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Posted Date: 24 October 2023

doi: 10.20944/preprints202310.1505.v1

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*Article*

# Diagnostic Accuracy of Multiplex PCR in Early Onset Neonatal Sepsis

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**Abstract:** Early onset neonatal sepsis is a significant contributor to neonatal morbidity and mortality. Although blood cultures remain the diagnostic gold standard, they detect pathogens in only a minority of suspected cases. This study compared the accuracy of blood cultures with a rapid multiplex PCR test. Newborns at risk for neonatal sepsis were prospectively screened as recommended per national guidelines. Evaluations included laboratory parameters (CrP, IL6, differential blood count), blood culture, and a molecular multiplex PCR test (ROCHE LightCycler SeptiFast®) identifying 20 common microbial agents. Blood samples were taken simultaneously from umbilical cord or venous sources on the first day of life. Of 229 infants included, 69% were born preterm. Blood culture and multiplex PCR sensitivity were 7.4% and 14.8% respectively. Specificity, negative and positive predictive values between methods showed no significant variance, though multiplex PCR had more false positives due to contamination. The limited sensitivity of blood cultures for early onset neonatal sepsis is concerning. Despite quicker results, multiplex PCR does not enhance diagnostic accuracy or antibiotic therapy guidance, thus it can not be recommended for this indication.

**Keywords:** neonatal sepsis; early onset sepsis; multiplex PCR; infection; neonate; diagnosis; pathogen detection

## 1. Introduction

Neonatal sepsis remains a significant challenge in neonatology, escalating both morbidity and mortality [1]. Annually, an alarming three million children succumb to this condition [2]. Defined as manifesting within the first 72 hours of life, early onset neonatal sepsis (EONS) primarily stems from infections ascending pre- or peripartum. This pathway elucidates why *Streptococcus agalactiae* (group B streptococci, GBS) and *Escherichia coli* are principal pathogens, attributing to nearly 70% of EONS cases. Documented incidence of culture-proven EONS ranges between 0.77 and 1 in 1000 deliveries, with mortality rates reaching 20% [1,3]. Notably, between 10-30% of pregnant women are colonized with *GBS*. Without maternal intrapartum antibiotic prophylaxis, neonates from these mothers display a colonization rate of 68%, and the risk of EONS attributed to *GBS* stands at 1.1%.[4-6].

One of the significant hurdles in neonatal sepsis diagnosis is its non-distinct and occasionally subtle symptoms, particularly in preterm infants. Reliance on individual laboratory tests remains precarious due to their variable sensitivity and specificity, compelling a multi-parameter approach [7-9]. Blood cultures, although a diagnostic gold standard, often yield inconclusive results, particularly in neonates. The inoculation of at least 1 mL of venous blood for a blood culture would increase sensitivity of pathogen detection but is rarely achieved in premature infants. Use of umbilical cord blood may be useful to ensure adequate blood volumes and can avoid skin punctures otherwise not indicated in the newborn but may be prone to contamination [10-12]. Moreover, the protracted 36-48 hour waiting period for results, coupled with its diminished sensitivity and specificity in EONS,

 doi:10.20944/preprints202310.1505.v1



renders it less ideal [7]. Previous maternal antibiotic treatment and GBS-targeted intrapartum antibiotic prophylaxis further compromise bacterial detection [13].

Molecular testing, especially polymerase chain reaction (PCR) and other nucleic acid amplification techniques like metagenomic next-generation sequencing (NGS) technologies, offers potential advantages in diagnosing and managing neonatal sepsis [14]. Conventional cultures may require several days for definitive results, while molecular assays can yield outcomes within hours. Such swift diagnostics can facilitate the timely initiation of targeted antibiotic therapy, potentially enhancing patient outcomes [15]. Additionally, molecular techniques can discern small quantities of bacterial or fungal DNA or RNA, thereby potentially detecting infections that conventional cultures might overlook. However, this precision may also introduce the risk of false positives from potential contamination. As a result, it has been concluded that multiplex PCR testing provides no additional benefit in nosocomial and late-onset infections of preterm infants [16,17]. If antibiotics have been administered to mother or infant, traditional cultures may fail to cultivate the causative organism. However, molecular assays can identify the pathogen's DNA or RNA even if the organism is rendered non-viable by antibiotics. Furthermore, some molecular tests can simultaneously identify a vast array of pathogens, enabling the detection of polymicrobial infections. As the specificity of the chosen test is contingent upon the pathogens incorporated in its panel, any organism not encompassed in the assay's design may remains undetected. A noteworthy advantage of molecular tests is their reduced smaller blood volume requirement compared to traditional cultures [18]. The majority of such tests however cannot ascertain antibiotic susceptibility, an essential insight to guide antimicrobial treatment that traditional cultures offer.

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This research aimed to assess whether direct molecular testing, specifically the ROCHE LightCycler SeptiFast®—a multiplex PCR test—surpasses blood culture in accuracy and pathogen detection rate in EONS. This test targets sequences between bacterial 16S-23S ribosomal RNA and fungal 18S-5.6S ribosomal RNA [16]. Its scope, though limited to 20 select microorganisms (Table 1), covers pathogens implicated in 90% of adult and pediatric bloodstream infections [13]. Notably, coagulase negative staphylococci, which are frequently detected as contaminant in blood cultures and PCR tests, are no typical cause of EONS.

**Table 1.** Pathogens included in the assay of the ROCHE LightCycler SeptiFast®[17].

| Gram-negative organisms | Gram-postive organisms | Fungi |
|---|---|---|
| *Escherichia coli* | *Staphylococcus aureus* | *Candida albicans* |
| *Klebsiella pneumoniae/oxytoca* | *Coagulase negative staphylococci*[1] | *Candida tropicalis* |
| *Serratia marcescens* | *Streptococcus pneumoniae* | *Candida parapsilosis* |
| *Enterobacter cloacae/aerogenes* | *Streptococcus ssp.*[2] | *Candida glabrata* |
| *Proteus mirabilis* | *Enterococcus faecium/faecalis* | *Candida krusei* |
| *Pseudomonas aeruginosa* | | *Aspergillus fumigatus* |
| *Acinetobacter baumannii* | | |
| *Stenotrophomonas maltophilia* | | |

[1] *S. epidermidis, S. haemolyticus, S. hominis, S. pasteuri, S. warneri, S. cohnii, S. lugdunensis, S. capitis, s. caprae, S. saprophyticus, and S. xylosus;* [2] *S. agalactiae, S. pyogenes, S. anginosus, S. bovis, S. constellatus, S. cristatus, S. gordonii, S. intermedius, S. milleri group, S. mitis, S. mutans, S. oralis, S. parasanguinis, S. salivarius, S. sanguinis, S. thermophilus, S. vestibularis, S. viridans group.*

Given the potential advantages and limitations, molecular testing may be a valuable tool in the diagnostic algorithm for EONS, particularly when used in conjunction with clinical judgement and routine laboratory parameters.

## 2. Materials and Methods

 doi:10.20944/preprints202310.1505.v1



Patient data were prospectively collected between March 2017 and September 2018. All neonates admitted to the neonatal intensive care unit at the Division of Neonatology of the Department of Pediatrics I at the University Hospital of Essen underwent a routine sepsis workup. Furthermore, healthy newborns screened for infection due to perinatal risk factors for neonatal sepsis, in accordance with the the national S2k guideline for prophylaxis of early onset neonatal sepsis by GBS [19], were also eligible. The study received approval from the ethics committee of the University of Duisburg-Essen (16-7306-BO), and written informed consent was obtained from the legal guardians of the infants.

Routine sepsis workup involved collecting a blood sample for culture, hematology and clinical chemistry. Samples were collected within the first 24 hours of life. For healthy newborns, umbilical cord blood was used to minimize the need for additional skin punctures for sampling. When neonates required peripheral blood sampling or the insertion of a peripheral venous catheter based on clinical indications, blood samples were primarily obtained from these venous sources. Blood collection followed disinfection using an alcohol-based antiseptic. Aerobic pediatric blood culture bottles (BD BACTEC Peds Plus /F® blood culture bottles; Benex Limited, Dun Laoghaire, Ireland) were inoculated with a minimum of 0.5 mL of blood and then incubated for at least 7 days. Positive blood cultures were Gram-stained, and detected microorganisms were further identified to the species level following standard microbiological methods. Additionally, 200 µL EDTA blood was employed for a compete blood count, and 300 µL of serum was used to determine CRP and Interleukin 6 levels. In addition, 100–200 µl EDTA bloodwas sampled for the multiplex-PCR (SeptiFast ®) in DNA-free Sarstedt Microvette® tubes (Nümbrecht, Germany). PCR analysis was commenced at the Institute of Medical Microbiology, University of Duisburg-Essen using the LightCycler® SeptiFast MGRADE system (Roche Diagnostics, Penzberg, Germany) with a modified DNA extraction protocol for small blood volumes [16,17]. The minimum dataset required was: blood culture, multiplex PCR and CRP/IL-6. Patients with incomplete datasets were excluded from the study. Multiplex PCR and blood culture were considered positive if they detected at least one pathogen. PCR results were made available to clinicians within 6 to 12 hours to guide therapeutic approaches in these patients.

To compare results of blood culture and multiplex PCR definition and confirmation of true sepsis in our patients was mandatory. Given the anticipated low sensitivity of blood cultures, a correspondingly low rate of blood culture-proven EONS was expected. While clinical late-onset or nosocomial sepsis have clear definitions based on both clinical and laboratory criteria (as outlined in the Surveillance Protocol NEOKISS by the Robert-Koch Institute (RKI), available at www.nrz-hygiene.de), a universally accepted definition for clinical EONS remains elusive. We therefore adapted the risk stratification published by Stocker et al. [20]. In this system, one point is allocated for the fulfillment of any criteria within the three categories: anamnestic risk factors, clinical signs, and laboratory findings. This results in a potential maximum "clinical EONS score" of three points (as detailed in Table 2). The necessary anamnestic information of patients and mothers, clinical symptoms of infection and laboratory parameters were extracted from medical records.

**Table 2.** Clinical EONS Score adapted from [20].

| Category | Criteria |
|---|---|
| anamnestic risk factors for EONS | chorioamnionitis (maternal fever >38.5°C, maternal or fetal tachycardia, uterine tenderness, malodorous vaginal discharge, and maternal leukocytosis ≥ 15 /nl) |
| | prolonged rupture of membranes > 18 hours |
| | prematurity (birth below 37 weeks of gestation) |
| | maternal colonization by GBS* |
| | GBS* bacteriuria during gravidity |
| | GBS* sepsis in a neonate from a previously pregnancy |
| clinical criteria | fever > 38 °C, hypothermia < 36.5 °C or temperature instability |
| | tachycardia > 200 /min or frequent bradycardia < 80 /min |

 doi:10.20944/preprints202310.1505.v1



| | |
|---|---|
| | arterial hypotension, poor perfusion (recapillarization time > 2s)<br>tachypnea > 60 /min, dyspnea, frequent desaturations, or apnea<br>vomiting, feeding intolerance<br>irritability or seizure<br>apathy, lethargy, mottled skin color, general instability |
| laboratory criteria | C-reactive protein (CrP) > 0.5 mg/dl<br>Interleukin-6 > 100 pg/ml<br>Ratio of immature to mature neutrophils (I/T ratio) > 0.2<br>White blood cells < 5 /nl |

* GBS – group B streptococci, Streptococcus agalactiae.

The patients were stratified into four distinct groups based on their blood culture results and clinical EONS score points (Table 3).

**Table 3.** Study groups based on blood culture result and EONS Score.

| EONS group | Blood culture | EONS Score Points |
|---|---|---|
| 1: culture proven sepsis | true positive | 1-3 |
| 2: sepsis likely | false positive or negative | 3 |
| 3: sepsis possible | false positive or negative | 2 |
| 4: sepsis unlikely | false positive or negative | 0-1 |

Results from blood cultures and PCR testing were verified by the attending physician; coagulase-negative staphylococci, Corynebacterium species, Bacillus species, Propionibacterium acnes, micrococci, and Neisseria species other than N. gonorrhoeae and fungi were defined as possible contaminants. In groups two through four, blood culture results were either negative or deemed as false positives. Patients in groups one and two, categorized as "culture proven sepsis" and "sepsis likely" respectively, were defined as having true EONS. The classifications were determined independent of multiplex PCR results. For groups one and two, positive PCR outcomes were viewed as potential true positives. Conversely, within groups three and four (“sepsis possible” or “sepsis unlikely”), all positive PCR outcomes were treated as false positives.

Data analysis and graphical displays were conducted using SPSS (version 28 for Mac) and Excel (version 16.53 for Mac). Continuous variables are presented as mean ± standard deviation (SD). Sensitivity, specificity, and positive and negative predictive values were derived using a confusion matrix.

## 3. Results

Between March 2017 and September 2018, a total of 2,728 neonates were born at the Clinic for Obstetrics and Gynecology of the University Hospital in Essen. Of these, 900 were admitted to the neonatal intensive care unit (NICU) and neonatal intermediate care units on their first day of life. Additionally, 20 outborn neonates were admitted to these units during the same period. After obtaining parental consent, 229 neonates with a complete laboratory set were included in the study (birth weight: 2,370g ± 879g, range: 400g to 4,750g; gestational age (GA): 35.3 ± 3.9 weeks, range: 23+4/7 to 41+4/7 weeks). The study cohort comprised 40 very low birth weight infants (<1,500g) and 159 preterm infants, of which 43 were very preterm infants born before 32 weeks of gestation. The distribution of patients by gestational age within the EONS groups is detailed in Table 4.

**Table 4.** Patient distribution in the EONS Study groups.

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| Study group | total | GA > 36+6/7 weeks (term) | GA 32+0/7 to 36+6/7 weeks (late preterm) | GA < 32+0/7 weeks (very preterm) |
|---|---|---|---|---|
| 1: culture proven sepsis | 2 (0.9%) | 1 | 0 | 1 |
| 2: sepsis likely | 25 (10.9%) | 7 | 7 | 11 |
| 3: sepsis possible | 37 (16.2%) | 15 | 12 | 10 |
| 4: sepsis unlikely | 165 (72.1%) | 47 | 97 | 21 |
| total | 229 | 70 (30.6%) | 116 (50.7%) | 43 (18.8%) |

*3.1. Results from blood cultures*

Of all blood cultures taken, eight tested positive. However, only two of these were deemed true positives. *Escherichia coli* was identified in one very preterm infant, and *Streptococcus agalactiae* in one term infant. Both these infants exhibited clinical and laboratory signs of infection. This results in an EONS rate of 0.9% in this cohort.

Four cultures were judged as contaminants because the corresponding infants did not manifest any clinical or laboratory signs of infection, did not receive antibiotic treatment, and were screened for infection solely due to anamnestic risk factors. Two of the assumed contaminated cultures revealed growth of *Staphylococcus epidermidis*, an organism not typically associated with EONS. One culture identified multiple pathogens (*Streptococcus mitis, Escherichia coli,* and *Enterococcus faecalis*), further suggesting contamination. One culture yielded *Klebsiella pneumoniae*, which could have been a potential true pathogen for EONS.

*3.2. Results from multiplex PCR tests*

Multiplex PCR testing failed to detect pathogens in the two instances of blood culture-proven sepsis. Both infants were born to mothers without premature or prolonged rupture of membranes, without clinical signs of amnion infection and without antibiotic treatment prior to giving birth.

Multiplex PCR testing identified pathogens in 19 cases from patients in EONS groups two to four. Of all multiplex PCR tests, only two were deemed potentially true positives, and both fell under the EONS category "sepsis likely".

Within the EONS group “sepsis likely”, which displayed clinical and laboratory signs of sepsis in conjunction with anamnestic risk factors, none of the blood cultures were positive, but four multiplex PCR tests were. Potentially true pathogens of EONS identified were *Staphylococcus aureus* in a very preterm infant and *Enterobacter cloacae* in a term infant. The preterm infant initially received antibiotic treatment with ampicillin and gentamycin. This was later adapted to a 3rd generation cephalosporin due to the discovery of resistant *Klebsiella pneumoniae* in the maternal vaginal swabs and a rising CrP level reaching 108 mg/l. The antibiotic treatment regimen was not adapted to specifically target *Staphylococcus aureus* despite the multiplex PCR result. The term infant, presenting with an initial IL6 level of 4,015 pg/ml and a CrP of 28 g/dl, was administered ampicillin for 8 days and gentamycin for 6 days. The treatment regimen remained unchanged even after obtaining the PCR result, notwithstanding the intrinsic resistance of *Enterobacter cloacae* to aminopenicillins owing to the production of constitutive AmpC β-lactamase. The other two tests detected *coagulase-negative staphylococci* and *Candida tropicalis*, both likely contaminants. In this EONS group five mothers had displayed signs of amnion infection with prolonged rupture of membranes > 18 hours and antibiotic treatment prior to giving birth (5/25 mothers, 20%). Only one of the five infants born to these mothers had a positive PCR result (the preterm infant mentioned above, *Staphylococcus aureus*).

For the EONS group “sepsis possible”, multiplex PCR detected positive results in four patients, all of whom had anamnestic risk factors for infection. Two tests identified *Candida krusei*, presumed to be contaminants. Neither of these patients received antimycotic treatment. One showed potential clinical signs but lacked laboratory evidence of infection, while the other displayed no clinical symptoms but had an elevated interleukin 6 level at 321 pg/ml as singular laboratory sign. The last

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two tests from this group were also considered contaminated: one identified *coagulase-negative staphylococci*, while the other detected both *coagulase-negative staphylococci* and *Staphylococcus aureus*. Notably, the latter very preterm infant had congenital cutaneous candidiasis and received appropriate systemic antimycotic treatment.

In the EONS group "sepsis unlikely", eleven patients returned positive results on the multiplex PCR test. Detected pathogens included *Candida albicans* (n=1) and *coagulase-negative staphylococci* (n=6), neither of which are typical for EONS. Other identified pathogens, namely *Streptococcus species, Staphylococcus aureus, Klebsiella*, and *Enterobacter cloacae*, have potential associations with EONS. However, none of these patients exhibited clinical symptoms, and none received antibiotic treatment.

### *3.3. Antibiotic treatment*

Classification into the EONS groups corresponded with the administration and duration of antibiotic therapy. All patients in EONS groups one and two (n=27) received antibiotics with a mean treatment duration of 7.2 ± 5.0 days. Notably, 12 out of 27 patients (44.4%) in were very preterm infants, who are often administered antibiotics more liberally. Within the "sepsis likely" EONS group two, the four patients with positive multiplex PCR tests had a longer treatment duration compared to those without pathogen detection (7.3 ± 2.2 days vs. 6.2 ± 3.3 days, $p = 0.002$). However, the chosen antibiotic regimen was not targeting the identified pathogens.

In the "sepsis likely" category of the EONS group, 91.9% of infants (34 out of 37) received antibiotics, with an average treatment duration of 4.8 ± 4.9 days. This group included 27% very preterm infants (10/37), all of whom were given antibiotic treatment. The duration of antibiotic therapy did not significantly differ between patients who had pathogen detection through PCR testing and those who did not (4.8 ± 1.7 days vs. 4.8 ± 5.2 days, respectively). All patients who tested positive in PCR were administered antibiotics.

For the EONS group with the lowest risk of infection, only 7.3% of patients (12 out of 165) underwent antibiotic treatment, with an average duration of 5.1 ± 9.0 days. Notably, 11 out of these 12 infants were treated for 3 days or less, reinforcing the belief that they likely did not have EONS. None of the patients with either a positive PCR test result or a positive blood culture received antibiotic treatment. This cohort was predominantly comprised of late preterm infants (97 out of 165, or 58.8%), with a smaller fraction being very preterm infants (21 out of 165, or 12.7%).

### *3.4. Comparison of reference and index test*

Defining patients in group 1 (positive blood culture) and patients in group 2 as goldstandard and having true EONS, the confusion matrix revealed no significant differences between the reference (blood culture) and the index test (multiplex PCR). As Table 5 illustrates, both tests exhibited very low sensitivity. Other metrics showed minimal variation between the two tests. Both achieved a specificity of over 90%, with the blood culture being marginally superior. The positive predictive value exhibited a broad confidence interval, rendering it an unreliable metric. The negative predictive values were identical for both tests. Although four multiplex PCR tests were positive in patients who were defined as having true EONS leading to a sensitivity of 14.8%, two of these were clinically judged as contaminations and only two detected potential pathogens for EONS.

**Table 5.** Comparison of sensitivity, specifity, negative and positive predictive values between reference test (blood culture) and index test (multiplex PCR).

| | Blood culture | | Multiplex PCR | |
|---|---|---|---|---|
| | Estimated value (%) | 95 % confidence interval (%) | Estimated value (%) | 95 % confidence interval (%) |
| Sensitivity | 7.4 | 1.3 – 25.8 | 14.8 | 5.9 – 23.7 |
| Specificity | 98.0 | 94.7 – 99.4 | 94.6 | 91.8 – 97.3 |

 doi:10.20944/preprints202310.1505.v1



| | | | | | |
|---|---|---|---|---|---|
| Positive predictive value | 33.3 | 6.0 – 75.9 | 26.7 | 7.9 – 45.4 | |
| Negative predictive value | 88.8 | 83.7 – 92.5 | 89.3 | 84.8 – 93.7 | |

## 4. Discussion

The incidence of culture-proven EONS in our study was 0.9%, which is higher than in the general neonatal population where it is 0.1% of all live births [3,21]. This discrepancy can be attributed to a selection bias since our study included a high proportion of preterm infants as well as infants who required admission to a neonatal intensive care unit. In cohorts comprising very low birth-weight infants, the incidence is anticipated to be around 1.3-1.4% [21,22]. Nonetheless, this selection bias likely does not affect the study's results.

The known low sensitivity of blood culture in EONS is significantly influenced by factors such as blood volume, the number of samples, timing, and maternal antibiotic treatment [23,24]. In our study, only two infants were diagnosed with culture-proven sepsis, both identified with typical pathogens: *Escherichia coli* and *Streptococcus agalactiae*. A limitation we encountered was that we likely did not consistently achieve the recommended pediatric blood culture volume of 1 ml, especially in very low birthweight infants. This exact volume was not documented. Our internal guidelines permitted 0.5 to 1 ml for venous samples in premature infants. While this can diminish the sensitivity of blood cultures, it likely mirrors prevalent clinical practice.

Notably, the multiplex PCR in our study failed to detect the pathogens identified in the blood culture. Previous studies suggest that molecular testing can yield reliable results with much smaller blood quantities, even in cases of low or intermittent neonatal bacteremia or previous maternal antibiotic treatment [17,18]. This potential advantage could account for the higher rate of false positives due to PCR sample contamination. In the EONS group labeled “sepsis unlikely”, four blood cultures and 11 PCR tests were deemed to be contaminants. A significant proportion of these patients were primarily screened for infection based on anamnestic risk factors, displayed no symptoms, and likely had samples drawn from their umbilical cord blood instead of venous blood. This method was preferred when there was no clinical justification for venous puncture in the newborn. While umbilical cord samples might be more voluminous due to easier access, less stringent aseptic conditions during sampling could lead to a higher contamination rate. A notable limitation is that the source of the blood was not tracked during our study. Although Diericks et al. [12] argued that “umbilical cord blood culture has higher sensitivity and comparable specificity for the diagnosis of neonatal early-onset sepsis,” such findings might not be directly applicable to this low-risk EONS group.

Sensitivity of the multiplex PCR test in adult studies ranges from 60 to 95 % and specifity between 74 and 99 % [13]. In children of all ages including neonates sensitivity ranges from 85-90.2 % and specificity between 72.9 to 93.5 % [15,17]. This can be confirmed by the present study reporting a specificity of 94.6 % for the multiplex PCR but with a higher specificity for the blood culture with 98.0%. In a study of late onset neonatal sepsis the false positive rate of the multiplex PCR test was as high as 27.1 to 35% [16,17]. Although the rate of false positive tests of 5.4% in our study of EONS is lower than this, the multiplex PCR achieved a sensitivity of only 14.8% and low positive and negative predictive values (26.7% and 89.3%). This does not make it a reliable test to diagnose or exclude EONS.

One theoretical advantage of multiplex PCR testing is the availability of results within 6 to 24 hours depending on the laboratory setting. Blood culture results can be safely interpreted after 36 to 48 hours, but the clinicians trust in sterile cultures is low [7,8]. In lack of other reliable laboratory indicators for EONS and overlapping of symptoms with non-infectious neonatal conditions like respiratory distress or hypoxia-ischemia this has potentially led to an overexposure to antibiotics. It is recommended to stop empirical antibiotic treatment if blood cultures remain sterile after 36 to 48 hours. In our high risk cohort 25 of 27 blood cultures (93%) returned sterile although the patients

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were clinically likely to have EONS (group 2). Three patients in this group received a positive multiplex PCR result, but the agents detected were not likely to cause EONS and none of the two patients with positive blood culture had a matching PCR result.

All neonates within EONS group "sepsis likely" received antibiotic therapy irrespective of negative blood culture results. The duration of antibiotic therapy correlated with the EONS risk group stratification. Of note is that 7.3% of patients in the group "sepsis unlikely" received antibiotics with a mean duration of 2.8 days. No antibiotic therapy was initiated because of a positive PCR, but in the duration of the antibiotic therapy was longer than in the patients without any microbiological proof, even in group 3 ("sepsis unlikely"). Overuse of antibiotics may have negative effects especially in preterm infants, in whom prolonged antibiotic therapy increases the mortality and risk for bronchopulmonary dysplasia, retinopathy, necrotizing enterocolitis and damage of periventricular white matter [11]. Multiplex PCR testing is not suitable to increase diagnostic security especially in a setting of low-risk infants that are only screened for infection (EONS group "sepsis unlikely").

The multiplex PCR test which was used in our study (LightCycler® SeptiFast MGRADE system, Roche Diagnostics) is no longer commercially available. Even as metagenomic NGS methods emerge that do not necessitate specific primer design, allowing for the detection of a broad spectrum of bacterial, fungal, and viral pathogens in one assay [25-27] and have the potential to detect antimicrobial resistance genes, we believe our study's results would remain largely consistent. Even though the multiplex PCR we employed already encompassed the most common pathogens associated with EONS, the diagnostic yield wasn't enhanced for culture-negative patients diagnosed with EONS based on clinical, laboratory, and anamnestic indicators (EONS group two).

The primary objective of any antibiotic stewardship program is threefold: to prevent the onset of unnecessary antibiotic therapy, to cease empirical antibiotic treatment once an infection can be confidently excluded, and to pinpoint and manage neonates with sepsis using precisely targeted antimicrobial therapy. Even with the promising advances in Next-Generation Sequencing (NGS) and other molecular techniques, the search for the perfect diagnostic marker persists. Our research underscores that, especially in the context of infection screening within a low-risk cohort, multiplex PCR testing is not optimal for guiding EONS diagnosis. Therefore, a swift marker boasting high sensitivity, specificity, and predictive value remains a sought-after aim.

**Author Contributions:** Conceptualization, AS, PR and UF; methodology, AS and PR; formal analysis, AS and DS; investigation, AS and DS; data curation, AS and DS.; writing—original draft preparation, DS; writing—review and editing, AS, PR and UF; visualization, AS; supervision, PR and UF; All authors have read and agreed to the published version of the manuscript.

**Funding:** This research received no external funding.

**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University of Duisburg-Essen (16-7306-BO, date of approval 05.09.2017).

**Informed Consent Statement:** Informed consent was obtained from all legal guardians of the neonates involved in the study.

**Data Availability Statement:** The dataset used and/or analyzed for the study is available from the corresponding author upon reasonable request.

**Acknowledgments:** We thank the midwives of the Department of Obstetrics and Gynecology in acquiring umbilical cord blood samples for analysis.

**Conflicts of Interest:** The authors declare no conflict of interest.

## List of Abbreviations

| | |
|---|---|
| CrP | C reactive protein |
| EDTA | Ethylendiamintetraacetat |
| EONS | early-onset neonatal sepsis |

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| | |
|---|---|
| GBS | group B streptococci, Streptococcus agalactiae |
| IL-6 | Interleukin 6 |
| PCR | Polymerase chain reaction |

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