Real-time PCR targeting the *sip* gene for detection of group B streptococcus colonization in pregnant women at delivery

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Group B streptococcus (GBS) is an important aetiological agent of serious neonatal infections. A rapid and sensitive method for the detection of GBS colonization in pregnant women at delivery could make intrapartum screening for GBS possible. A real-time PCR method targeting the *sip* gene of GBS in pregnant women at delivery has been evaluated. The performance of the real-time PCR was compared with optimized GBS culture. Separate vaginal and rectal swabs were collected from women hospitalized at the delivery department at St Olavs Hospital, Trondheim, Norway, from January 15 through May 2005. The specimens were cultured on selective blood agar plates and in selective broth and examined by real-time PCR. Of samples from 251 women, 87 (34.7 %) were GBS positive by culture and 86 (34.3 %) were positive by PCR. Using GBS culture as the 'gold standard', the sensitivity of real-time PCR was 0.97 (95 % confidence interval 0.90–0.99) and specificity was 0.99 (95 % confidence interval 0.97–1.00). In two women the PCR was positive and the culture negative. Additional analysis using *cy/E* PCR substantiates that these two women were true GBS carriers with negative GBS culture. The rate of GBS colonization was lower in vaginal specimens than in rectal specimens both by culture and PCR. The real-time PCR assay is fast, highly sensitive and specific for detecting GBS colonization in pregnant women at delivery, and has the potential for intrapartum detection of GBS colonization. Both vaginal and rectal samples are required to achieve highest possible detection rate.

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INTRODUCTION

Group B streptococcus (GBS) is an important cause of morbidity and mortality in newborns. The incidence of neonatal GBS infection ranges from 0.5 to > 2 per 1000 live births in different geographical areas (Lyytikäinen *et al.*, 2003; Persson *et al.*, 2004; Schrag *et al.*, 2002; Zangwill *et al.*, 1992). GBS neonatal disease is classified as either early-onset disease (< 7 days) or late-onset disease (> 7–90 days). Early onset disease accounts for 70–80 % of the cases.

GBS colonization in pregnant women is the single most important risk factor for early-onset newborn disease due to vertical transmission and colonization of the infant during delivery. Intrapartum antibiotic treatment lowers the incidence of early onset disease (Schrag *et al.*, 2002). There are two main strategies for prevention of vertical transmission of GBS from colonized women to the neonates, either screening all pregnant women, or assessment of the

risk factors for GBS. Screening of pregnant women for GBS at 35–37 weeks of gestation is recommended by the Centers for Disease Control and Prevention (CDC) in the US (Schrag *et al.*, 2002). Women colonized with GBS are offered antimicrobial prophylaxis intrapartum (after onset of labour or after rupture of membranes). As a result of the screening protocol, up to 30 % of pregnant women are given antibiotics at the time of delivery in the US. Most western European countries do not use a screening-based protocol, but offer antibiotics to women in cases with risk factors for neonatal group B streptococcal infection, including delivery at < 37 weeks of gestation, intrapartum fever, rupture of membranes > 18 h, a previous child with GBS disease and GBS urinary tract infection during pregnancy.

Some women are intermittent carriers of GBS, and the rate of GBS colonization may vary during pregnancy (Boyer *et al.*, 1983; Edwards *et al.*, 2002; Hansen *et al.*, 2004; Yancey *et al.*, 1996). Studies have shown that the predictive value of antenatal screening decreases significantly if it is performed more than a few weeks before delivery (Yancey *et al.*, 1996). Because of this fluctuation in colonization rate, intrapartum

Abbreviations: CI, confidence interval; C_t, cycle threshold; GBS, group B streptococcus.

screening of pregnant women for GBS would be preferable. However, since culture takes 24 to 72 h, this method would be of limited value as guidance for antimicrobial prophylaxis when specimens for culture are collected at delivery. A rapid test that could accurately detect GBS carriage at the time of labour may enhance the precision of such screening.

We have previously established a real-time PCR targeting the *sip* gene universally present across all serotypes of GBS. This assay is very sensitive, i.e. detection limit ≈ 1 c.f.u. per PCR reaction, and specific (Bergh *et al.*, 2004). In this study the performance of the real-time PCR on specimens from pregnant women at term was analysed and compared with optimized GBS culture.

METHODS

Design. A prospective study was conducted at St Olavs Hospital from January 15 through May 2005. Pregnant women were eligible if they presented to the delivery ward in labour after a minimum of 36 weeks gestation with no contraindication to vaginal examination, had not used systemic or topical (vaginal) antibiotic treatment in the week prior to admission, and did not need to proceed immediately to delivery.

Ethics. The study was approved by the Regional Committee for Medical Research Ethics, Norwegian Social Science Data Services and Directorate for Health and Social Affairs. A written consent for participation in the study was obtained from each woman.

Collection of specimens. Swabs (Transwab in Amies medium with charcoal; Medical Wire and Equipment) from lower vagina and rectum were collected and analysed separately. Specimens were taken either by the women themselves or by a midwife, and were stored at 4 °C for 1–48 h until further examination.

Culture of GBS. In the laboratory the same specimen was used for optimized GBS culture and for PCR. Each swab was twisted and twirled in 0.55 ml sterile saline; 50 μ l was seeded on selective blood agar (Columbia CNA agar with 10 mg nalidixic acid l^{-1} and 10 mg colistin l^{-1}) with 5% human blood, and 50 μ l was inoculated in selective GBS broth (Todd–Hewitt broth with 15 mg nalidixic acid l^{-1} and 8 mg gentamicin l^{-1}). The selective blood agar plates were incubated for 48 h and the broth was subcultured onto non-selective blood agar plates after 24 h of incubation. Blood agar plates were examined after 24 and 48 h and β -haemolytic and non-haemolytic, pyrrolidonyl arylamidase-negative colonies were identified as GBS using a commercial latex agglutination test (Pastorex Strep; Bio-Rad). Growth was semi-quantified as abundant/moderate (≥ 10 GBS colonies per plate), sparse (1–10 GBS colonies per plate) and growth only after enrichment in selective GBS broth.

PCR analyses. The sequences of primers and probes are shown in Table 1. For nucleic acid extraction, 300 μ l suspension was added to an equal volume of lysis solution [containing 15 μ l lysozyme (Sigma; 20 mg ml^{-1}), 6 μ l proteinase K (Sigma; 20 mg ml^{-1}), 6 μ l Mutanolysin (Sigma, 10 000 U ml^{-1}), 273 μ l TE buffer], and incubated for 15 min at 37 °C and 15 min at 65 °C. DNA was purified using DNeasy Tissue kit (Qiagen) and eluted in a volume of 100 μ l. A 2 μ l aliquot of the purified DNA solution was used as a template for PCR.

The *sip* gene encoding the Sip surface immunogenic protein was chosen as the target, with specific primers and TaqMan probe as previously described (Bergh *et al.*, 2004) (Table 1). The annealing temperature was raised from 50 °C in the original assay to 58 °C to ensure specificity without reduced sensitivity. Real-time PCR was performed using a LightCycler (Roche). These PCR conditions were applied: 0.5 μ M each primer, 0.2 μ M probe, 5 mM $MgCl_2$ and $1 \times$ LightCycler Fast-Start reaction mix HybProbe (Roche). A cycle threshold (C_t) value of ≤ 40 was used as the cut-off for positive fluorescence detection signal of target amplification. Two positive controls and one negative control were included in all runs. Purified DNA from a known GBS strain (12312/00) was used as positive control in two dilutions corresponding

Table 1. Sequences of primers and probes, and cycling conditions for different PCR assays

PCR-target	GenBank accession no.	Sequences of primers and probe (5'→3')	Cycling conditions	Reference
<i>sip</i>	AF 15135	<p><u>Primer 1:</u> ATC CTG AGA CAA CAC TGA CA (position 263–282)</p> <p><u>Primer 2:</u> TTG CTG GTG TTT CTA TTT TCA (position 340–320)</p> <p>TaqMan probe: 6-FAM–ATC AGA AGA GTC ATA CTG CCA CTT C–TAMRA (position 293–317)</p>	95 °C (5 min); 50 cycles of 96 °C (5 s), 58 °C (10 s) and 72 °C (20 s)	Bergh <i>et al.</i> (2004)
<i>cylE</i>	AF 157015	<p><u>Primer 1:</u> TGA CAT TTA CAA GTG ACG AAG (position 1541–1561)</p> <p><u>Primer 2:</u> TTG CCA GGA GGA GAA TAG GA (position 1808–1789)</p>	95 °C (10 min); 35 cycles of 95 °C (60 s), 55 °C (60 s) and 72 °C (120 s); final extension 72 °C (7 min)	This study
Human DNA	AL 133466	<p><u>Primer 1:</u> GTC ATA GGT AGT TGT GGT CG (position 10 4242–10 4223)</p> <p><u>Primer 2:</u> CAT AGA CTC ACC CTA GCA TGG (position 10 4006–10 4026)</p> <p>TaqMan probe: FAM–CAC TGA GCC TCT CTC TAT CC–TAMRA (position 10 4090–10 4071)</p>	95 °C (5 min); 45 cycles of 95 °C (5 s), 55 °C (10 s) and 72 °C (20 s)	This study

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine.

Table 2. Prevalence of GBS colonization in 251 pregnant women at delivery detected by optimized culture and real-time PCR

	Culture positive	PCR positive
Vaginal swabs	61 (24.3 %)	52 (20.7 %)
Rectal swabs	78 (31.1 %)	75 (29.9 %)
Vaginal and/or rectal swabs	87 (34.7 %)	86 (34.3 %)

to 10^4 and 10^2 bacterial genomes. As a negative control, sterile water was added instead of DNA template. Analysing the specimens with real-time PCR, including time for DNA extraction, required less than 2 h.

To control the specificity of the PCR method, selected specimens were analysed with a GBS-specific conventional PCR, targeting the *cylE* gene. The primer sequences are shown in Table 1. Amplification was performed in $1 \times$ PCR buffer II (Roche), 2.0 mM $MgCl_2$, 0.05 mM dNTP, 200 ng each primer, 1 U AmpliTaq Gold and dH_2O (2 μ l template in a final volume of 50 μ l), using a GeneAmp 2400 (Perkin Elmer). The PCR products were analysed and visualized by capillary electrophoresis (Agilent 2100 bioanalyser).

All PCR positive samples that were culture negative, were post-PCR analysed with capillary electrophoresis (Agilent 2100 bioanalyser) to ensure a correct amplicon size of the *sip* real-time PCR product. In addition, these specimens were analysed by PCR targeting the *cylE* gene. GBS isolates from specimens that were *sip*-gene PCR negative were recultured, and all isolates were confirmed as GBS phenotypically. PCR was carried out on these recultured strains and all were PCR positive, demonstrating the presence of the *sip* gene in all GBS strains.

In order to control for inhibition of PCR in clinical specimens a real-time PCR targeting human DNA was established. A sequence of DNA on chromosome 20 was chosen as target (Table 1). PCR conditions were similar to those described for the real-time PCR targeting the *sip* gene. In 49 samples from 252 women, human DNA could not be detected by the PCR. These samples were 'spiked' with GBS (the positive control) and the real-time PCR targeting the *sip* gene was run. All, but one of these samples were PCR positive, ensuring that inhibitors to PCR were not present. The negative sample was excluded from the study.

RESULTS AND DISCUSSION

Of 274 women asked to participate in the study, 252 gave their informed consent. One patient was excluded from the study because of PCR inhibition in her rectal specimen. Of the 251 women included, 87 (34.7 %) were identified as GBS

carriers based upon the culture results of rectal and/or vaginal swabs, while 86 (34.3 %) were identified as GBS carriers according to the PCR results (Table 2).

In this study the PCR results correlate well with the results of culture for detection of GBS in pregnant women, with a sensitivity of 0.97 [95 % confidence interval (CI) 0.90–0.99] and a specificity of 0.99 (95 % CI 0.96–1.00), when using culture as 'gold standard'. Five published studies, using real-time PCR assays in pregnant women, targeting the *cfb* gene or *ptsI* gene in GBS, and different methods for nucleic acid extraction, have demonstrated sensitivities from 0.45 to 1.0 compared to enrichment broth culture (Bergeron *et al.*, 2000; Davies *et al.*, 2004; Reglier-Poupert *et al.*, 2005; Uhl *et al.*, 2005; Chan *et al.*, 2006). Four of these studies have employed primers and probes targeting the *cfb* gene as described by Ke *et al.* (2000). Also, an assay based upon these nucleotide sequences has been made commercially available. In a multicenter study the mean sensitivity was 0.94, ranging from 0.85 to 0.99 at the five participating centres (Davies *et al.*, 2004). In the latter study 27 positive PCR results were categorized as false positive.

The *sip* PCR appeared somewhat less sensitive when analysing vaginal and rectal specimens separately. It more often failed to detect GBS colonization in vaginal swabs than in rectal swabs. In rectal specimens the PCR detected 71 of 78 GBS culture-positive specimens with a sensitivity of 0.91 (95 % CI 0.84–0.97) (Table 3, Fig. 1). In vaginal specimens the sensitivity of PCR was only 0.80 (95 % CI 0.69–0.89) (Table 3, Fig. 1). In three women the PCR was negative while GBS cultures were positive (Table 4). In these, growth was moderate, sparse and after enrichment, respectively (Table 4). Inhibition of PCR was not demonstrated either in the vaginal specimens or in the rectal specimens and could not explain the lower detection rate. It is important to recognize that from methodological reasons culture was favoured by a factor of ≈ 8 when comparing the original volume made accessible for culture versus PCR. Therefore, the overall sensitivity of the present real-time PCR must be considered very high.

In two women the PCR was positive and the culture was negative (Table 4). Additional tests, including electrophoresis of the *sip* real-time PCR product and conventional PCR targeting the *cylE* gene, demonstrated PCR products consistent with GBS. We therefore consider these two

Table 3. Comparison between optimized culture and real-time PCR for detection of GBS in separate vaginal and rectal swabs in 251 pregnant women at delivery

	Rectal swabs			Vaginal swabs		
	Culture positive	Culture negative	Total	Culture positive	Culture negative	Total
PCR positive	71	4	75	49	3	52
PCR negative	7	169	176	12	187	199
Total	78	173	251	61	190	251

Fig. 1. Detection of GBS colonization analysed by culture and PCR. The Venn diagram shows concordance and discrepancy between PCR and culture in vagina and rectal specimens, respectively.

women as true GBS carriers in whom culture failed to detect GBS.

DNA extraction from GBS is notoriously difficult. Of the published studies employing *cfb* real-time PCR the low sensitivity of 0.45 reported by Chan *et al.* (2006) is attributed to problems relating to the DNA extraction method. The other publications have employed commercial reagents. Prior to the establishment of the *sip* real-time PCR (Bergh *et al.*, 2004) great emphasis was a placed upon the optimization of a robust protocol for bacterial cell lysis. This protocol is considered essential for the high sensitivity achieved in the present study.

In addition to being more rapid than a conventional PCR, the real-time PCR allows a semi-quantification of the amount of specific DNA in the template through determination of the C_t . The median C_t value and the range of C_t values of the real-time PCR were compared with the semi-quantified growth of bacteria. As expected the median C_t in specimens with abundant/moderate growth was lower than in specimens with sparse growth and growth after enrichment. Surprisingly the median C_t was higher in specimens with sparse growth than in those of growth after enrichment (Fig. 2).

Fig. 2. Representative amplification curves in real-time PCR of templates containing GBS DNA in various concentrations. 1, Positive control (10^4 genomes); 2–4, samples with abundant/moderate, sparse growth and growth after enrichment only; 5, negative control. The arrows indicate range ($\leftrightarrow$) and median (vertical line) of C_t for the specimens investigated and correlation with growth by culture: A, positive controls (median C_t 30.8, range 30.8–30.88); B, abundant/moderate growth (median C_t 34.0, range 23.0–38.0); C, sparse growth (median C_t 38.0, range 30.0–40.0); D, growth after enrichment only (median C_t 36.0, range 31.0–39.0).

The GBS carriage of almost 35 % in the present study is higher than reported in the majority of published studies, ranging between 10 and 29 % (Boyer *et al.*, 1983; Brimil *et al.*, 2006; Lyytikäinen *et al.*, 2003; Schrag *et al.*, 2002; Valkenburg-van den Berg *et al.*, 2006; Werawatakul *et al.*, 2001; Yancey *et al.*, 1996). However, a similar prevalence of GBS colonization is described in a Danish study from 2000 (Hansen *et al.*, 2004) and a Canadian study from 2004 (Rallu *et al.*, 2006). The different prevalence rates may be explained by gestational age at culturing, differences in culture sites and culture techniques, a change of prevalence with time, or real differences of prevalence in different populations or ethnic groups (Valkenburg-van den Berg *et al.*, 2006). There may be a higher bacterial load in separate specimens compared to combined specimens, and collecting, processing and analysing vaginal and rectal specimens separately, as in this study, may lead to detection of more GBS colonized women.

Table 4. Comparison between optimized culture and real-time PCR for detection of GBS in vaginal and/or rectal swabs in 251 pregnant women at delivery

	Culture positive			Culture negative	Total
	Abundant/moderate growth	Sparse growth	Growth after enrichment		
PCR positive	57	14	13	2	86
PCR negative	1	1	1	162	165
Total	58	15	14	164	251

The pattern of GBS colonization as detected by vaginal and rectal sampling is complex. By analysing the vaginal and rectal swabs separately, we found that culture of vaginal swabs detected less GBS colonized women than rectal swabs (Table 2). The difference between vaginal and rectal carriage was also shown in previous studies, and suggests that the gastrointestinal tract is the primary reservoir of GBS, and that vaginal colonization represents dissemination from this source (Badri *et al.*, 1977; Philipson *et al.*, 1995; Schrag *et al.*, 2002; Valkenburg-van den Berg *et al.*, 2006). A previous study has shown that combined rectal and vaginal specimens detected approximately the same number of GBS colonized women as separate rectal specimens only (Bergeron *et al.*, 2000). However, in our study nine women had a positive vaginal culture and a negative rectal culture (Fig. 1). The complex pattern of detection of GBS when both culture and PCR are employed on separate vaginal and rectal swabs is shown in Fig. 1. Our findings clearly show that both vaginal and rectal specimens should be examined to demonstrate the true prevalence rate of GBS colonization in pregnant women at term.

Conclusion

The real-time *sip* PCR described is a fast, very sensitive and specific method for detection of GBS colonization in pregnant women at delivery. The assay has the potential for intrapartum detection of GBS colonization. Both vaginal and rectal specimens are required to ensure the highest possible GBS detection rate.

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