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 cylE 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.

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
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 μl 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 μl 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; BioRad). 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 μl suspension was added to an equal volume of lysis solution [containing 15 μl lysozyme (Sigma; 20 mg ml⁻¹), 6 μl proteinase K (Sigma; 20 mg ml⁻¹), 6 μl Mutanolysin (Sigma, 10,000 U ml⁻¹), 273 μl 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 μl. A 2 μl 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 μM each primer, 0.2 μM probe, 5 mM MgCl₂ and 1× LightCycler Fast-Start reaction mix HybProbe (Roche). A cycle threshold (Ct) 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

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

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

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Culture positive</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaginal swabs</td>
<td>61 (24.3%)</td>
<td>52 (20.7%)</td>
</tr>
<tr>
<td>Rectal swabs</td>
<td>78 (31.1%)</td>
<td>75 (29.9%)</td>
</tr>
<tr>
<td>Vaginal and/or rectal swabs</td>
<td>87 (34.7%)</td>
<td>86 (34.3%)</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Culture positive</th>
<th>PCR positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectal</td>
<td>Culture positive</td>
<td>71</td>
</tr>
<tr>
<td></td>
<td>Culture negative</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>240</td>
</tr>
<tr>
<td>Vaginal</td>
<td>Culture positive</td>
<td>49</td>
</tr>
<tr>
<td></td>
<td>Culture negative</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>216</td>
</tr>
</tbody>
</table>

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$_2$O (2 $\mu$L template in a final volume of 50 $\mu$L), using a GeneAmp 2400 (Perkin Elmer). The PCR products were analysed and visualized by capillary electrophoresis (Agilent 2100 bioanalyzer).

All PCR positive samples that were culture negative, were post-PCR analysed with capillary electrophoresis (Agilent 2100 bioanalyzer) 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 psl 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-Poupet 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 $\approx$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
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 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).

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; Lytykäinen 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

<table>
<thead>
<tr>
<th>Culture positive</th>
<th>Culture negative</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Abundant/moderate growth</td>
<td>Sparse growth</td>
</tr>
<tr>
<td>PCR negative</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>58</td>
<td>15</td>
</tr>
</tbody>
</table>
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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