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

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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.**

Group B streptococci (GBS),[4] or *Streptococcus agalactiae*, have remained the leading cause of bacterial sepsis and meningitis in neonates for the last two decades *(1)*. Recently, the incidence of perinatal group B streptococcal disease in the United States has been decreasing because intrapartum antibiotic prophylaxis has been widely used for prevention of GBS diseases *(2)*. Therefore, identification of GBS-colonized women is critical for prevention of neonatal GBS infections. Currently, prenatal screening culture, including broth culture in selective medium, is the gold standard method for detection of anogenital GBS colonization *(3, 4)*. However, the culture methods require up to 48 h to yield results and predict only 87% of women likely to be colonized by GBS at delivery *(5)*. A rapid, sensitive, and specific test for detection of GBS directly from clinical specimens would allow for a simpler and more efficient prevention program.

Rapid tests have been developed, such as the rapid antigen-based tests, but these tests are neither sensitive nor specific enough to substitute for bacterial culture *(6–9)*. Hybridization-based methods have been used successfully for the detection of GBS from broth cultures *(10–12)*, but they remain insufficiently sensitive for the

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[4] 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-Atkins-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 Médicine 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

The *cfb* gene sequences available in GenBank [*Streptococcus agalactiae* (X72754), *S. uberis* (U34322), and *S. pyogenes* (AF079502)] were aligned with the GCG package (Ver. 9.0; Genetics Computer Group) to compare the homology of these *cfb* sequences. The *cfb* sequences from two GBS strains described by Podbielski et al. *(20)* were also used to identify regions conserved in GBS only. PCR primers (Table 2) complementary to these conserved regions were analyzed using the Oligo primer analysis software (Ver. 5.0; National Biosciences). Primers were synthesized with a model 391 DNA synthesizer (Perkin-Elmer).

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 *Eco*RI (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.

Gram-positive aerobic bacteria
- *Abiotrophia adiacens* ATCC 49175[a]
- *A. defectiva* ATCC 49176[a]
- *Bacillus anthracis* ATCC 4229
- *B. cereus* ATCC 14579
- *Actinomyces pyogenes* ATCC 19411
- *Corynebacterium urealyticum* ATCC 43042
- *Enterococcus avium* ATCC 14025
- *E. casseliflavus* ATCC 25788
- *E. dispar* ATCC 51266
- *E. durans* ATCC 19432
- *E. faecalis* ATCC 29212[a]
- *E. faecium* ATCC 19434[a]
- *E. flavescens* ATCC 49996
- *E. gallinarum* ATCC 49573
- *E. hirae* ATCC 8043
- *E. mundtii* ATCC 43186
- *E. raffinosus* ATCC 49427
- *Lactobacillus casei* ATCC 393
- *L. crispatus* ATCC 33820
- *L. gasseri* ATCC 33323
- *L. reuteri* ATCC 23273
- *Lactococcus lactis* ATCC 19435[a]
- *Listeria monocytogenes* ATCC 15313[a]
- *Staphylococcus aureus* ATCC 25923[a]
- *S. capitis* ATCC 27840
- *S. epidermidis* ATCC 14990[a]
- *S. haemolyticus* ATCC 29970
- *S. hominis* ATCC 27844
- *S. lugdunensis* ATCC 43809
- *S. saprophyticus* ATCC 15305
- *S. simulans* ATCC 27848
- *S. warneri* ATCC 27836
- *S. acidominimus* ATCC 51726[a]
- *S. agalactiae* ATCC 27591[a]
- *S. anginosus* ATCC 33397[a]
- *S. bovis* ATCC 33317[a]
- *S. constellatus* ATCC 27823[a]
- *S. cricetus* ATCC 19624[a]
- *S. crista* ATCC 51100[a]
- *S. downei* ATCC 33748[a]
- *S. dysgalactiae* ATCC 43078[a]
- *S. equi* ATCC 9528[a]
- *S. ferus* ATCC 33477[a]
- *S. gordonii* ATCC 10558[a]
- *S. intermedius* ATCC 27335[a]
- *S. macacae* ATCC 35911[a]
- *S. mitis* ATCC 33399[a]
- *S. mutans* ATCC 25175[a]
- *S. oralis* ATCC 35037[a]
- *S. parasanguis* ATCC 15912[a]
- *S. parauberis* ATCC 6631[a]
- *S. pneumoniae* ATCC 6303[a]
- *S. pyogenes* ATCC 19615[a]
- *S. rattus* ATCC 19645[a]
- *S. salivarius* ATCC 7073[a]
- *S. sanguis* ATCC 10556[a]
- *S. sobrinus* ATCC 27352[a]
- *S. suis* ATCC 43765[a]
- *S. uberis* ATCC 19436[a]
- *S. vestibularis* ATCC 49124[a]

Gram-positive anaerobic bacteria[b]
- *Bifidobacterium breve* ATCC 15700[a]
- *Clostridium difficile* ATCC 9689[a]
- *Mobiluncus curtisii* ATCC 35242[a]
- *Peptococcus niger* ATCC 27731
- *Peptostreptococcus anaerobius* ATCC 27337
- *P. asaccharolyticus* ATCC 14963
- *P. lactolyticus* ATCC 51172[a]
- *P. magnus* ATCC 15794
- *P. prevotii* ATCC 9321
- *P. tetradius* ATCC 35098[a]
- *Propionibacterium acnes* ATCC 6919[a]

Gram-negative aerobic bacteria
- *Acinetobacter baumannii* ATCC 19606
- *A. haemolyticus* ATCC 17906
- *Bordetella pertussis* ATCC 9797
- *Bulkholderia cepacia* ATCC 25416
- *Citrobacter diversus* ATCC 27028
- *C. freundii* ATCC 8090
- *Enterobacter aerogenes* ATCC 13048
- *E. agglomerans* ATCC 27155
- *E. cloacae* ATCC 13047
- *E. coli* ATCC 25922[a]
- *G. vaginalis* ATCC 14018
- *Haemophilus ducreyi* ATCC 33940
- *H. haemolyticus* ATCC 33390
- *H. influenzae* ATCC 9007
- *H. parahaemolyticus* ATCC 10014
- *H. parainfluenzae* ATCC 7901
- *Hafnia alvei* ATCC 13337
- *Kingella indologenes* ATCC 25869
- *Klebsiella oxytoca* ATCC 13182
- *K. pneumoniae* ATCC 13883[a]
- *Moraxella atlantae* ATCC 29525
- *M. catarrhalis* ATCC 25240
- *M. osloensis* ATCC 19976
- *Morganella morganii* ATCC 25830
- *Neisseria caviae* ATCC 14659
- *N. elongata* ATCC 25295
- *N. gonorrhoeae* ATCC 35201
- *N. meningitidis* ATCC 13077
- *N. mucosa* ATCC 19696
- *Pasteurella aerogenes* ATCC 27883
- *Proteus mirabilis* ATCC 25933[a]
- *P. vulgaris* ATCC 13315
- *Providencia alcalifaciens* ATCC 9886
- *P. rettgeri* ATCC 9250
- *P. rustigianii* ATCC 33673
- *P. stuartii* ATCC 29914
- *Pseudomonas aeruginosa* ATCC 27853
- *P. fluorescens* ATCC 13525
- *P. stutzeri* ATCC 17588
- *Salmonella typhimurium* ATCC 14028
- *Serratia marcescens* ATCC 8100
- *Shigella flexneri* ATCC 12022
- *S. sonnei* ATCC 29930
- *Stenotrophomonas maltophilia* ATCC 13843
- *Yersinia enterocolitica* ATCC 9610

Gram-negative anaerobic bacteria[b]
- *Bacteroides fragilis* ATCC 25285[a]
- *Fusobacterium nucleatum* ATCC 10953[a]
- *Porphyromonas asaccharolytica* ATCC 25260[a]
- *Prevotella corporis* ATCC 35547
- *P. melaninogenica* ATCC 25845[a]
- *P. oris* ATCC 33573
- *P. oulorum* ATCC 43324
- *Veillonella muleris* ATCC 35243[a]

Fungi
- *C. albicans* ATCC 10231[a]
- *C. krusei* ATCC 34135

[a] Also tested with the real-time PCR assay.

[b] The anaerobic species tested are found in the vaginal or anal flora.

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 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-negative vaginal samples prepared for PCR using the IDI extraction kit were supplemented with various amounts of purified GBS genomic DNA (equivalent of 1–40 genome copies).

evaluation of sample preparation for pcr

The efficacy of the IDI DNA extraction kit to lyse GBS cells 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 (Culturette™; Beckon Dickinson) after excessive discharge was removed. The samples were obtained either before or after rupture of amniotic membranes. Ampoules containing 0.5 mL of Stuart's bacterial transport medium were crushed in the swab immediately after collection of samples. The swabs were transported at ambient temperature to the Centre de Recherche en Infectiologie de l'Université Laval and were tested within 24 h of collection. Upon receipt, the swabs were immersed in tubes prefilled with 1 mL of selective Todd-Hewitt broth containing nalidixic acid (15 mg/L) and gentamicin (8 mg/L; GNS broth) for at least 3 min. Approximately 0.5 mL of the specimen suspension and the tip of the swab were added to GNS broth for identification of GBS by the standard culture method recommended by the CDC *(3)*. Vaginal swabs were prepared for PCR amplification by using the IDI DNA extraction kit.

Table 2. Oligonucleotides used in this study.

Oligonucleotides	Nucleotide sequences
GBS-specific primers[a]	
Sag59	5′-TTTCACCAGCTGTATTAGAAGTA-3′
Sag190	5′-GTTCCCTGAACATTATCTTTGAT-3′
Adjacent hybridization probes specific for the GBS amplicon	
STB-F	5′-AAGCCCAGCAAATGGCTCAAA-fluorescein-3′
STB-C	5′-Cy5-GCTTGATCAAGATAGCATTCAGTTGA-phosphate-3′
Adjacent hybridization probes specific for the internal control amplicon	
IC-F	5′-TTATTGCAGCTTCGCCACAGGAA-fluorescein-3′
IC-C	5′-Cy5-GGTCCAGCAATGTGAAGAGGCAT-phosphate-3′

[a] The 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.

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

	Conventional PCR	Real-time PCR
Thermocycler	PTC-200 DNA Engine (MJ research)	LC-32 LightCycler (Idaho Technology)
Heating/cooling rate	3 °C/s	20 °C/s
Reaction vessel	0.2-mL plastic tube	Glass capillary
Reaction volume	20 μL	7 μL
Use of internal probes	No	Yes
Sample volume	2 μL	0.7 μL
Internal control	Coamplified	Amplified separately
PCR Master Mix	0.4 μmol/L primers	0.4 μmol/L primers
	200 μmol/L dNTP	200 μmol/L dNTP
	10 mmol/L Tris-HCl	10 mmol/L Tris-HCl
	2.5 mmol/L $MgCl_2$	3.5 mmol/L $MgCl_2$
	3.3 g/L BSA[a]	0.45 g/L BSA
	10 copies of pSTB	70 copies of pSTB[b]
	0.5 U of *Taq* polymerase[c]	0.5 U of Klentaq1[c]
	50 mmol/L KCl	16 mmol/L $(NH_4)_2SO_4$
		0.2 μmol/L fluorogenic probes
PCR program	Hold: 3 min/94 °C	Hold: 3 min/94 °C
	40 cycles: 1 s/95 °C	45 cycles: 0 s/95 °C
	30 s/55 °C	14 s/55 °C
	Hold: 2 min/72 °C	5 s/72 °C
Maximum reactions/run	96	32
Duration of amplification	65 min	35 min
Detection	Gel electrophoresis (25 min)	Fluorescence monitoring (real-time)

[a] BSA, bovine serum albumin.

[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.

[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.

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 *cfb* genes from GBS, *S. uberis*, and *S. pyogenes* (data not shown). Sequence comparison of streptococcal *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 DNAs 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 *E. coli*, *Candida albicans*, *Gardnerella vaginalis*, enterococci, coagulase-negative staphylococci, *Lactobacillus* spp., *Peptostreptococcus* spp., and *Bacteriodes* 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

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 performed in a separate capillary was detected for all PCR reactions, thereby showing the absence of significant PCR inhibition. As with the conventional PCR assay, the internal control signal progressively decreased as the amount of 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)*.

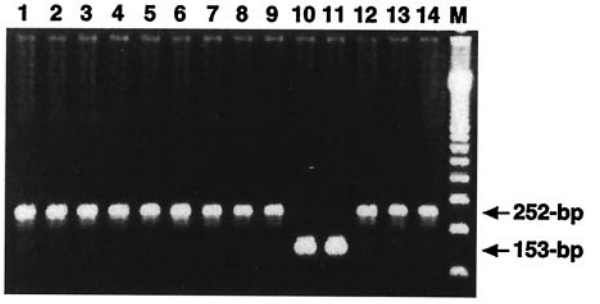

Fig. 1. Conventional multiplex PCR amplification with the GBS-specific PCR assay (*153-bp amplicon*) and the internal control (*252-bp amplicon*).

PCR assays were all performed with 1 μL of purified genomic DNA (0.1 ng/μL) from various bacteria. Note that the internal control was not amplified when target DNA was present because of competitive inhibition by amplification of GBS DNA. *Lane 2*, *S. aureus* ATCC 25923; *lane 3*, *S. epidermidis* ATCC 14990; *lane 4*, *E. faecalis* ATCC 19433; *lane 5*, *E. faecium* ATCC 19434; *lane 6*, *S. pneumoniae* ATCC 6303; *lane 7*, *S. pyogenes* ATCC 19615; *lane 8*, *S. uberis* ATCC 49124; *lane 9*, *L. reuteri* ATCC 23273; *lane 10*, GBS ATCC 13813; *lane 11*, GBS ATCC 27591; *lane 12*, *G. vaginalis* ATCC 14018; *lane 13*, *E. coli* ATCC 25922. *Lanes 1* and *14*, controls to which no DNA was added; *lane M*, 100-bp molecular size ladder.

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 (Gen-

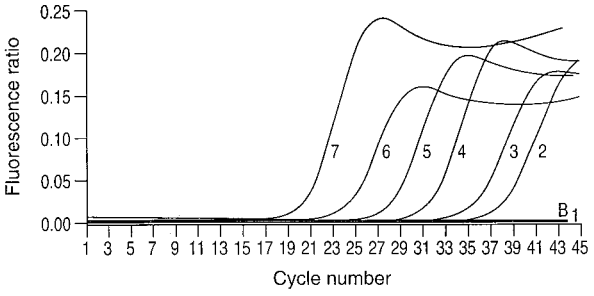

Fig. 2. Real-time PCR detection using the LightCycler.

Fluorescence ratio (Cy5/fluorescein) vs time plot for the GBS-specific PCR amplification using adjacent hybridization probes. Amplifications were performed from 0.5 fg (*curve 1*), 5 fg (*curve 2*), 50 fg (*curve 3*), 500 fg (*curve 4*), 5 pg (*curve 5*), 50 pg (*curve 6*), and 500 pg (*curve 7*) of genomic DNA from GBS ATCC 27591. *Curve B* represents a blank to which no DNA was added. Each amplification cycle was 35 s long on average, and 45 cycles were completed in 30 min. Based on the genome sizes of *S. pneumoniae* and *S. pyogenes*, 5 fg of GBS genomic DNA corresponds to two to three genome copies.

Probe) is suitable for the identification of GBS from cultures but is insufficiently sensitive for direct detection from the clinical specimens described *(10, 12)*. PCR has become one of the most widely used methods in molecular diagnosis. However, little work has been done on detection of GBS. A PCR assay for detection of the C-protein gene in GBS has been developed. However, this assay allowed the detection of only 63% of the GBS strains tested and therefore is not suitable for screening GBS colonization or infection *(15)*. Two nested PCR assays have been reported to detect GBS by amplifying the 16S rRNA gene *(13)* or the 16S-23S spacer region *(14)*. These assays allowed detection, from cerebrospinal fluid samples, of ~100 and 5 CFUs of GBS, respectively. However, nested PCR protocols often are limited by the increased risk of PCR carryover.

Rapid thermal cycling for PCR amplification coupled with real-time fluorescence monitoring of the PCR reactions *(17, 18)* provides a new tool for rapid detection of microorganisms. The detection of PCR products is achieved by monitoring the change of fluorescence on the basis of fluorescence resonance energy transfer when the two hybridization probes anneal to the target DNA (GBS-specific amplicons in this study) in close proximity. Using this technology, Woo et al. *(28)* detected *Leptospira* genospecies DNA with 45-cycle amplification requiring only 18 min. Because amplification and detection occur in the same reaction vessel and no postamplification sample transfer is needed, the LightCycler platform greatly reduces the risk of carryover, making it more suitable for use in the routine clinical laboratory than conventional heating-block thermocyclers coupled with agarose gel electrophoresis.

To adapt our PCR assay to the LightCycler platform, two fluorescently labeled adjacent hybridization probes complementary to part of the amplicon were used for specific hybridization to the GBS-specific amplification product. Analysis of 14 *cfb* sequences from Podbielski et al. *(20)* and from our own laboratory (data not shown), representing the major serotypes of GBS, indicates that the target sequences for the adjacent probes are highly conserved. This ensures the ability of the two adjacent probes to hybridize to all GBS-specific amplicons. The additional specificity provided by the combination of species-specific primers with internal probes ensures direct detection and identification of GBS from anovaginal specimens in which the typical vaginal and anal floras are microbiologically extremely complex and not well known *(23)*. The PCR assay with the LightCycler constantly detected as few as one to three genome copies as well as a comparable number of CFUs. Such sensitivity is suitable for direct detection of GBS from clinical specimens. This real-time PCR assay is able to quantify the GBS load of vaginal specimens by comparison to calibration curves with known amounts of GBS DNA or cells. However, no attempt to determine the degree of GBS colonization was made because both heavy and light colonization in mothers 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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