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Bacterial DNA detection in very preterm infants assessed for risk of early onset sepsis

https://doi.org/10.1515/jpm-2021-0184
Received April 16, 2021; accepted November 22, 2021; published online December 7, 2021

Abstract

Objectives: The aim of this study is to evaluate the diagnostic ability of multiplex real-time polymerase chain reaction (PCR) in very preterm infants assessed for risk of early onset neonatal sepsis (EOS).

Methods: Prospective observational cohort study. Blood samples of preterm neonates ≤32 weeks of gestation were evaluated by commercial multiplex real-time PCR within 2 h after delivery. The definition of EOS was based on positive blood culture and clinical signs of infection or negative blood culture, clinical signs of infection and abnormal neonatal blood count and serum biomarkers.

Results: Among 82 subjects analyzed in the study, 15 had clinical or confirmed EOS. PCR was positive in four of these infants (including the only one with a positive blood culture), as well as in 15 of the 67 infants without sepsis (sensitivity 27%, specificity 78%). Out of 19 PCR positive subjects, *Escherichia coli* was detected in 12 infants (63%). Statistically significant association was found between vaginal *E. coli* colonization of the mother and *E. coli* PCR positivity of the neonate (p=0.001). No relationship was found between neonatal *E. coli* swab results and assessment findings of bacterial DNA in neonatal blood stream.

Conclusions: Multiplex real-time PCR had insufficient diagnostic capability for EOS in high risk very preterm infants. The study revealed no significant association between PCR results and the diagnosis of clinical EOS. Correlation between maternal vaginal swab results and positive PCR in the newborn needs further investigation to fully understand the role of bacterial DNA analysis in preterm infants.

Keywords: bacterial DNA; early onset sepsis; preterm infant; real-time PCR.

Introduction

Early onset neonatal sepsis (EOS) remains a challenging issue accompanied by alarmingly high mortality and adverse outcomes [1, 2]. The diagnosis is based on a positive blood culture and the time of onset within 72 h after delivery [3]. It is considered to be the result of a vertical pathogen transmission [4]. A high risk of EOS is seen specifically in the case of prolonged premature rupture of membranes, clinical and/or laboratory signs of chorioamnionitis and presence of maternal urinary tract infection [4]. Gestational age of <34 weeks is another major risk factor increasing the likelihood of early infection up to tenfold [5]. The total rate of EOS is 0.98 per 1,000 live births, whilst in very preterm infants the rate is 10.96 per 1,000 live births [6].

Reported incidence of EOS generally includes culture-confirmed cases only, despite large numbers of infants treated with antibiotics for culture-negative sepsis [7]. In contrast, the ongoing development of the pediatric definition of sepsis is based on the assessment of organ dysfunction, as the failure to isolate the pathogen does not rule out sepsis [8, 9]. The incidence of culture-negative EOS is uncertain, though it could be 6 to 16 times higher than the number of confirmed cases [7]. The main reasons for this include blood culture limited sensitivity, maternal antimicrobial treatment and insufficient sample collection [3]. Moreover, an indeterminate number of culture-negative infants are possibly not infected [7]. This discrepancy between the standard of EOS diagnostics and clinical practice leads to efforts for alternative detection of causative agents, as more accurate and rapid diagnostic tools of EOS etiology are essential for the improvement of prognosis and rational use of antibiotics [2, 10].

Particularly, molecular diagnostics such as polymerase chain reaction (PCR) could offer faster results (within 12 h from sampling) and higher sensitivity compared to blood culture, even in the case of maternal exposure to antibiotic treatment [3, 11]. Multiple molecular assays were...
Blood culture

Blood cultures were investigated using the BacT/Alert automated blood culture monitoring system (Bact/Alert, BioMerieux, US). Immediately after sample collection, the blood samples were transported to the microbiology laboratory. Here they were loaded into the Bact/Alert culture instrument and were incubated for a standard period of 5 days (120 h) before being flagged as negative. Bottles flagged up as positive were subcultured on Columbia agar +5% sheep blood (BioMerieux), UriSelect™ 4 Medium (BioRad) and Schaedler agar +5% sheep blood (BioMerieux) plates and a microscopic slide for Gram stain was prepared. After 4–6 h of incubation, the streaked agar plates were inspected and in case of a detectable growth, the visible colonies were identified using the Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS) on a MicroFlex LT/SH smart platform (Bruker Daltonik GmbH).

Conventional microbiological methods

Bacterial DNA extraction

Bacterial DNA was extracted from citrated blood samples by using a QIAamp DSP DNA Mini Kit, according to the manufacturer’s protocol (QIAGEN, Hilden, Germany). Briefly, DNA was extracted from 500 µL of citrated blood and eluated into 50 µL of elution buffer. The eluates were stored at −20 °C until further analysis.

Real time PCR analysis

Two multiplex real time PCR assays were used during the study period to detect bacterial nucleic acid for the evaluation of infections in neonates. BactoFlexx Real-Time PCR Kit (KITGEN, Trinec, Czech Republic) and FTD Neonatal sepsis (Fast Track Diagnostics, Luxembourg) were used for the amplification and detection of the pathogens. Nucleic acid input was 2 µL for BactoFlexx and 10 µL for FTD Neonatal sepsis. Initial denaturation and thermocycling (°C/seconds) were 94/300 and 94/30, 58/30, 72/30 for BactoFlexx assay, for FTD Neonatal sepsis assay they were 94/180 and 94/8, 60/36 respectively. Cycles setting was 50 for BactoFlexx and 40 for FTD Neonatal sepsis. Cycles positivity assessment was s≤5 for both assays. The quantitative real-time PCR assays were processed on Rotor-Gene Q (QIAGEN, Hilden, Germany). BactoFlexx Real-Time PCR Kit was used until an assay validated for neonatal samples became available. It enables to detect a wide scale of bacterial pathogens: Acinetobacter baumannii, Enterobacter cloacae, Escherichia coli, Klebsiella oxytoca, Klebsiella pneumoniae, Proteus mirabilis, Pseudomonas aeruginosa/putida, Serratia marcescens, Stenotrophomonas maltophilia, Enterococcus faecalis, Enterococcus faecium, coagulase-negative staphylococci, Staphylococcus aureus, Streptococcus pneumoniae, Streptococcus
The number of cycles was used to quantify bacterial DNA load (BDL). The Real-Time thermocycler. Subsequently, Ct analysis was performed. Ct is a cycle threshold value (Ct) by the detection of an increase in observed fluorescence from the relevant reaction which is introduced into each sample and it also shows the correctness of the extraction process. The individual pathogen-specific PCR tests were performed using a dual-labeled probe, and was reported as a cycle threshold value (Ct) by the Real-Time thermocycler. Subsequently, Ct analysis was performed. The number of cycles was used to quantify bacterial DNA load (BDL).

Neonatal blood count and biomarkers

Blood counts were measured with a Coulter Micro Diff II (Coulter Electronics Ltd., Fullerton, US). CRP was measured by immunoturbidimetry (Cobas 6000, e501 module, Roche Diagnostics, Mannheim, Germany). Immunoluminometric assay (Lumitest PCT, Brahms, Germany) was used for PCT analysis. Luminescence was measured automatically in a Berlux Analyser (Behring Diagnostics, Germany). Serum IL-6 level was measured by electrochemiluminescence immunoassay (Cobas 6000, e601 module, Roche Diagnostics, Mannheim, Germany).

Early onset sepsis and neonatal outcome

Data were collected from neonatal and maternal medical records. The definition of early onset sepsis (EOS) was based on positive blood culture and clinical signs of infection or negative blood culture with manifested clinical symptoms and abnormal levels of two or more biomarkers in the first 72 h of life. The range of 5,000–20,000 white blood cells (WBC) in a microliter of blood was considered as normal. An I:T ratio of ≥0.2, CRP >10 mg/L, PCT >2 ng/mL and IL-6 >200 ng/L were considered as abnormal [3, 15, 16]. Clinical signs of an early onset infection included: hypothermia, respiratory instability (apnea, de-saturations, respiratory distress syndrome with ongoing mechanical ventilation), cardiac (cyanosis, bradycardia, poor peripheral perfusion, hypotension) and neurological (lightery, suspected seizures) symptoms [17]. Higher risk of EOS was considered in the presence of clinical and/or laboratory chorioamnionitis, Group B Streptococcus (GBS) and/or E. coli (E. coli) colonization of the mother, preterm premature rupture of membranes (PPROM) and preterm onset of labor [18]. Antibiotics were started by the attending physician according to the presence of risk factors, clinical and/or laboratory signs of infection. After 48 h, a decision was made whether to continue with antibiotic treatment according to blood culture findings and the dynamics of clinical signs, blood count and levels of serum biomarkers. Attending physicians were made aware of the PCR result once available, but the obtained result was not considered crucial for the treatment algorithm.

Other neonatal outcomes (respiratory distress syndrome, patent ductus arteriosus, intraventricular hemorrhage, necrotizing enterocolitis, periventricular leukomalacia, retinopathy of prematurity and bronchopulmonary dysplasia) were followed up according to the Vermont-Oxford definition [19].

Statistical analysis

Data were reported using descriptive statistical methods. Univariate analyses were performed using Chi-square, Fisher’s exact and Mann-Whitney U tests. All reported p-values are two-sided and not adjusted for multiplicity. A value of p<0.05 was considered statistically significant. Data analysis was performed using the IBM SPSS Statistics 25.0.0.0 software (IBM Corp., Armonk, NY).

Results

One hundred and three infants were eligible for the study. Twenty-one infants were excluded due to insufficient sample volumes, incomplete laboratory samples and unavailable PCR test. The characteristics of the study group (n=82) are summarized in Table 1.

PCR positive group

Twenty-nine infants were assessed by BactoPlex Real-Time PCR Kit, 53 by FTD Neonatal sepsis. Nineteen neonates were PCR positive. Seven positive results were assessed by BactoPlex Real-Time PCR Kit, 12 by FTD Neonatal sepsis. No difference was found in the capability to detect bacterial

| Table 1: Characteristics of the study group (n=82). |
|-----------------------------------------------|
| Gestational age, weeks | 27.0 ± 1.9 |
| Birth weight, g | 990 ± 311 |
| Sex, male | 54 (66) |
| Mode of delivery |  |
| Spontaneous | 15 (18) |
| C-section | 67 (82) |
| Clinical chorioamnionitis | 29 (35) |
| Clinical chorioamnionitis | 29 (35) |
| Histological chorioamnionitis | 35 (42) |
| Chorioamnionitis | 16 (20) |
| Chorioamnionitis and funisitis | 12 (15) |
| Preterm premature rupture of membrane | 26 (32) |
| Antibiotics in mothers | 50 (61) |
| Invasive prenatal procedures |  |
| None | 46 (56) |
| Amniocentesis | 32 (39) |
| Other | 4 (5) |
| Early onset sepsis |  |
| No sepsis | 67 (82) |
| Proven or clinical sepsis | 15 (18) |
| Positive serum bacterial DNA load | 19 (23) |
| Mortality | 8 (10) |

*Continuous variables are expressed as mean ± standard deviation. Categorical variables are presented as number (percent).

Histological examination was not performed in 25 cases.
DNA between both tests (24% vs. 23% respectively, p=0.99). *E. coli* was the most frequently detected strain (12 episodes, 63%). The remaining seven positive results included *Streptococcus* species (2 episodes), *S. aureus* (2), *S. maltophilia* (1), *Ureaplasma parvum* (1) and *Coagulase-negative Staphylococci* (1). The summary of microbiological and PCR findings of positive patients is expressed in Table 2. Only one patient had a positive blood culture with a corresponding PCR result (*E. coli*). Three patients regarded as infected had a negative blood culture and positive PCR result (*S. aureus*, *U. parvum*, *E. coli*). Eleven patients were treated as infected despite no pathogen detection. This shows a low PCR sensitivity of 27% for EOS. In two cases the clinical team decided to discontinue antibiotic treatment after 48 h despite a positive PCR result (*S. maltophilia*, *E. coli*). Forty-two patients received no antibiotic treatment for EOS and had no clinical and/or laboratory signs of early infection. Thirteen of them were positive for PCR. This shows a moderate specificity and a negative predictive value of 78% and 83%, respectively. The accuracy of the PCR test for EOS was 68%. We found no association of positive PCR results with clinical or histological chorioamnionitis and neonatal outcomes. WBC count was significantly lower in the PCR positive group, but it did not meet the EOS criteria. Comparison of clinical data and laboratory findings over the period of 2 h after delivery between PCR positive and negative patients are summarized in Table 3.

**E. coli** PCR positive sub-group

*E. coli* was the most frequent pathogen found in swabs of both mothers (16 cases, 20%) and neonates (13 cases, 16%), however there was no statistically significant link between them (p=0.71). No relationship was detected between neonatal *E. coli* swabs (ear, stomach, axilla) and bacterial PCR blood stream assessment results. Statistically significant association was revealed between vaginal *E. coli* colonization of mothers and *E. coli* PCR positivity in infants (44% PCR positive neonates of mothers with *E. coli* positive vaginal swabs vs. 8% PCR positive neonates of *E. coli* negative mothers, p=0.001), regardless of PPROM presence and mode of delivery. A positive *E. coli* vaginal swab result was not associated with higher frequency of chorioamnionitis and funisitis. No differences in neonatal morbidity, neonatal infection, mode of delivery and blood sampling procedure were found between *E. coli* PCR positive and negative subjects.

**Use of antibiotics**

Antibiotic treatment was commenced in 40 infants overall, for suspected EOS and the presence of risk factors. In 25 patients with negative blood culture results, the antibiotics were discontinued after 48 h. In 15 infants with persistent clinical and laboratory findings (including one case with positive blood culture), antibiotics were continued beyond 48 h.

**Discussion**

The latest Cochrane systematic review on molecular assays for the diagnosis of sepsis in neonates reveals a reasonable level of overall sensitivity and specificity. However, only five studies out of 35 in the summary of findings investigated preterm neonates alone and only two exclusively EOS. The reported quality of evidence regarding these studies was low [20].

Our observational study investigated solely very preterm infants at lower and higher risk of EOS [18]. Among the 19 PCR positive results, only one case can be considered truly positive for EOS, as defined by the current diagnostic gold standard [21]. Despite the statistical differences in levels of inflammatory biomarkers (WBC count, PCT) between the PCR positive and negative groups, 15 bacterial PCR positive cases showed insufficient association with clinical and/or other laboratory findings to meet the definition of suspected EOS. Moreover, the revealed statistically significant difference in WBC levels has no clinical impact (95% CI: 5.2–7.6 × 1,000/uL in DNA positive vs. 8.4–12.3 × 1,000/uL in DNA negative cases). PCT values were low in both groups and the difference had limited statistical significance.

An unexpected association between maternal *E. coli* positive vaginal swabs and *E. coli* DNA detection in neonatal blood was found (44% PCR positive neonates of mothers with *E. coli* positive vaginal swabs vs. 8% PCR positive neonates of *E. coli* negative mothers, p=0.001). We could not identify any link between *E. coli* PCR positivity and placental histological findings, PPROM, maternal antibiotic treatment or mode of delivery. We hypothesize that maternal colonization may lead to intra-amniotic invasion and transient blood stream DNA presence in some very preterm infants [14]. Contamination within sample handling is possible, but less likely under these circumstances.
Table 2: Microbiological and PCR summary of findings in positive patients.

| ID | GA, weeks | BW, g | PCR (organism) | PCR (BDL) | Number of cycles | PCR assay | Blood culture | Neonatal swabs (ear, axilla, stomach) | Vaginal swab | Neonatal infection | Comments |
|----|-----------|-------|----------------|-----------|-----------------|-----------|---------------|--------------------------------------|-------------|------------------|----------|
| 3  | 28        | 950   | CoNS           | 672       | 33 BactoPlex    | Negative  | Negative      | GBS, CoNS                            | CA, EF      | No sepsis         | No ATB   |
| 6  | 25        | 820   | Strep. spp.    | 6,760     | 30 BactoPlex    | Negative  | Negative      | CA, CoNS                            | No sepsis   | No ATB            | No ATB   |
| 7  | 25        | 700   | Strep. spp.    | 17,086    | 28 BactoPlex    | Negative  | Negative      | CA, EC, EF                          | No sepsis   | No ATB            | No ATB   |
| 9  | 24        | 490   | S. maltophilia | 36,194    | 27 BactoPlex    | Negative  | Negative      | CA, EF, EC                          | No sepsis   | No ATB            | No ATB   |
| 17 | 25        | 650   | E. coli        | 388       | 33 BactoPlex    | Negative  | Str. vestibul.| EC, KP                              | No sepsis   | No ATB            | No ATB   |
| 18 | 25        | 800   | E. coli        | 7,302     | 31 BactoPlex    | Negative  | Negative      | GC, KP                              | No sepsis   | No ATB            | No ATB   |
| 21 | 27        | 900   | E. coli        | 1,365     | 31 BactoPlex    | Negative  | Negative      | EC, KP                              | No sepsis   | No ATB            | No ATB   |
| 35 | 30        | 1,290 | E. coli        | 40,124    | 27 FTD neonatal sepsis | Negative  | SH            | Negative                            | Clinical sepsis | ATB 5 days | ATB 5 days |
| 38 | 27        | 1,180 | S. aureus      | 1,977     | 32 FTD neonatal sepsis | Negative  | SH            | Negative                            | Clinical sepsis | ATB 7 days | ATB 7 days |
| 39 | 30        | 1,410 | U. parvum      | 4,889     | 31 FTD neonatal sepsis | Negative  | Negative      | EC, CA, EF                          | Clinical sepsis | ATB 5 days | ATB 5 days |
| 47 | 30        | 1,650 | S. aureus      | 7,875     | 31 FTD neonatal sepsis | Negative  | Negative      | SV                                  | Clinical sepsis | ATB 5 days | ATB 5 days |
| 51 | 26        | 980   | E. coli        | 806       | 33 FTD neonatal sepsis | Negative  | Negative      | SV                                  | No sepsis   | No ATB            | No ATB   |
| 52 | 25        | 915   | E. coli        | 552       | 34 FTD neonatal sepsis | Negative  | Negative      | Negative                            | No sepsis   | No ATB            | No ATB   |
| 53 | 27        | 960   | E. coli        | 356       | 34 FTD neonatal sepsis | Negative  | Negative      | Negative                            | No sepsis   | No ATB            | No ATB   |
| 56 | 28        | 1,200 | E. coli        | 266       | 33 FTD neonatal sepsis | Negative  | Negative      | EC                                  | No sepsis   | No ATB            | No ATB   |
| 57 | 28        | 1,180 | E. coli        | 162       | 33 FTD neonatal sepsis | Negative  | Negative      | EC                                  | No sepsis   | No ATB            | No ATB   |
| 60 | 29        | 970   | E. coli        | 275       | 33 FTD neonatal sepsis | Negative  | Negative      | CA, EC, EB                          | No sepsis   | No ATB            | No ATB   |
| 63 | 25        | 920   | E. coli        | 321       | 33 FTD neonatal sepsis | Negative  | Negative      | EC, EF, EB                          | No sepsis   | No ATB            | No ATB   |
| 75 | 28        | 1,160 | E. coli        | 149,000   | 26 FTD neonatal sepsis | Negative  | EC            | Negative                            | No sepsis   | No ATB            | No ATB   |

ATB, antibiotics; BDL, bacterial DNA load; BW (g), birth weight (g); CA, Candida albicans; CoNS, coagulase-negative staphylococci; EB, Enterobacteriaceae; EC, Escherichia coli; EF, Enterococcus faecalis; GA (weeks), gestational age (weeks); GV, Gardnerella vaginalis; KP, Klebsiella pneumoniae; SH, Staphylococcus hominis; Str. vestibul., Streptococcus vestibularis; SV, Streptococcus viridans.
We defined a pragmatic cycle threshold value to avoid false positive results [22]. Nevertheless, one PCR true positive and eight false positive tests were identified, all of which had very low bacterial DNA load (<1,000). This indicates possible false positivity after exceeding 32 amplification cycles for both assays used.

The unfavorable ratio of the number of culture-negative to confirmed sepsis cases found, was corroborated by our study group. Eleven patients (17%) were exposed to prolonged antibiotic treatment despite missing proof of pathogen presence. This re-emphasizes the existing need for the definition of neonatal sepsis not to be restricted to conventional pathogen detection methods [2].

This study has a number of limitations: two commercial multiplex real time PCR assays were used and only one of them was validated for neonatal samples. The sample volume used (0.5 mL) for the PCR test was lower than recommended by manufacturers, which can cause false negative results. However, we followed recently published data where small sample volume was successfully used previously [13, 23]. The fact that only one of all study cases proved positive for both the PCR test and blood culture may seem surprising, although problems related to low rate of blood culture positivity in preterm infants are well known and remain a challenging issue. A negative blood culture result in the first hours of life is common and can be possibly attributed to a high rate of maternal antibiotic use (61%) in our maternal cohort [3].

### Conclusions

Multiplex real time PCR had insufficient diagnostic capability to detect bloodstream bacterial DNA in very preterm infants with high risk of EOS. Our study does not support the routine use of real time PCR assay in clinical practice. The correlation between vaginal swab results of the mothers and positive PCR results in newborns was unexpected and needs further investigation.

### Research funding:
This work was supported by the Czech Health Research Council Project (NV17-31403A).

### Author contributions:
All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

### Competing interests:
This work was supported by the Czech Health Research Council Project (NV17-31403A). The funding had no involvement in study design, the collection, analysis and interpretation of data, and writing of the report.

### Informed consent:
Written informed consent was obtained from parents of each enrolled infant.

### Ethical approval:
The study has been complied with all the relevant national guidelines, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by local Ethics Committee (2015/06-02-4) and local Committee on Human Research.
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