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Comparison of 16S rRNA Gene PCR and BACTEC 9240 for Detection of Neonatal Bacteremia

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Ten percent of infants born in the United States are admitted to neonatal intensive care units (NICU) annually. Approximately one-half of these admissions are from term infants (>34 weeks of gestation) at risk for systemic infection. Most of the term infants are not infected but rather have symptoms consistent with other medical conditions that mimic sepsis. The current standard of care for evaluating bacterial sepsis in the newborn is performing blood culturing and providing antibiotic therapy while awaiting the 48-h preliminary result of culture. Implementing a more rapid means of ruling out sepsis in term newborns could result in shorter NICU stays and less antibiotic usage. The purpose of this feasibility study was to compare the utility of PCR to that of conventional culture. To this end, a total of 548 paired blood samples collected from infants admitted to the NICU for suspected sepsis were analyzed for bacterial growth using the BACTEC 9240 instrument and for the bacterial 16S rRNA gene using a PCR assay which included a 5-h preamplification culturing step. The positivity rates by culture and PCR were 25 (4.6%) and 27 (4.9%) positive specimens out of a total of 548 specimens, respectively. The comparison revealed sensitivity, specificity, and positive and negative predictive values of 96.0, 99.4, 88.9, and 99.8%, respectively, for PCR. In summary, this PCR-based approach, requiring as little as 9 h of turnaround time and blood volumes as small as 200 μl, correlated well with conventional blood culture results obtained for neonates suspected of having bacterial sepsis.

Accurate clinical diagnosis of neonatal sepsis is often difficult, as signs and symptoms in the neonate may be subtle or vague (6, 12, 24, 35). Although the incidence of bacteremia is relatively low (one to eight cases/1,000 live births), the risk of mortality is quite high, ranging from 10 to 50% (10). As a result, between 4.4 and 10.5% of all newborns in the United States (~180,000 to 429,000 infants each year) receive systemic antibiotics (34).

Currently, blood culturing is considered to be the gold standard for diagnosing neonatal bacterial sepsis (33). However, even blood culturing techniques can have unacceptably low sensitivities (12). The reasons for this include intermittent seeding of low numbers of bacteria within the blood stream, the extremely small blood volumes obtained from infants for culturing, and the increasingly common practice of providing intrapartum antibiotics to mothers of high-risk deliveries (4, 30). It is well known that the smaller the volume of blood obtained for culturing, the lower the chances of recovering the organisms (1, 8). Although some infants may have a somewhat greater magnitude of bacteremia than adults (10 to 300 and 1 to 30 CFU/ml, respectively), the amount of blood sampled from newborn infants is significantly less than that taken from adults (0.5 to 1.0 and 10 to 30 ml, respectively). In fact, Kellogg et al. found that over half of the newborn infants demonstrated bacteremia with less than 10 CFU/ml (22).

Presently, intrapartum antibiotic therapy is considered the standard of care for women in labor with risk factors that are associated with neonatal sepsis (5, 7, 26, 31). These factors include premature delivery, prolonged rupture of membranes, fever, chorioamnionitis, and/or vaginal or rectal colonization with group B streptococci (GBS) (23, 30). The incidence of the last-named risk factor alone in all pregnant women is 5 to 35% (16, 23). The antibiotics received by women for GBS colonization can cross the placenta and achieve significant levels within the fetus (16). This practice has reduced significantly the incidence of GBS sepsis in the neonate. For example, at Magee-Women's Hospital (MWH), where the prevalence of GBS-positive cultures remains around 25 to 29% (23), the incidence of GBS sepsis went from 1.15 per 1,000 live births before the implementation of intrapartum antibiotic prophylaxis to 0.18 per 1,000 live births after its implementation (2). However, managing infants born to mothers receiving antibiotic prophylaxis within hours of delivery must include consideration of the effect that the drug may have on the bacterial cultures obtained from the infant. If the infant is infected and the particular bacterium is susceptible to the given drug, cultures may be falsely negative or slower to be detected (4, 30). The doubts surrounding the reliability of negative infant blood culture results may increase as more obstetricians implement antipartum screening and intrapartum prophlaxis against GBS (29).

Molecular techniques such as PCR have been used successfully to identify a wide range of organisms, including bacteria, yeasts, viruses, and protozoa (14, 20, 21, 27, 36). Unlike culture, these types of assays do not require growth of an organism for detection. This technology has proven to be quite useful in diagnosing nonculturable pathogens like human parvovirus B19 (20) or human papillomavirus (28) and has the potential for excellent sensitivity and a shorter turnaround time than those of culture-based protocols. Recently, bacterial DNA consensus sequences, e.g., the 16S rRNA gene, have been identified to define an organism as a bacterium. With such sequence information available, numerous DNA primers and probes have been described for use in PCR-based assays to diagnose bacterial sepsis (14, 17, 25, 27). These assays may prove useful for detecting or ruling out septicemia in term infants born to mothers who received intrapartum antibiotics. Although data describing the use of molecularly based testing

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for diagnosing viral, bacterial, or candidal infections in the adult and pediatric populations exist, the literature is nearly void for its application to the neonate population. The ability to rapidly and accurately rule out bacterial sepsis in a newborn infant might have a significant impact on the infant, the family, and the health care system.

For the foreseeable future, culture will not be superseded by PCR-based testing due to the requirement for purified culture isolates in antimicrobial susceptibility testing. However, if an amplification assay could reliably rule out neonatal sepsis in less time than bacterial culture, it would allow for the exclusive treatment of neonates with true infections, thus reducing the use of broad-spectrum antibiotics and the potential for infants who are not septic of acquiring drug-resistant bacteria. This approach would also permit shorter hospital stays within the neonatal intensive care unit (NICU) and reduce significantly the overall medical costs to the health care system as well as the emotional burdens of the families of these infants.

MATERIALS AND METHODS

Patient selection and sample rejection criteria. Our study received hospital institutional review board approval prior to its initiation. All infants admitted to the NICU for sepsis evaluation who had blood samples drawn for concomitant culture and a complete blood count (CBC) were eligible for inclusion in the study. No additional blood was collected from the infants for the purposes of PCR analyses. The discarded portions of the blood remaining after CBC testing were used for 16S rRNA gene PCR amplification. Sample rejection or exclusion criteria for PCR included blood volumes of <200 μl and grossly hemolyzed or clotted blood specimens.

Blood culture processing. Personnel at the study site used an automated continuous-monitoring blood culture system, BACTEC 9240 (Becton Dickinson, Sparks, Md.). The BACTEC system uses a fluorescent sensor for detecting microorganisms and relies primarily on the detection of CO_2 produced by actively metabolizing microorganisms. The pediatric-sample-sized, resin-containing blood culture bottles (Peds Plus; Becton Dickinson) were sent from the NICU prefilled with blood collected in a syringe from infants at their besides. Between 0.5 and 1.0 ml of whole blood was added per blood culture bottle. The bottles were incubated immediately upon receipt in the microbiology laboratory in accordance with the manufacturer's recommendation.

Whole-blood specimen preparation for PCR analysis. The entire remaining volume (200 to 500 μl) of discarded whole blood (pediatric-sample-sized EDTA-containing Vacutainer tubes) after CBC analysis was added to 4 ml of tryptic soy broth (TSB) (Difco Laboratories, Detroit, Mich.) and incubated for up to 5 h at 37°C in room air, with continuous shaking, after which the cellular fraction of the whole-blood sample was pelleted at 13,000 × *g* for 5 min at 4°C. One milliliter of 4°C lysis buffer, consisting of 0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, and 1% Triton X-100, was added to each cell pellet and left on ice for 5 min before the samples were again centrifuged as described above. To the remaining cell pellet, 100 μl of phosphate-buffered saline containing 50 U of mutanolysin (Sigma, St. Louis, Mo.) was added, and the pellet was incubated for 30 min at 37°C. Ten microliters of 10-mg/ml proteinase K (Sigma) was added and incubated for 30 min at 70°C. The proteinase K within the samples was inactivated by boiling for 10 min.

PCR amplification and product detection. Ten microliters of each prepared specimen was added to 90 μl of a PCR master mix consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM (each) dATP, dCTP, dGTP, and dUTP (Perkin-Elmer, Branchburg, N.J.), and either a 25 μM concentration of each of the primers RW01 and DG-74 (14) or a 100 μM concentration of each of the primers L1 and L2 (17), along with 2.5 U of *Taq* (Promega, Madison, Wis.) and 1 U of UNG (Perkin-Elmer). After sample addition, the PCR master mix was heated to 95°C for 10 min and subjected to 28 cycles of 1 min at 95°C and 2 min at 60°C (RW01 and DG-74 primers) or 2 min at 57°C (L1 and L2 primers) and 1 min at 72°C. Following amplification, the samples were held at 72°C until they were frozen at −20°C to inactivate the UNG enzyme. Twenty microliters of each amplified master mix was analyzed by gel electrophoresis using a 3% NuSieve–1% LE agarose gel (FMC BioProducts, Rockland, Maine). The 380-bp product was visualized under UV illumination after ethidium bromide staining.

Ultrafiltration of the PCR master mix. An ultrafiltration step, using an Amicon Centricon YM-100 centrifugal filter device (Millipore Corporation, Bedford, Mass.) was utilized for filtering the PCR master mix prior to sample inoculation (32). This filtration device, with a pore size that prevents the passage of proteins with a molecular weight of 100,000, holds back potential contaminating single-stranded DNA of greater than 300 bases or double-stranded DNA of greater than 125 bases when the master mix is spun at 1,000 × *g* for 1 h at 4°C but allows for passage of all components of the PCR master mix including the primers, *Taq*, and UNG. The filtration device was used as a safeguard to eliminate contaminating bacterial DNA that might be present in the reagents or the water used in preparing the PCR master mix (13, 32).

DNA dot blot hybridization. DNA dot blot hybridization analysis was carried out on all PCR-positive specimens to determine the nature of the bacteria for comparison to that determined by the blood culture results. Individually labeled oligonucleotide probes, RW03 (gram positive), DL04 (gram negative), and RDR245 (a universal probe), all previously described (14), were used to hybridize to the denatured 380-bp PCR product as follows. The resulting amplified PCR mixtures were heated to 95°C for 10 min to denature the double-stranded DNA before 20 μl of each mixture was applied in triplicate to the surfaces of prepared GeneScreen Plus membranes (catalog no. NEF-986; NEN-Life Science Products, Boston, Mass.), with subsequent cross-linkage using UV irradiation according to the recommendation of the manufacturer (SpectroLinker XL-1500; Spectronics Corp., Westbury, N.Y.). The prepared membranes were prehybridized for 1 h at 60°C in 5× SSPE (1× SSPE consists of 0.18 M NaCl, 10 mM Na_2PO_4, and 1 mM EDTA [pH 7.7]) containing 0.5% sodium dodecyl sulfate (SDS).

The oligonucleotide probes used were tail labeled with digoxigenin (DIG oligonucleotide tailing kit no. 1417231; Roche Molecular Biochemicals, Roche Diagnostics Corporation, Indianapolis, Ind.) using terminal transferase. Approximately 0.5 pmol of labeled probe was added per ml of 5× SSPE–0.5% SDS prehybridization solution before the membranes were incubated for 1 h at 60°C. The membranes were washed in 2× SSPE–0.1% SDS for 5 min at room temperature (RT), followed by an additional 5-min wash at RT in 3 M tetramethylammonium chloride–50 mM Tris-HCl (pH 8.0)–0.2% SDS and then by two 20-min washes at 65°C in the latter solution. The membrane was rinsed for 2 min at RT in a buffer consisting of 150 mM NaCl and 100 mM Tris-HCl (pH 7.5) before being incubated at RT for 30 min in the same buffer containing 1% blocking reagent (catalog no. 1096176; Roche Molecular Biochemicals). The hybridized product was detected using 150 mU of anti-digoxigenin-alkaline phosphatase Fab fragment (catalog no. 1093274; Boehringer Mannheim) (catalog no. 1093274; Roche Molecular Biochemicals) per ml and the chemiluminescent substrate CDP-Star (catalog no. 1685627; Roche Molecular Biochemicals) according to the manufacturer's recommendations, in combination with BioMax film (catalog no. 8689358; Eastman Kodak Company, Rochester, N.Y.).

RESULTS

PCR assay sensitivity. The sensitivity of the 16S rRNA PCR assay was determined using whole-blood samples spiked with known amounts of viable organisms, not purified bacterial DNA extracts. To this end, known numbers of CFU of *Streptococcus agalactiae*, calculated by agar dilution, were added to 0.5-ml volumes of whole blood containing EDTA.

The spiked whole-blood samples were prepared and amplified under exactly the same conditions as those described for the clinical blood specimens, with one exception. The inoculated TSB tubes were not incubated at 37°C for 5 h before sample preparation and extraction. Using this approach, as few as 13 CFU of bacteria could be detected after gel electrophoresis and ethidium bromide staining (data not shown).

Comparison of PCR and culture results for 548 paired blood samples from infants admitted to the NICU for sepsis evaluations. A total of 548 discarded blood samples obtained from infants admitted to the NICU for suspected sepsis were analyzed by PCR for the 16S rRNA gene, and results were compared to the concomitant blood culture results. The RW01 and DG-74 primers, previously described (14), were incorporated into the PCR master mix. Table 1 illustrates the comparison between the PCR results and those obtained using BACTEC 9240 and reveals a high level of agreement between the two methodologies, with sensitivity, specificity, and positive and negative predictive values of 96.0, 99.4, 88.9, and 99.8%, respectively.

Concordant culture-positive and PCR-positive samples. The positivity rates for blood specimens analyzed by culture and PCR were 25 (4.6%) and 27 (4.9%) positive specimens from a total of 548 tested, respectively. A review of the 24 concordant blood samples revealed detectable bacterial growth in Peds Plus blood culture bottles using BACTEC 9240. Using a combination of labeled oligonucleotide probes, it was possible to determine whether the 380-bp PCR amplimer originated from a gram-positive or a gram-negative organism. The types and

TABLE 1. Comparison of results of 16S rRNA gene PCR and BACTEC 9240 for detection of bacteria in neonatal blood samples[a]

PCR test result	No. of specimens with BACTEC 9240 result:		Total no. of specimens
	Pos	Neg	
Pos	24	3	27
Neg	1	520	521
Total	25	523	548

[a] Pos, positive; Neg, negative. PCR sensitivity, 96.0%; specificity, 99.4%; positive predictive value, 88.9%; negative predictive value, 99.8%.

numbers of organisms isolated by culture and further characterized by PCR are illustrated in Table 2.

At MWH, the most frequently isolated neonatal blood culture pathogens include coagulase-negative *Staphylococcus* species (41%), *Candida* species (17%), and *Staphylococcus aureus* (10%). Over the past 3 years, GBS were isolated from the blood cultures of three infants (1.6%), including two prior to the start of our study and one following its completion.

Concordant culture-negative, PCR-negative samples. The vast majority 94.9% (520 of 548 specimens) of neonatal blood samples lacked detectable levels of bacteria by both culture and PCR analyses. The results are illustrated in Table 1.

Culture-positive, PCR-negative discrepant samples. Table 1 illustrates results for a single blood sample that tested positive by culture but negative by PCR. The culture isolate was identified as a coagulase-negative *Staphylococcus* species. To ensure that the negative PCR result was not due to lack of primer sequence recognition, the purified DNA from the bacterial isolate was retested by PCR using the RW01 and DG-74 primers (14) and found to produce the predicted 380-bp product, which hybridized to both the universal and gram-positive probes (data not shown).

PCR-positive, culture-negative discrepant samples. Three specimens were PCR positive but culture negative (Table 1). They were collected from infants whose mothers were found to have negative GBS vaginal or rectal specimen cultures during their third trimester appointments. Two of these specimens were found to have 380-bp products which hybridized to the universal and gram-positive DNA probes, while the third specimen produced a 380-bp product which hybridized to the universal and gram-negative DNA probes (data not shown). Repeat PCR testing of these three discrepant samples using the L1 and L2 primer pair (17), which recognizes a distinct region of the 16S rRNA gene, resulted in identical findings compared to those obtained with the RW01–DG-74 primer pair (14).

TABLE 2. Bacterial isolates detected by both PCR and BACTEC 9240[a]

Culture ID	Probe reactivity	*n*
CoNS	Gram P	11
Staphylococcus aureus	Gram P	4
Enterococcus sp.	Gram P	4
Escherichia coli	Gram N	4
Klebsiella pneumoniae	Gram N	1
Bacillus sp.	Gram P	1

[a] ID, identification; *n*, number of isolates; CoNS, coagulase-negative *Staphylococcus* sp.; Gram P, gram-positive-bacterium-specific probe; Gram N, gram-negative-bacterium-specific probe. The size of each PCR amplification product was 380 bp.

Turnaround time for the 16S rRNA PCR assay. The approximate time required to complete the PCR assay described here is roughly 9 h. This includes 5 h of TSB incubation, 1.5 h for sample preparation, 2.0 h for DNA amplification, and 0.5 h for sample detection by gel electrophoresis.

DISCUSSION

This study was designed as a feasibility study, not as an outcome-based study, to compare the utility of a 16S rRNA PCR assay to that of the BACTEC 9240 system for detecting bacteria in blood obtained from neonates suspected of having bacterial sepsis. The comparison of 548 paired neonatal blood samples revealed a high level of agreement between the two methodologies, with sensitivity, specificity, and positive and negative predictive values of 96.0, 99.4, 88.9, and 99.8%, respectively, for PCR. The high negative predictive value that was calculated for the PCR assay compared to that of culture is indicative of the assay's usefulness in accurately ruling out the diagnosis of bacterial sepsis in the uninfected term neonate admitted to the NICU for such an evaluation.

This study reiterates the current problem; the vast majority of term infants admitted to the NICU for suspected sepsis are not infected but have symptoms consistent with those of other medical conditions that mimic sepsis, such as hypoglycemia, delayed transition, or transient tachypnea. Despite this fact, these term infants are treated with antibiotics for at least 48 h while awaiting the results of the preliminary blood culture report. Automated blood culturing systems are good given time, but if a laboratory test could be developed that would rule out bacterial septicemia in less time than blood culturing, those infants whose symptoms had resolved could be taken off of antibiotics and discharged from the NICU sooner.

NICU admissions and intravenous antibiotic therapies result in expensive hospital stays for infants that separate newborns from their mothers and create potential difficulties in successful bonding and breast-feeding, while exposing infants to antibiotics which are increasingly expensive and overused. At best these practices increase the financial burden on our health care system, and at worst they contribute to the increasingly serious problem of antibiotic resistance. Although blood culturing will not be completely replaced by a nucleic acid amplification technology anytime soon, as pure isolates remain essential for antimicrobial drug susceptibility testing, PCR does appear to be an excellent diagnostic test choice for a rapid means of ruling out bacterial sepsis in certain select patient populations.

There are numerous examples of PCR-based assays for detecting bacteria in blood, including *Streptococcus pneumoniae* DNA from whole blood (37) or inoculated Peds Plus bottles (11) and coagulase-negative *Staphylococcus* sp. from blood culture bottles (3). A different PCR-based assay that detected *Candida* sp. DNA directly from 26 of 27 blood samples obtained from neonates with culture-proven candidemia was developed (19). Another study illustrated the close agreement between PCR and bacterial culture in 15 of 16 culture-positive amniotic fluid samples obtained from women in premature labor (18). Recently, a multiplex approach was developed to detect neonatal sepsis, coamplifying portions of the 16S rRNA gene along with the housekeeping gene for GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (25). In that study, among the 33 newborn infants classified as being at risk for early-onset sepsis, Laforgia et al. was able to detect the 16S rRNA gene by PCR in all four of the culture-proven sepsis cases, as well as in two samples with negative culture results. Finally, a PCR assay using primers which recognize an 861-bp fragment of the 16S rRNA gene was suggested for use in

triaging bacterial sepsis (27). That study revealed the successful amplification of the rRNA gene from 12 different species of bacteria, including gram-negative and gram-positive organisms, without amplifying human genomic DNA.

Over the course of this study, a number of technical challenges were identified and successfully overcome. First of all, we came to appreciate the benefits of prefiltering the PCR master mix using the Centricon YM-100 centrifugal filter device (Millipore Corporation). Implementing this prefiltration step allowed the use of 28 cycles, compared to the originally indicated 25 cycles (14), without our experiencing the occasional false-positive result in the no-DNA control that had occurred with 28 and 30 cycles when prefiltering was not included. This change resulted in an increased assay sensitivity, permitting detection of 13 CFU/ml with 28 cycles, compared to 25 CFU/ml with 25 cycles (data not shown).

Second, a dramatic loss in sensitivity was observed when we attempted to detect the bacterial 16S rRNA gene from whole-blood specimens with volumes of appreciably less than 200 μl. More specifically, 19 culture-positive blood specimens whose paired samples for PCR testing had volumes ranging from 25 to 75 μl lacked detectable levels of the 16S rRNA gene (data not shown). This observation led us to establish a minimum blood volume requirement of 200 μl for 16S rRNA PCR testing.

Implementing a PCR-based assay for ruling out bacterial sepsis in the uninfected term neonate could potentially save significant health care dollars and reduce the emotional impact for families. In fact, Escobar et al. developed an algorithm for predicting which newborns admitted to the NICU to rule out sepsis are eligible for discontinuation of antibiotic treatment at 24 h based on 15 parameters, including clinical assessments, maternal risk factors, laboratory test results, and demographic data (9). In a retrospective analysis, their decision rule correctly identified the vast majority of babies with positive blood cultures, the persistently symptomatic babies with negative culture results, and a group of low-risk infants eligible for only 24 h of antibiotic treatment. More recently, Harrell et al. developed a clinical model for predicting outcomes for young infants which includes laboratory and diagnostic markers (15).

A similarly based algorithm containing a PCR-based assay for detecting the 16S rRNA gene in blood specimens might be used as an effective diagnostic tool in rapidly identifying uninfected term infants. The approximate time required to test neonatal blood for bacterial 16S rRNA gene is roughly 9 h. This includes 5 h of TSB incubation, followed by 1.5 h for sample preparation, 2 h for 28 cycles of DNA amplification, and 0.5 h for gel electrophoresis of the amplified PCR master mix. Attempts are under way to reduce still further the entire assay time, without sacrificing assay sensitivity.

The issue of organizing and managing sample processing is also important for minimizing the test turnaround time. Neonatal blood samples arrive in the laboratory continuously throughout the day. Samples should be set up immediately upon arrival into the laboratory, with two runs being completed each day. Blood samples inoculated into TSB by 8 a.m. could produce results by 5 p.m. Samples received after 8 a.m. would be incubated overnight in TSB, rather than the minimum 5 h and processed immediately the following morning, with final results being available before 12 noon. This approach would allow the NICU to receive finalized results on each neonatal blood sample from 9 to 28 h after receipt of the sample. On average, this work flow scheme could provide test results 1 full day earlier than the 48-h preliminary blood culture report would be received in the NICU.

For the term infant with a PCR-negative test result, discharge from the NICU might become possible if symptoms have resolved and vital signs have stabilized. In general, most term infants admitted to the NICU are there because of symptoms due to a poor transition immediately following birth and not because of bacterial sepsis. For these infants, symptoms usually resolve within the first 24 h of admission to the NICU.

In the three cases where the infants' blood specimens were found to be PCR positive but culture negative, the neonatalogists would have most likely treated them with a 7-day course of antibiotics for presumed sepsis, identical to that of an infant with a positive blood culture result.

In summary, the only way to prove the value of this PCR-based assay is to develop an algorithm with the NICU clinicians and to perform an outcome-based study. If found to be successful, this type of patient management could lead to shorter antibiotic courses and NICU stays for the otherwise healthy newborn infants. This approach is currently under evaluation at MWH for term infants suspected of having sepsis who are admitted to the NICU.

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