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

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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.

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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 evaluated for possible replacement of blood culture in the diagnosis of neonatal sepsis, but had insufficient sensitivity [12]. Nevertheless, real-time PCR has a reasonable sensitivity and specificity (both 0.96) to be used as an adjunctive diagnostic tool [12]. Multiplex real-time PCR was found to have a valuable additional role in the diagnosis of sepsis within the general neonatal population, however very limited data on preterm neonates are available [13].

The primary objective of this study was to analyze diagnostic capability of multiplex real-time PCR assay for the bacterial DNA detection in preterm infants at risk of EOS. Secondary outcome was to assess the ability of rapid PCR assay to support the decision making in whether to terminate or continue antibiotic treatment course.

Materials and methods

Subjects

This prospective study was conducted in a single tertiary neonatal intensive care unit from October 2017 until September 2018. Inclusion criteria were: preterm delivery ≤32 weeks of gestation, inborn neonates and collection of blood samples completed within the first 2 h after delivery. Exclusion criteria were: incomplete neonatal records, insufficient sample volume and congenital malformations. The study protocol has complied with all the relevant national guidelines, institutional policies and was 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. Written informed consent was obtained from parents of each enrolled infant.

Clinical and histological chorioamnionitis

Clinical chorioamnionitis was defined as: maternal body temperature ≥37.8 °C and a minimum of two other criteria from: maternal or fetal tachycardia, uterine tenderness, malodorous vaginal discharge and leukocytosis ≥15,000 mm^{-3}. Acute inflammatory lesions of the placenta were histologically characterized by the infiltration of neutrophils in the chorion and amnion (acute chorioamnionitis) or in the placenta (intervillositis, deciduitis, or villositis). Funisitis was referred to when the inflammatory process involved the umbilical cord (umbilical vein, umbilical artery, and the Wharton's jelly) [14].

Blood sampling and tests

Whole blood sample was collected aseptically from the neonate within the first 2 h after delivery through a newly inserted catheter (umbilical vein, umbilical artery or peripheral vein). Blood culture (1 mL) and bacterial DNA analysis were performed, whole blood count, C-reactive protein (CRP), procalcitonin (PCT) and interleukin 6 (IL-6) were determined. The real-time PCR sample was collected into a special tube (volume 1.6 mL – BD Vacutainer Plus Plastic with sodium citrate 0.129 M). The volume of blood collected was 500 μL.

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

High Vaginal Swabs were collected from all women with imminent preterm delivery. Neonatal swabs (ear, stomach and axilla) were taken immediately after admission to NICU. Cotton-tipped swabs were placed in AMIES agar (Micro-Trans Swab, Med-Lab Trade, Czech Republic), then inoculated to sheep blood agar, chocolate agar, and MacConkey agar and incubated aerobically. Organisms were identified by standard 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. BactoPlexx Real-Time PCR Kit (KITGEN, Třinec, 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 BactoPlex and 10 μL for FTD Neonatal sepsis. Initial denaturation and thermocycling (°C/seconds) were 94/300 and 94/30, 58/30, 72/30 for BactoPlex assay, for FTD Neonatal sepsis assay they were 94/180 and 94/8, 60/34 respectively. Cycles setting was 50 for Bactoplex and 40 for FTD Neonatal sepsis. Cycles positivity assessment was ≤35 for both assays. The quantitative real-time PCR assays were processed on Rotor-Gene Q (QIANGEN, Hilden, Germany). BactoPlex 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* *pyogenes*, *Streptococcus* spp. FTD Neonatal sepsis assay was used for the detection of *Group B Streptococcus/Streptococcus agalactiae*, *Listeria monocytogenes*, *E. coli*, *S. aureus*, *Chlamydia trachomatis* and *Ureaplasma urealyticum/parvum*. Each PCR test was performed in conjunction with three controls; positive control, negative control and an internal control. The assay uses *Streptococcus equi* as an internal control which is introduced into each sample and it also shows the correctness of the extraction process. The individual pathogen specific PCRs had been evaluated previously. Primer and probes used for each reaction are available from the manufacturer.

The presence of specific pathogen sequences in the reaction was detected by an increase in observed fluorescence from the relevant 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 Dif II (Coulter Electronics Ltd., Fullerton, US). CRP was measured by immunoturbidimetry (Cobas 6000, c501 module, Roche Diagnostics, Mannheim, Germany). Immunoluminometric assay (Lumitest PCT, Brahms, Germany) was used for PCT analysis. Luminescence was measured automatically in a Berilux Analyser (Behring Diagnostics, Germany). Serum IL-6 level was assessed 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 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 μg/L 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, desaturations, respiratory distress syndrome with ongoing mechanical ventilation), cardiac (cyanosis, bradycardia, poor peripheral perfusion, hypotension) and neurological (lethargy, suspected seizures) symptoms [17]. Higher risk of EOS was considered in the presence of clinical and/or laboratory chorioamnionitis, *Group B Streptococcal (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).[a]

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)
Histological chorioamnionitis[b]	
None	29 (35)
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)

[a]Continuous variables are expressed as mean ± standard deviation. Categorical variables are presented as number (percent).
[b]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, axila, stomach)	Vaginal swab	Neonatal infection	Comments
3	28	950	*CoNS*	672	33	BactoPlex	Negative	Negative	GBS	No sepsis	No ATB
6	25	820	*Strep. spp.*	6,760	30	BactoPlex	Negative	CoNS	CA, EF	No sepsis	No ATB
7	25	700	*Strep. spp.*	17,086	28	BactoPlex	Negative	Negative	CA, EC	No sepsis	No ATB
9	24	490	*S. maltophilia*	36,194	27	BactoPlex	Negative	EC, EF	Negative	No sepsis	ATB 48 h, death due to NEC/LOS at day 12 (*E. coli*)
17	25	650	*E. coli*	388	33	BactoPlex	Negative	Str. vestibul.	EC, KP	No sepsis	No ATB
18	25	800	*E. coli*	7,302	31	BactoPlex	Negative	Negative	EC, KP	No sepsis	No ATB
21	27	900	*E. coli*	1,365	31	BactoPlex	Negative	Negative	GV	No sepsis	No ATB
35	30	1,290	*E. coli*	40,124	27	FTD neonatal sepsis	EC	EC	EC	Proven sepsis	*E. coli* meningitis
38	27	1,180	*S. aureus*	1,977	32	FTD neonatal sepsis	Negative	SH	Negative	Clinical sepsis	ATB 5 days
39	30	1,410	*U. parvum*	4,889	31	FTD neonatal sepsis	Negative	Negative	EC, CA, EF	Clinical sepsis	ATB 7 days
47	30	1,650	*S. aureus*	7,875	31	FTD neonatal sepsis	Negative	Negative	Negative	No sepsis	No ATB
51	26	980	*E. coli*	806	33	FTD neonatal sepsis	Negative	Negative	SV	Clinical sepsis	ATB 5 days
52	25	915	*E. coli*	552	34	FTD neonatal sepsis	Negative	Negative	Negative	No sepsis	No ATB
53	27	960	*E. coli*	356	34	FTD neonatal sepsis	Negative	Negative	Negative	No sepsis	No ATB
56	28	1,200	*E. coli*	266	33	FTD neonatal sepsis	Negative	Negative	EC	No sepsis	No ATB
57	28	1,180	*E. coli*	162	33	FTD neonatal sepsis	Negative	Negative	EC	No sepsis	No ATB
60	29	970	*E. coli*	275	33	FTD neonatal sepsis	Negative	Negative	CA, EC, EB	No sepsis	ATB 48 h
63	25	920	*E. coli*	321	33	FTD neonatal sepsis	Negative	Negative	EC, EF, EB	No sepsis	No ATB
75	28	1,160	*E. coli*	149,000	26	FTD neonatal sepsis	Negative	EC	Negative	No sepsis	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*.

Table 3: Comparison of clinical data and laboratory findings between PCR positive and negative patients.[a]

	PCR negative (n=63)	PCR positive (n=19)	p-Values
Gestational age, weeks	27 ± 2 (24–31)	27 ± 2 (24–30)	0.95
Birth weight, g	985 ± 323 (440–1,900)	1,007 ± 277 (490–1,650)	0.66
Early onset sepsis			
No sepsis	52 (83)	15 (79)	0.73
Proven or clinical sepsis	11 (17)	4 (21)	
C-reactive protein, mg/L	1.0 (0.1–1.8)	4.2 (0–9.0)	0.82
Procalcitonin, µg/L	0.9 (0.4–1.4)	2.8 (0.1–5.4)	0.05
Interleukin 6, ng/L	561 (272–850)	592 (100–1,085)	0.96
WBC count (×1,000/uL)	10.4 (8.4–12.3)	6.5 (5.2–7.6)	0.02
I/T ratio	0.12 (0.09–0.16)	0.09 (0.03–0.14)	0.19
Use of antibiotics, days	2.0 ± 2.3 (0–10)	1.3 ± 2.2 (0–7)	0.15
Late onset sepsis	16 (25)	5 (26)	>0.99
Necrotizing enterocolitis	5 (8)	1 (5)	>0.99
Intraventricular hemorrhage ≥ grade III	7 (11)	2 (11)	0.94
Bronchopulmonary dysplasia	42 (72)	13 (77)	0.74
Retinopathy of prematurity (all stages)	13 (21)	5 (26)	0.60
Survival	56 (89)	18 (95)	0.45

[a]Continuous variables are expressed as mean ± standard deviation (range). Categorical variables are presented as number (percent). Laboratory data are presented as means (lower and upper bound 95% Confidence Interval). Statistically significant difference p<0.05 (Pearson Chi-Square test, Fisher's exact test and Mann-Whitney U test accordingly).

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.

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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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