing, PCR amplification, and gel electrophoresis was ~100 min. On the other hand, the time required for real-time PCR was ~45 min because thermal cycling is much faster and amplicon detection is performed in real time. Both PCR methods were able to identify GBS colonization in a much shorter turnaround time than the gold standard culture method.

Discussion
GBS can cause significant infections in human, especially in newborns (1, 3). Because GBS are generally susceptible to ampicillin, early detection and identification of the infection can effectively guide antibiotic therapy and thus prevent severe damage to the host (24). Currently, the gold standard method for detection of vaginal colonization with GBS is selective broth culture performed at 35–37 weeks of gestation, which is sensitive enough to allow detection of both light and heavy colonization, but identification results are not available until 48 h later (4). Therefore, these methods are not useful for identification of GBS at or near the time of delivery. Moreover, culturing at 35–37 weeks is not always indicative of carrier status at delivery because GBS colonization often is transient (3). Because rapid diagnosis of GBS colonization or infection is of importance in the prevention of neonatal sepsis and meningitis, many simple and rapid tests for GBS have been developed. The Gram stain smear is of limited clinical help in detecting GBS because of low sensitivity and poor specificity (6). Several special media have been introduced to rapidly detect GBS by pigment production (25–27). However, some GBS isolates lack the ability to produce pigment, and thus cannot be identified by these media. The sensitivities of latex agglutination tests and immunoassays for detection of GBS directly from clinical specimens vary from 19% to 82% when selective broth media are used as standards to recover GBS from specimens (9). Generally, these tests are not sufficiently sensitive for direct detection of GBS, and only women with heavy colonization can be readily identified by these methods (6–8).

In this study, we developed a conventional PCR assay that is rapid (~100 min), specific for GBS, and sensitive enough to detect a single genome copy of GBS. Importantly, there was no amplification with purified genomic DNA from 27 species of streptococci other than GBS and many members of the vaginal and anal flora. Furthermore, the assay was capable of efficiently amplifying DNA from 162 GBS strains from various geographic regions. Such an assay may be useful for the detection of GBS colonization directly from clinical specimens because of its high specificity and sensitivity. Although all GBS strains tested were positive for the CAMP test, the assays should be able to identify CAMP test-negative strains because the cfb gene is present in virtually all GBS isolates and is well conserved within this species according to phenotypic and molecular characterizations (19, 20).

Other molecular methods for identifying GBS have been described (10–12). The Accuprobe system (Geno...
Probes are associated with infantile GBS diseases (3). The LightCycler (LC-32; Idaho Technology) platform used in this study allowed monitoring of only two fluorescence signals in each capillary. Therefore, a second capillary was needed to verify the presence of PCR inhibitors in the clinical samples by the use of the other pair of adjacent hybridization probes to target the internal control template. A new generation LightCycler instrument and software developed recently by Roche allow monitoring of three fluorescence signals in the same capillary, permitting the use of two pairs of adjacent probes within the same reaction vessel.

The IDI DNA extraction kit allows simple, rapid, and efficient release of GBS DNA from vaginal specimens. In fact, there was a perfect correlation between the results of direct detection of GBS by culture and by both PCR assays. Furthermore, as demonstrated by sensitivity tests with vaginal samples to which purified genomic DNA had been added, the IDI DNA extraction kit prevents significant PCR inhibition. In addition, sensitivity tests performed with GBS cultures showed that the IDI protocol assures efficient GBS cell lysis.

We have found that much higher PCR inhibition is encountered in vaginal samples from pregnant women compared with samples obtained from nonpregnant women (unpublished data). Although a limited number of specimens were tested, our results indicate that both PCR assays are suitable for GBS screening in pregnant women. A clinical study in progress (29) indicates that the IDI lysis protocol is also suitable for direct detection of GBS from vaginal/anal specimens, which is the sample type recommended by the CDC for the screening of GBS carriers (3).

Rapid and reliable detection of GBS colonization would benefit parturient mothers, especially those with poor prenatal care during pregnancy, and permit more effective prevention of GBS infections. Improved diagnostic tools for GBS, such as our PCR assay using the
LightCycler, may lead to more rational use of antibiotics. Integration of rapid sample preparation with rapid amplification and detection technologies may help to improve the management and prevention of infectious diseases because clinicians will have rapidly in hand the clinical microbiology results.

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