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MAJOR ARTICLE

Yield of Targeted Polymerase Chain Reaction in Probable Early-Onset Sepsis: A Prospective Cohort Study in Term and Near-Term Neonates With Negative Blood Culture Results

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Background. Discriminating noninfected from infected neonatal cases remains challenging, and subsequently many neonates are treated with antibiotics in the first week of life. We aimed to study the additional value of a targeted polymerase chain reaction (PCR) for group B streptococcus (GBS) and *Escherichia coli* on leftover blood culture media from term and near-term neonates with probable early-onset sepsis (EOS).

Methods. Leftover blood culture material from neonates participating in the RAIN study was stored after 5 days of incubation. The RAIN study evaluated intravenous-oral antibiotic switch in probable bacterial infection, defined as risk factors and/or clinical signs and elevated inflammatory parameters but negative blood culture results. We applied 2 targeted PCRs for GBS and *E coli*, the main pathogens in EOS, and analyzed the samples batchwise in triplicate for each PCR.

Results. PCR was performed in triplicate on blood culture media from 284 neonates. In 23 neonates, the PCR result was positive (3 cycle threshold values <37) for GBS (n = 1) or *E coli* (n = 22). Inflammatory parameters did not discriminate for positive PCR result, nor did risk factors for sepsis, such as maternal GBS status and prolonged rupture of membranes. However, 96% of neonates with a positive PCR result were born vaginally vs 74% in the PCR-negative group ($P = .05$); furthermore, 96% vs 81% ($P = .21$) of neonates had clinical symptoms.

Conclusions. Blood culture–negative "probable" EOS in neonates is accompanied by an 8% rate of PCR positivity, suggesting low-grade bacteriemia after birth with yet unclear clinical consequences. Further research should focus on how PCR can contribute to more targeted antibiotic use of neonates, specifically in those highly suspected of infection but in the absence of a positive blood culture result.

Keywords. *E coli*; early-onset sepsis; group B streptococcus; PCR.

Neonatal bacterial infections are associated with substantial mortality and morbidity. Therefore, many neonates are exposed to antibiotics early in life. The most frequent pathogens in early-onset sepsis (EOS) in Western Europe are group B streptococcus (GBS) or *Escherichia coli* [1, 2]. However, the incidence of culture-proven EOS is low in late-preterm and term neonates (0.49/1000 live births). Significantly more patients receive prolonged therapy because of probable bacterial infection [2]. Those include neonates who show clinical signs and symptoms and/or elevated inflammatory markers but in whom blood culture results remain negative. These infants are likely a mix of those with infection and those with problems with adaptation to extrauterine life. Unfortunately, inflammatory markers do not always aid in this discrimination [3]. In terms of antibiotic stewardship, it is essential to discern those infants who benefit most from antimicrobial therapy, as exposure has been associated with gut microbiome dysbiosis and increased microbial resistance [4, 5]. Blood culture remains the gold standard for the diagnosis of bacterial infection and, when inoculated correctly, has a high discriminating power. Sensitivity is influenced by timing of collection, volume inoculated, and preanalytic time. Practically, the collection of an adequate sample (≥1 mL) can be difficult in neonates [6].

Low-grade bacteremia (<10 colony-forming units [CFU]/mL) has been reported in neonates, especially for *E coli* [7, 8]. This, with the frequently low inoculated volumes, could partly explain the high rates of culture-negative probable EOS in

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neonates. Molecular diagnostics could be a promising add-on tool to help discriminate infected from noninfected cases. These techniques have several advantages, including a short turnaround time (±6 hours vs 36–48 hours for culture-based methods) and the lower blood volume needed [9]. Only a few studies have evaluated the use of polymerase chain reaction (PCR) in EOS [10, 11]. In these studies, a multiplex PCR approach is used. Data are currently lacking on the use of a targeted PCR in term neonates with probable EOS. We aimed to study the proof of concept and additional yield of a targeted PCR for GBS and *E coli* on leftover blood culture media from term and near-term neonates with a probable bacterial infection.

METHODS

This study was a substudy of the multicenter RAIN study, in which we evaluated the efficacy and safety of oral antibiotic switch therapy after 48 hours of intravenous treatment in 510 neonates (≥35 weeks) with a probable bacterial infection (ie, clinical symptoms and/or risk factors and elevated inflammatory markers in the absence of a positive blood culture result). Clinical and laboratory details from those participants were gathered in a database. For most neonates, inflammatory parameters were measured at baseline/infection suspicion (T0) and 24 hours after the start of antibiotics (T24). More details can be found elsewhere [12]. In this substudy, 284 patients from 14 of 17 participating centers were included. Clinical symptoms were present in 82% of neonates in this substudy; all neonates had a C-reactive protein value >10 mg/dL, with a mean ± SD of 37 ± 23 mg/dL at 24 hours after infection suspicion. Blood cultures were obtained at the moment of infection suspicion, prior to initiation of antibiotics. All cultures were incubated for 5 days in the local hospital and remained negative. No additional cultures were performed. After 5 days of incubation, blood culture material was stored at −80 °C (2 × 2 mL of blood mixed with culture medium, transferred into cryovials under sterile conditions). Moreover, material from a patient ward was used as a positive control reference and stored at −80 °C; this consisted of 5 neonatal blood cultures: 3 positive for *E coli* and 2 positive for GBS. The study was approved by the Medical Ethics Committee of Erasmus MC (NL51888.078.16). Written informed consent was obtained for all participants.

PCR Development

We applied 2 real-time PCRs targeting GBS and *E coli*, as those are the most frequent pathogens in EOS, covering >50% of positive cultures [2]. The PCR for *E coli* was based on Pavlovic et al [13], whereas the GBS PCR involves a new design targeting the *dltS* gene. Both PCRs were able to detect down to an approximately 1-cell equivalent of DNA per PCR. The sample (1 mL) was processed with serum separator tubes (Becton Dickinson), and the interphase, containing white blood cells and bacterial cells, was carefully collected with a cotton swab and resuspended in 250 µL of phosphate-buffered saline. Phocine herpes virus was used as an internal process control [14]. Phosphate-buffered saline was used as negative process control. DNA was extracted on a MagNA Pure 96 platform in combination with the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Diagnostics) and eluted in 50 µL. PCR reactions (20 µL) were performed on a LightCycler 480 and contained 5 µL of DNA, primers, and probe (Supplementary Table 1) in 1× LightCycler 480 Probes Master (Roche Diagnostics). The amplification protocol consisted of an initial denaturation for 5 minutes at 95 °C, followed by 50 cycles of denaturation for 5 seconds at 95 °C and annealing/extension for 30 seconds at 60 °C, after which the samples were cooled down. Primers and probe concentrations were optimized for maximum sensitivity [15]. Known positive GBS and *E coli* PCR controls consisted of DNA isolated from blood culture bottles positive for GBS and *E coli*. All PCRs were performed in triplicate for GBS and *E coli* for all neonates batchwise. Cycle threshold (Ct) values (ie, the number of cycles at which the PCR turned positive) were determined with the user-independent second derivative maximum method.

Categories Based on PCR Outcomes

Participants were classified into 3 groups according to the probability of a true bacterial infection based on the triplicate PCR results. Individual PCR results (for GBS and *E coli* separately) were considered positive if the Ct value was <37.0. Cases were considered negative when Ct values in all 3 PCRs for either GBS or *E coli* were ≥37.0. Cases were considered possibly positive when 1 or 2 of the 3 PCR results were positive. Cases were considered definitely positive when all 3 PCR results for either GBS or *E coli* were positive. From spiking experiments, it was determined that with the given procedure, the Ct value of 37 corresponded to ∼700 cells for *E coli* and ∼5000 cells for GBS.

Data Analysis

Differences in neonatal baseline and infection characteristics among the 3 categories were assessed by chi-square tests for categorical data and analysis of variance test or nonparametric Kruskal-Wallis test for continuous data, depending on the data distribution. $P < .05$ was considered statistically significant. Statistical analyses were performed with SPSS version 28.0.1.0 (IBM).

Table 1. Baseline Characteristics of Participants by Group (N = 284)

Variable	PCR, No. (%), Median [IQR], or Mean ± SD			
	Definitely Positive (n = 23)	Possibly Positive (n = 19)	Negative (n = 242)	*P* Value
Female sex	9 (39.1)	5 (26.3)	97 (40.1)	.50
Gestational age, wk[a]	40.3 [39.4–41.1]	41.0 [40.1–41.4]	40.3 [39.4–41.1]	.04
Birth weight, g	3532 ± 534	3796 ± 528	3603 ± 517	.22
Nullipara	15 (65.2)	12 (63.2)	167 (69.0)	.89
Mode of delivery				.12
Vaginal	18 (78.3)	15 (78.9)	153 (63.2)	
Vaginal instrumental	4 (17.4)	1 (5.3)	27 (11.2)	
Caesarean section	1 (4.3)	3 (15.8)	62 (25.6)	
Mode of delivery combined				.05
Vaginal (natural + instrumental combined)	22 (95.7)	16 (84.2)	180 (74.4)	
Caesarean section	1 (4.3)	3 (15.8)	62 (25.6)	
Maternal GBS status[b]				.24
Screened: colonized	2 (8.7)	4 (21.1)	41 (16.9)	
Previous child with GBS disease	1 (4.3)	1 (5.3)	1 (0.4)	
Screened: not colonized	5 (21.7)	4 (21.1)	49 (19.8)	
Not screened	15 (65.2)	10 (52.6)	151 (62.4)	
Prolonged rupture of membranes >24 h[c]	5 (21.7)	4 (21.1)	88 (36.4)	.17
Maternal fever >38.0 °C	8 (34.8)	5 (26.3)	122 (50.4)	.06
Suspected intrauterine infection requiring maternal antibiotic treatment	7 (30.4)	3 (15.8)	72 (29.8)	.43
Clinical symptoms at onset of infection[d]	22 (95.7)	16 (84.2)	196 (81.0)	.21
Respiratory	20 (90.9)	15 (93.8)	157 (80.1)	
Circulatory	3 (13.6)	1 (6.3)	58 (29.6)	
Gastrointestinal	3 (13.6)	1 (6.3)	23 (11.7)	
Neurologic	4 (18.2)	1 (6.3)	27 (11.2)	
Temperature instability, <36.5; >38.0 °C	9 (40.9)	4 (25.0)	68 (34.7)	
Behavioral changes	1 (4.3)	3 (15.8)	46 (23.5)	
Requiring any respiratory support	9 (39.1)	10 (52.6)	92 (38.0)	.45
C-reactive protein, mg/L[e]				
T0 h	2.5 [1.0–4.0]	13.0 [2.0–24.0]	7 [0–17.4]	.15
T24 h	29.0 [18.0–49.0]	48.0 [21.0–56.0]	30.0 [14.0–48.0]	.72
Procalcitonin, ng/mL[f]				
T0 h	1.5 [0.6–23.0]	…	6.2 [3.3–14.3]	.12
T24 h	16.0 [4.8–38.8]	…	15.0 [1.2–28.9]	.80

P values represent differences among the 3 groups.

Abbreviations: GBS, group B streptococcus; PCR, polymerase chain reaction.

[a]Early-onset sepsis: infection suspicion <72 hours after birth.

[b]Colonization rates available at the moment of the start of antibiotic treatment.

[c]Timing of ruptured membranes was defined as the period between rupture of membranes and birth.

[d]Percentage of patients in whom a specific symptom has been reported among those who showed symptoms. Multiple symptoms were reported in a single patient; therefore, the total of percentages >100%.

[e]T0 missing in 5 patients and T24 missing in 17 patients.

[f]Procalcitonin levels were reported in 75 patients; elevated levels were based on the age-related nomogram.

RESULTS

In total, 284 patients from 14 of 17 participating centers were included. Table 1 describes the baseline characteristics of the participants subdivided per group.

The PCR result was definitely positive in 23 of 284 cases (8.1%); 22 *E coli* (7.7%) and 1 GBS (0.4%). There was no statistically significant difference among the 3 groups in baseline and infection-related characteristics (symptoms, risk factors, and elevated inflammation markers), although vaginal delivery was possibly associated with positive cases ($P = .05$). We measured inflammatory markers in all patients at least at 1 time point but preferably at 2 time points (C-reactive protein: T0, n = 279; T24, n = 269; procalcitonin optional based on availability in the hospital: T0, n = 78; T24, n = 74). Inflammatory markers were not different among the groups.

Specific Details Regarding PCR Positivity

In the *E coli*–positive blood culture control group (n = 3), the mean Ct value was 14.98 ± 0.78. In the definitely positive *E coli* group, the mean Ct values were higher when compared

with the blood culture positive control group, with a mean 33.25 ± 0.84.

In the GBS-positive blood culture control group (n = 2), the mean Ct value was 18.42 ± 3.55. In the study group, the PCR for GBS was definitely positive in 1 case (mean Ct value, 16.54 ± 0.08). This neonate was born vaginally with unknown maternal GBS colonization status and without risk factors. The neonate showed respiratory signs requiring noninvasive support. C-reactive protein increased to 65 mg/L at 24 hours after antibiotic initiation.

As PCR results were not available to the clinicians, all participants were treated for a probable EOS with a 7-day antibiotic course: either penicillin/gentamicin intravenous or penicillin/gentamicin for 2 days with intravenous-oral switch to amoxicillin/clavulanic acid for 5 days and discharge home according to the RAIN study protocol. No culture-proven infections or reinfections occurred in participants during 1-month follow-up after antibiotic cessation.

DISCUSSION

This study is the first to describe the yield of targeted PCR in probable EOS. Evidence for GBS and *E coli* was observed in the material of negative neonatal blood cultures in a substantial number of neonates (8.1%). Given the difficulties associated with neonatal blood cultures, as outlined in the introduction, and the unreliable nature of elevated inflammatory markers, clinicians require additional tools, such as PCR to guide antibiotic therapy in probable EOS effectively in the light of antibiotic stewardship.

As the Ct values of *E coli* in the study group reflect a roughly 1 million–fold lower number of bacteria when compared with the positive control reference, it can be questioned whether this number is too low to be detected by blood culture or if this originates from DNA isolated from nonviable bacteria. It is unknown how many bacteria are needed in a blood culture bottle to become positive; figures vary between 1 and 10 CFU/mL to 1000 and 10 000 CFU/mL [16]. However, this does not apply to the Ct value of GBS in the study patient, which was comparable to the Ct values of positive controls. Why this culture remained negative is unknown. There was no maternal pretreatment. Preanalytic time might have led to a detection failure or a low volume of blood inoculated [17, 18].

We were unable to distinguish positive from negative cases based on clinical and inflammatory-related characteristics, highlighting the imperfect diagnostic value of inflammatory markers for EOS. Nonetheless, in cases with a definite positive result, nearly all patients (22 of 23) experienced a wide array of clinical symptoms and were predominantly born through vaginal delivery. This suggests a potential scenario of perinatal translocation of maternal flora during labor, which could account for the detection of low-grade bacteremia and/or bacterial DNA in these cases. All neonates in our study received 7 days of antimicrobial treatment, so we could not determine the clinical significance of the positive blood PCR result. Its clinical significance, also in relation to Ct values, should be further studied. It remains uncertain whether a newborn's immune system can effectively combat a low bacterial load independently or if a brief course of antibiotics (<36 hours) is necessary. In a recent study, some asymptomatic neonates with risk factors for EOS experienced transient bacteriemia that resolved without treatment [19]. Ethical considerations prevented us from the inclusion of healthy newborns not being suspected for EOS; thus, the presence of PCR positivity in this group cannot be estimated. In future studies, PCR and blood culture can be used to study infants with an infection suspicion and whether a short course of treatment is sufficient in those with negative blood culture results but positive PCR results with high Ct values.

This study has several strengths. First, we used a unique cohort of neonates with a probable bacterial infection only. There are major concerns about overtreatment among these patients, and improving diagnostics is therefore urgently needed. Second, we developed a unique targeted PCR that can be used on leftover blood culture material, thereby reducing the burden for the individual patient, which is especially relevant in the case of neonates. Since the complexity of a PCR exponentially increases with the number of primers and probes used, a monoplex targeted PCR may prove more robust and specific than a highly multiplexed assay. Third, we performed the assay in triplicate to obtain more certainty about true positives and analyzed all samples in a single run. With regard to the interpretation of the data, we chose a strict cutoff Ct value of 37 and used triplicate results to prevent false-positive PCR results, and our data show that the mean Ct value in the positive group is 33. An inability to reproduce PCR results at high Ct values is a logical consequence of stochastic target distribution at very low concentrations. False positives, especially for *E coli*, can occur due to the presence of contaminating DNA in any of the DNA extraction or PCR reagents used. However, high Ct values (>40) are then expected and were also observed in negative controls.

Limitations concern differences with regard to blood culture sampling methods between sites, although sampling and storage procedures followed a predefined protocol. Culture bottle brands varied, as did the moment of incubation start after blood withdrawal, which might influence the detection rate [18]. A second limitation is that the inoculated blood volume was not recorded. It is well known that ≥1 mL should be inoculated; we do expect that lower volumes have been inoculated. Next, as we ran this assay on specimens incubated for 5 days, its clinical utility is limited at this time. Finally, we chose to target the 2 most important pathogens of neonatal infection in our setting, as we wanted the assay to be as sensitive as possible

and did not want to detect possible contaminants. However, we are aware of the fact that EOS can be caused by pathogens other than GBS and *E coli*, especially in non–high-income settings [20].

In conclusion, we developed a targeted PCR for GBS and *E coli* that can be used on blood culture material (medium with blood). We report a positive rate of 8% in neonates with probable EOS with negative blood culture results, especially *E coli* among vaginally born neonates. PCR might aid in treatment decisions in probable EOS and limiting neonatal antibiotic use. Additional research should focus on the use of PCR on whole blood or blood culture material, applying the PCR more early after infection suspicion to guide antibiotic treatment. However, the clinical significance of a positive PCR result in the absence of a positive blood culture result and the occurrence of low-grade bacteremia among suspected infection cases with low inflammatory markers should be further studied.

Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. F. M. K., C. H. W. K., R. F. K., M. v. W., and G. A. T.-S. made substantial contributions to the conception and design of the trial, the interpretation of data, and the funding acquisition. F. M. K., R. F. K., and G. A. T.-S. made significant contributions to the acquisition of the data. C. H. W. K. was responsible for the development and performance of the PCR. F. M. K., C. H. W. K., M. v. W., and G. A. T.-S. were responsible for the analysis of the data. F. M. K., C. H. W. K., and G. A. T.-S. directly accessed and verified the underlying data reported in the manuscript. F. M. K. and G. A. T.-S. drafted the manuscript. C. H. W. K., R. F. K., M. v. W., and G. A. T.-S. provided a critical revision for important intellectual content. All authors approved the final version to be published and agree to be accountable for all aspects of the work. F. M. K., C. H. W. K., and G. A. T.-S. had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest.

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