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# Non-culture detection of *Streptococcus agalactiae* (Lancefield group B *Streptococcus*) in clinical samples by real-time PCR

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A real-time PCR assay targeting the *cylB* gene was developed to detect *Streptococcus agalactiae* [Lancefield group B *Streptococcus* (GBS)] from clinical samples. A total of 110 blood culture-negative samples [75 cerebrospinal fluid (CSF) and 35 EDTA blood samples] from neonates with probable GBS sepsis or meningitis were analysed. Among these, 16 of the 75 CSF samples were positive [21.3 %, 95 % confidence interval (CI) 12.7–32.3 %] and two of the 35 EDTA blood samples were positive (5.7 %, 95 % CI 0.7–19.2 %). The proportion testing positive in the CSF samples was significantly higher than in the EDTA blood samples ($P$=0.05, Fisher's exact test). Overall, this real-time PCR assay was shown to be superior to culture methods for detection of GBS from CSF and EDTA blood samples.

## INTRODUCTION

*Streptococcus agalactiae* [Lancefield group B *Streptococcus* (GBS)] is the leading cause of sepsis, pneumonia and meningitis in neonates (Trijbels-Smeulders *et al.*, 2004). GBS is a commensal of the genitourinary and gastro-intestinal tract and is carried by 20–30 % of pregnant women in the UK (Bliss *et al.*, 2002). Vertical transmission to the infant occurs in 50 % of deliveries involving colonized women, and 1–3 % of colonized neonates go on to develop invasive disease (Verani & Schrag, 2010). The incidence of early-onset disease in the world ranges from 0.5 to four cases per 1000 live births. Severe early-onset disease (occurring in the first 6 days of life) usually presents as pneumonia, septicaemia or meningitis, and has a case fatality rate of around 12.1 % (Melin, 2011; Edmond *et al.*, 2012).

Currently, GBS can be differentiated into ten distinct serotypes based on capsular polysaccharides (Ia, Ib and II–IX) (Slotved *et al.*, 2007) and their distribution varies worldwide. The serotypes that cause human infections identified frequently in many parts of the world are III, Ia, Ib, II and V (Dogan *et al.*, 2005; Ippolito *et al.*, 2010; Madzivhandila *et al.*, 2011; Edmond *et al.*, 2012). Neonatal infections are most commonly caused by serotypes Ia, III and V (Pettersson, 2007).

Studies on clonal relatedness and the genetic population structures of GBS by multilocus enzyme electrophoresis have demonstrated two evolutionary clusters among serotype III (Musser *et al.*, 1989). Subsequently, multilocus sequence typing has classified GBS strains into several clones or sequence types (Jones *et al.*, 2003). Certain sequence types group together to form clusters or clonal complexes (CCs). The two clusters among serotype III confirmed by Musser *et al.* (1989) have been assigned to CC17 and CC19 by multilocus sequence typing. The CC17 strains of serotype III have been associated with neonatal disease in several populations and may have an enhanced ability to cause disease (Jones *et al.*, 2003, 2006; Bisharat *et al.*, 2005; Luan *et al.*, 2005; Lin *et al.*, 2006; Bohnsack *et al.*, 2008).

Diagnosis of GBS infection in the newborn by conventional culture methods is time-consuming and unreliable. In the absence of an available vaccine against GBS, rapid, sensitive and specific detection of the organism is crucial for effective management of disease and for reducing the risk of mortality, particularly in the newborn.

Abbreviations: CSF, cerebrospinal fluid; GBS, group B *Streptococcus*; IPC, internal positive control.


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In comparison with culture methods, PCR offers a vast reduction in time and has been described previously for the detection of GBS from maternal swabs. Several target genes (*cfb*, *sip* and *ssrA*) have been used (Ke *et al.*, 2000; Bergh *et al.*, 2004; Wernecke *et al.*, 2009) and several commercial assays have also been reported (Atkins *et al.*, 2006; Edwards *et al.*, 2008; Riedlinger *et al.*, 2010).

GBS haemolysin activity is regarded as an important virulence factor in the pathogenesis of neonatal infections. Expression of haemolysin is dependent on the *cyl* gene cluster (Spellerberg *et al.*, 1999), and the genes *cylA* and *cylB* encode the ATP-binding domain and the transmembrane protein of an ABC transporter, respectively. We have reported here a real-time PCR assay targeting the *cylB* gene region of the *cyl* locus for detection of GBS in clinical samples from neonates. A Roche LightCycler platform and fluorescence resonance energy transfer probes were used, and an internal processing control was included in the assay to highlight amplification failure of the PCR due to inhibition.

## METHODS

**Bacterial cultures.** *S. agalactiae* strain NCTC 11360 was used as a positive control for the assay. A panel of *S. agalactiae* NCTC strains and 50 clinical GBS isolates (epidemiologically unrelated) referred to the World Health Organization Streptococcus and Diphtheria Reference Unit representing the nine *S. agalactiae* capsular types were used to ensure that the assay detected all *S. agalactiae* capsular types (Tables 1 and 2). A specificity panel comprised 58 isolates (21 species of streptococci and 37 other bacterial species – common pathogens associated with sepsis) (Table 3).

**Study clinical samples.** A total of 110 blood culture-negative samples [35 EDTA blood samples and 75 cerebrospinal fluid (CSF) samples] from neonates with probable GBS sepsis or meningitis submitted to the World Health Organization Streptococcus and Diphtheria Reference Unit between 2006 and 2010 were analysed. DNA from a culture-confirmed and PCR-positive EDTA blood sample submitted for a separate study was also used as a positive control.

**Table 1.** NCTC strains representing nine capsular types

| Reference strain | Capsular type |
|---|---|
| NCTC 11360 | Ia |
| NCTC 9993 | Ia |
| NCTC 8187 | Ib |
| NCTC 11078 | Ia/c |
| NCTC 11079 | II |
| NCTC 11080 | III |
| R80/405 | IV |
| NCTC 11930 | IV |
| PRAGUE 1984 | V |
| NT 6 | VI |
| NCTC 7271 | VII |
| M9 | VIII |
| NCTC 9829 | V |

**Table 2.** Fifty clinical isolates representing seven *S. agalactiae* capsular types

| Serotype | No. of strains |
|---|---|
| Ia | 6 |
| Ia/c | 3 |
| Ib | 2 |
| Ib/c | 6 |
| II | 7 |
| II/c | 4 |
| III | 6 |
| III/X | 2 |
| IV | 2 |
| IV/c | 2 |
| V | 8 |
| VI/c | 1 |
| V/c | 1 |

**Extraction of DNA from bacterial cultures.** DNA from *S. agalactiae* strain NCTC 11360 was extracted using a Roche MagnaPure robot with a Pure LC Total Nucleic Acid Isolation kit (Roche Diagnostics) according to the manufacturer's instructions.

**Extraction of DNA from clinical specimens.** EDTA blood and CSF samples (200 µl) were extracted using a QiaAMP DNA Mini kit (Qiagen) following the manufacturer's instructions. The samples were eluted in 50 µl nuclease-free water (Sigma-Aldrich).

**Primers and probes.** A bioinformatics approach was used to try and rationally select genes specific to GBS. A script was designed to process each gene in one of the complete GBS genomes available in GenBank and search using BLASTN for the presence of a homologue (90 % cut-off) in each of the other available complete GBS genome sequences. Subsequently, each gene was used as a query in a BLASTN search against all the non-GBS genomes available. A putative GBS-specific gene was defined as that which had a homologue in all GBS genomes and no match in the non-GBS genomes and whose sequence length was >800 bp. This resulted in 210 putative GBS-specific genes. These candidates were then used as query sequences against the non-redundant GenBank database. Any genes that resulted in significant hits against any sequence other than GBS sequences were removed from the list. This process reduced the number of candidates to 162. A process of rational selection was used to identify five novel gene targets for evaluation by PCR: *arcD* (encoding an arginine/ornithine antiporter); *cpsL* capsule (encoding a capsular polysaccharide repeating unit transporter); *cylB* (encoding $\beta$-haemolysin and pigment production); *neuB* (encoding *N*-acetylneuramic acid synthetase); and *cfb* (used in commercially available assays and encoding complement factor B). Hybridization probes were optimized for each assay and tested, and the assay targeting the *cylB* gene was selected for use in this study.

Primers specific for the *cylB* gene of the *S. agalactiae cyl* gene cluster amplified a PCR product of 263 bp. Within this sequence, two probes were designed to a 61 bp region with a 2 bp gap located proximal to the 3′ end of the sense sequence. The sequences of the primers and probes are shown in Table 4.

**Construction of *cylB* internal positive control (IPC).** Primers for the construction of an IPC contained the *cylB* primer sequences external to sequences specific for a 619 bp portion of bacteriophage $\lambda$ DNA. A 652 bp fragment was amplified using the *cylB*-IPC-F and *cylB*-IPC-R primers (Table 4). PCR cycling conditions were 30 cycles of 95 °C for 30 s, 40 °C for 1 min and 72 °C for 10 min, with a final extension step at 72 °C for 10 min. The product size was confirmed


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**Table 3.** Specificity panel used in this study

| Species | NCTC strain no. | Species | NCTC strain no. |
|---|---|---|---|
| *Enterococcus durans* | 662 | *Acinetobacter baumannii* | 10303 |
| *Streptococcus dysgalactiae* | 4669 | *Aeromonas hydrophila* | 8049 |
| *Streptococcus zooepidemicus* | 4676 | *Burkholderia cepacia* | 10734 |
| *Streptococcus* sp. group F | 5389 | *Citrobacter freundii* | 6266 |
| *Lactococcus lactis* group N | 6681 | *Enterobacter aerogenes* | 10336 |
| *Aerococcus viridans* | 7592 | *Enterobacter cloacae* | 11572 |
| *Aerococcus viridans* | 7602 | *Enterococcus faecalis* | 12201 |
| *Aerococcus viridans* | 7764 | *Klebsiella oxytoca* | 8167 |
| *Aerococcus viridans* | 7777 | *Klebsiella pneumoniae* | 9633 |
| *Streptococcus sanguinis* | 7863 | *Morganella morganii* | 235 |
| *Streptococcus gordonii* | 7865 | *Pasteurella multocida* | 10322 |
| *Streptococcus bovis* II | 8177 | *Pleisomonas shigelloides* | 10363 |
| *Streptococcus equisimilis* | 8543 | *Proteus mirabilis* | 11938 |
| *Aerococcus viridans* | 8251 | *Proteus vulgaris* | 4175 |
| *Streptococcus* sp. group G | 9603 | *Serratia marcescens* | 10211 |
| *Streptococcus equi* subsp. *equi* | 9682 | *Shigella flexneri* | 8192 |
| *Streptococcus* sp. group P | 9824 | *Stenotrophomonas maltophilia* | 10258 |
| *Enterococcus avium* | 9938 | *Streptococcus pyogenes* | 2366 |
| *Streptococcus* sp. group H | 10231 | *Corynebacterium urealyticum* | 12011 |
| *Streptococcus pneumoniae* | 7465 | *Lactobacillus casei* | 10302 |
| *Streptococcus parasanguinis* | 12854 | *Listeria monocytogenes* | 4885 |
| *Streptococcus equinus* | 12969 | *Staphylococcus capitis* | 11045 |
| *Streptococcus agalactiae* group B | 12907 | *Staphylococcus hominis* | 11320 |
| *Streptococcus mitis* | 12261 | *Staphylococcus lugdunensis* | 7990 |
| *Streptococcus agalactiae* group B | 12906 | *Staphylococcus simulans* | 11046 |
| *Streptococcus canis* | 12191 | *Branhamella/Moraxella catarrhalis* | 11020 |
| *Streptococcus constellatus* | 13122 | *Fusobacterium nucleatum* | 12276 |
| *Streptococcus acidominimus* | 12957 | *Prevotella corporis* | 13065 |
| *Pediococcus pentosaceus* | 12956 | *Burkholderia cepacia* | 10744 |

by agarose gel electrophoresis. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and cloned into the pCR2.1-TOPO plasmid using a TOPO TA Cloning kit (Invitrogen) and transformed into *Escherichia coli*. Positive transformants were selected according to the manufacturer's instructions. Plasmid DNA was extracted using a Plasmid Mini kit (Qiagen). For use in the LightCycler assay, the IPC was diluted and used at approximately 30 copies per reaction. Amplification of the IPC was detected by a second set of hybridization probes targeting an internal region of the product, which were specific to the $\lambda$ sequence (Table 4).

**LightCycler assay.** A LightCycler assay was performed in 20 µl glass capillary tubes. The reaction mixture consisted of 1 × FastStart DNA Master PLUS HybProbe mixture (Roche Diagnostics), 1 mM $MgCl_2$ (Roche Diagnostics), 5 µM each *cylB*-F and *cylB*-R primers, 2 µM each *cylB*-DNR probe and *cylB*-AC probe, 0.25 µM each *cylB*-IPC-DNR probe and *cylB*-IPC-AC probe (Table 4), *cylB* IPC (~30 copies) and 1 U uracil DNA glycosylase (Roche Diagnostics). Five microlitres of extracted DNA was added to capillaries containing 15 µl of the above mix. The samples were run using neat, 1 : 10 and 1 : 100 dilutions. Each run contained a negative control (extracted water and/

**Table 4.** Primers and probes used in this study

Sequences of the *cylB* gene are shown in upper case and $\lambda$ sequences in lower case.

| Primer/probe | Target | Sequence (5′→3′) |
|---|---|---|
| *cylB*-F | *cylB* gene forward | ATTAGGTGCCTTTGGAGT |
| *cylB*-R | *cylB* gene reverse | ATCATCGCCGCAGATA |
| *cylB*-DNR | *cylB* gene donor probe | CTTGCTAGAGTATTCAGCTATGGATTATTTAACT-(FITC) |
| *cylB*-AC | *cylB* gene acceptor probe | (RED 640)-TTTAGGGATCGTTTCCTTAGGGACT-($PO_4$) |
| *cylB*-IPC-F | Amplification of IPC forward | ATTAGGTGCCTTTGGAGTctgacggtttctaac |
| *cylB*-IPC-R | Amplification of IPC reverse | ATCATCGCCGCAGATAagacatacggaaatag |
| *cylB*-IPC-DNR | IPC donor probe | AGCCGCTGGCGCATTGAGCA-(FITC) |
| *cylB*-IPC-AC | IPC acceptor probe | (RED 705)-TGCAGCGAACTGAGCGCGGT-($PO_4$) |


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or PBS) and six positive controls (serial dilutions of purified *S. agalactiae* strain NCTC 11360 DNA ranging from 100 pg to 50 fg).

The cycling conditions were: initial denaturation at 95 °C for 10 min (transition rate 20 °C $s^{-1}$; acquisition mode none); 45 cycles of denaturation at 95 °C for 0 s (transition rate 20 °C $s^{-1}$; acquisition mode none), annealing at 60 °C for 10 s (transition rate 20 °C $s^{-1}$; acquisition mode single) and elongation at 72°C for 10 s (transition rate 20 °C $s^{-1}$; acquisition mode none); melting curve cycle analysis of 95 °C for 0 s (transition rate 20 °C $s^{-1}$; acquisition mode none), 40 °C for 30 s (transition rate 20 °C $s^{-1}$; acquisition mode none) and 80 °C for 0 s (transition rate 0.1 °C $s^{-1}$; acquisition mode continuous); and cooling at 40 °C for 30 s (transition rate 20 °C $s^{-1}$; acquisition mode none). The results were analysed using Roche LightCycler software version 4.0. Samples were recorded as positive if the crossing point of the sample was less than or equal to the crossing point of the lowest standard (i.e. 50 fg). All positive samples were repeated for each respective assay to ensure reproducibility. If there was no specific amplification and the IPC did not amplify due to inhibition, the sample was retested by diluting to non-inhibitory levels.

**Statistical methods.** The exact binomial confidence interval (CI) was calculated for the proportion of CSF and EDTA blood samples that were PCR-positive for GBS. The difference between these proportions was assessed using Fisher's exact test.

## RESULTS AND DISCUSSION

A real-time PCR assay targeting the *cylB* gene was developed to detect GBS from clinical samples. Amongst the 110 blood culture-negative samples analysed from neonates with probable GBS sepsis or meningitis, 18 were positive (16.4 %, 95 % CI 10.0–24.6 %): 16 of the 75 CSF samples (21.3 %, 95 % CI 12.7–32.3 %) and two of the 35 EDTA blood samples (5.7 %, 95 % CI 0.7–19.2 %). The proportion testing positive in the CSF samples was significantly higher than in the EDTA blood samples ($P=0.05$, Fisher's exact test). Overall, the real-time PCR assay was shown to be superior to culture methods for detection of GBS from CSF and EDTA blood samples. The method is rapid, sensitive, specific and reproducible. In a multiplex assay with the IPC, the assay could reliably detect 50 fg positive-control DNA per reaction.

### Sensitivity

From the results obtained, the LightCycler PCR appeared to be more sensitive than culture because culture requires viable organisms to be present in the sample. In addition, a PCR can provide results in <2 h from receipt of sample. The sensitivity of the assay required at least ten bacterial cells to exist in 5 µl extracted sample [$2.0\times10^3$ organisms (ml extracted sample)$^{-1}$]. The difference in sensitivity rates between EDTA blood and CSF samples could be due to a number of reasons: the blood samples may have contained very low numbers of organisms, differences in DNA extraction methods together with small sampling volumes (especially from neonates) or differences in transportation to the laboratory.

### Specificity

The PCR was developed using purified *S. agalactiae* DNA and tested on blood culture-negative clinical samples (35 EDTA blood and 75 CSF samples) from neonates with probable GBS sepsis or meningitis. The assay demonstrated 100 % (95 % CI 93.8–100 %) specificity for GBS. The 21 other streptococcal species and the 37 related taxa tested were negative by PCR.

### PCR inhibition

With any molecular diagnostic method, the potential for false-negative results is of concern when analysing clinical samples. A false-negative result could be due to the presence of PCR inhibitors, poor target DNA recovery during extraction, degradation of target DNA before amplification, errors in setting up a reaction or degraded reagents (Al-Soud & Rådström, 2001). To determine the presence and absence of PCR inhibitors in the clinical samples, an IPC was developed and included in each reaction, which facilitated the detection of inhibition in the samples. Titration of the IPC gave reliable results at 30 copies per reaction. In this study, we observed that when amplification of the *cylB* gene was observed in 1 : 10 and 1 : 100 dilutions, no amplification was seen in the neat DNA sample, probably due to high inhibitor levels that blocked amplification.

### Conclusions

LightCycler amplification of the *cylB* gene was evidently more sensitive than culture for the detection of GBS mainly from CSF. Co-amplification of an IPC in the same reaction tube with the same primers as the target increases throughput and reduces cost. The rapid time to result of this molecular assay (<2 h from receipt of sample) could make it an attractive option to supplement culture-based tests. Validation of extraction methods to resolve sample inhibition and poor target DNA recovery during extraction would be beneficial but would require large numbers and volumes of clinical specimens. This method could be an invaluable tool in the rapid diagnosis of GBS and may improve our ability to detect and manage GBS, particularly in intrapartum and neonatal settings.

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