Evaluating the Near-Term Infant for Early Onset Sepsis

Progress and Challenges to Consider with 16S rDNA Polymerase Chain Reaction Testing

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Although the rate of early onset sepsis in the near-term neonate is low (one to eight of 1000 cases), the rate of mortality and morbidity is high. As a result, infants receive multiple, broad-spectrum antibiotic therapy, many for up to 7 days despite blood cultures showing no growth. Maternal intrapartum antibiotic prophylaxis and small blood volume collections from infants are cited as reasons for the lack of confidence in negative culture results. Incorporating an additional, more rapid test could facilitate a more timely diagnosis in these infants. To this end, a 16S rDNA polymerase chain reaction (PCR) assay was compared to blood culturing for use as a tool in evaluating early onset sepsis. Of 1751 neonatal intensive care unit admissions that were screened, 1233 near-term infants met inclusion criteria. Compared to culture, PCR demonstrated excellent analytical specificity (1186 of 1216, 97.5%) and negative predictive value (1186 of 1196, 99.2%); however, PCR failed to detect a significant number of culture-proven cases. These findings underscore the cautionary stance that should be taken at this time when considering the use of a molecular amplification test for diagnosing neonatal sepsis. The experience gained from this study illustrates the need for changes in sample collection and preparation techniques so as to improve analytical sensitivity of the assay. (*J Mol Diagn* 2006, 8:357–363; DOI: 10.2353/jmoldx.2006.050138)

Early onset sepsis (EOS) in the near-term infant (>34 weeks gestation) is difficult to diagnose because the infant's signs and symptoms may be subtle or may mimic other medical conditions such as hypoglycemia, delayed transition, or transient tachypnea.^{1–4} Even though the incidence of culture-positive sepsis is low (1 to 8 cases of

1000 live births), the risk of mortality is high, ranging from 15 to 50%.^{3,5} Thus, many infants are evaluated for EOS and receive multiple broad-spectrum antibiotics, although few have culture-proven sepsis. In fact, it is estimated that between 5% and 10% (189,000 to 489,000) of all newborns in the United States receive systemic antibiotics annually.⁶

Early-onset disease occurs before the first 5 to 7 days of life and presents as a fulminate, multisystemic illness.⁷ Typically, the infant has acquired the organism during the intrapartum period from the maternal genital tract. The most common etiologies of EOS include group B *Streptococcus*, *Escherichia coli*, *Enterococcus* sp., and *Listeria monocytogenes*.³

At Magee-Women's Hospital, a tertiary care center with a 65-bed neonatal intensive care unit (NICU), 70% of near-term infants undergo a sepsis evaluation on admission to the NICU although only ~1% of them have culture-proven sepsis. These infants are managed clinically with bacterial cultures, complete blood count (CBC), and combination antimicrobial therapy for presumed sepsis; ampicillin and gentamicin, or ampicillin and cefotaxime, are usually given to the infant.^{8–11} Although this regimen is effective in decreasing bacterial sepsis, there are significant risks associated with using multiple, broad-spectrum antibiotics. These risks include adverse drug reaction or toxicity, bacterial superinfection with drug resistant organisms, and/or overgrowth of yeast and/or fungi.

Among the diagnostic procedures used currently, growth in culture is considered the gold standard for detecting blood-borne bacteria. However, culturing requires significant time and has a low sensitivity.^{2,12} In addition, maternal intrapartum antimicrobial prophylaxis and/or small blood volume collections from infants are cited as possible reasons for the lack of confidence in negative culture results.^{13–16} At Magee-Women's Hospital, preliminary results are generated after 48 hours for

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blood cultures lacking detectable growth. However, final results for negative blood cultures are not finalized until after 5 days. Using a more rapid assay for ruling out sepsis in the near-term infant who is stable and asymptomatic would provide valuable information sooner for medical decision making.

Nucleic acid amplification tests such as the polymerase chain reaction (PCR) assay are rapid to perform and have been used successfully to diagnose a wide range of infectious diseases, including bacterial, yeast, viral, and protozoal infections.¹⁷ In fact, the nucleic acid amplification test is now considered the gold standard test for diagnosing neonatal meningitis due to herpes simplex virus.¹⁸ PCR detection of blood-borne bacteria using highly conserved targets within the *16S ribosomal RNA* gene (16S rDNA) has been described.^{19–25} Our own laboratory published the first large-scale study for detecting sepsis in neonates. The study tested 548 blood samples collected from NICU-based infants being evaluated for either EOS or hospital-acquired sepsis, regardless of their gestational age.²⁶ The results revealed a high level of overall agreement between PCR and culture (99.3%), with less time needed for reporting results.

The objective of the current study was to determine the robustness of the same PCR assay in a population with a very low rate of culture-proven sepsis: near-term infants (>34 weeks gestation) admitted to the NICU for EOS evaluation. The purpose of this study was to compare the performance of a 16S rDNA PCR assay to that of an automated blood culturing system for ruling out sepsis in the near-term infant (ie, negative predictive value). If the PCR assay could be shown to have a high level of agreement with culture, then using a more rapid test in conjunction with culture could provide valuable information sooner for medical decision making in the case of the uninfected, stable, and asymptomatic infant.

Materials and Methods

Patient Selection and Sample Rejection Criteria

We received hospital institutional review board approval before initiation of this study. To be eligible, an infant had to be >34 weeks gestation at the time of birth, be admitted to the NICU within a few hours of birth for EOS evaluation, and have both a blood culture and CBC ordered by the attending physician. The 16S rDNA PCR analyses were performed from the unused portions of each CBC. Sample rejection for PCR included blood volumes of <100 μ l or grossly hemolyzed and/or clotted specimens. Sample rejection occurred in only 10 specimens (0.8%). To be considered clinically stable, the infant needed to fulfill the following criteria for a minimum of 24 hours: adequate urine output (>1 cc/kg/hour), full volume oral feedings (>100 cc/kg/day), and breathing in room air (<60 breaths/minute).

Blood Culturing and Phenotypic Identification of Culture-Proven Cases

Blood for culture was collected either by a venous or arterial draw, with 0.5 to 1.0 ml being inoculated directly into a single pediatric sample-sized resin-containing blood culture bottle (Peds Plus; Becton Dickinson, Sparks, MD). Each blood culture bottle was sent immediately to the clinical microbiology laboratory where it was loaded into a Bactec 9240 blood culture instrument (Becton Dickinson) in 1 hour or less from the time of receipt in accordance with the manufacturer's recommendation. The Bactec 9240 instrument is an automated blood culture system that uses a fluorescent sensor for detecting growth of the microorganisms.

When the Bactec 9240 instrument detected bacterial growth, fluid from the bottle was withdrawn, gram-stained, and subcultured on the appropriate agar-based culture plates. After overnight incubation, the purified colonies were identified either using an automated identification system (MicroScan Dade, West Sacramento, CA) or manually using the appropriate reagents.

CBC Whole Blood Collection

Whole blood for CBC analysis was collected either by venous or arterial draw or by a heel stick method. A pediatric-sample-sized ethylenediaminetetraacetic acid-containing Vacutainer tube (Becton Dickinson) was filled with 300 to 900 μ l of whole blood. The specimens were sent immediately to the hospital's clinical hematology laboratory for analysis on a Coulter Counter instrument (Coulter Inc., South Beach, FL). The unused portion of blood was then released for 16S rDNA PCR analysis once the CBC result was finalized.

Whole Blood Enrichment and Sample Processing for 16S rDNA PCR

The protocol for sample enrichment and processing was described previously.²⁶ Briefly, the entire volume (100 to 600 μ l) of the remaining CBC was added to 4 ml of tryptic soy broth (Difco Laboratories, Detroit, MI) and incubated at 37°C for up to 5 hours. The pre-enriched specimen was treated with 4°C red cell lysis buffer (0.32 mol/L sucrose, 10 mmol/L Tris-HCl, pH 7.5, 5 mmol/L MgCl₂, and 1% Triton X-100). Nucleated cells were pelleted at 13,000 $\times g$ for 5 minutes at 4°C and treated with 100 μ l of phosphate-buffered saline (PBS) containing 50 U Mutanolysin (Sigma, St. Louis, MO) and 10 mg/ml of proteinase K for 30 minutes at 70°C. After enzymatic digestion, the crude cell lysate was heated to boiling for 10 minutes to inactivate the proteinase K.

Universal 16S rDNA PCR Assay

The PCR assay used the well-characterized and highly conserved RW01 and DG74 primers.^{19,26} The amplified 380-bp product was visualized using an ethidium-stained,

gel-based detection method, followed by Southern blot analysis using a digoxigenin-labeled RDR245 probe as described previously.²⁶ Two controls were included with each run, a β -globin control²⁶ and a spiking control. The former control tested for presence of DNA in the specimen whereas the latter control assessed the specimen for evidence of inhibitors. For spiking, 100 colony-forming units of group B *Streptococcus* were added to the 16S rDNA PCR master mix containing the specimen.

Initial 16S rDNA Sequencing Reaction

Each 380-bp amplicon generated by the 16S rDNA PCR was partially sequenced using RDR245 as the sequence primer (Pyrosequencing; Biotage, Uppsala, Sweden).²⁶ The sequence information obtained by pyrosequencing was used to provide partial identity for the bacteria contained within each PCR-positive blood sample as previously described.²⁷

Culture-Based Identification of the Staphylococcus sp.

Bacteria were grown on sheep blood agar plates (BBL Division, Becton Dickinson) to obtain purified colonies. The bacteria were gram-stained; those appearing gram-positive and resembling cocci in clusters under microscopic examination were tested for their catalase reaction using a 3% solution of H₂O₂. Catalase-positive colonies were further analyzed for their coagulase reaction using Staphaurex (Remel, Lenexa, KS). Coagulase-positive, catalase-positive, gram-positive cocci in clusters were identified as *Staphylococcus aureus* (SA), whereas the coagulase-negative, catalase-positive, gram-positive cocci in clusters were identified as coagulase-negative *Staphylococcus* species (CoNS).

Preparation of Crude Cell Lysates from Purified Isolates of Staphylococcus sp.

Individual colonies of SA and CoNS were used to prepare individual crude cell lysates for *Staphylococcus* sp.-specific 16S rDNA PCR analysis. An isolated colony was touched with a sterile loop and the resulting bacteria resuspended in a solution containing 89 μ l of PBS (pH 7.6) and 5 μ l (50 U) of Mutanolysin (Sigma) and then incubated for 30 minutes at 37°C. To this mixture, 5 μ l of proteinase K (10 mg/ml) (Sigma) and 1 μ l of 10% sodium dodecyl sulfate (Sigma) were added, and the solution was incubated for 30 minutes at 37°C and then heated to 95°C for 10 minutes. A 1- μ l volume of the resulting crude cell lysate was added to 99 μ l of the PCR master mix for amplification of the 16S rDNA *Staphylococcus* sp.-specific target.

Staphylococcus sp. 16S rDNA PCR Assay

The 16S rDNA PCR-positive specimens identified initially by pyrosequencing as *Staphylococcus* sp. were further

analyzed to distinguish SA from CoNS using a different set of primers targeting a separate region of the 16S rDNA gene. To accomplish this, GenBank database was queried for available *Staphylococcus* sp. 16S rRNA gene sequences. *Staphylococcus epidermidis* (AF397060 and AY167864), *Staphylococcus hominis* (X66101 and AY030318), *Staphylococcus haemolyticus* (X66100), *Staphylococcus caprae* (Y12593), *Staphylococcus capitis* (AY030321), and *S. aureus* (X68417) were aligned using the software program Sequencer (Gene Codes Inc., Ann Arbor, MI). The goal was to identify both a region within the 16S rRNA gene containing complete homology for primer recognition as well as a region with bp differences to differentiate SA from CoNS. The search revealed a 243-bp region within the 16S rRNA gene (bp no. 46 to 288, relative to GenBank reference number X70648). The PCR and sequencing primers were designed to recognize both SA and CoNS with an internal sequence containing differences in 3 of 23 bases to distinguish SA from CoNS.

The PCR primers included a biotinylated forward primer: 5'-TGC CTA ATA CAT GCA AGT CGA GCG-3' (bp no. 46 to 69) and an unlabeled reverse primer: 5'-GTT GCC TTG GTA AGC CGT TAC CTT-3' (bp no. 288 to 265). The PCR master mix consisted of 10 mmol/L Tris-HCl, pH 8.3; 2 mmol/L MgCl₂; 200 mmol/L each dATP, dCTP, dTTP, and dGTP; 0.2 mmol/L of each primer; and 2.5 U *Taq* polymerase (Promega, Madison, WI). A volume of 1 μ l of crude cell lysate was added to 99 μ l of master mix. The PCR cycling parameters consisted of an initial denaturing step (10 minutes at 95°C), followed by 40 cycles of the following: 1 minute at 95°C, 2 minutes at 60°C, and 1 minute at 72°C. After amplification, 10 μ l of material were analyzed by agarose gel electrophoresis. The ethidium-stained gel was evaluated for the presence of the 243-bp DNA fragment compared to a 100-bp ladder molecular weight standard (BioWhittaker, Rockland, ME).

Differentiation Between SA and CoNS Using Pyrosequencing

A 40- μ l aliquot of the *Staphylococcus* sp.-specific 16S rDNA PCR generated amplicon was purified using streptavidin-coated Sepharose beads (3 μ l) (Amersham, Piscataway, NJ) in an annealing buffer provided by Biotage. The unlabeled DNA strand was dissociated and discarded after alkaline denaturation according to the manufacturer's instructions. The pyrosequencing vacuum prep tool was used to perform the washing (10 mmol/L Tris acetate, pH 7.6) and bead transferring steps. The bead-bound, biotinylated DNA strand was added to a well in a 96-well microtiter plate along with annealing buffer and 0.5 μ mol/L of the complementary sequencing primer, 5'-GTG TTA CTC ACC CGT CCG CCG CTA-3' (bp no. 122 to 99). The microtiter plate containing the samples was heated to 95°C for 2 minutes and then allowed to cool to room temperature on the bench top, at which time the primer annealed to its complementary target.

Table 1. Comparison of Blood Culture and 16S rDNA PCR Results from Near-Term Infants Admitted to the NICU for EOS Evaluation

		Culture results		
		Positive	Negative	Total
PCR results	Positive	7	30	37
	Negative	10	1186	1196
	Total	17	1216	1233

The plate containing the annealed products was placed into the PSQ 96MA system (Biotage) where the direct sequencing reaction occurred using sequence analysis reagents (Biotage) and a cyclic dispensation program of nucleotides according to the manufacturer. The pyrosequencing reaction utilizes a four-enzyme cascade system that produces visible light read by a charge-coupled device camera. The light detected by the camera is displayed as peaks, with the height of the peak being proportional to the number of bases of a specific dNTP incorporated during the reaction. All four bases are added individually, one dNTP base at a time with the unincorporated nucleotide being destroyed enzymatically before the next NTP is added (dATP, dCTP, dTTP, and finally dGTP). The software program provides both a pyrogram figure and a text-based sequence for analysis.

Full-length sequencing of several SA and CoNS-based 243-bp amplicons was also performed using the ABI 3100 instrument for comparison (data not shown). The resulting sequences from both the ABI and pyrosequencing reactions were compared to one another and to the *Staphylococcus* sp. sequences found in GenBank, EMBL Nucleotide Sequence Database, and the DNA Data Bank of Japan^{28,29} using the BLAST tool³⁰ from the National Center for Biotechnology Information. Sequences were aligned using Sequencer software (Gene Codes Inc.).

Results

Enrollment Numbers for the Near-Term Infants Being Evaluated for EOS

In this study, a total of 1751 NICU admissions were screened between September 1, 2000 and April 1, 2004. Of the infants screened, 1233 met the inclusion criteria for enrollment and were included in this study. Compared to culture, the 16S rDNA PCR demonstrated a high negative predictive value for ruling out EOS in the asymptomatic and stable near-term infant.

In all, 1233 infants who had paired blood culture and 16S rDNA PCR results were enrolled (Table 1). The comparison revealed an overall level of agreement of 96.8% (1193 of 1233) between culture and PCR results. The percentage of near-term infants with culture-negative results was 98.6% (1216 of 1233), whereas those with PCR-negative results was 97% (1196 of 1233). Compared to blood culture, PCR demonstrated a high negative predictive value (99.2%) and analytical specificity (97.5%). The 1186 infants with culture-negative/PCR-

Table 2. Details of Culture-Positive/PCR-Positive Results

Culture-based phenotypic ID	Time to detect growth in Bactec 9240 (hours:minutes)
GBS	08:32
GBS	09:48
GBS	08:24
<i>E. coli</i>	09:40
<i>E. coli</i>	11:12
CoNS	17:15
CoNS	18:18

GBS, group B *Streptococcus*; CoNS, coagulase-negative *Staphylococcus* spp.

negative results had stabilized and were asymptomatic, having met the criteria listed for a minimum of 24 hours.

The 16S rDNA PCR Assay Failed to Detect a Significant Number of Culture-Proven Cases of EOS in Near-Term Infants

There were a total of 17 culture-proven cases of EOS detected in near-term infants enrolled in this study (Table 1); 5 cases have been previously described.²⁷ The culture-based identification of all 17 bacterial isolates included group B *Streptococcus* ($n = 6$), CoNS ($n = 6$), *E. coli* ($n = 2$), *Streptococcus viridans* ($n = 2$), and *Micrococcus* sp. ($n = 1$) (Tables 2 and 3) and are representative of bacteria responsible for EOS.⁷

The 16S rDNA PCR assay failed to detect 10 of the 17 cases of culture-proven sepsis. Dividing the culture-proven cases of EOS into concordant (Table 2) and discordant (Table 3) results revealed differences in the average times required to detect bacterial growth in culture: 12 hours 6 minutes for concordant results, and 23 hours 15 minutes for discordant results. In addition to the longer detection time needed for the discordant results, other issues including sample collection and preparation techniques are also responsible for the failure of PCR to detect bacterial DNA in these cases.

Table 3. Details of the Culture-Positive/PCR-Negative Results

Culture-based phenotypic ID	Time needed to detect growth in Bactec 9240 (hours:minutes)
GBS*	09:13
GBS*	10:00
GBS*	15:54
CoNS	27:48
CoNS	37:13
CoNS	23:15
CoNS	19:17
<i>S. viridans</i>	13:36
<i>S. viridans</i>	32:20
<i>Micrococcus</i> sp.	43:43

*All three infants with culture-proven GBS EOS were symptomatic with radiographic evidence of pneumonia and treated with a full course of antibiotics.

GBS, group B *Streptococcus*; CoNS, coagulase-negative *Staphylococcus* sp.

Table 4. Pyrosequencing Results of Blood Culture-Negative/PCR-Positive Discordant Results

Culture-negative/ PCR-positive (n)*	Pyrosequencing results using universal primer RDR245 ²⁷	Pyrosequencing results using <i>Staphylococcus</i> sp.-specific primer
20	<i>Staphylococcus</i> sp.	CoNS (19) SA (1)
5	<i>Streptococcus</i> sp.	
2	Enteric Gram-negative rod I	

*Three of the thirty culture-negative/PCR-positive samples lacked sufficient DNA to be analyzed by pyrosequencing.
n, Number of specimens analyzed by pyrosequencing; CoNS, coagulase-negative *Staphylococcus* species; SA, *Staphylococcus aureus*.

16S rDNA PCR Assay Detected Bacterial DNA from Blood Collected on a Significant Number of Culture-Negative Cases in Near-Term Infants

There were 30 culture-negative/PCR-positive results from near-term infants enrolled in this study. There was sufficient DNA in 27 of the 30 amplified samples to perform pyrosequencing using the RDR245 oligonucleotide as the sequencing primer as previously described.²⁷ Table 4 illustrates the pyrosequencing results for the 27 amplicons; they were identified as *Staphylococcus* sp. (n = 20), *Streptococcus* sp. (n = 5) and enteric gram-negative rods (n = 2).

Differentiation Between SA and CoNS by Direct Sequence Analysis

The *Staphylococcus* sp.-specific 16S rDNA PCR amplification and subsequent pyrosequencing reaction was useful in differentiating SA from CoNS. A total of 258 purified isolates of *Staphylococcus* sp. were analyzed. These isolates consisted of 106 *S. aureus*, including 26 methicillin-resistant *S. aureus*, and 152 coagulase-negative *Staphylococcus* sp. that had been identified using culture-based methods. Figure 1 illustrates representative pyrograms for the SA (Figure 1A) and CoNS (Figure 1B). The SA and CoNS sequences revealed three base

differences (underlined) that could be used to differentiate SA from CoNS. All of the 106 SA isolates tested had the following 23-bp sequence: 5'-ACA TCA GAG AAG CAA GCT TCT CG-3', whereas all of the 152 CoNS isolates tested had the following 23-bp sequence: 5'-ACG TCA G/A AG GAG CAA GCT CCT CG-3'. The seventh base varied in the group of CoNS, being either a guanine or an adenine.

The *Staphylococcus* sp.-specific 16S rDNA PCR assay using crude cell lysates from non-*Staphylococcal* bacteria failed to generate an amplicon (data not shown). The analysis included several isolates each of *Bacillus* sp., *Corynebacterium* sp., *Listeria monocytogenes*, *E. coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Enterococcus* sp., *Streptococcus pyogenes*, *Streptococcus agalactiae*, and *Streptococcus pneumoniae*.

The *Staphylococcus* sp. Detected by PCR and Identified by Pyrosequencing Were Mainly CoNS

Table 4 illustrates the success of using pyrosequencing directly from amplified clinical materials for differentiation between SA and CoNS. Nineteen of the twenty (95%) *Staphylococcus* sp. amplicons generated from PCR-positive/culture-negative blood samples had a pyrogram

Figure 1. Representative pyrograms of SA and CoNS. The SA (A) and CoNS (B) sequences revealed three base differences (underlined) that could be used to differentiate SA from CoNS. A: 5'-ACA TCA GAG AAG CAA GCT TCT CG-3'; and B: 5'-ACG TCA AAG GAG CAA GCT CCT CG-3'.

consistent with CoNS, whereas 1 of 20 (5%) was consistent with SA.

Discussion

To our knowledge, this is the largest study to date comparing the results of PCR to culture in a population of near-term infants being evaluated for EOS. Our data confirms the fact that EOS in the near-term infant occurs at a very low incidence, regardless of the method used to detect bacteria. More importantly however, the results from this study emphasize the need for improvements to study design and methodologies that must be incorporated into any future studies comparing PCR and culture before a molecular-based test can be considered for use as a screening tool for neonatal sepsis.

In retrospect, our study had several design flaws that compromised our efforts to adequately compare PCR to culture. The primary design flaw was using the remaining CBC sample for PCR analysis. This choice, however, was not made lightly; the time frame between an infant's delivery and his/her admission to the NICU for suspicion of EOS can be extremely short. This fact made obtaining parental consent onerous; in fact, the mother may still have been in recovery, having just left the labor suite. Therefore, to achieve adequate study participation of the near-term infant being evaluated for EOS, we felt justified in using the remaining CBC sample for PCR analysis.

A second weakness to our study design was variation in sample collection; a significant percentage of the CBC specimens were not collected concurrently with blood drawn for culture, and/or were collected using a heel-stick method. The former variation could have produced dissimilar levels of potential bacteria present in the two samples, whereas the latter variation could have introduced bacterial contamination into the sample used for PCR analysis. Using heel-stick collected blood for PCR analysis likely contributed to many of the 30 culture-negative/PCR-positive discrepancies that occurred in this study. Heel stick collection of blood for CBC analysis is a common practice in our NICU; up to 30% of CBC samples are collected using a heel-stick method, a practice that carries the risk of acquiring normal skin flora in the sample, which does not pose a problem for CBC analysis but could lead to false-positive PCR results. In fact, 20 of 30 (66.7%) of the culture-negative/PCR-positive discrepancies were identified using pyrosequencing to be *Staphylococcus* sp., with 19 of 20 (95%) being CoNS, organisms that can be considered skin contaminants.

For future studies, we strongly recommend using a dedicated blood sample for PCR analysis, one that is collected aseptically from an arterial or venous draw at the same time as the blood being drawn for culture. This approach will help to eliminate sample-to-sample variation and provide a more accurate comparison between PCR and culture methods. This approach, however, will require that parental consent be obtained. Therefore, to achieve adequate enrollment numbers, those infants being evaluated for acquired sepsis will be targeted for

enrollment rather than the near-term infants being evaluated for EOS. Here the time frame for obtaining consent will be more manageable, and the rate of sepsis significantly higher.

Improving analytical sensitivity of the PCR assay is critical. All 10 cases of culture-proven sepsis that PCR failed to detect were due to a gram-positive cocci; this observation points to a need to change sample preparation protocols. Effective lysis of bacterial cells to liberate their DNA is crucial in achieving an assay with adequate sensitivity. Because of their cell wall composition, gram-positive organisms are more difficult to lyse than gram-negative ones. Perhaps including an additional enzymatic treatment targeting gram-positive bacteria, using labiase or achromopeptidase³¹ or using glass beads to disrupt the cell wall more effectively would improve cell lysis.

Although the use of crude cell lysates did not appear to contain significant levels of PCR inhibitors, sample preparation protocols including a DNA extraction step would be an improvement; DNA extracted from whole blood using either a manual method (eg, QIAamp column; Qiagen Inc., Valencia, CA) or an automated one should be considered. A DNA purification method that can extract DNA efficiently from small sample volumes and elute DNA in small fraction volumes to facilitate sample concentration is recommended.

Nucleic acids released from whole blood contain not only possible bacterial DNA but also genomic DNA from the patient's white blood cells (WBCs) as well. Human genomic DNA from WBCs is normally present at much higher concentration than bacterial DNA, and this difference creates a competitive advantage for binding of human genomic DNA rather than bacterial DNA onto the column or bead matrix. Recently, we demonstrated that DNA contained in whole blood samples collected from infants with elevated white blood cell counts ($\geq 39 \times 10^3/\mu\text{l}$) out-competed the exogenously added bacterial DNA for binding to a Qiagen column, resulting in a false-negative PCR result.³²

The results from this study have allowed us to identify several key factors that likely contributed to the low analytical sensitivity of our PCR. The hurdles outlined above can be overcome. In fact, recent attempts to improve assay sensitivity have met with success when using purified DNA and a real-time PCR platform for testing.³² However, many challenges remain before PCR can be recommended for the diagnosis of sepsis, and the success of this approach must be proven on a much larger scale using multiple sites. Then, and only then, will we have confidence in the test. Implementing the modifications outlined above should improve the assay's analytical sensitivity, something that is essential before PCR can be considered a reliable and useful diagnostic tool in detecting neonatal sepsis.

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