

Rapid detection of group B streptococcus and *Escherichia coli* in amniotic fluid using real-time fluorescent PCR

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Objective: To establish reliability and validity of real-time fluorescent PCR for early detection of bacterial invasion of the amniotic cavity.

Methods: Amniotic fluid samples from 40 patients undergoing mid-trimester genetic amniocentesis were incubated for 6 h at 37°C and were cultured on media specific for group B streptococcus (GBS) and *E. coli*. Concurrently, samples were analyzed with real-time fluorescent PCR (Roche LightCycler) using DNA primers and probes designed to detect the CAMP factor encoding *cfb* gene and *uidA* gene of GBS and *E. coli*, respectively. For positive control and to simulate amniotic fluid colonization, 10⁴ cfu/ml of GBS and *E. coli* were inoculated on sterile amniotic fluid and incubated for 6 h. Bacterial genomic DNA for the two organisms was extracted and purified via the two-step precipitation method using a commercial kit. The real-time PCR assays were also tested against 25 non-GBS and non-*E. coli* bacterial species. The lower limit of detection for each pathogen was established using serial dilution of bacterial genomic DNA.

Results: All patient samples were negative for evidence of GBS and *E. coli* with both culture and real-time PCR methods. Amniotic fluid samples inoculated with GBS and *E. coli* were positive with real-time PCR whereas the 25 bacterial species other than GBS or *E. coli* tested negative with the assay. Average total sample processing time including the pre-enrichment step was 7 h 40 min. The average cost for DNA extraction and PCR testing was \$8.50 per test.

Conclusion: Real-time fluorescent PCR is a valid and reliable method for detection of specific pathogens in amniotic fluid. This technique is sensitive for low inoculation levels. Real-time fluorescent PCR has potential to impact clinical management as a rapid, reliable detection method for GBS and *E. coli* in chorioamnionitis.

Key words: REAL-TIME FLUORESCENT PCR; *E. COLI*; GBS

INTRODUCTION

Preterm birth occurs in approximately 11% of all pregnancies. Preterm labor may be initiated by infection in 33% of cases with intact membranes and in 40% with preterm premature rupture of membranes (pPROM). Early preterm birth (< 32

weeks gestation) has the strongest association with infection¹. Neonatal sepsis occurs more commonly with early preterm birth, accounting for a large portion of perinatal morbidity and mortality. Pathogens that have been isolated from amniotic fluid in these cases include *Ureaplasma urealyticum*, *Mycoplasma hominis*, fusobacterium species, prevo-

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tella species, *gardnarella vaginalis*, *streptococcus agalactiae*, anaerobic streptococci and *Escherichia coli*^{2,3}. In the pathogenesis of intra-amniotic infection, or chorioamnionitis, it appears that vaginal organisms overcome available cervical barrier and immunity factors and penetrate the chorioamnion to enter the amniotic fluid. Ultimately, some fetuses become infected⁴.

Identifying the gravid patient destined to deliver prematurely because of ascending genital tract infection is difficult. Many patients remain asymptomatic until they present in preterm labor, and most of them do not show signs of chorioamnionitis. Even patients with pPPROM often lack clinical evidence of infection upon presentation. Current clinical management of preterm labor and pPPROM does not universally include routine amniocentesis because there are no rapid, reliable diagnostic tests for intra-amniotic infection and because infection rates vary among clinical populations. Amniotic fluid culture takes 48 to 72 h, and may lack the sensitivity needed to reliably identify infection at a stage when intervention could alter outcome. Amniotic fluid glucose, Gram stain and leukocyte esterase assays provide rapid results, but have poor predictive value for infection-mediated preterm labor, and even less for identifying the neonate destined to develop sepsis. Ideally, accurate identification of bacterial invasion of the amniotic cavity might allow expedient and targeted management of infection-mediated preterm labor.

PCR is used to identify genetic material of pathogenic microorganisms by amplification, with highly specific and sensitive results, and has been shown to detect bacteria in amniotic fluid at a higher rate than standard microbial cultures^{3,5,6}. Unfortunately, PCR requires additional laboratory techniques to detect the amplified genomic signal, taking up to 1 day to obtain results. Real-time PCR employs the same biomolecular techniques that provide the sensitivity and specificity of traditional PCR, but detection of the pathogenic microorganism occurs during the amplification process, shortening the time to detection of pathogen.

The objective of our study was to develop a valid, reliable test for detection of GBS and *E. coli* in amniotic fluid.

MATERIALS AND METHODS

This study was initiated after approval by the Institutional Review Board at David Grant USAF Medical Center. For genetic analysis, 40 amniotic fluid samples were obtained from women undergoing mid-trimester amniocentesis. To test amniotic fluid samples for GBS and *E. coli* infection by standard culture and conventional and real-time PCR, 2 ml of each sample were incubated for 6 h at 37 C. Samples were frozen, stored at -70 C and batched for later analysis. An aliquot of each sample was cultured according to CDC recommendations for detection of GBS and *E. coli*. A second aliquot was processed for traditional and real-time fluorescent PCR using a Roche Light-Cycler. Bacterial DNA was extracted using the Genra Systems Puregene DNA isolation kit, modified by the addition of a second precipitation step.

Pathogen genomic targets

The real-time PCR probe for GBS targeted the *cfb* gene encoding the Christie-Atkins-Munch-Petersen (CAMP) factor⁷. Hybridization probes designed to anneal to an internal sequence of the 153 base pair (bp) amplicon were used. The *E. coli* target for the assay consists of a 186 bp sequence of the *uidA* gene encoding the β -glucuronidase protein. GBS or *E. coli* was detected in each sample in triplicate using the Hybridization (GBS) or Taqman (*E. coli*) probe systems in the Light-Cycler.

Specificity of assays

To assess the specificity of the Hybridization and Taqman probe assays, a battery of 25 non-GBS and non-*E. coli* bacterial strains were grown on blood agar plates, and 40 μ l of a 104 cfu/ml bacterial suspension of each strain were added to 500 μ l amniotic fluid sample and incubated for 6 h at 37 C. The sample was then tested for the presence of GBS and *E. coli* target DNA on the LightCycler, in duplicate.

Spiked samples

Streptococcus agalactiae (ATCC 12386) and *E. coli* (ATCC 35218) were grown in pure culture on blood agar plates and diluted to 104 cfu/ml. Then 40 μ l (25 cfu total) of each of the bacterial suspensions were added to 500 μ l aliquots of amniotic fluid, incubated at 37 C for 6 h and stored at -80 C until analyzed. PCR-grade distilled, de-ionized water was used as negative control for both traditional PCR and real-time PCR. The dilute bacterial suspensions served as the positive control for traditional and real-time PCR.

Limit of detection

To determine the lower limit of detection of the assays, pure genomic DNA extracted from GBS or *E. coli* cultured on sheep blood agar was obtained using the Gentra Puregene Isolation kit with the modification noted above. RNAse and proteinase K treatment steps after DNA isolation were added to remove contaminating proteins and RNA. The concentration of DNA was calculated by measuring absorbance at 260 and 280 nm. DNA concentration was adjusted to 1 μ M and serial dilution of DNA in Tris buffer was performed. GBS or *E. coli* was detected from each concentration in triplicate using the Hybridization (GBS) or Taqman (*E. coli*) probe systems in the LightCycler.

RESULTS

As was anticipated on the basis of the clinical histories, none of the 40 native amniotic fluid samples had evidence of *E.coli* or GBS by culture, conventional PCR or real-time PCR. All amniotic fluid samples inoculated with GBS or *E. coli* were uniformly positive by real-time PCR (Figure 1). PCR product results were confirmed by gel electrophoresis (Figure 2). Therefore, in our laboratory the real-time PCR system was 100% sensitive and specific for detecting GBS and *E. coli* in amniotic fluid. In the limit of detection studies we were able to consistently detect 25 genomic copies (50 cfu/ml) (Figure 3). The average time to complete the real-time PCR

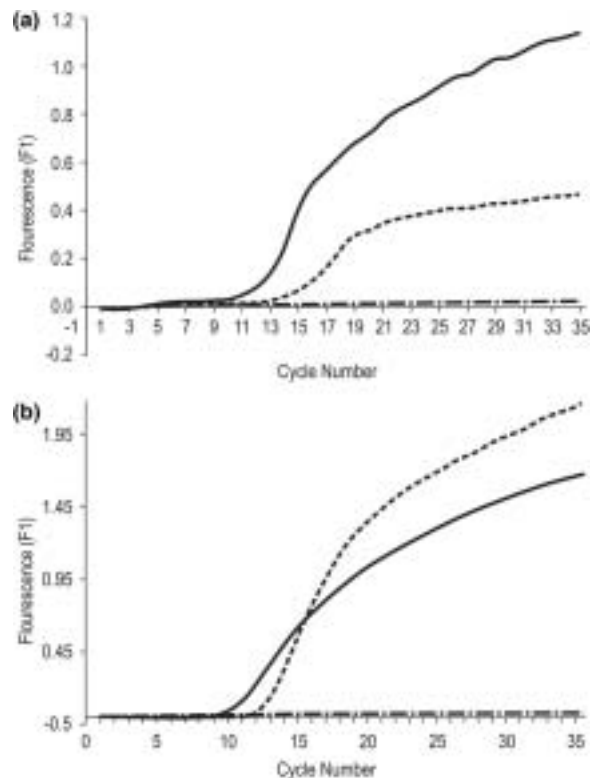


Figure 1 LightCycler™ PCR graph showing hybridization probe (GBS) and Taqman (*E. coli*) results, demonstrating (A) increased fluorescence for group B streptococci (solid) positive controls (dash), and (B) *E. coli* (solid) positive controls (dash). No fluorescence was observed for the negative control (PCR grade water) and patients' samples negative for GBS or *E. coli* (broken line)

testing was 1 h 40 min after the 6-h incubation period study protocol had elapsed.

DISCUSSION

We have shown that real-time PCR is a sensitive (100%) and rapid method for detecting pathogens in amniotic fluid that are commonly involved in the pathogenesis of preterm labor and adverse neonatal outcome. We consistently detected artificially created infected amniotic fluid samples and consistently detected greater than 50 cfu/ml of pathogen. We also proved that the real-time PCR system is highly specific (100%), by showing that the probes tested did not detect alternative bacterial species or produce false-positive results in control samples devoid of bacteria. Real-time PCR proved to be valid and reliable when

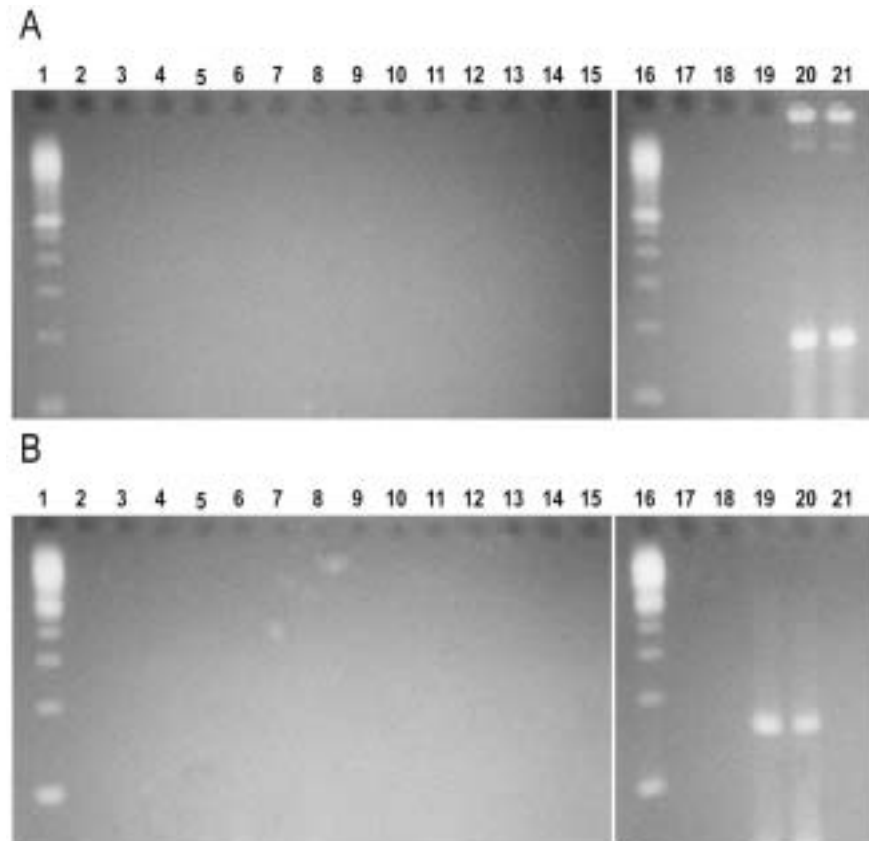


Figure 2 Results of real-time PCR were confirmed with agarose gel electrophoresis of PCR products. No amplification was observed for (A) GBS negative samples (A lanes 2–15 and 17) or (B) *E. coli* negative samples (B lanes 2–15 and 20–21). Negative control (PCR water, A18 and B19) tested appropriately. Positive control and spiked sample for GBS (A19–20) yielded the 153 bp amplification product whereas positive control and spiked sample for *E. coli* (B17–18) yielded the 186 bp PCR product

compared with standard culture and traditional PCR. The overall time required to obtain results from real-time PCR was 7 h 40 min, including the incubation time. Testing was completed at a material cost of \$8.50 per amniotic fluid sample. We have also shown with sensitive and specific biomolecular techniques that the amniotic cavity under uncomplicated clinical conditions is sterile with respect to GBS and *E. coli* in the mid-trimester.

If confirmed in future studies, the clinical implications of our data may lead to changes in diagnostic methods and clinical management of chorioamnionitis, preterm labor and pPROM. Future research should focus on validating this real-time PCR system in other clinical populations, including cases of suspected preterm labor,

pPROM or chorioamnionitis. We are currently planning such a study, to include patients with these diagnoses. We also plan to correlate PCR results with various adverse neonatal outcomes. With further study, it may become possible to more accurately and more rapidly identify earlier in gestation those pregnancies destined to deliver premature neonates that may develop sepsis. Early discovery of intra-amniotic infection may allow specific therapy *in utero* and maintenance of such pregnancies closer to term, as in the case of listeriosis. Conversely, early identification of intra-amniotic bacterial invasion may indicate expeditious delivery rather than ineffective maternal tocolytic or antibiotic therapy as the best strategy for reducing neonatal infectious morbidity and mortality.

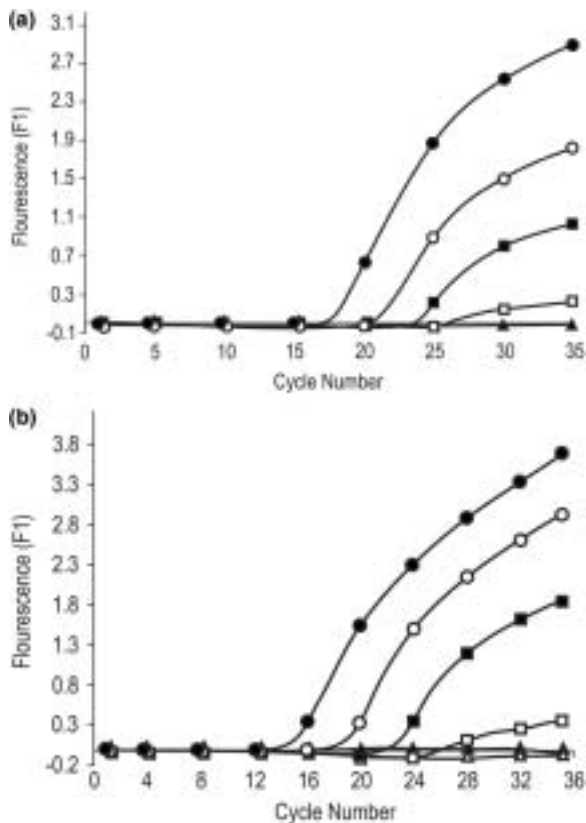


Figure 3 The limit of detection of the real-time Hybridization PCR assay for GBS and Taqman assay for *E. coli* were determined by serial dilution of extracted GBS or *E. coli* DNA before real-time PCR using LightCycler™. Each concentration was performed in triplicate and the average was used in the plot (solid circle, 10 ng/rxn; open circle, 1 ng/rxn; solid square, 100 pg/rxn; open square, 10 pg/rxn; closed triangle, 1 pg/rxn)

In summary, the results of our study suggest that real-time PCR technology is a promising method that may improve diagnostic accuracy and rapidity in identifying intra-amniotic infection, and may open new windows for applying therapy to prevent preterm delivery and neonatal morbidity and mortality from prematurity. However, before this technique can be applied clinically, further research is necessary. The sensitivity and specificity of real-time PCR needs to be evaluated and validated in the most important clinical target population, namely women at 24 to 32 weeks of gestation who are at increased risk for preterm delivery or pPROM or are suspected to have preterm labor. In addition, probes for other common and uncommon pathogens need to be tested.

Acknowledgement

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